Novel synthetic pathways for the preparation of ProTides as potential therapeutic agents

A thesis submitted in accordance with the conditions governing candidates for the degree of

Philosophiae Doctor in Cardiff University

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Summary

The phosphoroamidate (ProTide) approach is a prodrug technology aimed to circumvent metabolic bottlenecks in the activation of nucleoside-based drugs and optimise their intracellular delivery. The tremendous importance of the ProTide approach is highlighted by the approval of Sofosbuvir (Sovaldi®, HCV infections) and tenofovir alafenamide fumarate (TAF, Vemlidy®, HIV and HBV infections). A great deal of success is also demonstrated by many other compounds adopting this technology either in clinical trials or preclinical evaluations as antiviral and anticancer agents. Given the great impact of phosphor(n)oamidate nucleoside prodrugs in the antiviral arena and beyond, the application of this technology has grown dramatically.

Several procedures are present in the literature for the preparation of phosphoroamidate prodrugs of nucleosides. However, an efficient and inexpensive diastereoselective synthesis to prepare ProTides as single diastereoisomers is missing. Additionally, the phosphonoamidate cognate class, one of the most significant groups of antiviral drugs, presents many synthetic challenges. Recent literature reported the synthesis of novel acyclic nucleoside backbones including the phosphonate derivatives bearing a double bond in the aliphatic chain. However, the methodologies described for the preparation of ProTides on alkenyl acyclic nucleosides are scarce and inefficient. Beside phosphoroamidates and phosphonoamidates, many difficulties can also be encountered in the preparation of modified unnatural nucleosides and related prodrugs. One of them is the ProTide of 2'-deoxy-O^6-methylguanosine to be tested for mitochondrial DNA depletion syndrome.

In this context, the research discussed in this thesis is focused on addressing the synthetic problems related to unnatural nucleosides and their ProTides. This thesis aims to explore novel methodologies for the preparation of both phosphoroamidate and phosphonoamidate prodrugs of biologically relevant nucleosides in order to give easy access to novel ProTides to be evaluated for their potential therapeutic activites.

Publications

A part of the work presented in this thesis has been published in the following journals:

Elisa Pileggi, Michaela Serpi, Graciela Andrei, Dominique Schols, Robert Snoeck, and Fabrizio Pertusati. Expedient synthesis and biological evaluation of alkenyl acyclic nucleoside phosphonate prodrugs. *Bioorganic & Medicinal Chemistry*, 2018, 26, 3596-3609. https://doi.org/10.1016/j.bmc.2018.05.034

Elisa Pileggi, Michaela Serpi, Fabrizio Pertusati. Preparation of pyrimidine alkenyl acyclic nucleoside phosphonoamidates. *Current Protocols in Nucleic Acid Chemistry*, 2018, 74(1), e56. https://doi.org/10.1002/cpnc.56

Fabrizio Pertusati, **Elisa Pileggi**, Jennifer Richards, Mandy Wootton, Leentje Persoons, David De Coster, Xabier Villanueva, Dirk Daelemans, Hans Steenackers, Christopher McGuigan and Michaela Serpi. Drug Repurposing: Phosphate Prodrugs of Anticancer and Antiviral FDA-approved Nucleosides as Novel Antimicrobials. *Journal of Antimicrobial Chemotherapy* (under review).

Mark Vanden Avond, Hui Meng, Daniel C Helbling, Margaret Beatka, Mariah Prom, David Dimmock, Fabrizio Pertusati, Michaela Serpi, **Elisa Pileggi**, Patrick Crutcher, Stephen Thomas, Michael W. Lawlor. The Nucleotide Prodrug CERC-913 Improves mtDNA Content in Primary Hepatocytes from DGUOK-Deficient Rats. *Journal of Inherited Metabolic Disease* (under review).

The published articles are attached to the Appendix.

Table of Contents

Abbreviations and Acronyms

ABV - Abacavir

Ac - acetyl

ACV - Acyclovir

ACV - acyclovir

ADA - Adenosine deaminase

Ade - adenine

Ado - adenosine

AdoC - aristeromycin

AdoHcy - *S* - adenosylhomocysteine

AdoMet - *S* - adenosylmethionine

SUCLA2 – (ADP)-forming succinyl CoA ligase β - subunit

AK - adenosine kinase

Aldrithiol - 2,2 - dipyridyl disulphide

ALL - acute lymphocytic leukaemia

AML - Acute myeloid leukaemia

ANPp - acyclic nucleoside phosphonate diphosphate

ANPpp - acyclic nucleoside phosphonate triphosphate

ANPs - acyclic nucleoside phosphonates

ANPs - acyclic nucleoside phosphonates

ANs - acyclic nucleosides

Ar - Aryl

Ara - A - Vidarabine

Ara - C - Cytarabine

Ara-CMP – cytosine arabinoside 5'-monophosphate

Ara - G - arabinosylguanine

ATP - adenosine triphosphate

AZT - Zidovudine

BEN - *N*, *N*' - ethylenebis - benzaldimine -

Boc - *tert* - butylcarbonate

Boc_2O – di-*tert*-butyl dicarbonate

BSA - *N*, *O* - bistrimethylsilyl - acetamide

BTEA - Cl - benzyltriethylammonium chloride

BVDU - Brivudine

C10orf2 - twinkle mtDNA helicase gene

CAFdA - clofarabine

CAL - B - *Candida Antarctica* lipase B

cathepsin A catA - Aryloxy phosphoroamidate nucleoside prodrugs ProTides -

CDA - cytidine deaminase

CLL - chronic lymphocytic leukaemia

CM - cross - metathesis

CML - chronic myeloid leukaemia

CMV - cytomegalovirus

CNTs - concentrative nucleoside transporters

CPY - carboxypeptidase Y

CSP - polysaccharide - type chiral stationary phase

CycloSal - Cyclosaligenyl

Cyd - cytidine

CYP450 - cytochrome P_{450}

Cyt - cytosine

d4Ns - 2',3'-didehydro-2',3'-dideodxynucleosides

d4T - Stavudine

DAC - Decitabine

dAdo - deoxyadenosine

DBU - 1,8-diazabicyclo[5.4.0]undec-7-ene

dCK - deoxycytidine kinase

dCyt - deoxycytidine

ddC - Zalcitabine

ddI - Didanosine

ddNs - 2',3' - dideoxy - nucleosides

ddU - 2',3' - dideoxyuridine

de - diastereomeric excess

dfc - Deoxycoformycin

dFdC - Gemcitabine

dGK - deoxyguanosine kinase

dGK - deoxyguanosine kinase

dGuo - deoxyguanosine

DGUOK - deoxyguanosine kinase gene

DIAD - diisopropyl azodicarboxylate

DIBAL - H - diisobutyl aluminium hydride

Di*P*Pro - Diphosphate pronucleotide

DMAP - 4 - dimetilamminopiridina

DME - dimethoxyethane

DNA MT - DNA methyltransferases

dNKs - deoxyribonucleoside kinases

DPP - diphenyl phosphite

dr - diastereomeric ratio

dTMP - deoxythymidine monophosphate

dTTP - deoxythymidine tri - phosphate

dUMP - deoxyuridine monophosphate

dUTP - deoxyuridine tri - phosphate

DYKAT - catalytic dynamic kinetic asymmetric transformation

ENTs - equilibrative nucleoside transporters

ESI - MS Electrospray ionization - mass spectrometry -

ETV - Entecavir

F3T - Trifluridine

FA - Fludarabine

$FADH_2$ - flavin adenine dinucleotide

FCV - Famciclovir

FE - fast eluent

FTC - Emtricitabine

FUdR - floxuridine

G - Glycosyl group

GCV - Ganciclovir

GCV - ganciclovir

GDA - guanosine deaminase

GF - growth factors

Gua - guanine

Guo - guanosine

HATU - hexafluorophosphate azabenzotriazole tetramethyl uronium

HBV - Hepatitis B virus

HCL - hairy cell leukaemia

HCV - Hepatitis C virus

HDP - hexadecyloxypropyl

HINT-1 - histidine triad nucleotide-binding protein 1

HIV - human immunodeficiency virus

HPLC - reverse phase HPLC RP -

HPMP [3-hydroxy-2-phosphonomethoxypropyl]

HSQC - Heteronuclear Single Quantum Correlation

HSV - herpes simplex virus

hTMPK - human thymidylate monophosphate kinase

ICP - MS inductively coupled plasma mass spectrometry

IdU - idoxuridine

***L* - Glu** - *L* - Glutamic acid

***L* - Lys** - *L* - Lysine

***L* - Met** - *L* - Methionine

LdT - Telbivudine

$LiAlH_4$ - lithium aluminium hydride

LPC - lysophosphatidylcholine

m/z - mass - to - charge ratio

MDS - Mitochondrial DNA Depletion Syndrome

MDS - mitochondrial DNA depletion syndrome

MDS - myelodysplastic syndrome

MDS MINGIE - neurogastrointestinal encephalopathy

MTBE - *tert* - butyl methyl ether

mtDNA - Mitochondrial DNA

MW - Microwave

$NaBH_4$ - sodium borohydride

NADH - nicotinamide adenine dinucleotide

NAMP - nucleoside analogue monophosphate

NAs - nucleotide analogues

NATPs - nucleoside analogue 5' - triphosphate

NDPKs - nucleoside diphosphate kinases

NKs - nucleoside kinases

NMI - ***N*** - methyl imidazole

NMP - ***N*** - methyl - 2 - pyrrolidone

NMPKs - nucleoside monophosphate kinases

NOESY - Nuclear Overhauser effect spectroscopy

Np-A - neplanocin A

NT-5' - nucleotidase 5'

NTP - nucleoside 5' - triphosphate

NTs - nucleoside - specific membrane transport carriers

ODE - octadecyloxyethyl

P - Phosphorous group

P-Cl - Phosphorochloridate

PE2 - Penciclovir

PE2 - penciclovir

PEG - polyethylene glycol

PFP - pentafluorophenyl phosphate

PME [2 - phosphonomethoxyethyl]

PMP [2 - phosphonomethoxypropyl]

***p*NO_2P** - *p* - nitrophenyl phosphate

PNP - nucleoside phosphorylases

POC - isopropyloxymethyl carbonate

pol - γA - polymerase gamma catalytic subunit

POLG - DNA polymerase gamma gene

POM - Pivaloyloxymethyl

PPh_3 - triphenylphosphine

ppm - parts per million

RBV - Ribavirin

RCM - ring closing metathesis

RCMP - ring opening metathesis polymerisation

rNKs - ribonucleoside kinases

RNR - Ribonucleotide Reductase

RRM2B - ribonucleotide reductase M2 B unit

RSV - respiratory syncytial virus

SAR - structure - activity relationship

SATE - S-Acyl-2-thioethyl

SE - slow eluent

SFC - chiral supercritical fluid chromatography

SUCL - succinyl CoA ligase

SUCLG1 – (GDP)-forming succinyl CoA ligase α - subunit

T-ALL - T-cell acute lymphoblastic leukaemia

TAF - Tenofovir alafenamide fumarate

TBDMS - *tert* - butyldimethylsilyl

TBDMSCl - *tert* - butyldimethylsilyl chloride

***t*BuMgCl** - *tert* - butyl magnesium chloride

TDF - Tenofovir disoproxil fumarate

Thd - thymidine

Thy - thymine

TK1 - thymidine kinase 1

TK2 - thymidine kinase 2

TLC - analytical thin - layer chromatography

TLC - preparative thin - layer chromatography prep

TMSBr - trimethylsilyl bromide

TMSCl - trimethylsilyl chloride

TMSI - trimethylsilyl iodide

TMSOTf - trimethylsilyl triflate

TP - thymidine phosphorylase

TPANs - tri-phosphorylated acyclic nucleoside

t_R - Retention time

Tri*P*Pro - triphosphate pronucleotides

TS - thymidylate synthase

TYMP - thymidine phosphorylase gene

UCK1 - uridine - cytidine kinase 1

UCK2 - uridine - cytidine kinase 2

Ura - uracil

Urd - uridine

VACV - Valaciclovir

VGCV - Valganciclovir

VZV - varicella zoster virus

2' - MeG - 2' - methylguanosine

2CdA - Cladribine

3TC - Lamivudine

5 - AC - azacitidine

5 - FU - 5 - fluorouracil

6 - MP - mercaptopurine

6 - TG - thiopurines thioguanine

Chapter 1. Introduction

1.1 Nucleosides, Nucleotides and their analogues

1.1.1 Natural nucleosides

Nucleosides are a class of low molecular weight, intracellular compounds that participate in many biochemical processes. In particular, they are the biochemical precursors of nucleotides, the monomeric building blocks of nucleic acids, (DNA and RNA), the substances that carry genetic information.[1] Both nucleosides and nucleotides are formed by an heterocyclic nucleobase (purine or pyrimidine) linked to a 5-carbon sugar moiety (D-ribose or 2-deoxy-D-ribose) by a β-*N*-glycosidic bond.[2] The nucleotide form is characterized by the presence of one to three phosphate groups linked to the pentose carbohydrate via its 5'-oxygen (**Figure 1.1**).

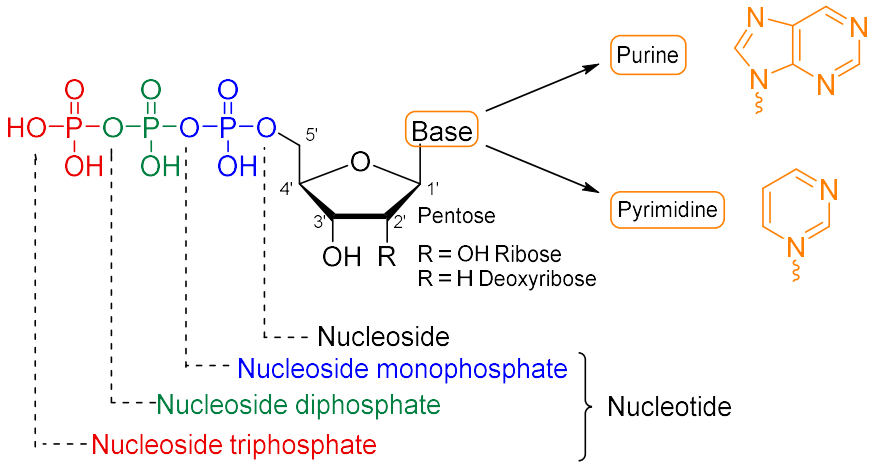


Figure 1.1 Basic structure of nucleosides and nucleotides.

Both the nucleic acids are polymers formed by nucleoside monophosphate monomers linked together via a phosphodiester bond between the 3'-OH of one nucleotide and the 5'-OH of the other nucleotide. The nitrogenous bases found in nucleosides are the pyrimidines cytosine (Cyt), thymine (Thy), and uracil (Ura), and the purines adenine (Ade) and guanine (Gua) (**Figure 1.2**). According to the nature of the sugar component, D-ribose or 2-deoxy-D-ribose, nucleosides can be categorised

respectively as ribonucleosides and deoxynucleosides (**Figure 1.1**). Ribonucleotides are the building blocks of the RNA polymer: adenosine (Ado), guanosine (Guo), cytidine (Cyd), uridine (Urd). Deoxynucleotides are instead the monomers forming the DNA polymer: deoxyadenosine (dAdo), deoxyguanosine (dGuo), deoxycytidine (dCyt) and thymidine (Thd).

To be incorporated in the nucleic acids, nucleosides need to be triphosphorylated at the 5'-position. The metabolic pathway that allows the conversion of nucleosides in active nucleotides is described in the following paragraph.

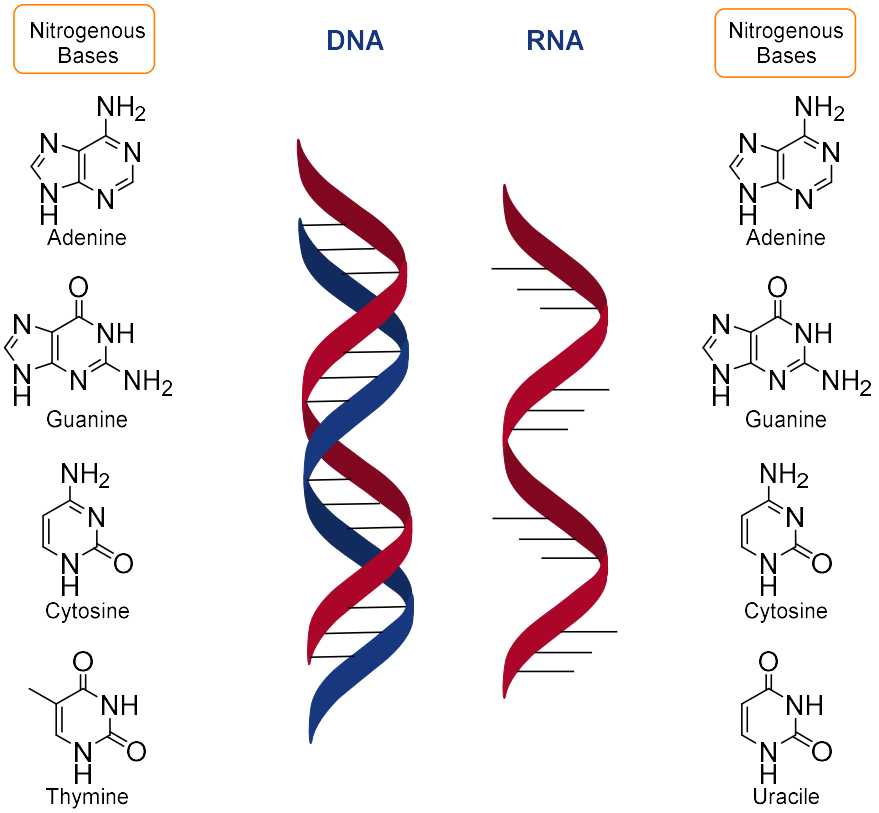


Figure 1.2 DNA and RNA nitrogenous bases.

1.1.2 Endogenous nucleoside metabolism

Endogenous nucleosides must be phosphorylated to their nucleoside 5'-triphosphate (NTP) active form to be incorporated into DNA or RNA strands. To this end, nucleosides can be synthesised *de novo* inside the cell and then phosphorylated to their active form, or they can be rescued by extracellular sources (salvage pathway). In the latter case nucleosides must enter cells, but their polarity hinders cell membrane passive diffusion. For this reason, they cross the membrane through the action of nucleoside-

specific membrane transport carriers (NTs). The role of NTs consists mainly of recycling nucleosides for anabolic purpose (nucleic acid synthesis).[3,4]

Two NT families have been identified: equilibrative nucleoside transporters (ENTs), based on a sodium-independent mechanism, and concentrative nucleoside transporters (CNTs), based on a sodium-dependent mechanism. ENTs facilitate nucleoside diffusion across membranes through a gradient-based reversible mechanism and are widely distributed across different cell types.[11,6,7] Otherwise, CNTs mediate influx only through an ATP-based mechanism and are limited to specialized cells (epithelia, intestinal).[3,6,8]

Once inside the cell, the nucleosides are phosphorylated to their 5'-triphosphate form by kinase enzymes, whose role consists of catalysing the transfer of a phosphate group from a donor to an acceptor (**Table 1.1**): the donor is usually the ATP γ-phosphate, while the acceptor can be the 5'-*OH* group of a nucleoside, or the α- or β-phosphate group of a nucleotide.[9]

Enzyme Name	Enzyme Class	Transfer Group	Reaction catalysed
Phosphorylase	Transferase	Glycosyl group (G)	G-B + P ⇌ G-P + B
Phosphatase	Hydrolase	-	P-G-B ⇌ P + G-B
Kinase	Transferase	Phosphorous group (P)	G-B + P ⇌ P-G-B

Table 1.1 Enzymes involved in biological phosphorylation and dephosphorylation processes. P: phosphate group, phosphonate group inorganic phosphate; G: glycosyl group; B: nucleobase.

The three subsequent phosphorylation steps are catalysed by nucleoside kinases (NKs), nucleoside monophosphate kinases (NMPKs), and nucleoside diphosphate kinases (NDPKs), respectively.

NKs exhibit different substrate specificities for the bases and the glycon moiety. For this latter, it is possible to distinguish two families: the deoxyribonucleoside kinases and the ribonucleoside kinases. The deoxyribonucleoside kinases (dNKs) catalyse the phosphorylation of deoxyribonucleosides to deoxyribonucleoside monophosphates. All the human enzymes have been identified and are known as deoxycytidine kinase (dCK), deoxyguanosine kinase (dGK), thymidine kinase 1 and 2 (TK1 and TK2). dCK and TK1 are cytosolic kinases, while dGK and TK2 are mitochondrial kinases.[10–12] The ribonucleoside kinases (rNKs) catalyse the phosphorylation of ribonucleosides to ribonucleoside monophosphates. Even though less is known about rNKs, uridine-cytidine kinase 1 and 2 (UCK1 and UCK2) and adenosine kinase (AK) have been identified.[10]

Once endogenous nucleosides are successfully converted into their 5'-monophosphate derivative, the second and third phosphotransferase processes can quickly lead to the conversion of the monophosphate nucleoside in the corresponding tri-phosphorylated form so that they can finally be incorporated into the nucleic acid chain.

However, dNTP pools need to be balanced to perform DNA replication and repair accurately. For this purpose, in addition to the mechanisms that lead to intracellular accumulation of dNTPs, nucleoside metabolism comprises two crucial families of catabolizing enzymes: deaminases and nucleotidases. These latter enzymes are also called phosphatases, and they act at the monophosphate level. Precisely, 5'-nucleotidase (5'-NT) catalyses the dephosphorylation of nucleoside monophosphates.[13] Deaminases are extra- and intra-cellular enzymes which convert nucleosides and nucleoside monophosphates into inactive compounds. Adenosine deaminase (ADA),[14] guanosine deaminase (GDA)[15] and cytidine deaminase (CDA)[16] catalyse the deamination of the corresponding nucleobase inducing the interconversion of adenosine, guanosine and cytidine to inosine, uridine and xanthine respectively.

Thanks to the functional cooperation of all these enzymes, both anabolic and catabolic, dNTP pools in cells can be fully balanced, so that DNA replication and repair can be correctly accomplished.

1.1.3 Nucleoside and Nucleotide analogues as therapeutic agents

Nucleoside and nucleotide compounds are involved not only in nucleic acid synthesis but also in several other physiological processes, including regulation of cardiovascular activity,[17] neurotransmission,[18] cell signalling,[19] enzyme regulation and metabolism.[20]

For this reason, nucleoside and nucleotide analogues (NAs) have been used as a versatile family of drugs that consists of chemically modified molecules synthesized to mimic the physiological behaviours of the natural counterparts. As shown in **Figure 1.3**, the sites where nucleosides can be subjected to modifications are many. This aspect represents the main reason for their success. Nucleobases can be replaced by pseudo-bases, in which functional group variations are applied to modify their biological interactions or to confer more stability towards catabolism and glycosidic bond cleavage.[21] Whereas, nucleoside analogues with sugar modifications have proved to be

effective and selective antiviral and anticancer agents.[21] Additionally, in the case of nucleotides, it is also possible to arrange modifications on the phosphate group.

In-depth research into nucleoside modifications showed that minor structural changes can have substantial effects on both mechanism of action and toxicity.[22] These considerations have extensively influenced nucleoside analogue design approaches.

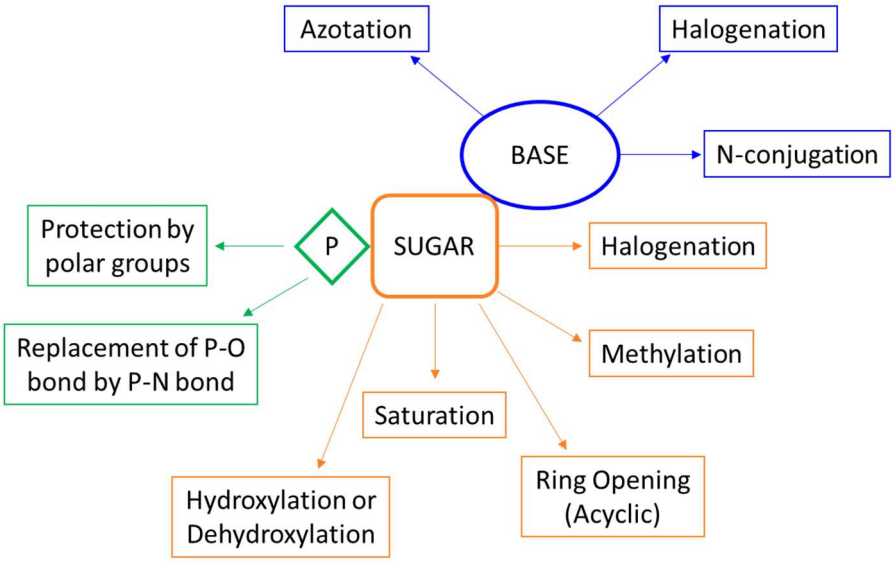


Figure 1.3 Modifications on nucleoside and nucleotide backbones.

Despite these variations, it is necessary that nucleoside and nucleotide analogues retain a close structural correlation with their endogenous counterparts, to undergo cellular metabolism and be incorporated into nucleic acids. Once incorporated, they can exert their therapeutic effects by inhibiting cellular division (anticancer activity)[23] and viral replication (antiviral activity).[24] Additionally, NAs can also interfere with various enzymes,[20] essential for the synthesis or the metabolism of natural nucleosides as largely explained in the next paragraph.

1.1.4 Mechanism of action of nucleoside-based drugs

The same metabolic pathway previously explained for natural nucleosides is exploited by nucleoside analogues. As their endogenous counterpart, NAs are phosphorylated to their nucleoside analogue 5'-triphosphate (NATPs) active form by kinase enzymes. The first phosphorylation step is generally the rate-limiting stage for the pharmacological activation of most nucleoside analogues: it is an irreversible reaction, although the monophosphate can be dephosphorylated to nucleoside by 5'-nucleotidases (5'-NT).[12,13]

Addition of the second and the third phosphate group to nucleoside monophosphate generally take place smoothly.[20]

When the NAs are administered as drugs, the success of the three phosphorylation steps leads to the accumulation of di- and tri-phosphorylated NAs in cancer or virus-infected cells (**Figure 1.4**).

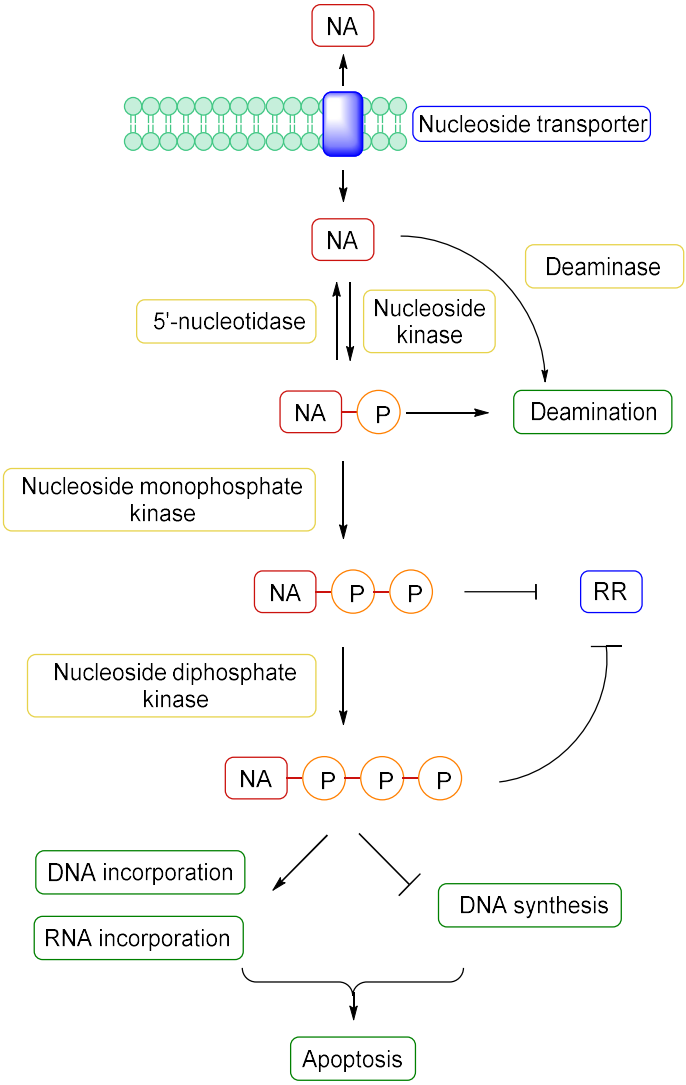


Figure 1.4 Mechanism of action of nucleoside analogues.[4]

At this point, NAs can express their therapeutic effects either through integration in nucleic acid strands in competition with their endogenous counterparts or by inhibition of cellular essential enzymes. The incorporation of NAs into DNA leads to replication fork blockage and chain termination. The DNA damage sensors recognize these events and activate survival mechanisms such as cell cycle arrest and DNA repair. However, if

these processes are overwhelmed by the DNA damage, the sensors can also trigger the signals for the apoptotic process.[23,25,26]

In addition, NATPs can be therapeutically exploited to interfere with the activities of key enzymes. For example, they can interact with viral (and human) polymerases, that catalyse the assembly of DNA and RNA. NAs can inhibit these enzymes preventing nucleic acid synthesis, and so replication.[27]

NAs can also affect purine and pyrimidine nucleoside phosphorylases (PNP), which catalyse the phosphorolysis of deoxy- and ribonucleotides (**Table 1.1**). These enzymes are more expressed in cancer cells than normal cells. Together with deaminases, these enzymes play a key role in nucleoside catabolism in the salvage pathway. Inhibition of PNP by NAs can lead to an increase of nucleoside analogue tri-phosphate intracellular levels.[28–30]

Another key enzyme that may be involved in NAs therapeutic activity is DNA methyltransferases (DNA MT), which catalyse the addition of a methyl group to the DNA. This process is implicated in the regulation of numerous cellular mechanisms: among them, the gene regulation associated with gene silencing that is responsible for human tumorigenesis.[31] The inhibition of DNA MT by NAs reduces the DNA methylation and leads to the activation of tumour suppressor genes, usually silenced as a consequence of the methylated DNA.[32]

Likewise, thymidylate synthase (TS) can be a NA target. TS catalyses the methylation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP). Inhibition of TS by NAs results in a depletion of dTMP leading to deoxythymidine tri-phosphate (dTTP) imbalance and deoxyuridine tri-phosphate (dUTP) increase. This latter might be misincorporated in the DNA along with the NAs and both can induce DNA damage.[33,34]

Finally, Ribonucleotide Reductase (RNR) is a crucial enzyme involved in *de novo* synthesis of dNTPs. Precisely, it catalyses the reduction of ribonucleotides to their deoxy form, thus improving dNTP pools.[35,36] As a target of NADPs and NATPs, RNR might be inhibited causing alteration in natural dNTP intracellular concentrations and thus promoting dNATP incorporation into DNA strand.[37]

Thanks to this wide range of potential targets, today NAs are successfully attested as first line drugs in both anticancer and antiviral treatments.

1.1.5 Anticancer nucleoside analogues

Nowadays, NAs represent a growing family of purine and pyrimidine nucleosides with activities against solid tumours and malignant states of the blood. There are currently fourteen nucleosides derived drugs approved as anticancer drugs: eleven nucleosides and three nucleobases (**Figure 1.5**, **Table 1.2**).

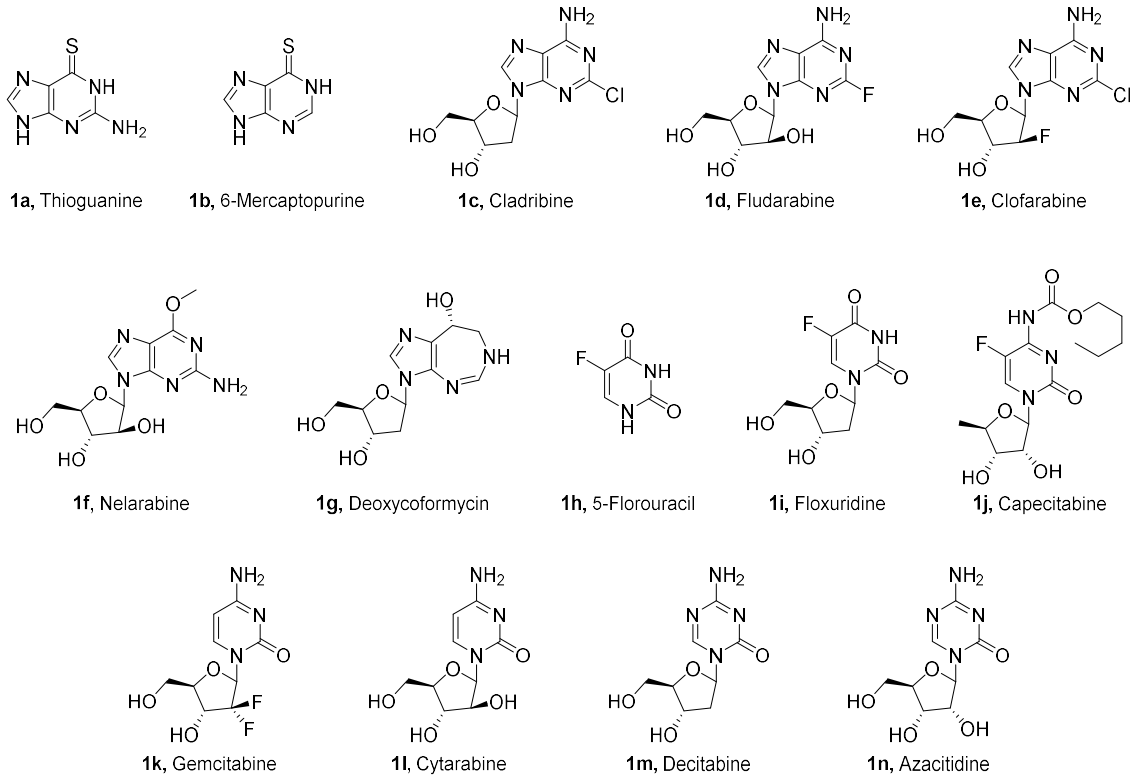


Figure 1.5 Anticancer nucleoside analogues.

1.1.5.1 Purine nucleobase and nucleoside analogues

The thiopurines thioguanine (6-TG, **1a**) and mercaptopurine (6-MP, **1b**) are nucleobases that display anti-leukaemia activities, particularly when used in acute lymphoblastic leukaemia in children, alone or in combination with other drugs. To be activated, they need to be first converted to the nucleotide by hypoxanthine-guanine phosphoribosyl transferase and then phosphorylated. As tri-phosphates, their cytotoxic activity is exerted mainly by their misincorporation into the DNA, but also by inhibition of enzymes involved in nucleoside metabolism.[38,39]

Among purine nucleosides, cladribine (2CdA, **1c**) and fludarabine (FA, **1d**) are deoxyadenosine derivatives resistant to deamination by ADA. Cladribine was approved for application in indolent lymphoid malignancies, while fludarabine is used for the

treatment of chronic lymphocytic leukaemia. Cladribine and fludarabine cytotoxicity can be accomplished both by DNA elongation termination caused by the incorporation of their triphosphate form in the DNA, and also by inhibition of RNR, leading to insufficient levels of dNTPs in the pool for DNA synthesis.[40,41]

These same mechanisms of action are also ascribed to clofarabine (CAFdA, **1e**), a next-generation deoxyadenosine analogue developed to improve FA and 2CdA efficacy. Indeed, in addition to FA and 2CdA effects, clofarabine has displayed inhibitory activity against DNA polymerase. CAFdA is used in the treatment of acute myeloid leukaemia.[42]

Nelarabine (**1f**) is a 6-methoxy prodrug of arabinosylguanine (Ara-G), 10-fold more soluble in water than the native nucleoside. Once administered, nelarabine is subjected to deaminase action and converted to Ara-G. After the latter enters the cell, it is phosphorylated and then incorporated into the DNA. The mismatch causes cell death. The drug is used for the treatment of T-cell lymphoblastic lymphoma and T-cell acute lymphoblastic leukaemia.[43]

Deoxycoformycin (dFC, **1g**) is another purine nucleoside analogue used in lymphoid malignancies, which exhibits a completely different mechanism of action. dFC does not need to be phosphorylated to exert its cytotoxicity, in fact it is a tight-binding inhibitor of ADA. The inhibition of the deaminase results in an increase of dAdo in plasma, and consequently dAdo tri-phosphates in cells. This strong imbalance in the dNTP pool has been correlated to spontaneously formed DNA breaks, which in turn cause activation of p53 leading to apoptosis machinery initiation.[41,44]

1.1.5.2 Pyrimidine nucleobase and nucleoside analogues

Among pyrimidine derivatives, 5-fluorouracil (5-FU, **1h**) is an uracil nucleobase analogue with activity against colorectal cancer. In cancer cells, 5-FU is in equilibrium with its nucleoside form, floxuridine (FUdR, **1i**), which has therapeutic effect. This equilibrium is mediated by thymidine phosphorylase. The key antiproliferative effect of these two drugs is thought to be the thymidylate synthase inhibition mediated by the monophosphorylated form of the nucleoside (FdUMP). The inhibition of the enzyme at issue causes a depletion of dTMP in the cell, which produces imbalance in the final dNTP pools complicated by an increase of dUTPs. The latter might be misincorporated into the DNA leading to cell death.[33,45] Among 5-FU prodrugs, the most active is Capecitabine (**1j**). The drug is converted to 5-FU through three enzymatic reactions catalysed in

sequence by carboxyl-esterase for the cleavage of the amidic bond, cytidine deaminase and thymidine phosphorylase.[46]

Concerning the deoxycytidine derivatives, gemcitabine (dFdC, **1k**) is currently one of the most potent antitumor drugs on the market. Both dFdC di-phosphate (dFdCDP) and tri-phosphate form (dFdCTP) are responsible for the drug cytotoxic activity. dFdCTP induces genome chain elongation termination by direct inhibition of DNA polymerase and misincorporation into the genome. The latter effect can be enhanced by dFdCDP action, which inhibits RNR leading to a decrease in natural deoxynucleosides intracellular concentrations.[47]

Cytarabine (Ara-C, **1l**) is an approved drug for application in haematological malignant diseases and, contrary to gemcitabine, does not show any activity against solid tumours. After the initial rate-limiting phosphorylation, cytidine deaminase catalyses the conversion of cytarabine monophosphate into the corresponding uracil derivative. The mechanism of action of the drug consists of the inhibition of DNA polymerase and incorporation of dUTPs, derived from its metabolism, into DNA leading to genome synthesis arrest.[38]

Decitabine (DAC, **1m**) and azacitidine (5-AC, **1n**) are deoxycytidine and cytidine analogues respectively, used for epigenetic cancer therapies.[48] After metabolic phosphorylation, DAC can be directly incorporated into DNA, while 5-AC needs to be converted into the corresponding deoxy-analogue. Although the structures are very similar to the deoxycytidine nucleoside drugs, these drugs induce different molecular changes. Indeed, they are known as DNA methyltransferase inhibitors. Once incorporated in the nucleic acid, the two drugs exhibit the same mechanism of action: the DNA methyltransferase recognises the azacytosine-guanine dinucleotides as natural substrates. The azanucleotide remains covalently bound to the enzyme, blocking its function. Additionally, DNA functionality is compromised and DNA damage signalling is triggered.[32,49]

Type of agent	Name	FDA approval	Indications
Purine nucleobase and nucleoside analogues	Thioguanine (6-TG, **1a**)[39]	1951	AML, ALL, CML
	6-Mercaptopurine (6-MP, **1b**)[39]	1953	ALL, CML
	Cladribine (2CdA, **1c**)[41]	1993	HCL
	Fludarabine (FA, **1d**)[41]	1991	CLL, AML
	Clofarabine (CAFdA, **1e**)[42]	2004	ALL

	Nelarabine (**1f**)[43]	2005	T-ALL
	Deoxycoformycin (dFC, **1g**)[44]	1991	HCL, CLL
Pyrimidine nucleobase and nucleoside analogues	5-Fluorouracil (5-FU, **1h**)[33]	1962	Colon, oesophageal, stomach, pancreatic, breast, cervical cancers
	Floxuridine (FUdR, **1i**)[45]	1970	Kidney, stomach, advanced colon cancers
	Capecitabine (**1j**)[46]	1998	Metastatic breast and colorectal cancers
	Gemcitabine (dFdC, **1k**)[47]	1996	Pancreatic, bladder, breast, non-small lung cancers
	Cytarabine (Ara-C, **1l**)[38]	1969	AML, ALL
	Decitabine (DAC, **1m**)[32]	2006	MDS, AML
	Azacitidine (5-AC, **1n**)[49]	2004	MDS

Table 1.2 Purine and pyrimidine anticancer nucleobase and nucleoside analogues FDA approved and their main indications. Acute myeloid leukaemia (AML), acute lymphocytic leukaemia (ALL), chronic myeloid leukaemia (CML), hairy cell leukaemia (HCL), chronic lymphocytic leukaemia (CLL), T-cell acute lymphoblastic leukaemia (T-ALL), myelodysplastic syndrome (MDS).

1.1.6 Antiviral nucleoside analogues

Since the approval of idoxuridine (IdU, **2a**) in 1962 against herpes simplex keratitis, NAs have been largely explored in the antiviral field (**Figure 1.6**).[20] Antiviral nucleoside analogues are structurally more diversified than anticancer nucleosides, as they consist not only of nucleosides, but also of acyclic nucleosides and nucleotides.

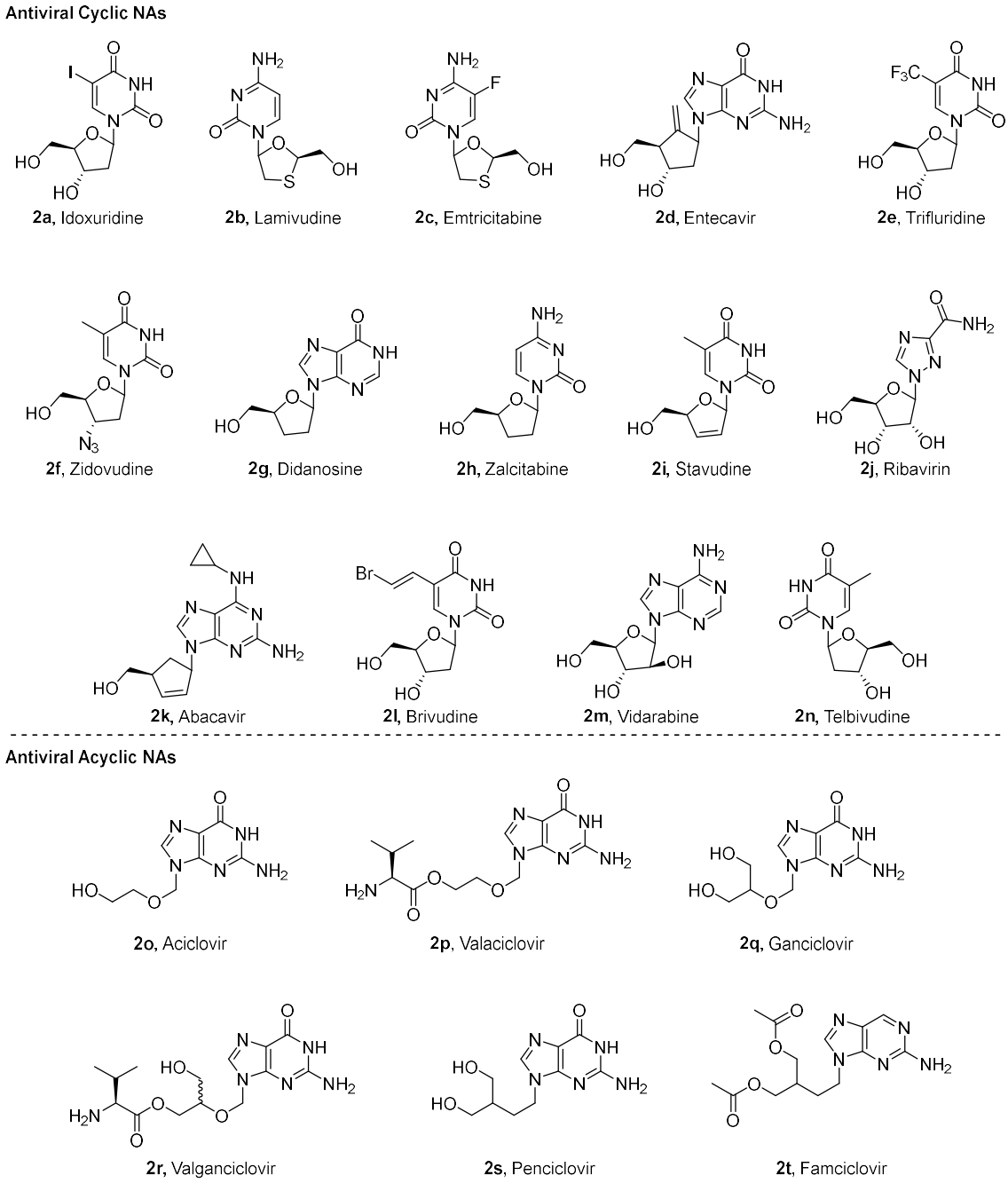


Figure 1.6 Cyclic and acyclic antiviral nucleoside analogues.

Among the currently approved antiviral drugs, there are fourteen cyclic and six acyclic NAs.[50] Many of them are 2',3'-dideoxynucleosides and share the same mechanisms of action. They are converted into triphosphates by viral or host-cell nucleoside kinases and thereby incorporated into the viral genome.[51] After phosphorylation, they can compete with the endogenous substrates in the RNA and DNA polymerisation reaction. The infected cells are characterised by a high rate of turnover, resulting in a rapid accumulation of multiple mutations. Since most of the antiviral nucleoside backbones are missing the 3'-hydroxyl group essential for attachment of the

incoming nucleotide for further chain elongation, they act as chain terminators.[52] In addition, they can also interfere with vital viral enzymes. Anti-HIV nucleoside analogues are inhibitors of the viral reverse transcriptase necessary for HIV replication.[53] Three of these agents, lamivudine (**2b**), emtricitabine (**2c**) and entecavir (**2d**), are *L*-enantiomer nucleoside analogues showing greater antiviral activities and more favourable toxicological profiles than their natural *D*-counterparts.[54] Moreover, the phosphorylated *L*-nucleosides are also metabolically stable displaying resistance to phosphorylases and deaminases. As triphosphates, these unnatural enantiomers of cytosine derivatives have proved to be able to interfere with the viral polymerase or to be incorporated into the viral DNA strands, resulting in blockage of the viral nucleic acid growth.[54] Today, their therapeutic affects are mainly used for the treatment of HIV and HBV.[53,55,56]

Among the variations that it are possible to design at the nucleoside backbone, the opening of the sugar ring was one of the most successful. These analogues are known as acyclic nucleosides and they find their main applications in the antiviral field. Among the most active antiviral agents discovered, acyclovir (**2o**), ganciclovir (**2q**) and penciclovir (**2s**) were approved by the FDA between 1982 and 1996 for the treatment of HSV, VZV and CMV. Since they displayed poor bioavailability due to low permeation properties, their prodrugs have been developed. Valaciclovir (**2p**), valganciclovir (**2r**) and famciclovir (**2t**) are the prodrugs of acyclovir (**2o**), ganciclovir (**2q**) and penciclovir (**2s**), respectively.[57] Nowadays, acyclic nucleotide analogues are a key class of antiviral drugs and this topic will be covered in more detail in chapter 3.

In **Table 1.3** all the antiviral nucleosides and their specific targets are reported.

Type of agent	Name	FDA approval	Indications
Cyclic Nucleoside Analogues	Idoxuridine (IdU, **2a**)[58]	1962	HSV, VZV
	Lamivudine (3TC, **2b**)[53]	1995	HIV, HBV
	Emtricitabine (FTC, **2c**)[55]	2003	HIV, HBV
	Entecavir (ETV, **2d**)[56]	2004	HBV
	Trifluridine (F3T, **2e**)[59]	1980	HSV
	Zidovudine (AZT, **2f**)[60]	1987	HIV
	Didanosine (ddI, **2g**)[61]	1991	HIV
	Zalcitabine (ddC, **2h**)[61]	1992	HIV
	Stavudine (d4T, **2i**)[62]	1994	HIV
	Ribavirin (RBV, **2j**)[63]	1986	HCV, RSV
	Abacavir (ABV, **2k**)[53]	1998	HIV
	Brivudine (BVDU, **2l**)[64]	1980	HSV, VZV
	Vidarabine (Ara-A, **2m**)[65]	1986	HSV, VZV
	Telbivudine (LdT, **2n**)[66]	2006	HBV

Acyclic Nucleoside Analogues	Acyclovir (ACV, **2o**)[67]	1982	HSV, VZV
	Valaciclovir (VACV, **2p**)[57]	1996	HSV, VZV, CMV
	Ganciclovir (GCV, **2q**)[68]	1989	CMV
	Valganciclovir (VGCV, **2r**)[57]	2001	CMV
	Penciclovir (PE2, **2s**)[69]	2002	HSV
	Famciclovir (FCV, **2t**)[57]	2007	HSV

Table 1.3 Cyclic and acyclic antiviral nucleoside analogues FDA approved and their indications. Hepatitis B and C viruses (HBV and HCV), human immunodeficiency virus (HIV), cytomegalovirus (CMV), herpes simplex virus (HSV), varicella zoster virus (VZV), respiratory syncytial virus (RSV).

Despite the wide range of potential targets that NAs have, their use is often limited by a few drawbacks that are intrinsic in their structures. These limitations and the strategies to overcome them are discussed below.

1.1.7 Overcoming limitations and resistance to nucleoside analogue treatments

Reduction of natural nucleoside pools and increase of NA intracellular concentrations are the main goals to be achieved in order to ensure a therapeutic effect of the nucleoside-based drugs. For this purpose, several biochemical modulations can be applied to overcome NA limitations.

Metabolic resistance to the effect of NAs is a significant clinical problem. It can be caused by somatic changes in cancer cells and specific mutations in viral genomes.[20] The general mechanisms of resistance to NAs are divided into three groups: 1) modifications of genes implicated in metabolism and intracellular accumulation of NATPs, such as membrane carriers, kinases, deaminases and 5'-nucleotidases; 2) alterations of NA targeted proteins. These proteins are DNA polymerases and ribonucleotide reductase; 3) variations in cellular responses involved in DNA repair and apoptotic mechanisms. Any of the aforementioned alterations might result in an imbalance of intracellular NATPs and decrease of the therapeutic activities of NA-based drugs.[40,70,71]

The membrane transporters are the first cellular proteins which drugs interact with, therefore they play significant roles in the manifestation of NA cytotoxicity.[70,71] *In vitro* assays have confirmed that cells deficient in nucleoside transporters are very resistant to nucleoside drugs.[72,73] To improve the NT-related NA efficacy, a measurement of the abundance of nucleoside carriers might be used as tool to predict the clinical outcome. For instance, NA-treated tumour patients with high level of hENT1(the most

abundantly expressed and detectable NT in human cells), showed longer survival times when compared with patients without detectable hENT1.[74] Furthermore, several studies have demonstrated that leukemic cells pre-treated with growth factors (GF), displayed an increase in nucleoside drug uptake associated with enhanced expression of hENT1.[75,76]

Another aspect that may affect the intracellular imbalance of NATPs is the overexpression of catabolizing enzymes. *In vitro* experiments have associated increased levels of both 5'-NUs and deaminases with nucleoside drug resistance.[40,70] The action of these deactivating enzymes results in a lower clinical efficacy of NA drugs.[77,78] Despite these deactivation mechanisms, the drug cytotoxicity might be potentiated when administered in combination with a 5'-nucleotide or deaminase inhibitors. Unfortunately, at present, no such inhibitors are clinically available: according to the literature, the main reason could be that many potential inhibitors are themselves NAs that may compete with the drug for their transport across the membrane.[79]

NAs resistance can be similarly manifested in the cases of overexpression of proteins such as DNA polymerase and RNR.[80,81] Regarding DNA polymerase, its overexpression is also associated with a decrease in affinity for NAs.[82] Both these factors contribute to reduce the inhibition of DNA synthesis as a NA therapeutic effect.[40] Similarly, large amounts of dNTPs originating from the overexpressed RNR activity are intended to be inserted into the DNA, completely excluding dNAs from DNA synthesis.[81] Additionally, boosted dNTP intracellular concentration inhibits the activity of kinases, reducing NA activation.[70] Therefore, compounds able to decrease the incorporation of dNTPs into the DNA strand by inhibiting DNA polymerase or RNR may enhance NA-drug cytotoxicites.[83,84]

The integration of dNAs into nucleic acid chains is recognised as DNA damage, therefore the p53 protein is expressed to trigger the apoptotic process, and the drug's action is accomplished. Besides, p53 appears to have an exonuclease activity, which is able to cleave the mismatched incorporated dNAs from the DNA chains.[85,86] This p53 role, together with defective cell death pathways, has a specific connection with NA resistance.[40] A proposed solution to facilitate the entry of the NAs into the DNA chain is the induction of DNA repair pathways through co-administration of the cytotoxic drug with DNA damaging agents. The body recognizes and repairs the injured patches resynthesizing the defective strand, creating opportunities for NAs insertion.[87]

Regarding the kinases, assays on different cell lines resistant to NAs have shown absence or decrease in the activities of these proteins.[88,89] This deficiency seems to be

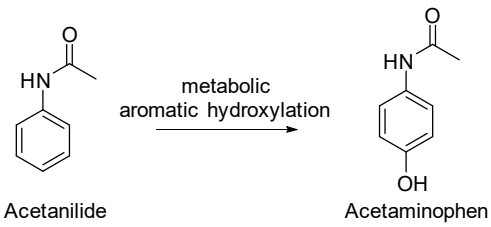
related to a partial or total deletion of the genes that express for the enzymes.[90–92] The direct delivery of nucleotides, instead of nucleoside analogues, might be a solution to circumvent the initial phosphorylation that is generally the rate-liming step.[93] Indeed, the NKs proved to be much more substrate selective than NMKs and NDKs, showing preferential phosphorylation of natural nucleosides.[94] Exceptions are AZT and clofarabine, whose limiting step is the conversion into their diphosphates.[95,96] However, the direct administration of the nucleoside analogue monophosphate (NAMP) brings to light another crucial problem: under physiological pH, the charged nature of the monophosphate impedes cell membrane passive diffusion. This issue might be easily overcome with an appropriate prodrug design that is able to mask the phosphate negative charges with lipophilic moieties.[97]

1.2 Drug design and Prodrug approach

The goal of every drug discovery project is the design of molecules that will have a very specific biological target thereby maximising the therapeutic effects and minimising the toxicity to the organism. To develop novel drugs which satisfy these fundamental requirements, three approaches are commonly applied: 1) molecules from a wide variety of sources can be screened for general pharmacological effect; 2) compounds can be designed to mimic natural substances which exert some therapeutic effect; 3) known drugs can be chemically modified to improve their properties or minimise unwanted effects. According to the third approach, either analogues or prodrugs of a lead compound can be prepared. When novel analogues are synthesised, the lead compound is chemically irreversibly modified leading to a different molecule. Contrarily, the application of a prodrug approach results in the parent drug molecule release *in vivo*.

1.2.1 Prodrug concept

According to the first definition introduced in 1958 by Adrien Albert, a prodrug is a "*therapeutic agent which is inactive per se but is transformed into one or more active metabolites*".[98] The first molecule recognised as a prodrug was acetanilide, used as an antipyretic agent.[99] After administration, acetanilide is hydroxylated to the pharmacologically active antipyretic acetaminophen (paracetamol) (**Scheme 1.1**).



Scheme 1.1 The prodrug acetanilide is metabolized to the pharmacologically active acetaminophen.

There may be barriers that impede the active molecule from reaching the target (**Figure 1.7**). These barriers in clinical drug applications can be of different natures: pharmaceutical (solubility, chemical stability, patient acceptance, drug formulation) or pharmacokinetic (oral absorption, site specificity, toxicity, duration of activity). The prodrug approach is a well-developed strategy to overcome some of the above problems by alteration of the drug's physico-chemical properties.[100,101]

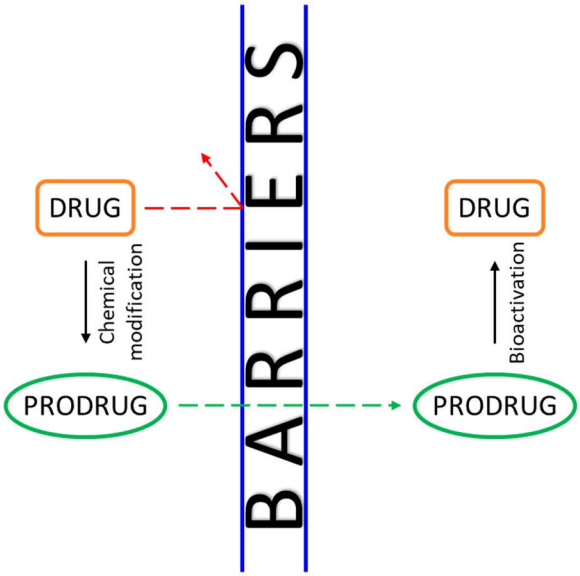


Figure 1.7 Representative picture of the prodrug concept.

The ideal prodrug should require adequate solubility and permeability, good enzymatic and chemical stability, high volume of distribution, low toxicity and efficient activation in the targeted cell.[102] Most of the prodrugs are simple chemically modified versions of the parent drug which require few chemical or enzymatic transformations to provide the therapeutic activity. Certainly, only a deep understanding of the biological pathways the drug is subjected to, allows the prodrug to be safe and efficient.[97] Moreover, a crucial factor to be evaluated in the prodrug design is the presence of functional groups

suitable for chemical derivatisations (carboxylic, hydroxyl, amine, phosphate/phosphonate, carbonyl groups to be converted into esters, carbonates, carbamates, amides, phosphates and oximes).

Prodrugs can be usefully classified in four major classes according to chemical criteria:

- *Carrier-linked prodrugs*. The pharmacologically active drug is temporary linked to a *promoiety*, a carrier group that can be enzymatically removed (reduction, hydrolysis, oxidation) releasing the active species.[99] The ideal promoiety should be nontoxic and rapidly removed from the body (**Figure 1.8**).[97]

  The prodrug may also consist of two active drugs coupled together (*co-drugs*). In this case, each molecule acts as a promoiety for the other.[103]

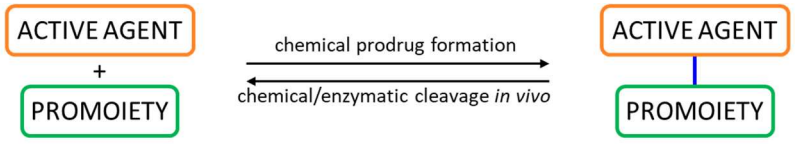


Figure 1.8 Representative picture of a carrier-linked prodrug.

- *Bioprecursor prodrugs* result from a chemical modification of the parent drug and do not contain a promoiety. The prodrug is converted metabolically or chemically (oxidation, hydration, reduction) into the active agent (**Figure 1.9**).[104]

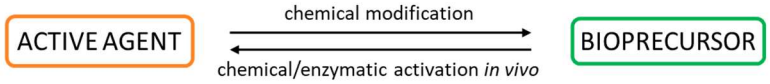


Figure 1.9 Representative picture of a bioprecursor prodrug.

- *Macromolecular prodrugs.* The drug is coupled with a macromolecule such as polyethylene glycol (PEG) aimed to improve the solubility and increase plasma half-life of the molecule leading to a sustained drug release (**Figure 1.10**).[105]

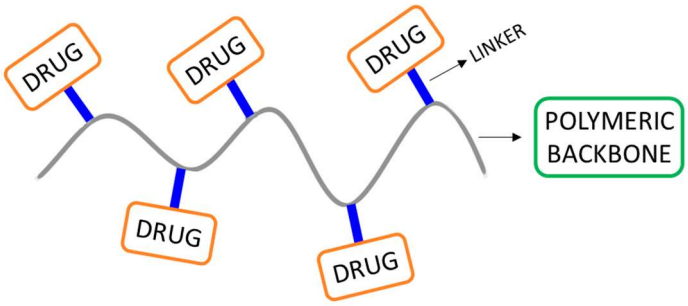


Figure 1.10 Representative picture of a macromolecular prodrug.

- *Drug-antibody conjugates*. The prodrug consists of an active agent covalently linked to a monoclonal antibody in order to increase target selectivity (**Figure 1.11**).[106]

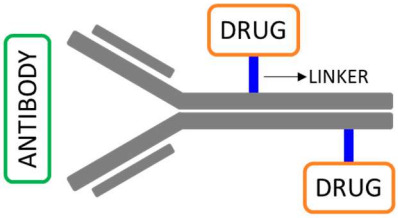


Figure 1.11 Representative picture of a drug-antibody conjugate prodrug.

These diversified approaches allow the prodrug strategy to be successfully applied to a wide range of drug molecules, bringing into clinical practise a number of compounds with better efficacies and safer profiles than the original parent drugs.

Concerning the NA-based drugs, the direct administration of the monophosphorylated agents was found to be a successful solution to bypass the first phosphorylation step. Unfortunately, the direct use of NAMP showed numerous disadvantages which could be overcome by the application of the carrier-linked prodrug approach. The problems related to the NAMP administration and the advantages brought by the application of the prodrug approach will be largely reviewed in the next paragraphs.

1.2.2 Pronucleotide monophosphate strategy

As previously explained in paragraph 1.1.7, the direct delivery of nucleoside monophosphates instead of nucleoside analogues might be a solution to circumvent the initial phosphorylation that is generally the rate-limiting stage. However, under

physiological pH, the monophosphate's charged nature impedes cell membrane passive diffusion. To overcome this issue, numerous prodrug approaches were developed by masking the phosphate negative charges with biolabile lipophilic moieties.[93] These NAMP prodrugs also known as pronucleotides are designed to successfully cross biological barriers to reach the targeted cells. In the specific case of a nucleotide prodrug under physiological conditions, two negatively charged phosphate oxygens need to be masked to achieve a neutral lipophilic phosphate ester that is able to cross the cell membrane (**Figure 1.12**). Accordingly, two chemical entities are necessary to increase the lipophilicity. The chemical masks can be the same moieties (symmetric compounds) or different moieties (asymmetric compounds).[20] In the last case, a chiral centre is generated at the phosphorus atom. Once inside the cell, the lipophilic groups are hydrolysed by enzymatic or chemical processes liberating the 5'-NAMP. At this stage, the NAMP is trapped inside the cell because of its polarity and it can be phosphorylated until achievement of the active form.

The pronucleotide concept was introduced for the first time in 1982 for the anticancer cytosine arabinoside 5'-monophosphate (Ara-CMP) which was evaluated as an alkyl phosphate ester.[107] Since then, many structural motifs have been investigated to identify the appropriate nature of the masking groups.[93,108–112]

Some of the most established monophosphate prodrug strategies will be described in the following sections. Particular attention will be paid to the nature of the masking moieties, their successful application to nucleotides and also the disadvantages experienced.

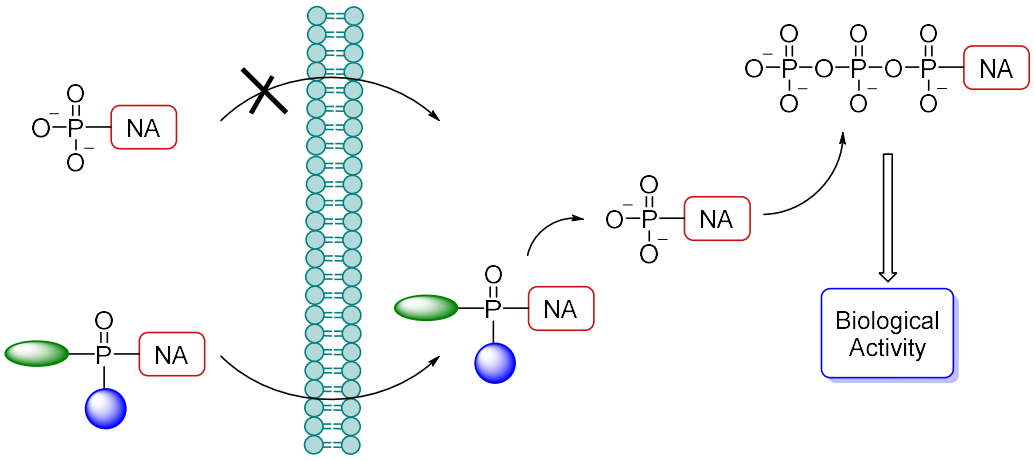


Figure 1.12 The pronucleotide strategy

1.2.2.1 Bis(carbonyloxymethyl) nucleotide prodrugs (POM and POC)

The bis(carbonyloxymethyl) masking group (**Figure 1.13**) was introduced as a nucleotide prodrug promoiety in 1983 by Farquhar and co-workers.[113]

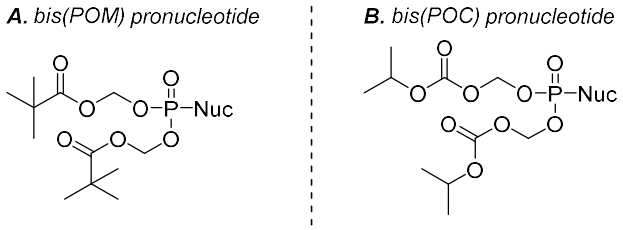


Figure 1.13 Generic structure of bis(POM) (**A**) and bis(POC) (**B**) pronucleotides.

This approach was applied to several nucleosides: FUdR,[114] 2',3'-dideoxyuridine (ddU),[115] thymidine,[116] AZT[117] and the acyclic nucleoside phosphonates adefovir[118] and tenofovir.[119] The anti-HBV adefovir dipivoxil [bis(pivaloyloxymethyl), POM (**3a**)][120] and the anti-HIV tenofovir disoproxil fumarate [bis(isopropyloxymethyl carbonate), POC, (**3b**)][121] are the only bis(carbonyloxymethyl) prodrugs approved by FDA (**Figure 1.14**). More recently, the bis(POM) prodrug of besifovir (LB80380, **3c**) completed the phase II clinical trial for the treatment of HBV.[122]

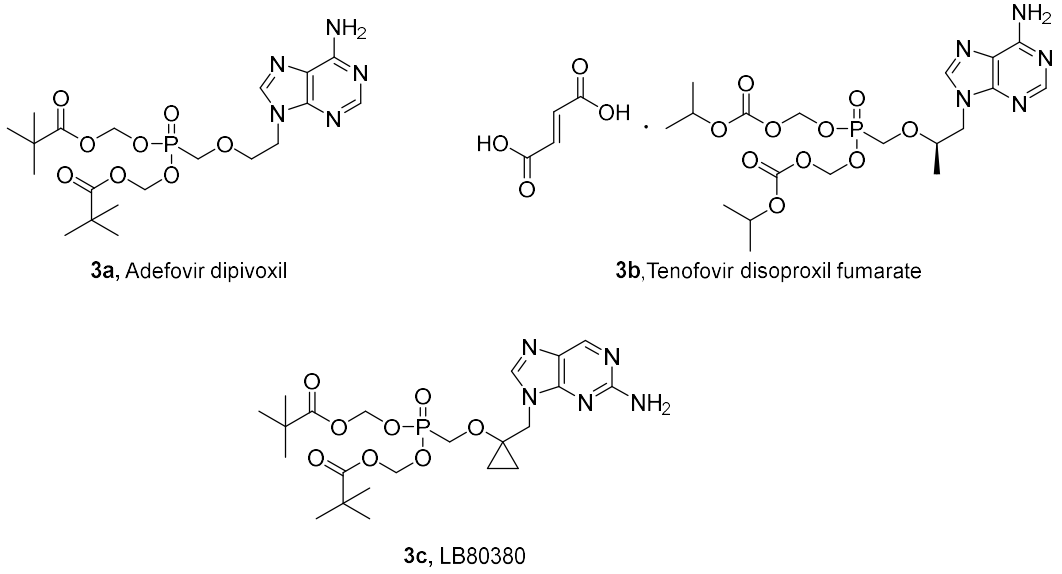
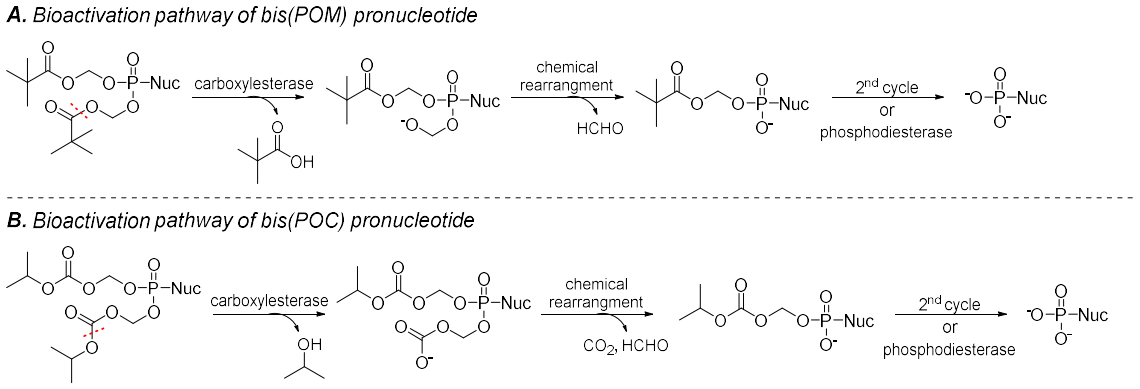


Figure 1.14 Structures of POM and POC nucleoside prodrugs.

The bis(POM)-prodrug bioactivation (**Scheme 1.2A**) involves firstly an enzymatic cleavage by carboxyesterase to give a molecule of pivalic acid and the unstable hydroxymethyl intermediate which spontaneously undergoes chemical degradation

releasing the mono-ester and formaldehyde. The final nucleoside monophosphate is freed by the repetition of these steps or direct cleavage carried out by a phosphodiesterase enzyme. In summary the delivery of a molecule of bis(POM)-prodrug in the body results in the liberation of two equivalents of formaldehyde and pivalinic acid. Because of the potential toxicity of these two metabolites, the bis(POC) technology was applied as a modification of the bis(POM) approach using a carbonate diester in the masking moiety. In the case of POC-prodrugs, the bioactivation (**Scheme 1.2B**) is similarly catalysed by a carboxylesterase enzyme leading to the monophosphate along with isopropanol, carbon dioxide and formaldehyde without pivalic acid release. Despite that, the POC-prodrug as well as the POM-prodrug, displayed susceptibility to serum-mediated esterase compromising the intake of the desired nucleoside inside the cell.



Scheme 1.2 Activation Mechanism of bis(POM) (**A**) and bis(POC) (**B**) pronucleotides.

1.2.2.2 S-Acyl-2-thioethyl (SATE) and S-[(2-Hydroxyethyl)sulfidyl]-2-thioethyl (DTE) nucleotide prodrugs

Gosselin and Imbach introduced in 1993 the nucleotide monophosphates bearing thioethyl chains.[123,124] The thiol can be masked as a thioester [S-Acyl-2-thioethyl (SATE)] (**Figure 1.15A**) or as dithioethanol [S-((2-hydroxyethyl)sulfidyl)-2-thioethyl (DTE)] (**Figure 1.15B**).

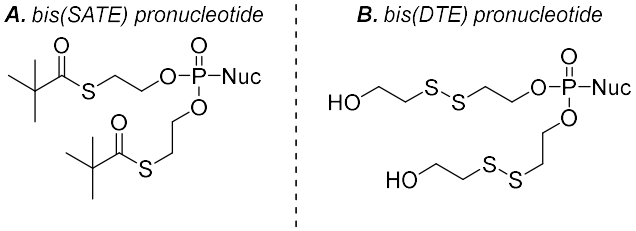


Figure 1.15 Generic structure of bis(SATE) (**A**) and bis(DTE) (**B**) pronucleotides.

Both the approaches have been applied to ddU,[124] AZT,[125] d4T[126] and ACV.[127] All of them proved to successfully deliver the parent nucleoside monophosphate inside the cell improving the antiviral activity. More recently, the bis(SATE) approach applied to Ara-C led to compound UA911 (**4a**, **Figure 1.16**) which is in preclinical evaluation for its anticancer activity on DCK-deficient cells.[128]

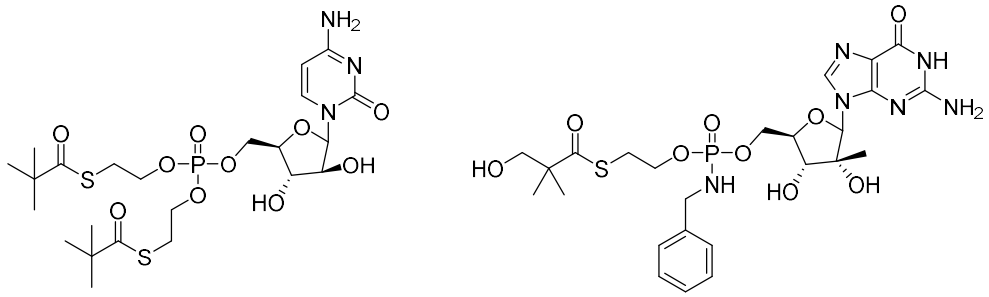
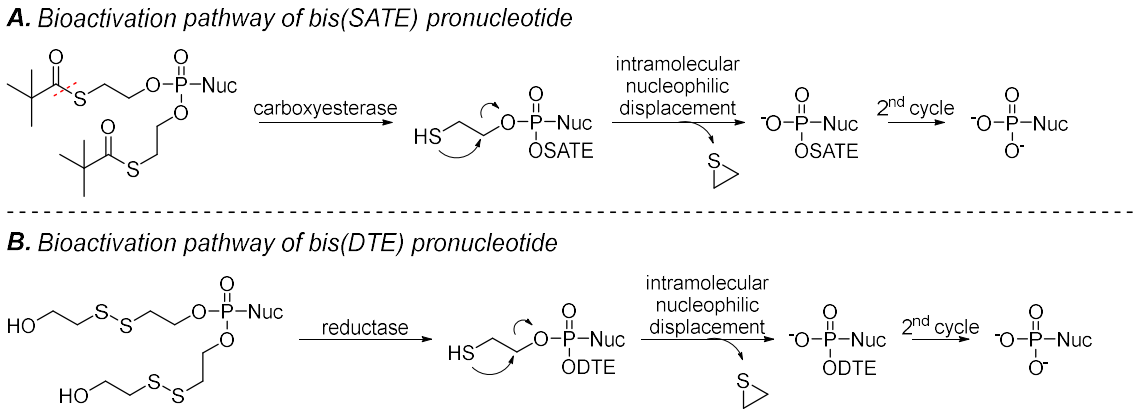


Figure 1.16 Structures of SATE nucleoside prodrugs

Bis(SATE) and bis(DTE) bioactivation (**Scheme 1.3**, **Figure 1.15**) is mediated by a carboxyesterase and a reductase enzyme respectively, generating the common unstable *O*-2-mercaptoethylphosphodiester intermediate. This latter intermediate is converted into a phosphomonoester with elimination of ethylene sulphide via a spontaneously intramolecular nucleophilic displacement. The same pathway allows the removal of the second SATE or DTE chain to release the nucleoside monophosphate. The poor clinical development of bis(thioethyl) nucleotide prodrugs has been correlated mainly with the ethylene sulphide liberated during the prodrug decomposition. Indeed, this by-product is a potent alkylating agent with high toxicity risk. For this reason, modified bis(SATE) (Mixed-SATE) have been evaluated.[109] The replacement of one of the two SATE chains with an aryl group (Aryl-SATE)[129] or an amine (aromatic, aliphatic or amino acid ester) (SATE-Phosphoroamidate diester)[130] turned out to be a successful strategy as well. Indeed, the application of a SATE-phosphorobenzylamine prodrug to a 2'-methylguanosine (2'-MeG) led to IDX184 (**4b**, **Figure 1.16**). This agent is the only example of a SATE-prodrug that reached clinical evaluation.[131] Unfortunately, it was halted in phase II for the treatment of HCV infections when three cases of severe liver damage occurred.[132]



Scheme 1.3 Activation Mechanism of bis(SATE) (**A**) and bis(DTE) (**B**) pronucleotides.

1.2.2.3 Cyclosaligenyl nucleotide prodrugs (cycloSal)

In the cyclosaligenyl phosphodiester approach (**Figure 1.17**), a salicylic alcohol is linked to the phosphate group. This prodrug strategy has been developed by Meier and co-workers[133] in 1997 and applied to numerous antiviral NAs:[134] BVDU,[135] d4T,[136] AZT,[137] ACV[138] and other acyclic nucleoside phosphonate derivatives.[139]

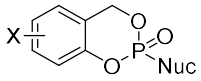
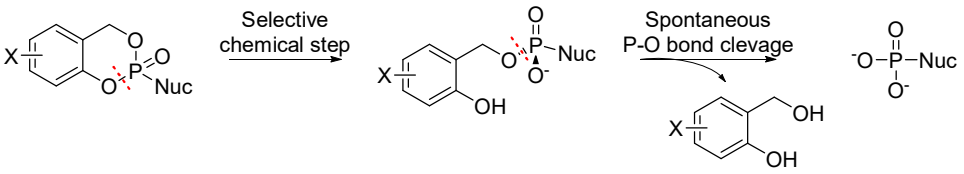


Figure 1.17 Generic structure of a cyclosaligenyl pronucleotide.

The first stage in the prodrug activation (**Scheme 1.4**) is a highly selective pH-dependent chemical hydrolysis of the phenyl ester bond, which is more labile than the benzyl ester because the charge can be delocalised on the aromatic ring.[108] The newly formed *ortho*-OH, a strong electron-donating group, induce the spontaneous benzyl phosphate ester rupture and the release of the nucleoside monophosphate.[140] Unfortunately, the chemical degradation is much more difficult to control and presents some limitations when compared with the enzymatic mechanisms.[141] Specifically, the chemically bioactivation is not fast enough to avoid the efflux of the lipophilic cycloSal prodrug through the membrane. Additionally, the masking group has a low hydrolytic stability and can be cleaved before reaching the target cells.



Scheme 1.4 Activation Mechanism of a cyclosaligenyl pronucleotide.

To bypass these issues, second and third cycloSal generations have been designed to block the prodrug inside the cell. These agents are referred to as "lock-in" cycloSal diesters and are characterised by an esterase cleavable group placed on the phenyl ring. Once the prodrug crosses the membrane, the cycloSal phosphate triester is trapped inside the cell due to a rapid enzymatic activation which impedes the back-diffusion.[141] Consequently, the desired nucleoside monophosphate can be intracellularly released by the designed, chemically induced pathway.

The esterase-cleavable groups of the second-generation class are mainly acyloxy systems and amino acid ester chains.[142] The enzymatic reaction is exploited to convert the carboxylic esters in acid residues which are then deprotonated at the physiological pH. Due to the negative charge, the compound is too polar to cross the membrane, then it is locked into the cell so that the chemical hydrolysis can take place. Notably, the ester residues are not directly installed on the aromatic ring. Indeed, their electron-withdrawing effect can induce a reduction of the molecule's chemical stability, thus the phosphate hydrolysis risks being triggered before the prodrug reach the target cells. To avoid that, an alkyl chain is used as a spacer between the ester function and the aromatic ring.[143]

The cyclosal third-generation exhibits geminal dicarboxylate[144] or acetoxyvinyl[145] groups. These functions are lipophilic carboxylesterase-sensitive residues with weak electron-withdrawing properties. Once inside the cell, they are enzymatically hydrolysed into polar acceptor moieties (ketones or aldehydes). As mentioned above, the presence of strong acceptor groups directly attached to the aromatic ring speeds up the elimination of the masking group by inducing a very fast phosphate hydrolysis. Then, the phosphodiester charged intermediate is rapidly generated, impeding the back diffusion of the compound. Finally, the desired nucleoside monophosphate is delivered intracellularly.[144]

These prodrug systems were mainly installed on the anti-HIV d4T nucleoside monophosphate (**Figure 1.18**). Although this prodrug strategy was also applied to the anticancer agent cladribine improving its cytotoxic activity in dCK deficient cells,[146] to date none of the cycloSal pronucleotides have reached clinical evaluation.

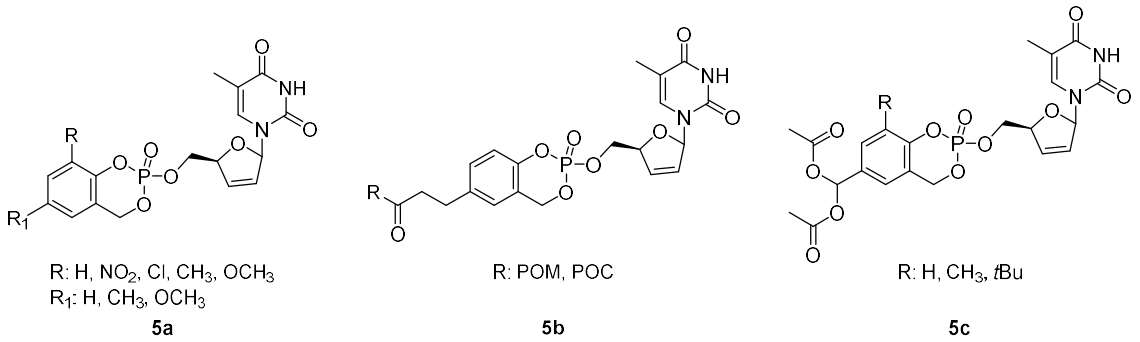


Figure 1.18 First (**5a**), second (**5b**) and third (**5c**) generation d4T cycloSal prodrugs.

1.2.2.4 HepDirect nucleotide prodrugs

This methodology was developed by Metabasis Therapeutics[147] in 2004 and consists of the introduction of X-aryl substituted cyclic 1,3-propanyl esters on the nucleotide (**Figure 1.19**).

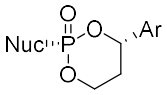
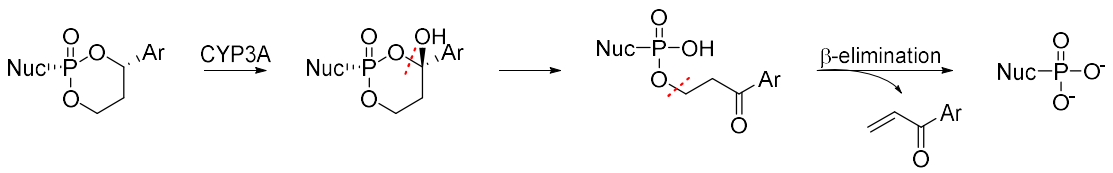


Figure 1.19 Generic structure of a HepDirect pronucleotide.

HepDirect prodrugs aim to selectively deliver the drug into the liver to treat infections that affects this organ without interfering with the others.[148] Specifically, the prodrug bioactivation is catalysed by cytochrome P_{450} (CYP450), an enzyme mainly expressed in the liver (**Scheme 1.5**). The masking group is oxidised to give an unstable cyclic hemiketal intermediate, which spontaneously undergoes ring opening, followed by a β-elimination to free the nucleotide. The vinyl ketone by-product is detoxified by glutathione *S*-transferase.[148]



Scheme 1.5 Activation mechanism of a HepDirect pronucleotide.

This prodrug strategy proved to be successful, with the HepDirect prodrugs of cytarabine (MB07133, **6a**)[149] and adefovir (pradefovir, **6b**)[150] in clinical trials (**Figure**

1.20). Both completed phase 2 for hepatocellular carcinoma and chronic hepatitis B respectively.

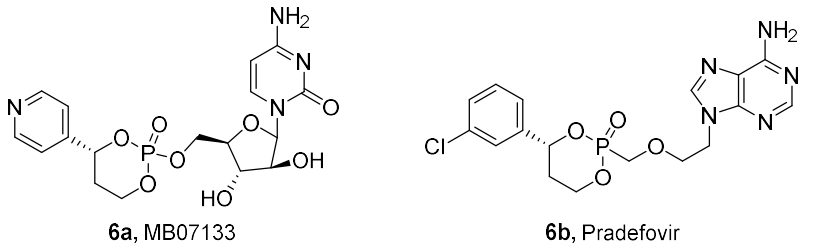


Figure 1.20 Structures of HepDirect nucleoside prodrugs.

1.2.2.5 Alkoxyalkyl monoester nucleotide prodrugs

This approach consists of a lipid phospho-conjugate linked to the NA. One of the two free -OH groups of the phosphate is masked by an hexadecyloxypropyl (HDP) or octadecyloxyethyl (ODE) group (**Figure 1.21**).

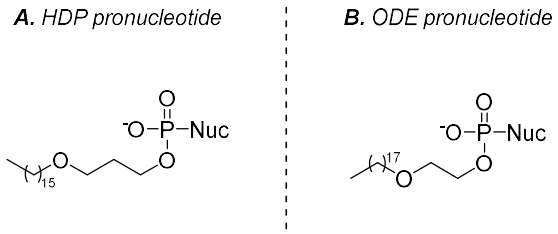
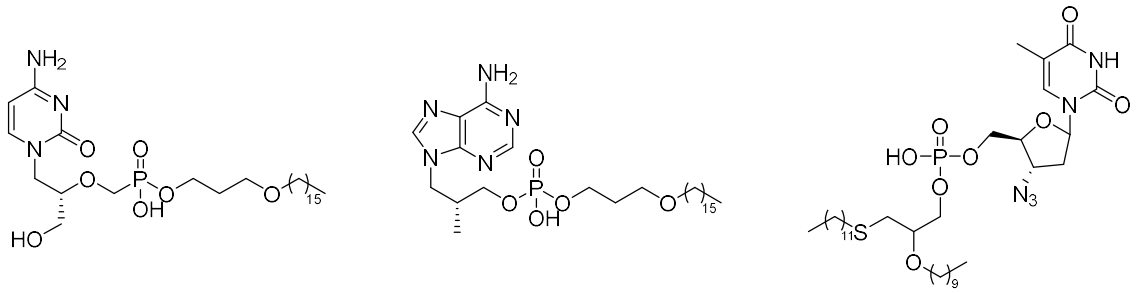


Figure 1.21 Generic structure of HDP (**A**) and ODE (**B**) pronucleotides.

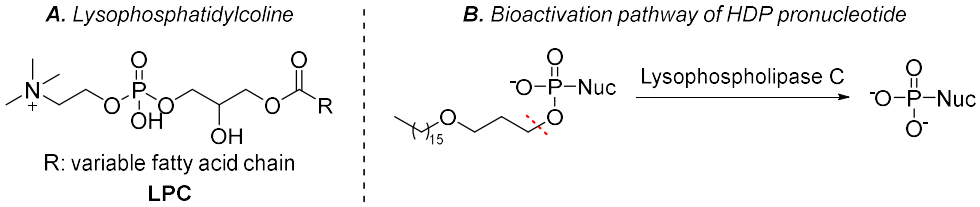
This strategy led Hostetler and co-workers to the discovery of numerous drugs currently in clinical trials (**Figure 1.22**).[151] Specifically, the HDP prodrug approach was successfully applied to the acyclic nucleoside analogues cidofovir and tenofovir yielding brincidofovir[152] (**7a**) and CMX157[153] (**7b**) respectively. The latter is an anti-HIV agent in phase 2, while brincidofovir is under clinical evaluation for CMV, Ebola and other viral infections. Due to their lipophilic nature and stability, these prodrugs showed high oral availability.[152]



7a, Brincidofovir **7b**, CMX-157 **7c**, Fozivudine tidoxil

Figure 1.22 Structures of alkoxyalkyl nucleoside prodrugs.

Their activation mechanism is based on the resemblance of the alkoxyalkyl monoester nucleotide prodrug to lysophosphatidylcholine (LPC) (**Scheme 1.6**). Therefore, the prodrug is recognised as a natural phospholipid and can easily reach the targeted cell following the LPC uptake pathway. Once inside the cell, phospholipase C cleaves the lipid mask and releases the nucleotide.



Scheme 1.6 Generic structure for LPC (**A**) and activation mechanism of a HDP pronucleotide (**B**).

A modified alkoxyalkyl ester prodrug with the phosphate masked by a thioether lipid carrier was installed on AZT yielding fozivudine tidoxil (**7c**, **Figure 1.22**). This AZT prodrug is an active anti-HIV agent in phase 2 trials.[154]

1.2.2.6 Aryloxy phosphoroamidate nucleoside prodrugs (ProTides)

The research project discussed here is mainly focused on the application of the phosphoroamidate prodrug strategy. For this reason, a more detailed description of the ProTide approach is necessary to lay the groundwork for the next chapters.

The aryloxy phosphoroamidate prodrug features an amino acid alkyl ester chain (phosphoroamidate motif) and an aryloxy group as masking moieties for the two negative charges of a monophosphate nucleoside (**Figure 1.23**). The ProTides are synthesised as diastereoisomeric mixtures.

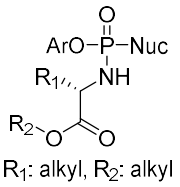
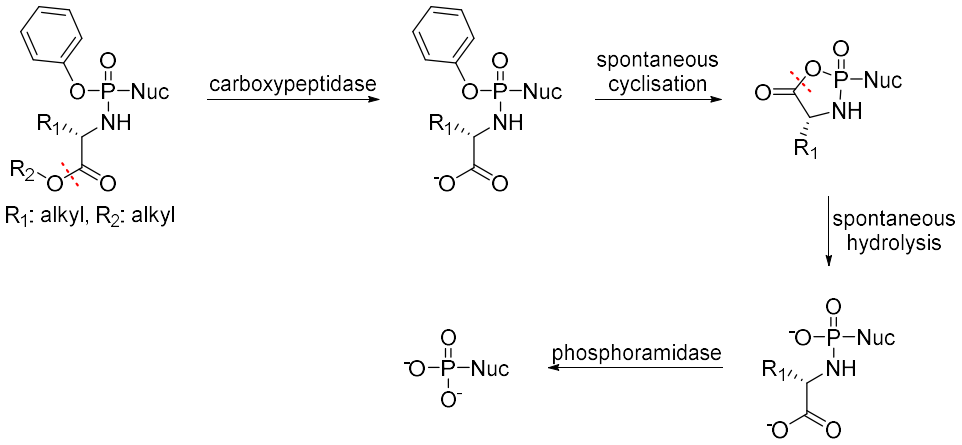


Figure 1.23 Generic structure of an aryloxy phosphoroamidate pronucleotide.

This prodrug was pioneered by Chris McGuigan *et al*. and reported for the first time in 1992 on AZT. [155,156] The strategy was the result of years of investigations into nucleotide prodrug approaches. First, McGuigan and co-workers developed a prodrug bearing bis(alkyloxy) and haloalkyloxy moieties on the AZT monophosphate.[157] Inside the cell, the bioactivation of these molecules hardly occurs because of their high stabilities. The replacement of one of the alkyl moieties with an amino acid ester chain led to the release of the nucleoside inside the cell, instead of the desired nucleotide.[158] The same outcome was detected in the presence of two aryl moieties on the phosphate.[159] Finally, when the amino acid ester and the aryloxy group were combined in the prodrug structure, an enhanced antiviral activity of the AZT ProTide was observed when compared with the parent nucleoside.[156] This prodrug motif demonstrated the ability to overcome the key resistance phenomena associated with NAs displaying activity also in nucleoside kinase and nucleoside transporter deficient cells.[160,161]

The ProTide bioactivation has been subjected to in depth studies over the years, but it is not yet fully confirmed by experimental data, and for this reason it is referred to as putative mechanism (**Scheme 1.7**).[162,163] It is initiated by the carboxypeptidase A, also called cathepsin A (catA).[164] This esterase cleaves the ester of the amino acid generating a carboxylate which spontaneously cyclises into a putative five membered ring.[165] As consequence of the intramolecular cyclisation, the aryl group is displaced. The unstable cyclic intermediate hydrolyses, either spontaneously, or by water attack on the phosphorus atom, leading to the ring opening.[166] Finally, either a phosphoramidase enzyme[167] or the histidine triad of the nucleotide-binding protein 1 (HINT-1)[168] cleaves the P-N bond and release the nucleoside monophosphate.



Scheme 1.7 Proposed activation mechanism of an aryloxy phosphoroamidate pronucleotide.

Over the years, an enzymatic experiment was modelled and optimised in McGuigan's laboratory to predict the activity of the prodrug based on its susceptibility toward cathepsin A (catA).[169] ProTides with numerous amino acid esters can be exposed to carboxypeptidase Y (CPY) which can be used as a surrogate of catA, since they are both C-type carboxypeptidases sharing similarities in the active site.[170] The reaction progress can be followed by ^{31}P NMR experiments as the example reported in **Figure 1.24**: the two diastereomeric signals of the starting ProTide disappear to give a downfield singlet which corresponds to the amino acyl phosphate monoester.

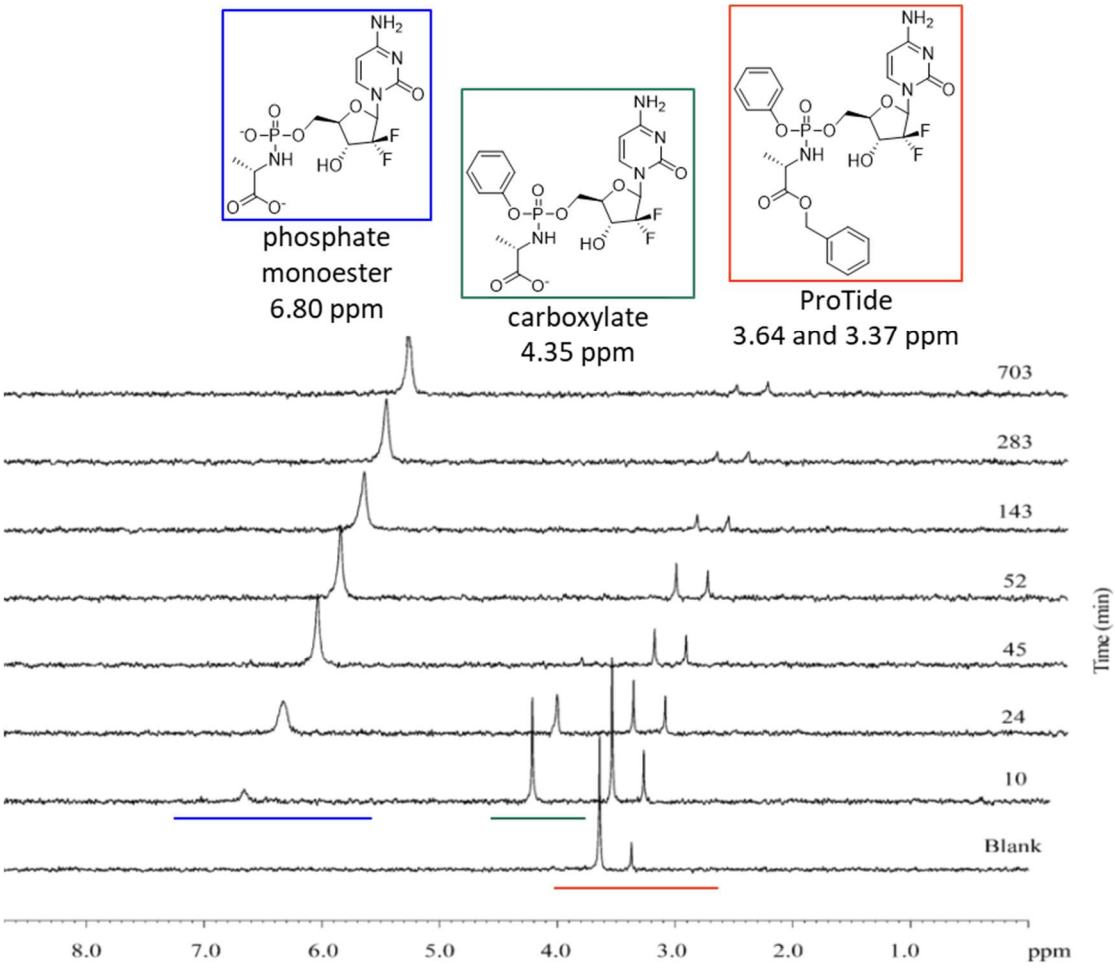


Figure 1.24 ^{31}P-NMR study of gemcitabine ProTide over time of carboxypeptidase Y digestion.[160] Starting from the right: the signals of the two diastereoisomers (in red), the carboxylate intermediate (in green), the final monoester metabolite (in blue).

Given the importance of the ProTide as a monophosphate prodrug approach, extensive SAR (structure-activity relationship) studies were conducted on the modifiable moieties (**Figure 1.25**):

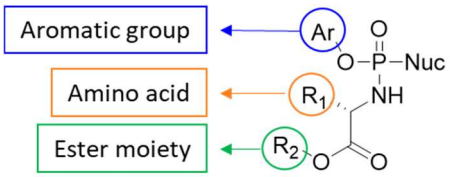


Figure 1.25 General structure of the modifiable residues of a Pronucleotide.

- Amino acid (R_1). The presence of the α-amino acid ester is essential for the biological performance of the prodrug. Indeed, both β-amino acid ester and simple alkyl amine chains exhibit total loss of activity.[171] Evaluations among the natural

α-amino acids proved that generally *L*-alanine is endowed with the greatest bioactivity, while *D*-alanine was found to be 30-fold less active than its laevorotary isomer.[172] Moreover, *L*-alanine esters were found to be hydrolysed faster than heavy branched amino acid such as leucine, valine and isoleucine bearing the same ester residues.[172–174] So far, all ProTides in clinical evaluation bear L-alanine as the amino acid moiety. However, recently publications showed that also phosphoroamidate prodrugs bearing aspartic acid esters exert good anticancer and antiviral activities.[175,176]

- Ester (R_2). A range of *L*-Ala amino acid esters was evaluated for the anti-HIV activity of phenoxy phosphoroamidate derivatives of d4T. It has been demonstrated that different esters can show different sensitivities to the carboxypeptidase enzyme, thus influencing the antiviral effect of the molecule. Specifically, a variety of linear, branched and aryl esters was examined. The tertiary alkyl esters, such as the *tert*-butyl chain, emerged to be poor esterase substrates, while aryl derivatives such as the benzyl group showed high potencies against HIV compared to primary and secondary alkyl analogues.[173,177,178]
- Aryl (Ar). This moiety plays a key role as the leaving group in the ProTide activation. Several substitutions on the phenol functionality have been investigated. The presence of electron-withdrawing groups on the aromatic ring, such as *p*-Br or *p*-$COOCH_3$ made them good leaving groups and the corresponding ProTide showed good potency.[179] Moreover, the replacement of the phenyl group with a naphthyl aryl derivative yielded interesting results when applied on BVDU[180] and FUdR,[181] whose ProTides reached clinical evaluation as anticancer agents.

To date, two ProTides Sofosbuvir (Sovaldi®, **8a**)[182] and Tenofovir alafenamide fumarate (TAF, Vemlidy®, **8b**)[183,184] are FDA approved drugs developed by Gilead Sciences (**Figure 1.26**). The former is a first line drug for the treatment of HCV since its approval in 2013. TAF is an acyclic nucleoside ProTide approved in 2015 against HIV infections and later in 2016 as an anti-HBV agent.

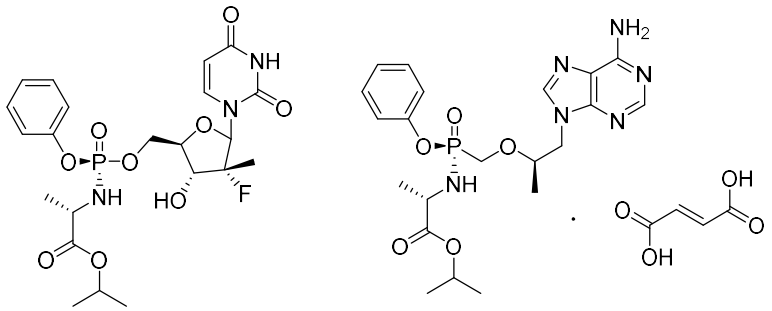


Figure 1.26 Structures of nucleoside ProTides that reached the market.

Remarkably, several antiviral and anticancer ProTides are currently under clinical evaluation (**Figure 1.27**). Among the antiviral ProTides, GS-9131[185] (**8c**) and stampidine[186] (**8d**) are respectively in phase 2 and 1 as anti-HIV agents. Against HCV infections, GS-6620[187] (**8e**) completed phase 1 trials and IDX-21437[188] (**8f**) is in phase 2, while the clinical progression of PSI-353661 (**8g**) and INX-08189 (**8h**) was halted due to their potential cardiotoxicity.[132] Finally, the C-nucleoside ProTide GS-5734 (**8i**) reached phase 1 for the treatment of Ebola virus.[189]

In recent years, ProTides are rapidly proving to be an important source of anticancer drugs. Among the anticancer ProTides, three out of four agents were developed by Nucana Biomed and are currently under clinical investigations for the treatment of advanced solid tumours: NUC-3373 (**8k**) and NUC-7738 (**8l**) are in phase 1 while NUC-1031 (**8j**) (*see infra*) is concluding phase 3 trials and it is approaching the market.[190] Thymectacin (**8m**) is in phase 2 for the treatment of colon cancer.[191]

Concerning NUC-1031 (Acelarin®), assay results showed that NUC-1031 is able to bypass the NK-mediated activation and carrier-dependent uptake to generate high intracellular concentrations of the 5'-triphosphosphorylated compound. Several *in vitro* and *in vivo* assays have strongly suggested that this ProTide expresses its anticancer activity with superior efficacy and better tolerability compared to the parent nucleoside gemcitabine (**1k**).[160] It yielded noteworthy reductions in tumour volumes in pancreatic cancer xenografts resistant to systemic gemcitabine treatment.[192] These encouraging efficacy signals clearly support the ProTide approach to develop promising new anticancer agents.[193,194]

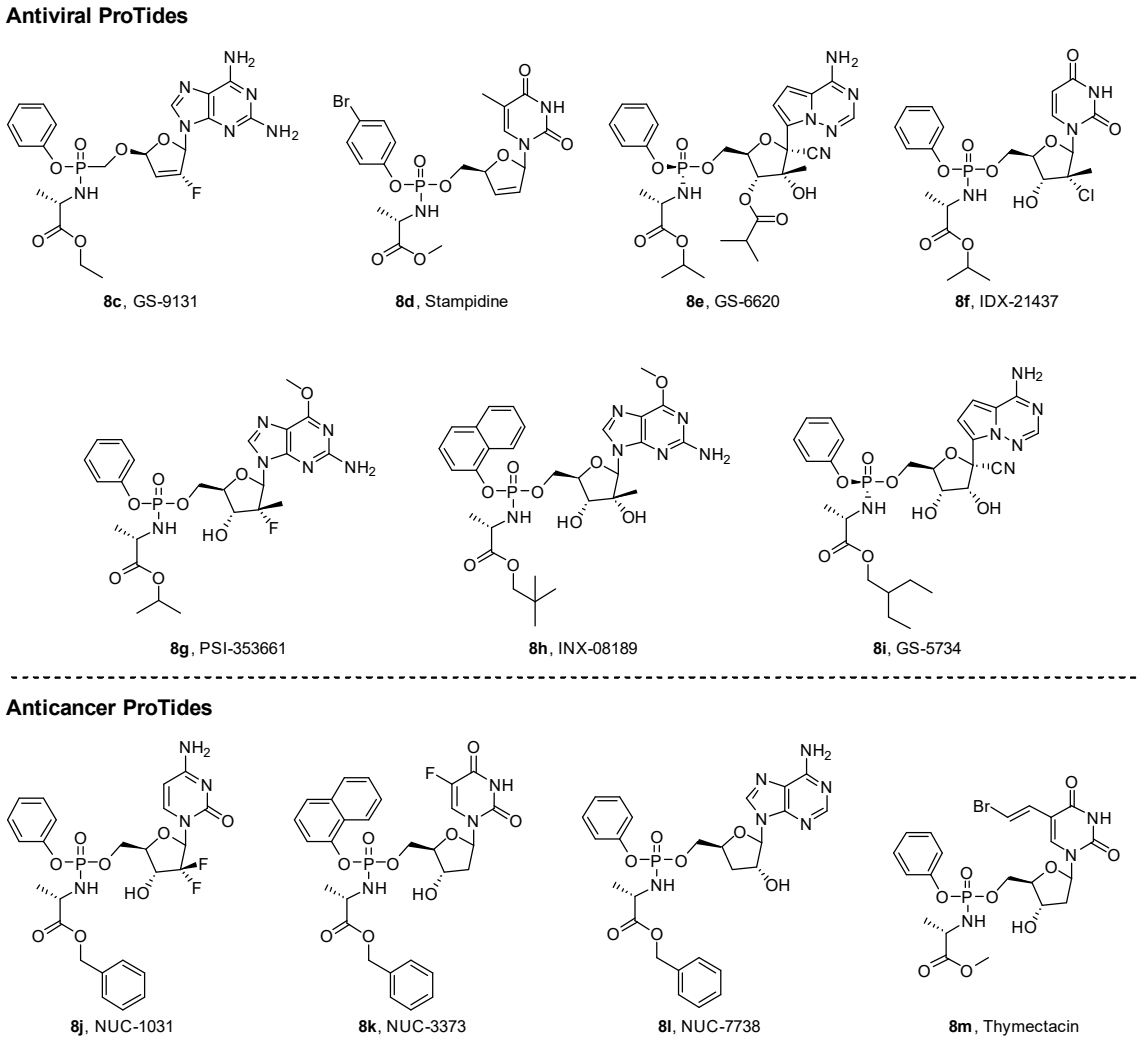


Figure 1.27 Structures of nucleoside ProTide that reached clinical evaluation.

1.2.2.7 Cyclic phosphate prodrugs

Gunic and co-workers developed in 2010 the 3',5'-cyclic phosphate ester nucleotide prodrug (**Figure 1.28**).[195]

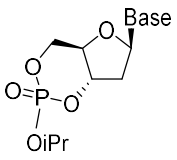
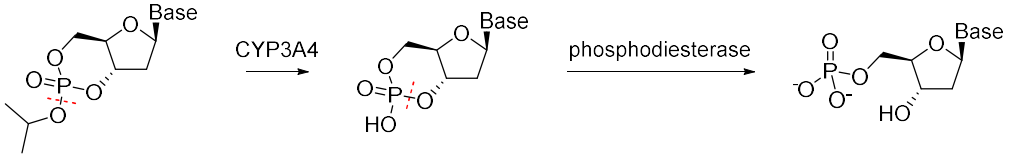


Figure 1.28 Generic structure of a cyclic phosphate pronucleotide.

The bioactivation consists of two consecutive enzymatic steps (**Scheme 1.8**): first the P-O hydrolysis catalysed by CYP3A4, then a phosphodiesterase cleaves the 3'O-P bond.[196]



Scheme 1.8 Activation mechanism of a cyclic phosphate pronucleotide.

This approach was successfully applied by Pharmasset to a modified guanosine derivative leading to the discovery of the anti-HCV PSI-352938 (**9a**).[196] The trial was halted in phase 1 due to hepatic side effects (**Figure 1.29**).[111]

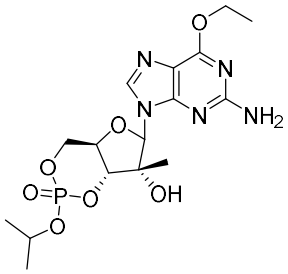


Figure 1.29 Structure of a cyclic phosphate nucleoside prodrug.

1.2.2.8 Phosphoroamidate and phosphorodiamidate nucleoside prodrugs

Both the phosphoroamidate and the phosphorodiamidate nucleoside prodrugs were developed as alternatives to the aryloxy phosphoroamidate approach (**Figure 1.30**).[197]

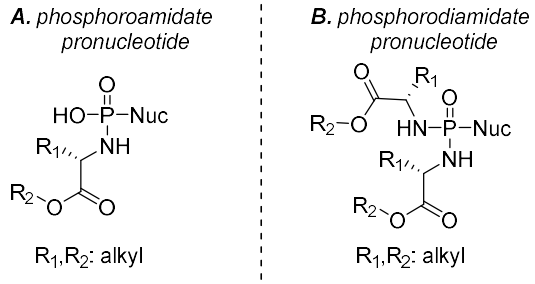
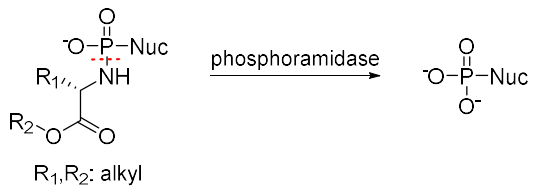


Figure 1.30 Generic structure of phosphoroamidate (**A**) and phosphorodiamidate (**B**) pronucleotides.

Masking one or two phosphate negative charges with amino acids avoids the release of the phenol moiety from the prodrug and abolishes the chirality at the phosphorus atom.

The monoamidate of a nucleotide is a prodrug strategy pioneered by Wagner and co-workers in 1994.[198] The bioactivation of the monoamidate prodrug involves only one step in which a phosphoramidase enzyme cleaves the amino acid moiety without the carboxylesterase intermediate action (**Scheme 1.9**).

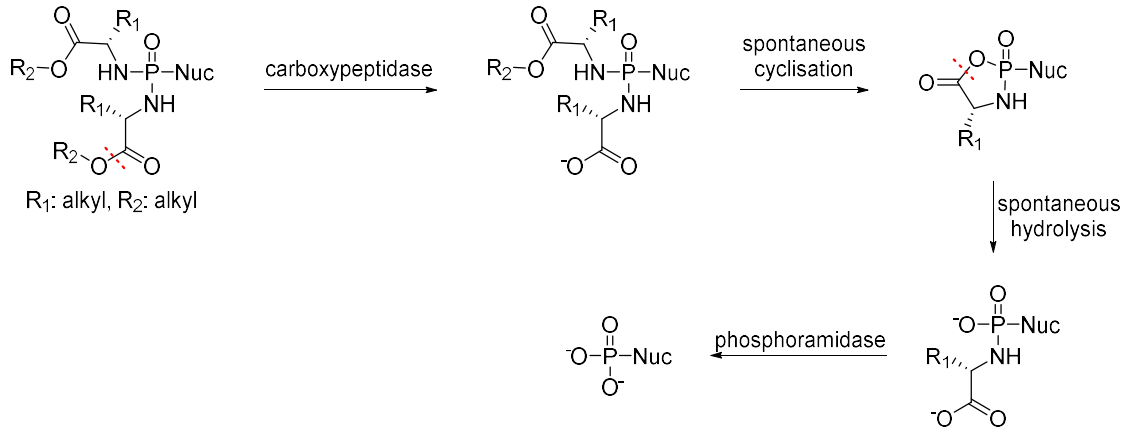


Scheme 1.9 Activation mechanism of a phosphoro(mono)amidate pronucleotide.

In recent years, Herdewijn *et al.* proved that a phosphoro(mono)amidate with a specific amino acid such as *L*-histidine or *L*-aspartic acid was able to mimic the nucleoside triphosphates, showing both electronic and structural properties required to bind the polymerase active site.[199] The monoamidate prodrug strategy was applied to both anticancer (FUdR and ara-C)[200] and antiviral (AZT)[201] nucleoside monophosphates. Although the prodrugs showed better pharmacokinetic profiles than the parent nucleosides, their poor oral bioavailability was a concerning issue.

A solution to this problem might be the phosphorodiamidate strategy developed by McGuigan and co-workers:[202] both the two negative charges of the phosphate are masked by an amino acid ester chain. This approach was originally applied on 3'-fluoro-3'-deoxythymidine (FdT)[203] and AZT.[204]

The proposed biodegradation of this prodrug seems to be closely related to the activation mechanism proposed for the ProTide. It is catalysed by a carboxypeptidase that hydrolases the ester of an amino acid generating a carboxylate ion, which in turn attacks the phosphorus atom cyclising to a five membered ring and releasing one of the amino acids. A spontaneous hydrolysis of the ring is followed by the action of a phosphoramidase that cleaves the second amino acid to free the NA (**Scheme 1.10**).[205]



Scheme 1.10 Activation mechanism of a phosphorodiamidate pronucleotide.

For a long period, the bisamidate prodrug was no longer investigated due to the success of the ProTide strategy. However, recently, our group successfully installed this structural motif on anticancer and antiviral nucleoside analogues (**Figure 1.31**). The phosphorodiamidate of 6-*O*-Me-2'-*C*-methylguanosine (**10a**) showed nanomolar activity against HCV versus the micromolar values observed with the parent nucleoside.[205] Similarly, the phosphorodiamidates of 2',3'-dideoxyadenosine (ddA, **10b**), ABC (**10c**) and ACV (**10d**) showed a great improvement of activity against HIV-1 and -2 compared with their parent nucleosides.[206]

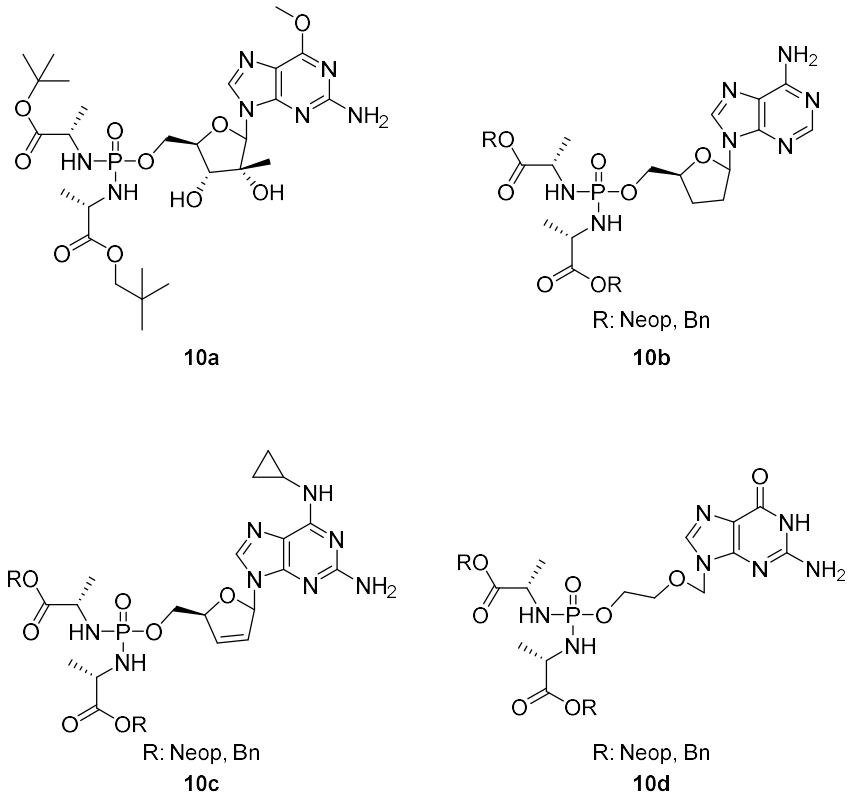


Figure 1.31 Structures of phosphoroamidate prodrugs of 6-O-Me-2'-C-Me-G (**10a**), ddA (**10b**), ABC (**10c**), ACV (**10d**).

1.2.3 Summary of the pronucleotide monophosphate strategies

A summary of all the monophosphate nucleoside prodrugs that reached the clinical evaluation along with their indications and development status is shown below (**Table 1.4**).

Prodrug	Name	Indications	Status	Ref
Bis(POM)	Adefovir Dipivoxil (**3a**)	HBV	FDA approved 2002	120
	LB80380 (**3c**)	HBV	Phase 2	122
Bis(POC)	Tenofovir Disoproxil Fumarate (**3b**)	HIV	FDA approved 2006	121
Bis(SATE)	UA911 (**4a**)	Cancer	Preclinic	128
SATE phosphoroamidate	IDX-184 (**4b**)	HCV	Halted	131
HepDirect	MB-07133 (**6a**)	Liver Cancer	Phase 2	149
	Pradefovir (**6b**)	HBV	Phase 2	150

Alkoxyalkyl ester	Brincidofovir (**7a**)	CMV, AdV, EBV, HHV6, BKV	Phase 3	152
		Ebola Virus	Phase 2	152
	CMX-157 (**7b**)	HIV	Phase 2	153
	Fozivudine Tidoxil (**7c**)	HIV	Phase 2	154
ProTide	Sofosbuvir (**8a**)	HCV	FDA approved 2013	182
	TAF (**8b**)	HIV	FDA approved 2015	183
		HBV	FDA approved 2016	184
	GS-9131 (**8c**)	HIV	Phase 2	185
	Stampidine (**8d**)	HIV	Phase 1	186
	GS-6620 (**8e**)	HCV	Phase 1	187
	IDX-21437 (**8f**)	HCV	Phase 2	188
	PSI-353661 (**8g**)	HCV	Halted	132
	INX-08189 (**8h**)	HCV	Halted	132
	GS-5734 (**8i**)	Ebola Virus	Phase 1	189
	NUC-1031 (**8j**)	Advanced solid tumours	Phase 3	190
	NUC-3373 (**8k**)	Advanced solid tumours	Phase 1	190
	NUC-7738 (**8l**)	Advanced solid tumours	Phase 1	190
	Thymectacin (**8m**)	Colon Cancer	Phase 2	191
Cyclic Phosphate ester	PSI-352938 (**9a**)	HCV	Halted	196

Table 1.4 Pronucleotide analogues in human clinical trials.

1.2.4 Pronucleotide di- and triphosphate strategies

Since the introduction of the pronucleotide concept in 1982,[107] several prodrug strategies were developed to deliver 5'-monophosphate nucleosides inside the cell by-passing the rate limiting step to NAMPs formation, while second and third phosphorylations proved to be efficient for most nucleosides. However, as reported in

paragraph 1.1.7, it is known that the limiting step in the activation of AZT[96] and clofarabine[95] is the second phosphorylation. Moreover, it was recently proved that the diphosphorylated forms of ddU and 2',3'-dideoxy-2'-3'-didehydrouridine (d4U) were minimally converted into the active triphosphates by NDPKs, demonstrating that even the third phosphorylation can be the bottleneck in the stepwise addition of phosphate groups.[207]

As a consequence, recently, the development of di- (Di*P*Pro) and triphosphate pronucleotides (Tri*P*Pro) (**Figure 1.32**) has become a major topic since some nucleosides are poor substrates for cellular NMPKs and NDPKs.

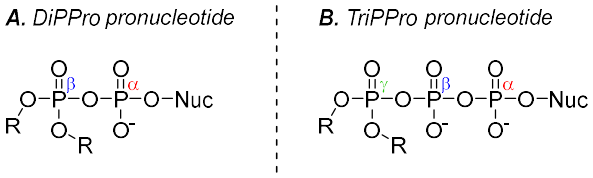
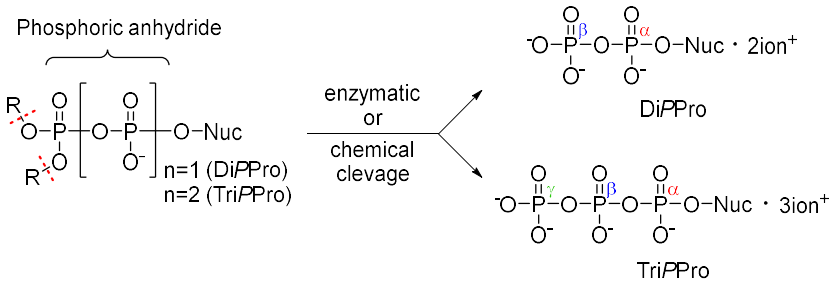


Figure 1.32 Generic structure of diphosphate (**A**) and triphosphate (**B**) pronucleotides.

Besides, di- and triphosphate pronucleotide approaches might display important advantages: the direct administration of the nucleoside bioactive form and the opportunity to by-pass some catabolic enzymatic processes (e.g. deamination).

The preparation of these kinds of prodrugs proved to be more challenging than the corresponding monophosphate compounds. Di*P*Pro and Tri*P*Pro are characterised by the presence of the phosphate anhydride bonds. These bonds are chemically unstable, and it is hard to achieve the cleavage of the phosphate ester bonds selectively. When the negative charges, both terminal and internal, are fully masked (neutralised nucleotide), a rapid hydrolysis of the anhydride bond occurs leading to the release of the monophosphate nucleoside. Contrarily, when only the terminal phosphate is neutralised by the presence of masking groups, the prodrug might be successfully delivered. The reason is that the anhydride bonds are stabilised by the negative charges for two reasons. First, they prevent a nucleophilic attack at the phosphate functions due to electrostatic repulsion, and second they make the phosphate moieties poor leaving groups that are difficult to displace (**Scheme 1.11**).[208,209]



Scheme 1.11 Activation mechanism of Di*P*Pro and Tri*P*Pro pronucleotide.

The literature reports examples of Di*P*Pro and Tri*P*Pro bearing acyl and alkyl glyceride moieties,[210] lipophilic acyl groups,[211] steroid and lipid chains[212] used to neutralise the phosphate terminal negative charges. For instance, the properly masked Di*P*Pro[207] and Tri*P*Pro[210] of AZT retained the antiviral activity of the parent compound by releasing directly the diphosphate and the triphosphate nucleoside, respectively, thus avoiding the intracellular accumulation of the monophosphate derivative which causes most of its side effects.[213]

Currently, the study of the prodrug strategies being applied on Di*P*Pro and Tri*P*Pro are ongoing. Unfortunately, the masking groups that proved to be successful in the monophosphate prodrug strategies, cannot be directly applied to the diphosphorylated and triphosphorylated nucleosides. Recently, the cycloSal prodrug approach used by Meier and co-workers to the diphosphorylated d4T, showed the disruption of phosphorus anhydride bonds leading to the release of the NAMP as the only metabolite.[142] However, the replacement of the cycloSal moiety with a *para*-acyloxybenzyl group (**Figure 1.33**) led to the delivery of the desired bioactive compound successfully.[214,215]

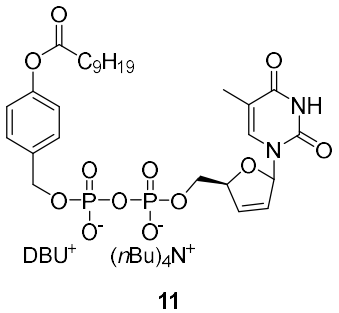


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Figure 1.33 Example of the diphosphate prodrug of d4T bearing a *para*-acyloxybenzyl moiety as the masking group.

1.3 General Aims

The ProTide technology has displayed a great deal of success in the antiviral field with two drugs on the market: Sofosbuvir (Sovaldi®, HCV infections) and tenofovir alafenamide fumarate (TAF, Vemlidy®, HIV and HBV infections). This success is also demonstrated by many other compounds adopting this technology either in clinical trial or preclinical evaluation as antiviral and anticancer agents. Since then, the application of the ProTide technology has grown dramatically and it has started to show very promising results as well as in other therapeutic areas.[216–219] In this context, the research presented herein was mainly focused on addressing the synthetic problems related to unnatural nucleosides and their ProTides. This thesis aimed to explore novel methodologies for the preparation of both phosphoroamidate and phosphonoamidate prodrugs of biologically relevant nucleosides (**Figure 1.34**).

Although there are several procedures to synthesize phosphoroamidates of nucleosides, there is currently not an efficient and inexpensive methodology to prepare ProTides as single diastereoisomers.[220–225] Sofosbuvir and TAF, are marketed as single stereoisomers at the phosphorus centre. Their biological activities have been correlated to the configuration of the phosphorus atom, with the S_P stereoisomer being the most biologically active. Because of the commercial importance of this aspect, part of the research was devoted to developing a catalytic methodology that would allow the preparation of the ProTide motif in a diastereoisomerically controlled fashion. The immediate target was the stereoselective synthesis of two therapeutically relevant ProTides: the antiviral drug Sofosbuvir (uridine-type ProTide), and the anticancer agent NUC-1031 (gemcitabine ProTide). After the optimisation of the procedure, the final intent was the extension of the methodology to nucleoside featuring different nucleobases. This part of the project will be largely discussed in Chapter 2.

Another area that presents synthetic challenges is the preparation of the phosphonoamidate cognate class, especially of acyclic nucleoside phosphonates (ANPs). ANPs play a key role in the treatment of antiviral infections, and it can be said that this class of compounds is one of the most significant groups of drugs in this field. The structure-activity relationship (SAR) studies on acyclic nucleosides clarified that the introduction of a rigid structural element such as the double bond is extremely important for the antiviral activity. The methodologies reported in the literature for the synthesis of ProTides on alkenyl acyclic nucleosides are scarce, inefficient and often low yielding.

For this reason, the second part of this PhD project was directed towards the optimisation of literature-based methodologies for the preparation of alkenyl acyclic nucleosides, and to investigate more effective synthetic approaches to give easy access to their ProTides. Additionally, the defined synthetic strategies were applied to the preparation of several families of acyclic nucleoside phosphonoamidates whose potential antiviral activities were then evaluated. This topic will be discussed in detail in Chapter 3.

Nowadays, synthetic procedures for the preparation of natural nucleosides are very well known and fully optimised methodologies. However, difficulties can be still encountered in the preparation of modified unnatural nucleosides and related prodrugs. Currently, Demeter Therapeutics is investigating ProTides of modified deoxyguanosine nucleosides for the potential pharmacological treatment for Mitochondrial DNA Depletion Syndrome (MDS). The ProTide of 2'-deoxy-O^6-methylguanosine (2'd-O^6Me-G) gave promising results. Unfortunately, the synthesis of both the nucleoside and its prodrug proved to be very challenging. A collaboration with this company was then established so that an efficient preparation of the demanding 2'd-O^6Me-G nucleoside and structurally related ProTides could be addressed. The final purpose was the design of a synthetic scheme suitable for a large scale approach, with the intent of evaluating both pharmacokinetic properties and *in vivo* analysis of the selected ProTides. Details of the project will be further reported in Chapter 4.

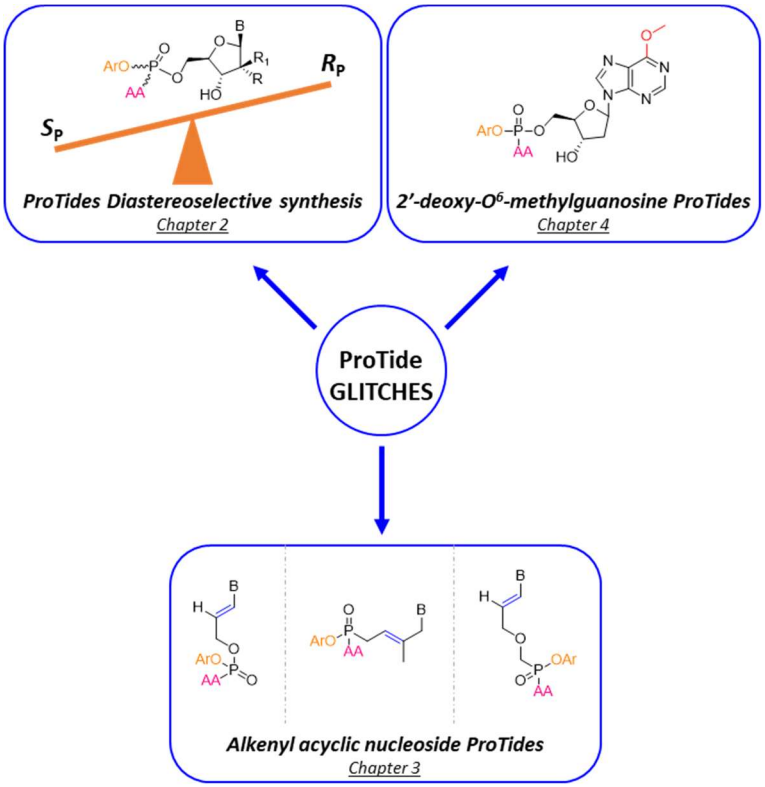

Figure 1.34 Aim of the project: resolution of challenging synthetic problems related to nucleoside and ProTide preparations.

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Chapter 2. ProTide diastereoselective synthesis

2.1 Drugs and chirality

Stereoisomers are molecules that have the same constitution but differ in the spatial arrangement of the atoms. They include enantiomers (non-superimposable mirror image isomers with one or more chiral centre), and diastereoisomers (not mirror image isomers with two or more chiral centres). Diastereoisomers are chemically and physically different and generally easily separated without chiral techniques.

Approximately more than half of the drugs in use are chiral compounds, and 90% of them are marketed as either enantiomeric or diastereoisomeric mixtures.[1] Although drug stereoisomers have the same chemical structure, they may show marked differences in biological activities. Indeed, they may exhibit different pharmacology, toxicology, metabolism and pharmacokinetics properties.[1] In the last few years, drug stereochemistry has become an important issue for both regulatory authorities and pharmaceutical industry. Currently, there are no mandatory requirements from any authority for the development of therapeutic compounds as single isomers, but the introduction of drugs as stereoisomer mixtures requires scientific justification.[2] Moreover, several compounds currently marketed as racemates (1:1 proportion of enantiomers) are subjected to re-evaluation as single isomers.[3] According to FDA guidelines, when isomers are biologically distinguishable, they should be considered different drugs. Until today, isomer separation was mostly an academic issue, but now the advanced technology available makes large-scale chiral separation and asymmetric synthesis possible.

A compound recurrently cited to support development of single isomer drugs, especially in the popular press, is thalidomide. Originally, the drug was introduced into the market in 1957 as a racemic mixture. Prescribed first as sedative, it was also used to treat nausea and morning sickness in pregnant women. That resulted in birth of thousands of infants with phocomelia (malformation of the limbs).[4] Further studies have reported that the *R*-enantiomer of the drug has hypnotic properties while its *S*-enantiomer is both teratogenic and hypnotic.[3] According to more recent investigations, it seems that thalidomide's biological activities may be due not only to the mother compound but also

to its several chiral and achiral metabolites. Therefore, an *in vivo* interconversion occurs and that makes it difficult to determine exactly the biological effect of each enantiomer.[1] Despite this, thalidomide is currently successfully used for the treatment of a range of adult conditions, including complications of leprosy and multiple myeloma.[4]

As a consequence of this tragedy, more severe drug regulation was required. Today's FDA policy aims to promote the chiral separation and the assessments of each isomer's activity in the body in order to find the optimal treatment for the patient.[1]

As far as phosphoroamidate prodrugs are concerned, the marketed S_P isomer Sofosbuvir is a significant example of a ProTide in which the stereochemistry has been correlated with its biological activity.[5] The chirality at the phosphorus atom can considerably alter the potency, toxicity and rate of metabolism of the prodrug. For this reason, the search for a synthetic strategy that is able to control the ProTide phosphorus configuration is a crucial topic that will be discussed in the following paragraphs.

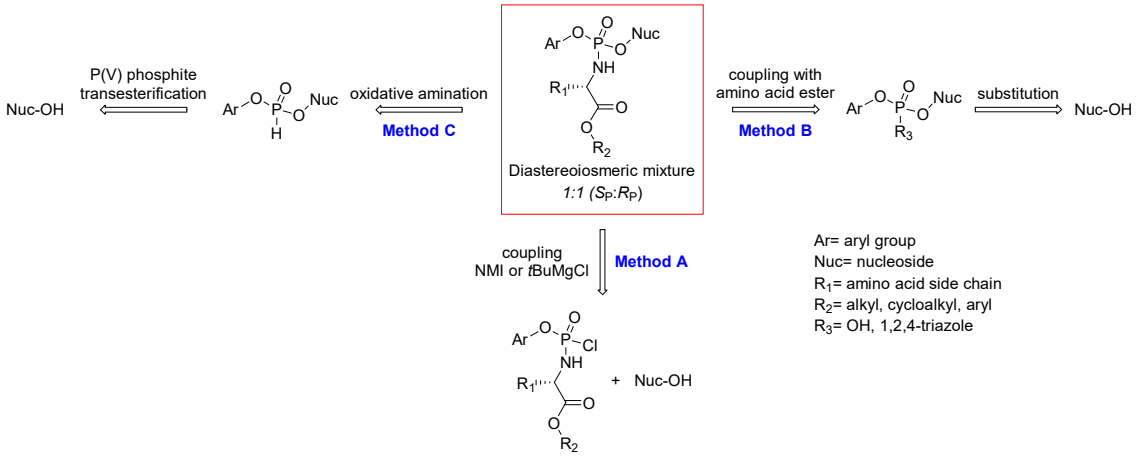
2.2 Current synthetic methods for ProTide preparation

Literature reviews on pronucleotide prodrugs generally focus on their improved biological activities and therapeutic potential neglecting their challenging synthetic preparation[6] such as the complete absence of control of the stereochemistry.[7] In fact, the common issue to all the methods for the aryloxy phosphoroamidate nucleoside synthesis is the formation of a new chiral centre at the phosphorus atom resulting in the formation of the final ProTide as a mixture of two diastereoisomers. So far, several methodologies have been used for the formation of diastereoisomeric aryloxyphosphoroamidates in the same ratio, but only recently, a few stereoselective strategies have been also investigated.

2.2.1 Non-stereoselective syntheses of phosphoroamidates and pronucleotide diastereoisomers and their separation

Three main strategies are present in the literature for the preparation of ProTides as 1:1 diastereoisomeric mixtures.[8] These methodologies differ according to the way the phosphoroamidate function is introduced at the 5'-*OH* of the nucleoside as depicted in the retrosynthetic scheme (**Scheme 2.1**). Method A consists of the phosphorylation of the nucleoside by a phosphoroamidating agent, such as a phosphorochloridate, in the presence of *tert*-butyl magnesium chloride (*t*BuMgCl)[9,10] or *N*-methyl imidazole

(NMI).[11,12] In method B, a coupling between a nucleoside aryl phosphate and an amino acid occurs. According to Method C, a diarylphosphite is coupled to a nucleoside forming an *H*-phosphonate intermediate, which is then subjected to oxidative amination.[13]



Scheme 2.1 General retrosynthetic approaches for the non-stereoselective syntheses commonly used to prepare nucleoside phosphoroamidate prodrugs.

Method A is the most common strategy applied for the preparation of ProTides and it has been employed for the preparation of several anticancer and antiviral aryloxy phosphoroamidate nucleoside analogs.[6] According to this strategy, two different activators can be used: *t*BuMgCl or NMI. Both procedures promote the coupling reaction between a nucleoside and an appropriately substituted phosphoroamidate source, such as a phosphorochloridate, but with completely different reaction mechanisms.

The *t*BuMgCl approach was developed by *Uchiyama et al.*[9] The Grignard reagent is used as a strong base that is able to deprotonate the 5'-*OH* of the nucleoside sugar moiety. As a consequence, the resulting alkoxide is nucleophilic enough to attack the phosphorus atom of the phosphoroamidate source to accomplish the coupling. However, the presence of other hydroxyl groups in the molecule, such as the 2'- and 3'-*OH* is detrimental, since they can also be deprotonated and consequently perform the nucleophilic attack. For this reason, to achieve selective 5'-*O*-phosphorylation, the other hydroxyl groups must be protected.

As an alternative, the NMI procedure developed by *Van Boom et al.* can be employed.[11] Contrarily to the Grignard regent, the imidazole derivative does not act as a base towards the nucleoside, but it is nucleophilic enough to attack the phosphorochloridate by displacing the chloride on the phosphorus atom. The resulting intermediate is a labile imidazolium derivative. As a consequence, the phosphorus atom

is more electrophilic thanks to the delocalisation of the positive charge on the imidazole ring making the NMI an excellent leaving group. Subsequently , the increased reactivity of the phosphorus towards nucleophiles makes possible the direct attack of the nucleoside hydroxyl group on the coupling counterpart. In this case, the phosphorylation occurs selectively on the 5'-position probably due to the steric hindrance provided by the imidazolium moiety on the phosphorus.

As aforementioned, both these coupling approaches are non-stereoselective procedures, consequently the prodrugs are usually obtained as 1:1 mixtures of diastereoisomers at the chiral phosphorus centre (R_P and S_P). As highlighted in paragraph 2.1, diastereoisomers may exhibit different pharmacology, toxicology, metabolism, and pharmacokinetics.[1] It was already mentioned that the marketed S_P isomer Sofosbuvir (**8a**, **Figure 2.1**)[5] is one of the most significant examples of a ProTide in which the stereochemistry has been correlated with the biological activity. After the synthesis of the drug as a diastereoisomeric mixture, the two isomers must be separated by reverse phase HPLC chromatography.[5] The slow eluting S_P isomer was demonstrated to be 18-fold more potent against HCV than the R_P isomer with EC_{90} values of 0.42 μM and 7.5 μM respectively. Not only Sofosbuvir but also TAF (**8b**, **Figure 2.1**) diastereoisomers have been separated by preparative HPLC and the more lipophilic S_P diastereoisomer was demonstrated to be 10-fold more active against HIV than the R_P compound.[14] These examples of pronucleotide drugs isolated as single diastereoisomers have shown that S_P isomers should be the more potent because of the greater accumulation of triphosphate drugs achieved in

cells.[14–17]

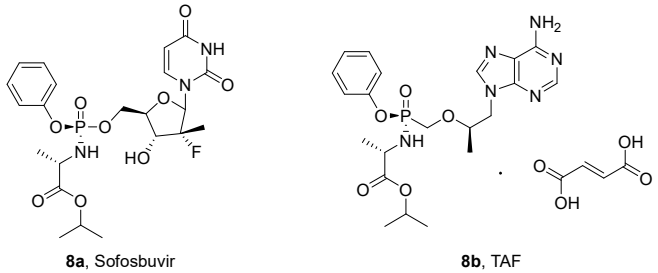


Figure 2.1 ProTides on the market as their S_P isomer.

Up to now, the large majority of nucleoside phosphoroamidate prodrugs have been progressed to the clinic as diastereoisomeric mixtures, but the search for the optimal treatment for the patient aims to achieve the development of drugs with well-identified

configurations at chiral centres.[18] For this reason, several techniques have been investigated for the separation of the two compounds. One of the properties that usually differentiate a pair of diastereoisomers is the solubility: crystallisation may be an effective method to induce their separation. However, a limit of this technique is that a crystal of pure isomer may be necessary to induce the precipitation of the desired diastereoisomer.[5]

Chromatographic separation of the two diastereoisomeric aryloxy phosphoroamidates could be tedious and time consuming.[19] Both chiral and reverse-phase HPLC have been evaluated. The latter proved to be efficient only in a few cases, whereas slightly better results have been obtained with a polysaccharide-type chiral stationary phase (CSP). After preliminary studies on a C_{18} column to optimise various operating parameters that affect the separation, the CSP column has been performed and has been shown to be useful in the resolution of the two diastereoisomers.[20] Moreover, an advanced molecularly imprinted HPLC stationary phase, a polymer capable of molecular recognition, has been developed.[21] The mentioned methods seem to be able to lay the foundations for a general approach to the separation of ProTide diastereoisomers. However, these techniques remain quite efficient analytical procedures that can hardly be applied at the industrial level where grams or kg quantities of material are necessary.

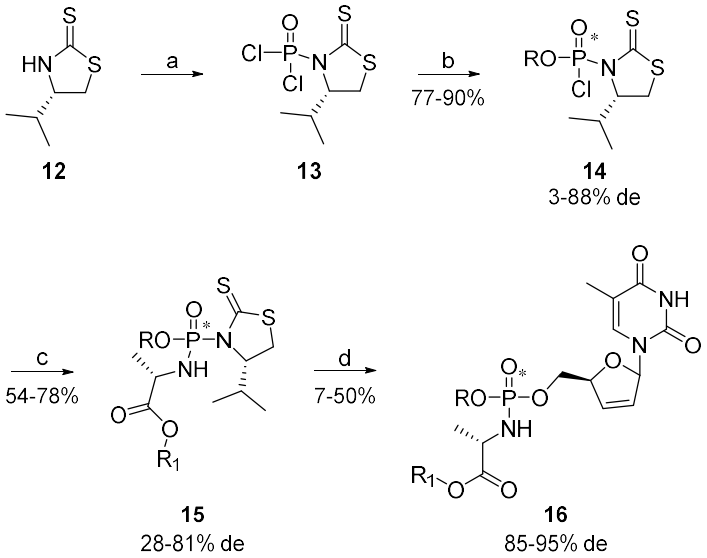
Due to the difficulty of separating the ProTide stereoisomers, recently, different stereoselective synthetic methodologies have been investigated to access the nucleoside prodrugs as single diastereoisomers.

2.2.2 Diastereoselective syntheses of phosphoroamidates

The ProTide stereogenic centre placed on phosphorus has proven to be challenging to prepare in an efficient stereoselective fashion.[6] Nevertheless, the diastereoselective synthesis of such compounds is an important target to achieve.[22] For this reason, several research groups have been active in finding an effective stereoselective synthesis for ProTide preparation.

One of the first attempts was settled by the Meier group through a multistep approach (**Scheme 2.2**).[22,23] This method involved the use of (*S*)-4-isopropylthiazolidine-2-thione **12** as a chiral auxiliary in order to obtain the desired stereochemistry on the phosphoroamidating reagent **15**, whose preparation was accomplished in three steps. The phosphorodichloridate **13** was prepared from the chiral auxiliary. Because compound **13** was unstable on silica gel, it was not isolated, but directly reacted with the desired aryl

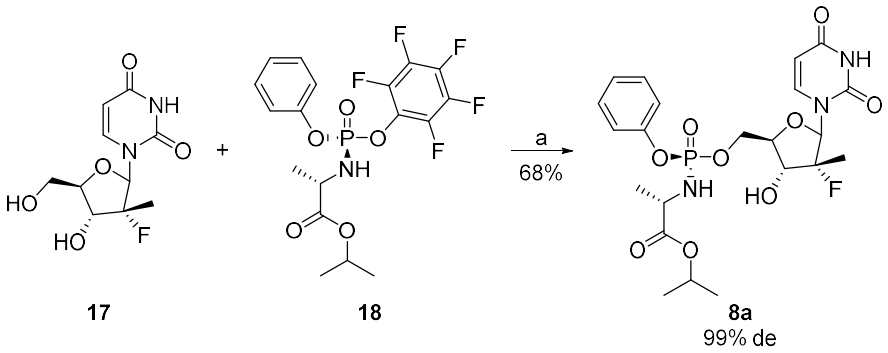
alcohol at -91°C in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU). Therefore, the stereochemistry was introduced at the phosphorus atom and compound **14** was obtained with 3-88% diastereomeric excess (de) depending on the aryl derivative used. The diastereomerically enriched mixture of **14** was then reacted with the amino acid ester hydrochloride salt to obtain the key phosphorodiamidate **15**. The diastereoisomers of **15** can be separated by flash chromatography on silica gel and used as stereochemically pure agents in the last step of the synthesis under Uchiyama conditions (*t*BuMgCl approach). This procedure was applied for the synthesis of d4T ProTides, which were obtained in 7-50% yield with 85-95% de. However, this method presents many limitations. It is important to highlight that the starting chiral auxiliary needs to be prepared, therefore, both its synthesis and purification are time-consuming. Separation of diastereomers is still necessary even though it was executed at the step just before the ProTide formation. Furthermore, the final step is associated with modest yield due to the fact that the starting material are not completely converted and an equilibrium in the reaction mixture is reached. Finally, the procedure was never applied on nucleoside substrates with competing hydroxyl groups in positions 2' and 3'. The reason could be the inefficient selective phosphorylation of the 5'-*OH*.



Scheme 2.2 Diastereoselective phosphoroamidate synthesis according to Meyer and co-workers. Reagents and conditions: a) $POCl_3$, Et_3N, CH_2Cl_2, 0°C to rt, 16h; b) ROH, DBU, acetone, -90°C, 25 to 45 min; c) amino acid ester hydrochloride salt, Et_3N, CH_2Cl_2, 0°C to rt, 16h; d) d4T (2i), *t*BuMgCl (1M in THF), THF, ACN, 0°C to rt, 120h.

Another study that aims to achieve diastereomerically pure aryloxyphosphoroamidate prodrugs was developed by Ross and co-workers.[24] They

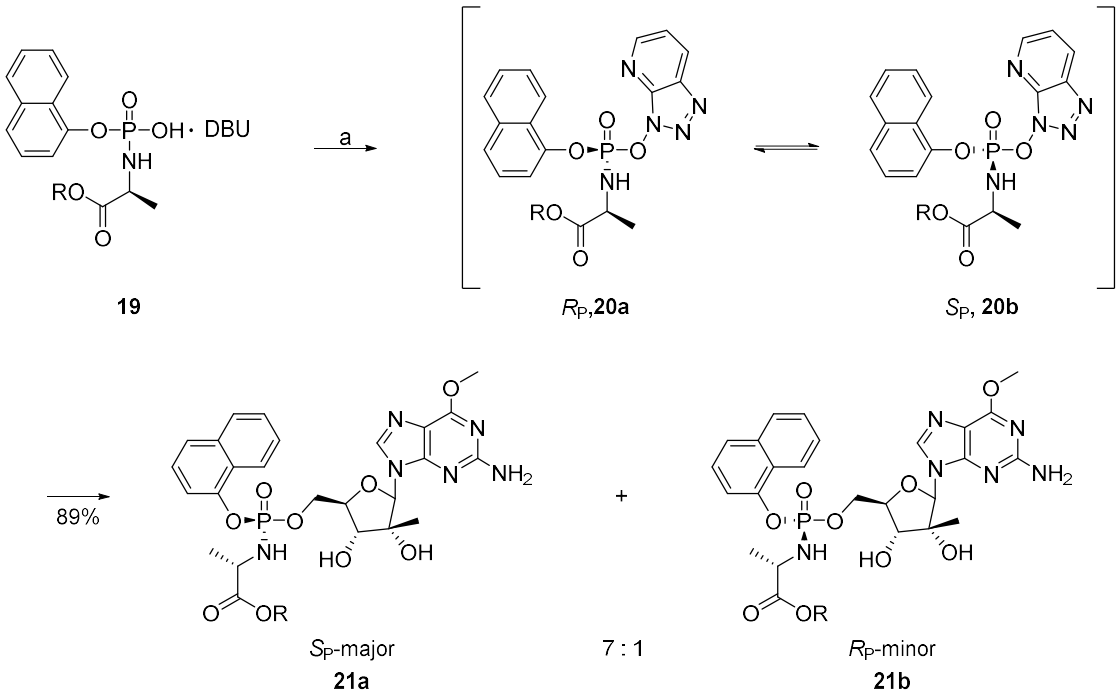
promoted the selective, nucleophilic phosphorylation of 5'-hydroxyl group using phosphoroamidating reagents with increased electrophilicity at the phosphorus atom compared to the phosphorochloridate. To do that, the chlorine was replaced by a better leaving group such as a phenolic derivative bearing electronegative substituents (*p*-nitro- or 2,3,4,5,6-pentafluorophenyl). These phosphoroamidating agents were prepared as diastereomerically pure compounds so that the individual diastereoisomers could be used for the coupling with the nucleoside. The single isomers of the phosphoroamidating reagents were isolated by crystallisation and additional supercritical fluid chromatography. The pentafluorophenyl reagent **18** was identified as the optimal reagent because it increased the electrophilicity at the phosphorus improving its reactivity, but still preserving a good discrimination between the 5'- and the 3'-*OH*. This method was successfully applied for the preparation of the marketed anti-HCV Sofosbuvir **8a** which features the S_P configuration. It was obtained with 68% yield by reacting the R_P diastereoisomer **18** with the nucleoside analogue **17**. According to the addition/elimination mechanism the inversion of configuration at the phosphorus chiral centre occurs.[25] Notably, only 5-8% of the 3',5'-bis product was observed, while only traces of the R_P product were present (**Scheme 2.3**).



Scheme 2.3 Procedure ideated by Ross and co-workers to prepare ProTides as single diastereoisomers. Reagents and conditions: a) *t*BuMgCl (1M in THF), THF, -5°C to 5°C, 17h.

Although this approach has the potential to be widely applied across a variety of substrates, and the synthesis of the pentafluorophenyl intermediate is quite easy to perform, the diastereoisomer separation remains an important issue. The methodology requires the reagent's purification and isolation of the desired stereo-pure diastereoisomer. In the case of Sofosbuvir, the individual diastereoisomer **18** was obtained by crystallisation, but a seed of the desired isomer was necessary to trigger the process.

Recently, kinetic resolution methodology has been gaining importance. According to this approach, two isomers react with different rates in the reaction with a chiral catalyst or reagent. As a result, the separation of the chiral intermediate is not required, and the product mixture is enriched with one of the two isomers. Based on this concept, a kinetic resolution procedure was recently developed by Tran and Eastgate (**Scheme 2.4**).[26] They investigated the use of a stable salt derivative of the phosphoroamidic acid **19**, which was then reacted with hexafluorophosphate azabenzotriazole tetramethyl uronium (HATU) to generate the active phosphate ester as 1:1 diastereomeric mixture (R_P **20a** and the S_P **20b**). The final P-O bond formation with the nucleoside occurred at different rates with the two diastereoisomers of the active esters, while the ratio between **20a** and **20b** remains unchanged (1:1). The reason is that the reaction is a dynamic kinetic resolution where the two diastereoisomers intermediates are in rapid interconversion.[27] The final products **21a** and **21b** were obtained with 89% yield and a diastereomeric ratio (dr) of 7:1 (S_P:R_P). The desired S_P compound **21a** was then isolated by crystallization with 57% yield and 50:1 dr. However, even this method encounters the intermediate purification issue: although diastereoisomer separation is not required, phosphoroamidic acid **19** is an oily substance that is difficult to purify. Moreover, in the reaction process there is the formation of a phosphorus anhydride by-product between the phosphate ester and the residual phosphoroamidic acid, which entails a reduction in yield.

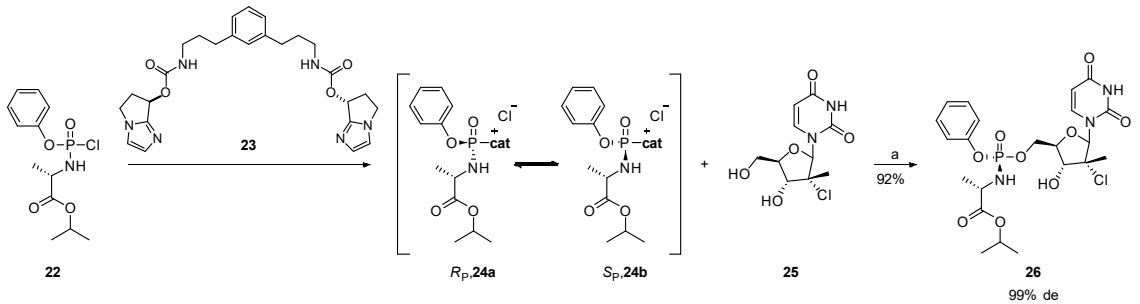


Scheme 2.4 Diastereoselective coupling ideated by Tran and Eastgate. Reagents and conditions: a) HATU, 2'-methyl-6-methoxyguanosine, quinine, THF, 50°C, 5h.

Recently, McGuigan's group envisaged a novel catalytic methodology for the diastereoselective synthesis of phosphoroamidate prodrugs. In particular, Pertusati developed the ProTide synthesis *via* a copper-catalysed reaction.[7] The hypothesis behind this work is based on the catalytic alcohol phosphorylation explored by Jones and co-workers.[28,29] Both the two investigations will be discussed in detail in paragraph 2.3 to better introduce the work correlated to the project discussed here.

In 2017, the application of the kinetic resolution approach to the ProTide synthesis was further explored for a new catalytic dynamic kinetic asymmetric transformation (DYKAT) by Merck industries.[30] As observed in the Tran and Eastgate strategy, Di Rocco *et al*. exploited the use of an interconverting mixture of chiral phosphorochloridate intermediate (**24a** and **24b**) to be coupled stereoselectively with the nucleoside in the presence of an adequate chiral catalyst. Additionally, the Merck researchers were interested in developing a strategy that is able to achieve the phosphoroamidation of the 5'-*OH* of the nucleoside regioselectively. To this purpose, their studies were based on the enzyme-mediated theory according to which the process must involve concomitant activation of both nucleophile by general base catalysis and phosphorus donor to lead to stereoselective enhancements. Therefore, they have developed an intramolecular cooperative catalytic system **23** able to mimic the complex function of an enzyme,

culminating in high stereoselective phosphoroamidation (**Scheme 2.5**). Specifically, they extensively studied the coupling reaction between the chlorophosphoroamidate **22** and the nucleoside **25** to generate the anti-HCV MK-3682 **26** (R_P) in a regio- and stereoselective fashion. They envisaged the use of the catalyst **23** that was thought to be able to activate the phosphorus atom generating the **24a** and **24b** interconverting mixture by binding one of the two imidazole functions of the catalyst (**Scheme 2.5**). The ratio between the two diastereoisomers (**24a** and **24b**) remained unchanged during the reaction progress proving that an equilibrium was established. Simultaneously, the other imidazole residue of the catalyst can interact with the 5'-*OH* of the nucleoside through general base catalysis. The imidazole function readily accepts the proton from the hydroxyl group of the nucleoside by hydrogen-bond and thus stabilises the transition state. By lowering the energy of the transition state, the bond formation between the activated phosphorus and the nucleoside can be generated. Additionally, the transition state was thought to be further stabilised by several interactions of the catalyst to the polarised P-O bond of the phosphoroamidating agent. Unfortunately, the catalyst is expensive and difficult to synthesise. Indeed, it can be purified only by preparative chiral supercritical fluid chromatography (SFC). However, the main drawback related to this procedure is the difficulty associated with obtaining a significative S_P diastereoselectivity. In fact, when the procedure was applied to different nucleosides, such as 5-FU, AZT, d4T and guanosine derivatives, only R_P compounds were synthesized under the stereo-controlled conditions.



Scheme 2.5 Stereoselective catalytic system for the synthesis of MK-3682 (**26**). Reagents and conditions: a) 2,6-lutidine, 1,3-dioxane, -10°C, 24h.

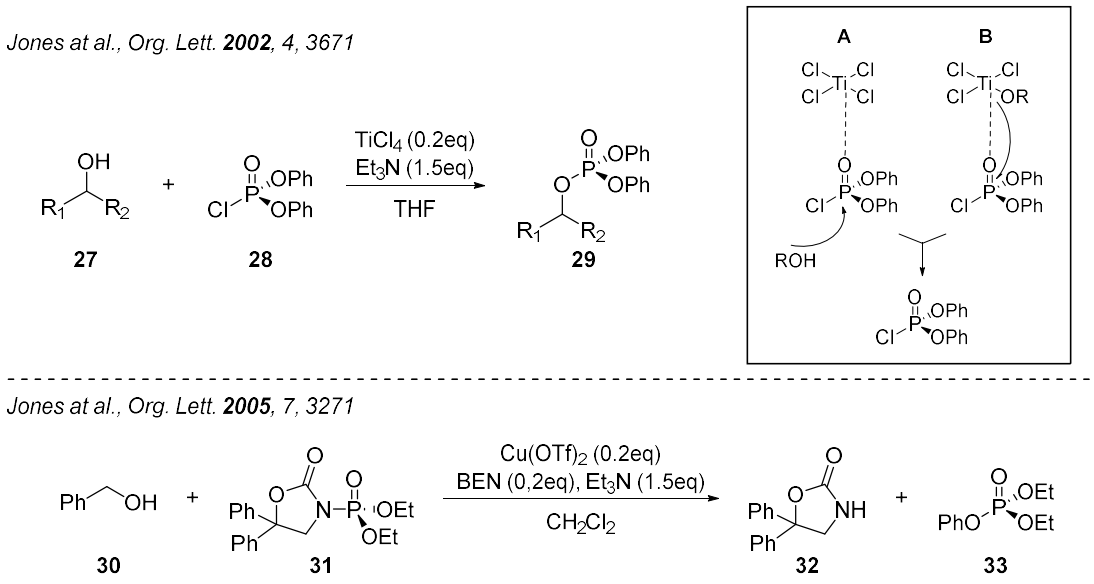
According to the above discussion, it is still desirable to pursue the development of a catalytic methodology which involves the use of easily accessible catalysts, ideally with high diastereoselective ratios, along with straightforward purifications of intermediates and final products. With this goal in mind this thesis explores a metal salt

catalytic procedure that may be applied on both purine and pyrimidine nucleosides to obtain aryloxyphosphoroamidate prodrugs with good diastereomeric ratios.

2.3 ProTide diastereoselective synthesis *via* metal salt catalyst reaction

Phosphate esters are common functionalising groups that are present in several biological molecules. To accomplish their preparation, one of the more widely used methodologies is the direct reaction of the alcohol substrate with a chlorophosphate. In 2002, Jones and co-workers introduced a catalytic route for the phosphorylation of numerous alcohols using chlorophosphate as the phosphoroamidating agent and $TiCl_4$ as catalyst (**Scheme 2.6**).[28] Even though the mechanism of the reaction is still unclear, Jones *et al*. suggested two pathways (**Scheme 2.6**). In the first (A), $TiCl_4$ coordinates to the oxygen (P=O) increasing phosphorus electrophilicity. The product is then released through intermolecular nucleophilic displacement. According to the second pathway (B), a titanium alkoxide generated in situ coordinates to the phosphoryl chloride and results into an intramolecular phosphate transfer.

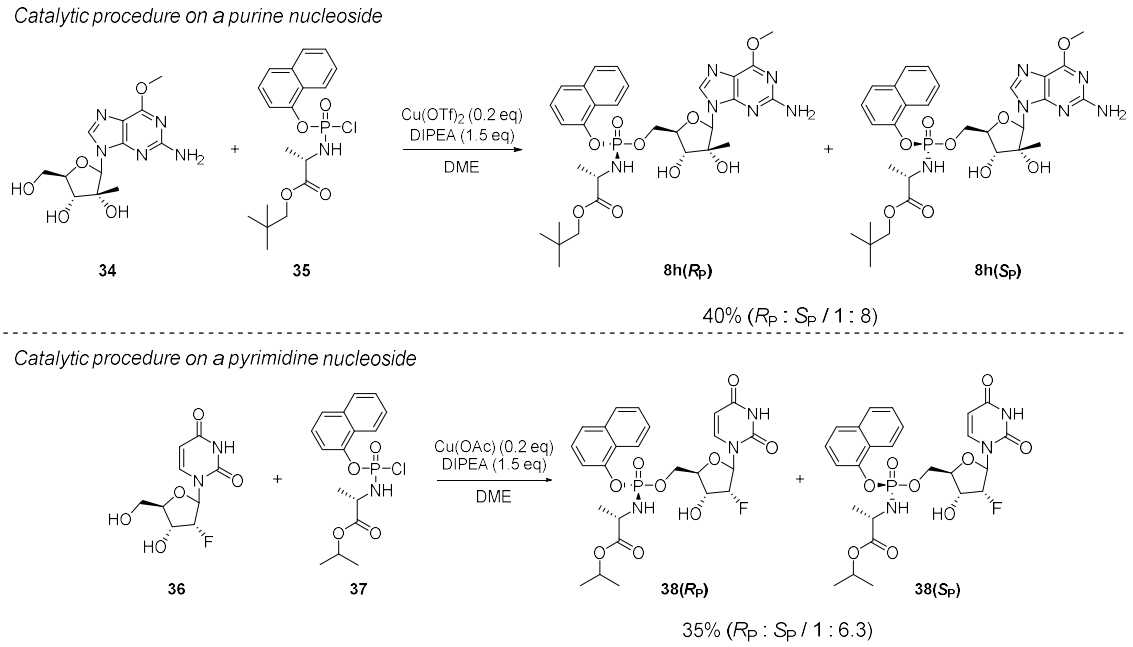
This work was followed in 2005 by a similar investigation: Jones and co-workers carried out an exploration of the use of Lewis acids as catalysts for phosphate ester preparation using *N*-phosphoryl oxazolidinones as phosphate sources (**Scheme 2.6**).[29]



Scheme 2.6 Jones alcohol phosphorylation.[28,29]

All the reactions were performed using benzyl alcohol (**30**) as a model substrate, Et_3N (1 eq) and oxazolidinone **31** (1 eq) in presence of a Lewis acid in a catalytic amount (0.2 eq). The catalytic activity of several Lewis acids, particularly triflate derivatives, were screened. No conversion of the starting alcohol was observed in any case. When the reaction was performed under identical conditions, but in the presence of a chelating agent (0.2 eq) successful outcome were observed. Indeed, a combination of $Cu(OTf)_2$ and *N*,*N*'-ethylenebis-(benzaldimine) (BEN) proved to be the optimal catalyst and ligand, respectively. The optimised reaction conditions were then applied to a range of primary, secondary and tertiary alcohols. Of particular interest was the application of this procedure to purine nucleosides: adenosine and guanosine were phosphorylated at the 5'-*OH* with excellent regioselectivities.

Since the catalytic procedure was successfully applied to two examples of nucleosides, and since the ProTide synthesis involves the use of chlorophosphoroamidate (analogues of Jones chlorophosphate), the catalytic activity of $Cu(OTf)_2$, $LiCl_4$ and other Lewis acids was then evaluated in our laboratory. The investigation began with the application of Jones' procedures to the synthesis of ProTide **8h** (**Scheme 2.7**).



Scheme 2.7 Synthesis of **8h** and **38** under metal catalysed conditions.[7]

The purine nucleoside **34** was reacted with the phosphorochloridate **35** in the presence of $TiCl_4$ or $Cu(OTf)_2$ (0.2 eq), BEN (1 eq) and Et_3N (1.5 eq) in THF at room temperature. The crude mixture was analysed by analytical RP-HPLC and after 12 hours **8h** was

obtained in low yield (12-14%) with a diastereomeric ratio of 1:2.5 in favour of the slowly eluting S_P diastereoisomer. After these first results, several other reaction parameters were investigated and surprisingly, when BEN was not included in the reaction mixture the product was obtained in 37% yield and in 1:6.2 (R_P/S_P) diastereomeric ratio. DIPEA (1.5 eq) and $Cu(OTf)_2$ (0.2 eq) in DME proved to be the optimal conditions to perform the phosphorylation of the purine nucleoside **34** with 40% yield and 1:8 (R_P/S_P) diastereomeric ratio. This successful protocol was then extended to other nucleosides such as the pyrimidine **36**. In this case, the conditions required to achieve the product were different: $Cu(OTf)_2$ and other triflates were less efficient in terms of diastereoselectivity, whereas Copper(I) acetate proved to be superior for pyrimidine nucleosides, giving ProTide **38** in 35% yield and 1:6.3 (R_P/S_P) diastereomeric ratio.

In summary, this methodology allowed the preparation of ProTide diastereomeric mixtures enriched in the compound featuring the S_P configuration. Interestingly, the use of the lanthanum triflate as catalyst resulted in the inversion of the diastereoselectivity with a diastereomeric ratio of 1.1:1 (R_P:S_P). Although the mixture was only slightly enriched with the R_P compound, the possibility to revert the stereochemistry at the phosphorus with the appropriate catalyst could be extremely useful in making this strategy highly versatile.

Despite the promising results of this methodology, further improvements were required. The final ProTides were isolated with moderate yields and modest diastereomeric ratios. Additionally, the chromatographic separation of the two final diastereoisomers was still necessary.

2.4 Aim

The main goal of this project is to develop a general procedure for the diastereoselective synthesis of phosphoroamidate prodrugs of nucleoside analogues.

The currently available methodologies, despite being sometimes very effective, still present some drawbacks such as the preparation of chiral auxiliary or complex organic catalyst and the problematic separation of the diastereoisomers at different stages of the synthetic strategy. These features make all these methods somewhat inefficient, high-priced, time consuming and hard to scale-up. For these reasons, there is a tremendous need for a process that is able to deliver only the desired diastereoisomer. The diastereoselective synthesis approach highlighted in this PhD project will have enormous potential to be applied to the wide areas of asymmetric phosphorus chemistry.

According to Pertusati's work,[7] the coupling between the nucleoside and the phosphorochloridate have the great potential to be accomplished in a stereo- and regio-selective fashion in the presence of selected copper catalysts. Therefore, this project aimed to improve and further optimise the preliminary results discussed in the previous paragraph. The main objectives can be summarised in the following list:

- The reaction conditions of Pertusati's work allow the successful regioselective phosphorylation of the 5'-*OH*, without the need to protect other hydroxyl groups in the nucleoside scaffold. Despite that, we were interested in evaluating the effects of the most common protecting groups used in nucleoside chemistry in 3'-position of the nucleosides on the stereo-outcome of this synthetic strategy.
- The impact of different metal catalysts, bases, ligands, solvents and temperature will be also considered during the evaluation of the yield and the stereo-outcome of this methodology.

To develop the investigation on a diastereoselective approach in a systematic and planned manner, the project started from the choice of specific nucleosides. Two reference phosphoroamidates (see *infra*), NUC-1031 (**8j**) and Sofosbuvir (**8a**), were chosen. Behind this decision, there is not only their therapeutic importance but also the opportunity to focus on nucleosides bearing pyrimidine nucleobases because of the lower yield and the inferior diastereomeric ratio observed with Pertusati's methodology. The syntheses of authentic samples of these prodrugs, including their 3'-protected analogues, were also necessary for our analytical evaluation of the diastereomeric excess via HPLC monitoring.

The coupling between a nucleoside and a phosphorochloridate is the most common procedure employed for ProTide preparation. Despite that, this project is not limited to the investigation of the best catalytic conditions of this commonly used approach. Therefore, to expand the scope of the work, transition metal catalytic system will be also evaluated in a different ProTide synthetic methodology. With this purpose in mind, the stereoselective preparation of a *H*-phosphonate as a key intermediate of an alternative ProTide synthesis will be further evaluated.

2.5 Reference compounds

The investigation of a diastereoselective approach was planned to start from the choice of specific nucleosides: Gemcitabine (dFdC, **1k**) and 2'-deoxy-2'-fluoro-2'-*C*-methyluridine (**17**). Behind this decision, there is the clinical relevance of the ProTides bearing these two nucleosides: NUC-1031 (**8j**) as **1k** phosphoroamidate prodrug and Sofosbuvir (**8a**) as ProTide of the uridine derivative **17** (**Figure 2.2**). Considering the increasing therapeutic importance that the nucleoside phosphoroamidate prodrugs are gaining in both anticancer and antiviral fields, the diastereoselective synthesis discussed in this project is predicted to have a great impact on the development of novel ProTides with potential therapeutic activities.

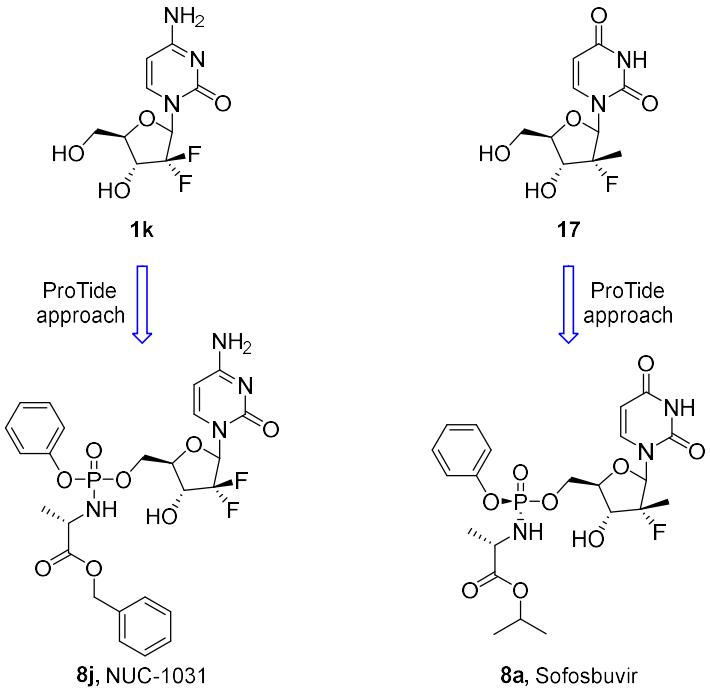


Figure 2.2 Reference compounds.

2.6 Results and Discussion

2.6.1 Preparation of authentic samples of 3'-protected ProTide

To optimise the preliminary results observed by Pertusati in his work,[7] the presence of a second hydroxyl group in the 3'-position of the selected nucleosides' sugar moieties provides the opportunity to investigate the impact of the nature of protecting groups on the stereo-outcome of the phosphoroamidate synthesis. The protecting groups were selected for their compatibility with the catalytic reactions and their potential utility in the resulting product. If a nucleophilic attack of the nucleoside on the phosphorochloridate is considered, it can take place from either side of the phosphorus atom with equal ease resulting in the diastereomeric mixture. The insertion of a hindered group on the sugar moiety could favour the attack of the 5'-*OH* on the most readily accessible side of the phosphorochloridate resulting in an enhanced formation of one of the two diastereoisomers. Additionally, the interaction between the metal catalyst and the protective group in the transition state of the reaction was also considered a possibility to achieve diastereoisomer resolution.

Before explaining the methodologies applied for nucleoside protection, it is necessary to clarify that the selected nucleosides are gemcitabine (**1k**, **Figure 2.3**), as previously established, and 2'-deoxy-2'-fluorouridine (**36**, 2'd-2'FU, **Figure 2.3**) as a model substrate for the related 2'-deoxy-2'-fluoro-2'-*C*-methyluridine **17** (**Figure 2.2**). The reason behind this decision is the high price (at the time this work was carried out) and long and difficult synthesis of nucleoside **17**, that would have been time consuming for the diastereoselective synthesis exploration.

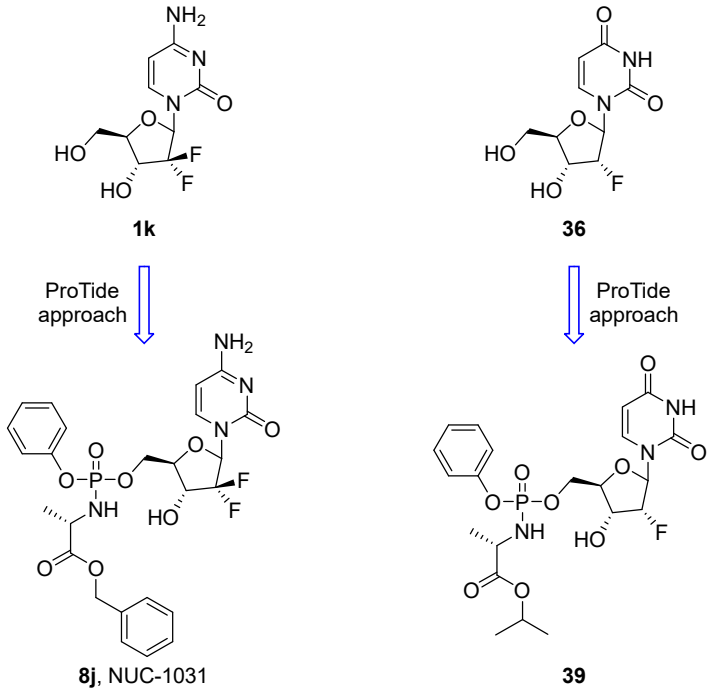


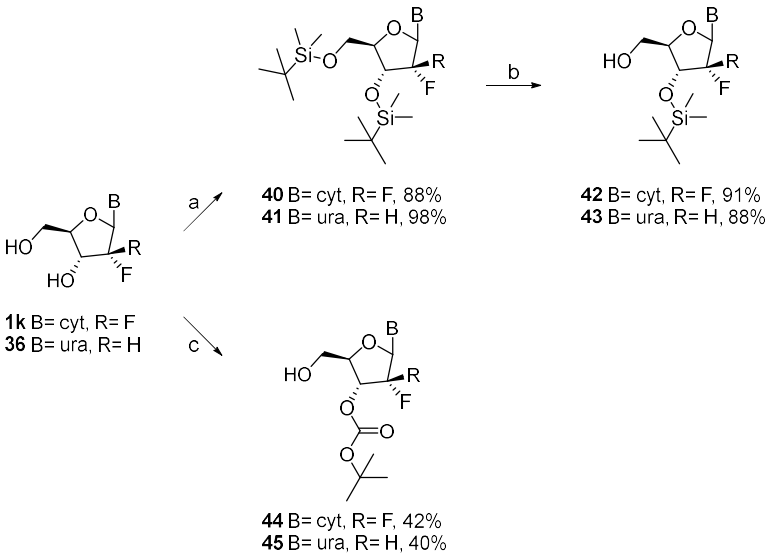
Figure 2.3 Nucleosides and corresponding ProTides selected for the diastereoselective synthesis investigations.

To evaluate the effects of a protecting group in the 3'-position on the catalytic ProTide synthesis, the project started from the preparation of the 3'-protected nucleosides. Among several protecting groups, we decided to explore *tert*-butyldimethylsilyl (TBDMS), *tert*-butylcarbonate (Boc) and acetyl (Ac). All of them were extensively reported in nucleoside chemistry and had the advantage that their removals were proved to be compatible with the ProTide motif. Therefore, we were interested in the possibility that the protecting groups may be able to exert some stereocontrol in the delivery of the phosphorus source used in the ProTide synthesis.

In relation to the protection with the silicon derivative, a well-known procedure was applied on both nucleosides **1k** and **36** (**Scheme 2.8**).[37,38] This strategy consists of two steps: silylation of both alcohol groups in positions 3' and 5', followed by the selective 5'-*O*-desilylation. For the first protection step, the nucleosides were treated with *tert*-butyldimethylsilyl chloride (TBDMSCl) in DMF at 50°C for 16h. The presence of imidazole and a catalytic amount of 4-dimethylaminopyridine (DMAP) were necessary for the formation of *tert*-butyldimethylsilylimidazole, which proved to be a very reactive silylating agent to accomplish the formation of the disilylated intermediates **40** and **41**. The following selective 5'-*O*-desilylation was performed in THF by addition of an aqueous solution of trichloroacetic acid at 0°C. Keeping the mixture in an ice/water bath

allowed the deprotection of the desired 5'-*OH* selectively yielding **42** (91%) and **43** (88%).

5'-Protection of **36** and **1k** with di-*tert*-butyldicarbonate was accomplished with a one-step reaction (**Scheme 2.8**).[39] To a solution of the nucleoside in dioxane and water, the $(Boc)_2O$ reagent was added in one portion and the mixture was left stirring for 16h at room temperature. It was found that the concentration of the reaction mixture was a crucial factor to achieve good yields. The use of 20 ml of dioxane/H_2O mixture (4:1 v/v) per 1g of nucleoside were identified as the best conditions to afford products **44** (42%) and **45** (40%). Otherwise, more dilute reaction mixture delivered the starting nucleoside as the major product with only traces of the protected derivatives.

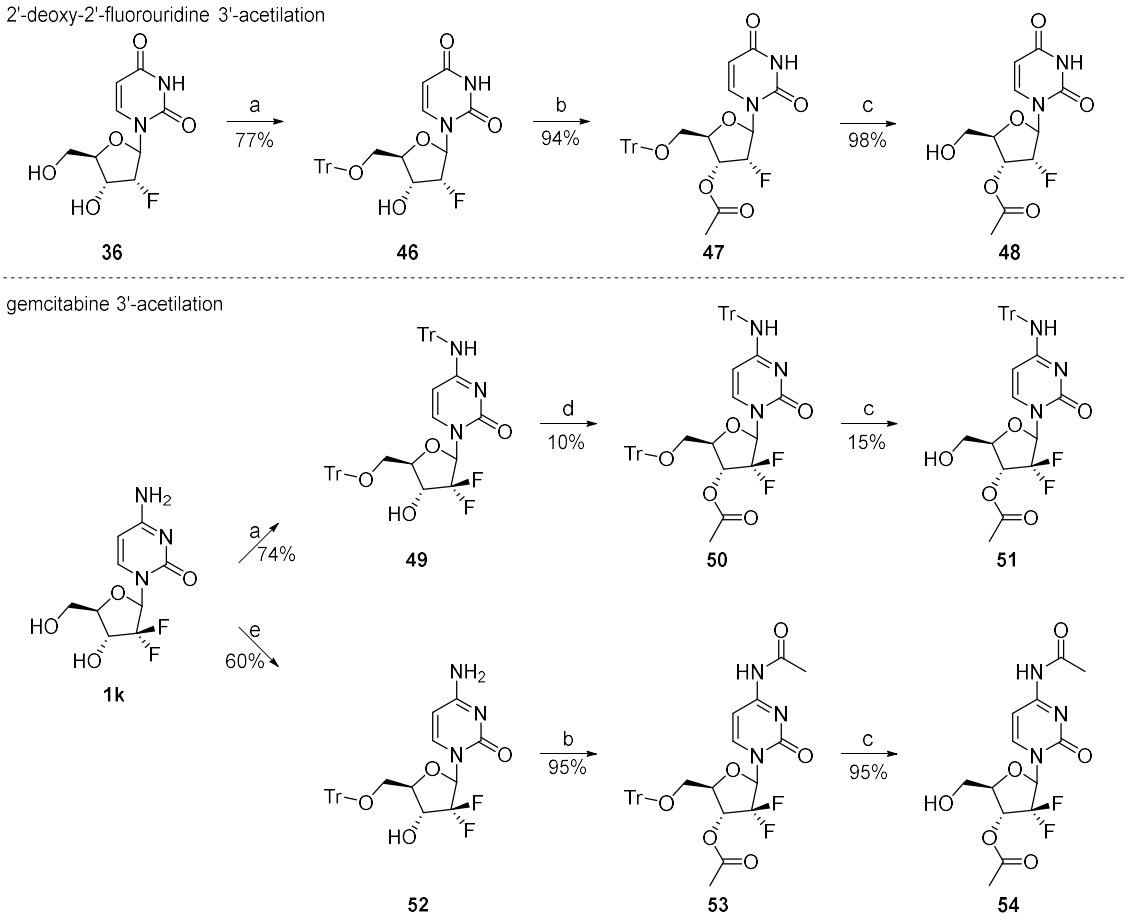


Scheme 2.8 Protection of the nucleosides 3'-*OH* with TBDMS and Boc groups. Reagents and conditions: a) TBDMSCl, DMAP, imidazole, DMF, 50°C, 16h; b) TCA in H_2O, THF, 0°C, 3h; c) Na_2CO_3, $(Boc)_2O$, Dioxane/H_2O (4:1, v/v), rt, 16h.

Selective acetylation of the 3'-*OH* for both nucleosides **1k** and **34** was accomplished in three steps: 1) protection of the 5'-*OH* with a trityl moiety; 2) acetylation of the desired secondary alcohol and 3) final deprotection of the 5'-*OH* (**Scheme 2.9**).[40]

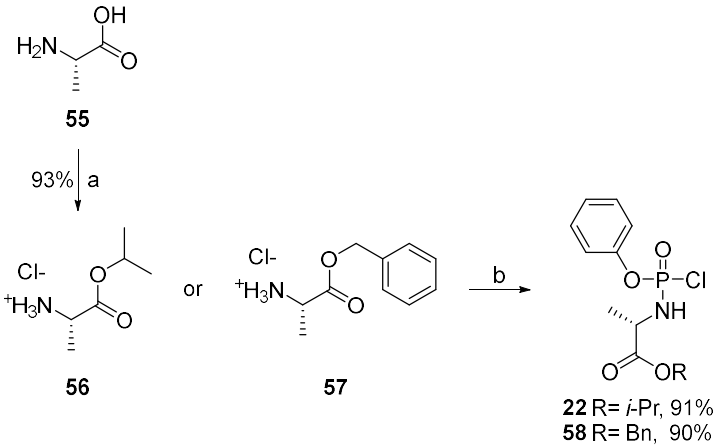
Briefly, nucleoside **36** was treated with trityl chloride and DMAP in pyridine at 85˚C to promote the selective protection of the 5'-*OH*. This reaction showed the formation of several side products, but after a careful purification via column chromatography, it led to the expected product **46** in 77% yield. The acetylated product **47** was prepared using an excess of acetic anhydride in pyridine at room temperature for 12h. Finally, according to the literature, the detritylation should be performed with glacial

acetic acid and MeOH.[41] Unfortunately, in our hands this procedure failed to return the desired nucleoside even after further addition of acetic acid, and **47** was detected as the only species present in the mixture. Pleasingly, the complete conversion of compound **47** into the monoacetylated product **48** was accomplished with a 1:1 mixture of TFA/CH_2Cl_2 (v/v) in 24h at room temperature with 98% yield. The same synthetic sequence was then applied to Gemcitabine **1k**. Tritylation was accomplished with trityl chloride and DMAP in pyridine at 85˚C leading to the tritylation of both the 5'-*OH* and the -NH_2 of the nucleobase (**49**) in 48h with 74% yield. The following acetylation step was performed according to literature procedure, with Ac_2O, DMAP and Et_3N in CH_3CN.[42] Because of the poor solubility of the starting material in the reaction solvent, the acetylated product **50** was obtained in 10% yield. The final detritylation step was initially performed with TFA/CH_2Cl_2 (1:1 v/v), but the reaction showed the formation of several side products alongside major compound **51** still bearing a trityl moiety on the amine group of the nucleobase. Consequently, it was necessary to change strategy to achieve the desired 3'-OAc protected gemcitabine. At first, the tritylation of the amino group was avoided by making small changes to the reaction conditions such as stirring the mixture at room temperature (rather than 85°C) and without the addition of DMAP. Under these conditions 5'-trityl gemcitabine **52** was obtained in 60% yield. The following acetylation step was performed with acetic anhydride in pyridine at room temperature and it led to the addition of the acetyl moiety both on the 3'-*OH* and the -NH_2 group of the nucleobase (**53**).[43] Finally, this substrate was de-tritylated with TFA/CH_2Cl_2 (1:1 v/v) in excellent yield (95%). Despite the presence of the *N*-acetyl group, the bis-acetylated nucleoside **54** was used anyway as a substrate of the diastereoselective ProTide synthesis. Indeed, it could be relevant to investigate the effect of the *N*-acetyl group on the metal catalysed phosphoroamidation. The protection of the amine on the nucleobase offered the possibility to have increased solubility of the nucleoside. Since metal catalysts proved in many cases to be poisoned by amine functional groups,[44,45] we planned the protection of the amine as further investigation. Fortunately, the simultaneously acetylation of the sugar moiety and the nucleobase provided an advantage in avoiding any potential poisoning effect of the nitrogen on the metal catalysts we were going to investigate.



Scheme 2.9 Protection of the nucleosides' 3'-*OH* groups with acetate group. Reagents and conditions: a) trityl chloride, DMAP, pyridine, 85°C, 48h; b) $(CH_3CO)_2O$, pyridine, rt, 12h; c) TFA/CH_2Cl_2 (1:1, v/v), 0°C to rt, 24h; d) $(CH_3CO)_2O$, Et_3N, DMAP, ACN, rt, 16h; e) trityl chloride, pyridine, rt, 48h.

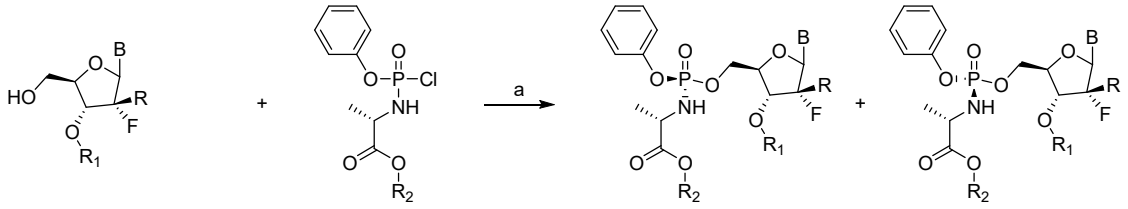
With the protected nucleosides in hand, the following steps consisted of the synthesis of the desired phosphoroamidating agent (**Scheme 2.10**).[46] At first, we prepared *L*-alanine isopropyl ester hydrochloride **56** using an excess of 2-propanol and thionyl chloride at reflux temperature. For the purification of this compound extensive trituration of the crude product with *n*-hexane was necessary to remove the great amount of 2-propanol and afford the pure amino acid ester salt **56** with 93% yield. Compound **56** was used in the synthesis of the corresponding aryloxy phosphorochloridate compound **22** using aryloxy dichlorophosphate and Et_3N in CH_2Cl_2 at -78˚C.



Scheme 2.10 Preparation of the aryloxyphosphorochloridates **22** and **58**. Reagents and conditions: a) $SOCl_2$, 2-propanol, 0°C to 70°C, 16h; b) phenyl dichlorophosphate, Et_3N, CH_2Cl_2, -78°C to rt, 2h.

Aryloxyphosphorochloridate **58** necessary for the preparation of the NUC-1031 molecule, was obtained with the same procedure, using commercial *L*-alanine benzyl ester hydrochloride **57**.

The protected nucleosides and the two phosphorochloridates previously synthesized were combined to obtain the corresponding 3'-protected ProTides (**Scheme 2.11**).



43 B= ura, R= H, R_1= TBMS
45 B= ura, R= H, R_1= BOC
48 B= ura, R= H, R_1= Ac
42 B= cyt, R= F, R_1= TBMS
44 B= cyt, R= F, R_1= BOC
54 B= cyt-NHAc, R= F, R_1= Ac

22 R_2= *i*-Pr
58 R_2= Bn

59 B= ura, R= H, R_1= TBMS, R_2= *i*-Pr 49%
60 B= ura, R= H, R_1= BOC, R_2= *i*-Pr 25%
61 B= ura, R= H, R_1= Ac, R_2= *i*-Pr 28%
62 B= cyt, R= F, R_1= TBMS, R_2= Bn 62%
63 B= cyt, R= F, R_1= BOC, R_2= Bn 70%
64 B= cyt-NHAc, R= F, R_1= Ac, R_2= Bn 48%

Scheme 2.11 Preparation of the 3'-protected ProTides. Reagents and conditions: a) *t*BuMgCl (1M in THF), THF, rt, 16h.

The coupling reactions consisted of the activation of the 5'-*OH* with the Grignard reagent *t*BuMgCl. It is an organometallic compound used as selective base for the activation of –*OH* groups. In fact, the use of this reagent consents to perform the coupling reaction without any kind of protection on the amino group, particularly in the cytosine nucleobase.

Nucleosides **42-45**, **48** and **54** were stirred for 30 min with the base, and then the phosphoroamidating agent was added dropwise over 15 min. Once the 1:1 diastereoisomeric mixtures of the 3'-protected ProTides were prepared, it was possible to proceed with their separation by HPLC. At first, analytical reverse phase HPLC (RP-HPLC) was used to screen the best separation conditions. Several analyses were carried out using different solvent gradients with ACN/H_2O or MeOH/H_2O. The best gradient able to separate the two single diastereoisomers was MeOH/H_2O from 10:90 to 100:0 in 30 minutes. To obtain the separation in shorter time, other conditions were evaluated. After extensive screening, three different isocratic solvent systems were discovered as the most efficient depending on the product to separate: MeOH/H_2O-60:40, MeOH/H_2O-70:30, MeOH/H_2O-80:20 (**Figure 2.4**). These solvents systems were able to separate diastereoisomers within 10 minutes of analysis.

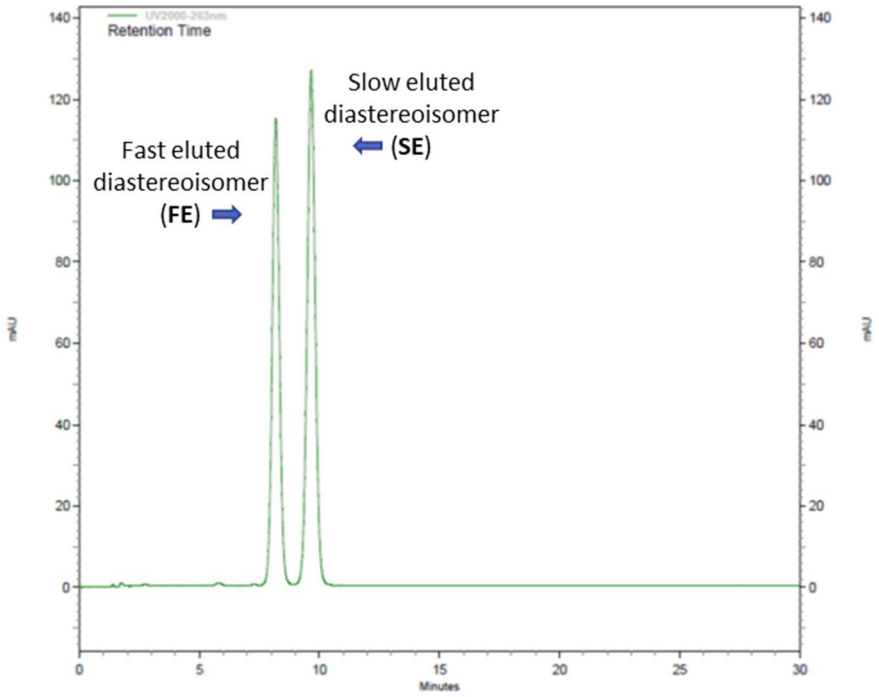


Figure 2.4 Example of RP-HPLC diastereoisomers separation. It represents the elution of the two diastereoisomers of **62** using the isocratic method MeOH/H_2O-80/20 in 30 minutes, 1 ml/min, λ = 263 nm.

The chromatographic conditions were then transferred into a preparative RP-HPLC to physically separate the fast eluted (FE) and slow eluted (SE) diastereoisomers of the 3'-protected ProTides. Each isomer (**59-64 FE** and **59-64 SE**) was collected and fully characterised. All these authentic samples were used as analytical references of the

diastereomeric excess via HPLC monitoring throughout diastereoselective ProTide synthesis evaluation.

2.6.2 ProTide diastereoselective synthesis assisted by transition metal catalysts.

The phosphoroamidate motif is a key functional group in numerous natural and pharmaceutical bioactive compounds.[47,48] Moreover, they are used in analytical chemistry to improve ionization efficiency in mass spectrometry[49] and in industry as flame retardants.[50] Given this wide use, several methods were developed for the preparation of the phosphoroamidate functional group. Two procedures for phosphoroamidate synthesis are further discussed here. One route implies a nucleophilic substitution of a phosphoryl halides with an amine in the presence of a base. This pathway is the most commonly applied for ProTide preparation and it is largely investigated in the following paragraph. The second route consists of the coupling between an *H*-phosphonate and an amine (see paragraph 2.6.4).

With the reference compounds and the related analytical data in our hands, it was possible to plan the diastereoselective synthesis investigations.

This project consisted, mainly, of the application of a transition metal catalyst approach on the two different procedures (**Figure 2.5**):

- Direct coupling between the nucleoside and the aryloxy phosphoroamidating agent.
- ProTide preparation *via H*-phosphonate intermediate.

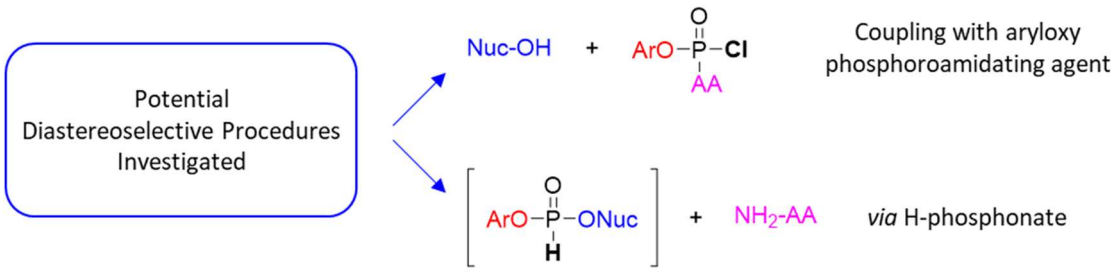


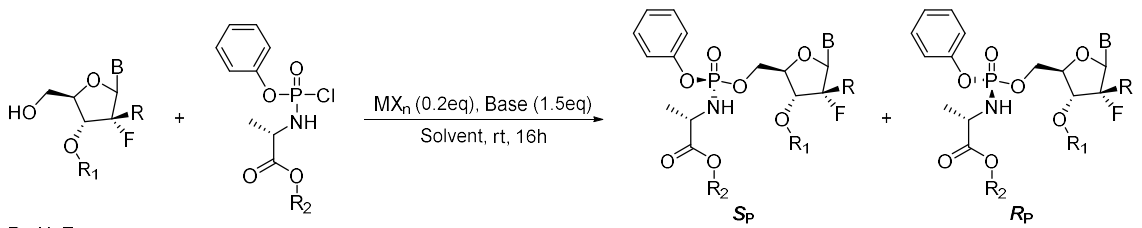
Figure 2.5 The two different general procedures for ProTide preparation on which a transition metal catalyst approach was applied to achieve diastereoselectivity.

All the results discussed here were established on the analysis of crude reaction mixtures via analytical HPLC using the reference peaks of both the starting nucleosides and the

ProTide diastereoisomers previously isolated. The S_P diastereoisomer proved to be the configuration that confers better activity to ProTides.[5,51] For this reason, this investigation aims to develop a stereo-controlled synthesis of the ProTide S_P isomer. Literature data demonstrated that the slow eluting compound is the desired ProTide featuring by the S_P configuration.[5,52]

2.6.3 Direct coupling between the nucleoside and the aryloxy phosphoroamidating agent

This methodology consisted of the investigation and improvement of the reaction conditions reported by Pertusati in 2015 and previously described here (**Scheme 2.7**, paragraph 2.3).[7] That protocol proved to be a successful catalytic system to deliver the S_P ProTide. The first part of this project aimed to study and overcome some limitations related to that procedure. Indeed, the S_P diastereoisomer was delivered only in moderate yield in Pertusati's protocol, and its application on pyrimidine nucleosides proved to be less efficient in terms of both yield and stereo-outcome. The formation of the ProTides discussed here (**8j** and **39**) was monitored by screening different reaction conditions. The general procedure consisted of the addition of a metal catalyst (0.2 eq) and a base (1.5 eq) to a solution of the nucleoside (50 mg, 1 eq) in an appropriate solvent (5 ml). The phosphorochloridate (1.2 eq) was then added dropwise (over 30 min) to the reaction mixture (**Scheme 2.12**).



R= H, F
B= pyrimidine nucelobase
R_1= TBDMS, Boc, Ac
R_2= *i*-Pr, Bn
MX_n= transition metal catalys

Scheme 2.12 Metal catalyst assisted diastereoselective synthesis of phosphoroamidate nucleosides.

After 16 hours at rt, the reaction progress was monitored by RP-HPLC. The starting nucleoside and the reference diastereomeric mixture HPLC traces were used as reference chromatograms. The picture reported in **Figure 2.6** is a general example of the analytical HPLC method applied for all the screenings discussed in the next paragraph. Based on

this chromatogram, we were able to determine the conversion of the starting nucleoside into the final product, and the diastereomeric ratio, by integration of the areas of the peaks of interest.

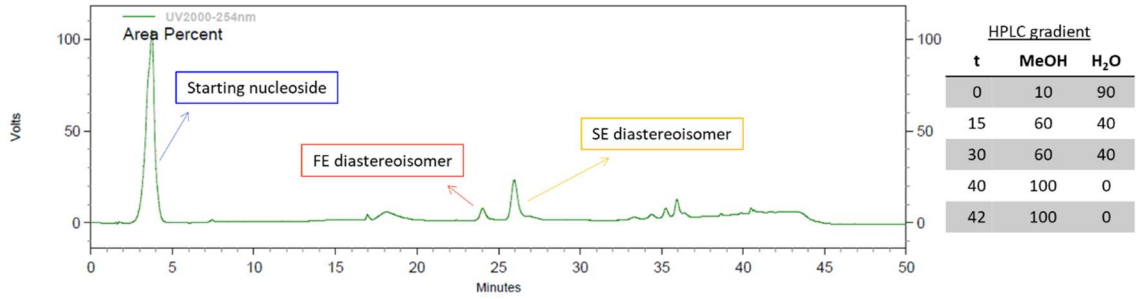
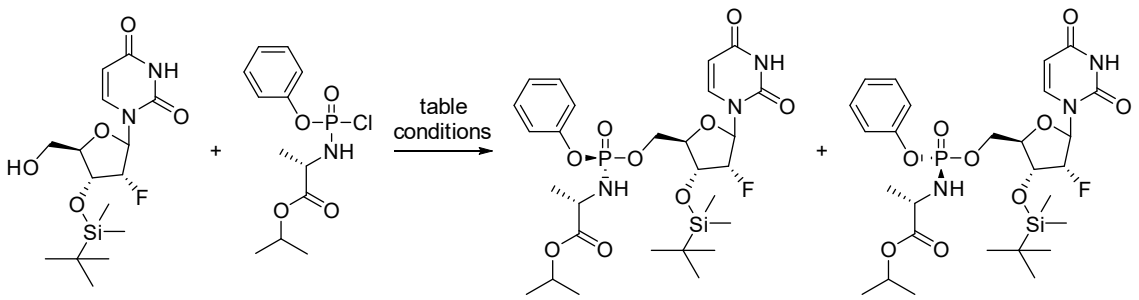


Figure 2.6 General example of an analytical HPLC trace of a crude mixture obtained by reacting a nucleoside with a phosphorochloridate in the presence of a transition metal catalyst and a base.

All the preliminary data were collected using the protected nucleosides **42-45**, **48** and **54** as substrates.

Metal catalysts screening

As an initial screening, a range of readily available metal catalysts was evaluated using the silylated 2'd-2'FU **43** as substrate for the delivery of the corresponding ProTide **59**. The metals selected presented different oxidation states as a parameter to evaluate. It is well known that divalent cations such as Mg^{2+} have a major role in the coordination of phosphorus compounds involved in biological processes. Among the metal ions evaluated, only Mg^{2+} (entry 5, **Table 2.1**) and Cu^{+} (entry 1 and 2, **Table 2.1**) were able to deliver **59**. According to these results, along with Pertusati's data, copper was the selected as the metal to be used for further investigations.



43 **22** **59-S_P** **59-R_P**

Entry	Catalyst	Base	Solvent	Conversion%*	FE/SE dr**
1	CuOAc	DIPEA	THF	6	1:2.4

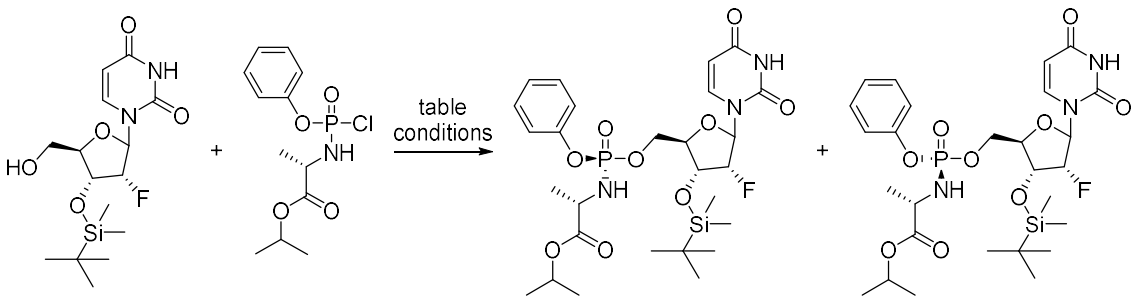
2	CuCl	DIPEA	THF	28	1:1.7
3	$RuCl_3 \cdot H_2O$	DIPEA	THF	0	-
4	$BiCl_3$	DIPEA	THF	0	-
5	$MgCl_2$	DIPEA	THF	12	1:2
6	$MgBr_2$	DIPEA	THF	0	-
7	$BH_3 \cdot NH(CH_3)_2$	DIPEA	THF	0	-

Table 2.1 Screening of different metal catalyst. Reagents and conditions: **43** (50mg, 1eq), **22** (1.2eq), catalyst (0.2eq), base (1.5eq), THF (5ml), rt, 16h. *Determined by HPLC analysis as ratio between the area of the peak of the starting nucleoside and the area of the peaks of the products (both diastereoisomers). **dr: diastereomeric ratio determined by HPLC ratio between the area of the Fast Eluting (FE) peak and area of the Slow Eluting (SE) peak.

Copper metal catalyst screening

We were interested in the evaluation of the oxidation state of the transition metal and the effect of the counterion on the catalytic activity. To this purpose, different copper (I) salts were investigated using the same reaction conditions applied in Pertusati's work, but focusing on the silylated 2'd-2'FU **43**.

Initially, we explored the use of both Cu(I) and Cu(II) and the corresponding results are reported in **Table 2.2** and **Table 2.3** respectively. Among Cu(I), using fluorotris(triphenylphosphine)copper and copper thiophenolate (entry 3 and 4, **Table 2.2**), both featured by the presence of hindered counterions, no conversion was observed. Copper (I) acetate (best copper catalyst for pyrimidine derivatives in Pertusati's work) and copper cyanide (entry 1 and 2, **Table 2.2**) delivered the final ProTide in low yields with poor diastereoselectivities. The use of copper (I) chloride (entry 5, **Table 2.2**) resulted into a moderate yield, but an inferior stereo-outcome was observed.



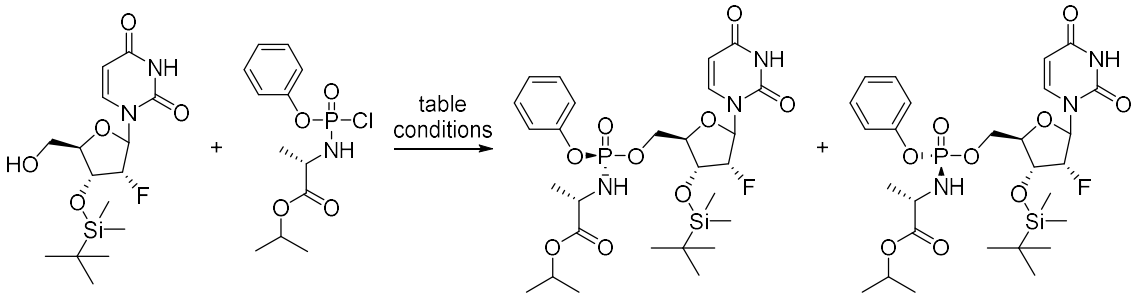
43 22 59-S_P 59-R_P

Entry	Catalyst	Base	Solvent	Conversion%*	FE/SE dr**
1	CuOAc	DIPEA	THF	6	1:2.4
2	CuCN	DIPEA	THF	8	1:2.2

3	$C_{54}H_{45}CuFP_3$	DIPEA	THF	0	-
4	C_6H_5SCu	DIPEA	THF	traces	-
5	CuCl	DIPEA	THF	28	1:1.7

Table 2.2 Screening of different Cu(I) salts. Reagents and conditions: **43** (50mg, 1eq), **22** (1.2eq), catalyst (0.2eq), base (1.5eq), THF (5ml), rt, 16h. *Determined by HPLC analysis as ratio between the area of the peak of the starting nucleoside and the area of the peaks of the products (both diastereoisomers). **dr: diastereomeric ratio determined by HPLC ratio between the area of the Fast Eluting (FE) peak and area of the Slow Eluting (SE) peak.

Concerning the Cu(II) species, we explored, copper monoxide, copper sulphate and its pentahydrate form (entry 7, 4 and 5, **Table 2.3**) but these did not lead to any product formation, with only starting material being observed in the crude mixtures. Low yields and poor diastereoselectivities were observed using copper (II) acetate and copper trifluoroacetate (entry 3 and 2, **Table 2.3**). Copper (II) triflate (best copper catalyst for purine derivatives in Pertusati's work) delivered the final product with moderate yield, but it was still the best one obtained in this category (entry 1, **Table 2.3**); whereas $Cu(HCO_2)_2$ (entry 6, **Table 2.3**) led to the greatest diastereoselectivity so far observed.

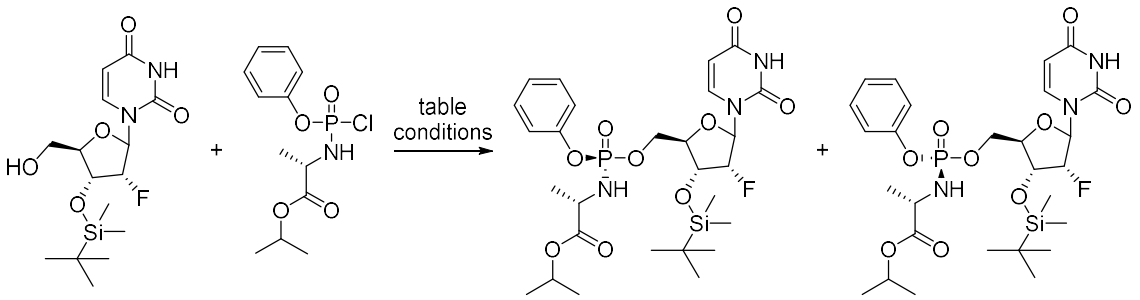


43 22 59-S_P 59-R_P

Entry	Catalyst	Base	Solvent	Conversion%*	FE/SE dr**
1	$Cu(OTf)_2$	DIPEA	THF	17	1:1.4
2	$Cu(CF_3CO_2)_2$	DIPEA	THF	4	1:2.2
3	$Cu(OAc)_2$	DIPEA	THF	12	1:2.1
4	$CuSO_4$	DIPEA	THF	0	-
5	$CuSO_4 \cdot 5H_2O$	DIPEA	THF	traces	-
6	$Cu(HCO_2)_2$	DIPEA	THF	4	1:3.3
7	CuO	DIPEA	THF	0	-

Table 2.3 Screening of different Cu(II) salts. Reagents and conditions: **43** (50mg, 1eq), **22** (1.2eq), catalyst (0.2eq), base (1.5eq), THF (5ml), rt, 16h. *Determined by HPLC analysis as ratio between the area of the peak of the starting nucleoside and the area of the peaks of the products (both diastereoisomers). **dr: diastereomeric ratio determined by HPLC ratio between the area of the Fast Eluting (FE) peak and area of the Slow Eluting (SE) peak.

Because of the better yield obtained with CuCl (entry 5, **Table 2.2**), other copper (I) halides were tested (**Table 2.4**). Except for the more electronegative fluoride atom (entry 3, **Table 2.4**), the other halides (entry 1, 2 and 4, **Table 2.4**) led to the desired product with improved yields when compared to the previously explored copper catalysts. Unfortunately, none of them showed a significant diastereoselectivity.



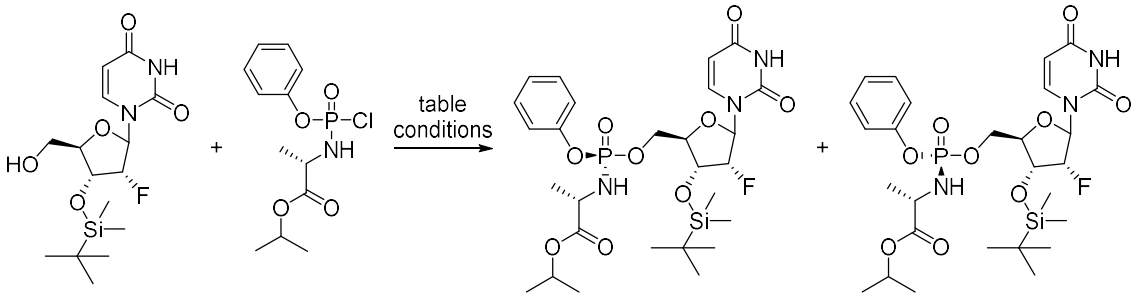
43 22 59-S_P 59-R_P

Entry	Catalyst	Base	Solvent	Conversion%*	FE/SE dr**
1	CuCl	DIPEA	THF	28	1:1.7
2	CuI	DIPEA	THF	25	1:1.6
3	CuF_2	DIPEA	THF	4	1:1.8
4	CuBr	DIPEA	THF	24	1:1.3

Table 2.4 Screening of different Cu halide. Reagents and conditions: **43** (50mg, 1eq), **22** (1.2eq), catalyst (0.2eq), base (1.5eq), THF (5ml), rt, 16h. *Determined by HPLC analysis as ratio between the area of the peak of the starting nucleoside and the area of the peaks of the products (both diastereoisomers). **dr: diastereomeric ratio determined by HPLC ratio between the area of the Fast Eluting (FE) peak and area of the Slow Eluting (SE) peak.

The stereoselectivity observed in Pertusati's work was obtained using ligandless copper (I) salts. However, ligands are reaction features commonly used to improve yields or stereoselectivities. According to the work of Jones and co-workers reported in paragraph 2.3, the phosphorylation of the starting alcohol was observed only when the Lewis acid was employed in presence of 0.2 equivalent of a chelating agent. A combination of $Cu(OTf)_2$ and *N*,*N*'-ethylenebis-(benzaldimine) (BEN) was demonstrated to be the optimal catalyst and ligand, respectively, in Jones methodology.[29] For this reason, the use of ligands and complexed copper catalysts was also investigated (**Table 2.5**). The addition of a ligand to the reaction mixture such as BEN (entry 1, **Table 2.5**) used in Jones' work,[29] resulted in the loss of activity of the copper acetate catalyst. Except for DABCO-CuCl (entry 3, **Table 2.5**), all the copper catalyst complexes explored were

able to deliver the final compound (2%-15% of conversion) with a poor stereo-outcome. Copper triflate benzene complex resulted the best among the complexed copper catalysts in terms of diastereoselectivity with FE/SE - 1:2.4 as the diastereomeric ratio (entry 2, **Table 2.5**).



43 22 59-S_P 59-R_P

Entry	Catalyst	Base	Solvent	Conversion%*	FE/SE dr**
1	CuOAc+BEN†	DIPEA	THF	0	-
2	Cu(OTf) C_6H_6	DIPEA	THF	15	1:2.4
3	DABCO-CuCl	DIPEA	THF	0	-
4	$Cu(CH_3CN)_4BF_4$	DIPEA	THF	10	1:1.7
5	$CuCl·(LiCl)_2$	DIPEA	THF	2	1:2.1
6	$Cu(NCCH_3)_4·CF_3SO_3$	DIPEA	THF	11	1:1.6

Table 2.5 Screening of different Cu catalysts complex. Reagents and conditions: **43** (50mg, 1eq), **22** (1.2eq), catalyst (0.2eq), base (1.5eq), THF (5ml), rt, 16h. *Determined by HPLC analysis as ratio between the area of the peak of the starting nucleoside and the area of the peaks of the products (both diastereoisomers). **dr: diastereomeric ratio determined by HPLC ratio between the area of the Fast Eluting (FE) peak and area of the Slow Eluting (SE) peak. †Addition of the ligand BEN (0.2eq).

In summary, the data obtained from the copper catalysts screening suggest that CuCl (entry 5, **Table 2.2**) and $Cu(HCO_2)_2$ (entry 6, **Table 2.3**) are the best copper salts in terms of conversion and diastereoselectivity, respectively.

Moreover, along with the catalysts, other conditions were examined, and the results are summarised here:

- Inert atmosphere. To evaluate how the copper oxidation state might influence the outcome, most of the reactions were performed both under argon atmosphere and in the presence of air. Literature data suggest that O_2 (present in air) may have a role as an oxidant in the copper catalytic cycle necessary for the successful outcome of the reaction.[53,54] However, in our investigation, no relevant differences were observed under the aerobic or inert conditions in terms of both

yield and stereoselectivity. As a consequence, an argon atmosphere was employed as a constant parameter to prevent the phosphorochloridate degradation.

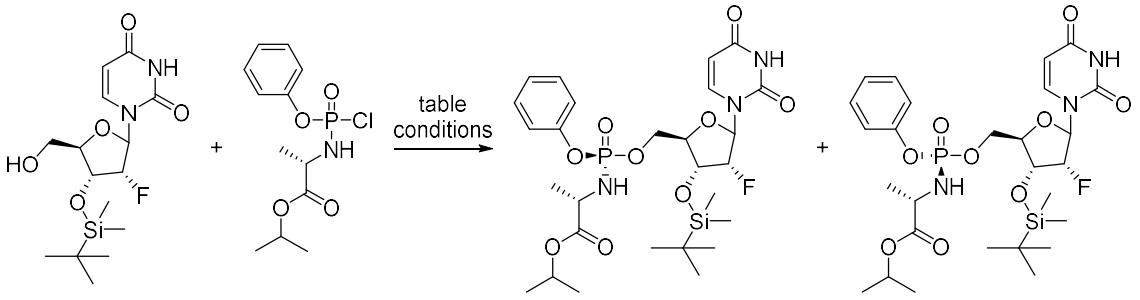
- Temperature. It is a parameter important to evaluate in connection with the solubility of the selected nucleosides. Moreover, given that different diastereoisomers may react at different rates, by tuning the temperatures we expected to enhance the stereo-selection of the phosphorochloridates. Both higher (up to 90°C) and lower (down to 0°C) temperatures were examined. Except for the phosphorochloridate's degradation over 70°C, only irrelevant variations were observed.
- Timing. Most of the reactions were checked using HPLC at different times while the reaction was ongoing. The term of 16h proved to be the most reliable threshold that ensure the reaction completion. Moreover, it was observed that the ratio between the diastereoisomers was not altered with longer reaction times.

From the data so far reported, CuCl was assessed as the catalyst able to deliver the final ProTide mixture with the best yield (28%). For this reason, further investigations were undertaken by keeping the copper halide as catalyst, while other parameters were explored.

Base screening

The base plays a crucial role in this metal catalysed phosphorylation. Its primary function is clearly to trap the HCl which inevitably is formed upon displacement of the chloride ion from the phosphorochloridate. Keeping CuCl as catalyst, different bases (**Table 2.6**) were tested in attempts to improve the diastereoselective ratio. As it is possible to observe from the table, different amines were employed considering their ability to coordinate the copper.

Among the bases, only using Et_3N (entry 2, **Table 2.6**) were comparable data to the ones previously obtained with DIPEA (entry 5, **Table 2.2**) observed. All the other bases tested, including the chiral cinchonidine, led only to traces of the desired product.



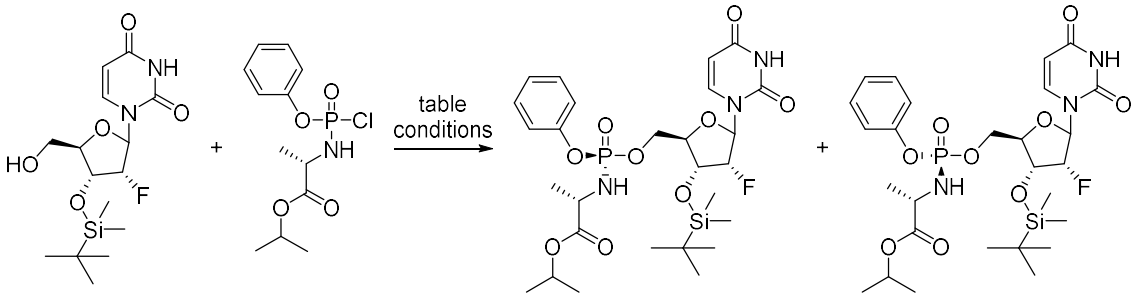
43 22 59-S_P 59-R_P

Entry	Catalyst	Base	Solvent	Conversion%*	FE/SE dr**
1	CuCl	DMAP	THF	3	1:1.2
2	CuCl	Et_3N	THF	27	1:1.2
3	CuCl	cinchonidine	THF	traces	-
4	CuCl	NMI	THF	3	1:1.2

Table 2.6 Screening of different bases using CuCl as catalyst. Reagents and conditions: **43** (50mg, 1eq), **22** (1.2eq), catalyst (0.2eq), base (1.5eq), THF (5ml), rt, 16h. *Determined by HPLC analysis as ratio between the area of the peak of the starting nucleoside and the area of the peaks of the products (both diastereoisomers). **dr: diastereomeric ratio determined by HPLC ratio between the area of the Fast Eluting (FE) peak and area of the Slow Eluting (SE) peak.

Solvent screening

Since phosphorylation reactions are usually performed using THF as solvent, we selected THF as the preferred reaction medium. However, the solvent may also play a crucial role in the stereochemical outcome of the reaction. *N*-donor coordinating solvents such as ACN or pyridine were explored to evaluate if the potential interaction between the medium and the metal cation may lead to some degree of stereoselectivity. Alternatively, also dimethoxyethane (DME) and dioxane were analysed. The solvent variations showed some diversification in terms of results. Indeed, both ACN and pyridine (entry 2 and 3, **Table 2.7**), which are able to complex the copper salts, led to greater conversions (30-35%), but inferior diastereoselective ratios (1:1.1) when compared with THF (entry 5, **Table 2.2**). Interestingly, the use of 1,4-dioxane (entry 4, **Table 2.7**) induced a slight reversion of the diastereoselectivity in favour of the FE isomer. Otherwise, DME performed as THF, and similar results were detected (entry 1, **Table 2.7**).



43 22 59-S_P 59-R_P

Entry	Catalyst	Base	Solvent	Conversion%*	FE/SE dr**
1	CuCl	DIPEA	DME	25	1:1.6
2	CuCl	DIPEA	ACN	30	1:1.1
3	CuCl	DIPEA	Pyridine	35	1:1.1
4	CuCl	DIPEA	Dioxane	5	1:0.7

Table 2.7 Screening of different solvents using CuCl as catalyst. Reagents and conditions: **43** (50mg, 1eq), **22** (1.2eq), catalyst (0.2eq), base (1.5eq), solvent (5ml), rt, 16h. *Determined by HPLC analysis as ratio between the area of the peak of the starting nucleoside and the area of the peaks of the products (both diastereoisomers). **dr: diastereomeric ratio determined by HPLC ratio between the area of the Fast Eluting (FE) peak and area of the Slow Eluting (SE) peak.

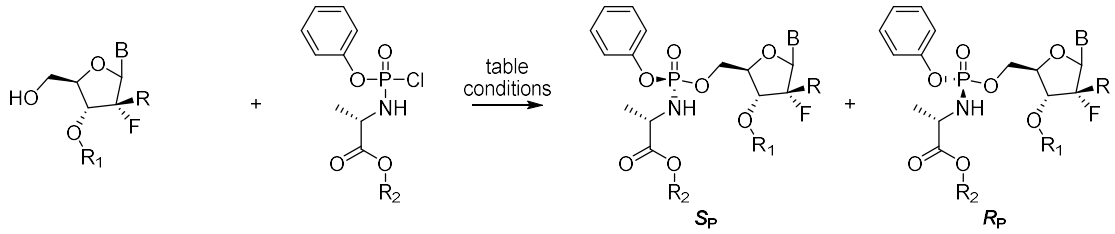
In summary, according to the results obtained, DIPEA and DME were the base and solvent selected for the following investigations.

Evaluation of the best copper catalysts with the protected nucleosides

All the parameters analysed up to this point (catalyst, base and solvent) were evaluated on the 2'd-2'FU 3'-sylilated **43**. A factor to be investigated was the diversification of the protecting group in the 3'-position of both the reference nucleosides (**1k** and **36**). To do that, four copper catalysts were selected:

- CuOAc and $Cu(OTf)_2$ were the catalysts of choice in Pertusati's work, and their ability to deliver the final ProTide was also confirmed in the current investigation.
- CuCl proved to lead to the greatest conversion of the nucleoside to the desired ProTide although with low diastereoselectivity.
- $Cu(HCO_2)_2$ was demonstrated to be able to increase the diastereoselective ratio in favour of the desired SE isomer, although it featured little conversion.

The four selected catalysts were then employed in the optimised reaction conditions (DIPEA, DME, 16h, rt) in the presence of **42-45**, **48** and **54** and the appropriate phosphorochloridate (**22** or **58**) (**Table 2.8**).



43 B= ura, R= H, R_1= TBMS
45 B= ura, R= H, R_1= BOC
48 B= ura, R= H, R_1= Ac
42 B= cyt, R= F, R_1= TBMS
44 B= cyt, R= F, R_1= BOC
54 B= cyt-NHAc, R= F, R_1= Ac

22 R_2= *i*-Pr
58 R_2= Bn

59 B= ura, R= H, R_1= TBMS, R_2= *i*-Pr
60 B= ura, R= H, R_1= BOC, R_2= *i*-Pr
61 B= ura, R= H, R_1= Ac, R_2= *i*-Pr
62 B= cyt, R= F, R_1= TBMS, R_2= Bn
63 B= cyt, R= F, R_1= BOC, R_2= Bn
64 B= cyt-NHAc, R= F, R_1= Ac, R_2= Bn

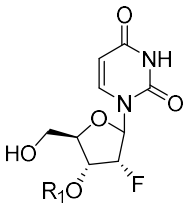
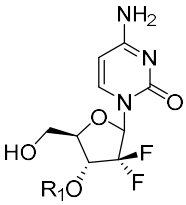
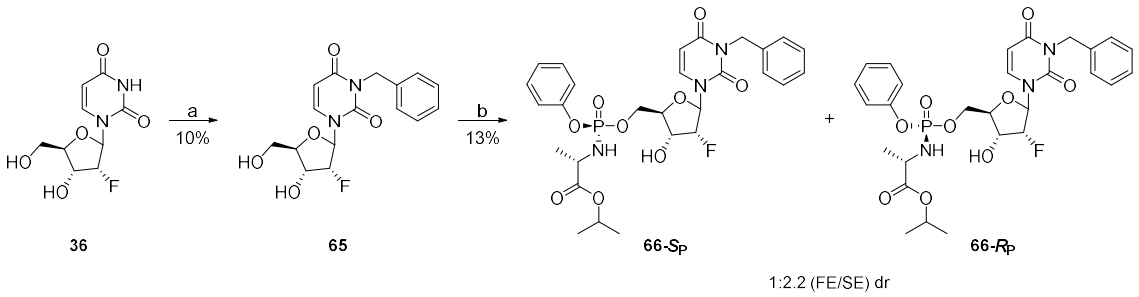
Nucleoside	3'-Protecting group(R_1)	Catalyst	Conversion%*	FE/SE dr**
 *2'd-2'FU*	TBMS (**43**)	CuOAc	6	1:2.4
		$Cu(OTf)_2$	17	1:1.4
		CuCl	28	1:1.7
		$Cu(HCO_2)_2$	4	1:3.3
	BOC (**45**)	CuOAc	10	1:2
		$Cu(OTf)_2$	15	1:1.2
		CuCl	18	1:1.8
		$Cu(HCO_2)_2$	traces	-
	Ac (**48**)	CuOAc	12	1:2
		$Cu(OTf)_2$	10	1:1.2
		CuCl	41	1:1.8
		$Cu(HCO_2)_2$	8	1:2.1
 *dFdC*	TBMS (**42**)	CuOAc	37	1:1.5
		$Cu(OTf)_2$	28	1:1.1
		CuCl	27	1:1.1
		$Cu(HCO_2)_2$	3	1:2.3
	BOC (**44**)	CuOAc	17	1:1.7
		$Cu(OTf)_2$	18	1:2
		CuCl	26	1:1.6
		$Cu(HCO_2)_2$	4	1:2.2
	Ac (**54**)	CuOAc	18	1:2.5
		$Cu(OTf)_2$	18	1:1.7
		CuCl	40	1:1.2
		$Cu(HCO_2)_2$	8	1:2.7

Table 2.8 Screening of different 3'-protecting groups on nucleoside **36** and **1k**. Reagents and conditions: 3'-protected nucleoside (50mg, 1eq), phosphorochloridate (1.2eq), catalyst (0.2eq), DIPEA (1.5eq), DME (5ml), rt, 16h. *Determined by HPLC analysis as ratio between the area of the peak of the starting nucleoside and the area of the peaks of the products (both diastereoisomers). **dr: diastereomeric ratio determined by HPLC ratio between the area of the Fast Eluting (FE) peak and area of the Slow Eluting (SE) peak.

These results showed that the nature of the nucleoside (uracil or cytosine) was not significantly influencing the yield or the stereo outcome of the reaction. Overall, the data suggested that the results gained with all three different protecting groups reflected the outcomes obtained previously using 3'-sylilated 2'd-2'FU **43**. Indeed, CuCl was confirmed to be the catalyst providing the best yield and $Cu(HCO_2)_2$ the one delivering the final ProTide with the best diastereoselectivity. Notably, in the presence of the less hindered acetyl protecting group, $Cu(HCO_2)_2$ led to a slight improvement of the conversion (8% for both **48** and **54**) although this was accompanied by an inferior diastereoselectivity concerning the uracil derivative **48**.

Moreover, it was possible to notice that the partial protection of the amino group obtained during the cytosine acetylation (**54**, **Scheme 2.9**) might not influence the activity of the catalysts. These data suggested that the nitrogen of the amino group was not exerting a poisoning effect on the metal catalyst. To further confirm this hypothesis, the protection of the *NH* group in the uracil nucleobase was also evaluated. The metal catalyst conditions were then applied on the 2'd-2'FU with the N^3 position protected with a benzyl group (**Scheme 2.13**).



Scheme 2.13 Preparation of the 2'd-2'FU N^3-protected nucleoside **65** and corresponding ProTide **66**. Reagents and conditions: a) benzyl bromide, K_2CO_3, DMF/Acetone (1:1, v/v), reflux temperature, 4h; b) phosphorochloridate **22** (1.2eq), CuOAc (0.2eq), DIPEA (1.5eq), DME rt, 16h.

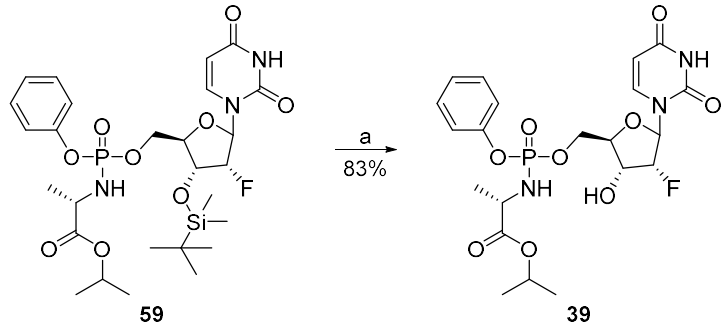
Using **65** as substrate, a conversion of 13% and a diastereomeric ratio of 1:2.2 (FE/SE) were detected, confirming that the *NH* group of the nucleobase was not significantly

affecting the catalyst activity. For this reason, no other N^3-protecting group were investigated.

Additionally, as a general summary of the above screenings, it was possible to assume that the presence of a protecting group in the 3'-position of the nucleoside did not positively affect either the yield or the stereo-outcome of the reaction. It can be supposed that the increasing of the steric hindrance around the 3'-position may impede the proper interaction of the metal catalyst or the copper-coordinated phosphoroamidate with the nucleoside.

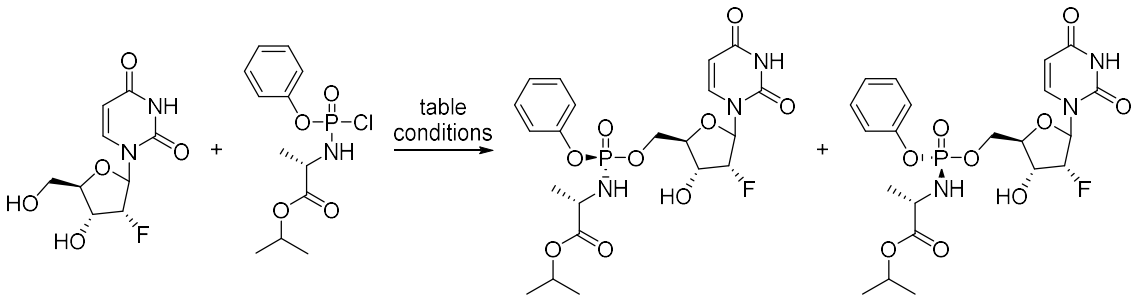
Base and solvent screening on unprotected nucleoside 36

Because of the presence of a protecting group in the 3'-position proved to be ineffective of improving both stereoselectivity and yield for the delivery of the final ProTides when compared with Pertusati's results on unprotected nucleosides, further parameter screening was performed on the unprotected uracil derivative **36**. For this reason, **59** was deprotected using TFA in dichloromethane (1:1, v/v) at room temperature to afford, after 2h, the diastereoisomeric mixture of the desired ProTide **39** in 83% yield in order to have a reference compound for successive HPLC analyses (**Scheme 2.14**).



Scheme 2.14 3'-Deprotection of ProTide **59**. Reagents and conditions: a) CH_2Cl_2:TFA (1:1, v/v), 0°C to rt, 2h.

Therefore, few bases were screened (**Table 2.9**). No significant differences were noticed with the results obtained on the 3'-sylilated protected nucleoside **43**. Therefore, DIPEA was still assessed as the preferred base.



36 22 39-S_P 39-R_P

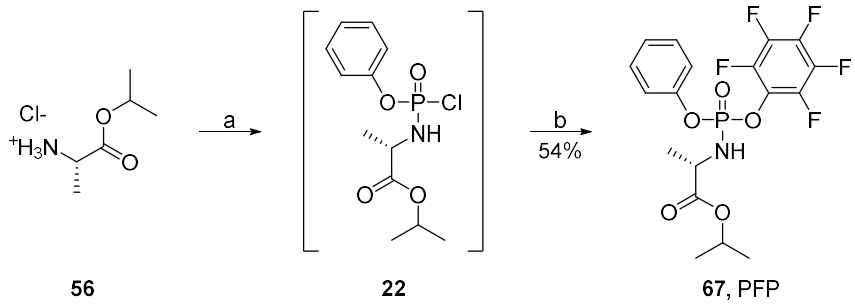
Entry	Catalyst	Base	Solvent	Conversion%*	FE/SE dr**
1	CuOAc	NMI	THF	8	1:1
2	CuOAc	DIPEA	DME	16	1:3
3	CuOAc	quinine	DME	20	1:1.4
4	CuOAc	Quinine+DIPEA	DME	25	1:1.1

Table 2.9 Screening of different bases using CuOAc as catalyst. Reagents and conditions: **36** (50mg, 1eq), **22** (1.2eq), catalyst (0.2eq), base (1.5eq), THF (5ml), rt, 16h. *Determined by HPLC analysis as ratio between the area of the peak of the starting nucleoside and the area of the peaks of the products (both diastereoisomers). **dr: diastereomeric ratio determined by HPLC ratio between the area of the Fast Eluting (FE) peak and area of the Slow Eluting (SE) peak.

Phosphoroamidating agents screening

Phosphorochloridate (P-Cl) was the agent until now adopted in the screenings. However, the phosphoroamidating agent itself is a parameter to be taken into account during the diastereoselective synthesis investigations. Indeed, both Ross[24] and Simmons[55] adopted the pentafluorophenyl phosphate (PFP) as a single diastereoisomer in their ProTide synthetic methodologies. With the purpose of testing different phosphoroamidating agents with increased electrophilicity at the phosphorus atom, PFP and *p*-nitrophenyl phosphate (pNO_2P) were first synthesised and then applied in the diastereoselective investigations here discussed.

PFP was prepared using pentafluorophenol and Et_3N at 0°C either directly from the phosphorochloridate **22** previously prepared, or from the amino acid ester **56**, synthetizing the phosphorochloridate *in situ* (**Scheme 2.15**).



56 22 67, PFP

Scheme 2.15 Preparation pf the phosphoroamidating agent PFP (**67**). Reagents and conditions: a) phenyl dichlorophosphate, Et_3N, CH_2Cl_2, -78°C 30 min to rt 1h; b) pentafluorophenol, Et_3N, CH_2Cl_2, 0°C, 3h.

The reactions monitored by ^{31}P NMR showed the presence of the two diastereoisomers in the mixture (in $CDCl_3$ δ_P -1.62, -1.69 ppm) with several by-products. The problem linked to this procedure was the work-up of the mixture. According to Ross and co-workers,[24] the precipitated triethylamine hydrochloride salt was filtered off and the filtrate was concentrated under reduced pressure and then treated with *tert*-butyl methyl ether (MTBE) to further precipitate triethylamine hydrochloride salt. After an additional filtration, the filtrate was evaporated affording the clean diastereoisomer mixture as a white solid. In our hands this work-up did not work. Under the above conditions we observed the decomposition of one of the two diastereoisomers (**Figure 2.7**, A). Nevertheless, diethyl ether was also used in the trituration step, but then again leading to the same result: poor yield of only one of the two diastereoisomers along with several by-products. Looking for an alternative work-up, a literature search brought to the light a patent of 2012. According to this procedure, a simple washing of the crude mixture with water was useful to remove the salts present in the reaction mixture.[56] However, also in this case the reported procedure did not give the desired result. After several attempts we discovered that only a very quick wash with water was necessary to avoid the degradation of one of the two diastereoisomers. (**Figure 2.7**, B).

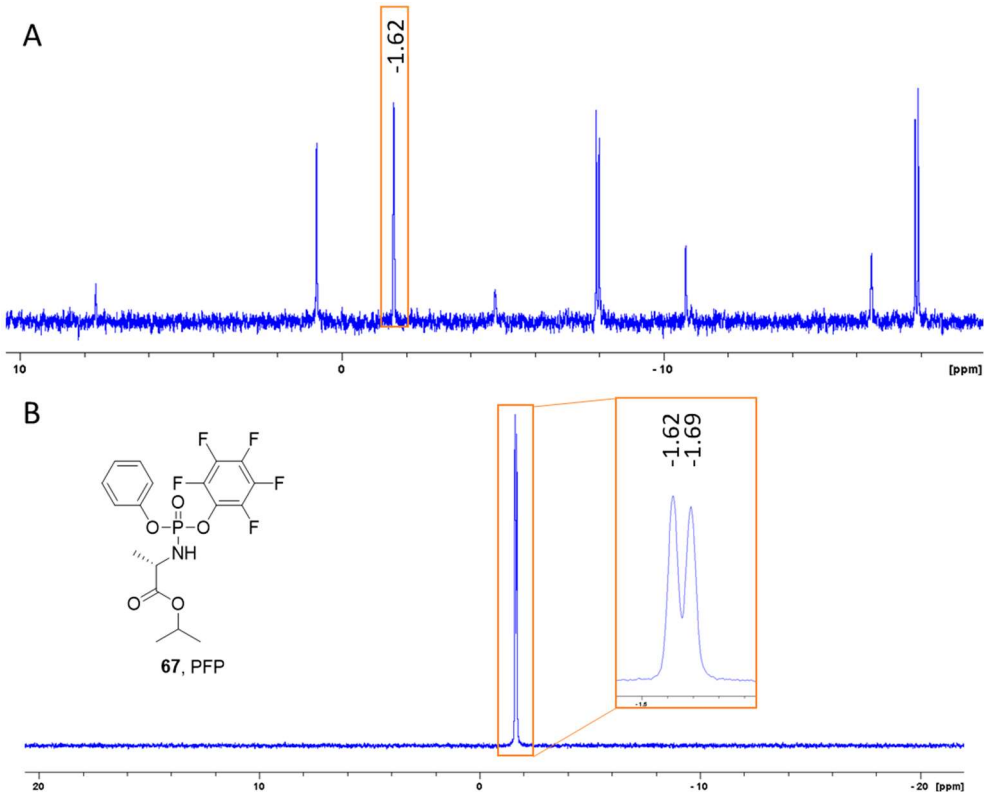
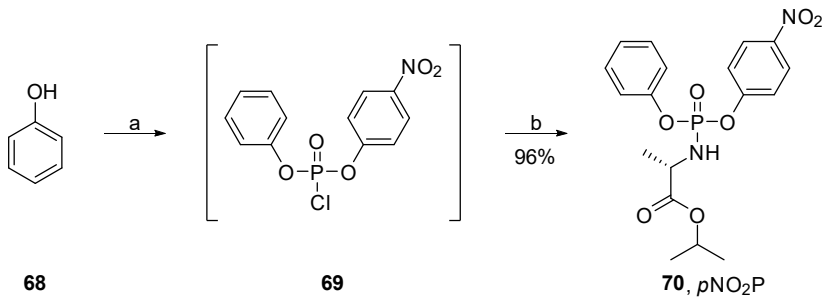


Figure 2.7 ^{31}P-NMR (202 MHz, $CDCl_3$) of PFP (**67**) after two different work ups. The upper spectrum A shows the result after the trituration with MTBE (only one diastereoisomer along with by-products). The bottom spectrum B shows the two clean diastereoisomers after a quick wash with water.

Contrarily to the PFP synthesis, the *p*NO_2P preparation was much more straightforward (**Scheme 2.16**). It started from the substitution of the commercially available *p*-nitrophenyl phosphorodichloridate with the appropriate phenol in the presence of anhydrous Et_3N.



Scheme 2.16 Preparation of the phosphoroamidating agent *p*NO_2P (**70**). Reagents and conditions: a) 4-nitrophenyl phosphorodichloridate, Et_3N, CH_2Cl_2, -78°C 30 min; b) *L*-alanine isopropyl ester hydrochloride salt, Et_3N, CH_2Cl_2, 0°C, 30 min.

The formation of the corresponding phosphorochloridate **69** was confirmed by ^{31}P NMR spectroscopy after 1h at -78°C. The addition of the amino acid ester salt and a further 2

equivalent of Et_3N led to the desired pNO$_2$P (in $CDCl_3$ the singlet of δ_P -5.63 ppm corresponding to **69** disappears and two new singlets at δ_P -3.11 and -3.14 ppm corresponding to **70** appear) after 1h at 0°C (**Figure 2.8**).

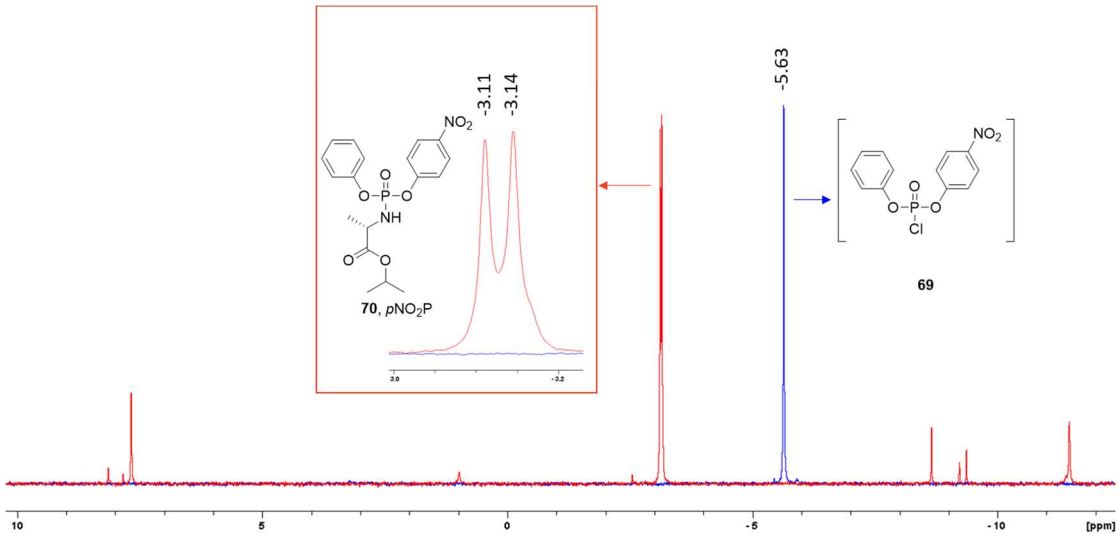
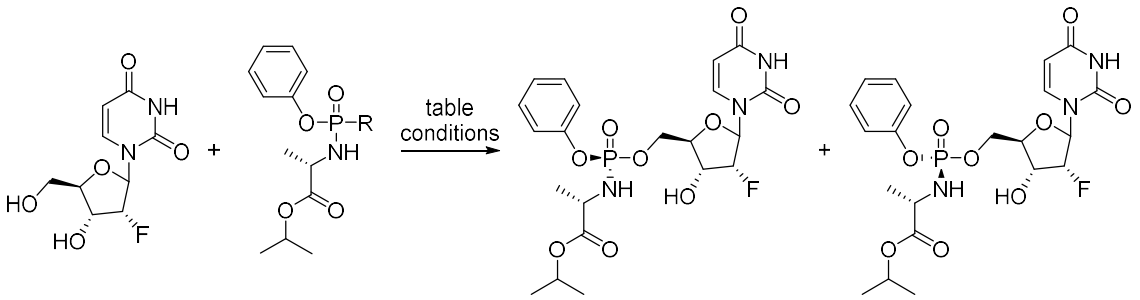


Figure 2.8 ^{31}P-NMR (202 MHz, $CDCl_3$) of the phosphorochloridate intermediate **69** (singlet in blue) and the final pNO$_2$P (**70**) diastereoisomers (spectrum in red).

With the phosphoroamidating agents PFP and pNO$_2$P (used as diastereoisomeric mixtures) in hand, the diastereoselective synthesis was explored using several reaction conditions. Surprisingly, when the common procedure (copper catalyst, DIPEA, DME, rt, 16h) was attempted (entry 1 and 2, **Table 2.10**) using two of the best catalysts until now tested (CuCl and CuOAc), no traces of the product were detected. For this reason, we focused our attention on a recent work reported by Simmons and co-workers.[55] They investigated the use of Lewis acid to activate the electrophilic phosphorylating reagent in the presence of weak bases, improving the regioselectivity of the reaction. They were able to maximize the yield of the desired product avoiding its consumption by the formation of the bis product by using Me_2AlCl and pyridine at rt for 16h. Looking for an improvement in the diastereoselectivity, we attempted the use of pyridine as both solvent and base. In the presence of CuOAc as catalyst, 5% of conversion was detected with PFP as phosphoroamidating agent (entry 3, **Table 2.10**), whereas only traces of the desired product were observed when pNO$_2$P was adopted (entry 4, **Table 2.10**). Under these same settings, Cu formate (entry 5 and 6, **Table 2.10**) did not lead to any product formation, and only starting material was detected in the crude mixtures. According to Simmons,[55] Me_2AlCl proved to be an interesting catalyst, able to deliver the final ProTide in the presence of pyridine. The paper's exact conditions were then applied on our substrates

(entry 7 and 8, **Table 2.10**) and only when PFP was selected as phosphate source, the desired product was detected with a very good conversion (40%). Entry 3 and entry 7 (**Table 2.10**) were the only reaction conditions that allowed the delivery of the desired product.



36 **67** R= PFPh, **70** R= *p*NO_2Ph **39-S_P** **39-R_P**

Entry	Catalyst	P-source	Base	Solvent	Conversion%*	FE/SE dr**
1	CuCl	PFP	DIPEA	DME	0	-
2	CuOAc	PFP	DIPEA	DME	0	-
3	CuOAc	PFP	-	Py	5	1:0.8
4	CuOAc	*p*NO_2P	-	Py	traces	-
5	Cu formate	PFP	-	Py	0	-
6	Cu formate	*p*NO_2P	-	Py	0	-
7	Me_2AlCl	PFP	-	Py	40	1:0.7
8	Me_2AlCl	*p*NO_2P	-	Py	0	-

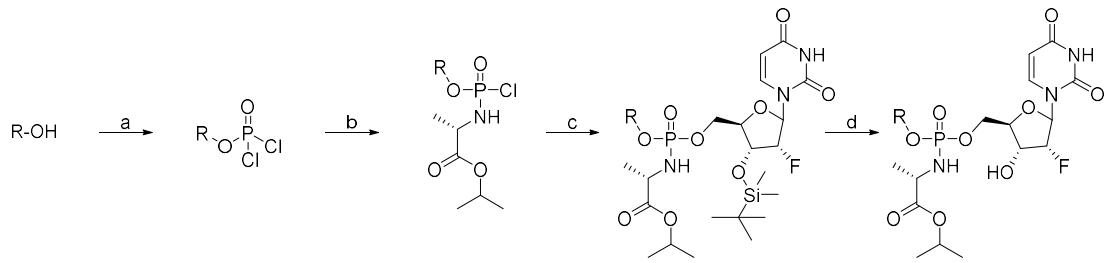
Table 2.10 Screening of PFP (**67**) and *p*NO_2P (**70**) as phosphoroamidating agents in different reaction conditions. Reagents and conditions: **36** (50mg, 1eq), phosphoroamidating agent (1.2eq), catalyst (0.2eq), base (1.5eq, where applicable), solvent (5ml), rt, 16h. *Determined by HPLC analysis as ratio between the area of the peak of the starting nucleoside and the area of the peaks of the products (both diastereoisomers). **dr: diastereomeric ratio determined by HPLC ratio between the area of the Fast Eluting (FE) peak and area of the Slow Eluting (SE) peak.

According to the data obtained from the screening of different phosphoroamidating agents, the phosphorochloridates (**22** and **58**) continued to be the phosphate source of choice in the ProTide diastereoselective synthesis investigation herein reported.

Aryl group screening

Once the unprotected nucleoside **36** was selected as the key substrate for further investigations, a direct comparison with Pertusati's data on the same nucleoside was

crucial. As the data suggested, although the same conditions (CuOAc, DIPEA, DME, rt, 16h) were applied, still both the yield and the stereo outcome of the reaction were inferior to Pertusati's published results. The only difference between the two works was evidently the aryl group in the ProTide moiety. According to this consideration, the investigation of the aromatic portion of the molecule was then undertaken to explore the influence of the aryl group on the stereo-outcome of the reaction. Indeed, the naphthyl group present in Pertusati's substrate was examined along with other hindered aromatic moieties, such as tetrahydro naphthyl and alkylated phenyl rings (**37**, **79-81**). The synthesis of these phosphoroamidating agents is shown in **Scheme 2.17**. The procedure for the phosphorochloridate preparation already explained in **Scheme 2.10** was applied, but in this case the desired substituted aryl phosphorodichloridate (**75-78**) was synthesised starting from the corresponding aryl alcohol (**71-74**).



71 R= CF_3-*o*-Ph	**75** R= CF_3-*o*-Ph 98%	**79** R= CF_3-*o*-Ph 92%	**82** R= CF_3-*o*-Ph 40%	**86** R= CF_3-*o*-Ph 63%
72 R= *t*Bu-*o*-Ph	**76** R= *t*Bu-*o*-Ph 84%	**80** R= *t*Bu-*o*-Ph 61%	**83** R= *t*Bu-*o*-Ph 42%	**87** R= *t*Bu-*o*-Ph 64%
73 R= THNaph	**77** R= THNaph 96%	**81** R= THNaph 87%	**84** R= THNaph 47%	**88** R= THNaph 66%
74 R= Naph	**78** R= Naph 98%	**37** R= Naph quant.	**85** R= Naph 44%	**38** R= Naph 85%

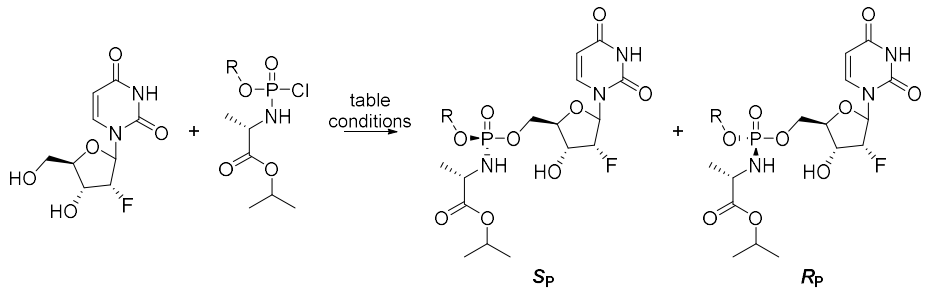
Scheme 2.17 Preparation of aryl substituted phosphorochloridates **37**, **79-81** and corresponding ProTides **38**, **86-88**. Reagents and conditions: a) $POCl_3$, Et_3N, Et_2O, -78°C to rt, 1h; b) *L*-alanine isopropyl ester hydrochloride salt, Et_3N, CH_2Cl_2, -78°C to rt, 2h; c) **43**, *t*BuMgCl (1M in THF), THF, rt, 16h; d) CH_2Cl_2:TFA (1:1, v/v), 0°C to rt, 2h.

After the conventional non-stereoselective procedure was applied using **37**, **79-81** to afford the final ProTides **38**, **86-88**, their HPLC reference peaks were recorded. The use of these phosphorochloridates was examined in the diastereoselective synthesis using the standard conditions (DIPEA, DME, rt, 16h), with CuOAc as catalyst of choice of Pertusati's work for pyrimidine nucleosides.

For what concern the phenyl moieties bearing an alkyl group (**79**, **80**), the presence of an electron withdrawing group as the CF_3 (entry 2, **Table 2.11**) provided a better yield when compared with the unsubstituted phenoxy (entry 1, **Table 2.11**). Whereas, the (mild) electron donor *t*-butyl group (entry 3, **Table 2.11**) was unfavourable for the delivery of the ProTide. The reason could be the increased electrophilicity of the phosphorus atom

when an electron withdrawing group is applied. However, both the phenoxy substituted systems led to inferior diastereomeric ratios.

When chloridate **37** bearing a naphthyl moiety (entry 4, **Table 2.11**) was finally used, much more similar results to Pertusati's data were obtained, proving the crucial role of the aryl moiety in the diastereoselective synthesis. Moreover, the poorer results observed using the 5,6,7,8-tetrahydro-1-naphthyl group **81** (entry 5, **Table 2.11**) demonstrated that the steric hinderance of the naphthyl moiety is crucial as well as its electronic contribution. The reason could be that both these parameters may play an important role in the potential interactions established during the transition state of the reaction, although a definitive explanation cannot be given.



36

22 R= Ph
79 R= CF_3-*o*-Ph
80 R= *t*Bu-*o*-Ph
81 R= THNaph
37 R= Naph

39 R= Ph
86 R= CF_3-*o*-Ph
87 R= *t*Bu-*o*-Ph
88 R= THNaph
38 R= Naph

Entry	P-Cl	Conversion%*	FE/SE dr**
1	**22**	16	1:3
2	**79**	25	1:1.8
3	**80**	5	1:2.5
4	**37**	14	1:5
5	**81**	7	1:4

Table 2.11 Screening of different phosphorochloridates diversified at the aromatic moiety. Reagents and conditions: **36** (50mg, 1eq), phosphoroamidating agent (1.2eq), CuOAc (0.2eq), DIPEA (1.5eq), DME (5ml), rt, 16h. *Determined by HPLC analysis as ratio between the area of the peak of the starting nucleoside and the area of the peaks of the products (both diastereoisomers). **dr: diastereomeric ratio determined by HPLC ratio between the area of the Fast Eluting (FE) peak and area of the Slow Eluting (SE) peak.

According to the aforementioned data, the naphthyl group was then selected as the preferred aryl moiety for the subsequent investigations.

Chiral bases screening

Chiral amines are commonly used as chiral bases for asymmetric synthesis or for resolving racemic mixtures.[57,58] Since the amine DIPEA was the base that provided the most successful delivery of the final ProTide, also three chiral amines were then evaluated in the diastereoselective ProTide synthesis (**Figure 2.9**).

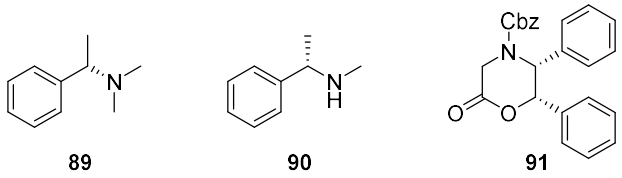
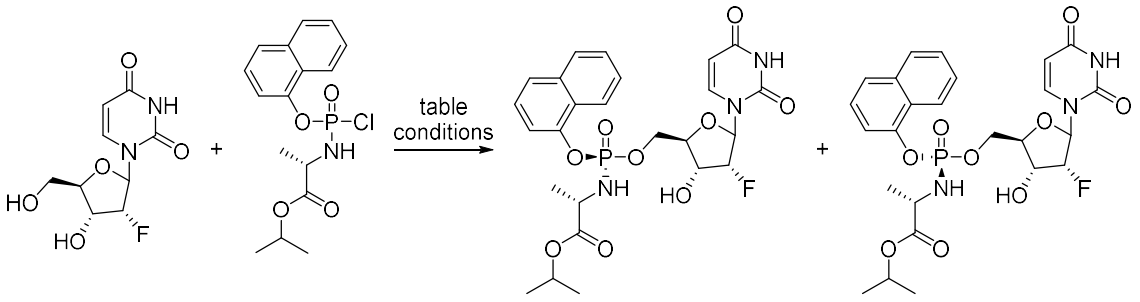


Figure 2.9 Chiral amines selected for diastereoselective investigations.

(*S*)-*N*,*N*-Dimethyl-1-phenylethylamine (**89**) was able to deliver the ProTide with good yield and a moderate FE/SE ratio (entry 1, **Table 2.12**). Whereas, (*S*)-*N*,α-dimethylbenzylamine (**90**) (entry 2, **Table 2.12**) gave poorer results in terms of both conversion and stereo outcome. Unfortunately, the cyclic carbamate (2*S*,3*R*)-*N*-*Z*-6-oxo-2,3-diphenylmorpholine (**91**) (entry 3, **Table 2.12**) was unsuccessful, affording only traces of the desired product. The 3'-protected nucleoside **43** was also used with the purpose of improving the diastereoselective ratio provided by **89** (entry 4, **Table 2.12**). The base was found to lose completely its activity when the hindered nucleoside was applied. Otherwise, a slight improvement in the FE/SE ratio was detected when **89** was evaluated in the presence of Cu formate (entry 5, **Table 2.12**).



Entry	Catalyst	Base	Solvent	Conversion%*	FE/SE dr**
1	CuOAc	**89**	DME	52	1:2.9
2	CuOAc	**90**	DME	10	1:1.7
3	CuOAc	**91**	DME	traces	-

†4	CuOAc	**89**	DME	traces	-
5	Cu formate	**89**	DME	14	1:4

Table 2.12 Screening of different chiral amines. Reagents and conditions: **36** (50mg, 1eq), **37** (1.2eq), CuOAc (0.2eq), DIPEA (1.5eq), DME (5ml), rt, 16h. *Determined by HPLC analysis as ratio between the area of the peak of the starting nucleoside and the area of the peaks of the products (both diastereoisomers). **dr: diastereomeric ratio determined by HPLC ratio between the area of the Fast Eluting (FE) peak and area of the Slow Eluting (SE) peak. †Attempt made on the 3'-OTBDMS protected **43**.

Miscellaneous attempts under microwave irradiation

Microwave (MW) assisted organic synthesis is today a powerful technology that can lead to cleaner and faster reactions when compared with conventional heating. Nowadays this technology is proving to be extremely useful and efficient when applied to stereoselective organic synthesis.[59]

With these considerations in mind, several reaction conditions already examined at room temperature or with conventional heating were also performed using microwave irradiations. **Table 2.13** shows the different conditions examined.

A crucial parameter for a successful procedure is the ability of reagents or solvents to respond to MW energy. For this reason, solvents suitable for MW irradiations and compatible with the reagents in use were examined. When polar solvents such as DMF or NMP (entry 1 and 2, **Table 2.13**) were applied, no product was detected. Contrarily, DME (entry 3, **Table 2.13**) proved again to successfully deliver the final ProTide with results very similar in terms of both yield and diastereoselectivity to those observed in conventional conditions. These data suggested that the microwave irradiation may not influence the reaction outcome since DME is not a very good microwave-absorbing solvent. Temperature (70°C) and timing (1h) were selected since ideally well tolerated by the phosphorochloridate, which is the component of the reaction mixture that the easiest might get degraded. For what concern the timing of the reaction, the microwave irradiations provided the maximum conversion reachable under these conditions in only 15 minutes (entry 4, **Table 2.13**). Higher temperatures were also evaluated (entry 5, **Table 2.13**). At 100°C no final ProTide was observed, but only starting nucleoside, maybe due to the degradation of the phosphoroamidating agent. The catalysts that were found to be the most successful under conventional conditions were also tested (entry 6-9, **Table 2.13**). None of them showed a significantly improved activity when exposed to microwave energy. Regarding the base, the chiral amine **89** showed a similar stereo outcome, but a much worse yield when compared with the results obtained using standard settings. The reaction was also performed in the presence of the PFP phosphoroamidating

agent. As expected, no product formation was detected when DIPEA and DME were applied (entry 11, **Table 2.13**), while a moderate FE/SE ratio was displayed using pyridine and Me_2AlCl (entry 12, **Table 2.13**).

Entry	Catalyst	Base	Solvent	Conditions	Conversion%*	FE/SE dr**
1	CuOAc	DIPEA	DMF	70°C, 1h	0	-
2	CuOAc	DIPEA	NMP	70°C, 1h	0	-
3	CuOAc	DIPEA	DME	70°C, 1h	10	1:2.5
4	CuOAc	DIPEA	DME	70°C, 15min	10	1:2.5
5	CuOAc	DIPEA	DME	100°C, 15min	0	-
6	$Cu(OTf)_2$	DIPEA	DME	70°C, 15min	7	1:2.3
7	Cu formate	DIPEA	DME	70°C, 15min	3	1:3.1
8	CuCl	DIPEA	DME	70°C, 15min	25	1:1.6
9	Me_2AlCl	DIPEA	DME	70°C, 15min	4	1:1.4
10	CuOAc	**89**	DME	70°C, 15min	7	1:2.9
11†	CuOAc	DIPEA	DME	70°C, 15min	0	-
12†	Me_2AlCl	DIPEA	DME	70°C, 15min	14	1:1.4

Table 2.13 Screening of different reaction conditions under MW irradiations. Reagents and conditions: **36** (50mg, 1eq), **22** (1.2eq), catalyst (0.2eq), base (1.5eq), solvent (2ml), MW irradiation. *Determined by HPLC analysis as ratio between the area of the peak of the starting nucleoside and the area of the peaks of the products (both diastereoisomers). **dr: diastereomeric ratio determined by HPLC ratio between the area of the Fast Eluting (FE) peak and area of the Slow Eluting (SE) peak. † PFP (**67**) was used as phosphoroamidating agent.

The MW irradiation gave a positive contribution to the ProTide synthesis only in terms of timing, which proved to be dramatically shorter than the conventional conditions (15 min instead of 16h). As far as the other parameters are concerned, no significant improvement was observed under microwave irradiations.

Summary

Several parameters have been discussed. Among the few metal catalysts, the copper salts proved to successfully deliver the final ProTide. Four were the preferred copper catalysts (CuOAC, $Cu(OTf)_2$, CuCl, Cu formate) for their ability to yield the phosphoroamidate

with a good conversion value or stereo-outcome. Most of the conditions were evaluated on both 3'-protected and unprotected nucleosides. The presence of a hindered moiety in the 3'-position showed a negative effect on the final result. Ligands were found to inactivate the catalysts, while DIPEA and DME were the base and solvent of choice respectively. The phosphorochloridate was evidently the preferred phosphoroamidating agent, particularly when it included the naphthyl group as its aryl moiety.

The application of the best diastereoselective conditions is summarised in the crude mixture HPLC trace in **Figure 2.10**, where the final ProTide was obtained with 14% yield and 1:5 (FE/SE) diastereoselective ratio.

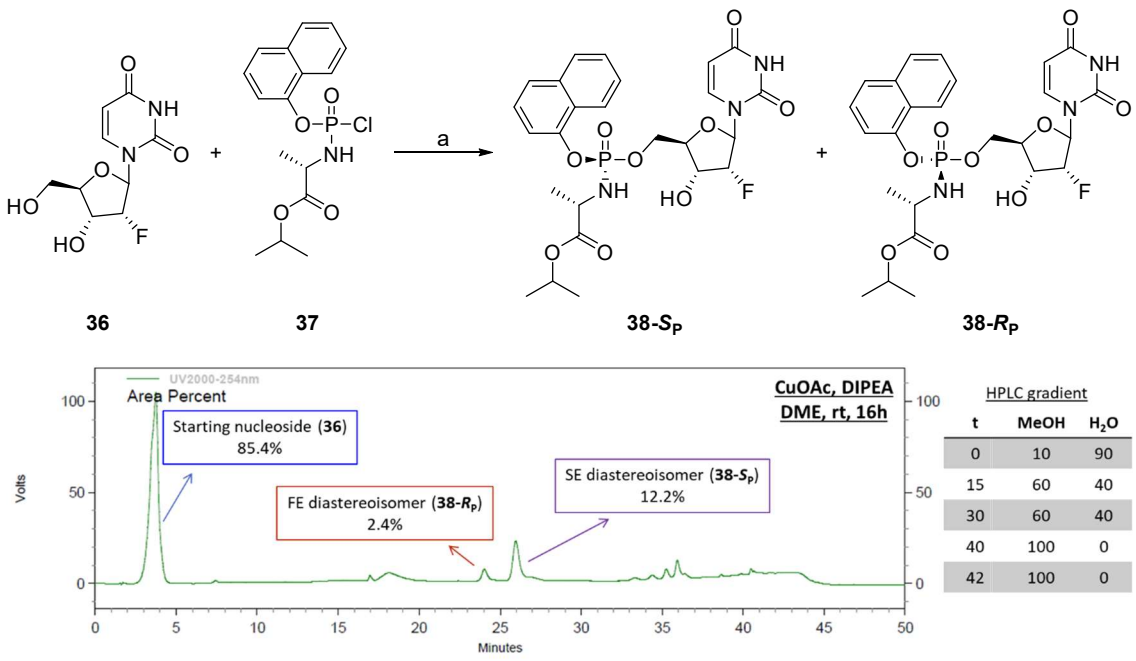


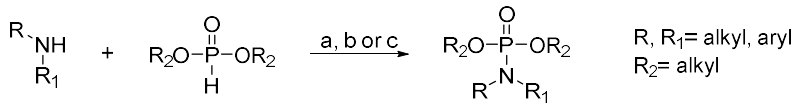
Figure 2.10 Analytical HPLC trace of the crude reaction mixture. Reagents and conditions: a) nucleoside **36** (50mg, 1eq), phosphorochloridate **37** (1.2eq), CuOAc (0.2eq), DIPEA (1.5eq), DME (2ml), rt, 16h. The percentage are determined by integration of the area of the selected peaks.

2.6.4 ProTide preparation via *H*-phosphonate intermediate

The nucleophilic substitution of a phosphoryl halide with an amine in the presence of a base is the most common route for ProTide preparation and it has been extensively investigated so far. Otherwise, as already discussed in paragraph 2.2.1, a second route for the phosphoroamidate synthesis consists of the coupling between a nucleoside aryl *H*-phosphonate and an amine.

The concept of oxidative cross-coupling applied to carbon-carbon or carbon-heteroatom bonds was inspiring for the development of novel methodologies.[60,61] Indeed,

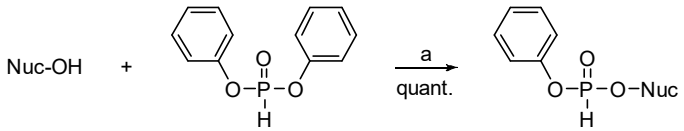
the copper-catalysed aerobic oxidative cross-coupling was successfully applied for the construction of P-N bonds starting from dialkyl *H*-phosphonates and nitrogen nucleophiles (**Scheme 2.18**).



Scheme 2.18 Copper-catalysed cross-coupling conditions present in literature. Reagents and conditions: a) CuI (20 mol%), amine, *H*-phosphonate, ACN, rt to 50°C, under air;[54] b) $Cu(OAc)_2$ (10 mol%), amine, *H*-phosphonate, K_2CO_3, toluene, rt to 80°C, under air;[62] c) CuBr (5 mol%), amine, *H*-phosphonate ,EtOAc, rt, under air.[53]

These procedures use a combination of inexpensive oxidant and catalyst systems. They are considered green reactions because they use oxygen from the air as oxidant and produce water as the by-product. The mechanism of the reaction is not yet determined, but it is believed that a Cu(I)-Cu(II)-Cu(III) catalytic cycle is involved. Therefore, some of the copper catalysts believed to be efficient for the P-N bond formation were examined according to the procedures disclosed in literature.[53,54,62]

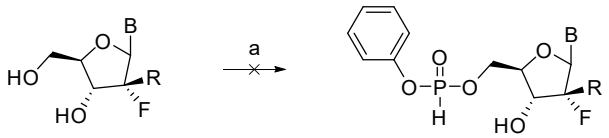
However, the application of these procedures first dealt with the preparation of the nucleoside aryl *H*-phosphonate, which was achieved by reacting a proper phosphonylating agent with a nucleoside.[63] Jankowska and co-workers proved that aryl *H*-phosphonate diesters were reactive enough to undergo quantitative reaction with nucleophiles. In particular, they discovered that diphenyl phosphite (DPP) may go through rapid transesterification with nucleosides affording the corresponding nucleoside aryl *H*-phosphonate (**Scheme 2.19**). The conventional protocol applied by Jankowska consisted of the solubilisation of the nucleoside in pyridine, followed by a dropwise addition of diphenyl phosphite (1 to 7 eq) under an inert atmosphere.[64]



Scheme 2.19 General reaction scheme reported by Jankowska and co-workers for the nucleoside aryl *H*-phosphonate preparation. Reagents and conditions: a) pyridine, rt, 15 min.

In this context, we were interested in the diastereoselective preparation of the phenyl *H*-phosphonates of the reference nucleosides **1k** and **36** (**Scheme 2.20**) by applying the metal catalytic conditions. To this purpose, several reaction parameters were

examined on both the reference nucleosides: temperature (-20°C to 40°C), timing (15min to 16h), solvents (pyridine, DMF and THF), reagent addition order (addition of the DPP to a nucleoside solution or nucleoside added to a DPP-solvent mixture), presence of 4 Å molecular sieves.



1k B= cyt, R= F
36 B= ura, R= H

92 B= cyt, R= F
93 B= ura, R= H

Scheme 2.20 Attempted preparation of phenyl *H*-phosphonate of reference nucleosides **1k** and **36**. Reagents and conditions: a) pyridine, rt, 15 min.

Unfortunately, none of the attempts proved to be successful and only initial nucleoside and the *H*-phosphonate monoester, obtained from the hydrolysis of the aryl ester, were detected at ESI-MS (**Figure 2.11**).

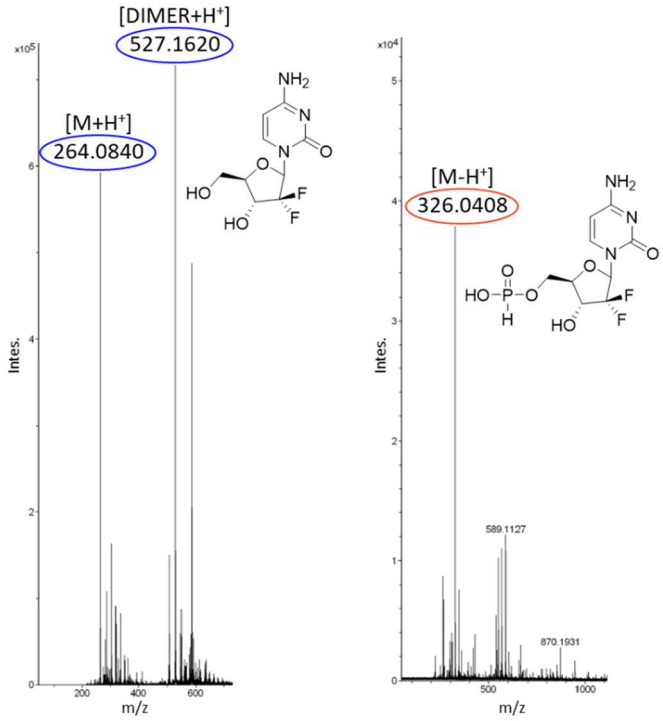
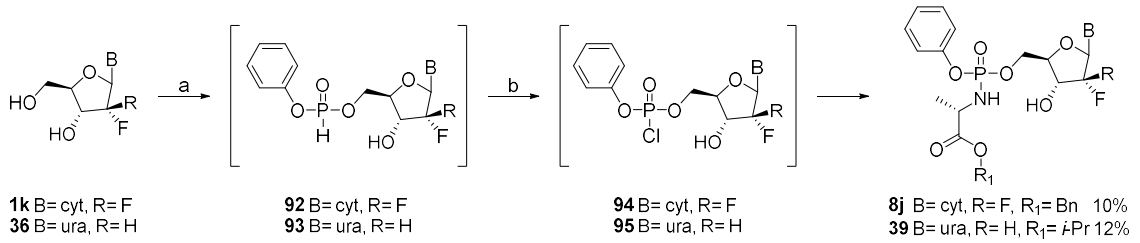


Figure 2.11 Example of a crude mixture analysis at MS(ESI). The MS(ESI+) spectrum on the left shows the presence of starting nucleoside (in blue). The MS(ESI-) spectrum on the right shows the presence of the hydrolysed product (in red).

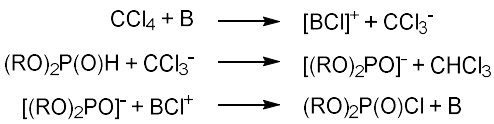
A thorough study of the literature brought to light a patent of 2016 in which processes for the preparation of Sofosbuvir and related analogues were investigated.[65] Indeed, one of

the procedures involved the formation of the phenoxy nucleoside *H*-phosphonate, which was generated *in situ* and not isolated. This procedure was applied on **36** and **1k** (**Scheme 2.21**).



Scheme 2.21 Preparation of ProTides **8j** and **39** via *H*-phosphonate intermediate and by the application of the Atherton-Todd reaction. Reagents and conditions: a) 4 Å molecular sieves, DPP, pyridine, -10°C, 1h; b) solution of *L*-alanine ester hydrochloride salt **56** or **57** in pyridine/ACN (1:8 v/v), CCl_4, Et_3N, 0°C to 30°C, 10min.

The nucleoside was solubilised in pyridine and stirred at -10°C under an argon atmosphere. Then, DPP was added dropwise and the mixture stirred for 1h at 0°C. At this point the disappearance of the starting nucleoside was observed by TLC and it was assumed that the phenoxy nucleoside *H*-phosphonate had formed quantitatively. The *H*-phosphonate intermediate was then subjected to the Atherton-Todd reaction conditions in the presence of CCl_4, which was the halogen source employed for the phosphorochloridate *in situ* formation.[66,67] According to the reaction mechanism, the first step consists of the formation of a salt between the base and the carbon tetrachloride. The trichloromethanide anion formed deprotonates the *H*-phosphonate and releases chloroform. The neo-generated phosphonate anion takes the chloride cation to form the chlorophosphate (**Scheme 2.22**).[67]

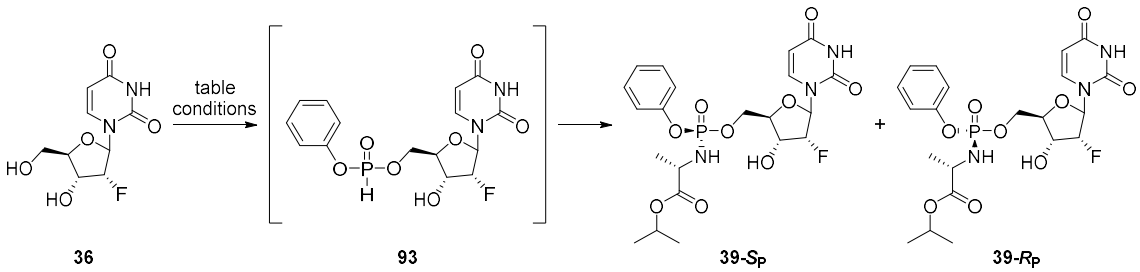


Scheme 2.22 Mechanism of the Atherton-Todd reaction where B= base .

After the formation of the phosphorochloridate intermediate, a solution of the *L*-alanine ester hydrochloride salt in pyridine and ACN (1:8 v/v) was injected dropwise into the reaction mixture. The formation of the final ProTides was immediate as confirmed by TLC after only 10 minutes. ProTides **39** and **8j** were then purified by flash chromatography to afford the pure compounds with 12% and 10% yields respectively.

Since the use of a stoichiometric amount of CCl_4 plays a limiting role in this method due to the toxic waste arising from its use, recently, significant efforts were applied to find more acceptable and greener alternatives as the copper catalysed conditions afore mentioned.[53,54,62] Because of the instability of the *H*-phosphonate here discussed, the catalyst was added to the reaction mixture after the intermediate formation. Unfortunately, none of the aforementioned procedures were successful and no final ProTide was detected in the HPLC analysis of the crude mixture.

After all the attempts performed to prepare the ProTides via *H*-phosphonate intermediate, only the pathway described in **Scheme 2.21** was found to be able to successfully deliver the final ProTide. Therefore, the additions of catalysts and other agents in the initial nucleoside-pyridine solution were investigated to accomplish a diastereoselective synthesis. The purpose clearly consisted in the generation of a *H*-phosphonate intermediate featured by a specific stereochemistry at the phosphorus centre. First, a general screening of copper salts was accomplished (**Table 2.14**). The data suggested that the presence of the catalyst did not significantly influence the level of conversion. Additionally, the stereooutcome of the reaction was completely unaffected by the use of the metal salts.



Entry	Catalyst	Base	Solvent	Conversion%*	FE/SE dr**
1	-	Et_3N	Py/ACN	18	1:1
2	CuOAc	Et_3N	Py/ACN	12	1:1
3	$Cu(OTf)_2$	Et_3N	Py/ACN	21	1:1
4	CuCl	Et_3N	Py/ACN	20	1:1
5	Cu formate	Et_3N	Py/ACN	10	1:1

Table 2.14 Screening of Copper salts in the synthesis of ProTide via *H*-phosphonate. Reagents and conditions: 4 Å molecular sieves (40 mg/1 mmol nucleoside), nucleoside **36** (1eq), catalyst (0.2eq), DPP (3eq), *L*-alanine *i*-Pr ester hydrochloride salt **56** (2.2eq) in pyridine/ACN (1/8, v/v) (4 ml/1 mmol nucleoside), CCl_4 (2 ml/1 mmol nucleoside), Et_3N (6.5eq), pyridine (1.2 ml/1 mmol nucleoside), -10°C to rt. *Determined by HPLC analysis as ratio between the area of the peak of the starting nucleoside and the area of the peaks of the products (both diastereoisomers). **dr: diastereomeric ratio determined by HPLC ratio between the area of the Fast Eluting (FE) peak and area of the Slow Eluting (SE) peak.

Since the addition of the catalyst proved to be ineffective in improving both yield and the stereo outcome of the reaction, the presence of a ligand was also examined (**Table 2.15**, **Figure 2.12**).

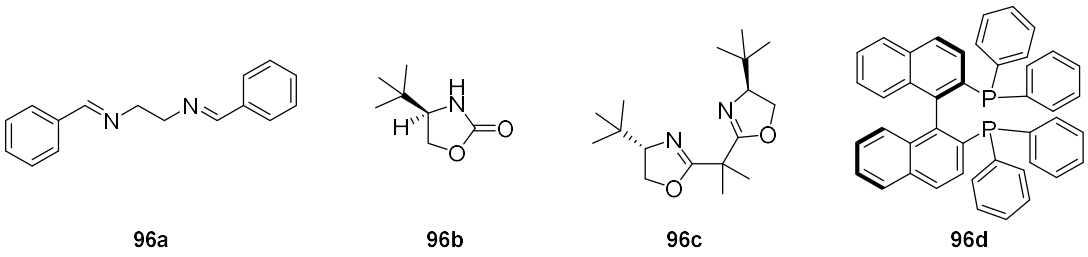
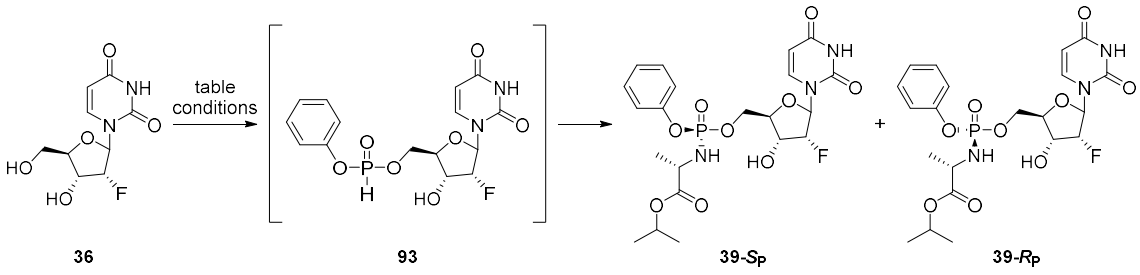


Figure 2.12 Ligands selected for diastereoselective investigations.

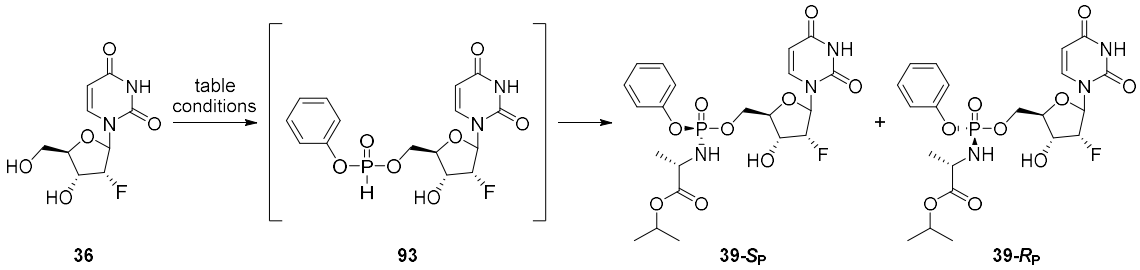
Contrarily to the procedure disclosed in **Scheme 2.7** and paragraph 2.3 where the addition of any ligand resulted into the loss of activity of the catalyst, the *H*-phosphonate approach proved to deliver the final ProTide in the presence of both achiral (entry1, **Table 2.15**) and chiral ligands (entry 2-5, **Table 2.15**). Although both small (entry 2, **Table 2.15**) and large (entry 3 and 5, **Table 2.15**) chiral ligands were employed, none of them showed a significant improvement in the FE/SE ratio. Moreover, the combinations of CuOAc/**96c** and $Cu(OTf)_2$/**96c** were evaluated also on the 3'-protected nucleoside, but no relevant difference was displayed in the yield and the stereo outcome when compared with the unprotected nucleoside.



Entry	Catalyst	Ligand	Base	Solvent	Conversion%*	FE/SE dr**
†1	CuOAc	**96a**	Et_3N	Py/ACN	18	1:0.8
2	CuOAc	**96b**	Et_3N	Py/ACN	27	1:0.8
†3	CuOAc	**96c**	Et_3N	Py/ACN	28	1:0.8
†4	$Cu(OTf)_2$	**96c**	Et_3N	Py/ACN	8	1:0.8
5	CuOAc	**96d**	Et_3N	Py/ACN	24	1:0.8

Table 2.15 Screening of ligands. Reagents and conditions: 4 Å molecular sieves (40 mg/1 mmol nucleoside), nucleoside **36** (1eq), catalyst (0.2eq), ligand (0.2eq), DPP (3eq), *L*-alanine *i*-Pr ester hydrochloride salt **56** (2.2eq) in pyridine/ACN (1/8, v/v) (4 ml/1 mmol nucleoside), CCl_4 (2 ml/1 mmol nucleoside), Et_3N (6.5eq), pyridine (1.2 ml/1 mmol nucleoside), -10°C to rt. *Determined by HPLC analysis as ratio between the area of the peak of the starting nucleoside and the area of the peaks of the products (both diastereoisomers). **dr: diastereomeric ratio determined by HPLC ratio between the area of the Fast Eluting (FE) peak and area of the Slow Eluting (SE) peak. †Conditions additionally applied on the 3'-Boc protected nucleoside **45**; furthermore, the addition of these catalyst and ligand combinations after the *H*-phosphonate formation was also evaluated.

Holding CuOAc as the preferred catalyst and **96c** as the ligand, both base (entry 1-3, **Table 2.16**) and solvent (entry 4-7, **Table 2.16**) variations were examined. It is noteworthy that the use of the chiral base **89** (**Figure 2.9**) proved to be unable to deliver the desired ProTide (entry 3, **Table 2.16**). Among the solvents, a Py/ACN combination remained the most efficient solvent mixture in terms of yield when compared with pyridine alone (entry 7, **Table 2.16**) or other solvents such as THF and DME (entry 5 and 6, **Table 2.16**). However, the diastereoselective ratio remained unchanged at FE/SE-1:0.8 ratio value.



Entry	Catalyst	Ligand	Base	Solvent	Conversion%*	FE/SE dr**
1	CuOAc	**96c**	quinine	Py/ACN	18	1:0.8
2	CuOAc	**96c**	DIPEA	Py/ACN	27	1:0.8
3	CuOAc	**96c**	**89**	Py/ACN	0	-
4	CuOAc	**96c**	Et_3N	Py/ACN	28	1:0.8
5	CuOAc	**96c**	Et_3N	THF	5	1:0.8
6	CuOAc	**96c**	Et_3N	DME	6	1:0.8
7	CuOAc	**96c**	Et_3N	Py	16	1:0.8

Table 2.16 Screening of bases and solvents. Reagents and conditions: 4 Å molecular sieves (40 mg/1 mmol nucleoside), nucleoside **36** (1eq), catalyst (0.2eq), ligand (0.2eq), DPP (3eq), *L*-alanine *i*-Pr ester hydrochloride salt **56** (2.2eq), CCl_4 (2 ml/1 mmol nucleoside), base (6.5eq), solvent (1.2 ml/1 mmol nucleoside), -10°C to rt. . *Determined by HPLC analysis as ratio between the area of the peak of the starting nucleoside and the area of the peaks of the products (both diastereoisomers). **dr: diastereomeric ratio determined by HPLC ratio between the area of the Fast Eluting (FE) peak and area of the Slow Eluting (SE) peak.

Summary

ProTide preparation via *H*-phosphonate intermediates was found to be an inappropriate procedure for the application of a catalytic diastereoselective approach. Despite the evaluation of several parameters, no conditions proved suitable for the delivery of the desired ProTide diastereoselectively. In summary, it is clear that the catalyst or its complex with a ligand do not actively contribute to the reaction outcome.

2.7 Conclusions and future works

To make up the lack of an efficient and inexpensive methodology to prepare ProTides as single diastereoisomers, a catalytic approach was explored. First of all, the synthesis of authentic samples of **8j** and **39**, and their corresponding 3'-protected analogs were prepared and the HPLC traces were recorded to be used as references. Transition metal catalysts were investigated by their application to two different procedures.

In the coupling between the nucleoside and the phosphorochloridate, CuOAc was found to be the catalyst that provided the best diastereoselective ratio (FE/SE-1:5) with 14% conversion, in the presence of DIPEA as the base, DME as the solvent and naphthyloxy phosphoroamidate as the phosphate source.

When the catalysts and their complexes with a ligand were employed in the ProTide preparation via a *H*-phosphonate intermediate, no improvement in the yields or stereo-outcomes of the reactions were noticed.

In the light of the results obtained, the assessment of a perfectly optimised diastereoselective synthesis appropriate to all nucleosides, suitable for scale up at an industrial level and able to deliver the diastereoisomer of choice, is still far away. A thorough reaction mechanism investigation was not addressed in this project. However, some assumptions have been formulated. According to the data gained and the literature statements,[55] the Lewis acids (as the transition metal salts) seem to be able to contribute catalytically to the regio- and diastereoselectivity of nucleoside 5'- phosphorylation. The catalyst might interact with the P=O group of the phosphoroamidating agent increasing the phosphorus electrophilicity. Although it has not been discussed until now, the coordination of the catalyst with other functional groups might be of interest. According to the results observed in this report, the absence of the amino acid moiety (see *H*-phosphonate intermediate) seems to lead to the delivery of the ProTide without diastereoselectivity. These considerations suggest that an additional interaction between the metal catalyst and the amino acid C=O group might be involved in the reaction mechanism. Besides, steric hinderance on the nucleoside 3'-position proved to negatively affect the reaction outcome albeit in a minor way.

To confirm these hypotheses, the X-ray analysis of the crystallised mixture of the phosphorochloridate with the metal catalyst could be performed to potentially elucidate the interactions between the reagent and the catalyst. Additionally, as further work, a detailed study of the mechanism of the reaction through kinetic studies might be

interesting and, accordingly, the reaction conditions can be systematically modulated to improve both the yields and the stereo-outcomes of these reactions.

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Chapter 3. Unsaturated acyclic nucleoside and nucleotide prodrugs

3.1 Acyclic nucleoside analogues

In the last decades, an extensive investigation took place to identify new classes of modified nucleoside able to overcome some catabolism processes responsible for the decomposition of known nucleoside drugs in patients' bodies.[1,2]

Among the structural design, modifications of the sugar moiety were first examined in depth. Starting from the removal of the 2' and 3'-*OH*, it was observed that the nucleoside could still retain activity with the additional excision of 2' and 3'-carbons. These molecules are dubbed acyclic nucleosides (ANs) and exhibit a broad spectrum antiviral activities.[3] Some of the most medically important antiviral molecules belong to this class of compounds: acyclovir (ACV, **2o**),[4] ganciclovir (GCV, **2q**)[5] and penciclovir (PE2, **2s**)[6] (**Figure 3.1**). All of them are guanosine analogues with some differences in the acyclic chain: compound **2o** presents both the 2' and 3'-carbon excised whereas **2q** only the 2' carbon. Compound **2s** lacks the 2' carbon and shows also the replacement of the oxygen with a methylene group.

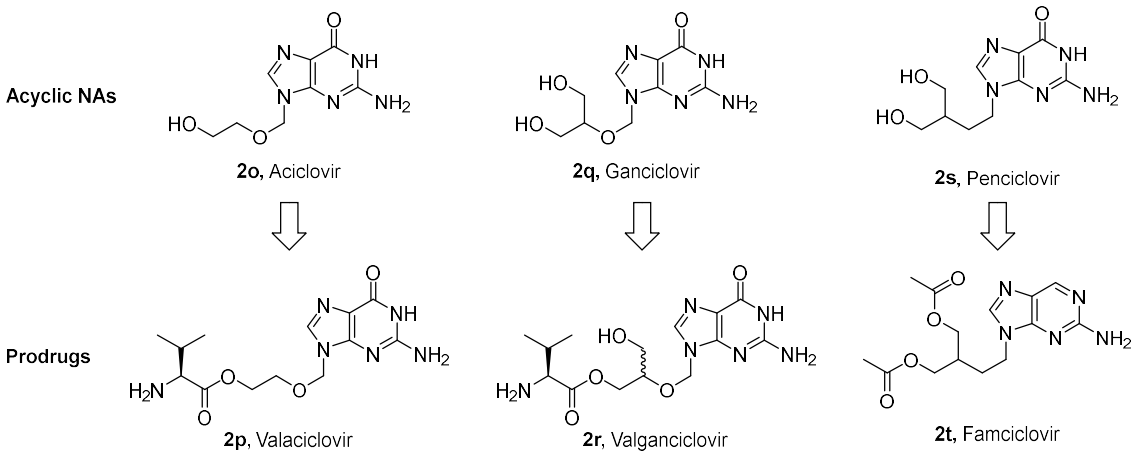


Figure 3.1 Structures of acyclic nucleoside analogues and their prodrugs.

Such nucleosides, as their cyclic parent, need to be phosphorylated since the tri-phosphorylated form represents the biologically active agent. Interestingly, the sp^3 character exhibited by the carbon atoms of the acyclic chain confers upon them flexibility to better adopt the appropriate conformation to bind the active sites of enzymes responsible for their phosphorylations.[7] Indeed, acyclic nucleosides are more readily recognised by viral thymidine kinases than the corresponding human TK, resulting in higher concentrations of tri-phosphorylated acyclic nucleoside (TPANs) in infected cells.[8] Additionally, TPANs are in turn more efficiently incorporated by viral polymerase into growing DNA chains, and thus, their antiviral activities are liked to be more specific.[9–11]

Unfortunately, acyclic nucleoside analogues display poor bioavailability. For this reason, prodrugs have been developed to increase lipophilicity (**Figure 3.1**). The oral bioavailability of acyclovir and ganciclovir was increased by the addition of a valine attached trough an ester bond to the 5'-*OH* of the nucleoside, to yield the prodrugs valacyclovir (**2p**)[12] and valganciclovir (**2r**),[13] respectively. They are converted *in vivo* to the active drugs by esterases. Famciclovir (**2t**) is a prodrug of penciclovir that features acetylation of the two hydroxyl groups and the removal of the carbonyl on the nucleobase.[14] Its *in vivo* activation consists of the oxidation of the purine ring and the hydrolysis of the acetate moieties.[15]

However, viruses might develop resistance to these nucleoside drugs due to mutations of the kinase enzymes responsible for the generation of the biologically active agents. When the genes that code for the kinases are partially or totally deleted, the enzymes' activity decreases and the compounds' phosphorylation fails leaving the drug inactived. As in the case of the cyclic analogues, the initial phosphorylation of acyclic nucleosides is generally the rate-limiting step. As a consequence, the use of acyclic nucleoside monophosphates has been seen as a solution to circumvent this problem.[16] However, these nucleotides have been demonstrated to be substrates of phosphatases, resulting in the removal of the phosphate function. For this reason, the phosphate moiety (P-O) was replaced by an isoelectronic and isosteric phosphonate group (P-C) (see *infra*).[17] These acyclic nucleotide analogues are known as acyclic nucleoside phosphonates (ANPs), and their antiviral potential was immediately attested. [1,18] Therefore, this new class of drugs was extensively investigated, and the following paragraph will review these interesting molecules.

3.2 Acyclic nucleoside phosphonates

ANPs are structural analogues of nucleoside monophosphates and are a key class of antiviral drugs.[19] These nucleotide analogues consist of a phosphonate group linked to pyrimidine or purine nucleobases *via* an acyclic side chain (**Figure 3.2**). Therefore, ANPs circumvent the first phosphorylation step, showing antiviral activity in nucleoside kinase-deficient cells where the classic acyclic nucleosides were inactive.[20].

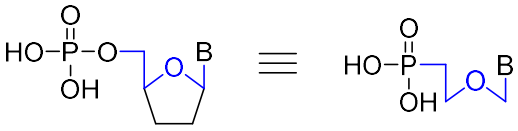


Figure 3.2 General structures for cyclic and acyclic nucleoside monophosphate analogues.

Their mechanism of action is based on two intracellular phosphorylations by nucleoside monophosphate and nucleoside diphosphate kinases to generate the diphosphate (ANPp) and the triphosphate (ANPpp) forms, respectively (**Figure 3.3**).[21] ANPpp act as triphosphorylated nucleoside analogues targeting viral DNA polymerases. ANPpp can be both inhibitors and substrates of the polymerase. In the first case they impede the DNA synthesis, in the second they are incorporated into the nucleic acid acting as chain terminators. The reason is that the 3'-hydroxyl group of the sugar moiety is not available to generate a phosphodiester bond with the incoming nucleoside.[22–25]

Figure 3.3 General phosphorylation scheme for ANPs.

Compared with cyclic nucleotides, ANPs have proved to be excellent templates for drug design for several reasons:

- Flexibility, as mentioned before (paragraph 3.1), is a key feature to improve the interactions with the desired enzymes.[7] Their higher affinities for viral kinases compared to the corresponding human enzymes, results in a greater concentration of the active tri-phosphorylated acyclic nucleoside in infected cells.[8] Moreover, they are easily incorporated into the nucleic chain by DNA polymerases.[9]

- Absence of the labile glyosidic bond, which can easily undergo chemical and biological degradation make these drugs resistant to nucleoside phosphorylases, which do not recognise the *N*-glycosidic bond in the absence of the sugar ring.[17]
- Replacement of the P-O bond (phosphate) with the more stable P-CH_2 (phosphonate) abolishes one of the catabolic processes that involves the phosphoroester bond cleavage catalysed by phosphatase enzymes.[17,26–28]

These common structural aspects of ANP molecules along with their unique spectrum of antiviral activities ensures their role as leading therapeutics in the antiviral field.[29]

ANPs can be classified into three different series based on the nature of the acyclic chain present in their backbone (**Figure 3.4**).[17]

HPMP [3-hydroxy-2-(phosphonomethoxypropyl)]. These derivatives are characterised by an hydroxymethyl chain that exhibits *S*-configuration, except for a 2,4-diamino-3-hydroxy pyrimidine derivative, whose *R* isomer demonstrated to inhibit adenovirus replication *in vitro*. The most representative compound of this series is cidofovir [(*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl) cytosine, HPMPC, Vistide®)] (**97**). It is used for the treatment of cytomegalovirus infections in immunosuppressed patients. Cidofovir (CDV) is a nucleotide analogue of deoxycytidine monophosphate (dCMP). Its triphosphate analogue is the active intracellular metabolite which acts as both an alternative substrate, in competition with dCTP, and an inhibitor for the viral DNA polymerase. As a result, the rate of viral nucleic acid synthesis is reduced.[30,31]

PME [2-(phosphonomethoxyethyl)]. The acyclic side chain of the PME series of ANPs is characterized by the lack of the hydroxymethyl group and they do not present any chiral carbon centres in their acyclic side chains. The PME block-buster compound is adefovir [(9-(2-phosphonylmethoxyethyl)adenine, PMEA)] (**98**). Adefovir is a broad-spectrum nucleotide analogue that is active against herpesvirus, hepadnavirus and retrovirus.[32] It is intracellularly converted into the active metabolite adefovir diphosphate, which competes with deoxyadenosine 5'-triphosphate for incorporation into viral DNA, then inhibits reverse transcriptase *via* viral DNA chain termination.[28,33]

PMP [2-(phosphonomethoxypropyl)]. The acyclic side chain of the PMP series is characterized by the lack of a hydroxyl group. PMP-like compounds have proved to be active in the *R* configuration. The most representative compound of this series is tenofovir (**99**). Tenofovir [(*R*)-9-(2-phosphonylmethoxypropyl)adenine), PMPA] shows the same mechanism of action of adefovir. It is active against hepadna- and retrovirus.

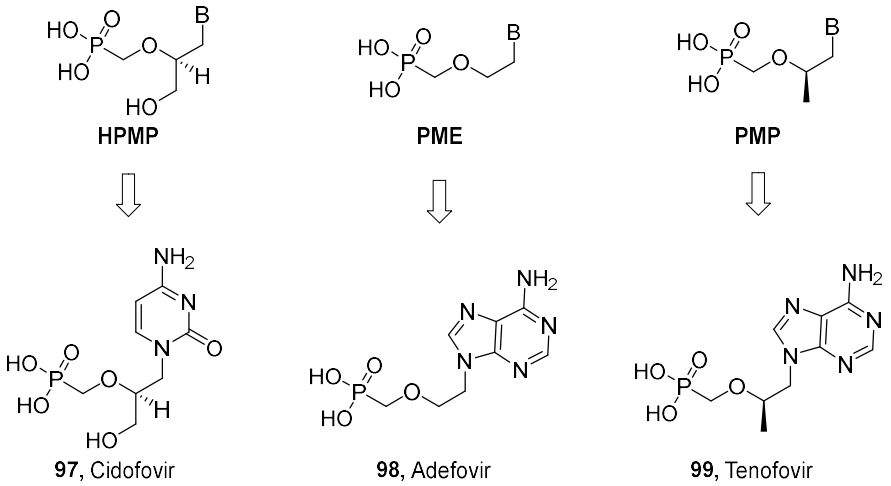


Figure 3.4 Classification of acyclic nucleoside phosphonates according to their chemical structures and corresponding drugs.

Interestingly, ANPs show longer antiviral responses when compared with the classic acyclic nucleosides. This was specifically proved for cidofovir whose antiviral response is prolonged for several days allowing a once a week administration, whereas acyclovir action lasts for only a few hours.[34] The reason is related to the long intracellular half-life of ANP metabolites (ANPp and ANPpp).[35] Moreover, ANPs exhibit exceptional affinities for viral DNA polymerases leading to minimal interference with cellular DNA synthesis.[29] For this reason, tenofovir displays a very low cytotoxicity and can be safely used for the treatment of HIV infections.[36]

3.3 ANP prodrugs and their clinical applications

Despite their great antiviral activity, pharmacokinetics studies showed that cidofovir, adefovir and tenofovir have poor oral bio-vailabilities along with scarce cell penetration due to the polar phosphonate moiety featuring two negative charges at physiological pH.[37] Therefore, several prodrug strategies were evaluated to overcome these limitations. Among them, bis(POM) and bis(POC) proved to be successful with the FDA's approval of adefovir dipivoxil **3a** and tenofovir disoproxil fumarate **3b** (**Figure 3.5**).

Adefovir dipivoxil {bis(pivaloyloxymethyl-[bis-(POM)] ester of PMEA, Hepsera®} (**3a**), is an adefovir prodrug approved for standard therapy of chronic Hepatitis B virus (HBV) infections.[38–41] It showed activity also against HIV, but the drug was not approved by FDA due to its nephrotoxicity at the required dose for the treatment of the disease.[42] The

HepDirect prodrug of adefovir (pradefovir, **6b**) is also under evaluation. Currently, it has completed the phase 2 trials for the treatment of HBV and displayed an improved *in vitro* activity along with an increased oral bioavailability and organ distribution.[43]

In analogy with adefovir dipivoxil, the bis(isopropyloxycarbonyloxymethyl-[bis-(POC)] ester of (*R*)-PMPA (tenofovir disoproxil, Viread®) (**3b**) was prepared to increase the oral bioavailability of tenofovir. This prodrug was then formulated as the fumarate salt.[44] Tenofovir disoproxil fumarate (TDF) was approved for the treatment of HIV infections in 2001 and for the treatment of HBV in 2008.[45] Moreover, the HDP prodrug of tenofovir (CMX157, **7b**) was designed to improve the delivery of the drug to the liver. It is currently an anti-HIV agent in phase 2 clinical trials as a promising drug for the treatment of drug-resistant HIV infections.[46]

Even though no cidofovir prodrug is in clinical use yet, brincidofovir (**7a**) is a successful HDP prodrug under clinical evaluation for CMV, Ebola and many other viral infections.[47–50] Notably, it displays a unique broad-spectrum activity that makes it a potential standard prophylaxis agent for double-stranded DNA viruses in patients with HIV.[51,52]

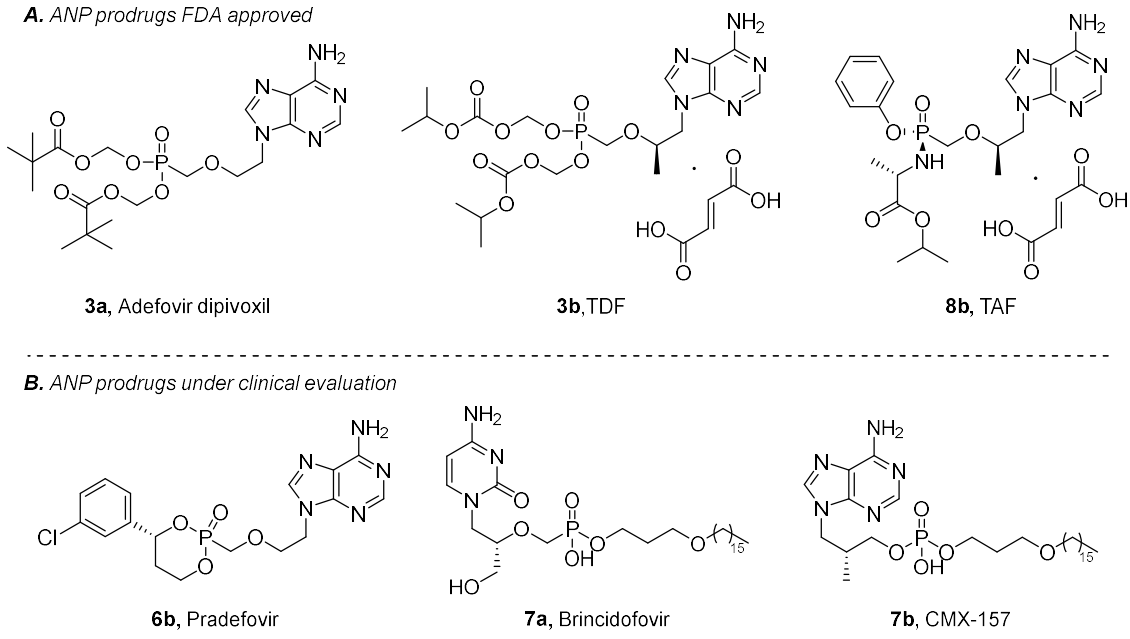


Figure 3.5 Structures of ANP prodrugs approved by FDA (**3a**, **3b** and **8b**) (**A**) and currently under clinical investigations (**6b**, **7a** and **7b**) (**B**).

Although TDF was considered as one of the most successful drugs for the treatment of HVB and HIV thanks to its high oral bioavailability and cell permeability, several studies indicated important negative effects.[44,53] Indeed, a significant reduction in

bone mineral density and an extensive loss of kidney function were detected in patients treated with this drug.[54,55] The main reason ascribed to these side effects is the prodrug susceptibility to serum-mediated esterases.[56,57] As a consequence, the high level of tenofovir in plasma was identified as the cause of the physiological disfunctions.[58] To overcome these limitations, Gilead Sciences developed an aryloxy phosphonoamidate prodrug (ProTide) of tenofovir (TAF, **8b**).[59,60] The success of TAF was immediately attested due to its selective intracellular cleavage, resulting in high intracellular levels of the active metabolite tenofovir.[59] The ProTide was approved by the FDA in 2015 for the treatment of HIV[61,62] and in 2016 as an active agent against HBV.[63,64]

3.4 Unsaturated cyclic nucleoside analogues

Beside the removal of the sugar ring, several other modifications of the carbohydrate function of the NAs were evaluated while searching for active antiviral agents. Among them, unsaturation of the sugar ring proved to be particularly successful with many unsaturated NAs endowed with effective antiviral activity. Unsaturated five-membered NAs are a prominent class of drugs active against several viral infections (**Figure 3.6**).[65] The path to their design started with the discovery of zidovudine (AZT, **2f**), the first NA approved for the treatment of HIV as a reverse transcriptase inhibitor. AZT proved that the absence of the 3'-*OH* group facilitates the nucleoside analogue role as DNA chain terminator. Based on these notions, further explorations revealed that 2',3'-dideoxy-nucleosides (ddNs) and 2',3'-didehydro-2',3'-dideodxynucleosides (d4Ns) are the most important classes of anti-HIV compounds.[66] Among them, stavudine (d4T, **2i**) was approved in 1994 by the FDA for the treatment of HIV,[67] while Elvucitabine (**100**) and 2',3'-didehydro-2',3'-dideodxy-2'-fluoro-β-L-cytidine (β-*L*-2'-F-d4C, **101**) are under clinical evaluation as anti-HIV and HBV agents.[68]

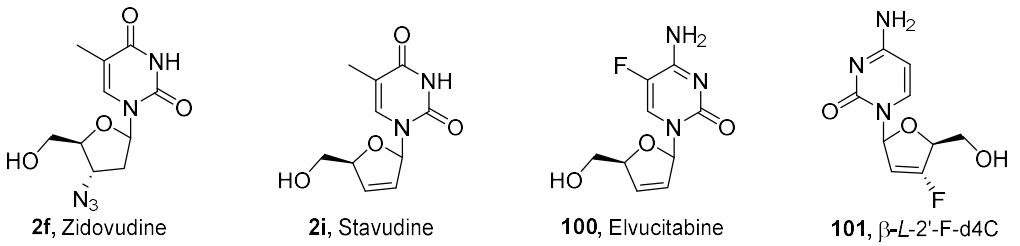


Figure 3.6 FDA approved (**2f**, **2i**) and under clinical evaluation (**100**, **101**) NA reverse transcriptase inhibitors.

The replacement of the furanose oxygen atom by a methylene group generated a new class of carbocyclic nucleosides.[69] These analogues showed increased chemical and metabolic stabilities because they are resistant to nucleoside hydrolase and phosphorylase enzymes, that are responsible for breaking the *N*-glycosidic bond of deoxy- and ribonucleotides. The conformational similarity between the cyclopentane and the tetrahydrofuran rings allows them to interact with target enzymes and to retain the biological properties of the original nucleosides.[69] As a consequence, several NAs featuring a cyclopentane ring were evaluated (**Figure 3.7**). The representative compound of this series is aristeromycin (C-Ado, **102**).[70] The further addition of a rigid structural element such a double bond in the 4'-position led to the development of a new class of NAs characterised by the presence of a cyclopentene ring, whose block-buster compound is neplanocin A (Np-A, **103**).[69] Both **102** and **103** are adenosine analogues with similar modes of action, although differing by the presence of a double bond in the carbocyclic ring. They are both inhibitors of *S*-adenosylhomocysteine (AdoHcy) hydrolase, whose activity is strictly correlated to viral replication. In particular, AdoHyc is a competitive inhibitor of *S*-adenosylmethionine (AdoMet)-dependent methyltransferases, which are required for the formation of the 5'-methylated cap structure necessary for viral replication.[71] Therefore, they proved to possess broad-spectrum antiviral activities. However, they were also found to be adenosine kinase substrates and, consequently, cytotoxic agents.[70,72,73] The main metabolic difference between the C-Ado and its unsaturated analogue is the ability of **102** to be additionally converted to the carbocyclic guanosine monophosphate, which acts as inhibitor of hypoxanthine phosphoribosyl transferase. The more rigid Np-A is a poor substrate of the deaminase and its conversion to the guanosine analogue was never observed. This dissimilarity in the metabolic effects of the two carbocyclic nucleosides confirmed that peculiar structural elements are required to be substrates of specific enzymes.[72] Further studies on carbocyclic nucleosides led to the development of more effective drugs such as abacavir (ABV, **2k**) and entecavir (ETV, **2d**). The first compound was a 2',3'-didehydro-2',3'-dideoxy carbocyclic nucleosides approved in 1998 for the treatment of HIV,[74] whereas ETV, which was approved in 2005, is an anti-HBV carbocyclic guanosine analogue featuring an exocyclic double bond on the 5'-position.[75,76]

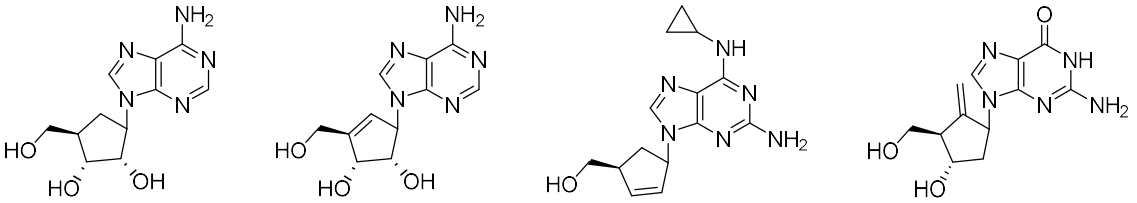


Figure 3.7 An antiviral carbocyclic nucleoside (**102**) and other unsaturated analogues (**103**, **2k**, **2d**).

In the search for new antiviral agents, also other 2',3'-unsaturated NAs, unsaturated 4'-thionucleosides, six-membered NAs, cyclohexyl derivatives, keto and exoxyclic methylene pyranonucleosides are under investigations as antiviral drugs.[65,66]

Due to the success of these unsaturated nucleosides, lately, the insertion of a double bond into the acyclic nucleoside side chains was found to be an effective structural element to boost their antiviral activities. The efficacy of these chemical structures was discovered when the unique structural backbone of ganciclovir (**2q, Figure 3.1**), the guanine derivative in which the 2'-*CHOH* was excised from the natural nucleoside, was combined with the potent carbocyclic unsaturated Np-A (**103**) structure to generate novel potent unsaturated carbocyclic analogues like **104** and **105** (**Figure 3.8**). These compounds displayed good *in vitro* activities as anti-HSV-1 and anti-HSV-2 agents.[77,78]

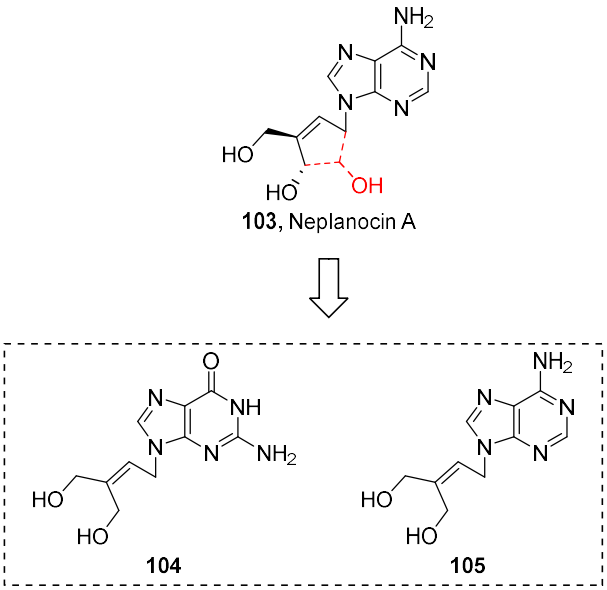


Figure 3.8 Example of antiviral unsaturated carbo-cyclic and -acyclic nucleoside analogues.

Since the removal of the sugar ring and the introduction of a rigid structural element such as the double bond proved to influence the ANs antiviral potency,[79,80] many unsaturated

acyclic nucleosides and nucleotides are currently under development as antiviral agents.[66] These explorations are reported in the following paragraphs.

3.5 A novel ANP structural motif: *trans*-alkene derivatives

ANPs play key roles in the treatment of viral infections, and this class of compounds can be regarded as one of the most significant groups of drugs in the antiviral field.[1,17] Discovered almost 30 years ago, a great wealth of research has been dedicated to the development of efficient synthetic methodologies to generate new ANPs.[81–85] These new structures were evaluated against a variety of infectious organisms and associated diseases[66,79] including parasites,[9,10,86–90] microbes,[91–94] and tuberculosis[95,96]

In 2011, a study on C5-substituted pyrimidine acyclic nucleoside phosphonates was published by Agrofoglio's group.[97] In this work nucleoside phosphonates bearing a double bond in the acyclic chain resulted in a substrate for the human thymidylate monophosphate kinase (hTMPK) that efficiently activates them to the diphosphate form.

The rational design behind this modification was the ability of the *trans*-alkene to mimic the three-dimensional geometry showed by ANP backbones (**Figure 3.9**).

Figure 3.9 General structures for acyclic and unsaturated acyclic nucleoside phosphonate analogues.

Additionally, the double bond can bring a similar electronic contribution to that provided by the oxygen atom.[98] Remarkably, the strong affinity of the *trans*-alkene acyclic nucleoside derivative to the active site of recombinant hTMPK was well demonstrated.[97] The interactions of the (*E*)- and (*Z*)-but-2-enyl-pyrimidine derivatives **106** and **107** (**Figure 3.10**) with the enzyme were evaluated through *in vitro* enzymatic assays. It was demonstrated that hTMPK worked better on ANP-substrates with a *trans* configuration. This was proved by X-ray analyses in which the affinity of the (*E*)-but-2-enyl side chain was compared with that of the natural substrate 2'-deoxyribose with hTMPK through co-crystallization. These data showed that the absence of the 3'-hydroxyl group in the acyclic motif represents a benefit that results in a productive binding of the ANP derivative in the active site of the enzyme.[97] Accordingly, the bis-

POM prodrugs of **(*E*)-106** and **(*E*)-107** exhibited in general good antiviral activities, with **(*E*)-108** showing inhibitory activity against VZV, HSV-1, HSV-2 (EC_{50} = 2.5-6.1 μM) and **(*E*)-109** against VV, HSV-1, HSV-2 and VZV (EC_{50} = 16-33 μM). As expected **(*Z*)-108** and **(*Z*)-109** exhibited little or no antiviral activities.[97]

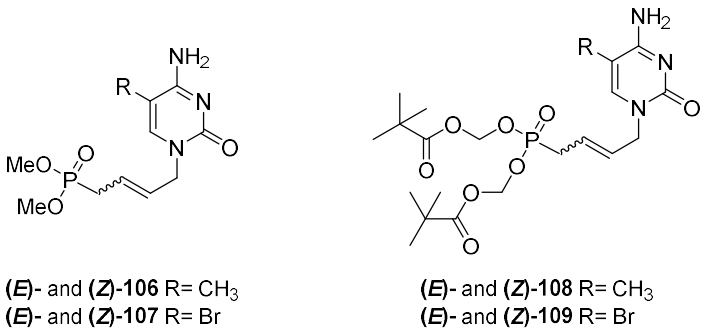
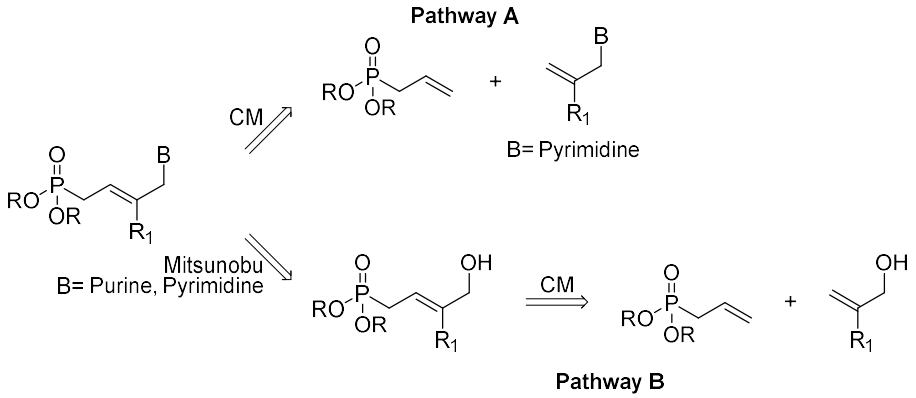


Figure 3.10 (*E*)- and (*Z*)-but-2-enyl-pyrimidine derivatives **106** and **107**, and corresponding bis-POM prodrugs **108** and **109**.

Agrofoglio's group elaborated two different retrosynthetic approaches for the preparation of alkenyl substituted ANPs according to the nucleobase nature (**Scheme 3.1**). The direct CM approach was applied for N^1-alkylated pyrimidine derivatives (**Scheme 3.1**, pathway A). To avoid the CM ruthenium catalyst poisoning effect caused by the nitrogens on purine scaffolds, a Mitsunobu coupling strategy on a previously prepared hydroxy alkenyl synthon was employed (**Scheme 3.1**, pathway B).[99] Interestingly, the olefin cross-metathesis methodology was also employed for the direct synthesis of a vast array of unsaturated ANPs prodrugs including bis-POM, bis-POC, and alkoxyesters analogues.[97,99–104]



Scheme 3.1 Retrosynthetic schemes applied for the preparation of novel alkenyl ANPs and alkenyl ANP prodrugs via olefin cross-metathesis (CM).

3.5.1 Basics of olefin cross-metathesis

Olefin metathesis is a proven synthetic route towards functionalise of olefins from simple alkenes.[105] There are three main types of metathesis: a) ring closing metathesis (RCM), in which the olefins involved react to form a ring;[106] b) ring opening metathesis polymerisation (ROMP), in which a double bond in a ring can be opened to generate polymers otherwise not accessible by other methods;[107] c) cross-metathesis (CM), that consists of a rearrangement of the connectivities of the olefins involved.[108]

In the last decade, olefin cross-metathesis has become a powerful tool for the preparation of regioselective unsaturated phosphonates.[109–112] It is a transalkylidenation of two terminal alkenes catalysed by metal carbenoids such as the ruthenium catalysts developed by Grubbs,[113] Nolan[114] and Hoveyda.[115] The mechanism of the reaction consists of a [2+2] cycloaddition (**Figure 3.11**) of a transition metal alkylidene (carbene complex) to an alkene double bond to generate a metallacyclobutane intermediate (metallacyclobutane I). The latter can undergo cycloelimination to give the original species (unproductive cleavage) or a new alkene (ethene) and alkylidene (productive cleavage). If the new carbene complex is found to have a comparable reactivity to the catalyst the reaction started with, then it can perform a second [2+2] cycloaddition with another alkene to generate again a metallacyclobutane intermediate (metallacyclobutane II). As before, the metallacyclobutane can undergo two alternative ways of break down, the productive one releases the cross-product and regenerates the carbene complex.[116]

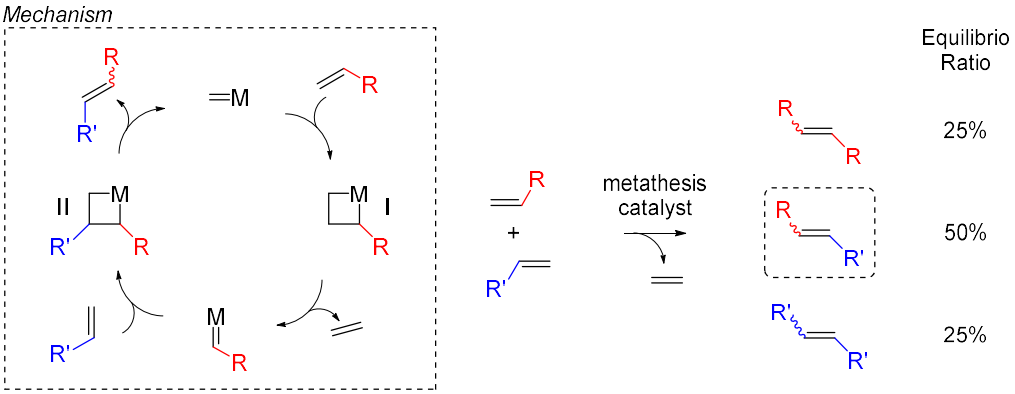


Figure 3.11 CM mechanism of reaction and its low product selectivity.

Statistically the reaction can generate three possible pairs of isomers: *E*/*Z* pairs of two homocouplings and one cross-coupling for a total of six products (**Figure 3.11**).[112] The selectivity of the reaction is still undergoing further studies, but it is already well known

that the choices of alkenes with different reactivities can give the cross-coupled product selectively and in excellent yield. Grubbs and co-workers collected all the literature data related to CM and developed an empirical categorisation of olefin reactivity which can be used to predict CM selectivity (**Figure 3.12**).[112] This classification is based on the olefin ability to homodimerise relative to other olefins. According to this classification, there are four type of olefins: a) type I olefins can homodimerise rapidly and the homodimers thus generated can be consumed in the CM; b) type II olefins undergo a slow homodimerization and their homodimers can be only moderately consumed in the CM; c) type III olefins are not able to homodimerise, but they can still participate in the CM by reacting with type II and II olefins; d) type IV olefins are just spectators of the reaction since they are not able to participate in the CM, but do not inhibit the catalyst. Outside this classification there are all the olefins that can deactivate the catalyst. Considering a reactivity gradient from type I olefin (the most active) to type IV olefin (the least active), it is possible to predict the product selectivity of the CM. Therefore, the reactions between olefin types I/II and olefin type III minimise the formation of homodimers of the starting olefins (undesirable CM side products) by avoiding their initial formation or by ensuring they are fully consumed in a secondary metathesis reaction.

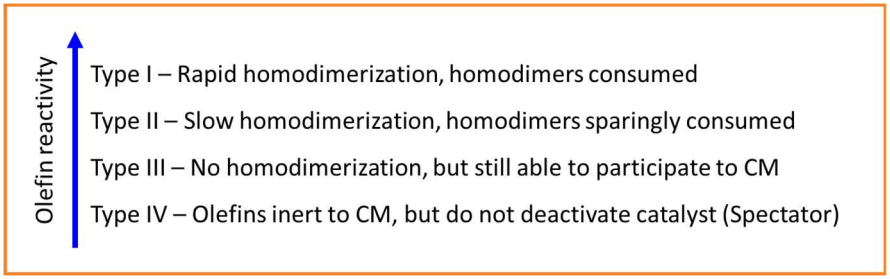


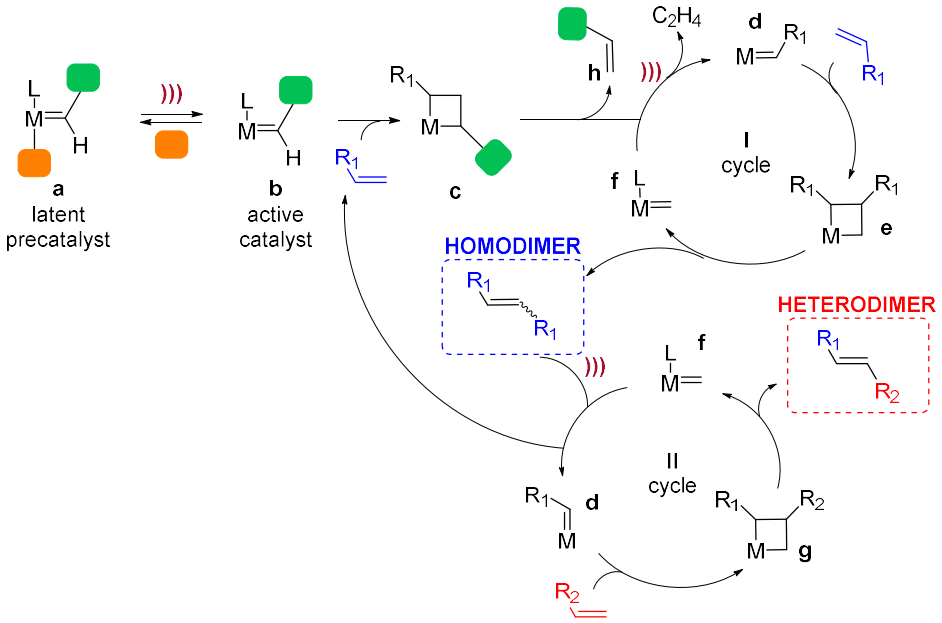
Figure 3.12 Olefin categorisation for CM selectivity.

For what concern the stereoselectivity problem (*E* or *Z* isomers) of this reaction remained unsolved until very recently. Selectivity for *E* products can often be achieved by allowing the reaction to reach equilibrium so that the *trans* thermodynamically favoured product is present in higher quantities. However, the difference in energy between *E* and *Z* isomers may be insignificant. Therefore, the increased *trans* ratio can be the result of the catalyst activity.[105] Ruthenium-complexes bearing *N*-heterocyclic carbenes have proven to have strong *trans* effect.[111]

Scheme 3.2 reports the accepted CM mechanism when an olefin type I (in blue) and type III (in red) are reacting together.[100,117,118] The pre-catalyst **a** needs to be activated with a

dissociative substitution that generates the active carbene complex **b** which undergoes the first [2+2] cycloaddition to afford the homo-coupling product of the more reactive olefin. The catalyst **f** regenerated from the first cycle can react with the homodimer through a second CM cycle leading to the formation of the desired hetero-coupling product. Since the reaction is an equilibrium, the *trans* thermodynamically favoured product is formed. This selectivity matches perfectly with this project's purpose to prepare the potentially more active *E* isomers of novel alkenyl ANP ProTides.

In the last few years, several publications reported improvements of the CM outcome caused by ultrasound irradiation.[100,119,120] Ultrasound waves can exert several functions: the mechanical force brought by the sonic waves on the metal catalyst not only might improve the ligand dissociation from the latent pre-catalyst to release the active form of the catalyst, but might also advantage the homodimer reaction toward the formation of the desired heterodimer product (**Scheme 3.2**). In addition, ultrasound exerts a degassing effect favouring the expulsion of the ethylene gas produced during the reaction, hence avoiding the regeneration of the terminal olefin.

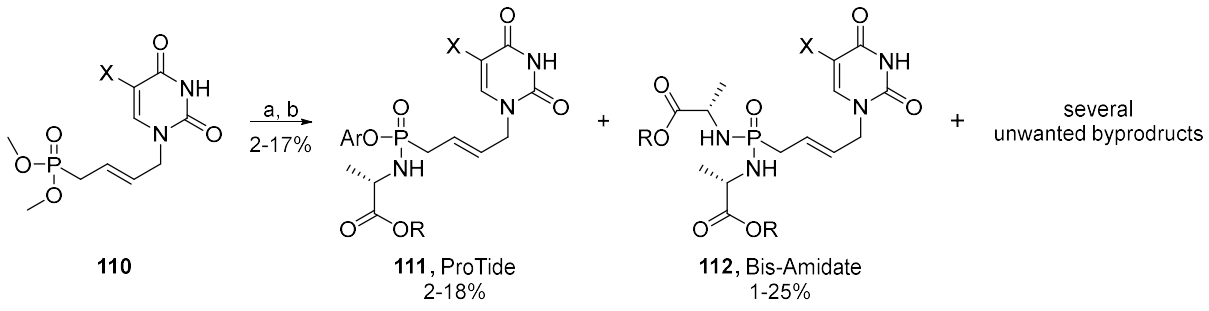


Scheme 3.2 Catalytic system for ultrasound olefin cross-metathesis reaction.

Quite recently, Pertusati's group applied the olefin cross-metathesis (CM) to the preparation of novel alkenyl ANP ProTides.

3.5.2 Preparation of alkenyl ANP phosphonoamidates

Our group extended the range of prodrugs of (*E*)-but-2-enylpyrimidine, by synthesising their ProTide and bis-amidate derivatives (**Scheme 3.3**).[121] This was accomplished using a one-pot procedure reported by Holy and co-workers[122] and adapted for the synthesis of adefovir and tenofovir phosphonoamidate prodrugs.[26] The phosphonic diester **110** was converted to the corresponding silyl ester by reacting with an excess of trimethylsilyl bromide (TMSBr). This silyl derivative was then activated by triphenylphosphine (PPh_3) and 2,2-dipyridyl disulphide (Aldrithiol) and reacted in the presence of the desired aryl alcohol (ArOH) and amino acid alkyl ester hydrochloride (see paragraph 3.7.1 for a detailed explanation of the reaction mechanism). In this study, ProTide technology was shown to be able to broaden the spectrum of antiviral activities when compared to other phosphonate prodrug approaches. Unfortunately, the preparation of phosphonoamidate prodrugs proved to be challenging, affording the compounds only in low yields (2-18%). The authors reported different reasons to explain such inefficient results. In particular, although 5 eq of phenol and only 1 eq of amino acid ester were employed the bis-amidate **112** (unwanted prodrug because usually less active than ProTides) was always the predominant compound in the reaction mixture. The triphenylphosphine was able to debrominate the C5-bromo to form an uracil derivative.[123] Additionally, the large excess of TMSBr employed was found able to brominate the alkenyl chain generating unstable brominated derivatives.



Scheme 3.3 Synthesis of phosphonoamidate (**111**) and phosphonodiamidate (**112**) prodrugs from an allyl phosphonate ester. Reagents and conditions: a) TMSBr, ACN, rt, 8h; b) amino acid ester hydrochloride salt, ArOH, PPh_3, aldrithiol-2, Et_3N, Pyridine, 50°C, 4-8h.

In general, the problem related to this class of compounds and their prodrugs is that there is not an efficient synthesis for their preparation. All the procedures reported in the literature are low yielding, long multi-step sequences and always accompanied by tedious purifications.[26,32,124]

3.6 Aim

While there are several efficient procedures to synthesize phosphoroamidate nucleosides, the phosphonoamidate cognate class, especially of alkenyl acyclic nucleoside phosphonates, lacks such a plethora of synthetic methodologies.[125] Indeed, the methodologies reported in the literature for the synthesis of ProTides on alkenyl acyclic nucleosides are scarce, inefficient and often low yielding.[121,124]

Therefore, part of this research project was directed to optimise these literature-based methodologies for the preparation of alkenyl acyclic nucleosides, and to investigate more effective synthetic approaches to give easy access to their ProTides. As a result, defined synthetic strategies could be applied for the preparation of potentially potent ANPs to study the impact of the ProTide motif on the antiviral activities of unsaturated ANPs. With this target in mind, two innovative procedures were optimised, and the best conditions were applied to the synthesis of two families of compounds: branched (trisubstituted) and linear (disubstituted) alkenyl ANP phosphonoamidates. Additionally, these two classes are characterised by a different position of the double bond on the aliphatic chain.

When the ProTide approach was applied to the linear (*E*)-but-2-enyl ANP scaffold (**Scheme 3.3**), the compounds proved to be active antiviral agents. Additionally, the phosphonoamidate technology was found to be able to broaden the spectrum of the antiviral activity when compared to other phosphonate prodrug approaches.[121] In this context, the promising anti-HIV-1 activity exhibited by the bis-POM prodrug of the (*E*)-2-methyl-but-2-enyl nucleoside backbone,[99] encouraged us to evaluate if the ProTide motif on branched alkenyl ANPs could exert the same positive effect shown by its application on the linear analogues. Recently, trisubstituted alkenyl acyclic nucleoside derivatives have been gaining increasing importance for their significant antiviral activities.[66,79] Surprisingly, almost all ANPs with biological activities have a purine nucleobase in their scaffold. The only pyrimidine ANP on the market is cidofovir (**97**). In the search for more potent pyrimidine ANP derivatives, the branched alkenyl ANP ProTides were investigated, including (*E*)-2-hydroxymethyl-but-2-enyl (**Figure 3.13**, scaffold A) and (*E*)-2-methyl-but-2-enyl backbones (**Figure 3.13**, scaffold B) on pyrimidine nucleobases. Both the scaffolds features a double bond in the 2'-position.

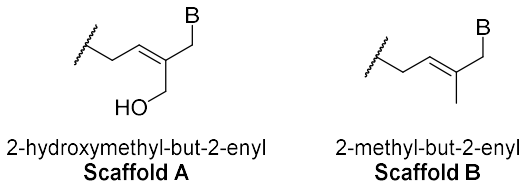
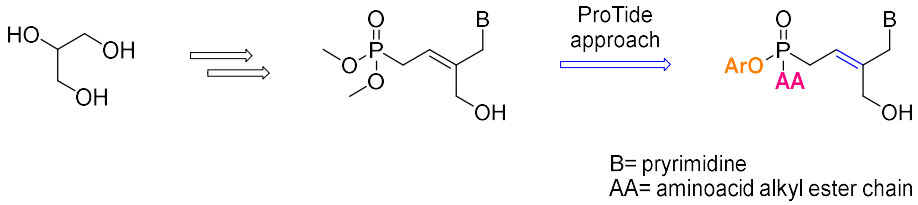


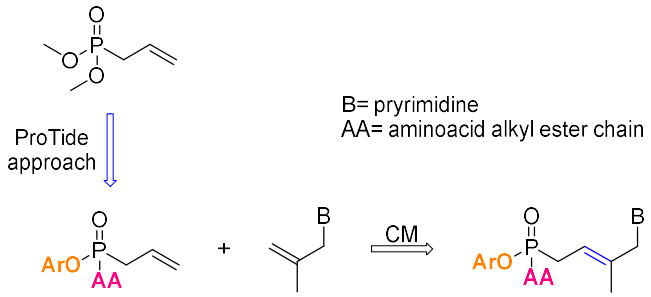
Figure 3.13 The two trisubstituted alkenyl ANP scaffolds investigated.

First, a known synthetic pathway was explored for the preparation of stereo-defined trisubstituted alkenyl nucleoside phosphonate (HPMP analogues),[100] followed by the application of the ProTide approach (**Scheme 3.4**).



Scheme 3.4 General approach for the preparation of a trisubstituted alkenyl nucleoside phosphonate HPMP analogue and application of the ProTide approach.

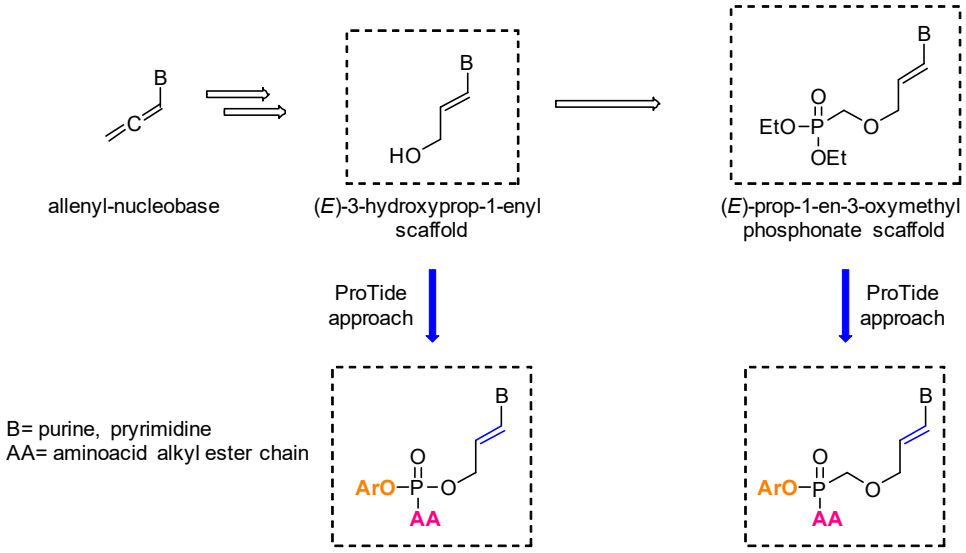
After the poor results obtained with this strategy most probably due to the presence of the hydroxymethyl moiety, the (*E*)-2-methyl-but-2-enyl scaffold was investigated and to accomplish its preparation, a short and innovative pathway was developed (**Scheme 3.5**). This route involved the synthesis of the novel aryloxy allylphosphonoamidate as the key intermediate to make the preparation of alkenyl ANP ProTides more effective by the direct application of the CM reaction.



Scheme 3.5 General approach for the preparation of the (*E*)-2-methyl-but-2-enyl ANP ProTide *via* aryloxy allylphosphonoamidate intermediate.

(*E*)-Prop-1-en-3-oxymethyl acyclic nucleoside and nucleotide prodrugs bearing both purine and pyrimidine bases were additionally investigated. Contrarily to the previous class of compounds, in this case the double bond is localised to the 1'-position. According to the great success of 6-methoxy purine analogues in another antiviral program,[126] this nucleobase was selected.

Employing the allenylnucleobase as a key intermediate, a synthetic strategy developed by Wei and co-workers[127] (see *infra*) was adapted for the preparation of (*E*)-prop-1-en-3-oxymethyl ANP phosphonoamidate prodrugs (**Scheme 3.6**). Furthermore, the application of this synthetic scheme allowed the preparation of (*E*)-prop-1-enyl ANP phosphoroamidate prodrug by the application of the ProTide approach on the (*E*)-3-hydroxyprop-1-enyl intermediate.

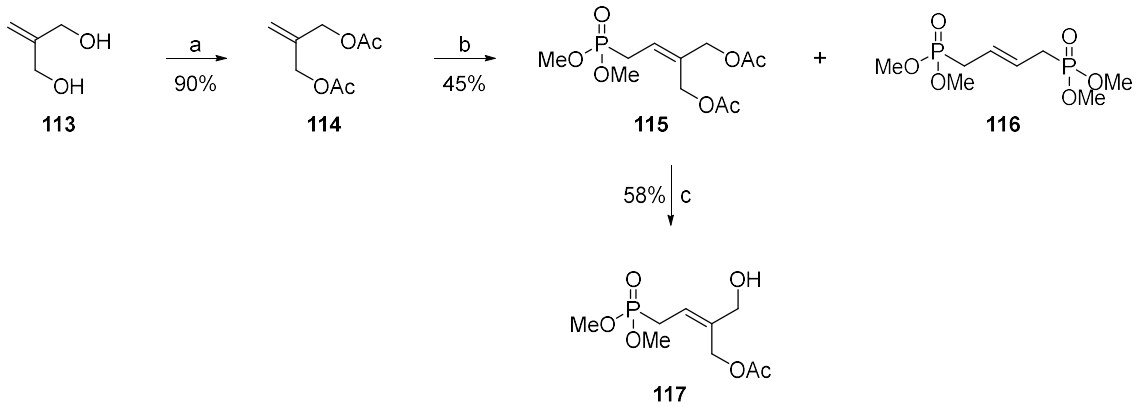


Scheme 3.6 General approach for the preparation of (*E*)-prop-1-enyl AN phosphoroamidate and (*E*)-prop-1-en-3-oxymethyl ANP phosphonoamidate prodrugs.

3.7 Results and Discussion

3.7.1 Preparation of (*E*)-2-hydroxymethyl-but-2-enyl ANP ProTides

At the beginning of the preparation of (*E*)-2-hydroxymethyl-but-2-enyl pyrimidine ProTides we attempted to adopt a reported synthetic pathway developed to afford stereo-defined trisubstituted alkenyl nucleoside phosphonates.[100] This synthetic approach (**Scheme 3.7**) started with the preparation of the olefin **114** from the commercially available 2-methylene-1,3-propanediol **113** under lipase-catalysed acetylation conditions in the presence of an excess of vinyl acetate as acetyl source. *Candida antarctica* lipase B (CAL-B) was the enzyme used to catalyse the acetylation of **113** in 90% yield.



Scheme 3.7 Synthetic scheme for the preparation of olefin **117**. Reagents and conditions: a) vinyl acetate, *Candida antarctica* lipase-B, CH_2Cl_2, rt, 24h; b) dimethyl allyl phosphonate, Hoveyda-Grubbs 2nd generation catalyst, CH_2Cl_2,))), 40°C, 24h; c) *Candida antarctica* lipase-B, phosphate buffer (0.1 M, pH 7), rt, 16h.

The fully acetylated olefin **114** was reacted with the dimethyl allylphosphonate through ultrasound-assisted cross-metathesis technology. Although the optimised literature conditions (1 eq of dimethyl allyl phosphonate and 2 eq of olefin **114** in CH_2Cl_2 sonicated for 24h at 40°C in the presence of 15 mol% of Hoveyda-Grubbs 2nd generation catalyst added in three equal portions)[100] were applied, a significant amount of homodimer by-product **116** was obtained along with the desired product **115**. A careful purification by flash chromatography was necessary to separate the two compounds and isolate compound **115** in 45% yield. The monoacetate **117** was then obtained by reacting the diacetyl compound **115** with CAL-B in phosphate buffer (0.1 M, pH 7). This enzyme was employed thanks to its excellent selective hydrolytic effect toward the *trans* acetyl group,

affording the desired (*Z*)-monoacetyl derivative **117** in 58% yield. Starting olefin **115** was recovered along with the fully hydrolysed compound, but no traces of the (*E*)-isomer were detected or isolated. The *cis* configuration of **117** was confirmed by NOESY (Nuclear Overhauser effect spectroscopy) experiment (**Figure 3.14**).

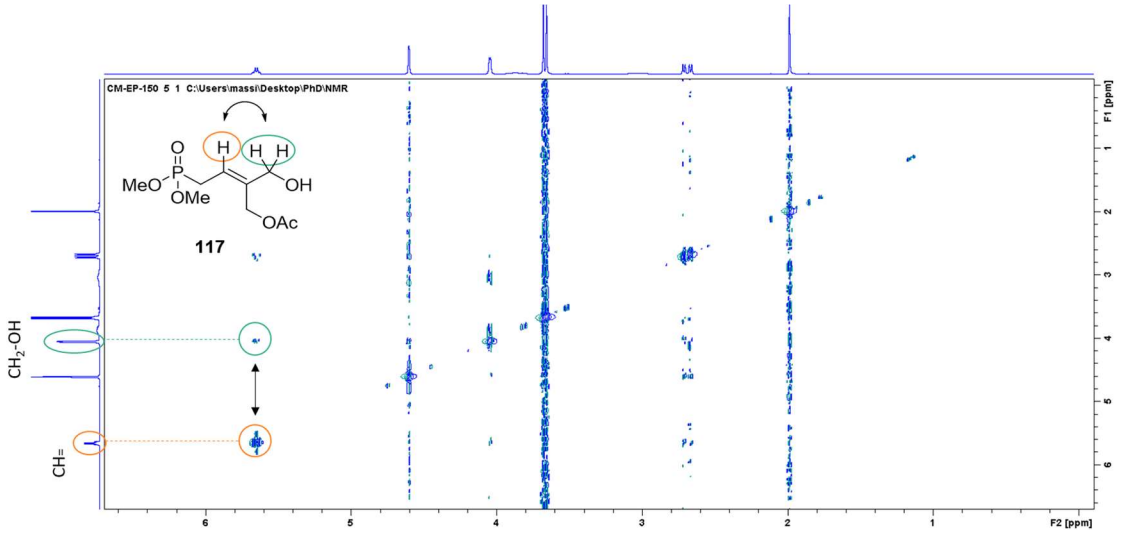


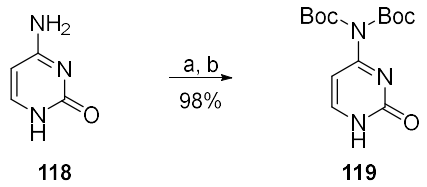
Figure 3.14 H-H 2D NOESY NMR experiment of **117** ($CDCl_3$). The cross-peaks between the *CH*= (in orange) and the CH_2-OH (in green) are highlighted. This correlation indicates that the protons are close in space laying on the same side of the double bond (*cis* configuration).

The NOESY is a bi-dimensional NMR experiment useful for determining which signals arise from protons that are close to each other in space even if they are not bonded. In this case, from **Figure 3.14** it is possible to observe the cross-peaks between the *CH*= and the CH_2-OH protons indicating they are located on the same side of the double bond and confirming the *cis* configuration.

Once **117** was prepared and its configuration confirmed, its coupling with cytosine nucleobase **118** was attempted. To avoid the interference of the cytosine NH_2 in the next reaction, **118** needed to be protected before being employed under the Mitsunobu conditions (**Scheme 3.8**).

The cytosine nucleobase was protected through a one-pot two steps procedure. Initially, the nucleobase was reacted with di-*tert*-butyl dicarbonate (Boc_2O) in the presence of a catalytic amount of 4-(dimethylamino)pyridine (DMAP), which forms a Boc-pyridinium species where DMAP is an excellent leaving group. Thus, the attack of the amines on the carbonyl site is accelerated affording the fully protected cytosine quantitatively. The compound was solubilised in MeOH and then treated with $NaHCO_3$ aqueous saturated

solution to afford the desired *N*-(Boc)$_2$ cytosine **119** in almost quantitative yield (98%) after 4h at stirring.[128]

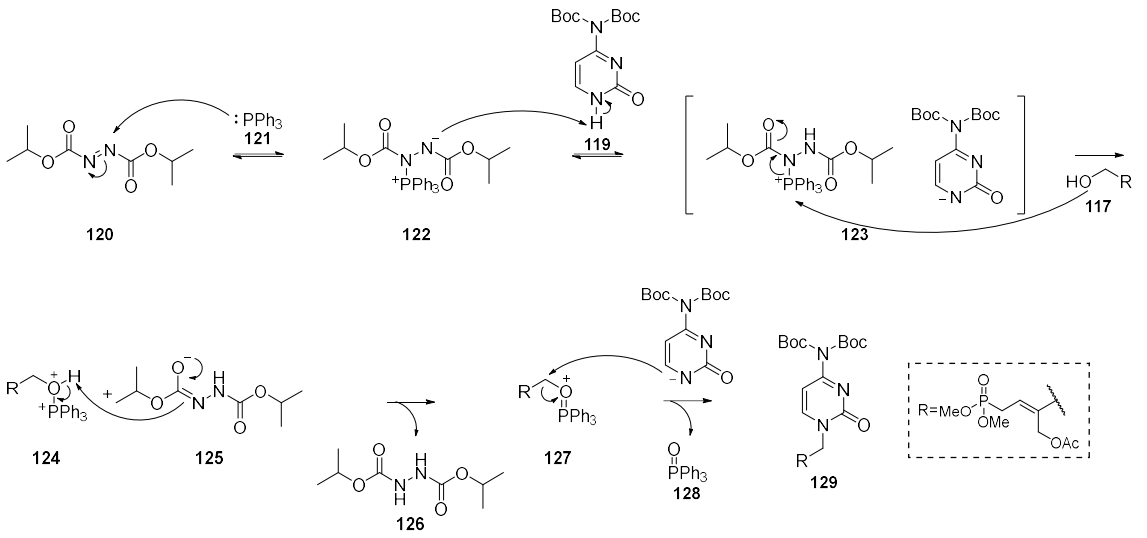


118 119

Scheme 3.8 Cytosine Boc-protection. Reagents and conditions: a) (Boc)$_2$O, DMAP, THF, rt, 16h; b) $NaHCO_3$ (aq.), MeOH, 60°C, 4h.

After their preparation, **117** and **119** were coupled under the Mitsunobu conditions (**Scheme 3.9**). The Mitsunobu reaction is a widely used method for the condensation of a pronucleophile (*NH*-nucleobase **119**) with a primary or secondary alcohol (**117**). The procedure requires a reducing phosphine reagent (triphenylphosphine, PPh_3, **121**) together with an oxidizing azo-reagent (diisopropyl azodicarboxylate, DIAD, **120**).

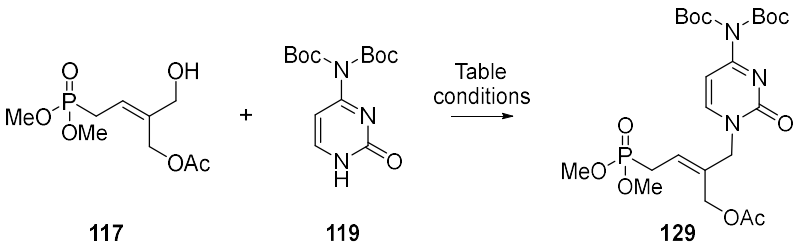
The reaction starts with the nucleophilic attack of PPh_3 (**121**) upon DIAD (**120**), which forms the zwitterionic intermediate **122**. Then the nucleobase is deprotonated by **122** to form the ion pair **123**. After the alcohol **117** binds to the phosphonium ion to form **124**, a rearrangement occurs to release saturated DIAD-H_2 (**126**) and the key oxyphosphonium ion **127**. The nucleophilic displacement of the nucleobase upon **127** completes the reaction and the new C-N bond is generated (**129**). The formation of the strong P=O bond in the triphenylphosphine oxide by-product **128** drives this reaction forward.[129]



120 122 123 124 125 126 127 128 129

Scheme 3.9 Mechanism of the Mitsunobu reaction using **117** and **119** as substrates to be coupled.

Several reaction conditions were attempted (**Table 3.1**). Firstly, the reaction was performed under the conditions reported in Agrofoglio's protocol (entry 1).[130] To a solution of the monoacetate **117** (1.5 eq), protected nucleobase **119** (1 eq) and PPh_3 (1.5 eq) in THF, DIAD (1.5 eq) was added dropwise at room temperature and the resulting mixture was heated at 70°C for 20h. Unfortunately, under these conditions, only multiple inseparable by-products were obtained, and no traces of the desired compound were detected by either ESI-MS or ^{1}H-NMR analyses of the crude mixture. The same result was observed when the conditions reported in entry 2 were attempted: PPh_3 (3 eq) and DIAD (3 eq) were stirred at room temperature in THF for 3h to allow the formation of the betaine intermediate before the nucleobase (1.7 eq) and the phosphonate (1 eq) addition. After a careful examination of the reaction conditions reported in the literature, it was noticed that the temperature at which the azo-compound is added could be a crucial factor.[131] In fact, the reaction did not work when DIAD was added at room temperature (entry 1 and 2). Otherwise, when the azo-compound was added dropwise to the stirred mixture of the two substrates and PPh_3 at 0°C, the final product **129** was finally isolated although in only 9% yield (entry 3). Pleasingly, when an excess of the phosphonate **117** (1.1 eq) was used, the formation of the desired product with 32% yield (entry 4) was observed.



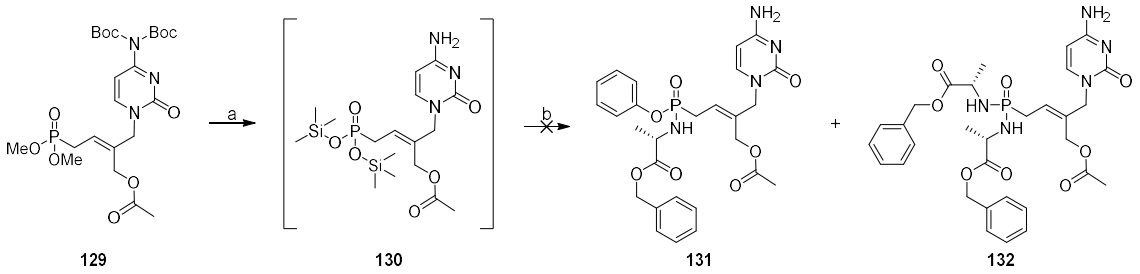
117 119 129

Entry	Nucleobase 119 (eq)	Alcohol 117 (eq)	PPh_3 (eq)	DIAD (eq)	T°C	Time	Yield
1[a]	1	1.5	1.5	1.5	rt to 70°C	20h	0%
2[b]	1.7	1	3	3	rt to 70°C	20h	0%
3[a]	1	0.6	1	1	0°C to 70°C	20h	9%
4[a]	1	1.1	1.1	1.1	0°C to 70°C	20h	32%

Table 3.1 Mitsunobu reaction optimisation. All the reactions were performed in THF. The yield was determined on the isolated product. [a]DIAD added dropwise to a stirring mixture of **117**, **119** and PPh_3. [b]DIAD and PPh_3 stirred 3h at room temperature before substrates addition.

With the phosphonate backbone **129** in hand, we moved to explore the application of the ProTide approach to this substrate. To prepare the phosphonoamidate prodrug of this scaffold, the one-pot procedure reported by Holy and co-workers,[26,122] and slightly modified by our group, was employed.[121] The modifications, which included the use of an amino acid ester (1 eq) and an excess of aryl alcohol (6 eq), allowed the synthesis of the phosphonoamidate (ProTide) prodrug along with the bis-amidate derivative.

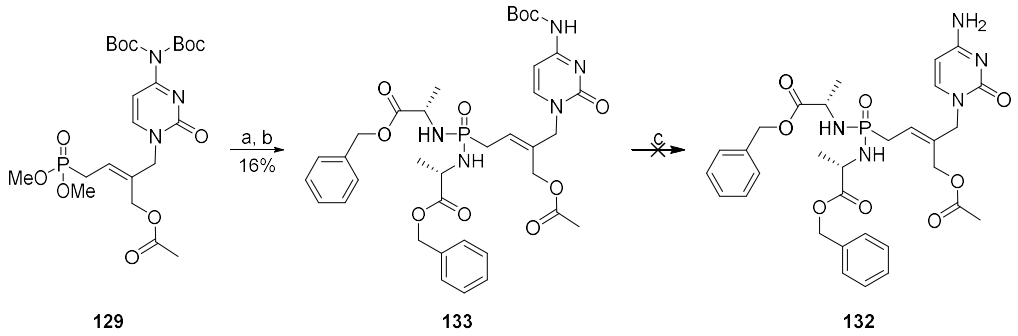
In **Scheme 3.10** is reported the application of this methodology on phosphonate **129**.



Scheme 3.10 Synthetic scheme attempted for the preparation of ProTide **131** and Bis-amidate **132**. Reagents and conditions: a) TMSBr, ACN, rt, 16h; b) *L*-alanine benzyl ester hydrochloride salt, phenol, Et_3N, aldrithiol-2, PPh_3, pyridine, 50°C, 16h.

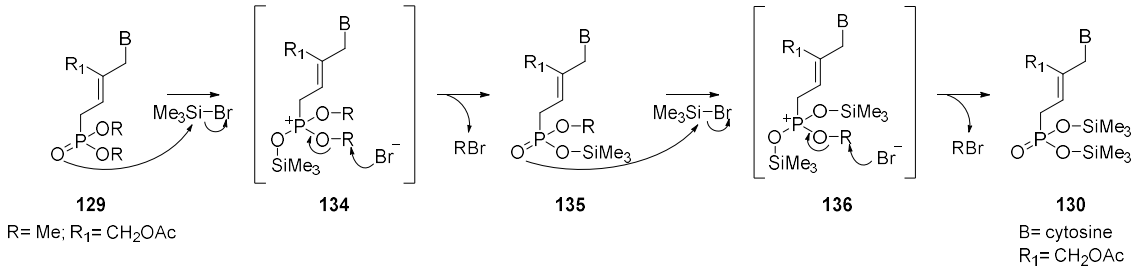
Briefly, the methyl phosphonate **129** was converted into the correspondent silyl ester **130** with an excess of trimethylsilyl bromide in dry acetonitrile solution. The reaction mixture was concentrated in vacuo without any contact with air and the silyl intermediate was not purified because of its sensitivity to moisture. The resulting crude reaction mixture was diluted in acetonitrile and immediately reacted with the amino acid (1 eq) and an excess of phenol (6 eq), in the presence of a solution of PPh_3 (6 eq) and Aldrithiol-2 (6 eq) in pyridine and triethylamine. Predictably, the use of TMSBr resulted in an acidic environment able to induce the *N*-cytosine Boc-deprotection. Therefore, even though the two deprotected prodrugs were identified by ESI-MS, the HPLC analysis of the reaction crude mixture showed numerous by-products. After an extensive flash chromatography purification and preparative RP-HPLC, only traces of both ProTide **131** and phosphonodiamidate **132** were detected. The small amount of phosphonoamidate obtained was attributed to the low reactivity of the substrate. Because of this inconvenience, the synthesis of the phosphonodiamidate prodrug **132** as the only product was attempted. When **129** was reacted with an excess of amino acid ester (3 eq) under the reaction conditions previously described (**Scheme 3.11**), it was possible to isolate only the mono-Boc derivative **133** (20 mg, 16%). Unfortunately, attempts to obtain the final

phosphonodiamidate **132**, *via* deprotection of **133** under acidic conditions (CH_2Cl_2/TFA) were not successful giving an inseparable mixture of several by-products.



Scheme 3.11 Synthetic scheme attempted for the preparation of the Bis-amidate **132**. Reagents and conditions: a) TMSBr, ACN, rt, 16h; b) *L*-alanine benzyl ester hydrochloride salt, Et_3N, aldrithiol-2, PPh_3, pyridine, 50°C, 16h; c) TFA/CH_2Cl_2 (1:1, v/v), 0°C to rt, 5h.

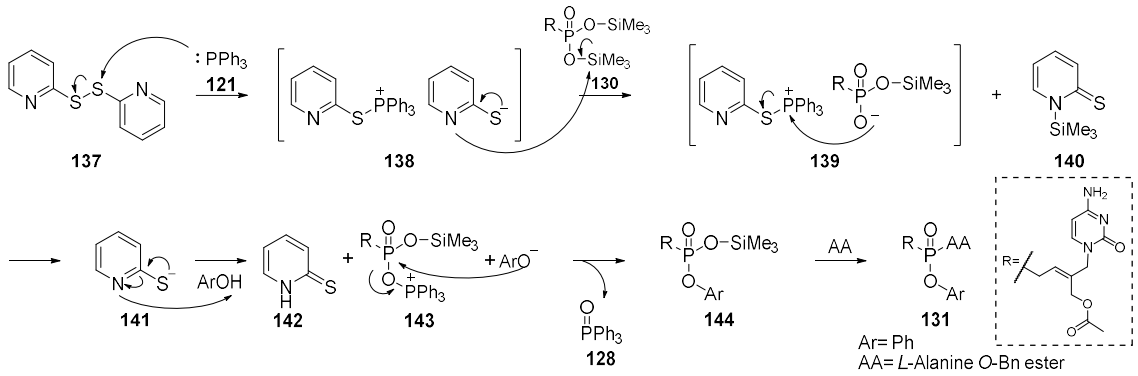
The application of the ProTide approach attempted consists in two steps one-pot procedure. The first step was the McKenna reaction (**Scheme 3.12**).[132]



Scheme 3.12 McKenna reaction mechanism.

According to the mechanism, the phosphonate terminal oxygen attacks the silicon atom, expelling a bromide anion from TMSBr and forming a phosphonium-like intermediate **134**. The P=O bond is quickly regenerated from one of the alkyloxy moieties upon the attack of the bromide anion on the neighbouring carbon. The just restored P=O then attacks a second molecule of TMSBr following the same pathway previously described to achieve the bis(trimethylsilyl)phosphonate **130**.[132] The mechanism for the second step is reported in **Scheme 3.13**. The sulphur of the aldrithiol-2 reagent **137** is attacked by the triphenylphosphine. A salt intermediate is generated. The pyridinethione anion attacks a silicon atom of **130** forming a new salt with a phosphonium anion and releasing the pyridinethione derivative **140**. An internal substitution occurs in the salt **139** and another pyridinethione anion (**141**) is generated, which is protonated by the alcohol. The

previously formed phosphoryloxyphosphonium salt **143** reacts with the aryloxy (or the amino acid ester) moiety and the release of the triphenylphosphine oxide **128** occurs. The monosubstituted nucleotide **144** undergoes the same reaction one more time with the amino acid ester affording the desired prodrug.



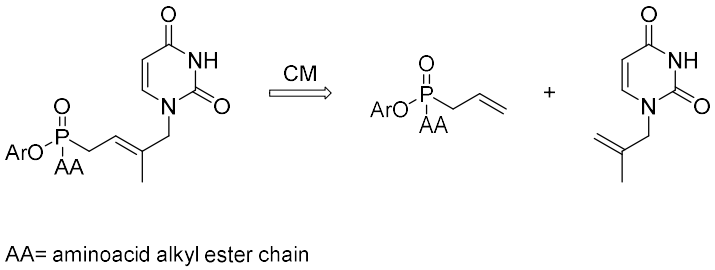
Scheme 3.13 Condensation of the bis(trimethylsilane)phosphonate with the adequate alcohol and amino acid ester.

It was clear from the results observed that the difficulty to obtain the final ProTide under these conditions was related not only to the weak nucleophilicity of the phenol compared with the more nucleophilic amino acid ester, but also to the formation of different products such as the isolated mono protected **133**.

These results, along with the side reactions often encountered in previous works during the application of this methodology (reduction of halogenated pyrimidine nucleobase, bromination of the double bond, phosphonodiamidate formation),[121] prompted us to explore other synthetic strategies, that would give better access to unsaturated pyrimidine ANP phosphonoamidates. The development of such strategy is the topic of the next paragraph.

3.7.2 Preparation of (*E*)-2-methyl-but-2-enyl ANP ProTides

Due to the difficulties encountered in the procedure reported in the previous paragraph, we turned our attention to the possibility to use CM to prepare branched alkenyl ANP prodrugs. (**Scheme 3.14**). Although in the literature there are several examples for the synthesis of ANP prodrugs by CM,[99] the application of this strategy for the ProTide preparation at the time we started our investigations was not reported.



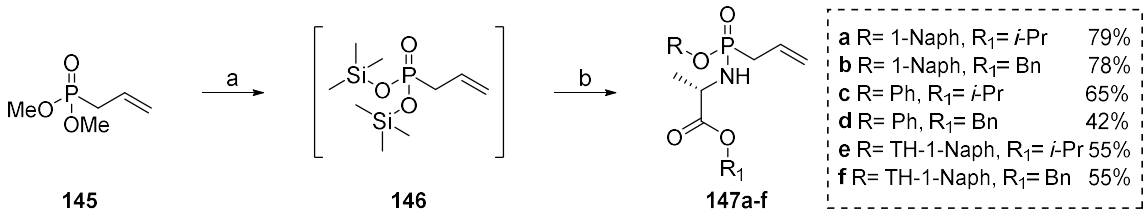
AA= aminoacid alkyl ester chain

Scheme 3.14 Retrosynthetic scheme for the preparation of new ANP ProTides via cross metathesis.

3.7.2.1 Synthesis of allylphosphonoamidate derivatives

To achieve this goal, and to find the best conditions to optimize this procedure we decided to prepare a simplified trisubstituted alkenyl phosphonate, starting from two easily accessible synthons: the marketed dimethyl allylphosphonate and the 2-methyl allyl pyrimidine, whose synthesis was reported as a one-step reaction directly from the desired nucleobase. We also decided to use uracil as nucleobase to avoid the catalyst poisoning effect of the amine function belonging to the cytosine and therefore avoiding -NH_2 protection and deprotection steps.

This research began with the synthesis of the aryloxy allylphosphonoamidate synthon. This stage of the project was the most innovative. The procedures reported in literature for the preparation of this kind of substrate were tedious and long multi-step sequences.[133,134] For this reason, we envisaged to apply to our advantage the previously described Holy's one-pot procedure[122] directly on the commercially available dimethyl allylphosphonate **145**, so that the aryloxy allylphosphonoamidate intermediates could be easily prepared (**Scheme 3.15**).



145 **146** **147a-f**

Scheme 3.15 Syntheses of *O*-aryl-(*L*-alanine ester)-allylphosphonate **147a-f**. Reagents and conditions: a) TMSBr, 2,6-lutidine, ACN, rt, 16h; b) amino acid ester hydrochloride salt, aryl alcohol, Et_3N, aldrithiol-2, PPh_3, pyridine, 50°C, 16h.

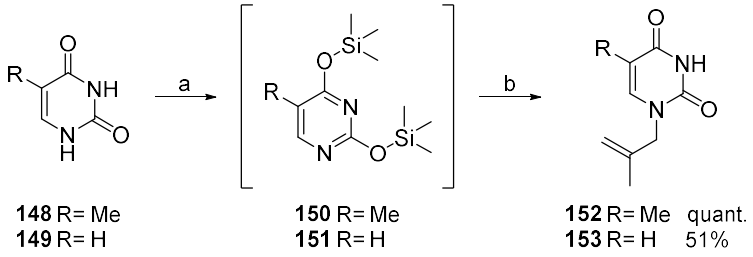
Commercial dimethyl allylphosphonate **145** was converted into the corresponding silyl ester **146**, by reaction with an excess of TMSBr (5.0 equivalents) (for the mechanism see **Scheme 3.12**). Due to the hydrolytically instability of this ester, **146** was not isolated but

immediately dissolved in a mixture of pyridine/Et_3N and treated with the *L*-alanine isopropyl ester hydrochloride (1.0 equivalents), an excess of 1-naphthol (6.0 equivalents), and a premade solution of PPh_3 (6.0 equivalents) and aldrithiol-2 (6.0 equivalents) in pyridine. After 16h, the crude mixture did not show the presence of either the desired product or phosphonodiamidate compound. This lack of reactivity was attributed to the decomposition of the disilylester **146** caused by the release of hydrobromic acid, due to the excess of TMSBr employed. Pleasingly, when the reaction was attempted in presence of 2,6-lutidine (4.0 equivalents) as acid scavenger, the formation of the desired product **147a** was observed by ^{31}P NMR and LC-MS analysis of the crude mixture. Quite surprisingly, no evidence of side reactions, including the phosphonodiamidate prodrug formation, were detected.[121] For this reason, the preparation of the aryloxy allylphosphonoamidate **147a** as the only product was achieved in very good yield (79%). With the above methodology, six different allyl phosphonate analogues were prepared (**147a-f**), and a variety of aryloxy groups was introduced in combination with two different amino acid esters (*L*-alanine isopropyl or benzyl esters). This method worked well with aryl alcohols with different steric requirements. Moreover, the allyl phosphonoamidates bearing the 5,6,7,8-tetrahydro-1-naphthol **147e** and **147f** were also prepared considering the remarkable antiviral activities of compounds bearing this promoiety in previous series.[26,121] This procedure is short and efficient, representing a great improvement over the literature methods.

With these allyl phosphonoamidates in hand, we moved to the synthesis of the alkene-functionalised nucleobases for the cross-metathesis reaction.

3.7.2.2 Synthesis of N^1-(methylbut-2-enyl) pyrimidines

The syntheses of N^1-(methylbut-2-enyl) pyrimidines **152** and **153**, selected as the other partners for the cross-metathesis reactions are reported in **Scheme 3.16**.



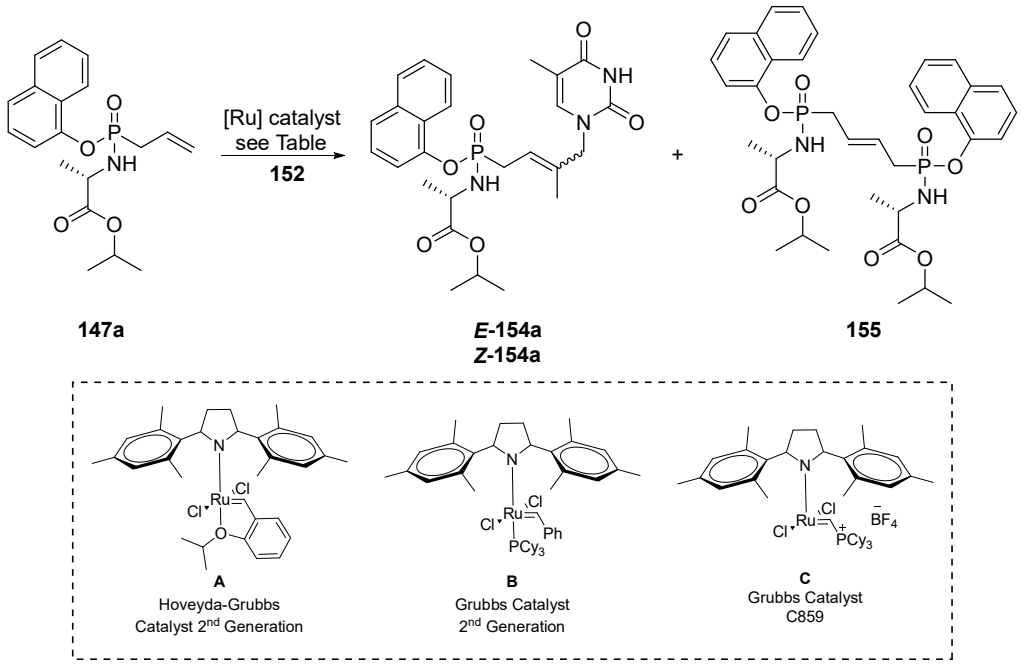
Scheme 3.16 Syntheses of N^1-(methylbut-2-enyl) pyrimidine **152** and **153**. Reagents and conditions: a) BSA, ACN, reflux, 30 min; b) 3-bromo-2-methylpropene, NaI, TMSCl, 55°C, 16h.

The nucleobase was persilylated using *N*,*O*-bis(trimethylsilyl)acetamide (BSA) to obtain intermediates **150** and **151** that were not isolated. The persilylation method is usually preferred to the direct alkylation of nucleobases. Intermediates **150** and **151** are much more soluble than the starting nucleobases, therefore milder reaction conditions are required for the alkylation, which is thus usually achieved in higher yields.[135,136] The starting nucleobase and the silylating agent were refluxed until the complete solubilisation of the compound was observed indicating the completion of the silylation. The alkylating reagent 3-bromo-2-methylpropene was directly added in the crude mixture in the presence of trimethylsilyl iodide (TMSI) generated *in situ* from trimethylsilyl chloride (TMSCl) and NaI. TMSI converts the alkyl bromine in the more reactive alkyl iodine agent that can easily be attacked by the more nucleophilic N^1 atom. [135–137] The final aqueous work up releases **152** and **153** in quantitative and 51% yields, respectively. Although thymine **148** and uracil **149** were not protected at the N^3-positions, the selective N^1-alkylation was easily performed without formation of the N^3,N^1-dialkylated derivative.

With both alkenyl derivatives in hand, the investigation of the cross-metathesis conditions between the two olefins (**147a-f** with **152** or **153**) could be approached.

3.7.2.3 Olefin cross-metathesis application to (E)-2-methyl-but-2-enyl ANP ProTides preparation

Taking into account all the considerations of the CM reaction discussed in paragraph 3.5.1, with both the aryloxy allylphosphonoamidate synthon and N^1-(methylbut-2-enyl) pyrimidines in our hands (type I and type III olefins respectively),[100,112] investigations into the cross-metathesis conditions between **147a** and **152** as model reaction were performed (**Table 3.2**).



Entry	catalyst	*E*-154a/155	*E*/*Z*-154a	154a (%)
1[a]	A	1:0.4	1:0.2	24
2[a]	B	1:1.4	1:0.1	11
3[a]	C	1:9	1:0.7	3
4[b]	A	1:0.3	1:0.2	26
5[b,c]	A	1:0.3	1:0.2	26

Table 3.2 Screened conditions for CM. Reagents and conditions: allyl phosphonoamidate **147a** (1.0 eq), olefin **152** (2.0 eq), ultrasound irradiation (37 MHz), reflux, CH_2Cl_2. Catalyst (5 mol%) added at t= 0, 2, 4 h. Ratio Het/Homo and *E*/*Z* determined by HPLC. [a]Reactions sonicated for 24 h. [b]Reactions sonicated for 36h. [c]Further addition of the catalyst (5 mol%) at t= 24h.

As a first attempt, the two olefins were sonicated in the presence of Hoveyda-Grubbs 2nd generation catalyst at 37 MHz in CH_2Cl_2 for 24h at reflux temperature (entry 1). [99] The reaction mixture, as expected, gave a mixture of *E*/*Z* isomers, which was isolated by column chromatography. The homodimer **155** was formed along with the *E*/*Z* derivatives (entry 1). Both ***E*-154a** and ***Z*-154a** isomers (24% and 3% yield, respectively) were isolated by preparative reversed phase-HPLC and their configurations were confirmed by NOESY experiments. The *E* geometry of isomer ***E*-154a** was strongly supported by the cross-peaks between the CH= and the CH_2-N protons indicating they are located on the same side of the double bond. Moreover, the *trans* configuration is also confirmed by the interaction between CH_2-P and CH_3 protons (**Figure 3.15**).

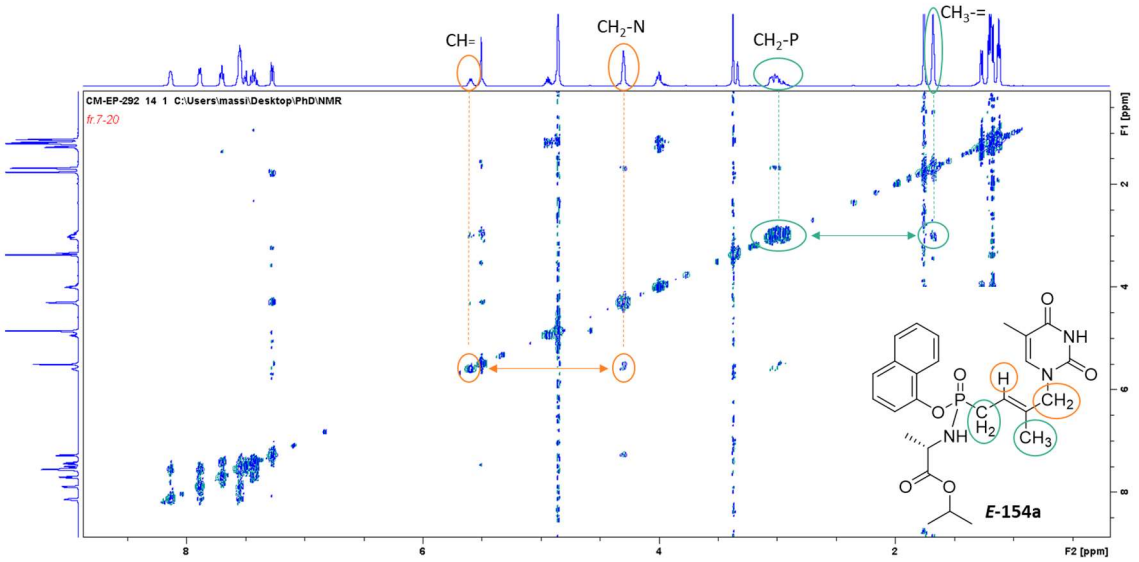


Figure 3.15 H-H 2D NOESY NMR experiment of ***E*-154a** ($CDCl_3$). The cross-peaks between the *CH*= and the *CH*$_2$-N (in orange) are highlighted as well as the cross peaks between *CH*$_2$-P and *CH*$_3$ (in green). This correlation indicates that the protons are close in space laying on the same side on the double bond (*trans* configuration).

To further confirm the above data, the NOESY spectra of ***Z*-154a** was also analysed (**Figure 3.16**). The cross-peaks between CH_2-N and CH_2-P protons were detected confirming the *cis* configuration of the compound.

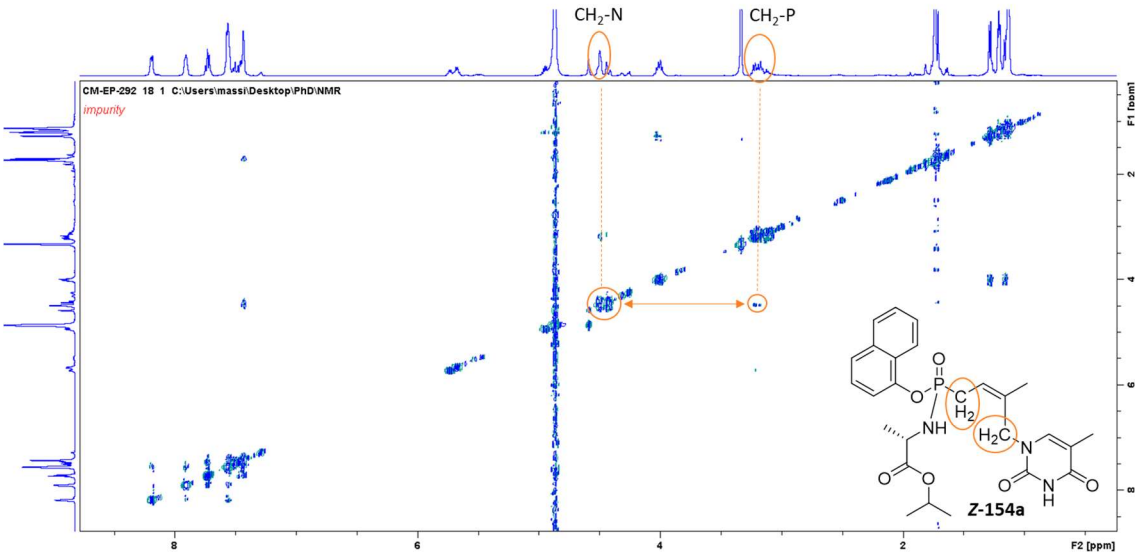


Figure 3.16 H-H 2D NOESY NMR experiment of ***Z*-154a** ($CDCl_3$). The cross-peaks between *CH*$_2$-N and *CH*$_2$-P (in orange) are highlighted. This correlation indicates that the protons are close in space laying on the same side on the double bond (*cis* configuration).

As depicted in **Figure 3.17**, the ratio Hetero/Homo and *E*/*Z* could be determined by direct HPLC analysis of the crude reaction mixture.

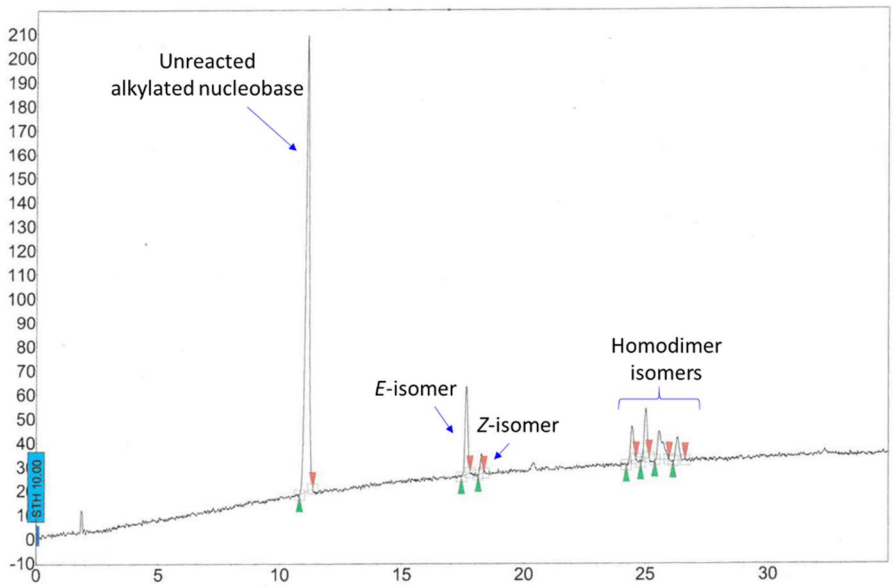
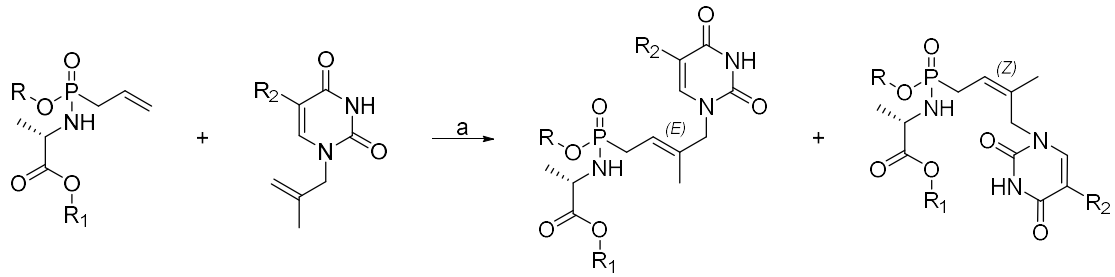


Figure 3.17 Analytical HPLC chromatogram of the CM reaction crude mixture (entry 1). Reverse phase HPLC eluting with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 minutes, 1ml/min, λ= 280 nm.

Any attempt to improve the reaction outcome (**Table 3.2**) using different catalysts (Hoveyda-Grubbs 2nd generation (A), Grubbs 2nd generation (B) and Grubbs catalyst C859 (C) was unsuccessful, providing **154a** in similar or lower yields and almost identical *E*/*Z* ratios (entries 2-3, **Table 3.2**).

Since catalyst A resulted the best in terms of product/homodimer ratio, further screenings were conducted using this catalyst. Prolonged reaction time (entry 4, **Table 3.2**) resulted in a slightly increased yield. However, the yield was not further improved with addition of more catalyst (entry 5, **Table 3.2**). Therefore, using the conditions of entry 1 in **Table 3.2**, different aryloxy phosphonoamidates of both thymine and uracil derivatives were prepared (**Scheme 3.17**). The *E*/*Z* mixtures were isolated by flash chromatography and then the two isomers were separated by preparative RP-HPLC to obtain the desired compounds (***E*-154a-f** and ***E*-156a-f**) in moderate yields (5%-36%). In a few cases *Z*-isomers (***Z*-154a**, ***Z*-154e**, ***Z*-154f**, ***Z*-156e**) were also isolated in 1% to 7% yield.

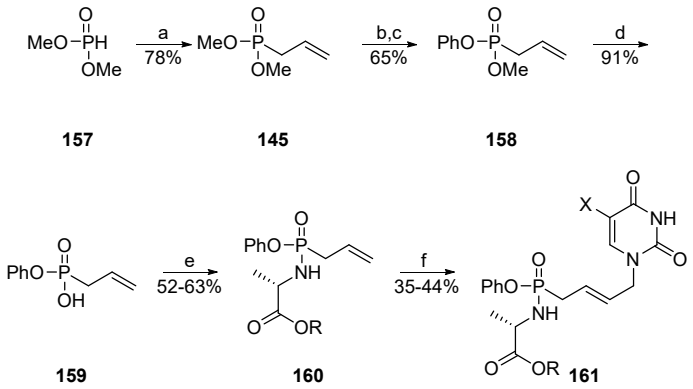


E-154 R_2= Me		*E*-156 R_2= H	
a R= 1-Naph, R_1= *i*-Pr	36%	**a** R= 1-Naph, R_1= *i*-Pr	14%
b R= 1-Naph, R_1= Bn	13%	**b** R= 1-Naph, R_1= Bn	5%
c R= Ph, R_1= *i*-Pr	10%	**c** R= Ph, R_1= *i*-Pr	10%
d R= Ph, R_1= Bn	23%	**d** R= Ph, R_1= Bn	18%
e R= TH-1-Naph, R_1= *i*-Pr	26%	**e** R= TH-1-Naph, R_1= *i*-Pr	11%
f R= TH-1-Naph, R_1= Bn	14%	**f** R= TH-1-Naph, R_1= Bn	5%

Scheme 3.17 ProTide synthesis via cross-metathesis. Reagents and conditions: a) Hoveyda-Grubbs 2nd generation catalyst, CH_2Cl_2, ultrasound irradiation, reflux temperature, 24h.

Since ruthenium catalyst was used during the synthesis, the measure of its residual amount in the final sample is, evidently, a crucial point that merits adequate consideration if any of the compounds need to be tested *in vivo*. The analytical technique commonly used for qualitative and quantitative trace element detection is ICP-MS (inductively coupled plasma mass spectrometry).[138] The analysis was performed on compound ***E*-156e** and showed ruthenium content of 0.116mg/g. According to the criteria defined by regulatory bodies, the residual metal present in a drug needs to be typically less than 0.01mg/g.[139] Therefore, further purification[140,141] will have to be considered if this methodology will be used for preparing compounds progressing to preclinical and clinical evaluation in order to comply the FDA recommended limits for residual metal catalyst in a drug.[139]

During this work,[142] a similar strategy for the synthesis of alkenyl ANP ProTides was developed and published by Bessieres and co-workers.[124] They reported the use of cross metathesis for the synthesis of ProTide derivatives of linear (*E*)-but-2-enyl nucleoside scaffolds (**Scheme 3.8**). Among the disadvantages of this strategy, the key allyl phosphonoamidate intermediate **160** was prepared through a long multi-steps sequence accompanied by several tedious purifications accounting for only 29% overall yield in four steps.



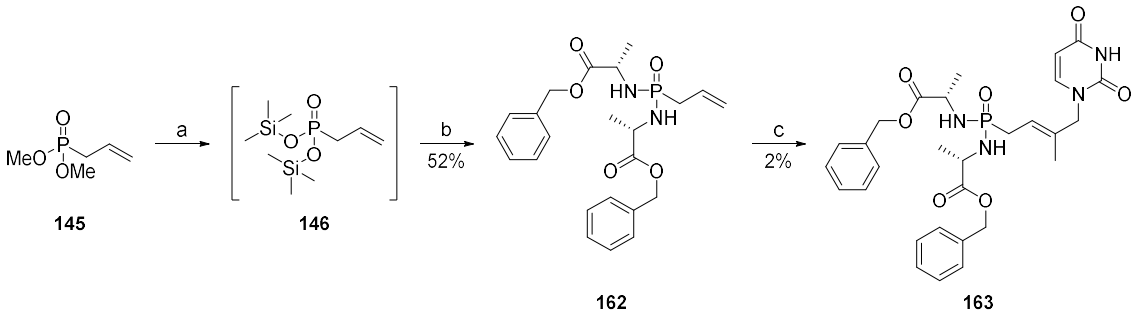
Scheme 3.18 Synthesis of the allyl aryloxy phosphonoamidate **160** and corresponding ANP ProTide **161**. Reagents and conditions: a) allyl bromide, K_2CO_3, TBAB, THF, 90°C, 12h; b) $(COCl)_2$, CH_2Cl_2, 50°C, 24h; c) Et_3N, phenol, CH_2Cl_2, 50°C, 48h; d) TMSBr, CH_2Cl_2, rt, 24h; e) $(COCl)_2$, DMF cat., Et_3N, CH_2Cl_2, amino acid ester hydrochloride salt, rt, 24h; f) N^1-crotylated uracil, Grubbs-II catalyst, H_2O,))), 55°C, 20h.

Therefore, the synthesis of allyl phosphonoamidate and its further application in olefin cross-metathesis for the synthesis of ANP ProTides[142] here reported proves still to be an effective and improved methodology when compared with the latest long, multi-step published procedure.[124]

Additionally, pleased to the outcome of our procedure, its application to phosphonodiamidate prodrug preparation was also investigated.

3.7.2.4 Synthesis of (E)-2-methyl-but-2-enyl ANP Bis-Amidate prodrugs

To explore the versatility of our optimised methodology toward the synthesis of novel trisubstituted alkenyl ANP phosphonoamidates, the same reaction conditions were applied for the synthesis of the symmetrical phosphonodiamidate **163**. Briefly, the desired bis-amidate intermediate **162** was obtained in 52% yield by treating the allyl phosphonate **145** with an excess of TMSBr (in presence of 4 eq of 2,6-lutidine) and the resulting silyl diester was reacted with an excess (5 eq) of benzyloxy *L*-alanine hydrochloride salt (**Scheme 3.19**). Compound **162** was then subjected to olefin cross-metathesis with the appropriate alkylated pyrimidine **153** under the conditions reported in **Scheme 3.19**. Phosphonodiamidate **163** was obtained as a mixture of the *E* and *Z* isomers. The *E*-isomer was isolated in 2% yield, after purification by preparative reverse phase-HPLC.

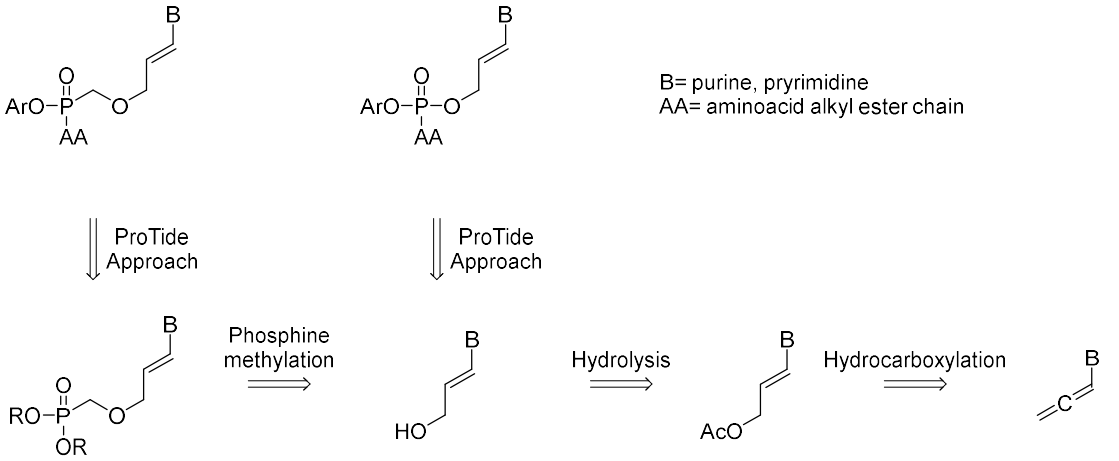


Scheme 3.19 Synthesis of symmetrical allyl phosphonodiamidate **163**. Reagents and conditions: a) TMSBr, 2,6-lutidine, ACN, rt, 16h; b) benzyloxy-L-alanine hydrochloride salt, Et_3N, aldrithiol-2, PPh_3, pyridine, 50°C, 16h; c) N^1-(methylbut-2-enyl) uracil **153**, Hoveyda-Grubbs 2nd generation catalyst, CH_2Cl_2 ultrasound irradiation, reflux temperature, 24h.

In conclusion, the developed synthetic pathway for the preparation of ANP prodrugs was largely successful for the synthesis of (*E*)-2-methyl-but-2-enyl uracil and thymine ProTides and bis-amidates.

3.7.3 Preparation of Linear Alkenyl ANP ProTides

Given the capacity of allenamines to be versatile building blocks in organic chemistry, this functional group was employed for the synthesis of alkenyl ANPs. Precisely, 9-allenyl-9*H*-purines were reacted with pronucleophiles for the introduction of different side chains on the N^9-purine nucleobase to afford acyclic nucleosides with high chemo-selectivities and *E*-selectivities.[127] To the best of our knowledge, no prodrug approaches have been applied to this interesting alkenyl acyclic purine scaffold. Therefore, the study on the disubstituted alkenyl ANP phosphonoamidate prodrugs was mainly focused on the installation of a (*E*)-prop-1-en-3-oxymethyl chain on both purine and pyrimidine nucleosides, following the retrosynthetic pathway reported in **Scheme 3.20**.



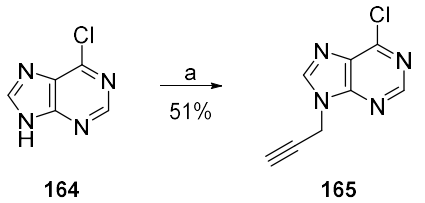
Scheme 3.20 Retrosynthetic scheme ideated for the preparation of the linear alkenyl acyclic nucleoside phosphonoamidate and phosphoroamidate prodrugs.

Essentially, a recently developed pathway for the preparation of acyclic nucleoside with diverse side chains *via* a common allene intermediate was applied.[127] The hydrocarboxylation of the allenylnucleoside followed by the hydrolysis of the acetyl group was envisaged as the proper route to achieve the hydroxyl intermediate. The primary alcohol could be used as substrate for the application of the ProTide approach to achieve the phosphoroamidate prodrug. Moreover, the hydroxyl intermediate could be additionally subjected to phosphine methylation so that the phosphonoamidate approach could be applied too.

As described in the following paragraph, the synthetic route started from the preparation of the key allene intermediate.

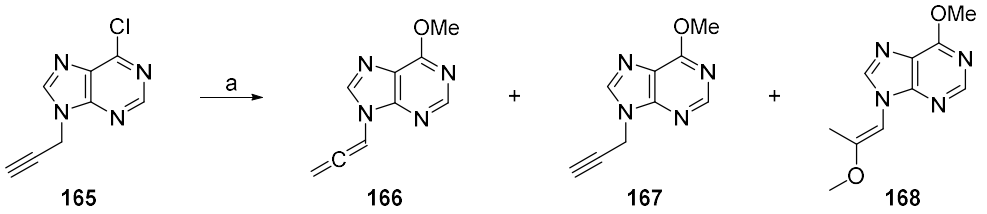
3.7.3.1 Preparation of 6-methoxy-9-allenylpurine

The synthetic route leading to the key allenylnucleoside started from the propargylation of the commercially available 6-chloropurine **164**, which was selected as starting material thanks to the easily replaceable chlorine atom in 6-position. The alkylation of the N^9-position was performed in the presence of propargyl bromide and NaH in DMF (**Scheme 3.21**).



Scheme 3.21 Synthesis of N^9-propargylpurine **165**. Reagents and conditions: a) propargyl bromide, NaH, DMF, rt, 24h.

The strong base was necessary to activate the purine deprotonating the N^9-postion to facilitate the nucleophilic substitution in the presence of the electrophilic propargyl bromide. The allene **166** was obtained from **165** in the presence of K_2CO_3 as base and MeOH as solvent under the conditions reported in **Table 3.3**.



Entry	Time	166 (yield %)	167 (yield %)	168 (yield %)
1	16h	3	-	70
2	1h	68	9	2
3	15 min	71	8	-

Table 3.3 Synthesis of N^9-allenylpurine **166**. Reagents and conditions: a) K_2CO_3, MeOH, reflux temperature.

Our first attempt was performed by refluxing a methanol solution of **165** and the base for 16h (entry 1, **Table 3.3**).[143] Under these conditions, the desired compound **166** was isolated in only 3% yield. From the chromatographic separation, an unknown compound was isolated. This major product (70% yield) was characterised by ^{1}H-NMR and mass spectrometry and corresponded to compound **168** (**Figure 3.18**).

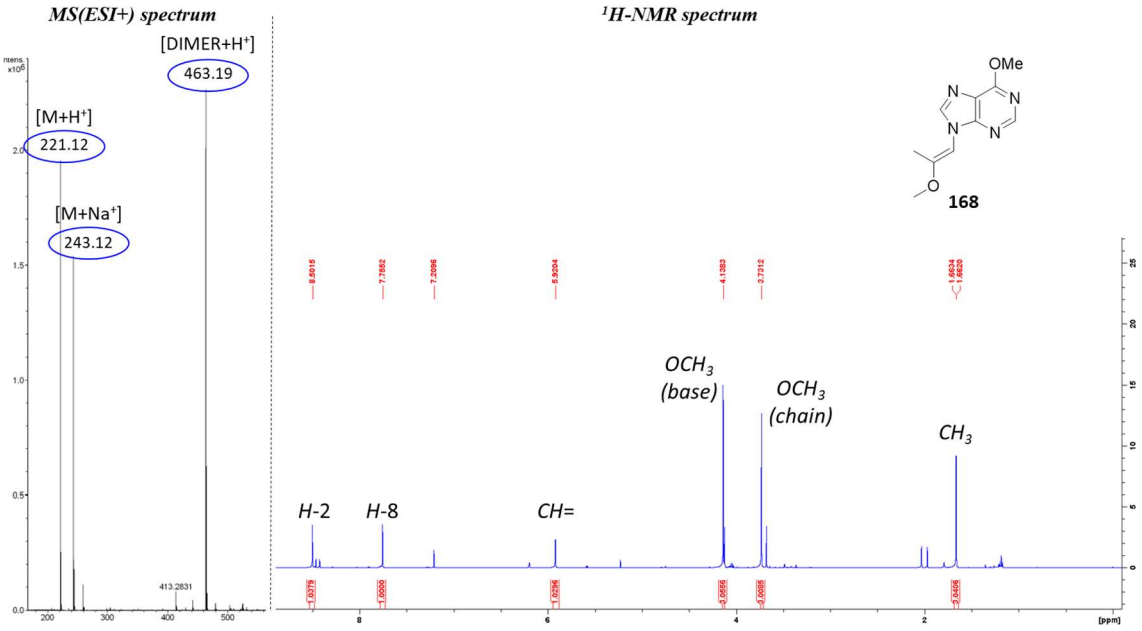
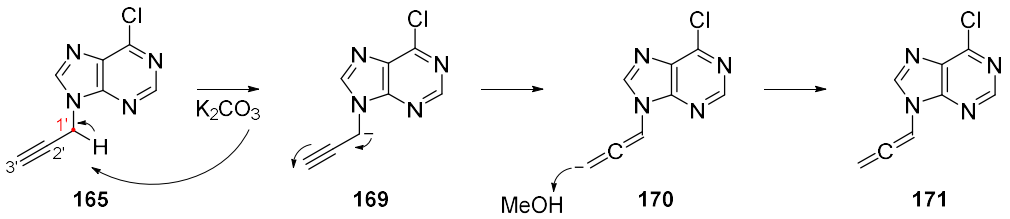


Figure 3.18 MS(ESI+) (left) and ^{1}H-NMR (500 MHz, $CDCl_3$) (right) spectra of compound **168**.

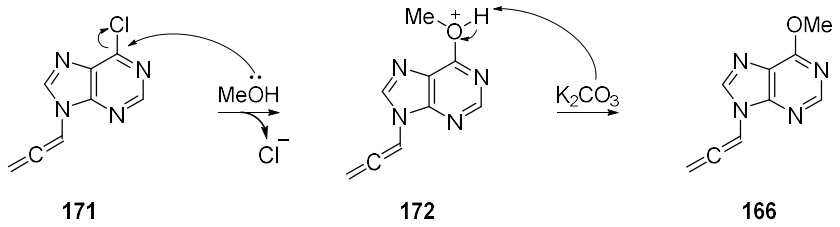
To minimise the formation of this product and increase the yield of the desired compound, the reaction conditions were changed. By refluxing the mixture for only 1h (entry 2, **Table 3.3**),[127] the desired product **166** was successfully isolated with 68% yield along with a small amount of the 6-methoxy-propargylpurine **167**. The reaction yield was further improved by reducing the reaction time to only 15 min (entry 3, **Table 3.3**), the time necessary to observe the disappearance of the starting material on TLC. In summary, the time was the crucial parameter to be optimised to make the reaction successful and obtain the desired key allene intermediate **166** in good yield.

This reaction was a key step in the synthetic pathway for the alkenyl nucleoside preparation. The aforementioned conditions allowed the formation of the double bond directly linked to the nucleobase by converting the propargyl group into an allene function. Moreover, the K_2CO_3/MeOH combination was also crucial for the replacement of the chlorine atom with the desired methoxy group.[144] Mechanistically (**Scheme 3.22**), the base (K_2CO_3) deprotonates the CH in 1'-position and the following electron rearrangement results in the formation of the carbene species in 3'-postion, which is readily protonated by the solvent.



Scheme 3.22 Reaction mechanism for the formation of the allene compound **171**.

At this stage, both potassium methoxide and methanol are capable to give the desired substituent in the purine nucleobase by attack the 6-postion. In this case, it can be assumed that methanol is the methoxy donor responsible for the attack on the 6-postion, mainly because it is present in a huge amount compared to potassium methoxide (**Scheme 3.23**).

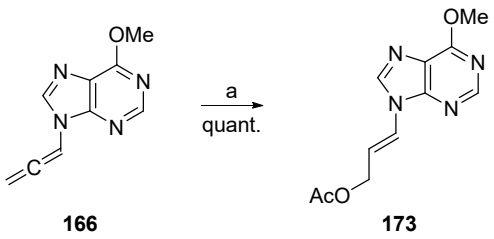


Scheme 3.23 Reaction mechanism for the formation of the oxymethyl derivative compound **166**.

Once the key allene intermediate was prepared, the (*E*)-3-hydroxyprop-1-enyl and (*E*)-prop-1-en-3-oxymethyl scaffolds, and corresponding ProTides, were prepared as reported in the following paragraphs.

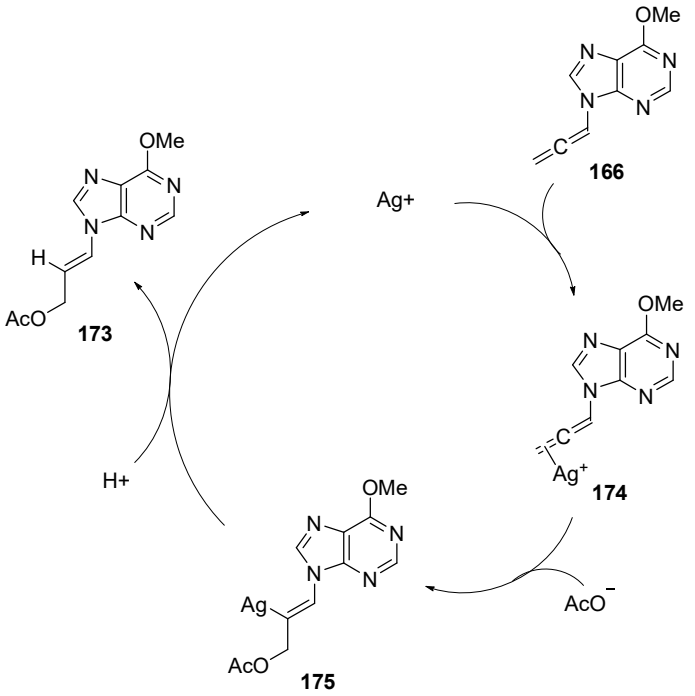
3.7.3.1 Synthesis of (E)-6-methoxy-9-(3-hydroxyprop-1-enyl)-9H-purine scaffold

Wei and co-workers performed extensive optimisation work to convert the allene-functionalised nucleobase into diverse side chains.[127] To accomplish this task, the allene **166** was converted into the acetyl derivative **173** by hydrocarboxylation. Compound **166** was treated with glacial acetic acid and ACN at 80°C for 16h to afford **173** in quantitative yield (**Scheme 3.24**). Crucial for the achievement of the desired outcome was the use of 5 mol% of silver carbonate as the Ag^+ source necessary to catalyse the reaction. Furthermore, this addition reaction exhibited *E*-selectivity and the *Z* isomer was not detected.



Scheme 3.24 Hydrocarboxylation reaction for the synthesis of (*E*)-6-methoxy-9-(3-acetoxyprop-1-enyl)-9H-purine **173**. Reagents and conditions: a) glacial acetic acid, Ag_2CO_3, ACN, 80°C, 16h.

The proposed reaction mechanism[127,145] (**Scheme 3.25**) involves the initial coordination of the Ag^+ cation on the allene function, thus forming intermediate **174**. The activation of the allene facilitates the attack by a nucleophile, such as acetate to accomplish the acetoxylation of the substrate, leading to the vinyl-silver **175**. Final protonation of compound **175**, released the product **173** with the desired *E* configuration.



Scheme 3.25 Proposed mechanism for the Ag^+ catalysed hydrocarboxylation of allenes.

The data in our hands were in accordance with the data reported in the background paper.[127] The *trans* configuration was confirmed by ^{1}H-NMR. Indeed, the $^3J_{H\text{-}H}$ coupling constant in an alkene scaffold is peculiar to the configuration. The *cis* isomer is typically features a smaller $^3J_{H\text{-}H}$ value with a range of 6-12 Hz; otherwise, the $^3J_{H\text{-}H}$ value of a *trans* alkene is generally contained in the range from 12 Hz to a maximum of 18 Hz.[146] As highlighted in the ^{1}H-NMR spectrum of compound **173** (**Figure 3.19**), the coupling

constant between N-*CH*= and CH_2-*CH*= presented a value of $^3J_{H\text{-}H}$ = 14.5 Hz, confirming the *E* configuration.

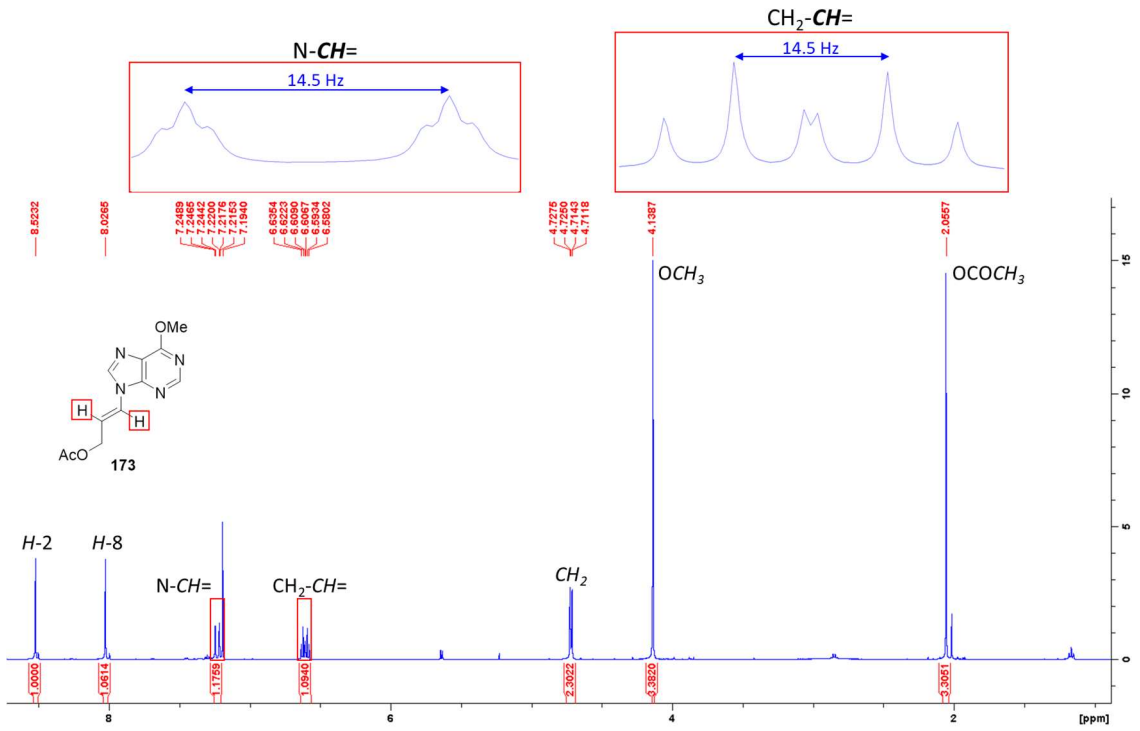
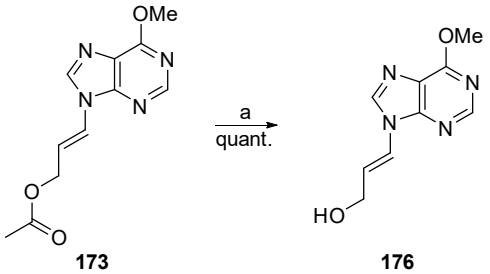


Figure 3.19 ^{1}H-NMR (500 MHz, $CDCl_3$) spectrum of compound **173**. The signal of N-*CH*= and CH_2-*CH*= are magnified to highlight their $^3J_{H\text{-}H}$ values, confirming the *E* configuration.

Therefore, once the *E* configuration of the desired compound was confirmed, the successive step was the hydrolysis of the acetate **173** to the alcohol (*E*)-6-methoxy-9-(3-hydroxyprop-1-enyl)-9H-purine (**176**) (**Scheme 3.26**).

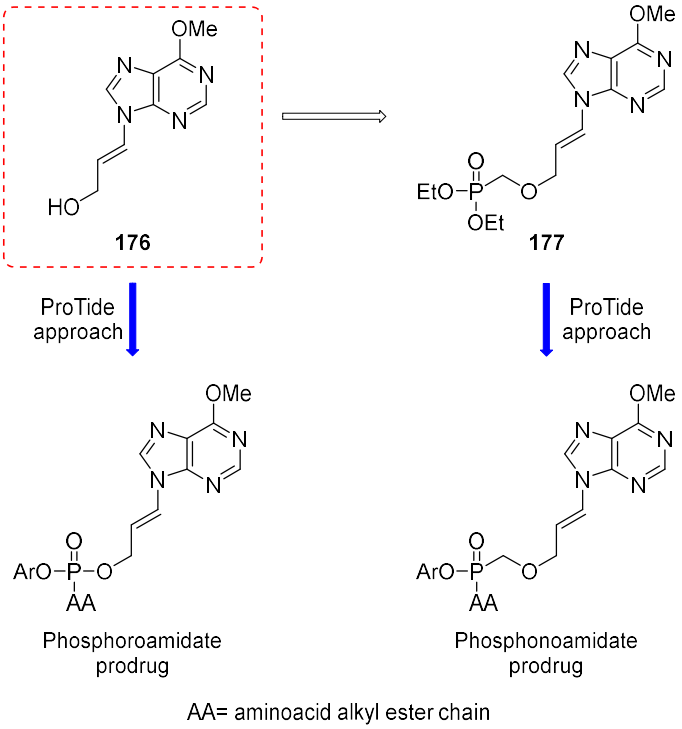


Scheme 3.26 Hydrolysis of compound **173** to give (*E*)-6-methoxy-9-(3-hydroxyprop-1-enyl)-9H-purine **176**. Reagents and conditions: a) KOH, MeOH, rt, 16h.

The hydrolysis takes place in the presence of potassium hydroxide attacking the carbonyl carbon to generate a tetrahedral alkoxide intermediate. The acetate is a good leaving group then it is expelled to give the desired product **176**. Due to the high water solubility

of this compound, solvent extraction was avoided, and the compound was directly dry loaded on an automatic flash chromatography cartridge to be purified.

The (*E*)-6-methoxy-9-(3-hydroxyprop-1-enyl)-9H-purine **176** was finally in our hands and it could be used not only as starting material for the preparation of (*E*)-prop-1-en-3-oxymethyl chain **177**, but also as a substrate to investigate the application of the ProTide approach for the synthesis of novel phosphoroamidate ANP prodrugs (**Scheme 3.27**).

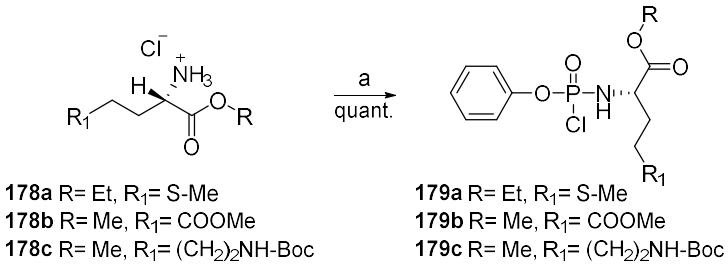


Scheme 3.27 General approach for the preparation of both phosphoroamidate and phosphonoamidate prodrugs starting from the alcohol derivative **176**.

3.7.3.2 Preparation of (E)-6-methoxy-9-(prop-1-enyl)-9H-purine ProTides

The synthesis of phosphoroamidate prodrugs of compound **176** was investigated. Firstly, the proper aryloxy amino phosphorochloridate derivatives to be coupled with the acyclic nucleoside needed to be synthesised. Beside *L*-alanine, which has been shown to be the preferred amino acid for this class of prodrugs in other programs, we were interested in the exploration of other amino acid moieties as recent literature reports started to highlight the efficacy of other amino acids as phosphoroamidate promoieties.[147,148] Therefore, together with the *L*-alanine iso-propoxy ester **22**, amino acids of different natures such as *L*-methionine (*L*-Met) ethoxy ester (hydrophobic/not

charged) (**178a**), *L*-glutamic acid (*L*-Glu) methoxy ester (negatively charged) (**178b**) and *L*-lysine (*L*-Lys) methoxy ester (positively charged) were also employed. Due to the presence of two potentially reactive amino groups present on *L*-lysine, the commercially available Nε-Boc protected derivative (**178c**), was used in order to direct the phosphorylation to the alpha-amino group. To accomplish their preparations, the hydrochloride salts of the selected amino acids ester (**178a-c**) were reacted with phenyloxy dichlorophosphate in presence of Et_3N (**Scheme 3.28**) as reported in Chapter 2 (paragraph 2.6.1).

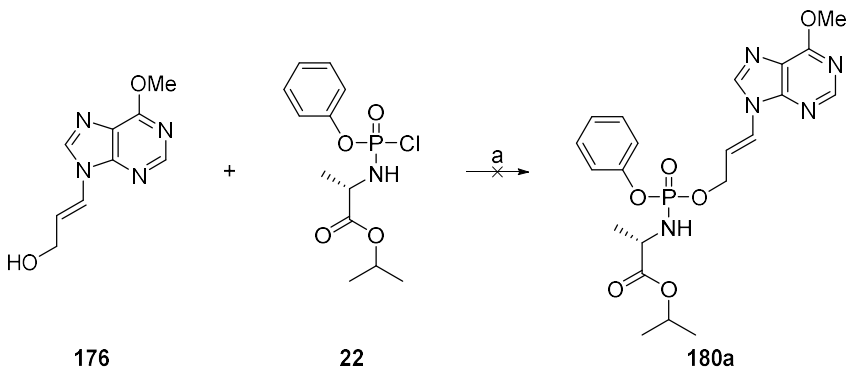


178a R= Et, R_1= S-Me
178b R= Me, R_1= COOMe
178c R= Me, R_1= $(CH_2)_2$NH-Boc

179a R= Et, R_1= S-Me
179b R= Me, R_1= COOMe
179c R= Me, R_1= $(CH_2)_2$NH-Boc

Scheme 3.28 Synthesis of the aryloxy amino phosphorochloridates **179a-c**. Reagents and conditions: a) phenyl dichlorophosphate, Et_3N, CH_2Cl_2, -78°C to rt, 2h.

All the new phosphorochloridates were successfully synthesised in quantitative yields. With these previously unexplored phosphorochloridates in hand, we proceeded to prepare the corresponding prodrugs of compound **176**. As first attempt, the synthetic protocol for the preparation of phosphate ProTides was applied.[149] According to this methodology, the coupling between the acyclic nucleoside and the phosphorochloridate **22** was expected by using the Grignard reagent *t*BuMgCl (**Scheme 3.29**) as discussed in Chapter 2 (paragraph 2.6.1).[150] Unfortunately, under these conditions, no traces of the desired product were detected.



176 **22** **180a**

Scheme 3.29 Attempted preparation of (*E*)-6-methoxy-9-(prop-1-enyl)-9H-purine ProTide **180a** using the Uchyiama approach. Reagents and conditions: a) *t*BuMgCl (1M in THF), THF, rt, 16h.

From the reaction mixture, one main species was isolated with a structure consistent with compound **181** where replacement of the hydroxy group with a chorine atom occurred (**Figure 3.20**). The reasons behind this unexpected substitution are unclear. However, it is possible to suppose that the 3'-CH_2 linked to the hydroxylic group showed a completely different reactivity compared to the 5'-CH_2OH of the nucleoside sugar moiety. The Cl- present in the reaction mixture might attack the 3'-CH_2 leading to the substitution of the hydroxylic function.

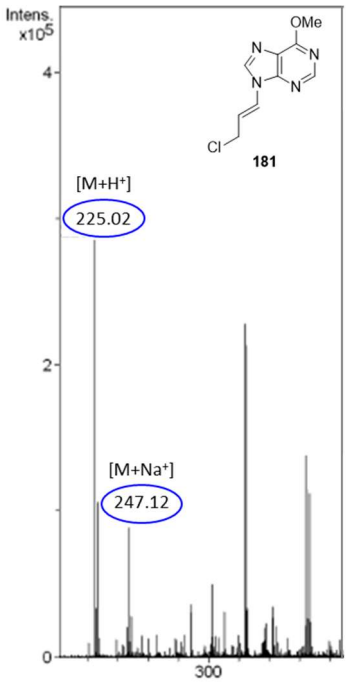
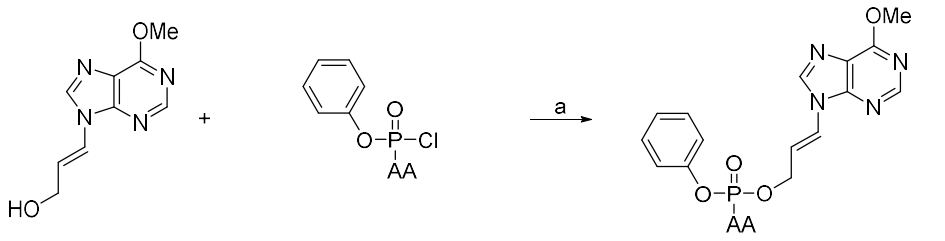


Figure 3.20 MS(ESI+) spectrum of compound **181** isolated from the reaction mixture.

However, ProTide preparations can also be accomplished using *N*-methyl imidazole (NMI) as disclosed in Chapter 2 (paragraph 2.2.1). Due to the different reaction mechanisms of the two methodologies, we additionally attempted the Van Boom approach for the desired phosphoroamidate preparation (**Scheme 3.30**).[151] A phospho-imidazolium intermediate is formed conferring higher reactivity to the phosphorus towards nucleophiles. This facilitates the attack on the phosphorus atom by the hydroxyl group without being converted into alkoxide. Pleasantly, the substitution of the hydroxylic group by the chloride, which was observed in the Grignard approach, proved to be overcome. Indeed, the desired ProTide **180a** was successfully synthesised and

isolated with 12% yield. Additionally, the same reaction conditions were applied using all the other phosphorochloridate agents previously prepared.



176

22 AA= *L*-Ala-*O*-*i*Pr
179a AA= *L*-Met-*O*-Et
179b AA= *L*-Glu-*O*-Et
179c AA= *L*-Lys-*N*ε-Boc-*O*-Et

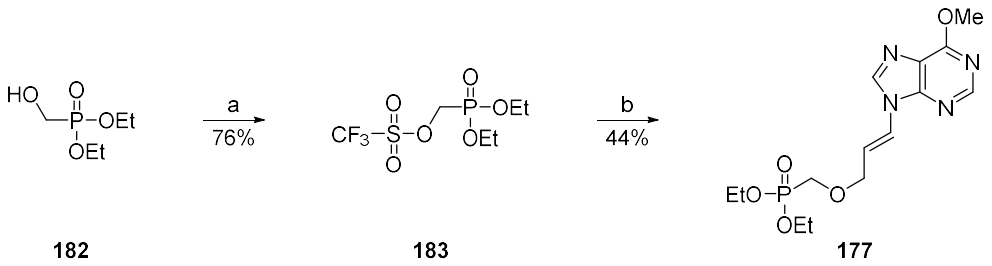
180a AA= *L*-Ala-*O*-*i*Pr 12%
180b AA= *L*-Met-*O*-Et 22%
180c AA= *L*-Glu-*O*-Et 19%
180d AA= *L*-Lys-*N*ε-Boc-*O*-Et 8%

Scheme 3.30 Preparation of (*E*)-6-methoxy-9-(prop-1-enyl)-9H-purine ProTides **180a-d** using the Van Boom approach. Reagents and conditions: a) NMI, THF, pyridine, rt, 16h.

In conclusion, the syntheses of novel acyclic nucleoside phosphoroamidate prodrugs was successfully developed and (*E*)-6-methoxy-9-(prop-1-enyl)-9H-purine ProTides **180a-d** featuring with diverse amino acid ester moieties was delivered as mixtures of diastereoisomers in 8%-22% yield (**Scheme 3.30**).

3.7.3.1 Preparation of (E)-6-methoxy-9-(prop-1-en-3-oxymethyl)-9H-purine phosphonoamidate prodrugs

According to the route described in **Scheme 3.27**, the hydroxyl group of compound **176** could be functionalised by a methylphosphonate moiety resulting in the preparation of novel (*E*)-prop-1-en-3-oxymethyl purine phosphonate. To accomplish this task, an effective reagent such as (diethoxyphosphoryl)methyl trifluoromethanesulfonate (**183**) for the insertion of the phosphonate group was synthesised (**Scheme 3.31**). The preparation of **183** was performed starting from the diethyl (hydroxymethyl)phosphonate (**182**) and trifluoromethanesulfonic anhydride, as the trifluoromethanesulfonyl source, in the presence of 2,6-lutidine.[127] The use of a base was necessary to activate **182** so that its oxygen could be able to attack the sulphur of the anhydride, whose addition was performed in a dropwise fashion at -50°C to keep under control the exothermic reaction. Moreover, the reaction was performed under strictly anhydrous conditions to avoid decomposition of triflic anhydride. A quick workup with H_2O and Et_2O as extraction solvents afforded **183** (76%) as a brown oil that, after complete characterisation, was stored at -20°C and used for the next step without further purification.

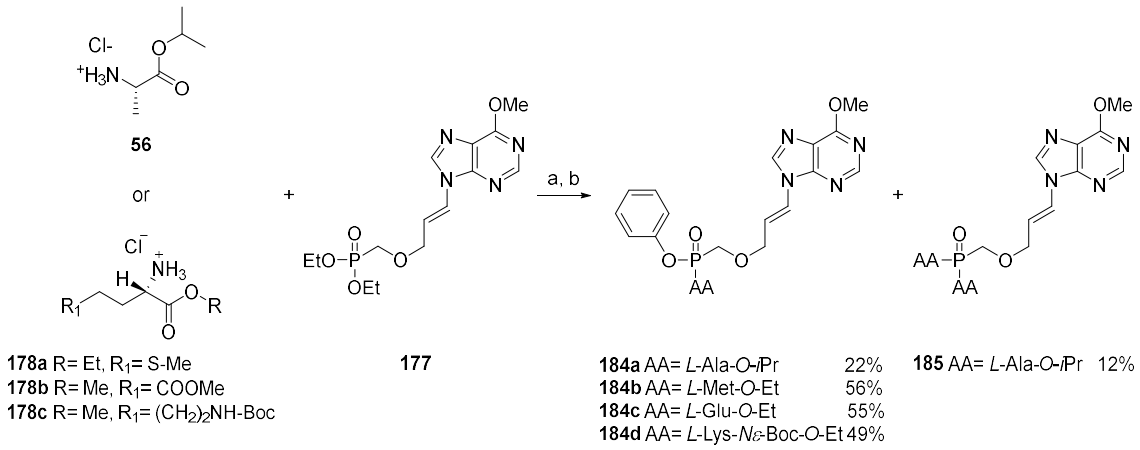


182 183 177

Scheme 3.31 Preparation of the diethylphosphonate **177**. Reagents and conditions: a) $(CF_3SO_2)_2O$, 2,6-lutidine, CH_2Cl_2, -50°C to 0°C, 1.5h; b) hydroxyl derivative **176**, NaH, THF, -20°C to rt, 16h.

For the preparation of the desired phosphonate **177**, a strong base such as NaH was necessary to deprotonate the hydroxyl group of **176**, so that the alkoxide could attack the previously prepared **183**, which was added dropwise to the reaction mixture at -20°C. The phosphonate group was readily inserted, and compound **177** isolated after flash chromatography purification with 44% yield (**Scheme 3.31**). The diethylphosphonate was available to be subjected to the ProTide approach and generate the phosphonoamidate prodrugs of the acyclic nucleoside.

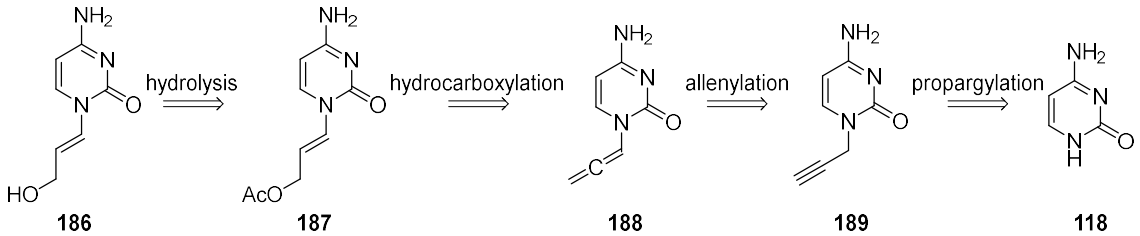
According to the previously described acyclic nucleoside project (paragraph 3.7.1), the synthesis of the desired prodrug starting from a diethylphosphonate could be accomplished by application of an optimised variant of Holy's one-pot procedure.[122] The first step, consisting of the McKenna reaction (for the reaction mechanism see **Scheme 3.12**) proved to be immediately successful thanks to the excess of 2,6-lutidine used as acid scavenger. The silyl ester thus generated was readily reacted with 1 equivalent of the desired amino acid ester hydrochloride (**56**, **178a-c**) and a large excess of phenol (6 equivalents) in the presence of PPh_3 and aldrithiol-2 in a pyridine/Et_3N mixture (for the reaction mechanism see **Scheme 3.13**). By applying this procedure, ProTides **184a-d** were successfully synthesised in moderate yields (22%-56%) (**Scheme 3.32**). Due to the high nucleophilicity of the amino acid compared to the phenol, it is also possible to observe the formation of the bis-amidate compound. The lower yield of compound **184a** can be explained by the formation of 12% of the phosphonodiamidate prodrug **185**. This reaction mixture required a more extensive purification to obtain both the two prodrugs with a >95% purity as attested from the HPLC analysis. However, only traces of the bis-amidate derivatives were detected in the crude mixture when other amino acids where employed. The reason could be that amino acids **178a-c** presented longer and more hindered alkyl chains compared to the methyl function in *L*-alanine **56**, making the attack of two molecules of amino acid on the phosphorous atom less favourable.



Scheme 3.32 Synthesis of (*E*)-6-methoxy-9-(prop-1-en-3-oxymethyl)-9H-purine phosphonoamidate (**184a-c**) and phosphonodiamidate (**185**) prodrugs. Reagents and conditions: a) TMSBr, 2,6-lutidine, ACN, rt, 16h; b) amino acid ester hydrochloride salt, phenol, Et_3N, aldrithiol-2, PPh_3, pyridine, 50°C, 16h.

3.7.3.2 Preparation of (E)-1-(3-hydroxyprop-1-enyl)cytosine scaffold

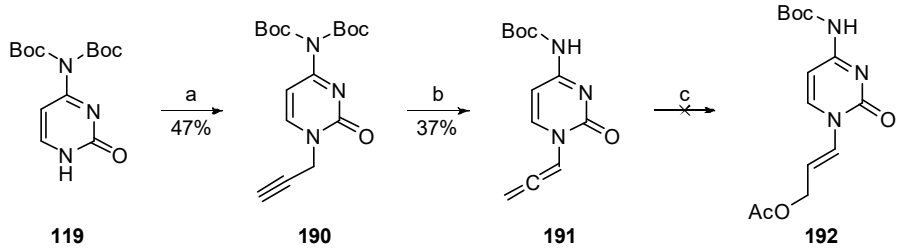
We planned to apply the retrosynthetic strategy previously reported for the 6-methoxy-9H-purine to the cytosine nucleobase analogue **118** to generate the scaffold **186** to be used for both the phosphoroamidation and phosphonoamidation as previously reported (**Scheme 3.33**).



Scheme 3.33 Retrosynthetic scheme for the preparation of the (*E*)-1-(3-hydroxyprop-1-enyl)cytosine scaffold (**186**).

From a synthetic point of view, the chemoselectivity toward the N^1-position could be a problem in the propargylation step. For this reason, complete protection of the primary amine was performed by using *tert*-butoxycarbonyl groups. The protected cytosine (**119**) was obtained through the one-pot, two steps procedure reported in paragraph 3.7.1. (**Scheme 3.8**). The propargylation of **119** yielded compound **190** (47%). Therefore, **190** was refluxed with K_2CO_3 in MeOH so that the allenamine could be readily synthesised under the previously optimised conditions. Indeed, 15 minutes proved again to be the necessary time to reach complete consumption of the starting material affording allene

191 in moderate yield (37%). However, the harsh basic conditions also caused the partial deprotection of the amine group, removing one of the *tert*-butoxycarbonyl functions (**Scheme 3.34**). Moreover, among the by-products, small amounts of NH-Boc-propargylcytosine (3%) and unprotected allenylcytosine (2%) were detected, thus explaining the moderate yield of **191**.



Scheme 3.34 Attempted synthesis of N^1-allylacetate derivative **192**. Reagents and conditions: a) propargyl bromide, NaH, DMF, rt, 24h; b) K_2CO_3, MeOH, reflux temperature, 15 minutes; c) glacial acetic acid, Ag_2CO_3, ACN, 80°C, 16h.

The crucial hydrocarboxylation reaction to build the acyclic chain with the double bond directly linked to the nucleobase was then performed with glacial acetic acid and Ag_2CO_3 in ACN at 80°C for 16h, following the mechanism aforementioned in **Scheme 3.25**.[127] Unfortunately, the application of this procedure proved unsuccessful on the cytosine substrate and only traces of the desired product were detected along with the starting material and numerous by-products (**Scheme 3.34**). A literature search was performed to attempt to understand the reason behind the reaction outcome: Silver (I) proved to be able to bind the N^3 atom of the nucleobase to afford an Ag^I-cytosine complex (**Figure 3.21**).[152–154] Therefore, this suggests that the silver (I) could not exert its catalytic role.

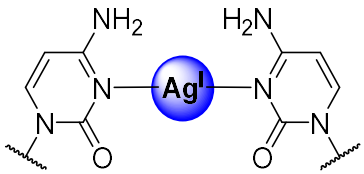
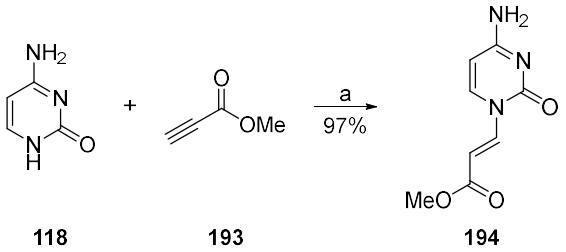


Figure 3.21 Structures of C–Ag^I–C base pair.

In light of these data, a further literature search was performed to find an alternative reaction to achieve the desired (*E*)-1-(3-hydroxyprop-1-enyl)cytosine (**186**) intermediate. According to Redwane and co-workers,[155,156] a Michael addition of a

nucleobase to an α-β unsaturated carbonyl group such as methyl propiolate, could be performed to generate the methyl acrylate **194** (**Scheme 3.35**). This reaction allows the insertion of an acyclic chain onto the nucleobase with the double bond directly linked to the cytosine. We could then explore the reduction of the ester to obtain the desired alcohol.



118 **193** **194**

Scheme 3.35 Preparation of the methyl acrylate **194**. Reagents and conditions: a) Cs_2CO_3, DMF, rt, 30min.

The coupling between the aliphatic chain and the nucleobase was performed by addition of the propiolate (**193**) to a suspension of cytosine and Cs_2CO_3 in DMF. After 30 minutes at room temperature, the complete consumption of the starting material was observed by TLC. The mixture was concentrated *in vacuo* and the residue was treated with H_2O and EtOAc. The precipitate obtained was filtered to give the solid product **194** in 97% yield. Pleasantly, the formation of the bis-acrylate was not observed and the presence of the free -NH_2 was confirmed by ^{1}H NMR (**Figure 3.22**). Moreover, the *trans* configuration of the double bond was confirmed by the coupling constant value ($^3J_{H\text{-}H}$ = 14.7 Hz), and no *cis* isomer was detected. This procedure showed the great advantage to be performed without any protection/deprotection steps of the cytosine exocyclic amine, demonstrating also excellent regioselectivity.

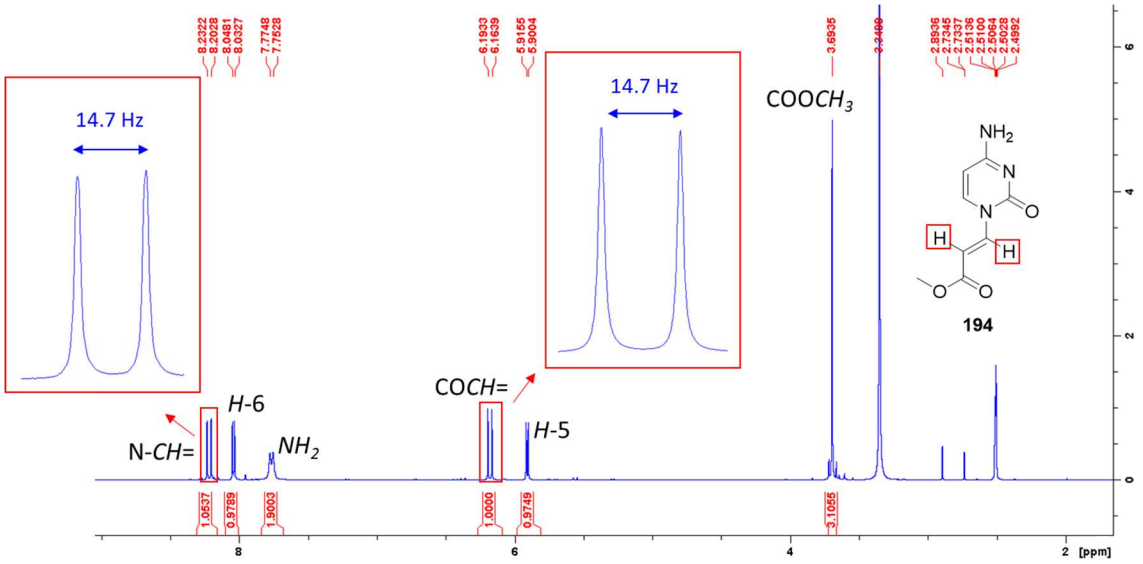


Figure 3.22 ^{1}H-NMR (500 MHz, DMSO-d_6) spectrum of compound **194**. The signal of N-*CH*= and CO*CH*= are magnified to highlight their $^{3}J_{H\text{-}H}$ values, confirming the *E* configuration.

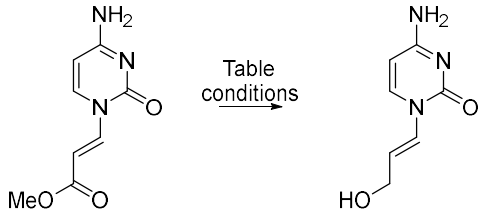
The synthesis proceeded by investigating the reactivity of the ester moiety in the presence of a reducing agent. The idea was that the amide function of the nucleobase should be less reactive and thus less susceptible to reduction than the terminal ester. The procedures commonly employed to reduce an ester to the corresponding alcohol are lithium aluminium hydride ($LiAlH_4$) and sodium borohydride ($NaBH_4$) (**Table 3.4**).[157,158]

In the first attempt, $LiAlH_4$ was investigated (entry 1, **Table 3.4**). This reducing agent was too harsh to be used. Although the temperature was kept at -78°C and only 1 equivalent of the reagent was employed, the crude reaction mixture after 1h, showed a mixture of the starting material and the cytosine nucleobase generated from the removal of the aliphatic chain. No presence of the desired product was observed.

As second attempt, $NaBH_4$ was investigated (entry 2 and 3, **Table 3.4**). Two equivalents of the reductive agent were added to a suspension of **194** in MeOH. After 72h at room temperature, the proton NMR of the reaction mixture showed only the presence of the starting material. To enhance the reduction efficiency of $NaBH_4$, a metallic ion can be employed. Indeed, $CaCl_2$ already proved to be a valid choice generating calcium borohydride as more effective reductive agent.[159] Under these conditions, only multiple inseparable by-products were detected (TLC) after only 30 minutes at room temperature.

Finally, diisobutyl aluminium hydride (DIBAL-H) (entry 4, **Table 3.4**), a reducing agent with more hindering substituents making it less reactive, was investigated. Compound **194** was dissolved in THF and the solution cooled down to -78°C. The hydride (4 eq) was added dropwise, and the resulting suspension was warmed to room

temperature. After 20 minutes stirring at room temperature, the crude mixture showed the complete consumption of the starting material. The excess of hydride was quenched by slow addition of water until H_2 evolution ended. After filtration on a small pad of Celite and a trituration with MeOH, the resulting solid was found to be the pure alcohol **186**, isolated in 87% yield.



194 **186**

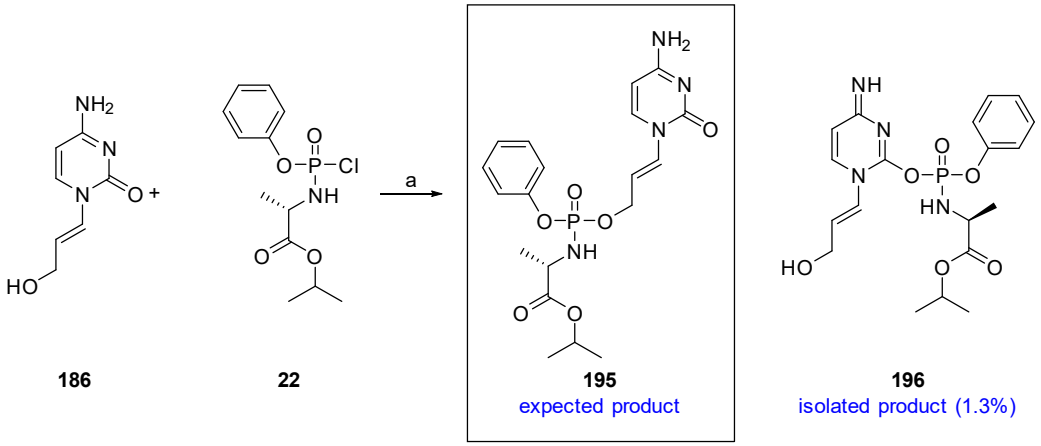
Entry	Reducing agent	Solvent	T°C	Time	Compound detected
1	$LiAlH_4$(1eq)	THF	-78°C	1h	**194**/C
2	$NaBH_4$(2eq)	MeOH	rt	72h	**194**
3	$NaBH_4$(2eq)/$CaCl_2$(2.5eq)	MeOH	rt	30min	-
4	DIBAL-H(4eq)	THF	-78°C to rt	20min	**186**(87%)

Table 3.4 Reagents and conditions for the ester reduction attempts.

3.7.3.3 Preparation of (E)-1-(prop-1-enyl)cytosine ProTides

The new synthetic route, allowed the preparation of the key (*E*)-1-(3-hydroxyprop-1-enyl)cytosine (**186**) which we wished to convert to the corresponding phosphoroamidate prodrug applying the same procedure developed for the purine analogues also on the cytosine derivative.

Phosphorochloridates (**22**, **179a-c**) were selected for the phosphoroamidation of the pyrimidine substrate. NMI was added to a mixture of **186** in THF/Pyridine followed by the addition of a solution of the phosphorochloridate in THF (**22**) and left stirring at room temperature. After 16h, only starting material was detected by ^{1}H-NMR of the crude reaction. The evident poor solubility of **186** was considered the potential cause. For this reason, the reaction conditions were slightly adjusted to enhance **186** solubility. NMI and phosphorochloridate **22** were added to a solution of the substrate in DMF and the resulting mixture was stirred for 16h at 50°C (**Scheme 3.36**).



Scheme 3.36 Attempt for the preparation of (*E*)-1-(prop-1-enyl)cytosine ProTide **195**, which instead led to compound **196**. Reagents and conditions: a) NMI, DMF, 50°C, 16h.

Although the starting material resulted the main product isolated, a very small amount (about 5 mg, 1.3%) of an unexpected compound was isolated and analysed. According to the mass spectrometry analysis, the isolated molecule could correspond to the desired product (**Figure 3.23**) but further analyses indicated to be instead compound **196**.

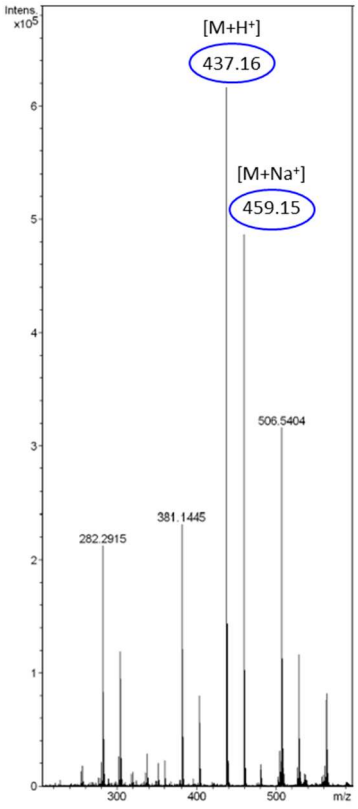


Figure 3.23 MS(ESI+) spectrum of compound **196** isolated from the reaction mixture.

Both **195** (expected product) and **196** (isolated product) presented the same molecular weight (Mol. Wt. = 436.40). The first doubts arise when some inconsistencies were noticed on the analysis of the NMR spectra. To begin, the ^{31}P NMR did not showe the usual two sharp peaks characteristic of a ProTide, but only one very broad peak, although the molecular shift (δ: 4.24 ppm, $CDCl_3$) could be consistent for a phosphoroamidate diester. The interpretation of the ^{1}H-NMR spectrum in $CDCl_3$ of the compound was complicated and it was difficult to discriminate the peaks related to the proton of the nucleobase. For this reason, numerous proton NMR analysis were performed using different solvents until the use of acetone-d_6 (**Figure 3.24**) proved to be able to make the two protons of the nucleobase (H-5 and H-6) sufficiently resolved to see the cytosine characteristic doublets. Additionally, the CH_2 of the aliphatic chain of **195** should have presented a complex multiplet due to the $^{3}J_{H\text{-}P}$ values to be added to the pre-existing coupling with the double bond protons. However, the highlighted peak in **Figure 3.24** shows that a simple doublet of doublets was detected, indicating no close relation with the phosphorus atom.

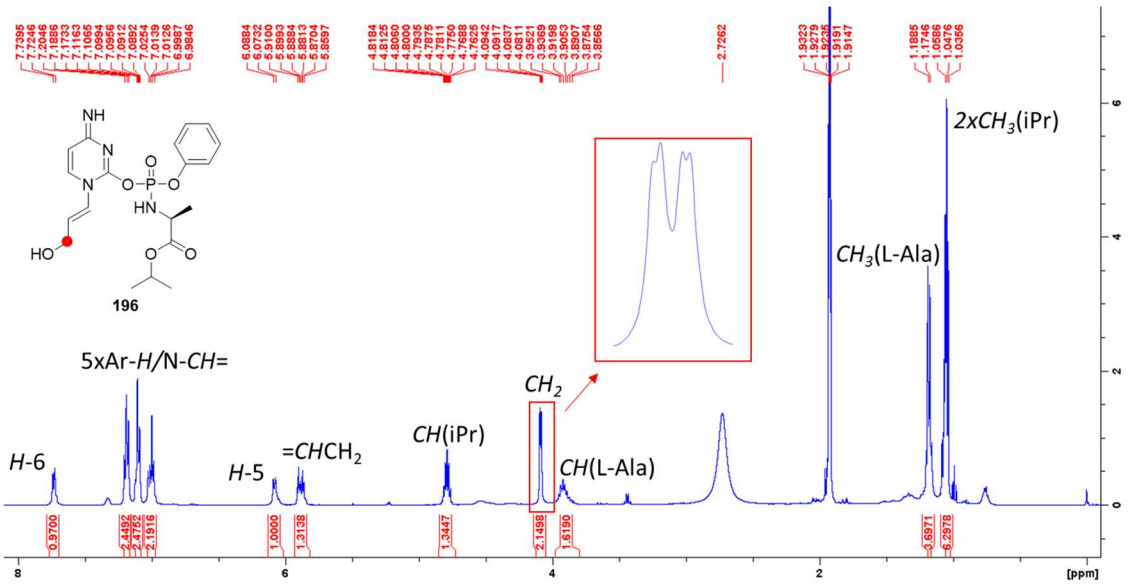


Figure 3.24 ^{1}H-NMR (500 MHz, Acetone-d_6) spectrum of compound **196**. The signal of the CH_2 of the aliphatic chain is magnified to highlight its multiplicity. A *dd* proved no close correlation to the phosphorus atom.

Similar results were already encountered in a previous project in our laboratory. According to that project,[160] the enolisable keto group of a nucleobase ring was phosphorylated. Literature data also offered support to this theory reporting the formation

of *O*-phosphoryl derivatives obtained by reacting hydroxypyrimidine with phosphorochloridates.[161]

The ^{1}H-NMR in DMSO-d$_6$ (**Figure 3.25**) showed the presence of a free -*OH*, which was further confirmed by the treatment of the solution with a drop of D_2O. The use of deuterated water caused the exchange of the hydroxylic proton with the deuterium ion causing the disappearance of the peak in the spectrum. Accordingly, it was confirmed that the phosphorylation did not happen on the desired hydroxylic group (as in compound **195**), although the expected *NH* (imino group of **196**) of the nucleobase was not detected in the spectrum.

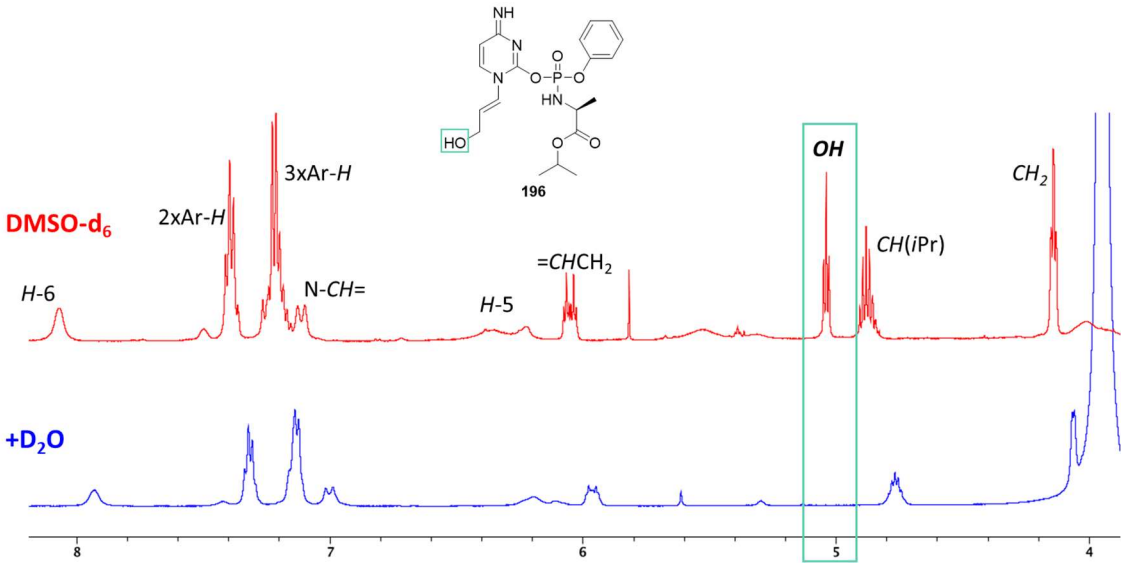


Figure 3.25 ^{1}H-NMR (500 MHz, DMSO-d$_6$) spectrum of compound **196** before (in red) and after (in blue) the addition of D_2O. The peak of the -*OH* disappeared after the addition of deuterated water.

Once it was established that **195** was not the reaction product, the confirmation of **196** could be performed only by ^{13}C-NMR. With the support of the HSQC (Heteronuclear Single Quantum Correlation) NMR experiment, that allows the correlation between the ^{1}H and the ^{13}C spectra, the signals present in the carbon NMR were assigned (**Figure 3.26**). From this spectrum, we expected to observe an indicative doublet around 65 ppm due to the $^{2}J_{C\text{-}P}$ coupling constant between the phosphorus and *C*-2 of the nucleobase. Unfortunately, all the *CH* and quaternary carbons of the nucleobase were not observed, including the key *C*-2 atom. As magnified in the picture, the CH_2 of the aliphatic chain proved once again to be not correlated to the phosphorus atom showing a singlet as the signal.

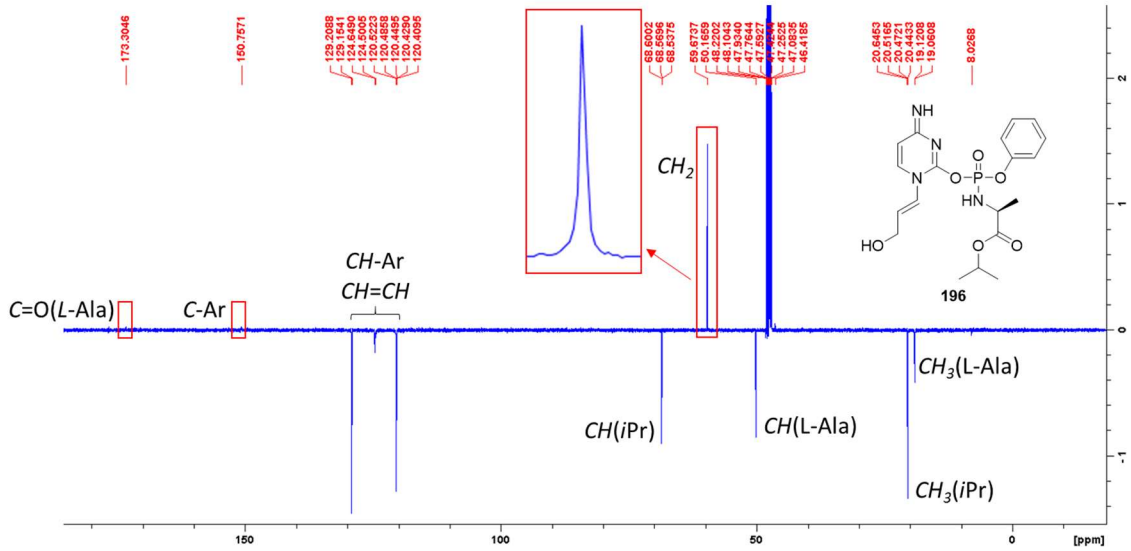


Figure 3.26 ^{13}C-NMR (126 MHz, MeOD) spectrum of compound **196**. The signal of the CH_2 of the aliphatic chain is magnified to highlight the singlet, hence no correlation with a phosphorous atom.

Literature searches were helpful to understand the reason for the undetectable peaks. From an NMR spectroscopy point of view, the nitrogen atom is a quadrupolar nucleus, meaning that is not a spherical nucleus but the charge is asymmetrically distributed. The interaction between the asymmetric nucleus and the electric field gradient may cause the generation of a quadrupolar moment Q, which in turn provokes a wider NMR signal in the spectrum, so that a peak is no longer detectable. The effect of the quadrupolar nitrogen atom seems to be particularly strong in its imino form and all the adjacent nuclei can be affected by its quadrupolar effect.[162]

In summary, due to the failure of the synthetic approach previously applied on a purine nucleobase, a different pathway needed to be designed to afford (*E*)-1-(3-hydroxyprop-1-enyl)cytosine (**186**). Once the latter was successfully prepared, the application of the ProTide approach was investigated to generate (*E*)-1-(prop-1-enyl)cytosine phosphoroamidate prodrugs. Unfortunately, the *O*-phosphorylated imino nucleobase was detected as the only product. These discouraging results prevented us from further investigating the phosphonylation of this substrate.

3.8 Biological evaluation

All the ProTide derivatives synthesised were evaluated against a panel of DNA and RNA viruses. None of the compounds were active against herpes simplex virus-1 (KOS) (HVS-1), herpes simplex virus-2 (G) (HVS-2), thymidine kinase deficient herpes simplex virus-1 (KOS Acyclovir-resistant strain) (TK- HSV-1), vaccinia virus (VV), adenovirus-2 (AV-2), human coronavirus (HCoV-229E) in HEL cells, parainfluenza-3 virus (HPIV-3), reovirus-1 (REO-1), vesicular stomatitis virus (VSV), respiratory syncytial virus (RSV) in HeLa cells, influenza A/H1N1, influenza A/H3N2 or influenza B in MDCK cells. As shown in **Table 3.5**, thymine derivatives **E-154a-f** showed weak antiviral activity against varicella-zoster virus (VZV TK+ and TK-) and human cytomegalovirus (HCMV AD-169 strain and Davis strain) with EC_{50} ranging from 20 to 76 μM, whereas uracil derivatives **E-156a-c** were mostly inactive against these viruses with the exception of **E-156a** (EC_{50}=20 μM VZV TK+) and **E-156b** (EC_{50}=58 μM VZV TK-). Interestingly, uracil derivatives **E-156e-f**, bearing the 5,6,7,8-tetrahydro-1-napthol moiety, were slightly active against VZV (both TK+ and TK- strains), confirming once again the biological potential of this promoiety. Remarkably, all the Z isomers isolated (**Z-154a,e,f** and **Z-156e**) showed to some extent antiviral activity against both AD-169 and Davis HCMV strains. Furthermore, compound **Z-154e** was found to be weakly active against Sindbis Virus (SINV), coxsackie virus B4, Punta Toro virus (PTV) and yellow fever virus (YFV) in Vero cells with EC_{50} values in the range of 20-58 μM. None of the compounds showed significant cytotoxicity.

Cpds	EC_{50}(HEL cells)(μM)				MCC (HEL cells) (μM)	EC_{50}(Vero cells)(μM)				MCC (Vero cells)(μM)
	VZV		HCMV			SINV	Coxsackie Virus B4	PTV	YFV	
	TK^+	TK^-	AD-169	Davis						
***E*-154a**	44.72	>100	>100	>100	>100	>100	>100	>100	>100	≥20
***E*-154b**	34.2	55.27	>100	>100	>100	>100	>100	>100	>100	>100
***E*-154c**	76.47	>100	>100	>100	>100	>100	>100	>100	>100	≥20
***E*-154d**	55.7	46.66	>100	>100	>100	>100	>100	>100	>100	>100
***E*-154e**	58.48	53.48	>100	>20	>100	>100	>100	>100	>100	≥20
***E*-154f**	50.17	47.19	>100	>100	>100	>100	>100	>100	>100	≥100
***E*-156a**	20	>100	>100	>100	>100	>100	>100	>100	>100	>100
***E*-156b**	100	58.48	>100	>100	>100	>100	>100	>100	>100	>100
***E*-156c**	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
***E*-156d**	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
***E*-156**	29.91	71.52	>100	>100	>100	>100	>100	>100	>100	>100
***E*-156f**	55.7	52.53	>100	>100	>100	>100	>100	>100	>100	≥100

***Z*-154a**	39.86	41.57	>20	44.72	100	>100	>100	>100	>100	≥20
***Z*-154e**	>20	>20	44.72	>20	100	45	58	45	58	>100
***Z*-154f**	17.03	65.1	76.47	76.47	>100	>100	>100	>100	>100	≥20
***Z*-156e**	58.48	100	>20	54.69	100	>100	>100	>100	>100	>100
Acyclovir	3.55	14.87	-	-	>440	-	-	-	-	-
Brivudin	0.012	0.57	-	-	>300	-	-	-	-	-
Ganciclovir	-	-	11.43	2.29	-	-	-	-	-	-
Cidofovir	-	-	1.24	0.76	-			-	-	-
DS-10.000	-	-	-	-	-	20	7.6	7.6	34	>100
Ribavirin	-	-	-	-	-	>250	>250	126	>250	>250
Mycophenolic acid	-	-	-	-	-	4	>100	6.1	4	>100

Table 3.5 Antiviral activity of alkenyl ANP ProTides **154a-f** and **156a-f**. EC_{50}: 50% effective concentration or concentration required inhibiting viral induced cytopathic effect (HSMV, SINV, coxsackie virus B4, PTV and YFV) or plaque formation (VZV) by 50%. MCC: minimal cytotoxic concentration that causes a microscopically alteration of cell morphology.

Being able to inhibit VZV, ProTides of allylphosphonate pyrimidine showed a broader antiviral activity than the corresponding bis-POM prodrugs.[97] On the contrary, linear alkenyl derivatives such as the ProTides prepared by our group,[121] showing higher EC_{50} against VZV, perform better than branched systems, suggesting that a more substituted double bond is detrimental for the antiviral activity. The metabolic activation of phosphonoamidates follows the same two-enzymatic steps involved in the activation of the phosphoroamidates.[163] Although the use of 5,6,7,8-tetrahydro-1-naphthol as aryloxy group in the ProTides is quite recent, it was already shown that its metabolic activation occurs via carboxypeptidase Y.[26] To prove the stability of this class of compound we have performed stability assays of compound **E-154e** monitored by ^{31}P-NMR, in rat and human sera. These assays showed that the compound was not susceptible to serum-mediated esterolysis over to 12 hours. As an example, the assay performed in human serum is reported in **Figure 3.27**.

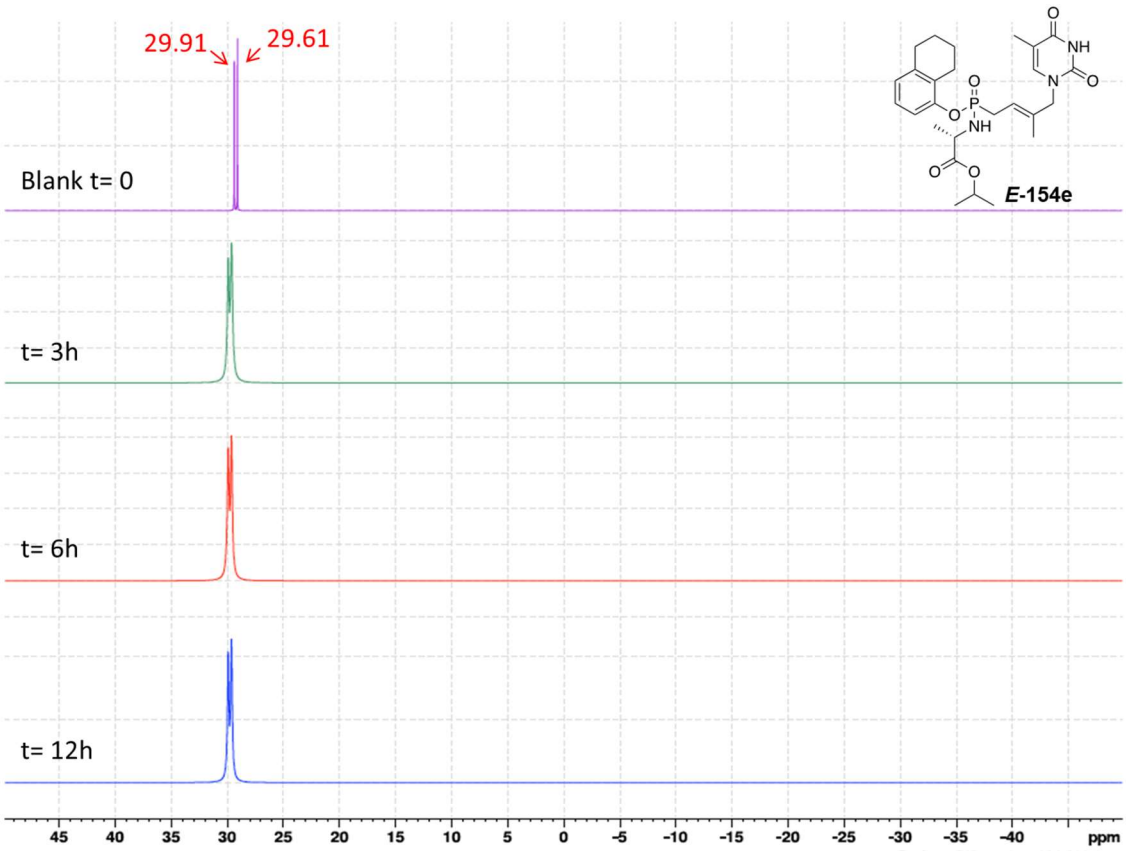


Figure 3.27 Stability assay of ***E*-154e** in human serum at 37°C monitored by ^{31}P-NMR (202 MHz, DMSO-d_6/H_2O) at different time.

For what concern the (*E*)-6-methoxy-9-(prop-1-enyl)-9H-purine phosphoroamidates (**180a-d**), (*E*)-6-methoxy-9-(prop-1-en-3-oxymethyl)-9H-purine phosphonoamidate (**184a-d**) and phosphonodiamidate (**185**) prodrugs, none of them resulted active. Considering the uncommon amino acid used for their preparation, an enzymatic assay to verify their capability to be activated should have been performed.

3.9 Conclusions and future work

In this chapter, the preparation of two categories of alkenyl ANP ProTides was discussed. Although, these two classes are feature different aliphatic chains, they can be more easily categorised as branched (trisubstituted) and linear (disubstituted) alkenyl ANP.

First, a successful one-pot two-steps synthesis of a family of allyl phosphonoamidates intermediates intended for the preparation of the branched alkenyl ANP ProTides was reported. This methodology was an important improvement of a recently reported strategy[124] allowing the synthesis of these substrate in a shorter synthetic sequence and with an overall higher yield. We also extended this protocol to the synthesis of hitherto unknown allyl phosphonodiamidates. It was also proved that both synthons are capable of undergoing alkene cross-metathesis with alkene-functionalized uracil and thymine nucleobases, although the yields need to be further optimised, especially in the case of phosphonodiamidates. These phosphonoamidate prodrugs were evaluated for their biological activities against a panel of DNA and RNA viruses. None of the compounds, showed significant cytotoxicity. ProTides of allylphosphonate pyrimidine showed broader antiviral activities than the corresponding bis-POM prodrugs against VZV infected cells. It was also demonstrated, once again, that the introduction of the 5,6,7,8-tetrahydro-1-naphthyl moiety into the ProTide scaffold increases the antiviral activity of the prodrug. Finally, not only the *E*-isomers showed some biological activity, but also all the *Z* isomers (**Z-154a,e,f** and **Z-156e**) showed to some extent antiviral activity against both AD-169 and Davis HCMV strains. Further studies directed to the optimisation of the cross-metathesis procedure, especially for the allyl phosphonodiamidates, need to be pursued. Moreover, the family of trisubstituted alkenyl ANP ProTides could be expanded by applying the optimised synthetic methodology towards purine derivatives.

Concerning the second class of acyclic nucleosides investigated, a multistep synthesis of the linear (*E*)-6-methoxy-9-(3-hydroxyprop-1-enyl)-9H-purine and the following oxymethyl phosphonate derivative were successfully reported going through an allene intermediate. The ProTide technology was applied on these scaffolds to generate both phosphoroamidate and phosphonoamidate prodrugs featuring diverse amino acid ester chains, some of which were completely new to the ProTide field. Purine and pyrimidine nucleosides showed completely different reactivities when subjected to the

same reaction conditions. The presence of the double bond directly linked to the nucleobase was found to be a problematic function, particularly for the cytosine nucleobase. Indeed, a totally different synthetic pathway needed to be designed for the preparation of the (*E*)-1-(3-hydroxyprop-1-enyl)cytosine scaffold. Moreover, the NMI procedure, which already proved to be able to afford the final phosphoroamidate of the alkenylated purine analogue, failed when applied on a cytosine nucleobase. The *O*-phosphorylated imino nucleobase was detected as the only product.

However, the final purine products that were obtained were tested against a panel of DNA and RNA viruses, but none of the compounds were active. It could be interesting to perform an enzymatic assay to evaluate the capability of the ProTide to be activated. Additionally, considering the increasingly importance of these classes of compounds in other therapeutic fields,[91–96] especially as antiparasitic agents,[9,10,86–90] further assays need to be planned to examine their activities as antimalarial agents. Indeed, literature evidence suggest that compounds structurally related to the ones synthesised in these projects may inhibit the *Plasmodium falciparum*.[156] Furthermore, this family of linear alkenyl ANP ProTides could be still largely expanded. Other pyrimidine nucleobases such as uracil and thymine could be evaluated under the two synthetic pathways explored, and some *Z*-isomers could be prepared to confirm their supposed inactivity.

3.10 References

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Chapter 4. dGMP ProTides for MDS treatment

4.1 Mitochondria and their biological function

Mitochondria are cellular organelles, which play a crucial role in numerous essential processes for cell physiology. The Krebs cycle, pyruvate oxidation, metabolism of steroids, fatty acids and amino acids are some of the tasks performed by mitochondria. However, the energy production in aerobic cells is probably the best-known function of these organelles. Their structure consists of four compartments: the intermembrane, the inner membrane, the matrix (region inside the inner membrane) and the outer membrane. Energy is generated as adenosine triphosphate (ATP), by means of an oxidative phosphorylation which takes place in the mitochondrial inner membrane. The oxidative phosphorylation system consists of multimeric protein complexes (I-V) and electron carriers (ubiquinone and cytochrome *c*) (**Figure 4.1**). This system can catalyse the transfer of electrons along the complexes to molecular oxygen, while hydrogen ions obtained from the reduction of nicotinamide adenine dinucleotide (NADH) or flavin adenine dinucleotide ($FADH_2$) are pumped from the matrix to the mitochondrial intermembrane. As a result, water is produced, and an electrochemical gradient is generated. As a consequence, the influx of the protons back to the mitochondrial matrix is used by complex V (ATP synthase) to phosphorylate ADP and generate ATP.[1,2] The entire system is also known as the mitochondrial respiratory chain.

Mitochondrial dysfunction is clearly associated with severe energy deficiency.

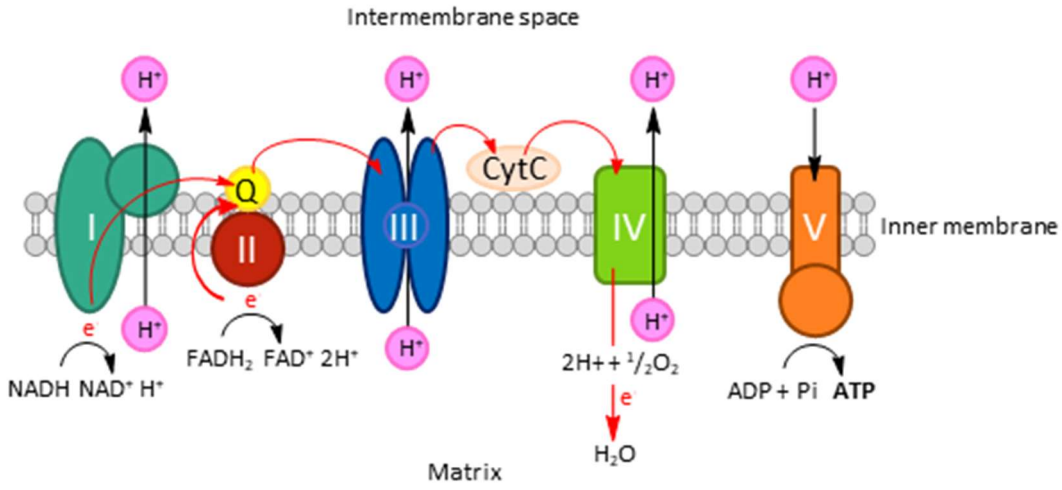


Figure 4.1 The mitochondrial respiratory chain.

Besides the nucleus, mitochondria contain also DNA. Mitochondrial DNA (mtDNA) is a closed, circular double-stranded molecule. Numerous copies of mtDNA are localised in the matrix. Contrary to the DNA present in the nucleus, mtDNA replication is independent of cell division, therefore continuous replication is required to balance mitochondrial dNTP pools. mtDNA encodes 37 genes which are all involved, directly or indirectly, in ATP production. Mutations in nucleus-encoded genes can either directly interfere with mtDNA replication and repair processes or be implicated in mitochondrial dNTPs metabolism. In both cases, the result is a reduction of mtDNA copy number, then mitochondrial dysfunction in affected tissues.[3,4] This condition is known as mitochondrial DNA depletion syndrome (MDS), which is the topic of the next paragraph.

4.2 Mitochondrial depletion syndromes: clinical and molecular features

MDS are rare diseases which consist of a group of severe, autosomal recessively inherited disorders with onset in infancy. As afore mentioned, it is characterised by a profound decrease of mtDNA copy numbers. This depletion results in an insufficient synthesis of key respiratory chain factors required for adequate energy production.[5] This condition may concern a specific tissue, commonly muscle, liver or brain; otherwise a combination of tissues and organs may be affected (muscle, liver, brain and kidney).[3] MDS are clinically and genetically heterogenous disorders usually classified as

encephalomyopathic, hepatocerebral, myopathic and neurogastrointestinal encephalopathy by correlation with mutations at different nuclear genes (**Table 4.1**).[4] The defective genes encode proteins, which can be involved in either mtDNA replication machinery or dNTPs synthesis.

Encephalomyopathic MDS is characterised by neonatal onset with pronounced neurological features and hypotonia.[6] It is caused by mutations in guanosine diphosphate (GDP)-forming succinyl CoA ligase α-subunit (SUCLG1), adenosine diphosphate (ADP)-forming succinyl CoA ligase β-subunit (SUCLA2) or ribonucleotide reductase M2 B unit (RRM2B). The latter encodes p53R2, a p53-dependent subunit of ribonucleotide reductase. This cytosolic enzyme catalyses the reduction of ribonucleoside diphosphates in the final step of the *de novo* synthesis of deoxyribonucleosides.[7] SUCLG1 and SUCLA2 encode two subunits of succinyl CoA ligase (SUCL). This enzyme mediates the conversion of succinyl CoA and GDP (or ADP) to succinate and guanosine triphosphate (or adenosine triphosphate) in the Krebs cycle. Additionally, SUCL is able to form a complex with the nucleoside diphosphate kinase present in the mitochondria, facilitating the kinase activity.[8] The lack of this complex, as well as the absence of p53R2 results in a decreased dNTPs pool for mtDNA synthesis.

In hepatocerebral MDS, the onset is in the first 6 months after birth with liver dysfunction and progressive neurological symptoms.[3] It is caused by mutations in deoxyguanosine kinase (DGUOK), DNA polymerase gamma (POLG) or twinkle mtDNA helicase (C10orf2) genes. DGUOK encodes mitochondrial deoxyguanosine kinase (dGK) which catalyses the first phosphorylation step of purine nucleosides in the salvage pathway. An impaired synthesis of mitochondrial deoxy-purine nucleoside triphosphates, as mtDNA building blocks, is detected when DGUOK is mutated.[9] Conversely, POLG and C10orf2 are genes implicated in mtDNA replication. The polymerase gamma catalytic subunit (pol-γA) is encoded by POLG. This gamma polymerase is the polymerase that is able to replicate and repair mtDNA.[10] C10orf2 encodes the twinkle protein, which mediates the function of a DNA helicase required for DNA replication.[11] The result of these defective genes is insufficient synthesis of mtDNA. Additionally, mutations to MPV17 gene are also associated to hepatocerebral MDS. This gene encodes a small inner mitochondrial membrane protein whose function and involvement in the disorder is still unclear.[12]

Myopathic MDS mainly features muscle weakness and hypotonia. Most affected children manifest the disease before the age of two years.[13] It is caused by mutations in thymidine kinase 2 (TK2) gene, which encodes the homonym enzyme. This protein catalyses the first phosphorylation step of pyrimidine nucleosides in the salvage pathway.[14] Decreased mtDNA is detected when TK2 is mutated, due to an insufficient synthesis of mitochondrial deoxy-pyrimidine nucleoside triphosphates.

In neurogastrointestinal encephalopathy MDS (MINGIE), the onset is usually between the first and the fifth decades of age. The symptoms mainly consist of peripheral neuropathy and progressive gastrointestinal dysmotility.[15] The defected gene that provokes the disorder is thymidine phosphorylase (TYMP). The protein encoded by this gene is the cytosolic thymidine phosphorylase (TP), which mediates the conversion of thymidine and deoxyuridine to thymine and uracil, respectively. When the enzyme activity is altered, accumulation of thymidine and deoxyuridine occurs generating an imbalanced cytosolic dNTP pool. This condition can lead to an impaired mitochondrial dNTP pool, which relies on cytosolic dNTPs for the salvage pathway.[16]

Syndrome	Mutated gene	Altered protein	Clinical manifestation	Age of onset
EM-MDS	SUCLG1	SUCL α-subunit	Brain, muscle	Infancy
	SUCLA2	SUCL β-subunit		
	RRM2B	p53R2	Muscle	Neonatal-infancy
HC-MDS	DGUOK	dGK	Brain, liver	Neonatal
	POLG	pol-γA		Early childhood
	C10orf2	twinkle		Neonatal-infancy
	MPV17	unknown		Infancy-childhood
M-MDS	TK2	TK2	Muscle	Infancy-early childhood
MINGIE	TYMP	TP	Nerve, muscle, GI, brain	Late childhood-adolescence

Table 4.1 Clinical manifestations of different mitochondrial DNA depletion syndromes. EM: encephalomyopathic; HC: hepatocerebral; M: myopathic; MINGIE: neurogastrointestinal encephalopathy.

Overall, MDS are characterised by poor prognosis in most of the patients. The affected individuals usually die during infancy or childhood. The existing therapies

provide symptomatic management, but no efficacious curative treatment is available.[4] Liver transplantation in patients suffering from hepatocerebral MDS is the only current therapeutic option. However, its application is still controversial because it involves multiple-organs and gives marginal survival benefit.[17] Additionally, exogenous stem cell therapy or gene therapy are challenging, but promising treatments.[18,19]

Alternatively, among the potential therapeutic options for MDS caused by defects in dNTP metabolism, direct administration of deoxyribonucleosides may prevents mtDNA depletion by supplementation of deficient mitochondrial dNTPs. This aspect is herein further discussed.

4.3 Implementation of the deoxyribonucleosides salvage pathway as a pharmacological approach

4.3.1 Mitochondrial deoxyribonucleotide synthesis

As afore mentioned, a subgroup of MDS is caused by defects in genes related to deoxyribonucleotide metabolism. dNTP pool homeostasis regulation is crucial for both nuclear and mitochondrial DNA replication and repair processes. The precursor for both genomes' building blocks can be obtained by two different metabolic sources: the salvage pathway and cytosolic *de novo* synthesis. The latter relies on two cytosolic enzymes, thymidylate synthase (TS), which coverts deoxyuridine monophosphate to deoxythymidine monophosphate, and ribonucleotide reductase (RNR), which mediates the reduction of the ribonucleoside diphosphates to the corresponding deoxyribonucleoside diphosphates. The dNTPs generated in the cytosol are transported to both the nucleus and the mitochondria in order to be incorporated in the two genomes. Indeed, it has been largely proved that the cytosol and mitochondria are different compartments that are able to actively communicate.[20]

However, mitochondrial dNTPs are mainly formed by the salvage pathway as a result of the activities of four different kinases, which catalyse the phosphorylation of deoxyribonucleosides to deoxyribonucleoside monophosphates. Two of them, deoxycytidine kinase (dCK) and thymidine kinase 1 (TK1) are cytosolic enzymes. Their products need to enter mitochondria to be available for mtDNA synthesis. Otherwise, the other two enzymes involved in the salvage pathway are deoxyguanosine kinase (dGK) and thymidine kinase 2 (TK2). Both are mitochondrial enzymes, which make

deoxycytidine-, thymidine-, deoxyguanosine- and deoxyadenosine monophosphates immediately available for the incorporation into the mtDNA.

As explained in the previous paragraph, lots of MDS defective genes encode proteins directly or indirectly involved in deoxyribonucleoside synthesis (TK2, SUCLA2, SUCLG1, DGUOK, RRM2B and TYMP). Therefore, mutation in any of these genes provoke an imbalance in cytosolic and mitochondrial dNTP pools, which affects mtDNA stability and replication.[4]

4.3.2 Strategies to increase deoxyribonucleoside availability

A molecular bypass therapy seems to be a promising treatment to modulate dNTP pools by deoxyribonucleosides supplementation in specific MDS patients. The final aim is to restore the dNTP pools in mitochondrial deoxyribonucleosides deficient cells.[18] Addition of the purine nucleoside dGuo demonstrated to prevent mtDNA depletion in cultured cells derived from patients with defected DGUOK gene.[2,21] The supplementation of dGuo may enhance its phosphorylation by dCK or by any residual activity of dGK.[22] Likewise, the supplementation of deoxycytidine showed a partial reduction of mtDNA depletion in an *in vitro* MNGIE model. The incrementation of the dCTP precursor is able to counterbalance the excess of cytosolic thymidine caused by TYMP defected gene.[23] Therefore, as in the viral and anticancer fields, nucleoside analogs may represent a valuable approach to regulate dNTP pool homeostasis in individuals affected by MDS.[24]

Unfortunately, a few drawbacks were detected in trying to selectively increase the level of the deficient nucleoside. Supplementation of deoxyguanosine or deoxycytidine may partially improve all the kinases' activities, increasing all four components of the dNTP pool. Consequently, a negative regulatory effect on the kinase and the RNR enzymes takes place leading to further imbalance of the dNTP pool composition.[25,26] Additionally, this condition may result in genetic and biochemical disturbances due to mutagenesis of both mitochondrial and nuclear genomes.[27] Alternatively, an easier option for supplying the nucleoside triphosphate precursors is the direct administration of the missing product of the defective kinase, so that an implementation of the kinases activity is not required. The addition of dGMP to cell culture medium was found to be effective in improving the mtDNA levels.[28] Nucleoside monophosphates are charged molecules at physiological pH. Therefore, they are not

able to passively cross the cell membrane. For this reason, they remain localised extracellularly, where they can be dephosphorylated, and only afterwards cross the membrane using the specific transporters.[29] As already extensively observed in anticancer and antiviral projects, the application of a prodrug strategy appears to be an advantageous solution and it may allow the correction of precise biochemical deficiencies.[30,31]

Moreover, because catabolic enzymes play crucial roles in maintaining both cytosolic and mitochondrial dNTP pool balances, inhibitors able to interfere with dNTP degradation may represent a further alternative to improve the amount of genomes building blocks and consequently reduce mtDNA depletion.[32]

These potential therapeutic strategies may potentially slow the progression of the diseases and improve the patients' health conditions. To date, the only pharmacological treatment reaching clinical trial for the treatment of MDS is thymidine. The nucleoside is now in phase 1/2 as a supplement for TK2 deficient myopathic MDS patients.[33] However, further research is needed to establish whether manipulation of the salvage pathway can serve as a successful solution in the regulation of the composition and the size of the mitochondrial dNTP pool, avoiding new imbalances and mutagenesis.

4.4 Background of the project

There is the need for new therapies that can effectively provide the deficient building blocks to mitochondrial DNA. The application of a prodrug approach to mask the negative charges of the phosphate proved to be an advantageous solution when administration of nucleoside monophosphates is required for anticancer and antiviral purposes. Among the different prodrug moieties, ProTide was found to be one of the most successful.[34] Therefore, Demeter Therapeutics patented numerous dNMP ProTides, synthesised with the purpose of treating mitochondrial DNA depletion syndromes.[35] The compounds were tested in patient-derived fibroblasts with DGUOK deficiency. Interestingly, two of the ProTides (**197** and **198**, **Figure 4.2**) significantly increase the mtDNA copy number relative to dGMP, which was used as a control.[35]

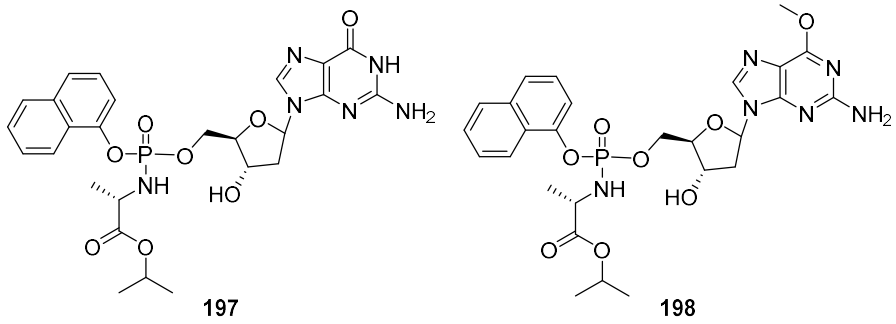
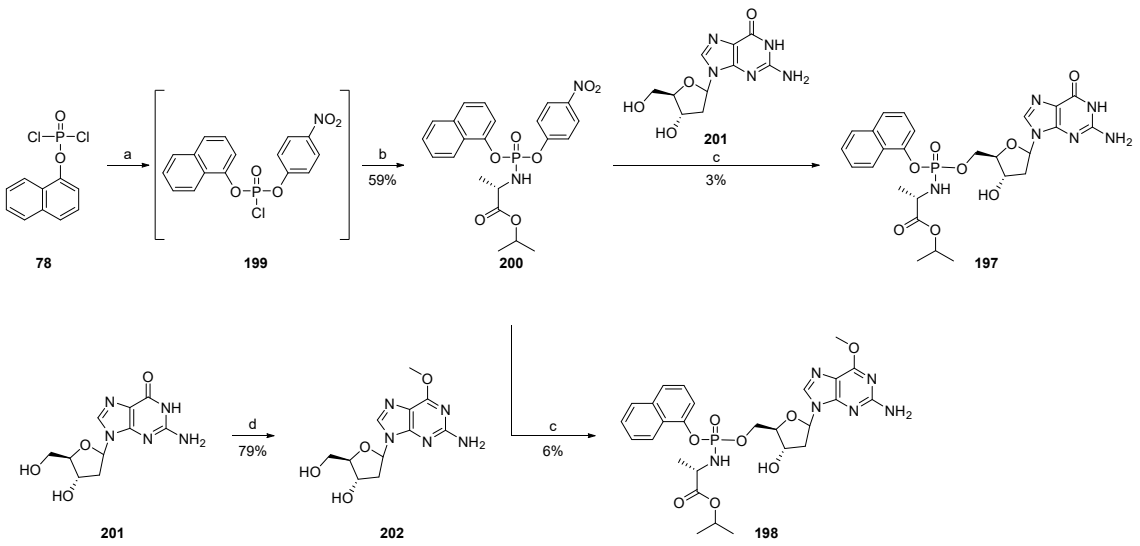


Figure 4.2 The two 2'-deoxyguanosine ProTides found active in DGUOK deficient cells by Demeter Therapeutics.

Both ProTides **197** and **198** were prepared by the company following the procedure reported in **Scheme 4.1**. First, the phosphoroamidating agent **200** was prepared by reacting 4-nitrophenol with naphthyl dichlorophosphate at -78°C in the presence of Et_3N to form the corresponding phosphorochloridate **199**. The addition of the amino acid with further Et_3N at 0°C led to the desired *p*NO_2P, which was used as phosphoroamidating source for both the ProTides. Concerning the preparation of the alkylate nucleoside **202**, starting from the natural 2'-deoxyguanosine **201**, an excess of diazomethane etherate was added at -20°C to accomplish the methylation in 6-position. Therefore, ProTides **197** and **198** were prepare using a Grignard reagent in THF and *N*-methyl-2-pyrrolidone (NMP) as solvents at rt for 16h.[35]



Scheme 4.1 Synthesis of ProTides **197** and **198** according to Demeter Therapeutics patent. Reagents and conditions: a) *p*-nitrophenol, Et_3N, CH_2Cl_2, -78°C to rt, 2h; b) *L*-alanine isopropyl ester, Et_3N, CH_2Cl_2, 0°C to rt, 2h; c) *t*BuMgCl (1M in THF), THF, NMP, 0°C rt, 16h; d) CH_2CN_2, MeOH, -20°C, 4h.

However, the ProTides were obtained in very low yields (3% and 6%) and the preparation of the 6-methoxy-2'-deoxyguanosine (2'-d-O^6-Me-G) with the diazo compound is considered too hazardous to be employed on an industrial scale.[36] For these reasons, an optimisation of the synthesis of **197** and **198** was necessary to carry on with the pharmacokinetics studies of the two compounds.

4.5 Aim

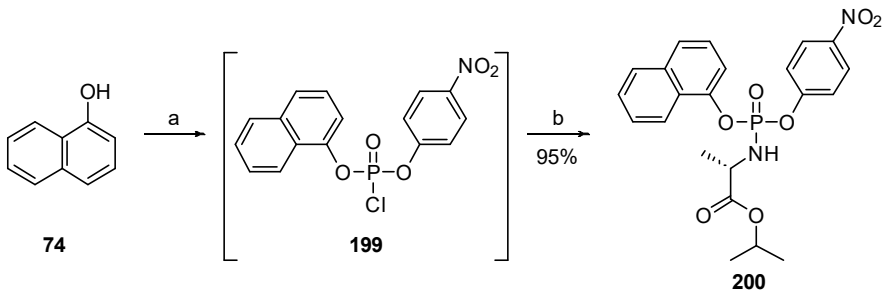
In the frame of a research project in collaboration with Demeter Therapeutics we were interested in studying further ProTides **197** and **198** for the treatment of MDS. To achieve this goal our first objective was the optimisation of the syntheses so that their scale up could be performed to carry on the pharmacokinetic studies. The limitations which needed to be overcome in their synthesis were numerous. The nucleosides displayed instability to a moderate acid environment, leading to the disruption of the glycosidic bond. Additionally, an alternative procedure for the methoxylation of the nucleoside needed to be envisaged to avoid the use of the hazardous diazomethane. In this challenging context, looking for an effective methylation of the natural nucleoside, a deep investigation of the reaction conditions was needed along with the exploration of a nucleoside protection/deprotection strategy.

Therefore, in accordance with the general aim of the project, an optimised synthetic methodology for the preparation of the demanding 2'd-O^6-Me-G nucleoside along with its ProTides was researched.

4.6 Results and discussion

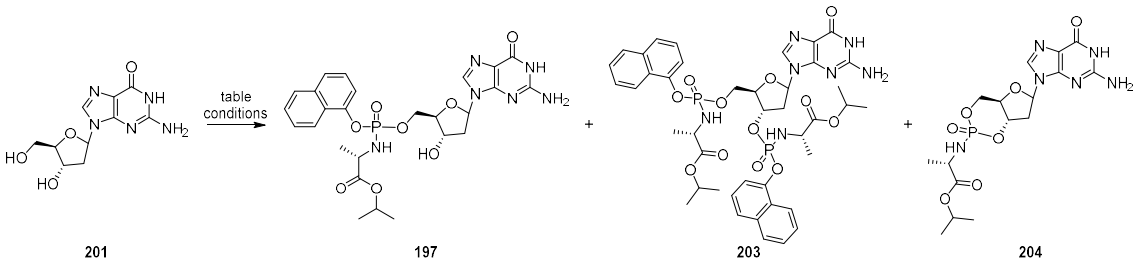
4.6.1 Preparation of 2'-dG ProTide

First, we focused on the optimisation of the coupling conditions, therefore we attempted the synthesis of ProTide **197**. The synthetic work for the preparation of the selected ProTide started with the synthesis of the aryloxy *p*-nitrophenyl phosphoroamidate **200**. The procedure starting from the commercial *p*-nitrophenol and phenyl dichlorophosphate disclosed in the patent resulted in 59% yield of the desired phosphoroamidating agent. Despite the decent yield, we decided to seek an improved route, that would make an industrial scale production of this reagent more convenient. A slightly different procedure was then employed to achieve the final compound in almost quantitative yield (**Scheme 4.2**). Instead of synthesising 1-naphtyl dichlorophosphate, we reacted the 1-naphthol with the *p*-nitrophenyl dichlorophosphate in presence of Et_3N at -78°C. After 30 minutes the formation of the corresponding aryloxy *p*-nitrophenyl phosphorochloridate **199** was detected by ^{31}P NMR spectroscopy (in $CDCl_3$, singlet at δ_P -5.65 ppm). Although **199** presents a chiral centre at the phosphorus atom, only one peak was observed in the spectrum since the enantiomers are not differentiated by NMR without using a chiral environment. The addition of the amino acid ester salt and further two equivalents of Et_3N at 0°C allowed the formation of the free amino acid with increased activity towards the phosphorus atom compared to the hydrochloride salt. After 30 minutes, the ^{31}P NMR spectroscopy showed the completion of the reaction by the disappearance of the singlet corresponding to **199** while the two peaks of the diastereoisomers of **200** appeared at δ_P -2.84 ppm and -2.88 ppm ($CDCl_3$). This procedure applied for the preparation of **200** (95% yield) proved to be more efficient than the methodology disclosed in the patent (59% yield). The reason can be found in the different preparation of the intermediate **199**. As reported in **Scheme 4.2**, preparation of **199** by reacting 1-naphthol (**74**) and *p*-nitrophenyl dichlorophosphate may lead to an improved formation of the key intermediate **199**. It can be supposed that the presence of the $-NO_2$ on the phenyl ring as a strong electron withdrawing group, may increase the electrophilicity at the phosphorus atom facilitating the attack of the naphthalenolate.



Scheme 4.2 Preparation of the phosphoroamidating agent naphthyloxy *p*-nitrophenyl phosphoroamidate **200**. Reagents and conditions: a) *p*-nitrophenyl dichlorophosphate, Et_3N, CH_2Cl_2, -78°C, 30 min; b) *L*-alanine isopropyl ester hydrochloride salt, Et_3N, CH_2Cl_2, 0°C, 30 min.

Once the phosphoroamidating agent was synthesized, the application of the ProTide technology was attempted using different coupling conditions: the Grignard approach, which is the procedure reported in the original patent,[35] and NMI (**Table 4.2**). The investigations started with the application of the patent conditions (entry 1, **Table 4.2**).



Entry	Activator	Conditions	197	203	204
1	*t*BuMgCl	THF, NMP rt, 16h	traces	ND	ND
2	NMI*	THF, Pyridine 40°C, 16h	traces	ND	ND
3	*t*BuMgCl	DMF 55°C, 6h	2%	5%	3%
4	*t*BuMgCl	DMF rt, 16h	32%	36%	ND

Table 4.2 Optimisation of the reaction conditions for the preparation of 2'-dG ProTide **197** without protection in 3'-position. The yields reported are based on HPLC analysis of the crude mixtures. The reactions are performed in presence of the phosphoroamidating agent **200**. ND: Not Detected. *Different mechanism is operating with this molecule.

In our hands, the use of tBuMgCl at room temperature for 16h in THF and NMP showed formation of the product only in traces. Even though the *m/z* (mass-to-charge

ratio) corresponding to the desired ProTide was observed in the MS(ESI+) analysis of the crude reaction (**Figure 4.3**), the amount of the product was so low that its isolation by flash chromatography could not be achieved. A similar result was observed when NMI was used in place of the Grignard reagent (entry 2, **Table 4.2**). Because of the nucleoside's poor solubility in THF and pyridine mixture (2:3, v/v), the reaction was performed at 40°C to enhance the starting material solubilization. Unfortunately, after 16h at this temperature only traces of the final ProTide were detected at the MS(ESI+) analysis.

In both cases the starting nucleoside was the main species in the crude mixtures along with traces of the desired compound and several by-products (**Figure 4.3**).

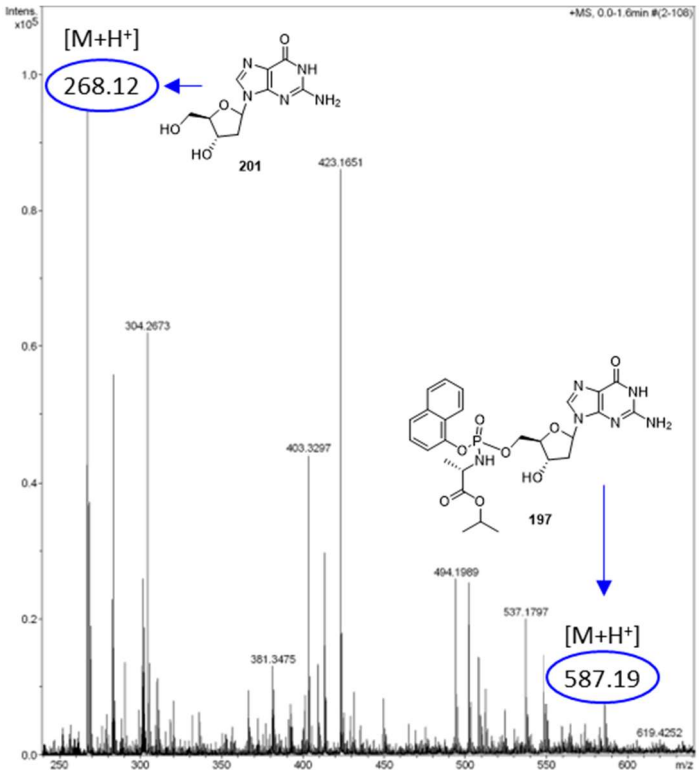


Figure 4.3 MS(ESI+) spectrum of the crude mixture obtained from the reaction conditions reported in entry 1, **Table 4.2**.

We then attempted to increase 2'-deoxyguanosine's solubilization and promote the coupling, by using the polar solvent DMF performing the reaction at 55°C (entry 3, **Table 4.2**). After only 6h, the TLC showed the disappearance of the starting nucleoside and the formation of three new spots with higher retention factors. The DMF was then evaporated from the reaction crude, which was directly purified by flash chromatography. The purification of the mixture was found to be difficult: three normal

phase and two reverse phase flash chromatography columns were necessary to successfully obtain the desired ProTide with a purity greater than 95% (determined by HPLC). Unfortunately, **197** was obtained only in 2% yield, therefore no improvements upon the patent conditions were realised.

Additionally, the other two main species detected were isolated as enriched fractions. A combination of ^{1}H-NMR and mass spectroscopy (**Figure 4.4**) allowed the identification of all species. Starting from the compound with higher retention factor (R_f: 0.32, CH_2Cl_2:MeOH-9:1) recognized as the expected bis-ProTide **203** featured by the phosphoroamidate moiety in both 3'- and 5'-positions. The desired ProTide **197** was identified as the compound with the middle retention factor (R_f: 0.25, CH_2Cl_2:MeOH-9:1), and finally, the least lipophilic product (R_f: 0.15, CH_2Cl_2:MeOH-9:1) proved to be the cyclic derivative **204**.

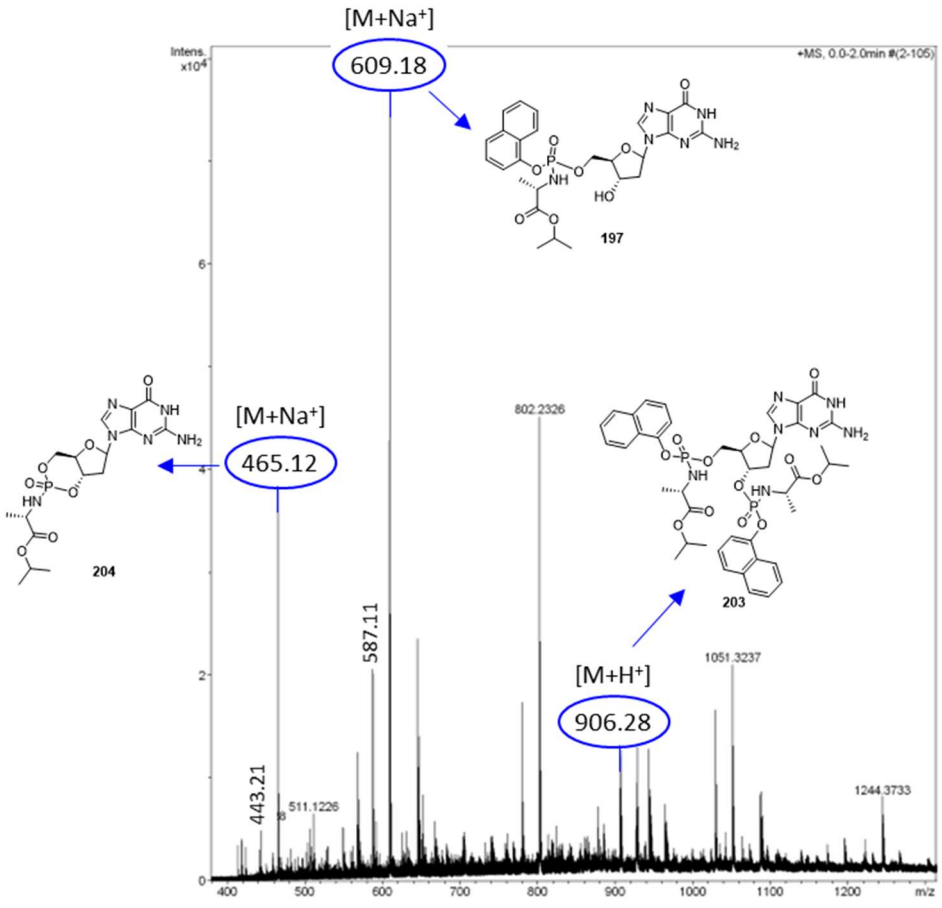
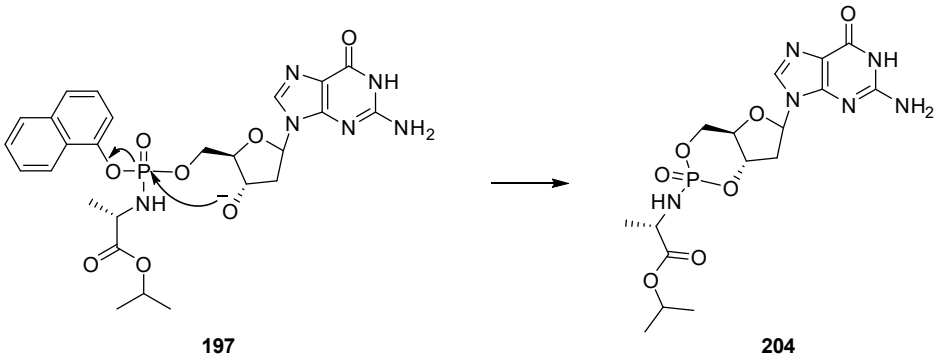


Figure 4.4 MS(ESI+) spectrum of the crude mixture obtained from the reaction conditions reported in entry 3, **Table 4.2**.

These data demonstrated that the activated 3'-*OH* is able to attack the phosphorus atom of a new molecule of the phosphoroamidating agent (intermolecular attack) or the one

of the ProTide moiety (intramolecular attack). In the last case, the naphthalenolate is a good leaving group and can be easily displaced so that a cyclic derivative is generated (**Scheme 4.3**). The HPLC chromatograms of each of the three species isolated are here reported (**Figure 4.5**).



Scheme 4.3 Proposed mechanism for the formation of the cyclic phosphate **204**.

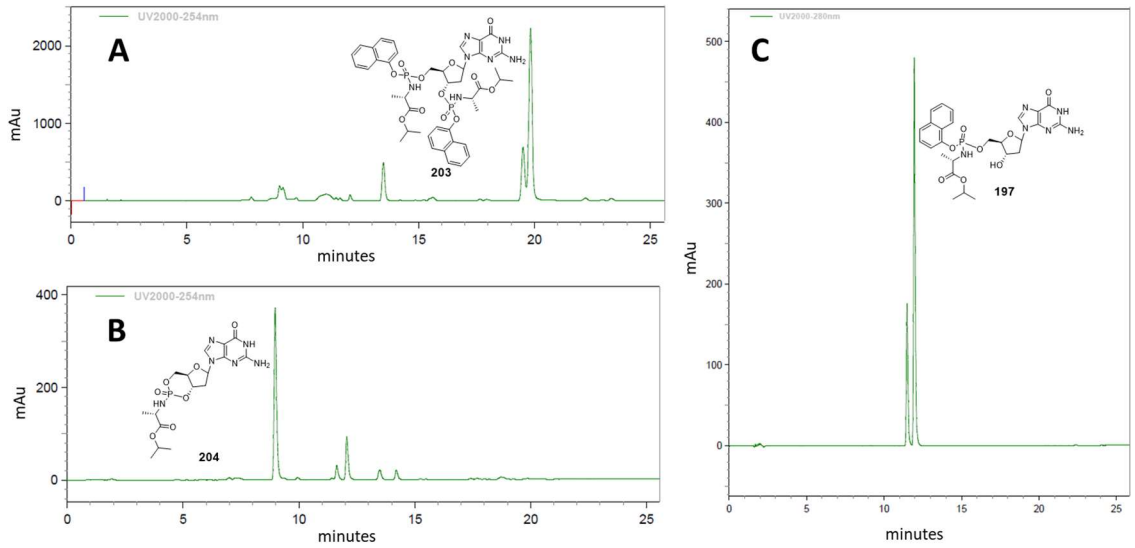


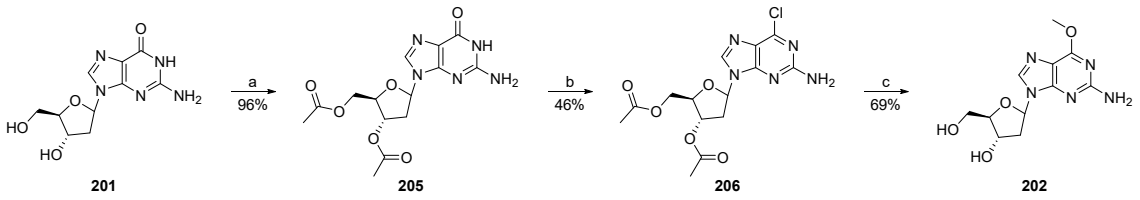
Figure 4.5 Analytical reverse phase HPLC eluting with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 minutes, 1ml/min, λ= 254 nm. **A**: isolated fraction enriched in bis-ProTide **203** (t_R: 19.5, 19.9 min.); **B**: isolated fraction enriched in cyclic derivative **204** (t_R: 9.3 min.); **C** isolated fraction of pure ProTide **197** (t_R: 11.5, 11.9 min.).

When the reaction was performed in DMF at room temperature (entry 4, **Table 4.2**), after 16h, no formation of the cyclic derivative **204** was detected, but still the formation of the bis-ProTide proved to be predominant (36%) compared to the desired ProTide (32%).

Despite the numerous difficulties encountered in the synthesis and isolation of ProTide **197**, a great improvement of the ProTide yield was reached and we were able to obtain it in a sufficient amount for the pharmacokinetic studies.

4.6.2 First strategy adopted for the preparation of 2'-d-O^6-Me-G ProTide

For the synthesis of 2'-d-O^6-Me-Guanosine the original patent reported the use of CH_2N_2 in MeOH at -20°C,[35] In order to avoid employing this or other diazo compounds, whose use is not appealing for industrial scale synthesis, we devised a new synthetic pathway for the synthesis of the targeted 2'-d-O^6-Me-G nucleoside. The strategy consisted of the preparation of the unprotected nucleoside with a three steps approach (**Scheme 4.4**).[37]

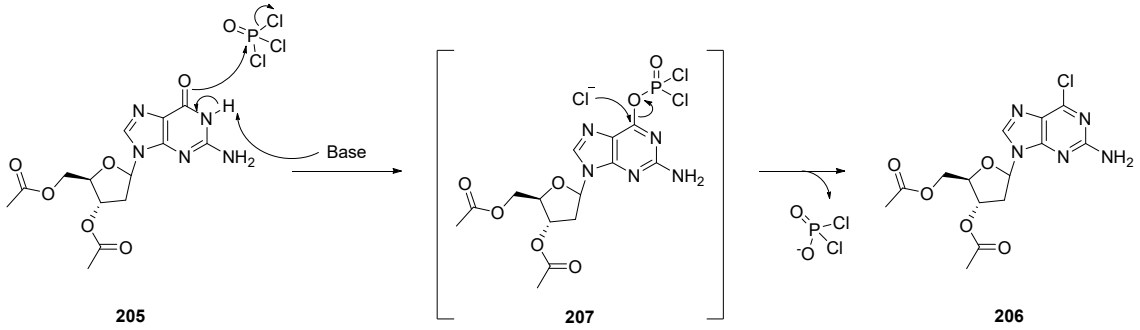


Scheme 4.4 Synthetic pathway adopted for the preparation of the 3'-unprotected 2'-d-O^6-Me-G **202**. Reagents and conditions: a) acetic anhydride, Et_3N, DMAP, ACN, rt, 16h; b) $POCl_3$, *N*,*N*-dimethylaniline, BTEA-Cl, ACN, 0°C to rt, 10 min., reflux, 20 min.; c) $NaOCH_3$ (1M in MeOH), MeOH, 0°C to rt, 6h.

Commercially available 2'-dG **201** was acetylated to obtain the diacetylated analogue **205**. Briefly, **201** was suspended in ACN and treated with an excess of acetic anhydride and Et_3N in the presence of a catalytic amount of DMAP. The addition of DMAP to the acetylating system was found able to improve the reaction yields.[38] DMAP reacts with the acyl donor (acetic anhydride) to generate an acylpyridinium cation which is attacked by the alcohol. Then, Et_3N acts as auxiliary base able to regenerate the catalyst.[39] After 16h at room temperature, the desired product was precipitated by addition of MeOH affording pure **205** in almost quantitative yield.

To insert a methoxy group in the 6-position of the nucleobase, the strategy adopted required the replacement of the carbonyl function with a chlorine atom as an excellent leaving group. To do that, $POCl_3$ was added dropwise to a suspension of protected **205** in ACN in the presence of *N*,*N*-dimethylaniline at 0°C. The reaction mixture was stirred at room temperature for 10 minutes and then refluxed for 1h in a preheated oil bath. Afterwards, the solution was immediately cooled in an ice-bath and quickly

concentrated to dryness with the addition of ice to the rotary evaporator bath. The excess of phosphoryl chloride was slowly hydrolysed by addition of cold water keeping the flask at 0°C. The solution was left stirring for 20 minutes and then extracted with EtOAc.[40] Mechanistically, the chlorination proceeds with the attack of the oxygen of the carbonyl function in the 6-postion on the phosphorus of $POCl_3$ leading to the elimination of chloride. The phosphorodichloridate moiety of the intermediate **207** (an excellent leaving group) can be easily replaced by the chloride present in the reaction mixture so that 3'5'-di-*O*-acetyl-6-deoxo-6-chloro-2'-deoxyguanosine **206** is readily obtained (**Scheme 4.5**).

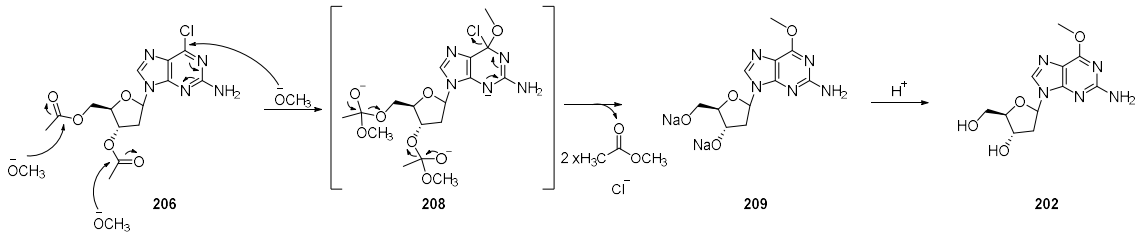


Scheme 4.5 Mechanism of deoxychlorination of **205** by $POCl_3$.

A tertiary amine is often used as catalyst and/or acid scavenger.[41] As a catalyst, *N*,*N*-dimethylaniline is required for the deprotonation of N^1 to increase the carbonyl oxygen nucleophilicity. Additionally, an excess of amine can be also used to quench the hydrochloric acid formed in the reaction mixture. Indeed, the omission of *N*,*N*-dimethylaniline proved to provoke the cleavage of the nucleoside.[42] Unfortunately, in our hands, despite the excess of amine employed, nucleoside degradation occurred. Robins *et al.* demonstrated that the conversion of **207** into the final compound can be very slow.[41] That may require a prolonged refluxing time leading to the breakage of the glycosidic bond. However, this problem can be overcome by incrementing the concentration of chloride. The introduction of ammonium chloride as an external source of anions should facilitate the conversion of the intermediate **207** into **206** before decomposition occurrs.[41] Therefore, the procedure was repeated in the presence of benzyltriethylammonium chloride (BTEA-Cl) and **206** was pleasantly obtained in 46% yield. Unfortunately, the scale up of the reaction mixture (from 500mg to 5g) gave

much lower yield (15%). The reason could be that it is more difficult to control the reaction temperature inside the flask working with large amount of solvents.

Once the chlorine was successfully installed in 6-postion of the nucleobase, the next step consisted of its replacement with a methoxy group. The final methoxylation was accomplished by slow addition of a freshly prepared 1M solution of $NaOCH_3$ in MeOH to a dispersion of **206** in MeOH cooled at 0°C. After 6h at room temperature, TLC showed completion of the reaction by disappearance of the starting nucleoside. The chloride is replaced by an addition-elimination mechanism initiated by the nucleophilic attack of the methoxide to the carbon bearing the halogen (addition). The subsequent loss of the chloride (elimination) followed by the ring aromaticity restoration led to the formation of the nucleoside methoxylated in 6-position (**Scheme 4.6**). Additionally, the large excess (5 equivalents) of the $NaOCH_3$ used provided the deprotection of the sugar 3'- and 5'-*OH* by attack of the methoxide to the acetyl groups generating a negatively charged nucleoside neutralised by the sodium cation present in the reaction mixture (**Scheme 4.6**). To isolate the final nucleoside **202**, the concentrated crude mixture was dissolved in excess of water and the pH adjusted to a value of 7 by addition of acetic acid.

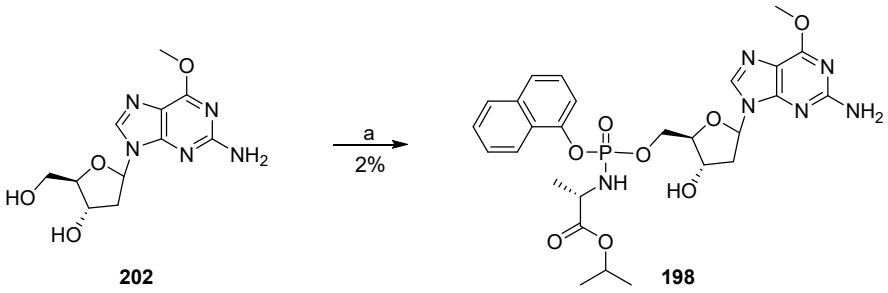


Scheme 4.6 Mechanism for the methoxylation of the guanine ring and simultaneous deprotection of the 3'- and 5'-*OH* by treatment with $NaOCH_3$.

The aqueous solution was extracted with ethyl acetate several times, but these efforts proved ineffective since most of the compound was still in the aqueous phase due to the hydrophilicity of the unprotected nucleoside. For this reason, an alternative work up was necessary to avoid the loss of compound. An appealing option seemed to be the use of ion-exchange resins, which being insoluble cross-linked polymer matrix can be easily removed after use. In this case, a cation-exchange resin, such as acid amberlite was required, which conventionally consists of a negatively charged matrix (SO_3^-) and exchangeable positive ions (H^+).[43] By eluting the reaction mixture though a column of resin, the nucleoside Na^+ cations would be exchanged with the resin H^+ cations, so that

the 3'- and 5'-*OH* of the compound could be restored and the resin neutralised. However, in this case, to limit the exposure of the compound to the acid resin, small amounts of amberlite were added to the crude colourless solution checking the pH value of the mixture while high stirring was operating. Unfortunately, the control of the pH proved to be very difficult to manage and after few additions of the resin, the solution became brown in colour, an indication that glycosidic bond cleavage had occurred. After resin filtration and solvent evaporation, the degradation was confirmed by NMR spectroscopy. Due to this failure, another attempt with the acetic acid was pursued, but this time the crude mixture was solubilised in the minimum amount of water. When pH 7 was achieved, precipitation occurred. The precipitated white solid was filtered off and recovered while the filtrate was extracted multiple times with EtOAc. The recovered residue was then solubilised in the minimum amount of water and extracted with both EtOAc and CH_2Cl_2 until TLC showed no presence of the nucleoside in the water phase. All the combined organic layers were concentrated to dryness and the final pure 2'-d-O^6-Me-G nucleoside **202** was obtained in 69% yield.

With **202** in hand, it was possible to accomplish the desired ProTide synthesis. Based on our experience with 2'-dG ProTide synthesis previously performed, it was decided to prepare **198** via Grignard methodology (**Scheme 4.7**) using the best conditions identified for the synthesis of ProTide **197** (entry 4, **Table 4.2**).

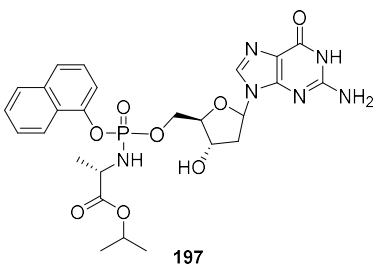


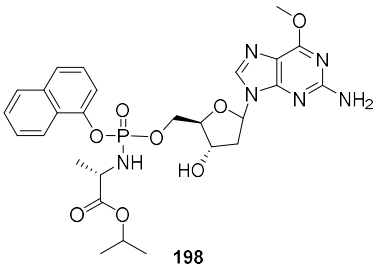
Scheme 4.7 Synthesis of 2'-d-O^6-Me-G ProTide **198** without protection in the 3'-position. Reagents and conditions: a) *t*BuMgCl (1M in THF), naphthyloxy *p*-nitrophenylphosphoroamidate **200**, DMF, rt, 16h.

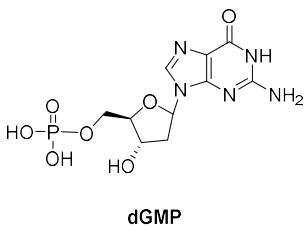
Although both 5'-mono- and 3',5'-bisphosphoroamidates were formed, careful and extensive flash chromatography allowed the isolation of a mixture enriched in the desired 5'-ProTide, which only after a reverse phase chromatography was isolated in a sufficient amount to carry on with the pharmacokinetic studies.

4.6.3 Summary of the result of Pharmacokinetics studies.

Pharmacokinetic properties of ProTides **197** and **198** along with 2'-deoxyguanosine monophosphate (dGMP) were evaluated, and the results are reported in **Table 4.3**.







Physicochemical Property	197	198	dGMP
Kinetic Solubility[a]	166 µM	194 µM	ND
Log P[b]	1.1	3.0	< -1.4
Caco-2 P_{app} ($x10^{-6}$)[c]	0.1/1.8	2.0/5.7	0.3/0.5

Table 4.3 Pharmacokinetic studies results. [a]pH: 7.4; [b]pH: 11.0; [c]A-B/B-A with efflux inhibitors (verapamil, fumitremorgin).

Concerning the solubility of the molecules, the kinetic solubility parameter was examined by measuring the compound's concentration in a phosphate buffered saline solution at the physiological pH 7.4, after removal of insoluble residues. As expected, dGMP could be detected only in its charged deprotonated form, which impede the compound's intracellular delivered. Otherwise, both **197** and **198** showed nice kinetic solubility values with a concentration of 166µM and 194µM, respectively.

The lipophilicity of ProTides is a crucial parameter to consider. Indeed, the compounds need to be lipophilic enough to cross the cell membrane. According to the druglikeness predictions, the optimal value of logP should be in the range between 2 and 3, or in larger terms it should not exceed -1.4 and +5.6 values.[44] It is possible to notice from the table that dGMP showed a negative value of logP indicating the high hydrophilicity of the compound. Predictably, the addition of the phosphoroamidate moiety to the nucleoside conferred improved lipophilicity upon the molecule. Notably, 2'-d-O^6-Me-G ProTide **198** showed an optimal 3.0 value, indicating its higher potential ability to pass the biological membranes.

Finally, the *in vivo* absorption of the compounds across the gut wall was predicted through the *in vitro* Caco-2 permeability assay.[45] This is an established

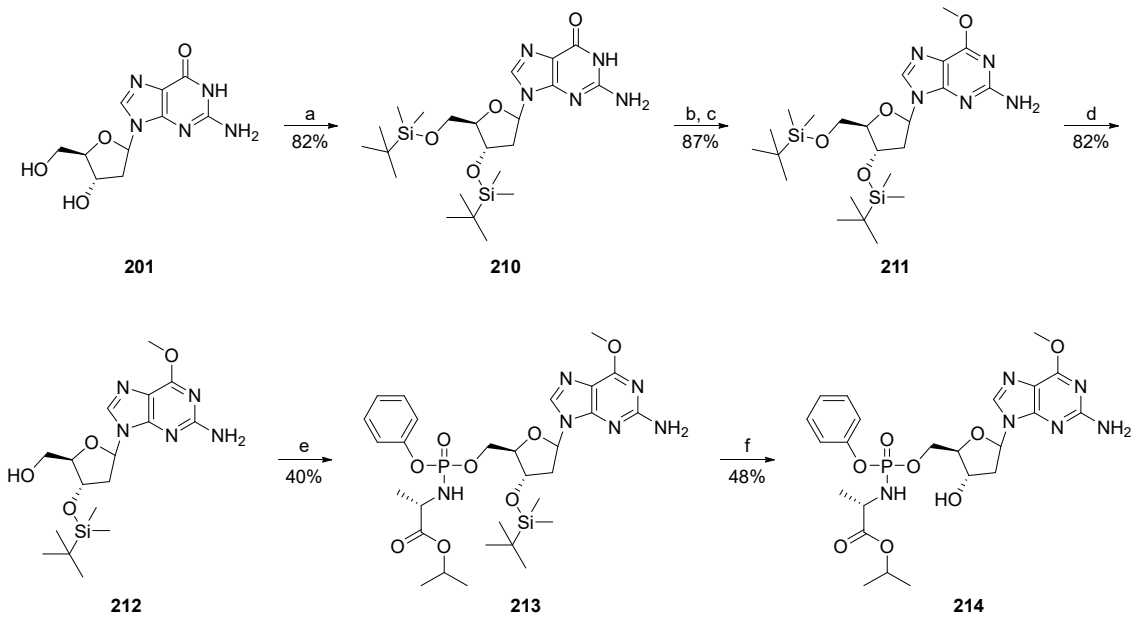
method to measure the rate of the compound transported across the intestinal epithelial cells in both the two possible directions: basolateral to apical (B-A) and apical to basolateral (A-B). Additionally, the values reported in the table are referring to the assay performed in the presence of efflux inhibitors, such as the verapamil (P-glycoprotein inhibitor) and fumitremorgin (Breast Cancer Resistance Protein-BCRP inhibitor), so that any potential active efflux could be excluded. The resulting data suggested that the order of the predicted intestinal absorption followed the same trend as previously described for the compounds lipophilicity: **198** > **197** > dGMP. Once again, the phosphoroamidate moiety proved to be of pivotal importance to improve the nucleoside monophosphate pharmacokinetic properties.

According to these data, the alkylation at the 6-position of the nucleobase was found to be an efficient variation of the natural 6-oxo derivative. Compound **198** demonstrated good kinetic solubility, improving both logP and predicted intestinal permeability values. For these reasons, a more efficient synthetic pathway toward the 2'-d-O^6-Me-G nucleoside and its corresponding ProTide preparation needed to be designed in order to make it suitable for an industrial scale up. Moreover, ProTide **198** was not the only targeted compound, but also the aryloxy variant with the phenyl moiety was additionally synthesised to be evaluated as a potential MDS treatment, showing the optimal calculated logP value of 2.7.

4.6.4 Second strategy adopted for the preparation of 2'-d-O^6-Me-G ProTide

The synthesis of 2'-d-O^6-Me-G nucleoside (**Scheme 4.4**) showed some important limitations. The highly instability of the nucleoside in acid conditions made the chlorination step crucial. Moreover, the scale up of the reaction caused a dramatic decrease in the amount of product afforded. Additionally, the use of the unprotected nucleoside resulted in a very low yielding ProTide formation due to the starting material's poor solubility and the absence of selectivity in the phosphorylation of the 5'-position. Furthermore, an increase in the reaction temperature may result in the formation several by-products. Accordingly, these problems make that the synthetic pathway unattractive for industry. To overcome these limitations, a new strategy for the preparation of the 2'-d-O^6-Me-G nucleoside was designed (**Scheme 4.8**). The presence of a protecting group in the 3'-position proved to be essential to achieve a good

outcome in the ProTide reaction. For this reason, a protection/deprotection strategy was envisaged so that a selective 5'-phosphorylation could be accomplished.



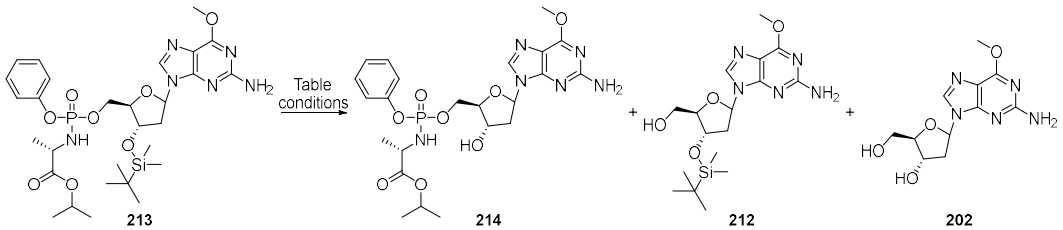
Scheme 4.8 Synthesis of 2'-d-O^6-Me-G ProTide **214** using the 3'-protected nucleoside **212**. Reagents and conditions: a) TBDMSCl, imidazole, pyridine, room temperature, 12h; b) $(CF_3CO)_2O$, pyridine, 0°C, 15 min.; c) $NaOCH_3$ in MeOH (0.17 M), room temperature, 20h; d) TFA:H_2O:THF (1:1:4, v/v/v), 0°C, 30 min.; e) *t*BuMgCl (1M in THF), phenyloxy *L*-Alanine *iso*-propyl ester phosphorochloridate **22**, THF, rt, 16h; f) CH_3SiOTf, THF, 0°C, 5 min., Al_2O_3.

The first step in the designed synthetic pathway consisted of the protection of both the 3'- and 5'-*OH* of **201** with *tert*-butyldimethylsilyl groups (TBDMS). The protected nucleoside was obtained in 82% yield by reacting **201** with the silylating agent TBDMSCl and imidazole as catalyst in dry pyridine at room temperature for 24h.[46] The methoxylation of compound **210** was then achieved by a one-pot, two steps procedure first using trifluoroacetic anhydride in pyridine to form a 6-pyridyl compound at 0°C. The reaction is complete in less than 15 minutes (monitored by TLC) and it is not accompanied by degradation. The subsequent addition of the alkoxide ($NaOCH_3$, 0.17M in MeOH) allowed slow displacement of the pyridyl moiety at room temperature to obtain **211**. Literature data suggested that the methoxylation step can be affected by the concentration of the alkoxide solution added, which is then kept below 0.18M to obtain higher yield.[47] Indeed, higher concentration of alkoxide may cause the cleavage of the glycosidic bond leading to a brown suspension and determining a drastic reduction in the reaction outcome. Due to this limit, the methoxylation is slow and 20h were necessary to afford **211** in 87% yield. Compared to the procedure previously adopted

(overall yield 30%), the methoxylation step was largely improved and it could be successfully applied for the industrial scale up.

We proceeded to the selective deprotection of the 5'-*OH*. To accomplish this task, an established procedure consisting in a mixture of aqueous TFA in THF was employed.[48] Despite the use of the acid catalyst necessary to trigger the hydrolysis, no nucleoside degradation was observed. Keeping the reaction temperature at 0°C the hydrolysis of the primary alcohol was selectively accomplished and compound **212** was successfully afforded with 82% yield. The 3'-protected nucleoside was finally ready to be subjected to the desired phosphoroamidation. By applying this synthetic strategy, no formation of the bis-prodrug was expected resulting in an improved yield of the mono-ProTide. Compound **213** was obtained by using *t*BuMgCl as base in the presence of the phenyloxy *L*-Alanine *iso*-propyl ester phosphorochloridate. After 16h at room temperature, the desired ProTide was formed. Pleasantly, only one normal phase flash chromatography column was necessary to successfully isolate it in 40% yield. Therefore, the presence of the protecting group on the 3'-*OH* proved to be helpful not only in avoiding by-product formation, but also in increasing the nucleoside lipophilicity resulting in a compound more soluble in organic solvents and easier to purify.

The final ProTide deprotection was a problematic step. Several conditions were attempted and evaluated by checking the reaction through HPLC analysis. All the results are collected in **Table 4.4**.



Entry	Reagent	Solvent	T°C	Time	214*	212*	202*	213*
1	TFA	CH_2Cl_2	0°C	5 min.	degradation			
2	HF·pyridine	THF	0°C	2h	16%	12%	4%	56%
			rt	2h	23%	12%	39%	9%
			rt	12h	15%	26%	51%	8%
3	TMSOTf (2eq)	CH_2Cl_2	-78°C	15 min.	50%	ND	ND	50%
				1h	56%	ND	ND	44%
				2h	68%	ND	ND	32%

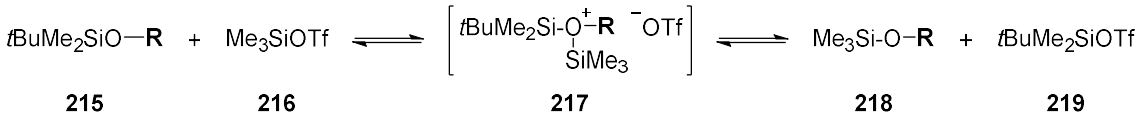
4	TMSOTf (2eq)	CH_2Cl_2	-40°C	15 min	58%	ND	ND	31%
5	TMSOTf (5eq)	CH_2Cl_2	-40°C	10 min.	5%	ND	ND	90%
6	TMSOTf (5eq)	CH_2Cl_2	0°C	5 min.	78%	2%	7%	13%

Table 4.4 Screening of different deprotection conditions. *Evaluated by RP-HPLC analysis: the percentage areas of product and by-products are reported. ND: Not Detected.

The first condition attempted was the most common procedure applied in our laboratory for ProTide 3'-TBDMS deprotection (entry 1, **Table 4.4**). The starting compound **213** was solubilised in CH_2Cl_2 and an equivalent volume of TFA was added dropwise keeping the mixture temperature at 0°C. After only a few minutes, the colourless solution became a brown suspension indicating nucleoside degradation. The main by-products were identified by MS (ESI+) and HPLC analysis. The 3'-protected nucleoside without the ProTide moiety (**212**) and the 3',5'-unsubstituted nucleoside (**202**) were also recognised. All the compounds present in the reaction mixture, including the starting **213**, were analysed by HPLC so that their corresponding peaks could be taken as reference for the evaluation of the final deprotection outcome as the example reported in **Figure 4.6**.

As an alternative procedure, we then attempted a fluoride-based deprotection strategy using HF·pyridine (entry 2, **Table 4.4**).[49] The presence of the organic base in the complex allows the reduction of the acidity of the system. The driving force of the deprotection is the formation of Si-F bond, which is greatly preferred to the Si-O bond because it is stronger. After 2h at 0°C only 16% of product was formed and lots of starting material was still unreacted (56%). When the reaction was left stirring at room temperature an increased amount of product (23%) was observed along with the by-products **212** (12%) and **202** (39%). Unfortunately, after 12h the desired ProTide yield decreased (15%) in favour of the 3'- and 5'-unprotected nucleoside **202** (51%). These data suggested that the strong basicity of the fluoride ion may be incompatible with the ProTide feature.

A potential safer deprotection strategy involving trimethylsilyl triflate (TMSOTf) was evaluated.[50] This desilylating reagent works through an exchange reaction between trimethylsilyl triflate (**216**) and *tert*-butyldimethylsilyl ethers (**215**) *via* a bis-silyloxonium ion intermediate (**217**) by reversible equilibria (**Scheme 4.9**).[51] The resulting trimethylsilyl ether **218** is highly prone to hydrolysis and it is readily cleaved by addition of either methanol or water to release the unprotected hydroxyl group.



Scheme 4.9 Exchange reaction between *tert*-butyldimethylsilyl ethers **215** and trimethylsilyl triflate **216**.

Different reaction conditions were evaluated to achieve the targeted unprotected ProTide in good yields. Firstly, 2 equivalents of TMSOTf were employed at -78°C (entry 3, **Table 4.4**) and -40°C (entry 4, **Table 4.4**). It was observed that at -78°C, by-products **212** and **202** were never detected. After 2h at this temperature 68% of desired product was observed. At -40°C, 58% of product formation was reached after 15 minutes, with some deprotected nucleoside **202** being formed (31%). When, 5 equivalents of TMSOTf were evaluated at -40°C (entry 5, **Table 4.4**), after 10 minutes, only the 5% of product was formed. Pleasantly, when the same amount of triflate was employed at 0°C (entry 6, **Table 4.4**), the 78% of **214** was formed along with only small amounts of undesired by-products (2-7%) (**Figure 4.6**).

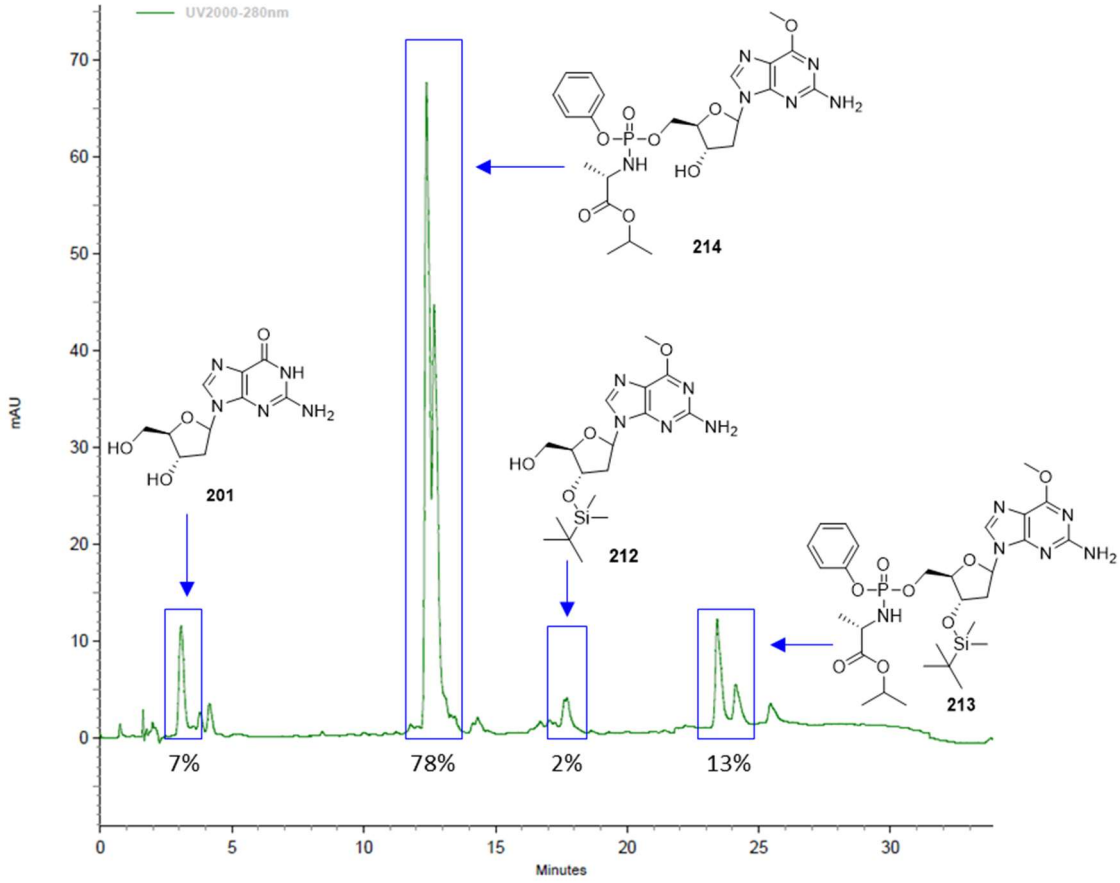


Figure 4.6 Analytical reverse phase HPLC chromatography of the crude of the final deprotection step (entry 6, **Table 4.4**). Elution performed with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 minutes, 1ml/min, λ= 280 nm.

The conditions of entry 6 (**Table 4.4**) were selected as the best reaction settings to obtain the desired 3'-unprotected ProTide in very good yield (78%).

Although we had established an efficient procedure for the synthesis of **214**, we encountered several problems in the work up of this reaction. The dilution of the mixture with MeOH should allow not only the equilibria to be shifted to the right but also the reaction to be quenched. Indeed, the addition of MeOH is able to hydrolyse the excess of TMSOTf. Despite that, when MeOH was added directly to the whole reaction mixture, complete degradation was observed. It was supposed that the great amount of triflic acid generated as by-product could be the cause of glycosidic cleavage. For this reason, the literature suggested the addition of a solution of $NaHCO_3$ (5% in MeOH) to the mixture so that the acid could be neutralised. Unfortunately, also in this case degradation occurred.[51] Compound **214** proved to be both acid and base sensitive. In the latter case the phosphoroamidate moiety was hydrolysed, whereas the glycosidic bond was cleaved in the acidic media. Therefore, the use of alternative desilylating methods

under neutral conditions was highly desirable. Addition of neutral alumina to the reaction mixture was found to be an effective strategy to remove the silyl protecting groups under very mild conditions. The deprotection take places when neutral alumina is mixed with the silyl ethers. The desilylation rate depends on the steric bulkiness of the silicon substituents. Therefore, the unhindered and less stable trimethylsilyl ether **218**, resulting from the treatment with TMSOTf, could be readily hydrolysed.[52] Finally, the mixture was filtered through a pad of alumina using CH_2Cl_2:MeOH (9:1, v/v) as eluent, and the filtrate was purified by flash chromatography to isolate the final pure ProTide **214** in 48% yield.

In summary, an effective strategy for the preparation of 2'-d-O^6-Me-G ProTide was designed and successfully optimised. The 3'-protection envisaged to increase the nucleoside solubility and improve mono-ProTide formation was successful, affording **213** with 40% yield. As expected, the most insidious step, the final desilylation, was largely explored due to the presence of both basic and acid sensitive moieties in the molecule. Neutral alumina proved to be an effective mild deprotecting agent to accomplish the preparation of the final ProTide in good yields. Moreover, it is a readily available and low-priced substance which is be easily handled in industry scale-up. The ProTide **214** is currently under evaluation as a potential MDS treatment.

4.7 Conclusions and future work

In this chapter, the syntheses of 2'-dG and 2'-d-O^6-Me-G ProTides were discussed. Their synthetic pathways were optimised to make them suitable for a big scale approach to allow further testing of their efficacy against MDS.

Initially, several conditions for the ProTide preparation of the 3'-unprotected 2'-dG nucleoside were investigated. As a result, the ProTide was synthesised in very low yield and the bis-prodrug was identified as the major product.

For the 6-methoxy derivative, two different strategies were explored for the preparation of the 2'-d-O^6-Me-G nucleoside.

According to the first strategy, 2'-d-O^6-Me-G was synthesised by using a three step procedure via a 6-chlorine derivative, and then the ProTide approach was applied to the 3'-unprotected nucleoside. This methodology showed important limitations: the chlorination was crucial because the reaction conditions affected the nucleoside stability; additionally, the scale up of the reaction caused a dramatic decrease in the amount of product afforded. Moreover, regarding the ProTide formation, it resulted in a very low yield due to the poor solubility of the nucleoside and the absence of selectivity in the phosphorylation as expected when a 3'-unprotected nucleoside is employed.

According to the pharmacokinetics data, the alkylation in the 6-position was an effective variation of the natural 6-oxo derivative. 2'-d-O^6-Me-G ProTide showed increased logP and predicted intestinal absorption values while maintaining a good value of kinetic solubility. For these reasons, a more efficient synthetic pathway for 2'-d-O^6-Me-G ProTide preparation needed to be designed.

In the second strategy explored, the methoxylation was successfully accomplished by a one-pot two steps procedure consisting of the formation of a 6-pyridyl intermediate followed by methoxylation. Furthermore, the 3'-TBDMS protection of the nucleoside was envisaged to increase the compound solubility and improve mono-ProTide formation. As expected, the most insidious step was the final desilylation, which was largely explored due to the presence of both basic and acid sensitive moieties in the molecule. Finally, treatment of the 3'-protected ProTide with TMSOTf followed by neutral alumina, was found to be an effective methodology to accomplish the desired ProTide desilylation in good yields.

In conclusion, an efficient synthetic pathway for the preparation of 2'-dG and 2'-d-O^6-Me-G ProTides was successfully optimised. The compounds are currently being subjected to a big scale-up so that the *in vivo* test can be performed.

In the future, having this synthetic procedure in hand, novel modifications at the nucleobase or ProTide moiety could be envisaged to further investigate the SAR of this class of compounds as potential therapeutic MDS options.

4.8 References

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Chapter 5. Experimental

5.1 General experimental details

All solvents used were anhydrous and used as supplied by Sigma-Aldrich. All commercially available reagents were supplied by either Sigma-Aldrich or Fisher and used without further purification. All nucleosides and solid reagents were dried for several hours under high vacuum prior to use.

All glassware was oven-dried at 130°C for several hours or overnight and allowed to cool in a desiccator or under a steam of dry nitrogen.

For analytical thin-layer chromatography (TLC), precoated aluminium-backed plates (60 F-54, 0.2 mm thickness; supplied by E. Merck AG, Darmstadt, Germany) were used and developed by an ascending elution method. For preparative thin-layer chromatography (prep TLC), preparative TLC plates (20 cm x 20 cm, 500-2000 μm) were purchased from Merck. After solvent evaporation, compounds were detected by quenching of the fluorescence, at 254 nm upon irradiation with a UV lamp.

Column chromatography purifications were carried out by means of manual flash chromatography or automatic Biotage Isolera One. Fractions containing the product were identified by TLC and pooled, and the solvent was removed in vacuo.

^{1}H, ^{31}P, ^{13}C and ^{19}F NMR spectra were recorded in a Bruker Avance 500 spectrometer at 500 MHz, 202 MHz, 125 MHz and 407 MHz respectively and auto-calibrated to the deuterated solvent reference peak in case of ^{1}H and ^{13}C NMR and 85% H_3PO_4 for ^{31}P NMR experiments. All ^{31}P and ^{13}C NMR spectra were proton-decoupled.

Chemical shifts are given in parts per million (ppm) and coupling constants (*J*) are measured in Hertz. The following abbreviations are used in the assignment of NMR signals: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), bs (broad singlet), dd (doublet of doublet), ddd (doublet of doublet of doublet), dt (doublet of triplet). The assignment of the signals in ^{1}H NMR and ^{13}C NMR was done based on the analysis of coupling constants and additional two-dimensional experiments (COSY, HSQC).

All analytical high-performance liquid chromatography (HPLC) experiments were done on a Thermo Fisher Spectra System SCM1000 provided with a System Controller

SN4000, a pump Spectra System P4000 and a Spectra UV2000 detector set or Varian Prostar (LC Workstation-Varian Prostar 335 LC detector) using a C-18Varian Pursuit (150 x 4.6 mm, 5 μm) reverse phase column. All final compounds were isolated with purity ≥ 95%.

Low resolution mass spectrometry was performed on a Bruker Daltonics microTof-LC system (atmospheric pressure ionization, electron spray mass spectroscopy) in positive mode.

5.2 Serum Stability assays

The appropriate substrate (5 mg, ~0.008 mmol) was dissolved in 100 μl of DMSO-d_6 and 300 μl of D_2O. A ^{31}P NMR (202 MHZ, 256 scans) was conducted as a reference (blank, t = 0). To this mixture, 300 μl of a stock solution of rat/human serum (purchased from SIGMA) was added. ^{31}P (256 scans) were carried out with 1 minute of delay between experiments for 14 hours at 37°C.

5.3 Standard procedures

General procedure A for the synthesis of ProTides with Grignard reagent

Under an argon atmosphere, the appropriate nucleoside (1 eq) was dissolved in THF (8 ml / mmol of nucleoside). *t*BuMgCl (1.0 M in THF, 1.1 eq) was then added in a dropwise fashion. A solution of the phosphorochloridate (1.2 eq) in THF (2 ml per 1 mmol of phosphorochloridate) was added and the resulting mixture was stirred at room temperature for 18-24 hours. After this period, the solvent was evaporated to dryness and the crude product was purified by silica gel chromatography or Biotage Isolera One. In some cases further purification by preparative TLC was necessary. The title compound was usually obtained as a white foamy solid.

General procedure B for the transition metal catalysed synthesis of ProTides

Under an argon atmosphere, to a solution of the nucleoside (1 eq) in THF (28 ml/mmol of nucleoside), DIPEA (1.5 eq) was added. After 30 minutes also the catalyst MX_n (0.2 eq) was added and the reaction mixture was allowed to stir for 1h. After this period, a solution of the appropriate phosphorochloridate (1.2 eq) in THF (7 ml/mmol of

phosphorochloridate) was added dropwise over 30 minutes. After 16 h, 25 µl of the crude mixture were collected, diluted with MeOH (4 ml), filtered and analyzed by RP HPLC.

General procedure C for the synthesis of *O*-Aryl-(*L*-Alanine-ester)-allylphosphonate

Under an argon atmosphere, 2,6-Lutidine (4 eq) and trimethylsilyl bromide (TMSBr, 5 eq) were added to a solution of dimethyl allylphosphonate (1 eq) in anhydrous acetonitrile (8 ml / mmol of allylphosphonate). The mixture was stirred for 16 h at room temperature and then the volatiles were evaporated without any contact with air. Then the flask was charged with dry aminoacid ester hydrochloride (1 eq), dry aryl-alcohol (6 eq), dry triethylamine (15 eq) and dry pyridine (3 ml / mmol of allylphosphonate) and heated to 50˚C to obtain a homogenous solution. To this mixture was then added a solution of Aldrithiol-2 (6 eq) and triphenylphosphine (6 eq) in dry pyridine (3 ml / mmol of allylphosphonate) under argon atmosphere. The resulting mixture was stirred at 50˚C for 16 h. After evaporating all the volatiles, the residue was purified by Biotage Isolera One.

General procedure D for the synthesis of N^1-2'-methylallylpyrimidine

Under an argon atmosphere, to a solution of the nucleobase (1 eq) in anhydrous acetonitrile (2 ml / mmol of nucleobase) was added BSA (2.5 eq). The mixture was refluxed until clear solution was observed (usually 5 min). 3-bromo-2-methylpropene (2.0 eq), NaI (1.1 eq) and TMSCl (1 eq) were then added to the reaction mixture. The solution was refluxed for 16 h and then evaporated under reduced pressure. The residue was dissolved in EtOAc, washed with $NaHCO_3$ (aqueous saturated solution), Na_2SO_4 (aqueous saturated solution), H_2O, brine and dried over $MgSO_4$. The resulting mixture was evaporated and the residue was purified by Biotage Isolera One.

General procedure E for the synthesis of (*E*)-N^1-(4'-*O*-Aryl-(*L*-Alanine-ester)-phosphinyl-2'-methyl-but-2'-enyl)pyrimidine

To a solution of *O*-Aryl-(*L*-Alanine-ester)-allylphosphonate (1 eq) and N^1-2'-methylallylpyrimidine (2 eq) in dry CH_2Cl_2 (20 ml / mmol allylphosphonate), was added Hoveyda-Grubbs 2nd generation catalyst (15 mol%). The catalyst was added in three equal portions of 5 mol% at t = 0, 2, 4 h over the course of the reaction. The solution was sonicated under an argon atmosphere for 24 h. Volatiles were then evaporated, and the residue was purified by Biotage Isolera One. Also, a reverse phase chromatography was necessary to gain pure final products.

General procedure F for the synthesis of *O*-Aryl-phosphorodichloridate

Under an argon atmosphere, to a solution of the adequate substituted aryl alcohol (1 eq) in anhydrous diethyl ether (8 ml / mmol of aryl alcohol) cooled at - 78°C in a dry-ice/acetone bath, $POCl_3$ (1 eq) was added. After stirring 15 minutes, E_3N (1 eq) was added dropwise over 15 minutes at -78°C. The suspension was stirred for 1h at room temperature. The precipitated triethylammonium chloride salt was filtered off and solid residue washed with diethyl ether. The filtrate was reduced to dryness on a rotary evaporator protected with an argon atmosphere to give the compound as a clear oil.

General procedure G for the synthesis of *O*-Aryl-(isopropyloxy-L-Alanine)-phosphorochloridate

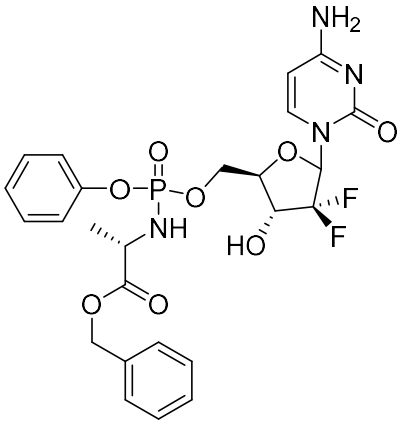
Under an argon atmosphere, *L*-Alanine isopropyl ester hydrochloride salt (1 eq) was dissolved in anhydrous CH_2Cl_2 (15 ml/ mmol of amino acid). To this solution the aryl dichlorophosphate (1 eq) was then added. The mixture was cooled to -78˚C in a dry-ice/acetone bath. Et_3N (2 eq) was added dropwise over 15 minutes and the reaction mixture was stirred at -78˚C for 15 minutes. After this period the suspension was allowed to reach room temperature and stirred for further 2 h. When the reaction was judged completed (^{31}P NMR), the solvent was evaporated under reduced pressure. The resulting triethylammonium chloride salt residue was triturated with anhydrous Et_2O and the filtrate concentrated to give the final product as a clear oil.

General procedure H for the silyl deprotection of ProTides

The 3'-TBDMS protected ProTide (1 eq) was dissolved in anhydrous CH_2Cl_2 (10 ml / mmol ProTide). The solution was cooled at 0˚C in a ice/water bath and TFA (10 ml / mmol ProTide) was then added in a dropwise fashion. The mixture was stirred at rt for 2 h. The solvent was concentrated under reduced pressure, then diluted with $NaHCO_3$ (saturated solution) and extracted with CH_2Cl_2. The organic phases were dried over $MgSO_4$ and evaporated *in vacuo*. The residue was purified by Biotage Isolera One.

5.4 Experimental details

Benzyl ((((2*R*,3*R*)-5-(4-amino-2-oxopyrimidin-1(2H)-yl)-4,4-difluoro-3-hydroxytetrahydrofuran-2-yl)methoxy)(phenoxy)phosphoryl)-*L*-alaninate 8j

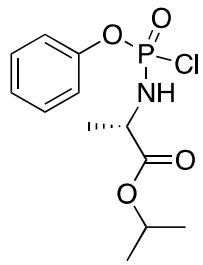


Method 1. Under an argon atmosphere, gemcitabine **1k** (100 mg, 379.9 µmol) was dissolved in pyridine (600 µl) and stirred in the presence of 4 Å molecular sieves (20 mg) for 5 minutes at -10°C in a dry-ice/acetone bath, followed by the addition of diphenylphosphite (145.5 µl, 759.8 µmol). After the consumption of the starting material as monitored by TLC (~1h), a solution of the amino acid alkyl ester hydrochloride **57** (180.2 mg, 853.8 µmol) in pyridine (250 µl) and acetonitrile (2 ml) was added to the reaction mixture at 0°C. After stirring for 5 minutes, CCl_4 (953.0 µl, 9.88 mmol) and Et_3N (344.2 µl, 2.47 mmol) were added to the reaction, mixture at 0°C and then stirred at 30°C for 10 minutes. After the completion of the reaction monitored by TLC, the solvent was evaporated under reduced pressure. The crude product was purified by Biotage Isolera One (10 g SNAP cartridge ULTRA, 36 ml/min, gradient eluent system MeOH/CH_2Cl_2 2% 1CV, 2-20% 12CV, 20% 2CV) to give **8j** as a white foamy solid (25 mg, 11%).

Method 2. Prepared according to the standard procedure **H** using protected ProTide **42** (200 mg, 288.3 µmol) in anhydrous CH_2Cl_2 (3 ml) and TFA (3 ml). After evaporation, the mixture was purified by Biotage Isolera One (25 g SNAP cartridge KP-SIL, 50 ml/min, gradient eluent system MeOH/CH_2Cl_2 2% 1CV, 2-20% 12CV, 20% 2CV) to give **8j** as a white foamy solid (106 mg, 63%). R_f = 0.28 (CH_2Cl_2/MeOH – 9:1). **^{31}P NMR (202 MHz, CD_3OD) δ_P**: 3.82, 3.65. **^{19}F NMR (470 MHz, CD_3OD) δ_F**: -117.88 (d, *J* = 103.9 Hz), -118.37 (d, *J* = 106.0 Hz), -119.71 (bs), -120.24 (bs). **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 7.48 (d, *J* = 7.6 Hz, 0.5H, *H*-6), 7.44 (d, *J* = 7.5 Hz, 0.5H, *H*-6), 7.30-7.23 (m, 7H, Ar*H*), 7.18-7.13 (m, 3H, Ar*H*), 6.20-6.15 (m, 1H, *H*-1'), 5.80 (d, *J* = 7.6 Hz, 0.5H, *H*-5), 5.77 (d, *J* = 7.5 Hz, 0.5H, *H*-5), 5.12-5.04 (m, 2H, *CH_2*Ph), 4.41-4.35 (m, 1H, *H*-3'), 4.21-4.23 (m, 1H, *H*-4'), 4.17-4.09 (m, 1H, *H_a*-5'), 3.99-3.93 (m, 2H, *H_b*-5', *CH*CH_3 L-Ala), 1.92 (d, *J* = 6.9 Hz, 1.5H, CH*CH_3* L-Ala), 1.76 (d, *J* = 6.9 Hz, 1.5H, CH*CH_3* L-Ala). **^{13}C NMR (125 MHz, CD_3OD) δ_C**: 173.4 (d, $^3J_{C\text{-}P}$ = 3.8 Hz, *C*=O, ester), 173.1 (d, $^3J_{C\text{-}P}$ = 3.8 Hz, *C*=O, ester), 166.2 (*C*-4), 156.35 (*C*-2), 156.32 (*C*-2), 150.7 (*C*-O, Ph), 150.6 (*C*-O, Ph), 141.0 (*C*-6), 140.8 (*C*-6), 135.82 (*C*-Ar), 135.8 (*C*-Ar), 129.47

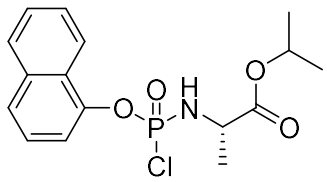
(*CH*-Ar), 129.45 (*CH*-Ar), 129.0 (*CH*-Ar), 128.2 (*CH*-Ar), 127.98 (*CH*-Ar), 127.97 (*CH*-Ar), 127.95 (*CH*-Ar), 127.8 (*CH*-Ar), 126.8 (*CH*-Ar), 126.6 (*CH*-Ar), 124.9 (*CH*-Ar), 122.1 (t, $^{1}J_{C\text{-}F}$ = 256.0 Hz, *C*-2'), 122.0 (t, $^{1}J_{C\text{-}F}$ = 256.1 Hz, *C*-2'), 120.0 (d, $^{3}J_{C\text{-}P}$ = 5.2 Hz, *CH*-Ar), 119.8 (d, $^{3}J_{C\text{-}P}$ = 5.0 Hz, *CH*-Ar), 95.28 (*C*-5), 94.24 (*C*-5), 84.5 (t, $^{2}J_{C\text{-}F}$ = 29.7 Hz, *C*-1'), 78.9 (*C*-4'), 78.8 (*C*-4'), 69.9 (d, $^{2}J_{C\text{-}F}$ = 29.9 Hz, *C*-3'), 69.5 (d, $^{2}J_{C\text{-}F}$ = 29.9 Hz, *C*-3'), 66.6 (*CH*$_2$Ph), 66.3 (*CH*$_2$Ph), 64.3 (d, $^{2}J_{C\text{-}P}$ = 4.9 Hz, *C*-5'), 64.1 (d, $^{2}J_{C\text{-}P}$ = 5.1 Hz, *C*-5'), 50.4 (*CH*CH$_3$ L-Ala), 50.2 (*CH*CH$_3$ L-Ala), 19.0 (d, $^{3}J_{C\text{-}P}$ = 7.6 Hz, CH*CH$_3$* L-Ala), 18.8 (d, $^{3}J_{C\text{-}P}$ = 7.6 Hz, CH*CH$_3$* L-Ala). **(ES+) m/z**, found: 681.5 [M+H$^+$] and 603.5 [M+Na$^+$], $C_{25}H_{27}F_2N_4O_8P$ required: 680.15 [M]. **HPLC**: Reverse phase HPLC eluting with gradient method CH$_3$CN/H$_2$O from 10/90 to 100/0 in 30 minutes, 1ml/min, λ = 254 nm and 263 nm, showed two peaks with t_R 12.11 min and 12.41 min.

Isopropyl (chloro(phenoxy)phosphoryl)-L-alaninate 22



L-Alanine isopropyl ester hydrochloride salt **56** (2g, 11.93 mmol) was dissolved in anhydrous CH_2Cl_2 (100 ml) under an argon atmosphere. To this solution the aryl dichlorophosphate (1.78 ml, 11.93 mmol) was then added. The mixture was cooled to -78˚C in a dry-ice/acetone bath. Et_3N (3.33 ml, 23.86 mmol) was added dropwise over 15 minutes and the reaction mixture was stirred at -78˚C for 15 minutes. After this period the suspension was allowed to reach room temperature and stirred for further 1.5 h. When the reaction was judged completed (^{31}P NMR), the solvent was evaporated under reduced pressure. The resulting white residue was triturated with anhydrous Et_2O and the filtrate concentrated to give **22** as a clear oil (3.3 g, 91%). **^{31}P NMR (202 MHz, CDCl$_3$) δ$_P$**: 8.49, 8.25; **^{1}H NMR (500 MHz, CDCl$_3$) δ$_H$**: 7.30-7.03 (m, 5H, Ar*H*), 4.98 (septet, *J* = 6.1 Hz, 0.5H, *CH*(CH$_3$)$_2$), 4.89 (septet, *J* = 6.1 Hz, 0.5H, *CH*(CH$_3$)$_2$), 4.02 (q, *J* = 7.1 Hz, 1H, *CH*CH$_3$ L-Ala), 1.51 (d, *J* = 7.1 Hz, 1.5H, CH*CH$_3$* L-Ala), 1.50 (d, *J* = 7.1 Hz, 1.5H, CH*CH$_3$* L-Ala), 1.26-1.30 (m, 6H, CH(*CH$_3$*)$_2$).

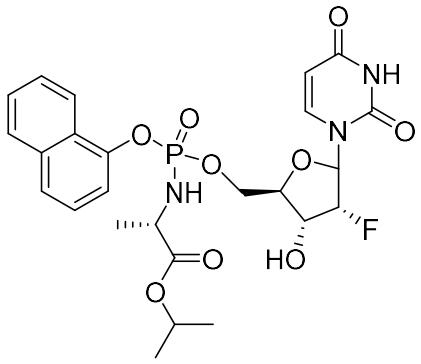
Isopropyl (chloro(naphthalen-1-yloxy)phosphoryl)-*L*-alaninate 37



Prepared according to procedure **G** using *L*-Alanine isopropyl ester hydrochloride salt **56** (5.3 g, 20.30 mmol) in anhydrous CH_2Cl_2 (200 ml), the dichlorophosphate **78** (3.4 g, 20.30 mmol), Et_3N (5.66 ml, 40.61 mmol). After evaporation, compound **37** was obtained as a clear oil (7.2 g, quant.). **^{31}P NMR (202 MHz, CDCl$_3$)**

δ_P: 8.37, 8.09.**^{1}H-NMR (CDCl$_3$, 500 MHz) δ_H**: 8.02-7.98 (m, 1H, Ar*H*), 7.79-7.77 (m, 1H, Ar*H*), 7.65-7.63 (m, 1H, Ar*H*), 7.54-7.44 (m, 3H, Ar*H*), 7.36-7.33 (m, 1H, Ar*H*), 5.06-4.95 (m, 1H, *CH*(CH$_3$)$_2$), 4.54-4.44 (m, 1H, *NH* L-Ala), 4.21-4.11 (m, 1H, *CH*CH$_3$ L-Ala), 1.46 (d, *J* = 7.4 Hz, 1.5H, CH*CH$_3$* L-Ala), 1.44 (d, *J* = 7.8 Hz, 1.5H, CH*CH$_3$* L-Ala), 1.22-1.11 (m, 6H, CH(*CH$_3$*)$_2$).

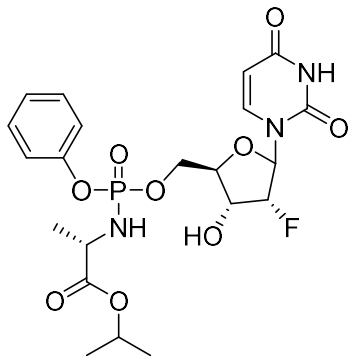
Isopropyl ((((2*R*,3*R*,4*R*)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-fluoro-3-hydroxytetrahydrofuran-2-yl)methoxy)(naphthalen-1-yloxy)phosphoryl)-*L*-alaninate 38



Prepared according to the standard procedure **H** using protected ProTide **85** (170 mg, 255.4 μmol) in anhydrous CH_2Cl_2 (4 ml) and TFA (4 ml). After evaporation, the mixture was purified by Biotage Isolera One (10 g SNAP cartridge ULTRA, 36 ml/min, gradient eluent system MeOH/CH_2Cl_2 2% 1CV, 2-20% 12CV, 20% 2CV) to give **38** as a white foamy solid (123 mg, 85%). R_f= 0.23 (CH_2Cl_2/MeOH – 9:1). **^{31}P NMR (202 MHz, CDCl$_3$) δ_P**: 3.54, 3.38. **^{19}F NMR (470 MHz, CDCl$_3$) δ_F**: -202.68, -205.04. **^{1}H NMR (500 MHz, CDCl$_3$) δ_H**: 9.90 (bs, 0.6H, *NH*), 9.85 (bs, 0.4H, *NH*), 8.00-7.98 (m, 1H, Ar*H*), 8.73-7.70 (m, 1H, Ar*H*), 7.54-7.52 (m, 1H, Ar*H*), 7.45-7.37 (m, 3H, Ar*H*), 7.029-7.24 (m, 1.6H, Ar*H*, *H*-6), 7.10 (d, *J* = 8.1 Hz, 0.4H, *H*-6), 5.80-5.72 (m, 1H, *H*-1'), 5.42 (d, *J* = 8.1 Hz, 0.4H, *H*-5), 5.29 (d, *J* = 8.1 Hz, 0.6H, *H*-5), 4.85-4.73 (m, 2H, *H*-2', *CH*(CH$_3$)$_2$), 4.67-4.61 (m , 1H, *NH* L-Ala), 4.45-4.06 (m, 3H, *H*-5', *H*-3'), 4.10 (bs, 1H, *H*-4'), 3.95-3.80 (m, 1H, *CH*CH$_3$ L-Ala), 1.23 (d, *J* = 7.0 Hz, 3H, CH*CH$_3$* L-Ala), 1.08 (d, *J* = 6.3 Hz, 1.8H, CH(*CH$_3$*)$_2$), 1.06 (d, *J* = 6.2 Hz, 1.8H, CH(*CH$_3$*)$_2$), 1.03 (d, *J* = 6.4 Hz, 1.2H, CH(*CH$_3$*)$_2$), 1.01 (d, *J* = 6.4 Hz, 1.2H, CH(*CH$_3$*)$_2$). **^{13}C NMR (125 MHz, CDCl$_3$) δ_C**: 173.1 (d, $^3J_{C\text{-}P}$ = 6.5 Hz, *C*=O, ester), 173.0 (d, $^3J_{C\text{-}P}$ = 6.7 Hz, *C*=O, ester), 163.5 (*C*-4), 163.4 (*C*-4), 150.2 (*C*-2), 150.1 (*C*-2), 146.4 (d, $^2J_{C\text{-}P}$ = 7.1 Hz, *C*-O, Ph), 146.3 (d, $^2J_{C\text{-}P}$ = 7.1 Hz, *C*-O, Ph), 140.5 (*C*-6), 139.2 (*C*-6), 134.7 (*C*-Ar), 127.98 (*CH*-Ar), 127.94 (*CH*-Ar), 126.8 (*CH*-Ar), 126.7 (*CH*-Ar), 126.6 (*CH*-Ar), 126.5 (*CH*-Ar), 126.17 (d, $^3J_{C\text{-}P}$ = 6.8 Hz *C*-Ar), 126.12 (d, $^3J_{C\text{-}P}$ = 6.2 Hz *C*-Ar), 125.5 (*CH*-Ar), 125.0 (*CH*-Ar), 124.9 (*CH*-Ar), 121.1 (*CH*-Ar), 114.9 (d, $^3J_{C\text{-}P}$ = 2.6 Hz, *CH*-Ar), 114.8 (d, $^3J_{C\text{-}P}$ = 2.6 Hz, *CH*-Ar), 102.5 (*C*-5), 93.3 (d, $^1J_{C\text{-}F}$ = 190.3 Hz, *C*-2'), 92.5 (d, $^1J_{C\text{-}F}$ = 191.7 Hz, *C*-2'), 89.2 (d, $^2J_{C\text{-}F}$ = 35.3 Hz, *C*-1'), 88.4 (d, $^2J_{C\text{-}F}$ = 34.9 Hz, *C*-1'), 81.2 (d, $^3J_{C\text{-}P}$ = 6.6 Hz, *C*-4'), 81.0

(d, $^{3}J_{C\text{-}P}$ = 6.2 Hz, *C*-4'), 69.54 (*CH*(CH_3)$_2$), 69.50 (*CH*(CH_3)$_2$), 68.3 (d, $^{2}J_{C\text{-}F}$ = 14.1 Hz, *C*-3'), 68.1 (d, $^{2}J_{C\text{-}F}$ = 13.8 Hz, *C*-3'), 65.1 (d, $^{2}J_{C\text{-}P}$ = 4.3 Hz, *C*-5'), 64.7 (d, $^{2}J_{C\text{-}P}$ = 4.2 Hz, *C*-5'), 50.53 (*CH*CH_3 L-Ala), 50.50 (d, $^{2}J_{C\text{-}P}$ = 1.4 Hz, *CH*CH_3 L-Ala), 21.7 (CH(*CH_3*)$_2$), 21.5 (CH(*CH_3*)$_2$), 20.9 (d, $^{3}J_{C\text{-}P}$ = 3.6 Hz, CH*CH_3* L-Ala), 20.7 (d, $^{3}J_{C\text{-}P}$ = 3.6 Hz, CH*CH_3* L-Ala). **(ES+) m/z**, found: 566.5 [M+H^+], $C_{25}H_{29}FN_3O_9P$ required: 565.16 [M]. **HPLC**: Reverse phase HPLC eluting with gradient method MeOH/H_2O from 10/90 to 100/0 in 45 minutes, 1ml/min, λ = 254 nm and 263 nm, showed two peaks with t_R 24.56 min and 26.84 min.

Isopropyl ((((2*R*,3*R*,4*R*)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-fluoro-3-hydroxytetrahydrofuran-2-yl)methoxy)(phenoxy)phosphoryl)-*L*-alaninate 39

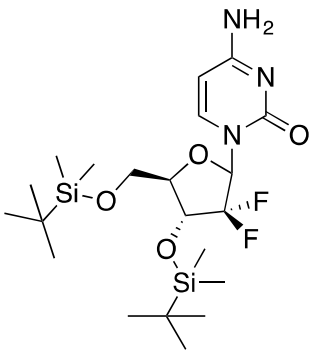


Method 1. The 3'-TBDMS protected ProTide **59** (170 mg, 276.1 μmol) was dissolved in anhydrous CH_2Cl_2 (2 ml) under an argon atmosphere. The solution ws cooled at 0˚C in a ice/water bath and TFA (2 ml) was then added in a dropwise fashion. The mixture was stirred at rt for 2 h. The solvent was concentrated under reduced pressure, then diluted with $NaHCO_3$ (saturated solution) and extracted with EtOAc (3 x 20 ml). The organic phases were dried over $MgSO_4$ and evaporated *in vacuo*. The residue was purified by Biotage Isolera One (10 g SNAP cartridge ULTRA, 36 ml/min, gradient eluent system MeOH/CH_2Cl_2 2% 1CV, 2-20% 12CV, 20% 2CV) to give **39** as a white solid (121 mg, 85%). R_f = 0.44 (CH_2Cl_2/MeOH – 95:0.5).

Method 2. Under an argon atmosphere, the nucleoside **36** (100 mg, 406.1 μmol) was dissolved in pyridine (600 μl) and stirred in presence of 4 Å molecular sieves (20 mg) for 5 minutes at -10℃ in a dry-ice/acetone bath, followed by the addition of diphenylphosphite (156.2 μl, 812.3 μmol). After the consumption of the starting material as monitored by TLC (~1h), a solution of the amino acid alkyl ester hydrochloride **56** (150.0 mg, 893.6 μmol) in pyridine (250 μl) and acetonitrile (2 ml) was added to the reaction mixture at 0℃. After stirring 5 minutes, CCl_4 (1.02 ml, 10.56 mmol) and Et_3N (368.1 μl, 2.64 mmol) were added to the reaction mixture at 0℃ and then stirred at 30℃ for 10 minutes. After the completion of the reaction monitored by TLC, the solvent was evaporated under reduced pressure. The crude product was purified by Biotage Isolera One (10 g SNAP cartridge ULTRA, 36 ml/min, gradient eluent system MeOH/CH_2Cl_2 2% 1CV, 2-20% 12CV, 20% 2CV) to give **39** as a white foamy solid (22 mg, 12%). **^{31}P**

NMR (202 MHz, $CDCl_3$) δ_P: 3.56, 3.40. **^{19}F NMR (470 MHz, $CDCl_3$) δ_F**: -202.58, -203.54. **^{1}H NMR (500 MHz, $CDCl_3$) δ_H**: 9.90 (bs, 1H, *NH*), 7.35-7.29 (m, 5.4H, Ar*H*, *H*-6), 5.79-5.73 (m, 1H, *H*-1'), 5.40 (d, J = 8.1 Hz, 0.4H, *H*-5), 5.27 (d, J = 8.1 Hz, 0.6H, *H*-5), 4.88-4.74 (m, 2H, *H*-2', *CH*$(CH_3)_2$), 4.66-4.62 (m , 1H, *NH* L-Ala), 4.46-4.08 (m, 3H, *H*-5', *H*-3'), 4.09 (bs, 1H, *H*-4'), 3.95-3.80 (m, 1H, *CH*CH_3 L-Ala), 1.21 (d, J = 7.1 Hz, 3H, CH*CH_3* L-Ala), 1.09 (d, J = 6.3 Hz, 1.8H, CH(*CH_3*)$_2$), 1.07 (d, J = 6.2 Hz, 1.8H, CH(*CH_3*)$_2$), 1.05 (d, J = 6.4 Hz, 1.2H, CH(*CH_3*)$_2$), 1.03 (d, J = 6.4 Hz, 1.2H, CH(*CH_3*)$_2$). **^{13}C NMR (125 MHz, $CDCl_3$) δ_C**: 173.2 (d, $^3J_{C\text{-}P}$ = 6.4 Hz, *C*=O, ester), 173.1 (d, $^3J_{C\text{-}P}$ = 6.8 Hz, *C*=O, ester), 163.6 (*C*-4), 163.5 (*C*-4), 150.2 (*C*-2), 150.1 (*C*-2), 146.34 (d, $^2J_{C\text{-}P}$ = 7.0 Hz, *C*-O, Ph), 146.32 (d, $^2J_{C\text{-}P}$ = 7.0 Hz, *C*-O, Ph), 140.6 (*C*-6), 139.0 (*C*-6), 129.7 (*CH*-Ar), 124.8 (*CH*-Ar), 124.9 (*CH*-Ar), 120.2 (d, $^3J_{C\text{-}P}$ = 4.6 Hz, *CH*-Ar), 119.9 (d, $^3J_{C\text{-}P}$ = 4.6 Hz, *CH*-Ar), 102.6 (*C*-5), 93.1 (d, $^1J_{C\text{-}F}$ = 189.3 Hz, *C*-2'), 92.4 (d, $^1J_{C\text{-}F}$ = 188.7 Hz, *C*-2'), 89.1 (d, $^2J_{C\text{-}F}$ = 35.1 Hz, *C*-1'), 88.5 (d, $^2J_{C\text{-}F}$ = 34.8 Hz, *C*-1'), 81.1 (d, $^3J_{C\text{-}P}$ = 6.7 Hz, *C*-4'), 81.0 (d, $^3J_{C\text{-}P}$ = 6.3 Hz, *C*-4'), 69.55 (*CH*$(CH_3)_2$), 69.51 (*CH*$(CH_3)_2$), 68.3 (d, $^2J_{C\text{-}F}$ = 14.0 Hz, *C*-3'), 68.2 (d, $^2J_{C\text{-}F}$ = 13.7 Hz, *C*-3'), 65.1 (d, $^2J_{C\text{-}P}$ = 4.6 Hz, *C*-5'), 64.8 (d, $^2J_{C\text{-}P}$ = 4.2 Hz, *C*-5'), 50.5 (*CH*CH_3 L-Ala), 50.4 (d, $^2J_{C\text{-}P}$ = 1.2 Hz, *CH*CH_3 L-Ala), 21.6 (CH(*CH_3*)$_2$), 21.5 (CH(*CH_3*)$_2$), 20.8 (d, $^3J_{C\text{-}P}$ = 3.6 Hz, CH*CH_3* L-Ala), 20.7 (d, $^3J_{C\text{-}P}$ = 3.5 Hz, CH*CH_3* L-Ala). **(ES+) m/z**, found: 516.7 [M+H$^+$], $C_{21}H_{27}FN_3O_9P$ required: 515.15 [M]. **HPLC**: Reverse phase HPLC eluting with gradient method MeOH/H_2O from 10/90 to 100/0 in 45 minutes, 1ml/min, λ = 254 nm and 263 nm, showed two peaks with t_R 20.77 min and 22.022 min.

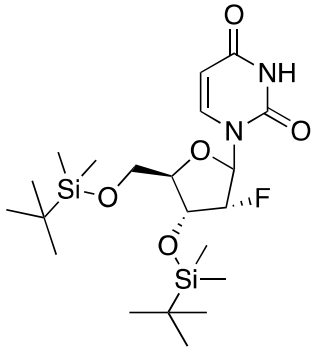
4-Amino-1-((4*R*,5*R*)-4-((*tert*-butyldimethylsilyl)oxy)-5-(((*tert*-butyldimethylsilyl)oxy)methyl)-3,3-difluorotetrahydrofuran-2-yl)pyrimidin-2(1H)-one 40[1]



Gemcitbine **1k** (5 g, 19.00 mmol) was dissolved in anhydrous DMF (40 ml) under an argon atmosphere and TBDMSCl (6.30 g, 41.79 mmol), DMAP (696.26 mg, 5.70 mmol) and imidazole (6.47 g, 94.98 mmol) were then added. The solution was stirred at 50˚C overnight. The solvent was evaporated *in vacuo* and the residue was dissolved in water (50 ml) and extract with EtOAc (3 x 20 ml). The organic phase was washed with NH_4Cl (aqueous saturated solution, 3 x 10 ml), dried over $MgSO_4$ and concentrated under reduced pressure to give **40** as a white solid (8.2 g, 88%). R_f = 0.33 (CH_2Cl_2/MeOH

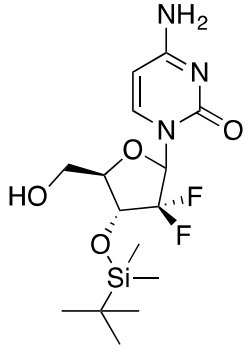
- 9:1). **^{1}H NMR (500 MHz, $CDCl_3$) δ_H**: 7.49 (d, *J* = 7.5 Hz, 1H, *H*-6), 6.18 (t, $^3J_{H\text{-}F}$ = 7.8 Hz, 2H, *H*-1'), 5.76 (d, *J* = 7.5 Hz, 1H, *H*-5), 4.15-4.19 (m, 1H, *H*-3'), 3.86 (d, *J* = 11.4 Hz, 1H, H_a-5'), 3.75 (d, *J* = 8.2 Hz, 1H, *H*-4'), 3.68 (dd, *J* = 11.4, *J* = 2.0, 1H, H_b-5'), 0.82 (s, 9H, *t*Bu), 0.79 (s, 9H, *t*Bu), 0.02 (s, 3H, CH_3), 0.00 (s, 6H, 2 x CH_3), -0.02(s, 3H, CH_3).

1-((3*R*,4*R*,5*R*)-4-((*tert*-Butyldimethylsilyl)oxy)-5-(((*tert*-butyldimethylsilyl)oxy)methyl)-3-fluorotetrahydrofuran-2-yl)pyrimidine-2,4(1H,3H)-dione 41[2]



2'-deoxy-2'-fluorouridine **36** (3 g, 12.19 mmol) was dissolved in anhydrous DMF (25 ml) under an argon atmosphere and TBDMSCl (4.6 g, 30.46 mmol), DMAP (446.6 mg, 3.66 mmol) and imidazole (4.15 g, 60.93 mmol) were then added. The solution was stirred at 50˚C overnight. The solvent was evaporated *in vacuo* and the residue was dissolved in water (50 ml) and extract with EtOAc (3 x 20 ml). The organic phase was washed with NH_4Cl (aqueous saturated solution, 3 x 20 ml), dried over $MgSO_4$ and concentrated under reduced pressure to give **41** as a white solid (5.7 g, 98%). R_f = 0.75 (CH_2Cl_2/MeOH - 9:1). **^{1}H NMR (500 MHz, $CDCl_3$) δ_H**: 7.79 (d, *J* = 8.1 Hz, 1H, *H*-6), 5.94 (dd, $^3J_{H\text{-}F}$ = 15.3 Hz, *J* = 1.7 Hz, 1H, *H*-1'), 5.57 (d, *J* = 8.1 Hz, 1H, *H*-5), 4.65 (ddd, $^2J_{H\text{-}F}$ = 52.4, *J* = 4.3 Hz, *J* = 1.6 Hz, 1H, *H*-2'), 4.16 (ddd, $^3J_{H\text{-}F}$ = 19.0 Hz, *J* =7.2 Hz, *J* = 4.2 Hz, 1H, *H*-3'), 3.95 (dd, *J* = 7.2 Hz, *J* = 1.8Hz, 1H, H-4'), 3.93 (dd, *J* = 11.9 Hz, *J* = 1.8 Hz, 1H, *H*-5$_a$'), 3.65 (dd, *J* = 11.9 Hz, *J* = 1.8 Hz, 1H, *H*-5$_b$'), 0.80 (s, 9H, *t*Bu), 0.78 (s, 9H, *t*Bu), -0.00 (s, 6H, CH_3), -0.01 (s, 3H, CH_3), -0.02 (s, 3H, CH_3).

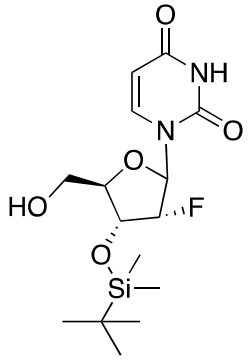
4-Amino-1-((4*R*,5*R*)-4-((*tert*-butyldimethylsilyl)oxy)-3,3-difluoro-5-(hydroxymethyl)tetrahydrofuran-2-yl)pyrimidin-2(1H)-one 42[1]



3',5'-(Bis-*O*-*tert*-butyldimethylsilyl)-gemcitabine **40** (3 g, 6.10 mmol) was dissolved in anhydrous THF (30 ml) under an argon atmosphere and the solution was cooled at 0˚C in an ice/water bath. A solution of trichloroacetic acid (16 g, 97.86 mmol) in H_2O (9 ml) was added to the flask and allowed to stirr for 3 h at 0˚C. The solution was carefully neutralized by addition of $NaHCO_3$ (aqueous saturated solution) and then extracted with CH_2Cl_2 (3 x 20 ml). The

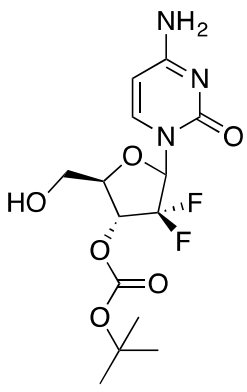
organic phases were combined, dried over $MgSO_4$ and concentrated under reduced pressure to give the product as a withe solid (2.10 g, 91%). R_f = 0.10 (CH_2Cl_2/MeOH - 9:1). **^{1}H NMR (500 MHz, MeOD) δ_H**: 7.78 (d, *J* = 7.6 Hz, 1H, *H*-6), 6.16 (t, $^3J_{H\text{-}F}$ = 8.1 Hz, 1H, *H*-1'), 5.87 (d, *J* = 7.6 Hz, 1H, *H*-5), 4.32.4.36 (m, 1H, *H*-3'), 3.89 (d, *J* = 12.8 Hz, 1H, H_a-5'), 3.83 (dt, *J* = 8.0 Hz, *J* = 2.9 Hz, 1H, *H*-4'), 3.68 (dd, *J* = 12.8 Hz, *J* = 2.9 Hz, 1H, H_b-5'), 0.88 (s, 9H, *t*Bu), 0.10 (s, 3H, CH_3), 0.09 (s, 3H, CH_3).

1-((3*R*,4*R*,5*R*)-4-((*tert*-Butyldimethylsilyl)oxy)-3-fluoro-5-(hydroxymethyl)tetrahydrofuran-2-yl)pyrimidine-2,4(1H,3H)-dione 43[2]



2'-deoxy-2'-fluoro-3',5'-(Bis-*O*-*tert*-butyldimethylsilyl)-uridine **41** (3 g, 6.32 mmol) was dissolved in anhydrous THF (30 ml) under an argon atmosphere and the solution was cooled at 0°C in an ice/water bath. A solution of trichloroacetic acid (16.52 g, 101.11 mmol) in H_2O (9.2 ml) was added to the flask and allowed to stirr for 6 h at 0°C. The solution was carefully neutralized by addition of $NaHCO_3$ (aqueous saturated solution) and then extracted with CH_2Cl_2 (3 x 20 ml). The organic phases were combined, dried over $MgSO_4$ and concentrated under reduced pressure to give the product **43** as a withe solid (2.0 g, 88%). R_f = 0.56 (CH_2Cl_2/MeOH - 9:1). **^{1}H NMR (500 MHz, $CDCl_3$) δ_H**: 9.07 (bs, 1H, *NH*), 7.50 (d, *J* = 7.8 Hz, 1H, *H*-6), 5.69-5.64 (m, 2H, *H*-1', *H*-5), 5.61 (d, $^2J_{H\text{-}F}$ = 53.5 Hz, 1H, H-2'), 4.38-4.36 (m, 1H, *H*-3'), 4.01-3.96 (m, 1H, *H*-4'), 3.89 (d, *J* = 11.4 Hz, 1H, H_a-5'), 3.65 (d, *J* = 11.4 Hz, 1H, H_b-5'), 0.81 (s, 9H, *t*Bu), 0.02 (s, 3H, CH_3), -0.00 (s, 3H, CH_3).

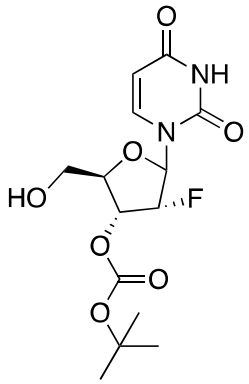
(2*R*,3*R*)-5-(4-Amino-2-oxopyrimidin-1(2H)-yl)-4,4-difluoro-2-(hydroxymethyl)tetrahydrofuran-3-yl *tert*-butyl carbonate 44[3]



To a solution of Gemcitabine **1k** (2 g, 7.60 mmol) and Na_2CO_3 (4.28 g, 38 mmol) in Dioxane / H_2O (40 ml, 4:1, v/v) was added $(Boc)_2O$ (1.66 g, 7.60 mmol) in one portion. After stirring the mixture for 16 h, the solution was concentrated under reduced pressure, diluted with water (50 ml) and extracted with EtOAc (3 x 20 ml). The organic phases were combined, dried over $MgSO_4$ and concentrated under *vacuum*. The residue was purified by Biotage Isolera One (50 g SNAP cartridge ULTRA, 100 ml/min, gradient eluent system MeOH/CH_2Cl_2 2% 1CV, 2-20% 12CV, 20% 2CV), yielding the title

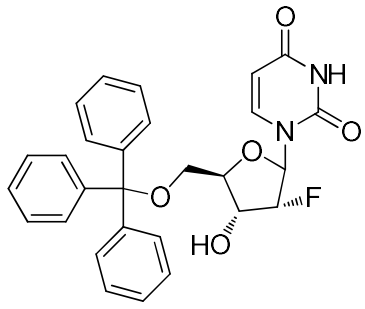
compound as a white solid (1.16 g, 42%). R_f = 0.64 (CH_2Cl_2/MeOH - 8:2). **^{1}H NMR (500 MHz, DMSO) δ_H**: 7.65 (d, *J* = 7.5 Hz, 1H, *H*-6), 7.45 (bs, 2H, *NH*$_2$), 6.22 (t, $^3J_{H\text{-}F}$ = 9.1 Hz, 1H, *H*-1'), 5.82 (d, *J* = 7.5 Hz, 1H, *H*-5), 5.27 (t, 1H, *J* = 5.5 Hz, *OH*-5'), 5.20-5.15 (m, 1H, *H*-3'), 4.15 (dt, *J* = 7.0 Hz, *J* = 3.3 Hz, 1H, *H*-4'), 3.76-3.74 (m, 1H, H_a-5'), 3.67-3.61 (m, 1H, H_b-5'), 1.46 (s, 9H, *t*Bu).

tert-Butyl ((2*R*,3*R*,4*R*)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-fluoro-2-(hydroxymethyl)tetrahydrofuran-3-yl) carbonate 45[3]



To a solution of the nucleoside **36** (2 g, 8.12 mmol) and Na_2CO_3 (4.3 g, 40.6 mmol) in Dioxane / H_2O (40 ml, 4:1, v/v) was added $(Boc)_2O$ (1.77 g, 8.12 mmol). After stirring 16 h, the solution was concentrated under reduced pressure, diluted with H_2O (50 ml) and extracted with EtOAc (3 x 20 ml). The organic phases were combined, dried over $MgSO_4$ and concentrated under *vacuum*. The residue was purified by Biotage Isolera One (120 g ZIP cartridge KP-SIL, 100 ml/min, gradient eluent system MeOH/CH_2Cl_2 2% 1CV, 2-20% 12CV, 20% 2CV), yielding the title compound as a white solid (1.10 g, 40%). R_f = 0.55 (CH_2Cl_2/MeOH - 9:1). **^{1}H NMR (500 MHz, DMSO) δ_H**: 11.46 (bs, 1H, *NH*), 7.86 (d, *J* = 8.0 Hz, 1H, *H*-6), 5.96 (dd, $^3J_{H\text{-}F}$ = 18.4 Hz, *J* = 3.3 Hz, 1H, *H*-1'), 5.69 (d, *J* = 8.0 Hz, 1H, *H*-5), 5.44 (ddd, $^2J_{H\text{-}F}$ = 52.6 Hz, *J* = 5.2 Hz, *J* = 3.3 Hz, 1H, *H*-2'), 5.29 (bs, 1H, *OH*-5'), 5.10 (ddd, $^3J_{H\text{-}F}$ = 13.2 Hz, *J* = 6.4 Hz, *J* = 5.2 Hz, 1H, *H*-3'), 4.14-4.12 (m, 1H, *H*-4'), 3.69 (d, *J* = 12.2 Hz, 1H, H_a-5'), 3.60 (d, *J* = 12.2 Hz, 1H, H_b-5'), 1.45 (s, 9H, *t*Bu).

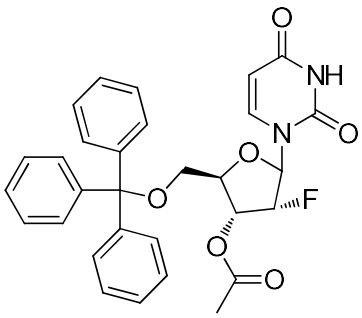
1-((3*R*,4*R*,5*R*)-3-Fluoro-4-hydroxy-5-((trityloxy)methyl)tetrahydrofuran-2-yl)pyrimidine-2,4(1H,3H)-dione 46[4]



The nucleoside **36** (250 mg, 1.02 mmol) was dissolved in anhydrous pyridine (15 ml) under an argon atmosphere. Trityl chloride (990 mg, 3.55 mmol) and DMAP (99.25 mg, 812.37 µmol) were then added. The solution was stirred at 85˚C for 48 h. The solvent was evaporated *in vacuo* and purified by Biotage Isolera One (120 g ZIP cartridge KP-SIL, 100 ml/min, gradient eluent system MeOH/CH_2Cl_2 1% 1CV, 1-10% 10CV, 10% 2CV) to give **46** as a white solid (380 mg, 77%). R_f = 0.37 (CH_2Cl_2/MeOH –

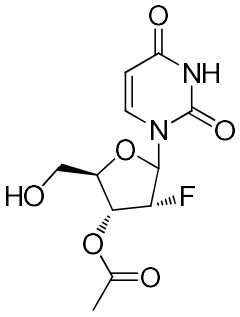
9.5:0.5). **^{1}H NMR (500 MHz, DMSO) δ_H**: 11.44 (bs, 1H, *NH*), 7.78 (d, *J* = 8.0 Hz, 1H, *H*-6), 7.41 (d, *J* = 7.6 Hz, 6H, Ar*H*), 7.35 (t, *J* = 7.8 Hz, 6H, Ar*H*), 7.29 (t, *J* = 7.2 Hz, 3H, Ar*H*), 5.91 (d, $^3J_{H\text{-}F}$ = 19.7 Hz, 1H, *H*-1'), 5.70 (d, *J* = 7.0 Hz, 1H, *OH*-3'), 5.32 (d, *J* = 8.0 Hz, 1H, *H*-5), 5.14 (dd, $^2J_{H\text{-}F}$ = 53.2 Hz, *J* = 4.1 Hz, 1H, *H*-2'), 4.43-4.34 (m, 1H, *H*-3'), 4.05-4.02 (m, 1H, *H*-4'), 3.36-3.30 (m, 2H, *H*-5').

(2*R*,3*R*,4*R*)-5-(2,4-Dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-fluoro-2-((trityloxy)methyl)tetrahydrofuran-3-yl acetate 47[4]



2'-deoxy-2'-fluoro-5'-(*O*-trityl)-uridine **46** (360 mg, 716.4 µmol) was dissolved in anhydrous pyridine (20 ml) under an argon atmosphere and acetic anhydride (216.8 µl, 2.29 mmol) was then added. The solution was stirred at rt for 12 h. The solvent was evaporated *in vacuo* and co-evaporated with toluene and MeOH to give **47** as a white solid (360 mg, 94%). R_f = 0.56 (AcOEt/Hexane - 6:4). **^{1}H NMR (500 MHz, DMSO) δ_H**: 11.47 (bs, 1H, *NH*), 7.78 (d, *J* = 7.9 Hz, 1H, *H*-6), 7.39-7.33 (m, 12H, Ar*H*), 7.28 (t, *J* = 6.9 Hz, 3H, Ar*H*), 5.88 (dd, $^3J_{H\text{-}F}$ = 22.6 Hz, *J* = 1.7 Hz, 1H, *H*-1'), 5.54 (ddd, $^2J_{H\text{-}F}$= 52.9 Hz, *J* = 5.1 Hz, *J* = 1.7 Hz, 1H, *H*-2'), 5.53 (d, *J* = 7.9 Hz, 1H, *H*-5), 5.33 (ddd, $^3J_{H\text{-}F}$ = 18.3 Hz, *J* = 7.9 Hz, *J* = 5.1 Hz, 1H, *H*-3'), 4.20 (dt, *J* = 7.9 Hz, *J* = 3.8 Hz, 1H, H-4'), 3.33-3.31 (m, 2H, *H*-5'), 2.07 (s, 3H, CH_3).

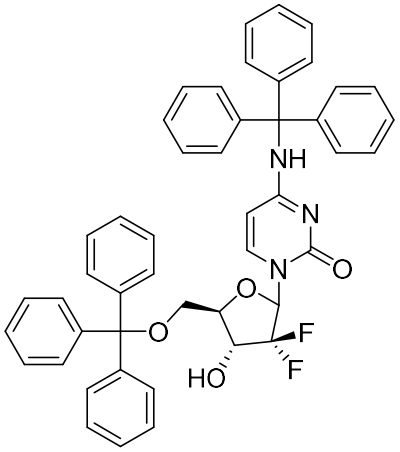
(2*R*,3*R*,4*R*)-5-(2,4-Dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-fluoro-2-(hydroxymethyl)tetrahydrofuran-3-yl acetate 48[4]



2'-deoxy-2'-fluoro-3'-(*O*-acetyl)-5'-(*O*-trityl)-uridine **47** (857 mg, 1.62 mmol) was dissolved in anhydrous CH_2Cl_2 (15 ml) under an argon atmosphere. The solution was cooled at 0˚C in a ice/water bath and TFA (15ml) was added in a dropwise fashion. The mixture was stirred at rt for 24 h. The solvent was concentrated under reduced pressure, then diluted with $NaHCO_3$ (aqueous saturated solution) and extracted with EtOAc (3 x 10 ml). The organic phases were dried over $MgSO_4$ and evaporated *in vacuo*. The residue was purified by Biotage Isolera One (100 g SNAP cartridge KP-SIL, 100 ml/min, gradient eluent system MeOH/CH_2Cl_2 2% 1CV, 2-20% 10CV, 20% 2CV) to give **48** as a white solid (850 mg, 98%). R_f = 0.39 (CH_2Cl_2/MeOH – 9:1). **^{1}H NMR (500 MHz, DMSO) δ_H**: 11.45 (bs, 1H, *NH*), 7.87 (d, *J*

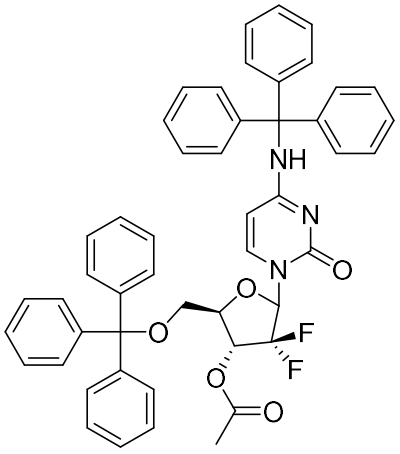
= 8.2 Hz, 1H, *H*-6), 5.96 (dd, $^{3}J_{H\text{-}F}$ = 18.8 Hz, *J* = 3.0 Hz, 1H, *H*-1'), 5.69 (d, *J* = 8.2 Hz, 1H, *H*-5), 5.41 (ddd, $^{2}J_{H\text{-}F}$= 52.2 Hz, *J* = 5.3 Hz, *J* = 3.0 Hz, 1H, *H*-2'), 5.27 (t, *J* = 4.9 Hz, 1H, *OH*-5'), 5.21 (ddd, $^{3}J_{H\text{-}F}$ = 14.5 Hz, *J* = 6.8 Hz, *J* = 5.3 Hz, 1H, *H*-3'), 4.14 (dt, *J* = 6.8 Hz, *J* = 3.0 Hz, 1H, H-4'), 3.70 (dt, *J* = 11.8 Hz, *J* = 4.9 Hz, 1H, H-5_a'), 3.59 (dt, *J* = 11.8 Hz, *J* = 4.9 Hz, 1H, H-5_b'), 2.12 (s, 3H, CH_3).

1-((4*R*,5*R*)-3,3-difluoro-4-hydroxy-5-((trityloxy)methyl)tetrahydrofuran-2-yl)-4-(tritylamino)pyrimidin-2(1H)-one 49[4]



Gemcitabine **1k** (250 mg, 949.8 µmol) was dissolved in anhydrous pyridine (15 ml) under an argon atmosphere and trityl chloride (926.8 mg, 3.32 mmol) and DMAP (92.83 mg, 759.8 µmol) were then added. The solution was stirred at 85°C for 48 h. The solvent was evaporated *in vacuo* and purified by Biotage Isolera One (25 g SNAP cartridge ULTRA, 100 ml/min, gradient eluent system MeOH/CH_2Cl_2 2% 1CV, 2-20% 10CV, 20% 2CV) to give **49** as a yellow solid (528 mg, 74%). R_f = 0.67 (CH_2Cl_2/MeOH - 9:1). **^{1}H NMR (500 MHz, DMSO) δ_H**: 8.71 (bs, 1H, *NH*), 7.58 (d, *J* = 7.4 Hz, 1H, *H*-6), 7.41-7.17 (m, 30H, Ar*H*), 6.30-6.27 (m, 2H, *H*-5, *OH*-3'), 6.07 (t, $^{3}J_{H\text{-}F}$ = 7.8 Hz, 1H, *H*-1'), 4.27-4.24 (m, 1H, *H*-3'), 3.97-3.94 (m, 1H, *H*-4'), 3.48-3.46 (m, 1H, *H*-5'), 3.15-3.11 (m, 1H, *H*-5').

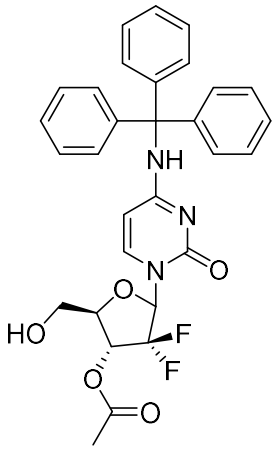
(2*R*,3*R*)-4,4-Difluoro-5-(2-oxo-4-(tritylamino)pyrimidin-1(2H)-yl)-2-((trityloxy)methyl)tetrahydrofuran-3-yl acetate 50[4]



The bis-trityl nucleoside **49** (500 mg, 668.6 µmol) was suspended in anhydrous actonitrile (25 ml) under an argon atmosphere and acetic anhydride (75.8 µl, 802.3 µmol), DMAP (4 mg, 33.4 µmol) and Et_3N (116.6 µl, 835.7 µmol) were then added. The solution was stirred at rt for 16 h. The solvent was evaporated *in vacuo* and co-evaporated with toluene and MeOH and purified by Biotage Isolera One (25 g SNAP cartridge KP-SIL, 50 ml/min, gradient eluent system MeOH/CH_2Cl_2 2% 1CV, 2-20% 10CV, 20% 2CV) to give **50** as an orange oil (55 mg, 10%). R_f = 0.87

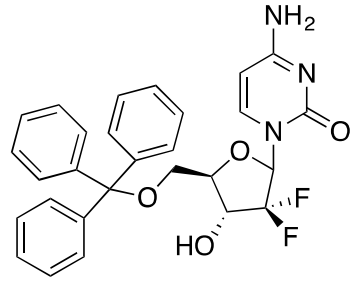
(CH_2Cl_2/MeOH - 9:1). **^{1}H NMR (500 MHz, DMSO) δ_H**: 8.76 (bs, 1H, *NH*), 7.51 (d, *J* = 7.6 Hz, 1H, *H*-6), 7.40-7.20 (m, 30H, Ar*H*), 6.33 (d, *J* = 7.6 Hz, 1H, *H*-5), 6.10 (bs, 1H, *H*-1'), 5.42-5.35 (m, 1H, *H*-3'), 4.20 (bs, 1H, *H*-4'), 3.46-3.430 (m, 2H, *H*-5'), 2.09 (s, 3H, *CH_3*).

(2*R*,3*R*)-4,4-Difluoro-2-(hydroxymethyl)-5-(2-oxo-4-(tritylamino)pyrimidin-1(2H)-yl)tetrahydrofuran-3-yl acetateacetate 51[4]



Compound **50** (456 mg, 3.39 mmol) was dissolved in anhydrous CH_2Cl_2 (5 ml) under an argon atmosphere. The solution ws cooled at 0˚C in a ice/water bath and TFA (5 ml) was then added in a dropwise fashion. The mixture was stirred at rt for 24 h. The solvent was concentrated under reduced pressure, then diluted with $NaHCO_3$ (saturated solution) and extracted with EtOAc (3 x 20 ml). The organic phases were dried over $MgSO_4$ and evaporated *in vacuo*. The residue was purified by Biotage Isolera One (50 g SNAP cartridge KP-SIL, 100 ml/min, gradient eluent system EtOAc/Hexane 20% 1CV, 20-100% 10CV, 100% 2CV) to give **51** as a white solid (28 mg, 15%). R_f = 0.32 (CH_2Cl_2/MeOH – 9.5:0.5). **^{1}H NMR (500 MHz, DMSO) δ_H**: 8.92 (bs, 1H, *NH*), 7.91 (d, *J* = 7.7 Hz, 1H, *H*-6), 7.23-7.11 (m, 15H, Ar*H*), 6.24 (t, $^3J_{H\text{-}F}$ = 8.8 Hz, 1H, *H*-1'), 6.09 (d, *J* = 7.7 Hz, 1H, *H*-5), 5.44-5.34 (m, 1H, *H*-3'), 4.71-4.61 (m, 3H, *H*-5', *OH*-5'), 4.57-4.54 (m, 1H, *H*-4'), 2.08 (s, 3H, *CH_3*).

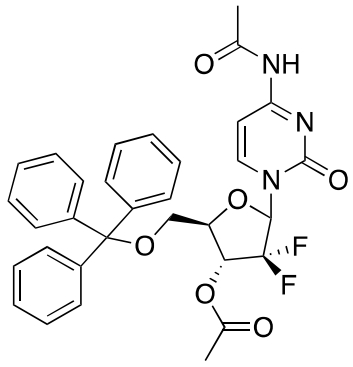
4-Amino-1-((4*R*,5*R*)-3,3-difluoro-4-hydroxy-5-((trityloxy)methyl)tetrahydrofuran-2-yl)pyrimidin-2(1H)-one 52[4]



Gemcitabine **1k** (3 g, 11.40 mmol) was dissolved in anhydrous pyridine (50 ml) under an argon atmosphere and trityl chloride (6.3 g, 22.80 mmol) was then added. The solution was stirred at rt for 48 h. The solvent was evaporated *in vacuo* and purified in two times by Biotage Isolera One (50 g SNAP cartridge ULTRA, 100 ml/min, gradient eluent system MeOH/CH_2Cl_2 2% 1CV, 2-20% 10CV, 20% 2CV) to give **52** as a white solid (3.42 g, 60%). R_f = 0.42 (CH_2Cl_2/MeOH - 9:1). **^{1}H NMR (500 MHz, DMSO) δ_H**: 7.64 (d, *J* = 7.5 Hz, 1H, *H*-6), 7.41-7.28 (m, 17H, Ar*H*, *NH_2*), 6.33 (d, *J* = 6.7 Hz, 1H,

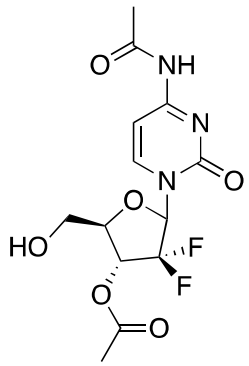
O*H*-3'), 6.20 (t, $^{3}J_{H\text{-}F}$ = 7.87 Hz, 1H, *H*-1'), 5.66 (d, *J* = 7.5 Hz, 1H, *H*-5), 4.32-4.27 (m, 1H, *H*-3'), 4.01-3.98 (m, 1H, *H*-4'), 3.37-3.31 (m, 2H, *H*-5')

(2*R*,3*R*)-5-(4-Acetamido-2-oxopyrimidin-1(2H)-yl)-4,4-difluoro-2-((trityloxy)methyl)tetrahydrofuran-3-yl acetate 53[5]



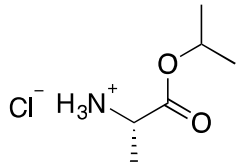
5'-(*O-trityl*)-gemcitabine **52** (1.71 g, 3.38 mmol) was dissolved in anhydrous pyridine (30 ml) under an argon atmosphere and acetic anhydride (1.92 ml, 20.30 mmol) was then added. The solution was stirred at rt for 12 h. The solvent was evaporated *in vacuo* and co-evaporated with toluene and MeOH to give **53** as a yellow foamy solid (1.9 g, 95%). R_f = 0.84 (CH_2Cl_2/MeOH - 9:1). **^{1}H NMR (500 MHz, DMSO) δ_H**: 11.00 (bs, 1H, *NH*), 7.98 (d, *J* = 7.6 Hz, 1H, *H*-6), 7.31-7.05 (m, 16H, Ar*H*, *H*-5), 6.22 (t, $^{3}J_{H\text{-}F}$ = 7.9 Hz, 1H, *H*-1'), 5.42-5.44 (m, 1H, *H*-3'), 4.27-4.24 (m, 1H, *H*-4'), 3.38-3.32 (m, 1H, *H*-5'), 2.05 (s, 3H, *CH*$_3$), 2.04 (s, 3H, *CH*$_3$).

(2*R*,3*R*)-5-(4-Acetamido-2-oxopyrimidin-1(2H)-yl)-4,4-difluoro-2-(hydroxymethyl)tetrahydrofuran-3-yl acetate 54[5]



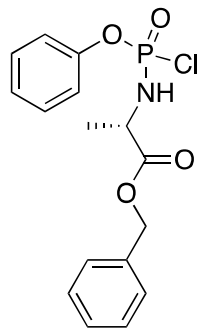
3'-(*O*-acetyl)-5'-(*O*-trityl)-*N*^{4}-acetyl-gemcitabine **53** (3.5 g, 6.39 mmol) was dissolved in anhydrous CH_2Cl_2 (20 ml) under an argon atmosphere. The solution ws cooled at 0°C in a ice/water bath and TFA (15ml) was then added in a dropwise fashion. The mixture was stirred at rt for 24 h. The solvent was concentrated under reduced pressure, then diluted with $NaHCO_3$ (saturated solution) and extracted with EtOAc (3 x 20 ml). The organic phases were dried over $MgSO_4$ and evaporated *in vacuo*. The residue was purified by Biotage Isolera One (100 g SNAP cartridge KP-SIL, 100 ml/min, gradient eluent system EtOAc/Hexane 20% 1CV, 20-100% 10CV, 100% 2CV) to give **54** as a white solid (420 mg, 19%). R_f = 0.26 (CH_2Cl_2/MeOH – 9.5:0.5). **^{1}H NMR (500 MHz, DMSO) δ_H**: 11.20 (bs, 1H, *NH*), 8.34 (d, *J* = 7.6 Hz, 1H, *H*-6), 7.43 (d, *J* = 7.6 Hz, 1H, *H*-5), 6.44 (t, $^{3}J_{H\text{-}F}$ = 8.3 Hz, 1H, *H*-1'), 5.54-5.50 (m, 1H, *H*-3'), 5.47 (t, *J* = 5.7 Hz, 1H, *OH*-5'), 4.40-4.37 (m, 1H, *H*-4'), 3.95-3.92 (m, 1H, *H*-5$_a$'), 3.83-3.78 (m, 1H, *H*-5$_b$'), 2.31(s, 3H, *CH*$_3$), 2.26 (s, 3H, *CH*$_3$).

_L_-Alanine isopropyl ester hydrochloride salt 56[6]



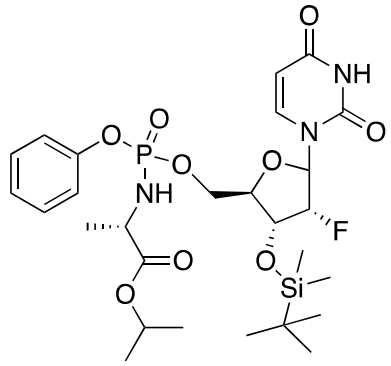
2-propanol (60 ml, 841.81 mmol) was cooled at 0˚C in ice/water bath and $SOCl_2$ (8.2 ml, 112.24 mmol) was added dropwise under an argon atmosphere. After stirring for 30 minutes at 0˚C the solution was allowed to reach rt. L-Alanine (5 g, 56.12 mmol) was quickly added and the reaction mixture was stirred at reflux for 16 h. The solvent was then removed under *vacuum* and co-evaporated with hexane. The L-alanine-*O*-isopropyl ester hydrochloride salt **56** was obtained as a white solid in 93 % yield (8.7 g). **^{1}H NMR (500 MHz, MeOD) δ_H**: 5.13 (septet, *J* = 6.2 Hz, 1H, *CH*(CH_3)$_2$), 4.08 (q, *J* = 7.2 Hz, 1H, *CH*CH_3 L-Ala), 1.56 (d, *J* = 7.2 Hz, 3H, CH*CH$_3$* L-Ala), 1.33 (d, *J* = 6.2 Hz, 3H, CH(*CH$_3$*)$_2$), 1.32 (d, *J* = 6.2 Hz, 3H, CH(*CH$_3$*)$_2$).

Benzyl (chloro(phenoxy)phosphoryl)-*L*-alaninate 58



The L-Alanine benzyl ester hydrochloride salt **57** (3.08 g, 14.28 mmol) was dissolved in anhydrous CH_2Cl_2 (150 ml) under an argon atmosphere. To this solution the aryl dichlorophosphate (2.13 ml, 14.28 mmol) was then added. The mixture was cooled to -78˚C in a dry-ice/acetone bath. Et_3N (3.99 ml, 28.56 mmol) was added dropwise over 15 minutes and the reaction mixture was stirred at -78˚C for 15 minutes. After this period the suspension was allowed to reach room temperature and stirred for further 1.5 h. When the reaction was judged completed (^{31}P NMR), the solvent was evaporated under reduced pressure. The resulting white residue was triturated with anhydrous Et_2O and the filtrate concentrated to give **58** as a clear oil (5.0 g, 98%). **^{31}P NMR (202 MHz, $CDCl_3$) δ_P**: 8.12, 7.92; **^{1}H NMR (500 MHz, $CDCl_3$) δ_H** : 7.27-7.21 (m, 7H, Ar*H*), 7.16-7.11 (m, 3H, Ar*H*), 5.11 (AB app t, J_{AB} = 12.7 Hz, 1H, *CH$_2$*Ph), 5.09 (AB app t, J_{AB} = 12.2 Hz, 1H, *CH$_2$*Ph), 4.67 (bs, 1H, *NH* L-Ala), 4.13 (bs, 1H, *CH*CH_3 L-Ala), 1.42 (d, *J* = 7.2 Hz, 3H, CH*CH$_3$* L-Ala), 1.40 (d, *J* = 7.2 Hz, 3H, CH*CH$_3$* L-Ala).

Isopropyl ((((2*R*,3*R*,4*R*)-3-((*tert*-butyldimethylsilyl)oxy)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-fluorotetrahydrofuran-2-yl)methoxy)(phenoxy)phosphoryl)-*L*-alaninate 59



Prepared according to the standard procedure **A** for the synthesis of ProTides using 2'-deoxy-2'-fluoro-3'-(*O*-*tert*-butyldimethylsilyl)-uridine **43** (232 mg, 643.63 μmol) in anhydrous THF (4 ml), tBuMgCl (708 μl, 708 μmol), Phenyl-(isopropyloxy-L-Alanine)-phosphorochloridate **22** (236 mg, 772.35 μmol) in anhydrous THF (1 ml). After evaporation, the mixture was purified by two preparative TLC (CH_2Cl_2/MeOH – 9.5:0.5), to afford the title compound as a white foamy solid (200 mg, 49%). R_f = 0.61 (CH_2Cl_2/MeOH – 9.5:0.5). **^{31}P NMR (202 MHz, $CDCl_3$) δ_P**: 2.79, 2.85. **^{19}F NMR (470 MHz, $CDCl_3$) δ_F**: -201.71, -202.04. **^{1}H NMR (500 MHz, $CDCl_3$) δ_H**: 9.40 (bs, 1H, *NH*), 7.37 (d, *J* = 8.1 Hz, 0.6H, *H*-6), 7.35 (d, *J* = 8.1 Hz, 0.4H, *H*-6), 7.21-7.17 (m, 2H, Ar*H*), 7.09-7.07 (m, 2H, Ar*H*), 7.04 (t, *J* = 7.3 Hz, 1H, Ar*H*), 5.80 (dd, $^{3}J_{H\text{-}F}$ = 17.0 Hz, *J* = 2.7 Hz, 0.4H, *H*-1'), 5.74 (dd, $^{3}J_{H\text{-}F}$ = 17.0 Hz, *J* = 2.7 Hz, 0.6H, *H*-1'), 5.60 (d, *J* = 8.1 Hz, 0.4H, *H*-5), 5.53 (d, *J* = 8.1 Hz, 0.6H, *H*-5), 4.91-4.84 (m, 1H, *CH*(CH_3)$_2$), 4.77 (ddd, $^{2}J_{H\text{-}F}$ = 52.6 Hz, *J* = 4.8 Hz, *J* = 2.7 Hz, 0.6H, *H*-2'), 4.66 (ddd, $^{2}J_{H\text{-}F}$ = 52.6 Hz, *J* = 4.8 Hz, *J* = 2.7 Hz, 0.4H, *H*-2'), 4.37-4.31 (m, 1H, H_a-5'), 4.28-4.19 (m, 1H, *H*-3'), 4.16-4.10 (m, 1H, H_b-5'), 4.03 (bs, 1H, *H*-4'), 3.91-3.81 (m, 2H, *NH* L-Ala, *CH*CH_3 L-Ala), 1.24 (d, *J* = 7.0 Hz, 1.8H, CH*CH*$_3$ L-Ala), 1.20 (d, *J* = 6.4 Hz, 1.2H, CH*CH*$_3$ L-Ala), 1.10 (d, *J* = 6.2 Hz, 1.2H, CH(*CH*$_3$)$_2$), 1.09 (d, *J* = 6.2 Hz, 3.6H, CH(*CH*$_3$)$_2$), 1.08 (d, *J* = 6.2 Hz, 1.2H, CH(*CH*$_3$)$_2$), 0.78 (s, 5.4H, *t*Bu), 0.77 (s, 3.6H, *t*Bu), 0.01 (s, 1.8H, *CH*$_3$), -0.00 (s, 1.8H, *CH*$_3$), -0.02 (s, 1.2H, *CH*$_3$), -0.03 (s, 1.2H, *CH*$_3$). **^{13}C NMR (125 MHz, $CDCl_3$) δ_C**: 173.1 (d, $^{3}J_{C\text{-}P}$ = 7.3 Hz, *C*=O, ester), 172.9 (d, $^{3}J_{C\text{-}P}$ = 7.3 Hz, *C*=O, ester), 163.24 (*C*-4), 163.21 (*C*-4), 150.56 (d, $^{2}J_{C\text{-}P}$ = 6.8 Hz, *C*-O, Ph), 150.53 (d, $^{2}J_{C\text{-}P}$ = 6.6 Hz, *C*-O, Ph), 150.0 (*C*-2), 140.6 (*C*-6), 140.3 (*C*-6), 129.8 (*CH*-Ar), 125.2 (*CH*-Ar), 125.1 (*CH*-Ar), 120.1 (d, $^{3}J_{C\text{-}P}$ = 4.6 Hz, *CH*-Ar), 119.9 (d, $^{3}J_{C\text{-}P}$ = 4.6 Hz, *CH*-Ar), 102.8 (*C*-5), 102.7 (*C*-5), 91.9 (d, $^{1}J_{C\text{-}F}$ = 192.9 Hz, *C*-2'), 91.8 (d, $^{1}J_{C\text{-}F}$ = 193.4 Hz, *C*-2'), 89.6 (d, $^{2}J_{C\text{-}F}$ = 34.6 Hz, *C*-1'), 89.1 (d, $^{2}J_{C\text{-}F}$ = 34.6 Hz, *C*-1'), 82.06 (d, $^{3}J_{C\text{-}P}$ = 5.6 Hz, *C*-4'), 82.03 (d, $^{3}J_{C\text{-}P}$ = 5.6 Hz, *C*-4'), 69.4 (*CH*(CH_3)$_2$), 69.3 (d, $^{2}J_{C\text{-}F}$ = 16.1 Hz, *C*-3'), 69.1 (d, $^{2}J_{C\text{-}F}$ = 16.1 Hz, *C*-3'), 64.9 (d, $^{2}J_{C\text{-}P}$ = 5.1 Hz, *C*-5'), 64.2 (d, $^{2}J_{C\text{-}P}$ = 5.1 Hz, *C*-5'), 50.4 (*CH*CH_3 L-Ala), 50.3 (d, $^{2}J_{C\text{-}P}$ = 1.2 Hz, *CH*CH_3 L-Ala), 25.6 (C(*CH*$_3$)$_3$), 25.5 (C(*CH*$_3$)$_3$), 21.7 (CH(*CH*$_3$)$_2$), 21.6

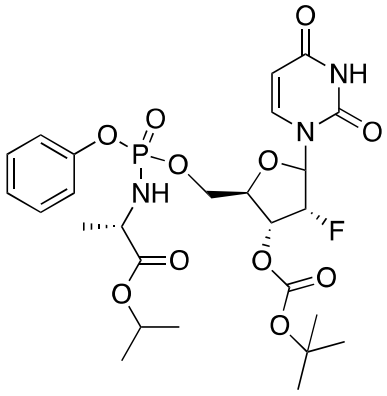
(CH(*CH*$_3$)$_2$), 21.1 (d, $^3J_{C\text{-}P}$ = 4.5 Hz, CH*CH*$_3$ L-Ala), 20.9 (d, $^3J_{C\text{-}P}$ = 4.5 Hz, CH*CH*$_3$ L-Ala), 18.0 (*C*(CH$_3$)$_3$), -4.7 (*CH*$_3$), -4.8 (*CH*$_3$), -5.0 (*CH*$_3$), -5.1 (*CH*$_3$). **(ES+) m/z**, found: 630.7 [M+H$^+$], $C_{27}H_{41}FN_3O_9PSi$ required: 629.23 [M]. **HPLC**: Reverse phase HPLC eluting with isocratic method MeOH/H_2O-80/20 in 30 minutes, 1ml/min, λ = 254 nm and 263 nm, showed two peaks with t_R 7.19 min. and t_R 8.10 min.

59 Fast eluting. ^{31}P NMR (202 MHz, CDCl$_3$) δ$_P$: 2.73. **^{19}F NMR (470 MHz, CDCl$_3$) δ$_F$**: -201.94. **^{1}H NMR (500 MHz, CDCl$_3$) δ$_H$**: 7.38 (d, *J* = 8.1 Hz, 1H, *H*-6), 7.24 (t, *J* = 7.9 Hz, 2H, Ar*H*), 7.11 (d, *J* = 8.1 Hz, 2H, Ar*H*), 7.08 (t, *J* = 7.3 Hz, 1H, Ar*H*), 5.81 (dd, $^3J_{H\text{-}F}$ = 17.1 Hz, *J* = 2.5 Hz, 1H, *H*-1'), 5.64 (d, *J* = 8.1 Hz, 1H, *H*-5), 4.91 (sept, *J* = 6.1 Hz, 1H, *CH*(CH$_3$)$_2$), 4.70 (ddd, $^2J_{H\text{-}F}$ = 53.1 Hz, *J* = 5.0 Hz, *J* = 2.5 Hz, 1H, *H*-2'), 4.38 (ddd, *J* = 11.8 Hz, $^3J_{H\text{-}P}$= 5.3 Hz, *J* = 1.9 Hz, 1H, *H*$_a$-5'), 4.27 (ddd, $^3J_{H\text{-}F}$ = 15.1 Hz, *J* = 6.7 Hz, *J* = 5.0 Hz, 1H, *H*-3'), 4.17 (ddd, *J* = 12.0 Hz, $^3J_{H\text{-}P}$= 6.1 Hz, *J* = 2.5 Hz, 1H, *H*$_b$-5'), 4.05 (bs, 1H, *H*-4'), 3.92-3.85 (m, 1H, *CH*CH$_3$ L-Ala), 3.63-3.68 (m, 1H, *NH* L-Ala), 1.23 (d, *J* = 6.9 Hz, 3H, CH*CH*$_3$ L-Ala), 1.14 (d, *J* = 6.1 Hz, 3H, CH(*CH*$_3$)$_2$), 1.12 (d, *J* = 6.1 Hz, 3H, CH(*CH*$_3$)$_2$), 0.80 (s, 9H, *t*Bu), 0.01 (s, 3H, *CH*$_3$), -0.00 (s, 3H, *CH*$_3$). **^{13}C NMR (125 MHz, CDCl$_3$) δ$_C$**: 173.0 (d, $^3J_{C\text{-}P}$ = 7.6 Hz, *C*=O, ester), 162.5 (*C*-4), 150.5 (d, $^2J_{C\text{-}P}$ = 7.0 Hz, *C*-O, Ph), 149.7 (*C*-2), 140.4 (*C*-6), 129.8 (*CH*-Ar), 125.2 (*CH*-Ar), 120.0 (d, $^3J_{C\text{-}P}$ = 4.8 Hz, *CH*-Ar), 102.8 (*C*-5), 91.7 (d, $^1J_{C\text{-}F}$ = 193.5 Hz, *C*-2'), 89.2 (d, $^2J_{C\text{-}F}$ = 34.6 Hz, *C*-1'), 82.0 (d, $^3J_{C\text{-}P}$ = 7.7 Hz, *C*-4'), 69.5 (*CH*(CH$_3$)$_2$), 69.0 (d, $^2J_{C\text{-}F}$ = 16.0 Hz, *C*-3'), 64.2 (d, $^2J_{C\text{-}P}$ = 5.0 Hz, *C*-5'), 50.4 (d, $^2J_{C\text{-}P}$ = 2.0 Hz, *CH*CH$_3$ L-Ala), 25.5 (C(*CH*$_3$)$_3$), 21.7 (CH(*CH*$_3$)$_2$), 21.6 (CH(*CH*$_3$)$_2$), 21.0 (d, $^3J_{C\text{-}P}$ = 4.0 Hz, CH*CH*$_3$ L-Ala), 18.0 (*C*(CH$_3$)$_3$), -4.8 (*CH*$_3$), -5.1 (*CH*$_3$). **(ES+) m/z**, found: 630.7 [M+H$^+$], $C_{27}H_{41}FN_3O_9PSi$ required: 629.23 [M].

59 Slow Eluting. ^{31}P NMR (202 MHz, CDCl$_3$) δ$_P$: 2.51. **^{19}F NMR (470 MHz, CDCl$_3$) δ$_F$**: -201.44. **^{1}H NMR (500 MHz, CDCl$_3$) δ$_H$**: 7.36 (d, *J* = 8.1 Hz, 1H, *H*-6), 7.19 (t, *J* = 7.9 Hz, 2H, Ar*H*), 7.07 (d, *J* = 8.6 Hz, 2H, Ar*H*), 7.04 (t, *J* = 7.3 Hz, 1H, Ar*H*), 5.72 (dd, $^3J_{H\text{-}F}$ = 17.4 Hz, *J* = 2.4 Hz, 1H, *H*-1'), 5.53 (d, *J* = 8.1 Hz, 1H, *H*-5), 4.88 (sept, *J* = 6.2 Hz, 1H, *CH*(CH$_3$)$_2$), 4.77 (ddd, $^2J_{H\text{-}F}$ = 52.8 Hz, *J* = 4.9 Hz, *J* = 2.4 Hz, 1H, *H*-2'), 4.33 (ddd, *J* = 11.7 Hz, $^3J_{H\text{-}P}$= 6.9 Hz, *J* = 2.1 Hz, 1H, *H*$_a$-5'), 4.26 (ddd, $^3J_{H\text{-}F}$ = 15.8 Hz, *J* = 6.7 Hz, *J* = 4.9 Hz, 1H, *H*-3'), 4.11 (ddd, *J* = 12.1 Hz, $^3J_{H\text{-}P}$= 6.7 Hz, *J* = 3.6 Hz, 1H, *H*$_b$-5'), 4.02 (bs, 1H, *H*-4'), 3.87-3.75 (m, 1H, *CH*CH$_3$ L-Ala), 3.76-3.78 (m, 1H, *NH* L-Ala), 1.23 (d, *J* = 6.9 Hz, 3H, CH*CH*$_3$ L-Ala), 1.10 (d, *J* = 6.2 Hz, 6H, CH(*CH*$_3$)$_2$), 0.78 (s, 9H, *t*Bu), 0.01 (s, 3H, *CH*$_3$), -0.00 (s, 3H, *CH*$_3$). **^{13}C NMR (125 MHz, CDCl$_3$) δ$_C$**: 172.9 (d, $^3J_{C\text{-}P}$ = 7.6 Hz, *C*=O, ester), 162.7 (*C*-4), 150.5 (d, $^2J_{C\text{-}P}$ = 6.8 Hz, *C*-O, Ph), 149.8 (*C*-2),

140.4 (*C*-6), 129.8 (*CH*-Ar), 125.2 (*CH*-Ar), 120.0 (d, $^{3}J_{C\text{-}P}$ = 4.8 Hz, *CH*-Ar), 102.8 (*C*-5), 91.9 (d, $^{1}J_{C\text{-}F}$ = 192.8 Hz, *C*-2'), 89.8 (d, $^{2}J_{C\text{-}F}$ = 34.5 Hz, *C*-1'), 82.0 (d, $^{3}J_{C\text{-}P}$ = 6.3 Hz, *C*-4'), 69.5 (*CH*$(CH_3)_2$), 69.3 (d, $^{2}J_{C\text{-}F}$ = 16.3 Hz, *C*-3'), 64.8 (d, $^{2}J_{C\text{-}P}$ = 4.5 Hz, *C*-5'), 50.3 (d, $^{2}J_{C\text{-}P}$ = 1.8 Hz, *CH*CH_3 L-Ala), 25.6 (C(*CH_3*)$_3$), 21.7 (CH(*CH_3*)$_2$), 21.6 (CH(*CH_3*)$_2$), 21.2 (d, $^{3}J_{C\text{-}P}$ = 4.5 Hz, CH*CH_3* L-Ala), 18.0 (*C*$(CH_3)_3$), -4.7 (*CH_3*), -5.0 (*CH_3*). **(ES+) m/z**, found: 630.7 [$M+H^+$], $C_{27}H_{41}FN_3O_9PSi$ required: 629.23 [M].

Isopropyl ((((2*R*,3*R*,4*R*)-3-((*tert*-Butoxycarbonyl)oxy)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-fluorotetrahydrofuran-2-yl)methoxy)(phenoxy)phosphoryl)-*L*-alaninate 60



Prepared according to the standard procedure **A** for the synthesis of ProTides using 2'-deoxy-2'-fluoro-3'-(*O*-*tert*-butoxy)-uridine **45** (200 mg, 557.51 µmol) in anhydrous THF (4 ml), tBuMgCl (635.27 µl, 635.27 µmol), Phenyl-(isopropyloxy-L-Alanine)-phosphorochloridate **22** (265 mg, 866.27 µmol) in anhydrous THF (1 ml). After evaporation, the mixture was purified by Biotage Isolera One (120 g ZIP cartridge KP-SIL, 100 ml/min, gradient eluent system MeOH/CH_2Cl_2 1% 1CV, 1-10% 12CV, 10% 2CV), to afford the title compound as a white foamy solid (89mg, 25%). R_f = 0.34 (CH_2Cl_2/MeOH – 9.5:0.5). **^{31}P NMR (202 MHz, $CDCl_3$) δ_P**: 2.70, 2.56. **^{19}F NMR (470 MHz, $CDCl_3$) δ_F**: -202.08, -202.16. **^{1}H NMR (500 MHz, $CDCl_3$) δ_H**: 8.64 (bs, 0.5H, *NH*), 8.58 (bs, 0.5H, *NH*), 7.35 (d, *J* = 8.1 Hz, 1H, *H*-6), 7.27-7.24 (m, 2H, Ar*H*), 7.15-7.08 (m, 3H, Ar*H*), 5.88 (dd, $^{3}J_{H\text{-}F}$ = 12.8 Hz, *J* = 2.7 Hz, 0.5H, *H*-1'), 5.84 (dd, $^{3}J_{H\text{-}F}$ = 12.8 Hz, *J* = 2.7 Hz, 0.5H, *H*-1'), 5.66 (d, *J* = 8.1 Hz, 0.5H, *H*-5), 5.55 (d, *J* = 8.1 Hz, 0.5H, *H*-5), 5.20-5.19 (m, 0.5H, *H*-2'), 5.13-5.04 (m, 1.5H, *H*-2', *H*-3'), 4.96-4.91 (m, 1H, *CH*$(CH_3)_2$), 4.44-4.39 (m, 1H, H_a-5') 4.32-4.28 (m, 1.5H, *H*-4', H_b-5'), 4.26-4.21 (m, 0.5H, H_b-5'), 3.96-3.87 (m, 1H, *CH*CH_3 L-Ala), 3.81-3.84 (m, 1H, *NH* L-Ala), 1.43 (s, 4.5H, *t*Bu), 1.41 (s, 4.5H, *t*Bu), 1.30 (d, *J* = 6.8 Hz, 1.5H, CH*CH_3* L-Ala), 1.24 (d, *J* = 7.0 Hz, 1.5H, CH*CH_3* L-Ala), 1.17 (d, *J* = 6.2 Hz, 1.5H, CH(*CH_3*)$_2$), 1.16 (d, *J* = 6.2 Hz, 3H, CH(*CH_3*)$_2$), 1.14 (d, *J* = 6.2 Hz, 1.5H, CH(*CH_3*)$_2$). **^{13}C NMR (125 MHz, $CDCl_3$) δ_C**: 173.8 (d, $^{3}J_{C\text{-}P}$ = 6.5 Hz, *C*=O, ester), 172.8 (d, $^{3}J_{C\text{-}P}$ = 6.5 Hz, *C*=O, ester), 162.57 (*C*-4), 162.52 (*C*-4), 152.08 (*C*=O, Boc), 152.02 (*C*=O, Boc), 150.5 (d, $^{2}J_{C\text{-}P}$ = 5.6 Hz, *C*-O, Ph), 149.8 (*C*-2), 149.7 (*C*-2), 140.3 (*C*-6), 129.86 (*CH*-Ar), 129.82 (*CH*-Ar), 125.2 (*CH*-Ar),

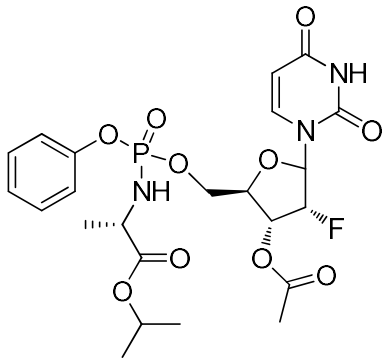
120.1 (d, $^{3}J_{C\text{-}P}$ = 4.8 Hz, *CH*-Ar), 120.0 (d, $^{3}J_{C\text{-}P}$ = 4.8 Hz, *CH*-Ar), 103.1 (*C*-5), 103.0 (*C*-5), 90.6 (d, $^{1}J_{C\text{-}F}$ = 194.6 Hz, *C*-2'), 90.5 (d, $^{1}J_{C\text{-}F}$ = 194.6 Hz, *C*-2'), 89.5 (d, $^{2}J_{C\text{-}F}$ = 34.4 Hz, *C*-1'), 89.4 (d, $^{2}J_{C\text{-}F}$ = 34.4 Hz, *C*-1'), 84.1 (*C*(CH_3)$_3$), 84.0 (*C*(CH_3)$_3$), 79.5 (d, $^{3}J_{C\text{-}P}$ = 6.7 Hz, *C*-4'), 79.4 (d, $^{3}J_{C\text{-}P}$ = 6.7 Hz, *C*-4'), 71.2 (d, $^{2}J_{C\text{-}F}$ = 14.8 Hz, *C*-3'), 71.1 (d, $^{2}J_{C\text{-}F}$ = 14.8 Hz, *C*-3'), 69.5 (*CH*(CH_3)$_2$), 69.4 (*CH*(CH_3)$_2$), 64.6 (d, $^{2}J_{C\text{-}P}$ = 4.8 Hz, *C*-5'), 64.3 (d, $^{2}J_{C\text{-}P}$ = 4.8 Hz, *C*-5'), 50.4 (d, $^{2}J_{C\text{-}P}$ = 2.1 Hz, *CH*CH_3 L-Ala), 50.3 (d, $^{2}J_{C\text{-}P}$ = 2.1 Hz, *CH*CH_3 L-Ala), 27.6 (C(*CH_3*)$_3$), 27.5 (C(*CH_3*)$_3$), 21.7 (CH(*CH_3*)$_2$), 21.6 (CH(*CH_3*)$_2$), 21.1 (d, $^{3}J_{C\text{-}P}$ = 4.5 Hz, CH*CH_3* L-Ala), 21.0 (d, $^{3}J_{C\text{-}P}$ = 4.5 Hz, CH*CH_3* L-Ala). **(ES+) m/z**, found: 616.5 [M+H$^+$], $C_{26}H_{35}FN_3O_{11}P$ required: 615.20 [M]. **HPLC**: Reverse phase HPLC eluting with isocratic method MeOH/H_2O-70/30 in 35 minutes, 1ml/min, λ = 254 nm and 263 nm, showed two peaks with t_R 6.40 min. and t_R 7.15 min.

60 Fast eluting. ^{31}P NMR (202 MHz, $CDCl_3$) δ_P: 2.66. **^{19}F NMR (470 MHz, $CDCl_3$) δ_F**: -202.05. **^{1}H NMR (500 MHz, $CDCl_3$) δ_H**: 7.35 (d, *J* = 8.1 Hz, 1H, *H*-6), 7.50 (t, *J* = 7.8 Hz, 2H, Ar*H*), 7.14 (d, *J* = 8.5 Hz, 2H, Ar*H*), 7.09 (t, *J* = 7.0 Hz, 1H, Ar*H*), 5.86 (dd, $^{3}J_{H\text{-}F}$ = 17.3 Hz, *J* = 2.7 Hz, 1H, *H*-1'), 5.66 (d, *J* = 8.1 Hz, 1H, *H*-5), 5.17 (ddd, $^{2}J_{H\text{-}F}$ = 40.1 Hz, *J* = 5.2 Hz, *J* = 2.7 Hz, 1H, *H*-2'), 5.11-5.09 (m, 1H, *H*-3'), 4.93 (sept, *J* = 6.2 Hz, 1H, *CH*(CH_3)$_2$), 4.41 (ddd, *J* = 12.6 Hz, $^{3}J_{H\text{-}P}$= 5.8 Hz, *J* = 2.7 Hz, 1H, H_a-5'), 4.32-4.28 (m, 2H, *H*-4', H_b-5'), 3.96-3.87 (m, 1H, *CH*CH_3 L-Ala), 3.75-3.71 (m, 1H, *NH* L-Ala), 1.41 (s, 9H, *t*Bu), 1.24 (d, *J* = 7.0 Hz, 3H, CH*CH_3* L-Ala), 1.16 (d, *J* = 6.2 Hz, 3H, CH(*CH_3*)$_2$), 1.14 (d, *J* = 6.2 Hz, 3H, CH(*CH_3*)$_2$. **^{13}C NMR (125 MHz, $CDCl_3$) δ_C**: 173.1 (d, $^{3}J_{C\text{-}P}$ = 6.1 Hz, *C*=O, ester), 162.2 (*C*-4), 152.0 (*C*=O, Boc), 150.5 (d, $^{2}J_{C\text{-}P}$ = 6.6 Hz, *C*-O, Ph), 149.6 (*C*-2), 140.3 (*C*-6), 129.8 (*CH*-Ar), 125.2 (*CH*-Ar), 120.1 (d, $^{3}J_{C\text{-}P}$ = 4.9 Hz, *CH*-Ar), 103.1 (*C*-5), 90.5 (d, $^{1}J_{C\text{-}F}$ = 193.9 Hz, *C*-2'), 89.5 (d, $^{2}J_{C\text{-}F}$ = 34.7 Hz, *C*-1'), 84.0 (*C*(CH_3)$_3$), 79.4 (d, $^{3}J_{C\text{-}P}$ = 7.5 Hz, *C*-4'), 71.1 (d, $^{2}J_{C\text{-}F}$ = 16.3 Hz, *C*-3'), 69.5 (*CH*(CH_3)$_2$), 69.5 (*CH*(CH_3)$_2$), 64.2 (d, $^{2}J_{C\text{-}P}$ = 5.4 Hz, *C*-5'), 50.4 (d, $^{2}J_{C\text{-}P}$ = 1.9 Hz, *CH*CH_3 L-Ala), 27.5 (C(*CH_3*)$_3$), 21.7 (CH(*CH_3*)$_2$), 21.6 (CH(*CH_3*)$_2$), 21.1 (d, $^{3}J_{C\text{-}P}$ = 4.2 Hz, CH*CH_3* L-Ala). **(ES+) m/z**, found: 616.5 [M+H$^+$], $C_{26}H_{35}FN_3O_{11}P$ required: 615.20 [M].

60 Slow eluting. ^{31}P NMR (202 MHz, $CDCl_3$) δ_P: 2.51. **^{19}F NMR (470 MHz, $CDCl_3$) δ_F**: -201.95. **^{1}H NMR (500 MHz, $CDCl_3$) δ_H**: 7.35 (d, *J* = 8.1 Hz, 1H, *H*-6), 7.20 (t, *J* = 7.8 Hz, 2H, Ar*H*), 7.13 (d, *J* = 8.4 Hz, 2H, Ar*H*), 7.10 (t, *J* = 7.4 Hz, 1H, Ar*H*), 5.84 (dd, $^{3}J_{H\text{-}F}$ = 17.6 Hz, *J* = 2.5 Hz, 1H, *H*-1'), 5.55 (d, *J* = 8.1 Hz, 1H, *H*-5), 5.15 (ddd, $^{2}J_{H\text{-}F}$ = 51.8 Hz, *J* = 4.8 Hz, *J* = 2.7 Hz, 1H, *H*-2'), 5.07 (ddd, $^{3}J_{H\text{-}F}$ = 13.9 Hz, *J* = 6.4 Hz, *J* = 5.1 Hz, 1H, *H*-3'), 4.94 (sept, *J* = 6.1 Hz, 1H, *CH*(CH_3)$_2$), 4.42 (ddd, *J* = 12.6 Hz, $^{3}J_{H\text{-}P}$= 6.6

Hz, J = 2.1 Hz, 1H, H_a-5'), 4.30 (bs, 1H, H-4'), 4.23 (ddd, J = 11.9 Hz, $^3J_{H\text{-}P}$= 6.3 Hz, J = 3.0 Hz, 1H, H_b-5'), 3.94-3.85 (m, 1H, CHCH$_3$ L-Ala), 3.79-3.75 (m, 1H, *NH* L-Ala), 1.43 (s, 9H, *t*Bu), 1.30 (d, J = 7.0 Hz, 3H, CHCH_3 L-Ala), 1.16 (d, J = 6.1 Hz, 6H, CH(CH_3)$_2$). **^{13}C NMR (125 MHz, CDCl$_3$) δ_C**: 172.8 (*C*=O, ester), 162.2 (*C*-4), 152.0 (*C*=O, Boc), 150.5 (d, $^2J_{C\text{-}P}$ = 6.7 Hz, *C*-O, Ph), 149.6 (*C*-2), 140.3 (*C*-6), 129.8 (*CH*-Ar), 125.2 (*CH*-Ar), 120.0 (d, $^3J_{C\text{-}P}$ = 4.6 Hz, *CH*-Ar), 103.0 (*C*-5), 90.6 (d, $^1J_{C\text{-}F}$ = 194.3 Hz, *C*-2'), 89.5 (d, $^2J_{C\text{-}F}$ = 34.4 Hz, *C*-1'), 84.1 (*C*(CH$_3$)$_3$), 79.4 (d, $^3J_{C\text{-}P}$ = 7.6 Hz, *C*-4'), 71.1 (d, $^2J_{C\text{-}F}$ = 15.2 Hz, *C*-3'), 69.5 (*CH*(CH$_3$)$_2$), 69.5 (*CH*(CH$_3$)$_2$), 64.5 (d, $^2J_{C\text{-}P}$ = 4.5 Hz, *C*-5'), 50.3 (d, $^2J_{C\text{-}P}$ = 1.9 Hz, *CH*CH$_3$ L-Ala), 27.6 (C(CH_3)$_3$), 21.7 (CH(CH_3)$_2$), 21.6 (CH(CH_3)$_2$), 21.1 (d, $^3J_{C\text{-}P}$ = 4.7 Hz, CHCH_3 L-Ala). **(ES+) m/z**, found: 616.5 [M+H$^+$], $C_{26}H_{35}FN_3O_{11}P$ required: 615.20 [M].

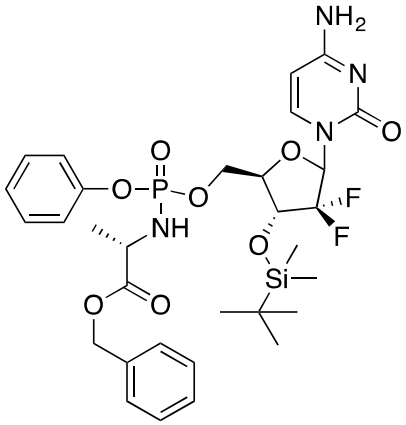
Isopropyl ((((2*R*,3*R*,4*R*)-3-acetoxy-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-fluorotetrahydrofuran-2-yl)methoxy)(phenoxy)phosphoryl)-*L*-alaninate 61



Prepared according to the standard procedure **A** for the synthesis of ProTides using 2'-deoxy-2'-fluoro-3'-(*O*-acetyl)-uridine **48** (200 mg, 693.89 µmol) in anhydrous THF (4 ml), tBuMgCl (736.28 µl, 736.28µmol), Phenyl-(isopropyloxy-L-Alanine)-phosphorochloridate **22** (254.54 mg, 832.66 µmol), in anhydrous THF (1 ml). After evaporation, the mixture was purified by Biotage Isolera One (50 g SNAP cartridge KP-SIL, 100 ml/min, gradient eluent system MeOH/CH_2Cl_2 2% 1CV, 2-20% 12CV, 20% 2CV), to afford the title compound as a white foamy solid (109 mg, 28%). R_f = 0.63 (CH_2Cl_2/MeOH – 9:1). **^{31}P NMR (202 MHz, CDCl$_3$) δ_P**: 2.80, 2.68. **^{19}F NMR (470 MHz, CDCl$_3$) δ_F**: - 201.75, - 201.93. **^{1}H NMR (500 MHz, CDCl$_3$) δ_H**: 8.99 (bs, 0.5H, *NH*), 8.92 (bs, 0.5H, *NH*), 7.37 (d, J = 8.1 Hz, 0.5H, *H*-6), 7.36 (d, J = 8.1 Hz, 0.5H, *H*-6), 7.26 (t, J = 7.9 Hz, 2H, Ar*H*), 7.14 (d, J = 7.5 Hz, 2H, Ar*H*), 7.10 (t, J = 7.3 Hz, 1H, ArH), 5.90 (dd, $^3J_{H\text{-}F}$ = 17.6 Hz, J = 2.4 Hz, 0.5H, *H*-1'), 5.86 (dd, $^3J_{H\text{-}F}$ = 17.6 Hz, J = 2.8 Hz, 0.5H, *H*-1'), 5.67 (dd, J = 8.1 Hz, $^4J_{NH,CH}$ = 2.0 Hz, 0.5H, *H*-5), 5.56 (dd, J = 8.1 Hz, $^4J_{NH,CH}$ = 2.0 Hz, 0.5H, *H*-5), 5.19-5.13 (m, 1.5H, *H*-3', *H*-2'), 5.07-5.04 (m, 0.5H, *H*-2'), 4.96-4.90 (m, 1H, *CH*(CH$_3$)$_2$), 4.43-4.39 (m, 1H, H_a-5'), 4.29-4.25 (m, 1.5H, *H*-4', H_b-5'), 4.24-4.19 (m, 0.5H, H_b-5'), 3.95-3.85 (m, 2H, *CH*CH$_3$ L-Ala, *NH* L-Ala), 2.08 (s, 1.5H, COCH_3), 2.06 (s, 1.5H, COCH_3), 1.29 (d, J = 6.3 Hz, 1.5H, CHCH_3 L-Ala), 1.24 (d, J = 6.5 Hz, 1.5H,

CH*CH*$_3$ L-Ala), 1.17 (d, *J* = 6.2 Hz, 1.5H, CH(*CH*$_3$)$_2$), 1.16 (d, *J* = 6.2 Hz, 3H, CH(*CH*$_3$)$_2$), 1.14 (d, *J* = 6.2 Hz, 1.5H, CH(*CH*$_3$)$_2$). **^{13}C NMR (125 MHz, CDCl$_3$) δ$_C$**: 173.1 (d, $^3J_{C\text{-}P}$ = 7.7 Hz, *C*=O, ester), 172.9 (d, $^3J_{C\text{-}P}$ = 6.6 Hz, *C*=O, ester), 169.8 (*CO*CH$_3$), 169.7 (*CO*CH$_3$), 162.76 (*C*-4), 162.70 (*C*-4), 150.49 (*C*-O, Ph), 150.44 (*C*-O, Ph), 149.9 (*C*-2), 149.8 (*C*-2), 140.2 (*C*-6), 140.1 (*C*-6), 129.89 (*CH*-Ar), 129.88 (*CH*-Ar), 125.28 (*CH*-Ar), 125.26 (*CH*-Ar), 120.06 (d, $^3J_{C\text{-}P}$ = 4.9 Hz, *CH*-Ar), 120.01 (d, $^3J_{C\text{-}P}$ = 4.7 Hz, *CH*-Ar), 103.2 (*C*-5), 103.1 (*C*-5), 90.5 (d, $^1J_{C\text{-}F}$ = 194.3 Hz, *C*-2'), 90.4 (d, $^1J_{C\text{-}F}$ = 194.1 Hz, *C*-2'), 89.4 (d, $^2J_{C\text{-}F}$ = 32.3 Hz, *C*-1'), 89.1 (d, $^2J_{C\text{-}F}$ = 32.3 Hz, *C*-1'), 79.6 (d, $^3J_{C\text{-}P}$ = 7.7 Hz, *C*-4'), 79.5 (d, $^3J_{C\text{-}P}$ = 7.8 Hz, *C*-4'), 69.53 (*CH*(CH$_3$)$_2$), 69.50 (*CH*(CH$_3$)$_2$), 69.3 (d, $^2J_{C\text{-}F}$ = 14.9 Hz, *C*-3'), 69.2 (d, $^2J_{C\text{-}F}$ = 15.0 Hz, *C*-3'), 64.6 (d, $^2J_{C\text{-}P}$ = 4.56 Hz, *C*-5'), 64.3 (d, $^2J_{C\text{-}P}$ = 4.8 Hz, *C*-5'), 50.4 (d, $^2J_{C\text{-}P}$ = 1.8 Hz, *CH*CH$_3$ L-Ala), 50.3 (*CH*CH$_3$ L-Ala), 21.7 (CH(*CH*$_3$)$_2$), 21.6 (CH(*CH*$_3$)$_2$), 21.1 (d, $^3J_{C\text{-}P}$ = 4.6 Hz, CH*CH*$_3$ L-Ala), 21.0 (d, $^3J_{C\text{-}P}$ = 5.1 Hz, CH*CH*$_3$ L-Ala), 20.47 (CO*CH*$_3$), 20.43 (CO*CH*$_3$). **(ES+) m/z**, found: 558.4 [M+H$^+$], $C_{23}H_{29}FN_3O_{10}P$ required: 557.16 [M]. **HPLC**: Reverse phase HPLC eluting with isocratic method MeOH/H_2O-60/40 in 35 minutes, 1ml/min, λ = 254 nm and 263 nm, showed two peaks with t_R 6.07 min. and t_R 7.33 min.

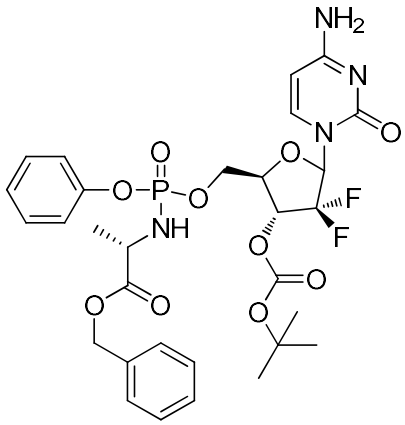
Benzyl ((((2*R*,3*R*,5*R*)-5-(4-Amino-2-oxopyrimidin-1(2H)-yl)-3-((*tert*-butyldimethylsilyl)oxy)-4,4-difluorotetrahydrofuran-2-yl)methoxy)(phenoxy)phosphoryl)-*L*-alaninate 62



Prepared according to the standard procedure **A** for the synthesis of ProTides using 3'-(*O*-*tert*-butyldimethylsilyl)-gemcitabine **42** (200 mg, 529.85 µmol) in anhydrous THF (4 ml), tBuMgCl (582.84 µl, 582.84 µmol), Phenyl-(benzyloxy-L-Alanine)-phosphorochloridate **58** (244 mg, 635.82 µmol) in anhydrous THF (1 ml). After evaporation, the mixture was purified by flash chromatography (eluent system CH_2Cl_2/MeOH – 9:1), to afford the title compound as a white foamy solid (244 mg, 66%). R_f = 0.56 (CH_2Cl_2/MeOH - 9:1). **^{31}P NMR (202 MHz, CDCl$_3$) δ$_P$**: 2.50, 2.40. **^{19}F NMR (470 MHz, CDCl$_3$) δ$_F$**: -114.81 (d, *J* = 238.9 Hz), -115.00 (d, *J* = 240.0 Hz), -117.10 (bs), -117.68 (bs). **^{1}H NMR (500 MHz, CDCl$_3$) δ$_H$**: 7.30-7.18 (m, 8H, Ar*H*, *H*-6), 7.10-7.05 (m, 3H, Ar*H*), 6.23-6.17 (m, 1H, *H*-1'), 5.59 (d, *J* = 7.5 Hz, 0.4H, *H*-5), 5.55 (d, *J* = 7.5 Hz, 0.6H, *H*-5), 5.06, 5.00 (ABq, J_{AB} = 12.1 Hz,

0.8H, CH_2Ph), 5.02 (AB app t, J_{AB} = 12.1 Hz, 1.2H, CH_2Ph), 4.38-4.33 (m, 0.4H, *H*-3'), 4.31-4.27 (m, 0.6H, *H*-3'), 4.15-4.03 (m, 1H, *H*-4'), 4.00-3.89 (m, 2H, H_a-5', *CH*CH_3 L-Ala), 3.87-3.84 (m, 1H, H_b-5'), 1.30 (d, *J* = 6.3 Hz, 1.8H, CHCH_3 L-Ala), 1.29 (d, *J* = 6.6 Hz, 1.2H, CHCH_3 L-Ala), 0.79 (s, 5.4H, *t*Bu), 0.78 (s, 3.6H, *t*Bu), 0.02 (s, 1.8H, CH_3), 0.00 (s, 1.8H, CH_3), -0.01 (s, 1.2H, CH_3). -0.03 (s, 1.2H, CH_3). **^{13}C NMR (125 MHz, $CDCl_3$) δ_C**: 173.2 (d, $^3J_{C\text{-}P}$ = 5.4 Hz, *C*=O, ester), 173.1 (d, $^3J_{C\text{-}P}$ = 5.4 Hz, *C*=O, ester), 165.5 (*C*-4), 155.2 (*C*-2), 150.5 (*C*-O, Ph), 150.6 (*C*-O, Ph), 141.6 (*C*-6), 141.3 (*C*-6), 135.1 (*C*-Ar), 135.0 (*C*-Ar), 129.85 (*CH*-Ar), 129.81 (*CH*-Ar), 128.7 (*CH*-Ar), 127.6 (*CH*-Ar), 128.3 (*CH*-Ar), 128.2 (*CH*-Ar), 125.2 (*CH*-Ar), 125.1 (*CH*-Ar), 121.48 (t, $^1J_{C\text{-}F}$ = 256.9 Hz, *C*-2'), 121.42 (t, $^1J_{C\text{-}F}$ = 256.9 Hz, *C*-2'), 120.1 (d, $^3J_{C\text{-}P}$ = 4.5 Hz, *CH*-Ar), 120.0 (d, $^3J_{C\text{-}P}$ = 5.0 Hz, *CH*-Ar), 95.0 (*C*-5), 94.9 (*C*-5), 84.3 (bs, *C*-1'), 79.8 (bs, *C*-4'), 79.4 (bs, *C*-4'), 71.2 (bs, *C*-3'), 67.4 (CH_2Ph), 67.3 (CH_2Ph), 64.1 (d, $^2J_{C\text{-}P}$ = 4.5 Hz, *C*-5'), 63.9 (d, $^2J_{C\text{-}P}$ = 4.9 Hz, *C*-5'), 50.5 (*CH*CH_3 L-Ala), 50.3 (*CH*CH_3 L-Ala), 25.52 (C(CH_3)$_3$), 25.50 (C(CH_3)$_3$), 21.0 (d, $^3J_{C\text{-}P}$ = 4.6 Hz, CHCH_3 L-Ala), 20.9 (d, $^3J_{C\text{-}P}$ = 5.0 Hz, CHCH_3 L-Ala), 18.0 (*C*(CH_3)$_3$), 17.9 (*C*(CH_3)$_3$), -5.2 (CH_3), -5.6 (CH_3). **(ES+) m/z**, found: 695.2 [M+H$^+$], $C_{31}H_{41}F_2N_4O_8PSi$ required: 694.24 [M]. **HPLC**: Reverse phase HPLC eluting with isocratic method MeOH/H_2O-80/20 in 30 minutes, 1ml/min, λ = 254 nm and 263 nm, showed two peaks with t_R 8.18 min. and t_R 9.66 min.

Benzyl ((((2*R*,3*R*)-5-(4-Amino-2-oxopyrimidin-1(2H)-yl)-3-((*tert*-butoxycarbonyl)oxy)-4,4-difluorotetrahydrofuran-2-yl)methoxy)(phenoxy)phosphoryl)-*L*-alaninate 63



Prepared according to the standard procedure **A** for the synthesis of ProTides using 3'-(*O*-*tert*-butoxy)-gemcitabine **44** (200 mg, 550.48 μmol) in anhydrous THF (4 ml), tBuMgCl (605.53 μl, 605.53 μmol), Phenyl-(benzyloxy-L-Alanine)-phosphorochloridate **58** (233.67 mg, 660.58 μmol) in anhydrous THF (1 ml). After evaporation, the mixture was purified by Biotage Isolera One (50 g SNAP cartridge KP-SIL, 100 ml/min, gradient eluent system MeOH/CH_2Cl_2 2% 1CV, 2-20% 12CV, 20% 2CV), to afford the title compound as a white foamy solid (264mg, 70%). R_f = 0.44 (CH_2Cl_2/MeOH - 9:1). **^{31}P NMR (202 MHz, $CDCl_3$) δ_P**: 2.57, 2.50. **^{19}F NMR (470 MHz, $CDCl_3$) δ_F**: -114.93 (d, *J* = 244.6 Hz), -115.18 (d, *J* = 244.6 Hz), -119.65 (bs). **^{1}H NMR**

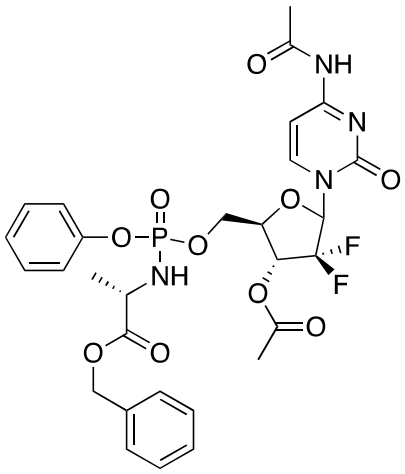
(500 MHz, $CDCl_3$) δ_H: 7.29-7.21 (m, 8H, Ar*H*, *H*-6), 7.13-7.05 (m, 3H, Ar*H*), 6.26 (bs, 1H, *H*-1'), 5.68 (d, *J* = 7.5 Hz, 0.5H, *H*-5), 5.59 (d, *J* = 7.5 Hz, 0.5H, *H*-5), 5.09-5.02 (m, 3H, CH_2Ph, *H*-3'), 4.40-4.31 (m, 1H, H_a-5'), 4.27-4.18 (m, 1H, *H*-4') 4.16-4.10 (m, 1H, H_b-5'), 4.04-3.96 (m, 1H, *CH*CH_3 L-Ala), 1.41 (s, 9H, *t*Bu) 1.34 (d, *J* = 6.6 Hz, 1.5H, CHCH_3 L-Ala), 1.32 (d, *J* = 6.6 Hz, 1.5H, CHCH_3 L-Ala). **^{13}C NMR (125 MHz, $CDCl_3$) δ_C**: 173.3 (d, $^3J_{C\text{-}P}$ = 6.7 Hz, *C*=O, ester), 173.1 (d, $^3J_{C\text{-}P}$ = 6.2 Hz, *C*=O, ester), 165.8 (*C*-4), 165.7 (*C*-4), 155.28 (*C*-2), 155.27 (*C*-2), 151.6 (*C*=O, Boc), 151.5 (*C*=O, Boc), 150.5 (d, $^2J_{C\text{-}P}$ = 4.5 Hz, *C*-O, Ph), 150.4 (d, $^2J_{C\text{-}P}$ = 4.5 Hz, *C*-O, Ph), 141.4 (*C*-6), 135.2 (*C*-Ar), 129.8 (*CH*-Ar), 129.7 (*CH*-Ar), 128.6 (*CH*-Ar), 128.53 (*CH*-Ar), 128.50 (*CH*-Ar), 125.26 (*CH*-Ar), 125.22 (*CH*-Ar), 125.1 (*CH*-Ar), 120.52 (t, $^1J_{C\text{-}F}$ = 256.7 Hz, *C*-2'), 120.59 (t, $^1J_{C\text{-}F}$ = 256.7 Hz, *C*-2'), 120.2 (d, $^3J_{C\text{-}P}$ = 4.5 Hz, *CH*-Ar), 120.18 (d, $^3J_{C\text{-}P}$ = 4.5 Hz, *CH*-Ar), 95.7 (*C*-5), 95.6 (*C*-5), 84.6 (*C*$(CH_3)_3$), 83.9 (bs, *C*-1'), 77.3 (bs, *C*-4'), 77.2 (bs, *C*-4'), 72.8-72.3 (m, *C*-3'), 67.3 (CH_2Ph), 67.2 (CH_2Ph), 64.29 (*C*-5'), 50.4 (*CH*CH_3 L-Ala), 50.3 (*CH*CH_3 L-Ala), 27.6 (C(CH_3)$_3$), 27.5 (C(CH_3)$_3$), 20.74 (d, $^3J_{C\text{-}P}$ = 4.5 Hz, CHCH_3 L-Ala), 20.73 (d, $^3J_{C\text{-}P}$ = 5.2 Hz, CHCH_3 L-Ala). **(ES+) m/z**, found: 681.1 [M+H$^+$], $C_{30}H_{35}F_2N_4O_{10}P$ required: 680.21 [M]. **HPLC**: Reverse phase HPLC eluting with isocratic method MeOH/H_2O-70/30 in 35 minutes, 1ml/min, λ = 254 nm and 263 nm, showed two peaks with t_R 9.08 min. and t_R 11.40 min.

63 Fast Eluting. ^{31}P NMR (202 MHz, $CDCl_3$) δ_P: 2.54. **^{19}F NMR (470 MHz, $CDCl_3$) δ_F**: -115.05 (d, *J* = 247.0Hz), -119.49 (bs), -119.88 (bs). **^{1}H NMR (500 MHz, $CDCl_3$) δ_H**: 7.41 (d, *J* = 7.2 Hz, 1H, *H*-6), 7.27-7.20 (m, 7H, Ar*H*), 7.11-7.06 (m, 3H, Ar*H*), 6.31-6.24 (m, 1H, *H*-1'), 5.75 (d, *J* = 7.2 Hz, 1H, *H*-5), 5.09-5.02 (m, 3H, *H*-3', CH_2Ph), 4.41-4.37 (m, 1H, H_a-5'), 4.22-4.27 (m, 1H, H_b-5'), 4.18 (bs, 1H, *H*-4'), 4.06-3.98 (m, 1H, *CH*CH_3 L-Ala), 3.94 (bs, 1H, *NH* L-Ala), 1.42 (s, 9H, *t*Bu), 1.33 (d, *J* = 6.9 Hz, 3H, CHCH_3 L-Ala). **^{13}C NMR (125 MHz, $CDCl_3$) δ_C**: 173.2 (d, $^3J_{C\text{-}P}$ = 6.7 Hz, *C*=O, ester), 164.9 (*C*-4), 154.7 (*C*-2), 151.5 (*C*=O, Boc), 150.4 (d, $^2J_{C\text{-}P}$ = 6.9 Hz, *C*-O, Ph), 141.9 (*C*-6), 135.4 (*C*-Ar), 129.8 (*CH*-Ar), 128.6 (*CH*-Ar), 128.5 (*CH*-Ar), 128.2 (*CH*-Ar), 125.2 (*CH*-Ar), 120.4 (t, $^1J_{C\text{-}F}$ = 258.7 Hz, *C*-2'), 120.1 (d, $^3J_{C\text{-}P}$ = 5.2 Hz, *CH*-Ar), 95.6 (*C*-5), 84.6 (*C*$(CH_3)_3$), 83.7 (bs, *C*-1'), 72.6 (bs, *C*-4'), 72.6-72.3 (m, *C*-3'), 67.3 (CH_2Ph), 64.7 (d, $^2J_{C\text{-}P}$ = 4.8 Hz, *C*-5'), 50.4 (*CH*CH_3 L-Ala), 27.5 (C(CH_3)$_3$), 20.7 (d, $^3J_{C\text{-}P}$ = 4.8 Hz, CHCH_3 L-Ala). **(ES+) m/z**, found: 681.1 [M+H$^+$], $C_{30}H_{35}F_2N_4O_{10}P$ required: 680.21 [M].

63 Slow Eluting. ^{31}P NMR (202 MHz, $CDCl_3$) δ_P: 2.53. **^{19}F NMR (470 MHz, $CDCl_3$) δ_F**: -115.45 (d, *J* = 246.9 Hz), -119.86 (bs), -120.35 (bs). **^{1}H NMR (500 MHz, $CDCl_3$) δ_H**: 7.39-7.32 (m, 8H, Ar*H*, *H*-6), 7.22 (d, *J* = 8.6 Hz, 2H, Ar*H*), 7.18 (t, *J* = 7.3 Hz, 1H,

Ar*H*), 6.41-6.38 (m, 1H, *H*-1'), 5.60 (d, *J* = 7.5 Hz, 1H, *H*-5), 5.19, 5.15 (ABq, J_{AB} = 12.3 Hz, 2H, CH_2Ph), 5.14-5.10 (m, 1H, *H*-3'), 4.42 (ddd, *J* = 11.9 Hz, $^3J_{H\text{-}P}$= 6.7 Hz, *J* = 2.4 Hz, 1H, H_a-5'), 4.28 (ddd, *J* = 11.9 Hz, $^3J_{H\text{-}P}$= 7.0 Hz, *J* = 3.8 Hz, 1H, H_b-5'), 4.22 (bs, 1H, *H*-4'), 4.13-4.05 (m, 1H, *CH*CH_3 L-Ala), 3.92 (t, *J* = 9.9 Hz, 1H, *NH* L-Ala), 1.52 (s, 9H, *t*Bu), 1.43 (d, *J* = 7.0 Hz, 3H, CHCH_3 L-Ala). **^{13}C NMR (125 MHz, $CDCl_3$) δC**: 173.1 (d, $^3J_{C\text{-}P}$ = 6.4 Hz, *C*=O, ester), 165.5 (*C*-4), 155.1 (*C*-2), 151.6 (*C*=O, Boc), 150.4 (d, $^2J_{C\text{-}P}$ = 5.7 Hz, *C*-O, Ph), 141.8 (*C*-6), 135.2 (*C*-Ar), 129.8 (*CH*-Ar), 128.6 (*CH*-Ar), 128.5 (*CH*-Ar), 128.2 (*CH*-Ar), 125.1 (*CH*-Ar), 120.4 (t, $^1J_{C\text{-}F}$ = 258.7 Hz, *C*-2'), 120.1 (d, $^3J_{C\text{-}P}$ = 4.6 Hz, *CH*-Ar), 95.0 (*C*-5), 84.6 (*C*$(CH_3)_3$), 83.6 (bs, *C*-1'), 77.4 (bs, *C*-4'), 72.6-72.3 (m, *C*-3'), 67.3 (CH_2Ph), 64.3 (d, $^2J_{C\text{-}P}$ = 4.1 Hz, *C*-5'), 50.3 (*CH*CH_3 L-Ala), 27.5 (C(CH_3)$_3$), 20.8 (d, $^3J_{C\text{-}P}$ = 5.1 Hz, CHCH_3 L-Ala). **(ES+) m/z**, found: 681.1 [M+H$^+$], $C_{30}H_{35}F_2N_4O_{10}P$ required: 680.21 [M].

Benzyl ((((2*R*,3*R*)-5-(4-Acetamido-2-oxopyrimidin-1(2H)-yl)-3-acetoxy-4,4-difluorotetrahydrofuran-2-yl)methoxy)(phenoxy)phosphoryl)-*L*-alaninate 64



Prepared according to the standard procedure **A** for the synthesis of ProTides using 3'-(*O*-acetyl)-*N*^{4}-acetyl-gemcitabine **54** (200 mg, 575.91 µmol) in anhydrous THF (4 ml), tBuMgCl (633.50 µl, 633.50 µmol), Phenyl-(benzyloxy-L-Alanine)-phosphorochloridate **58** (244.47 mg, 691.10 µmol) in anhydrous THF (1 ml). After evaporation, the mixture was purified by Biotage Isolera One (25 g SNAP cartridge ULTRA, 75 ml/min, gradient eluent system MeOH/CH_2Cl_2 1% 1CV, 1-100% 12CV, 10% 2CV), to afford the title compound as a white foamy solid (182mg, 48%). R_f = 0.38 (CH_2Cl_2/MeOH – 9.5:0.5). **^{31}P NMR (202 MHz, $CDCl_3$) δP**: 2.94, 2.85. **^{19}F NMR (470 MHz, $CDCl_3$) δF**: -115.09 (d, *J* = 245.9 Hz), -115.16 (d, *J* = 247.4 Hz), -119.06 (bs), -119.54 (bs). **^{1}H NMR (500 MHz, $CDCl_3$) δH**: 7.69 (d, *J* = 7.5 Hz, 0.7H, *H*-6), 7.51 (d, *J* = 7.5 Hz, 0.3H, *H*-6), 7.38 (d, *J* = 7.5 Hz, 0.7H, *H*-5), 7.26-7.19 (m, 7.3H, *H*-5, Ar*H*), 7.14-7.06 (m, 3H, Ar*H*), 6.33-6.38 (m, 0.7H, *H*-1'), 6.30 (m, 0.3H, *H*-1'), 5.29-5.22 (m, 1H, *H*-3') 5.11-5.02 (m, 2H, CH_2Ph), 4.42-4.30 (m, 2H, *H*-5'), 4.22 (bs, 0.7H, *H*-4'), 4.16 (bs, 0.3H, *H*-4'), 4.07-3.96 (m, 1H, *CH*CH_3 L-Ala), 2.14 (s, 3H, CH_3), 2.06 (s, 3H, CH_3), 1.37 (d, *J* = 8.7 Hz, 0.9H, CHCH_3 L-Ala), 1.36 (d, *J* = 7.3 Hz, 2.1H, CHCH_3 L-Ala). **^{13}C NMR (125 MHz, $CDCl_3$) δC**: 173.3 (*C*=O, ester), 173.2 (*C*=O,

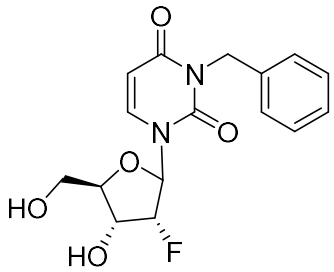
ester), 171.2 (*CO*CH_3), 169.0 (*CO*CH_3), 168.9 (*CO*CH_3), 163.4 (*C*-4), 163.3 (*C*-4), 154.7 (*C*-2), 150.49 (*C*-O, Ph), 150.44 (*C*-O, Ph), 144.8 (*C*-6), 135.3 (*C*-Ar), 135.2 (*C*-Ar), 129.89 (*CH*-Ar), 129.82 (*CH*-Ar), 128.6 (*CH*-Ar), 128.46 (*CH*-Ar), 128.44 (*CH*-Ar), 128.1 (*CH*-Ar), 125.3 (*CH*-Ar), 125.2 (*CH*-Ar), 120.4 (*CH*-Ar), 120.4 (t, $^1J_{C\text{-}F}$ = 260.3 Hz, *C*-2'), 120.3 (t, $^1J_{C\text{-}F}$ = 264.9 Hz, *C*-2'), 120.07 (d, $^3J_{C\text{-}P}$ = 4.7 Hz, *CH*-Ar), 97.5 (*C*-5), 97.4 (*C*-5), 78.3 (bs, *C*-1'), 77.3 (bs, *C*-4'), 70.2-69.9 (m, *C*-3'), 67.2 (*CH_2*Ph), 64.1 (d, $^2J_{C\text{-}P}$ = 4.5 Hz, *C*-5'), 63.9 (d, $^2J_{C\text{-}P}$ = 4.5 Hz, *C*-5'), 50.5 (*CH*CH_3 L-Ala), 50.4 (*CH*CH_3 L-Ala), 24.8 (CO*CH_3*), 20.6 (d, $^3J_{C\text{-}P}$ = 5.4 Hz, CH*CH_3* L-Ala), 20.5 (d, $^3J_{C\text{-}P}$ = 5.5 Hz, CH*CH_3* L-Ala), 20.3 (CO*CH_3*). **(ES+) m/z**, found: 665.6 [M+H^+], $C_{29}H_{31}F_2N_4O_{10}P$ required: 664.17 [M]. **HPLC**: Reverse phase HPLC eluting with isocratic method MeOH/H_2O-70/30 in 35 minutes, 1ml/min, λ = 254 nm and 263 nm, showed two peaks with t_R 5.37 min. and t_R 7.02 min.

64 Fast Eluting. ^{31}P NMR (202 MHz, $CDCl_3$) δ_P: 2.62. **^{19}F NMR (470 MHz, $CDCl_3$) δ_F**: -115.36 (d, *J* = 246.0 Hz), -119.24 (bs), -119.72 (bs). **^{1}H NMR (500 MHz, $CDCl_3$) δ_H**: 9.30 (bs, 1H, *NH*$COCH_3$), 7.71 (d, *J* = 7.4 Hz, 1H, *H*-6), 7.38 (d, *J* = 7.4 Hz, 1H, *H*-5), 7.27-7.20 (m, 7H, Ar*H*), 7.11-7.06 (m, 3H, Ar*H*), 6.38-6.34 (m, 1H, *H*-1'), 5.29-5.23 (m, 1H, *H*-3'), 5.09,5.05 (ABq, J_{AB} = 12.2 Hz, 2H, *CH_2*Ph), 4.40-4.37 (m, 1H, H_a-5'), 4.32-4.28 (m, 1H, H_b-5'), 4.22 (bs, 1H, *H*-4'), 4.09-4.01 (m, 1H, *CH*CH_3 L-Ala), 3.85-3.81 (m, 1H, *NH* L-Ala), 2.16 (s, 3H, *CH_3*), 2.09(s, 3H, *CH_3*), 1.34 (d, *J* = 7.0 Hz, 3H, CH*CH_3* L-Ala). **^{13}C NMR (125 MHz, $CDCl_3$) δ_C**: 173.2 (d, $^3J_{C\text{-}P}$ = 6.4 Hz, *C*=O, ester), 170.4 (*CO*CH_3), 168.9 (*CO*CH_3), 163.0 (*C*-4), 154.7 (*C*-2), 150.4 (d, $^2J_{C\text{-}P}$ = 7.1 Hz, *C*-O, Ph), 145.0 (*C*-6), 135.2 (*C*-Ar), 129.8 (*CH*-Ar), 128.6 (*CH*-Ar), 128.5 (*CH*-Ar), 128.2 (*CH*-Ar), 125.2 (*CH*-Ar), 120.4 (t, $^1J_{C\text{-}F}$ = 260.8 Hz, *C*-2'), 120.0 (d, $^3J_{C\text{-}P}$ = 4.9 Hz, *CH*-Ar), 97.3 (*C*-5), 78.4 (bs, *C*-1'), 77.2 (bs, *C*-4'), 70.4-70.0 (m, *C*-3'), 67.3 (*CH_2*Ph), 64.1 (d, $^2J_{C\text{-}P}$ = 4.5 Hz, *C*-5'), 50.5 (*CH*CH_3 L-Ala), 24.9 (CO*CH_3*), 20.8 (d, $^3J_{C\text{-}P}$ = 5.4 Hz, CH*CH_3* L-Ala), 20.3 (CO*CH_3*). **(ES+) m/z**, found: 665.6 [M+H^+], $C_{29}H_{31}F_2N_4O_{10}P$ required: 664.17 [M].

64 Slow eluting. ^{31}P NMR (202 MHz, $CDCl_3$) δ_P: 2.56. **^{19}F NMR (470 MHz, $CDCl_3$) δ_F**: -115.39 (d, *J* = 246.2 Hz), -119.37 (bs), -119.85 (bs). **^{1}H NMR (500 MHz, $CDCl_3$) δ_H**: 8.83 (bs, 1H, *NH*$COCH_3$), 7.54 (d, *J* = 7.4 Hz, 1H, *H*-6), 7.30-7.27 (m, 8H, *H*-5, Ar*H*), 7.15-7.08 (m, 3H, Ar*H*), 6.38-6.26 (m, 1H, *H*-1'), 5.26-5.21 (m, 1H, *H*-3'), 5.11,5.06 (ABq, J_{AB} = 12.1 Hz, 2H, *CH_2*Ph), 4.38-4.34 (m, 1H, H_a-5'), 4.23-4.18 (m, 1H, H_b-5'), 4.15 (bs, 1H, *H*-4'), 4.05-3.97 (m, 1H, *CH*CH_3 L-Ala), 3.82-3.77 (m, 1H, *NH* L-Ala), 2.16 (s, 3H, *CH_3*), 2.10(s, 3H, *CH_3*), 1.36 (d, *J* = 7.0 Hz, 3H, CH*CH_3* L-Ala). **^{13}C NMR**

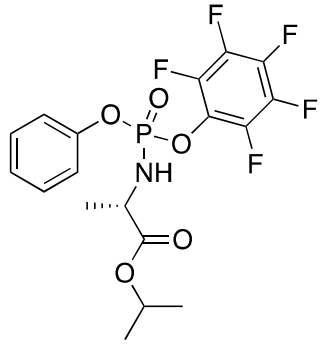
(125 MHz, $CDCl_3$) δ_C: 173.1 (d, $^3J_{C\text{-}P}$ = 6.4 Hz, *C*=O, ester), 170.1 (*CO*CH_3), 169.0 (*CO*CH_3), 162.7 (*C*-4), 154.7 (*C*-2), 150.4 (d, $^2J_{C\text{-}P}$ = 6.6 Hz, *C*-O, Ph), 145.0 (*C*-6), 135.2 (*C*-Ar), 129.9 (*CH*-Ar), 128.6 (*CH*-Ar), 128.5 (*CH*-Ar), 128.2 (*CH*-Ar), 125.4 (*CH*-Ar), 120.2 (t, $^1J_{C\text{-}F}$ = 260.9 Hz, *C*-2'), 120.0 (d, $^3J_{C\text{-}P}$ = 4.6 Hz, *CH*-Ar), 97.0 (*C*-5), 78.4 (bs, *C*-1'), 77.2 (bs, *C*-4'), 70.3-69.9 (m, *C*-3'), 67.3 (*CH_2*Ph), 63.9 (d, $^2J_{C\text{-}P}$ = 4.2 Hz, *C*-5'), 50.3 (*CH*CH_3 L-Ala), 25.0 (CO*CH_3*), 20.8 (d, $^3J_{C\text{-}P}$ = 5.8 Hz, CH*CH_3* L-Ala), 20.3 (CO*CH_3*). **(ES+) m/z**, found: 665.6 [$M+H^+$], $C_{29}H_{31}F_2N_4O_{10}P$ required: 664.17 [M].

3-benzyl-1-((3*R*,4*R*,5*R*)-3-fluoro-4-hydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)pyrimidine-2,4(1H,3H)-dione 65



Under an argon atmosphere, nucleoside **36** (300 mg, 1.22 mmol) and anhydrous K_2CO_3 (286.3 mg, 2.07 mmol) were added to a solution of DMF (1.5 ml) and acetone (1.5 ml). Benzyl bromide (217.4 µl, 2.07 mmol) was added dropwise to the solution, and the resulting mixture was refluxed for 4h. The mixture was evaporated to dryness and purified by Biotage Isolera One (25 g SNAP cartridge KP-SIL, 50 ml/min, gradient eluent system MeOH/CH_2Cl_2 2% 1CV, 2-20% 10CV, 20% 2CV) to give **65** as a colourless oil (38 mg, 10%). R_f = 0.18 (CH_2Cl_2/MeOH – 9:1). **^{1}H NMR (500 MHz, DMSO) δ_H**: 7.6 (d, *J* = 7.7 Hz, 1H, *H*-6), 7.31-7.29 (m, 2H, Ar*H*), 7.19-7.11 (m, 3H, Ar*H*), 5.79 (dd, $^3J_{H\text{-}F}$ = 17.2 Hz, *J* = 2.3 Hz, 1H, *H*-1'), 5.66 (d, *J* = 8.0 Hz, 1H, *H*-5), 5.00-4.85 (m, 3H, *CH_2*, *H*-2'), 4.30-4.25 (m, 1H, *H*-3'), 4.00-3.85 (m, 3H, H-4', H-5_a', *OH*-3'), 3.69-3.67 (m, 2H, H-5_b', *OH*-5').

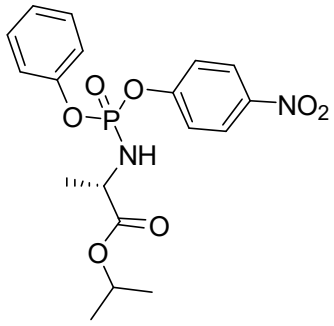
Isopropyl ((perfluorophenoxy)(phenoxy)phosphoryl)-*L*-alaninate 67[7]



Phenyl-(isopropyloxy-L-Alanine)-phosphorochloridate **22** (1.5 g, 4.91 mmol) and 2,3,4,5,6-pentafluoro phenol (903.18 mg, 4.91 mmol) were dissolved in anhydrous CH_2Cl_2 (11 ml) under an argon atmosphere. The solution was cooled at 0˚C in a ice/water bath and Et_3N (684 µl, 4.91 mmol) was then added in a dropwise fashion. After 16 h, the reaction mixture was washed very quickly with H_2O (10 ml), dried over $MgSO_4$ and evaporated *in vacuo.* The residue was purified by Biotage Isolera One (120 g ZIP cartridge KP-SIL, 100 ml/min, gradient eluent system MeOH/CH_2Cl_2 0% 1CV, 0-4% 10CV, 4% 2CV) to give **67** as a white solid (1.2 g, 54%). R_f = 0.81 (CH_2Cl_2/MeOH –

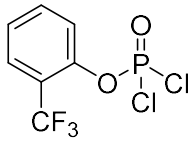
9.8:0.2). **^{31}P NMR (202 MHz, $CDCl_3$) δ_P**: -1.65, -1.72; **^{19}F NMR (470 MHz, $CDCl_3$) δ_F**: -153.16 (d, *J* = 19.7 Hz, 0.8 F), -153.26 (d, *J* = 19.7 Hz, 1.2 F), -159.31 (t, *J* = 22.0 Hz, 0.4 F), -159.47 (t, *J* = 21.7 Hz, 0.6 F), -162.00-(-162.18) (m, 2F). **^{1}H NMR (500 MHz, $CDCl_3$) δ_H**: 7.30-7.26 (m, 2H, Ar*H*), 7.22-7.12 (m, 3H, Ar*H*), 5.03-4.93 (m, 1H, *CH*(CH_3)$_2$), 4.11-4.02 (m, 1H, *CH*CH_3 L-Ala), 3.97-3.88 (m, 1H, *NH* L-Ala), 1.38 (d, *J* = 6.6 Hz, 1.8H, CH*CH$_3$* L-Ala), 1.36 (d, *J* = 6.9 Hz, 1.2H, CH*CH$_3$* L-Ala), 1.20-1.16 (m, 6H, CH(*CH$_3$*)$_2$).

Isopropyl ((4-nitrophenoxy)(phenoxy)phosphoryl)-*L*-alaninate 70[7]



A solution of phenol **68** (1.47 g, 15.62 mmol) and anhydrous triethylamine (2.4 ml, 17.19 mmol) in anhydrous dichloromethane (35 ml) was added dropwise to a solution of *p*-nitrophenyl phosphorodichloridate (4.00 g, 15.62 mmol) in anhydrous dichloromethane (35 ml) under argon atmosphere at -78°C. The resulting mixture was stirred at that temperature for 30 minutes and after that period, when ^{31}P NMR confirmed completion of the reaction ($CDCl_3$, a singlet at -6.00 ppm corresponding to desired phosphorochloridate was observed), the reaction mixture was added to a cold solution (0°C) of *L*-alanine isopropyl ester hydrochloride (2.62 g, 15.62 mmol) in anhydrous dichloromethane (35 ml). Subsequently, anhydrous triethylamine (4.6 ml, 32.82 mmol) was added dropwise and the mixture was stirred at 0°C for further 30 minutes. Once ^{31}P NMR confirmed completion of the reaction dichloromethane was evaporated under reduced pressure without any contact with air. The residue was suspended in diethyl ether and stirred at 0°C for 30 minutes. The white solid was filtered off and the filtrate was concentrated under reduced pressure on rotary evaporator without any contact with air to obtain **70** as yellow oil (6.12 g, 96%). **^{31}P NMR (202 MHz, $CDCl_3$) δ_P**: -3.11, -3.14. **^{1}H-NMR ($CDCl_3$, 500 MHz) δ_H**: 8.30-8.16 (m, 2H, Ar*H*) 7.44-7.12 (m, 7H, Ar*H*), 5.02-4.89 (m, 1H, *CH*(CH_3)$_2$), 4.12-4.02 (m, 2H, *CH*CH_3 L-Ala, -*NH*), 1.42-1.31 (m, 3H, CH*CH$_3$* L-Ala), 1.25-1.20 (m, 6H, CH(*CH$_3$*)$_2$).

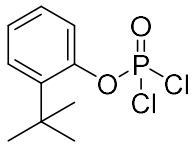
2-(Trifluoromethyl)phenyl phosphorodichloridate 75



Prepared according to the standard procedure **F** using 2-(trifluoromethyl)phenol **71** (1 g, 6.17 mmol) in anhydrous diethyl ether (50 ml), $POCl_3$ (575 µl, 6.17 mmol), E_3N (860 µl, 6.17mmol). After

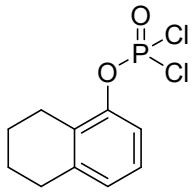
evaporation, compound **75** was obtained as clear oil (1.7 g, 98%). **^{31}P NMR (202 MHz, $CDCl_3$) δ_P**: 3.82.**^{1}H-NMR ($CDCl_3$, 500 MHz) δ_H**: 7.76 (d, *J* = 7.7 Hz, 1H, Ar*H*), 7.70-7.63 (m, 2H, Ar*H*), 7.44 (t, *J* = 7.3 Hz, 1H, Ar*H*). **^{19}F NMR (470 MHz, $CDCl_3$) δ_F**: -61.16.

2-(*tert*-Butyl)phenyl phosphorodichloridate 76



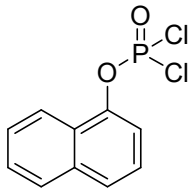
Prepared according to the standard procedure **F** using 2-(*tert*-butyl)phenol **72** (1 ml, 6.66 mmol) in anhydrous diethyl ether (50 ml), $POCl_3$ (620 µl, 6.66 mmol), E_3N (927 µl, 6.66 mmol). After evaporation, compound **76** was obtained as clear oil (1.5 g, 84%). **^{31}P NMR (202 MHz, $CDCl_3$) δ_P**: 2.81.**^{1}H-NMR ($CDCl_3$, 500 MHz) δ_H**: 7.32 (d, *J* = 7.8 Hz, 1H, Ar*H*), 7.25-7.20 (m, 2H, Ar*H*), 7.10-7.05 (m, 1H, Ar*H*), 1.45 (s, 9H, C(CH_3)$_3$).

5,6,7,8-Tetrahydronaphthalen-1-yl phosphorodichloridate 77



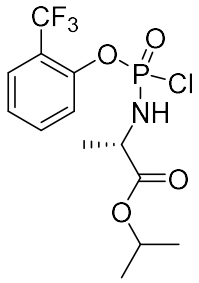
Prepared according to the standard procedure **F** using 5,6,7,8-tetrahydronaphthalen-1-ol **73** (1 g, 6.75 mmol) in anhydrous diethyl ether (50 ml), $POCl_3$ (629 µl, 6.75 mmol), E_3N (940 µl, 6.75 mmol). After evaporation, compound **77** was obtained as clear oil (1.7 g, 96%). **^{31}P NMR (202 MHz, $CDCl_3$) δ_P**: 3.55. **^{1}H-NMR ($CDCl_3$, 500 MHz) δ_H**: 7.45-7.10 (m, 2H, Ar*H*), 7.02-6.98 (m, 1H, Ar*H*), 2.83-2.59 (m, 2H, Ar*H*), 1.96-1.65 (m, 2H, Ar*H*).

Naphthalen-1-yl phosphorodichloridate 78



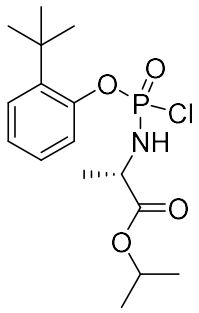
Prepared according to the standard procedure **F** using naphthalen-1-ol **74** (1 g, 6.94 mmol) in anhydrous diethyl ether (50 ml), $POCl_3$ (648 µl, 6.94 mmol), E_3N (968 µl, 6.94 mmol). After evaporation, compound **78** was obtained as clear oil (1.78 g, 98%). **^{31}P NMR (202 MHz, $CDCl_3$) δ_P**: 3.81.**^{1}H-NMR ($CDCl_3$, 500 MHz) δ_H**: 8.01 (d, *J* = 8.1 Hz, 1H, Ar*H*), 7.81-7.80 (m, 1H, Ar*H*), 7.71 (d, *J* = 8.2 Hz, 1H, Ar*H*), 7.54-7.45 (m, 3H, Ar*H*), 7.38-7.35 (m, 1H, Ar*H*).

Isopropyl (chloro(2-(trifluoromethyl)phenoxy)phosphoryl)-*L*-alaninate 79



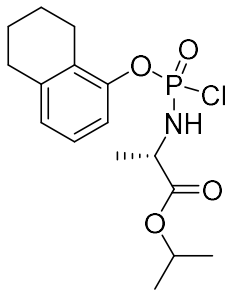
Prepared according to procedure **G** using *L*-Alanine isopropyl ester hydrochloride salt **56** (1 g, 5.97 mmol) in anhydrous CH_2Cl_2 (100 ml), the dichlorophosphate **75** (1.66 g, 5.97 mmol), Et_3N (1.66 ml, 11.93 mmol). After evaporation, compound **79** was obtained as a clear oil (2.05 g, 92%). **^{31}P NMR (202 MHz, $CDCl_3$) δ_P**: 7.26, 6.71. **^{1}H-NMR ($CDCl_3$, 500 MHz) δ_H**: 7.80-7.50 (m, 3H, Ar*H*), 7.36-7.32 (m, 1H, Ar*H*), 5.13-4.96 (m, 1H, *CH*$(CH_3)_2$), 4.52-4.39 (m, 1H, *NH* L-Ala), 4.23-3.99 (m, 1H, *CH*CH_3 L-Ala), 1.52 (d, *J* = 7.4 Hz, 1.5H, CH*CH$_3$* L-Ala), 1.50 (d, *J* = 7.3 Hz, 1.5H, CH*CH$_3$* L-Ala), 1.31-1.21 (m, 6H, CH(*CH$_3$*)$_2$). **^{19}F NMR (470 MHz, $CDCl_3$) δ_F**: -62.12, -62.23.

Isopropyl ((2-(*tert*-butyl)phenoxy)chlorophosphoryl)-*L*-alaninate 80



Prepared according to procedure **G** using *L*-Alanine isopropyl ester hydrochloride salt **56** (750 mg, 4.47 mmol) in anhydrous CH_2Cl_2 (50 ml), the dichlorophosphate **76** (1.19 g, 4.47 mmol), Et_3N (1.25 ml, 8.95 mmol). After evaporation, compound **80** was obtained as a clear oil (1 g, 61%). **^{31}P NMR (202 MHz, $CDCl_3$) δ_P**: 6.39, 6.06.**^{1}H-NMR ($CDCl_3$, 500 MHz) δ_H**: 7.79-7.68 (m, 3H, Ar*H*), 7.30-7.28 (m, 1H, Ar*H*) 5.12-4.97 (m, 1H, *CH*$(CH_3)_2$), 4.39-4.18 (m, 2H, *NH*, *CH*CH_3 L-Ala), 1.52 (d, *J* = 6.8 Hz, 1.5H, CH*CH$_3$* L-Ala), 1.50 (d, *J* = 7.1 Hz, 1.5H, CH*CH$_3$* L-Ala), 1.43 (s, 4.5H, C(*CH$_3$*)$_3$), 1.42 (s, 4.5H, C(*CH$_3$*)$_3$), 1.30-1.22 (m, 6H, CH(*CH$_3$*)$_2$).

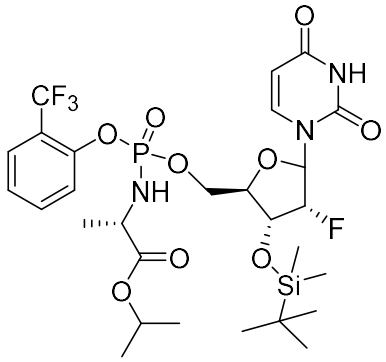
Isopropyl (chloro((5,6,7,8-tetrahydronaphthalen-1-yl)oxy)phosphoryl)-*L*-alaninate 81



Prepared according to procedure **G** using *L*-Alanine isopropyl ester hydrochloride salt **56** (1.1 g, 6.56 mmol) in anhydrous CH_2Cl_2 (100 ml), the dichlorophosphate **77** (1.74 g, 6.56 mmol), Et_3N (1.83 ml, 13.12 mmol). After evaporation, compound **81** was obtained as a clear oil (2 g, 87%). **^{31}P NMR (202 MHz, $CDCl_3$) δ_P**: 7.71, 7.60. **^{1}H-NMR ($CDCl_3$, 500 MHz) δ_H**: 7.38-7.08 (m, 2H, Ar*H*), 7.00-6.93 (m, 1H, Ar*H*), 5.11-4.91 (m, 1H, *CH*$(CH_3)_2$), 4.42-4.11 (m, 1H, *NH* L-Ala), 4.09-3.85 (m, 1H, *CH*CH_3 L-Ala), 2.63-2.59 (m, 2H, Ar*H*), 1.63-1.55 (m, 2H, Ar*H*), 1.48 (d, *J* = 6.9

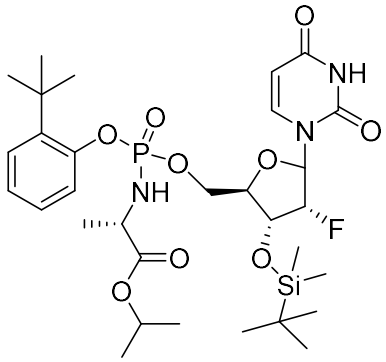
Hz, 1.5H, CH*CH*$_3$ L-Ala), 1.46 (d, *J* = 7.0 Hz, 1.5H, CH*CH*$_3$ L-Ala), 1.37-1.13 (m, 6H, CH(*CH*$_3$)$_2$).

Isopropyl ((((2*R*,3*R*,4*R*)-3-((*tert*-butyldimethylsilyl)oxy)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-fluorotetrahydrofuran-2-yl)methoxy)(2-(trifluoromethyl)phenoxy)phosphoryl)-*L*-alaninate 82



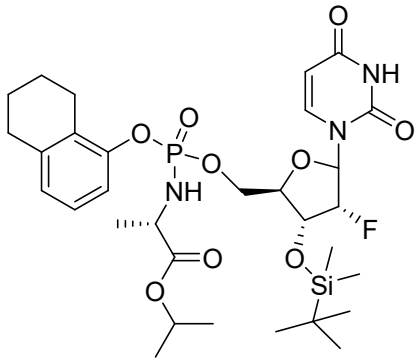
Prepared according to the standard procedure **A** using nucleoside **43** (100 mg, 277.43 µmol) in anhydrous THF (4 ml), tBuMgCl (554.8 µl, 554.8 µmol), phosphorochloridate **79** (207.3 mg, 554.8 µmol) in anhydrous THF (1 ml). After evaporation, the residue was purified by Biotage Isolera One (10 g SNAP cartridge ULTRA, 36 ml/min, gradient eluent system MeOH/CH_2Cl_2 2% 1CV, 2-20% 12CV, 20% 2CV) to give **82** as a colourless oil (78 mg, 40%). R_f = 0.41 (CH_2Cl_2/MeOH – 9.5:0.5). **^{31}P NMR (202 MHz, $CDCl_3$) δ_P**: 3.78, 3.83. **^{19}F NMR (470 MHz, $CDCl_3$) δ_F**: -201.71, -202.04, -61.14, -61.22. **^{1}H NMR (500 MHz, $CDCl_3$) δ_H**: 9.49 (bs, 1H, *NH*), 7.82-7.54 (m, 3H, Ar*H*), 7.43-7.40 (m, 1H, Ar*H*), 7.32 (d, *J* = 7.8 Hz, 0.6H, *H*-6), 7.31 (d, *J* = 7.7 Hz, 0.4H, *H*-6), 5.82-5.71 (m, 1H, *H*-1'), 5.66 (d, *J* = 7.8 Hz, 0.6H, *H*-5), 5.52 (d, *J* = 7.7 Hz, 0.4H, *H*-5), 4.89-4.80 (m, 1H, *CH*(CH_3)$_2$), 4.78-4.65 (m, 1H, *H*-2'), 4.38-4.33 (m, 1H, H_a-5'), 4.22-4.19 (m, 1H, *H*-3'), 4.11-4.07 (m, 1H, H_b-5'), 3.99 (bs, 1H, *H*-4'), 3.89-3.75 (m, 2H, *NH* L-Ala, *CH*CH_3 L-Ala), 1.22 (d, *J* = 7.2 Hz, 1.8H, CH*CH*$_3$ L-Ala), 1.19 (d, *J* = 6.9 Hz, 1.2H, CH*CH*$_3$ L-Ala), 1.11-0.95 (m, 6H, CH(*CH*$_3$)$_2$), 0.75 (s, 4.5H, *t*Bu), 0.73 (s, 4.5H, *t*Bu), 0.01 (s, 1.5H, *CH*$_3$), -0.00 (s, 1.5H, *CH*$_3$), -0.01 (s, 1.5H, *CH*$_3$), -0.03 (s, 1.5H, *CH*$_3$).

Isopropyl ((2-(*tert*-butyl)phenoxy)(((2*R*,3*R*,4*R*)-3-((*tert*-butyldimethylsilyl)oxy)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-fluorotetrahydrofuran-2-yl)methoxy)phosphoryl)-*L*-alaninate 83



Prepared according to the standard procedure **A** using nucleoside **43** (100 mg, 277.43 μmol) in anhydrous THF (4 ml), tBuMgCl (554.8 μl, 554.8 μmol), phosphorochloridate **80** (200.7 mg, 554.8 μmol) in anhydrous THF (1 ml). After evaporation, the residue was purified by Biotage Isolera One (10 g SNAP cartridge ULTRA, 36 ml/min, gradient eluent system MeOH/CH_2Cl_2 2% 1CV, 2-20% 12CV, 20% 2CV) to give **83** as a colourless oil (80 mg, 42%). R_f = 0.32 (CH_2Cl_2/MeOH – 9.5:0.5). **^{31}P NMR (202 MHz, $CDCl_3$) δ_P**: 2.85, 2.46. **^{19}F NMR (470 MHz, $CDCl_3$) δ_F**: -202.81, -203.02. **^{1}H NMR (500 MHz, $CDCl_3$) δ_H**: 8.00 (bs, 0.5H, *NH*), 7.99 (bs, 0.5H, *NH*), 7.51 (d, *J* = 8.1 Hz, 0.5H, *H*-6), 7.47 (d, *J* = 8.0 Hz, 0.5H, *H*-6), 7.44-7.35 (m, 1H, Ar*H*), 7.31-7.19 (m, 2H, Ar*H*), 7.06-6.92 (m, 1H, Ar*H*), 5.79-5.74 (m, 1H, *H*-1'), 5.56 (d, *J* = 8.0 Hz, 1H, *H*-5), 5.02 (bs, 0.5H, *H*-2'), 4.91 (bs, 0.5H, *H*-2'), 4.83-4.75 (m, 1H, *CH*(CH_3)$_2$), 4.42-4.30 (m, 2H, H_a-5', *H*-3'), 4.21-4.07 (m, 1H, H_b-5'), 4.03-4.00 (m, 1H, *H*-4'), 3.83-3.65 (m, 2H, *NH* L-Ala, *CH*CH_3 L-Ala), 1.27 (s, 4.5H, C(*CH_3*)$_3$), 1.26 (s, 4.5H, C(*CH_3*)$_3$), 1.20 (d, *J* = 7.1 Hz, 1.5H, CH*CH_3* L-Ala), 1.17 (d, *J* = 6.9 Hz, 1.5H, CH*CH_3* L-Ala), 1.04-1.01 (m, 6H, CH(*CH_3*)$_2$), 0.78 (s, 9H, *t*Bu-Si), 0.00 (s, 6H, *CH_3*).

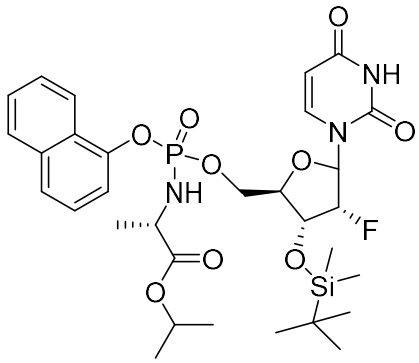
Isopropyl ((((2*R*,3*R*,4*R*)-3-((*tert*-butyldimethylsilyl)oxy)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-fluorotetrahydrofuran-2-yl)methoxy)((5,6,7,8-tetrahydronaphthalen-1-yl)oxy)phosphoryl)-*L*-alaninate 84



Prepared according to the standard procedure **A** using nucleoside **43** (100 mg, 277.43 μmol) in anhydrous THF (4 ml), tBuMgCl (554.8 μl, 554.8 μmol), phosphorochloridate **81** (199.6 mg, 554.8 μmol) in anhydrous THF (1 ml). After evaporation, the residue was purified by Biotage Isolera One (10 g SNAP cartridge ULTRA, 36 ml/min, gradient eluent system MeOH/CH_2Cl_2 2% 1CV, 2-20% 12CV, 20% 2CV) to give **84** as a colourless oil (89 mg, 47%). R_f = 0.35 (CH_2Cl_2/MeOH – 9.5:0.5). **^{31}P NMR (202 MHz, CD_3OD) δ_P**: 3.80, 3.63.

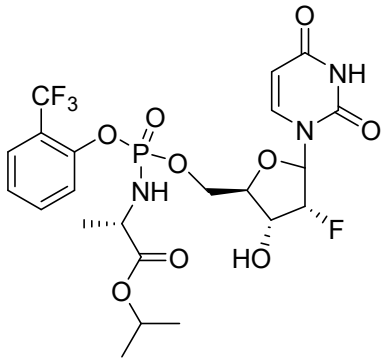
^{19}F NMR (470 MHz, $CDCl_3$) δ_F: -201.81, -201.06. **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 7.53 (d, *J* = 8.1 Hz, 0.5H, *H*-6), 7.47 (d, *J* = 8.0 Hz, 0.5H, *H*-6), 7.01-6.98 (m, 1H, Ar*H*), 6.90-6.86 (m, 1H, Ar*H*), 6.75-6.71 (m, 1H, Ar*H*), 5.79-5.75 (m, 1H, *H*-1'), 5.53 (d, *J* = 8.1 Hz, 0.5H, *H*-5), 5.48 (d, *J* = 8.0 Hz, 0.5H, *H*-5), 4.97-4.89 (m, 1H, *H*-2'), 4.85-4.79 (m, 1H, *CH*$(CH_3)_2$), 4.39-4.23 (m, 2H, H_a-5', *H*-3'), 4.13-4.07 (m, 1H, H_b-5'), 3.97 (bs, 1H, *H*-4'), 3.83-3.72 (m, 1H, *CH*CH_3 L-Ala), 2.59 (bs, 2H, Ar*H*), 1.62-1.59 (m, 2H, Ar*H*), 1.21 (d, *J* = 7.0 Hz, 1.5H, CH*CH*$_3$ L-Ala), 1.19 (d, *J* = 7.2 Hz, 1.5H, CH*CH*$_3$ L-Ala), 1.09-1.04 (m, 6H, CH(*CH*$_3$)$_2$), 0.78 (s, 4.5H, *t*Bu), 0.76 (s, 4.5H, *t*Bu), 0.00 (s, 3H, *CH*$_3$), -0.02 (s, 1.5H, *CH*$_3$), -0.04 (s, 1.5H, *CH*$_3$).

Isopropyl ((((2*R*,3*R*,4*R*)-3-((*tert*-butyldimethylsilyl)oxy)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-fluorotetrahydrofuran-2-yl)methoxy)(naphthalen-1-yloxy)phosphoryl)-*L*-alaninate 85



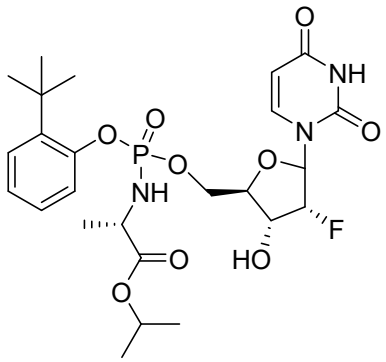
Prepared according to the standard procedure **A** using nucleoside **43** (200 mg, 554.8 μmol) in anhydrous THF (8 ml), tBuMgCl (1.11 ml, 1.11 mmol), phosphorochloridate **37** (236.8 mg, 665.8 μmol) in anhydrous THF (1 ml). After evaporation, the residue was purified by Biotage Isolera One (25 g SNAP cartridge ULTRA, 50 ml/min, gradient eluent system MeOH/CH_2Cl_2 2% 1CV, 2-20% 12CV, 20% 2CV) to give **85** as a colourless oil (168 mg, 44%). R_f = 0.38 (CH_2Cl_2/MeOH – 9.5:0.5). **^{31}P NMR (202 MHz, $CDCl_3$) δ_P**: 3.13, 2.85. **^{19}F NMR (470 MHz, $CDCl_3$) δ_F**: -201.80, -201.03. **^{1}H NMR (500 MHz, $CDCl_3$) δ_H**: 8.22 (bs, 0.5H, *NH*), 8.18 (bs, 0.5H, *NH*), 8.05-8.01 (m, 1H, Ar*H*), 7.78-7.49 (m, 1H, Ar*H*), 7.60-7.59 (m, 1H, Ar*H*), 7.51-7.41 (m, 3H, Ar*H*), 7.36-7.30 (m, 1H, Ar*H*), 7.13 (d, *J* = 8.0 Hz, 0.5H, *H*-6), 7.08 (d, *J* = 8.1 Hz, 0.5H, *H*-6), 5.84-5.77 (m, 1H, *H*-1'), 5.40 (d, *J* = 8.2 Hz, 0.5H, *H*-5), 5.39 (d, *J* = 7.8 Hz, 0.5H, *H*-5), 5.12-5.01 (m, 1H, *H*-2'), 4.95-4.86 (m, 2H, *H*-3', *CH*$(CH_3)_2$), 4.49-4.41 (m, 1H, H_a-5', *H*-3'), 4.38-4.33 (m, 1H, H_b-5'), 4.30-4.26 (m, 1H, *H*-4'), 4.01-3.85 (m, 1H, *CH*CH_3 L-Ala), 3.82-3.73 (m, 1H, *NH* L-Ala), 1.22 (d, *J* = 7.1 Hz, 1.5H, CH*CH*$_3$ L-Ala), 1.18 (d, *J* = 7.1 Hz, 1.5H, CH*CH*$_3$ L-Ala), 1.10-1.02 (m, 6H, CH(*CH*$_3$)$_2$), 0.78 (s, 9H, *t*Bu), 0.00 (s, 3H, *CH*$_3$), -0.02 (s, 1.5H, *CH*$_3$), -0.04 (s, 1.5H, *CH*$_3$).

Isopropyl ((((2*R*,3*R*,4*R*)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-fluoro-3-hydroxytetrahydrofuran-2-yl)methoxy)(2-(trifluoromethyl)phenoxy)phosphoryl)-*L*-alaninate 86



Prepared according to the standard procedure **H** using protected ProTide **82** (100 mg, 143.3 μmol) in anhydrous CH_2Cl_2 (2 ml) and TFA (2 ml). After evaporation, the mixture was purified by preparative TLC (CH_2Cl_2/MeOH – 9.5:0.5), to afford the title compound as a white foamy solid (53 mg, 63%). R_f = 0.28 (CH_2Cl_2/MeOH – 9:1). **^{31}P NMR (202 MHz, $CDCl_3$) δ_P**: 3.84, 3.75. **^{19}F NMR (470 MHz, $CDCl_3$) δ_F**: -201.81, -202.06, -61.53, -61.64. **^{1}H NMR (500 MHz, $CDCl_3$) δ_H**: 9.53 (bs, 1H, *NH*), 7.79-7.34 (m, 3H, Ar*H*), 7.31-7.25 (m, 1H, Ar*H*), 7.23 (d, *J* = 6.9 Hz, 0.5H, *H*-6), 7.21 (d, *J* = 7.1 Hz, 0.5H, *H*-6), 5.81-5.73 (m, 1H, *H*-1'), 5.58 (d, *J* = 6.9 Hz, 0.5H, *H*-5), 5.51 (d, *J* = 7.1 Hz, 0.5H, *H*-5), 4.88-4.79 (m, 1H, *CH*(CH_3)$_2$), 4.76-4.60 (m, 1H, *H*-2'), 4.38-4.33 (m, 1H, H_a-5'), 4.21-4.10 (m, 1H, *H*-3'), 4.09-4.03 (m, 1H, H_b-5'), 3.99-3.95 (m, 1H, *H*-4'), 3.89-3.73 (m, 2H, *NH* L-Ala, *CH*CH_3 L-Ala), 1.25 (d, *J* = 7.1 Hz, 1.5H, CH*CH$_3$* L-Ala), 1.20 (d, *J* = 6.8 Hz, 1.5H, CH*CH$_3$* L-Ala), 1.10-0.98 (m, 6H, CH(*CH$_3$*)$_2$). **(ES+) m/z**, found: 584.2 [M+H$^+$], $C_{22}H_{26}F_4N_3O_9P$ required: 583.13 [M]. **HPLC**: Reverse phase HPLC eluting with gradient method MeOH/H_2O from 10/90 to 100/0 in 45 minutes, 1ml/min, λ = 254 nm and 263 nm, showed two peaks with t_R 21.98 min and 24.43 min.

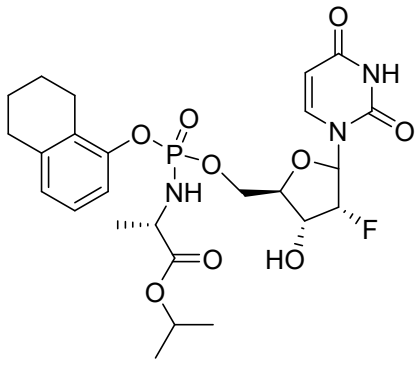
Isopropyl ((2-(*tert*-butyl)phenoxy)(((2*R*,3*R*,4*R*)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-fluoro-3-hydroxytetrahydrofuran-2-yl)methoxy)phosphoryl)-*L*-alaninate 87



Prepared according to the standard procedure **H** using protected ProTide **83** (80 mg, 116.6 μmol) in anhydrous CH_2Cl_2 (2 ml) and TFA (2 ml). After evaporation, the mixture was purified by preparative TLC (CH_2Cl_2/MeOH-9.5:0.5), to afford the title compound as a white foamy solid (43 mg, 64%). R_f = 0.25 (CH_2Cl_2/MeOH – 9:1). **^{31}P NMR (202 MHz, CD_3OD) δ_P**: 2.96, 2.63. **^{19}F NMR (470 MHz, CD_3OD) δ_F**: -202.64, -203.28. **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 7.53 (d, *J* = 7.8 Hz, 0.5H, *H*-6), 7.49 (d, *J* = 8.0 Hz, 0.5H, *H*-6), 7.40-7.27

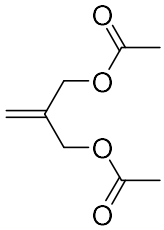
(m, 2H, Ar*H*), 7.08-6.98 (m, 2H, Ar*H*), 5.87-5.79 (m, 1H, *H*-1'), 5.46 (d, *J* = 7.8 Hz, 0.5H, *H*-5), 5.37 (d, *J* = 8.0 Hz, 0.5H, *H*-5), 5.03-5.01 (m, 0.5H, *H*-2'), 4.93-4.90 (m, 0.5H, *H*-2'), 4.88-4.78 (m, 1H, *CH*$(CH_3)_2$), 4.48-4.38 (m, 1H, H_a-5'), 4.34-4.20 (m, 2H, H_b-5', *H*-3'), 4.14-4.08 (m, 1H, *H*-4'), 3.89-3.78 (m, 1H, *CH*CH_3 L-Ala), 1.30 (s, 9H, C(*CH*$_3$)$_3$), 1.26 (d, *J* = 7.9 Hz, 1.5H, CH*CH*$_3$ L-Ala), 1.24 (d, *J* = 7.5 Hz, 1.5H, CH*CH*$_3$ L-Ala), 1.12-1.04 (m, 6H, CH(*CH*$_3$)$_2$). **(ES+) m/z**, found: 572.3 [M+H^+] and 594.2 [M+Na^+], $C_{25}H_{35}FN_3O_9P$ required: 571.21 [M]. **HPLC**: Reverse phase HPLC eluting with gradient method MeOH/H_2O from 10/90 to 100/0 in 45 minutes, 1ml/min, λ = 254 nm and 263 nm, showed two peaks with t_R 34.23 min and 35.37 min.

Isopropyl ((((2*R*,3*R*,4*R*)-5-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-fluoro-3-hydroxytetrahydrofuran-2-yl)methoxy)((5,6,7,8-tetrahydronaphthalen-1-yl)oxy)phosphoryl)-L-alaninate 88



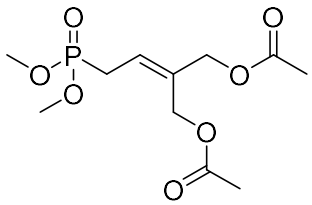
Prepared according to the standard procedure **H** using protected ProTide **84** (90 mg, 131.6 μmol) in anhydrous CH_2Cl_2 (2 ml) and TFA (2 ml). After evaporation, the mixture was purified by preparative TLC (CH_2Cl_2/MeOH – 9.5:0.5), to afford the title compound as a white foamy solid (45 mg, 66%). R_f = 0.21 (CH_2Cl_2/MeOH – 9:1). **^{31}P NMR (202 MHz, CD_3OD) δ_P**: 3.84, 3.78. **^{19}F NMR (470 MHz, CD_3OD) δ_F**: -202.80, -202.03. **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 7.59 (d, *J* = 8.1 Hz, 0.5H, *H*-6), 7.51 (d, *J* = 7.9 Hz, 0.5H, *H*-6), 7.04-6.92 (m, 2H, Ar*H*), 6.80-6.79 (m, 1H, Ar*H*), 5.89-5.76 (m, 1H, *H*-1'), 5.54 (d, *J* = 7.9 Hz, 0.5H, *H*-5), 5.46 (d, *J* = 8.1 Hz, 0.5H, *H*-5), 4.99-4.94 (m, 1H, *H*-2'), 4.89-4.84 (m, 1H, *CH*$(CH_3)_2$), 4.46-4.32 (m, 1H, H_a-5'), 4.42-4.18 (m, 2H, H_b-5', *H*-3'), 4.11-4.03 (m, 1H, *H*-4'), 3.84-3.78 (m, 1H, *CH*CH_3 L-Ala), 2.65-2.60 (m, 2H, Ar*H*), 1.68-1.57 (m, 2H, Ar*H*), 1.26 (d, *J* = 7.2 Hz, 1.5H, CH*CH*$_3$ L-Ala), 1.23 (d, *J* = 7.1 Hz, 1.5H, CH*CH*$_3$ L-Ala), 1.13-1.04 (m, 6H, CH(*CH*$_3$)$_2$). **(ES+) m/z**, found: 592.2 [M+Na^+], $C_{25}H_{33}FN_3O_9P$ required: 569.19 [M]. **HPLC**: Reverse phase HPLC eluting with gradient method MeOH/H_2O from 10/90 to 100/0 in 45 minutes, 1ml/min, λ = 254 nm and 263 nm, showed two peaks with t_R 27.27 min and 31.03 min.

2-Methylenepropane-1,3-diyl diacetate 114[8]



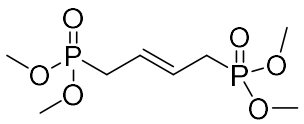
Under an argon atmosphere, to a solution of 2-methylene-1,3-propendiol (462 µl, 5.67 µmol) and vinyl acetate (105.0 µl, 1.13 µmol) in anhydrous CH_2Cl_2 (2.5 ml) was added immobilised *Candida Antarctica* lipase-B (50 mg) at room temperature. After stirring for 24 h, the mixture was filtered to remove the lipase. The filtrate was concentrated in vacuo to obtain the desired compound **114** (880 mg, 90%) as colourless oil. R_f = 0.84 (CH_2Cl_2/MeOH – 9:1). **^{1}H NMR (500 MHz, $CDCl_3$) δ_H**: 5.02 (s, 2H, =*CH_2*), 4.52 (s, 4H, 2 x *CH_2*), 2.21 (s, 6H, 2 x *CH_3*)

2-(2-(Dimethoxyphosphoryl)ethylidene)propane-1,3-diyl diacetate 115[8]



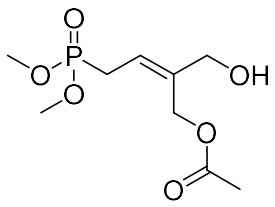
To a solution of dimethyl allyl phosphonate **114** (500mg, 3.33 mmol) and 1,3-diacetoxy-2-methylenepropane (1.15 g, 6.66 mmol) in anhydrous CH_2Cl_2 (33 ml) was added Hoveyda-Grubbs 2nd generation catalyst (15 mol%). The catalyst was added in three equal portions of 5 mol% at t = 0, 2, 4 h over the course of the reaction. The solution was sonicated at 37 MHz under argon atmosphere for 24 h at 50°C. Volatiles were then evaporated, and the residue was purified by Biotage Isolera One (25 g SNAP cartridge KP-SIL, 50 ml/min, gradient eluent system MeOH/CH_2Cl_2 1% 1CV, 1-10% 12CV, 10% 2CV), to afford the desired compound **115** (450 mg, 45%) as a brown oil. R_f = 0.37 (CH_2Cl_2/MeOH – 95:0.5). **^{31}P NMR (202 MHz, $CDCl_3$) δ_P**: 28.53. **^{1}H NMR (500 MHz, $CDCl_3$) δ_H**: 5.80 (q, *J* = 7.9 Hz, 1H, =*CH*), 4.67 (d, *J* = 2.2 Hz, 1H, O*CH_2*), 4.61 (d, *J* = 3.5 Hz, 1H, O*CH_2*), 3.76 (d, $^3J_{H\text{-}P}$ = 10.9 Hz, 6H, 2 x O*CH_3*), 2.79 (dd, *J* = 22.5 Hz, $^2J_{H\text{-}P}$ = 8.0 Hz, 2H, P-*CH_2*), 2.08 (s, 3H, *CH_3*), 2.07 (s, 3H, *CH_3*). **(ES+) m/z**, found: 295.1 [M+H$^+$], $C_{11}H_{19}O_7P$ required: 294.09 [M].

From the flash chromatography was also isolated the homodimer by-product **tetramethyl but-2-ene-1,4-diyl(*E*)-bis(phosphonate) 116**. R_f = 0.28 (CH_2Cl_2/MeOH – 95:0.5).



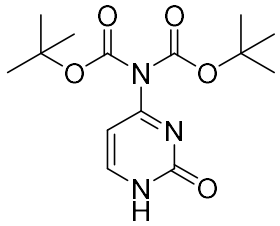
^{31}P NMR (202 MHz, $CDCl_3$) δ_P: 29.24. **^{1}H NMR (500 MHz, $CDCl_3$) δ_H**: 5.64-5.61 (m, 2H, 2 x =*CH*), 3.76 (d, $^3J_{H\text{-}P}$ = 10.6 Hz, 12H, 4 x O*CH_3*), 2.66-2.61 (m, 4H, 2 x P-*CH_2*). **(ES+) m/z**, found: 273.1 [M+H$^+$] and 295.1 [M+Na$^+$], $C_8H_{18}O_6P_2$ required: 272.06 [M].

(*Z*)-4-(dimethoxyphosphoryl)-2-(hydroxymethyl)but-2-en-1-yl acetate 117[8]



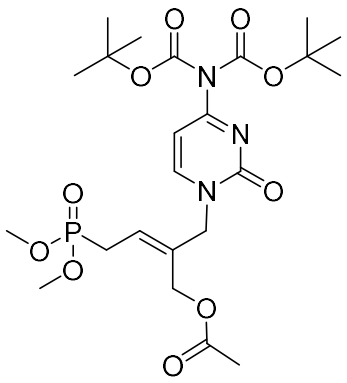
Under an argon atmosphere, to a suspension of the diacetate **115** (462 µl, 5.67 µmol) in phosphate buffer (pH 7) was added immobilised *Candida Antarctica* lipase-B (50 mg) at room temperature. After stirring for 16 h, the mixture was filtered to remove the lipase. The filtrate was concentrated in vacuo and purified by Biotage Isolera One (25 g SNAP cartridge KP-SIL, 50 ml/min, gradient eluent system MeOH/CH_2Cl_2 1% 1CV, 1-10% 12CV, 10% 2CV), to obtain the desired compound **117** (250 mg, 58%) as brown oil. R_f = 0.28 (CH_2Cl_2/MeOH – 95:5).**^{31}P NMR (202 MHz, $CDCl_3$) δ_P**: 29.72. **^{1}H NMR (500 MHz, $CDCl_3$) δ_H**: 5.65 (q, *J* = 7.3 Hz, 1H, =*CH*), 4.60 (s, 2H, O*CH*$_2$), 4.04 (s, 2H, *CH*$_2$OH), 3.66 (d, $^3J_{H\text{-}P}$ = 10.8 Hz, 6H, 2 x O*CH*$_3$), 2.68 (dd, *J* = 22.4 Hz, $^2J_{H\text{-}P}$ = 8.1 Hz, 2H, P-*CH*$_2$), 1.98 (s, 3H, *CH*$_3$). **(ES+) m/z**, found: 275.1 [M+Na^+], $C_9H_{17}O_6P$ required: 252.08 [M].

***tert*-Butyl (*tert*-butoxycarbonyl)(2-oxo-1,2-dihydropyrimidin-4-yl)carbamate 119**[9]



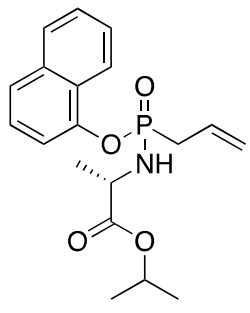
To a stirred suspension of cytosine **118** (1g, 9.0 mmol) in argon atmosphere in anhydrous THF (30 ml), DMAP (1.10 g, 9.0 mmol) and di-tert-butyl decarbonate (7.86 g, 36.0 mmol) were added. After 16 h stirring at room temperature, the mixture was diluted with EtOAc (30 ml). The solution was extracted with water (2 x 50 ml). The combined organic phases were washed with brine, dried over $MgSO_4$, and concentrated in vacuo. The resulting tris-Boc cytosine was used for the next step. To a solution of the tris-Boc cytosine in MeOH (50 ml) was added saturated $NaHCO_3$ solution (23 ml) at room temperature. After stirring 4 h at 60°C, the solvent was removed in vacuo. The mixture was diluted with EtOAc (30 ml), quenched with water (20 ml) and finally extracted with EtOAc (2 x 20 ml). The combined organic phases were washed with brine, dried over $MgSO_4$, and concentrated to dryness. The residue was purified by Biotage Isolera One (100 g SNAP cartridge KP-SIL, 50 ml/min, gradient eluent system MeOH/CH_2Cl_2 2% 1CV, 2-20% 12CV, 20% 2CV), to obtain the desired compound **119** (2.75 g, 98%) as white solid. R_f = 0.32 (CH_2Cl_2/MeOH – 9:1). **^{1}H NMR (500 MHz, $CDCl_3$) δ_H**: 13.17 (bs, 1H, *NH*), 7.79 (d, *J* = 7.1 Hz, 1H, *H*-6), 7.12 (d, *J* = 7.1 Hz, 1H, *H*-5), 1.56 (s, 18H, 2 x *t*Bu).

(*Z*)-2-((4-(bis(*tert*-Butoxycarbonyl)amino)-2-oxopyrimidin-1(2H)-yl)methyl)-4-(dimethoxyphosphoryl)but-2-en-1-yl acetate 129



Under an argon atmosphere, the monoacetate phosphonate **117** (326.7 mg, 1.17 mmol) was dissolved in anhydrous THF (16 ml) at room temperature. Bis-boc cytosine **119** (330 mg, 1.06 mmol) and triphenylphosphine (305.8 mg, 1.17 mmol) were added sequentially and the reaction cooled down to 0°C in an ice-bath. DIAD (229.6 µl, 1.17 mmol) was added dropwise to the solution and the mixture left stirring at 0°C for 5 minutes. The ice bath was removed and the reaction stirred at 70°C for 20 h. Volatiles were evaporated and the mixture purified by Biotage Isolera One (100 g SNAP cartridge KP-SIL, 100 ml/min, gradient eluent system MeOH/CH_2Cl_2 1% 1CV, 1-10% 12CV, 10% 2CV), to obtain the desired compound **129** (195 mg, 32%) as brown oil. R_f= 0.43 (CH_2Cl_2/MeOH – 95:5). **^{31}P NMR (202 MHz, $CDCl_3$) δ_P**: 25.61. **^{1}H NMR (500 MHz, $CDCl_3$) δ_H**: 7.50 (d, *J* = 7.3 Hz, 1H, *H*-6), 6.95 (d, *J* = 7.3 Hz, 1H, *H*-5), 5.68 (q, *J* = 8.0 Hz, 1H, =*CH*), 4.55 (d, *J* = 2.1 Hz, 2H, *CH_2*), 4.50 (d, *J* = 3.4 Hz, 2H, *CH_2*), 3.68 (d, $^{3}J_{H\text{-}P}$ = 10.7 Hz, 6H, 2 x O*CH_3*), 2.69 (dd, *J* = 22.2 Hz, $^{2}J_{H\text{-}P}$ = 7.7 Hz, 2H, P-*CH_2*), 1.95 (s, 3H, *CH_3*). 1.47 (s, 18H, 2 x *t*Bu). **^{13}C NMR (125 MHz, $CDCl_3$) δ_C**: 170.5 (*C*=O, Ac), 162.1 (*C*-4), 1654.8 (*C*-2), 149.4 (*C*=O, Boc), 147.2 (*C*-6), 132.9 (d, $^{3}J_{C\text{-}P}$ = 13.7 Hz, *C*=), 125.4 (d, $^{2}J_{C\text{-}P}$ = 11.3 Hz, *CH*=), 96.2 (*C*-5), 84.8 (*C*$(CH_3)_3$),59.8 (d, $^{4}J_{C\text{-}P}$ = 1.5 Hz, *CH_2*), 53.8 (d, $^{2}J_{C\text{-}P}$ = 6.5 Hz, O*CH_3*), 53.0 (d, $^{4}J_{C\text{-}P}$ = 1.6 Hz, *CH_2*), 27.5 (C(*CH_3*)$_3$), 26.45 (d, $^{1}J_{C\text{-}P}$ = 139.5 Hz, P-*CH_2*), 20.6 (*CH_3*). **(ES+) m/z**, found: 268.1 [M+Na^+], $C_{23}H_{36}N_3O_{10}P$ required: 545.21 [M].

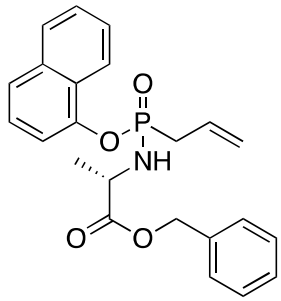
Isopropyl (allyl(naphthalen-1-yloxy)phosphoryl)-*L*-alaninate 147a



Prepared according to the standard procedure **C** for the synthesis of ProTide allylphosphonate using dimethyl allylphosphonate (500 mg, 3.33 mmol), 2,6-Lutidine (1.55 ml, 13.32 mmol), TMSBr (2.20 ml, 16.65 mmol) in anhydrous acetonitrile (25 ml). For the second step we used dry isopropyloxy-*L*-Alanine hydrochloride (558 mg, 3.33 mmol), dry 1-Naphthol (2.88 g, 19.98 mmol), dry triethylamine (6.9 ml, 49.96 mmol) in dry pyridine (10 ml) and a solution of Aldrithiol-2 (4.40 g, 19.98 mmol) and triphenylphosphine (5.24 g, 19.98 mmol) in dry pyridine (10 ml). After evaporation, the mixture was purified by Biotage Isolera One (100 g SNAP

cartridge ULTRA, 100 ml/min, gradient eluent system EtOAc/Hexane 10% 1CV, 10-100% 12CV, 100% 2CV), to afford the title compound as a yellow oil (940 mg, 79%). R_f = 0.58 (EtOAc/Hexane - 4:6). **^{31}P NMR (202 MHz, CD_3OD) δ_P**: 30.01, 29.43. **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 8.19 (d, *J* = 7.2 Hz, 1H, Ar*H*), 7.89 (d, *J* = 7.9 Hz 1H, Ar*H*), 7.71-7.69 (m, 1H, Ar*H*), 7.57-7.40 (m, 4H, Ar*H*), 6.07-5.91 (m, 1H, *CH*=), 5.38-5.28 (m, 2H, CH_2=), 5.01-4.82 (m, 1H, *CH*(CH_3)$_2$), 3.99-3.97 (m, 1H, *CH*CH_3 L-Ala), 3.10-2.95 (m, 2H, CH_2P), 1.25 (d, *J* = 7.8 Hz, 1.5H, CHCH_3 L-Ala), 1.21-1.06 (m, 7.5H, CHCH_3 L-Ala, CH(CH_3)$_2$). **^{13}C NMR (125 MHz, CD_3OD) δ_C**: 173.5 (d, $^3J_{C\text{-}P}$ = 4.2 Hz, *C*=O, ester), 173.1 (d, $^3J_{C\text{-}P}$ = 4.2 Hz, *C*=O, ester), 146.4 (d, $^2J_{C\text{-}P}$ = 8.5 Hz, *C*-O, Ph), 146.3 (d, $^2J_{C\text{-}P}$ = 8.5 Hz, *C*-O, Ph), 134.9 (*C*-Ar), 127.4 ($^2J_{C\text{-}P}$ = 9.3 Hz, *CH*=), 123.3 ($^2J_{C\text{-}P}$ = 10.9 Hz, *CH*=), 126.9 (d, $^3J_{C\text{-}P}$ = 5.6 Hz *C*-Ar), 126.8 (d, $^3J_{C\text{-}P}$ = 4.9 Hz *C*-Ar), 126.3 (*CH*-Ar), 125.95 (*CH*-Ar), 125.90 (*CH*-Ar), 125.1 (*CH*-Ar), 125.0 (*CH*-Ar), 124.3 (*CH*-Ar), 124.2 (*CH*-Ar), 121.6 (*CH*-Ar), 121.4 (*CH*-Ar), 119.7 (d, $^3J_{C\text{-}P}$ = 14.2 Hz CH_2=), 119.6 (d, $^3J_{C\text{-}P}$ = 13.8 Hz CH_2=), 115.4 (d, $^3J_{C\text{-}P}$ = 4.1 Hz *CH*-Ar), 115.2 (d, $^3J_{C\text{-}P}$ = 3.4 Hz *CH*-Ar), 68.6 (*CH*(CH_3)$_2$), 68.5 (*CH*(CH_3)$_2$), 49.6 (*CH*CH_3 L-Ala), 49.4 (*CH*CH_3 L-Ala), 33.7 (d, $^1J_{C\text{-}P}$ = 129.0 Hz CH_2P), 33.5 (d, $^1J_{C\text{-}P}$ = 129.6 Hz CH_2P), 20.5 (CH(CH_3)$_2$), 20.4 (CH(CH_3)$_2$), 20.3 (CH(CH_3)$_2$), 19.7 (d, $^3J_{C\text{-}P}$ = 5.4 Hz, CHCH_3 L-Ala), 19.1 (d, $^3J_{C\text{-}P}$ = 5.4 Hz, CHCH_3 L-Ala).

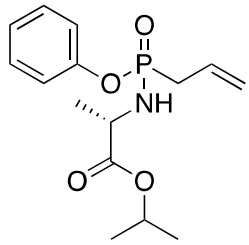
Benzyl (allyl(naphthalen-1-yloxy)phosphoryl)-*L*-alaninate 147b



Prepared according to the standard procedure **C** for the synthesis of ProTide allylphosphonate using dimethyl allylphosphonate (500 mg, 3.33 mmol), 2,6-Lutidine (1.55 ml, 13.32 mmol), TMSBr (2.20 ml, 16.65 mmol) in anhydrous acetonitrile (25 ml). For the second step we used dry benzyloxy-*L*-Alanine hydrochloride (718 mg, 3.33 mmol), dry 1-Naphthol (2.88 g, 19.98 mmol), dry triethylamine (6.9 ml, 49.96 mmol) in dry pyridine (10 ml) and a solution of Aldrithiol-2 (4.40 g, 19.98 mmol) and triphenylphosphine (5.24 g, 19.98 mmol) in dry pyridine (10 ml). After evaporation, the mixture was purified by Biotage Isolera One (100 g SNAP cartridge ULTRA, 100 ml/min, gradient eluent system EtOAc/Hexane 10% 1CV, 10-100% 12CV, 100% 2CV), to afford the title compound as a yellow oil (1.1 g, 78%). R_f = 0.58 (EtOAc/Hexane - 4:6). **^{31}P NMR (202 MHz, CD_3OD) δ_P**: 30.09, 29.48. **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 8.17 (s, 1H, Ar*H*), 7.86 (s, 1H, Ar*H*), 7.71-7.65 (m, 1H, Ar*H*), 7.52-7.22 (m, 9H, Ar*H*), 5.99-5.89 (m, 1H, *CH*=), 5.30-5.17 (m,

2H, CH_2=), 5.09, 5.03 (ABq, J_{AB} = 12.1 Hz, 1H, CH_2Ph), 4.97, 4.93 (ABq, J_{AB} = 12.1 Hz, 1H, CH_2Ph), 4.09-4.07 (m, 1H, CHCH$_3$ L-Ala), 2.95-2.86 (m, 2H, CH_2P), 1.26 (d, J = 6.8 Hz, 1.5H, CHCH_3 L-Ala), 1.16 (d, J = 6.8 Hz, 1.5H, CHCH_3 L-Ala). **^{13}C NMR (125 MHz, CD$_3$OD) δ$_C$**: 173.7 (d, $^3J_{C\text{-}P}$ = 3.9 Hz, C=O, ester), 173.2 (d, $^3J_{C\text{-}P}$ = 4.0 Hz, C=O, ester), 146.4 (d, $^2J_{C\text{-}P}$ = 9.7 Hz, C-O, Ph), 146.3 (d, $^2J_{C\text{-}P}$ = 10.0 Hz, C-O, Ph), 135.8 (C-Ar), 135.7 (C-Ar), 134.9 (C-Ar), 128.17 (CH-Ar), 128.12 (CH-Ar), 127.9 (CH-Ar), 127.8 (CH-Ar), 127.48 (CH-Ar), 127.42 (CH-Ar), 127.3 ($^2J_{C\text{-}P}$ = 11.3 Hz, CH=), 127.2 ($^2J_{C\text{-}P}$ = 11.0 Hz, CH=), 126.8 (d, $^3J_{C\text{-}P}$ = 5.0 Hz C-Ar), 126.7 (d, $^3J_{C\text{-}P}$ = 5.3 Hz C-Ar), 126.3 (CH-Ar), 125.98 (CH-Ar), 125.93 (CH-Ar), 125.18 (CH-Ar), 125.10 (CH-Ar), 124.3 (CH-Ar), 124.2 (CH-Ar), 121.6 (CH-Ar), 121.4 (CH-Ar), 119.7 (d, $^3J_{C\text{-}P}$ = 15.2 Hz CH_2=), 119.6 (d, $^3J_{C\text{-}P}$ = 14.9 Hz CH_2=), 115.4 (d, $^3J_{C\text{-}P}$ = 3.9 Hz CH-Ar), 115.2 (d, $^3J_{C\text{-}P}$ = 3.9 Hz CH-Ar), 66.5 (CH_2Ph), 66.3 (CH_2Ph), 49.6 (CHCH$_3$ L-Ala), 49.4 (CHCH$_3$ L-Ala), 33.7 (d, $^1J_{C\text{-}P}$ = 129.2 Hz CH_2P), 33.5 (d, $^1J_{C\text{-}P}$ = 129.7 Hz CH_2P), 19.6 (d, $^3J_{C\text{-}P}$ = 5.3 Hz, CHCH_3 L-Ala), 19.0 (d, $^3J_{C\text{-}P}$ = 5.8 Hz, CHCH_3 L-Ala).

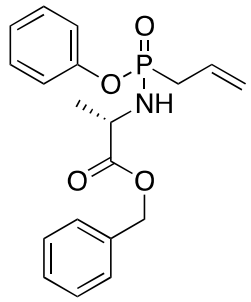
Isopropyl (allyl(phenoxy)phosphoryl)-*L*-alaninate 147c



Prepared according to the standard procedure **C** for the synthesis of ProTide allylphosphonate using dimethyl allylphosphonate (500 mg, 3.33 mmol), 2,6-Lutidine (1.55 ml, 13.32 mmol), TMSBr (2.20 ml, 16.65 mmol) in anhydrous acetonitrile (25 ml). For the second step we used dry isopropyloxy-*L*-Alanine hydrochloride (558.3 mg, 3.33 mmol), dry Phenol (1.88 g, 19.98 mmol), dry triethylamine (6.9 ml, 49.96 mmol) in dry pyridine (10 ml) and a solution of Aldrithiol-2 (4.40 g, 19.98 mmol) and triphenylphosphine (5.24 g, 19.98 mmol) in dry pyridine (10 ml). After evaporation, the mixture was purified by Biotage Isolera One (100 g SNAP cartridge ULTRA, 100 ml/min, gradient eluent system EtOAc/Hexane 10% 1CV, 10-100% 12CV, 100% 2CV), to afford the title compound as a yellow oil (670 mg, 65%). R_f = 0.37 (EtOAc/Hexane - 6:4). **^{31}P NMR (202 MHz, CDCl$_3$) δ$_P$**: 26.77, 26.35. **^{1}H NMR (500 MHz, CDCl$_3$) δ$_H$**: 7.31-7.28 (m, 2H, ArH), 7.22-7.19 (m, 2H, ArH), 7.13-7.09 (m, 1H, ArH), 5.95-5.82 (m, 1H, CH=), 5.31-5.24 (m, 2H, CH_2=), 5.00-4.93 (m, 1H, CH(CH$_3$)$_2$), 4.13-3.96 (m, 1H, CHCH$_3$ L-Ala), 3.51 (dd, $^2J_{H\text{-}P}$, $^3J_{NH,CH}$ = 10.3 Hz, 0.5H, NH L-Ala), 3.41 (dd, $^2J_{H\text{-}P}$, $^3J_{NH,CH}$ = 10.7 Hz, 0.5H, NH L-Ala), 2.84-2.72 (m, 2H, CH_2P), 1.29 (d, J = 7.2 Hz, 1.5H, CHCH_3 L-Ala), 1.22-1.19 (m, 7.5H, CHCH_3 L-Ala, CH(CH_3)$_2$). **^{13}C NMR (125 MHz, CDCl$_3$) δ$_C$**: 173.5 (d, $^3J_{C\text{-}P}$ = 4.7 Hz, C=O, ester), 173.1 (d, $^3J_{C\text{-}P}$ = 4.7 Hz, C=O, ester), 150.6 (d, $^2J_{C\text{-}}$

$_P$ = 9.1 Hz, *C*-O, Ph), 150.5 (d, $^2J_{C\text{-}P}$ = 9.4 Hz, *C*-O, Ph), 129.4 (*CH*-Ar), 129.3 (*CH*-Ar), 127.5 ($^2J_{C\text{-}P}$ = 11.3 Hz, *CH*=), 127.4 ($^2J_{C\text{-}P}$ = 11.3 Hz, *CH*=), 124.6 (*CH*-Ar), 124.5 (*CH*-Ar), 120.8 (d, $^3J_{C\text{-}P}$ = 4.0 Hz *CH*-Ar), 120.6 (d, $^3J_{C\text{-}P}$ = 4.0 Hz *CH*-Ar), 119.6 (d, $^3J_{C\text{-}P}$ = 14.6 Hz CH_2=), 119.6 (d, $^3J_{C\text{-}P}$ = 14.6 Hz CH_2=), 68.59 (*CH*(CH_3)$_2$), 68.57 (*CH*(CH_3)$_2$), 49.6 (*CH*CH_3 L-Ala), 49.7 (*CH*CH_3 L-Ala), 33.8 (d, $^1J_{C\text{-}P}$ = 129.3 Hz CH_2P), 33.6 (d, $^1J_{C\text{-}P}$ = 129.7 Hz CH_2P), 20.86 (CH(*CH_3*)$_2$), 20.82 (CH(*CH_3*)$_2$), 20.81 (CH(*CH_3*)$_2$), 20.75 (CH(*CH_3*)$_2$), 20.0 (d, $^3J_{C\text{-}P}$ = 5.3 Hz, CH*CH_3* L-Ala), 19.5 (d, $^3J_{C\text{-}P}$ = 5.1 Hz, CH*CH_3* L-Ala).

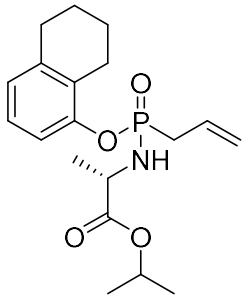
Benzyl (allyl(phenoxy)phosphoryl)-*L*-alaninate 147d



Prepared according to the standard procedure **C** for the synthesis of ProTide allylphosphonate using dimethyl allylphosphonate (500 mg, 3.33 mmol), 2,6-Lutidine (1.55 ml, 13.32 mmol), TMSBr (2.20 ml, 16.65 mmol) in anhydrous acetonitrile (25 ml). For the second step we used dry benzyloxy-*L*-Alanine hydrochloride (718 mg, 3.33 mmol), dry Phenol (1.88 g, 19.98 mmol), dry triethylamine (6.9 ml, 49.96 mmol) in dry pyridine (10 ml) and a solution of Aldrithiol-2 (4.40 g, 19.98 mmol) and triphenylphosphine (5.24 g, 19.98 mmol) in dry pyridine (10 ml). After evaporation, the mixture was purified by Biotage Isolera One (100 g SNAP cartridge ULTRA, 100 ml/min, gradient eluent system EtOAc/Hexane 10% 1CV, 10-100% 12CV, 100% 2CV), to afford the title compound as a yellow oil (500 mg, 42%). R_f = 0.22 (EtOAc/Hexane - 4:6). **^{31}P NMR (202 MHz, CD_3OD) δ_P**: 29.62, 29.01. **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 7.35-7.28 (m, 7H, Ar*H*), 7.22-7.13 (m, 3H, Ar*H*), 5.92-5.81 (m, 1H, *CH*=), 5.27-5.18 (m, 2H, CH_2=), 5.14, 5.12 (ABq, J_{AB} = 12.5 Hz, 1H, CH_2Ph), 5.06 (s app, 1H, CH_2Ph), 4.12-4.01 (m, 1H, *CH*CH_3 L-Ala), 2.82-2.75 (m, 2H, CH_2P), 1.31 (d, *J* = 7.2 Hz, 1.5H, CH*CH_3* L-Ala), 1.22 (d, *J* = 7.5 Hz, 1.5H, CH*CH_3* L-Ala). **^{13}C NMR (125 MHz, CD_3OD) δ_C**: 172.3 (d, $^3J_{C\text{-}P}$ = 4.1 Hz, *C*=O, ester), 171.9 (d, $^3J_{C\text{-}P}$ = 3.99 Hz, *C*=O, ester), 149.0 (d, $^2J_{C\text{-}P}$ = 9.5 Hz, *C*-O, Ph), 148.9 (d, $^2J_{C\text{-}P}$ = 9.5 Hz, *C*-O, Ph), 134.37 (*C*-Ar), 134.34 (*C*-Ar), 127.84 (*CH*-Ar), 127.81 (*CH*-Ar), 126.75 (*CH*-Ar), 126.73 (*CH*-Ar), 126.5 (*CH*-Ar), 126.49 (*CH*-Ar), 126.46 (*CH*-Ar), 125.8 ($^2J_{C\text{-}P}$ = 10.1 Hz, *CH*=), 125.7 ($^2J_{C\text{-}P}$ = 10.1 Hz, *CH*=), 123.1 (*CH*-Ar), 123.0 (*CH*-Ar), 119.2 (d, $^3J_{C\text{-}P}$ = 4.3 Hz *CH*-Ar), 119.0 (d, $^3J_{C\text{-}P}$ = 4.3 Hz *CH*-Ar), 118.2 (d, $^3J_{C\text{-}P}$ = 14.5 Hz CH_2=), 118.0 (d, $^3J_{C\text{-}P}$ = 14.6 Hz CH_2=), 65.0 (CH_2Ph), 64.9 (CH_2Ph), 48.09 (*CH*CH_3 L-Ala), 47.9 (*CH*CH_3 L-Ala), 32.1 (d, $^1J_{C\text{-}P}$ =

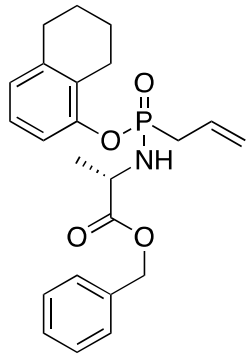
129.7 Hz CH_2P), 31.9 (d, $^1J_{C\text{-}P}$ = 129.7 Hz CH_2P), 18.2 (d, $^3J_{C\text{-}P}$ = 5.3 Hz, CHCH_3 L-Ala), 17.7 (d, $^3J_{C\text{-}P}$ = 5.3 Hz, CHCH_3 L-Ala).

Isopropyl (allyl((5,6,7,8-tetrahydronaphthalen-1-yl)oxy)phosphoryl)-*L*-alaninate 147e



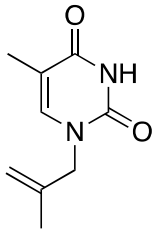
Prepared according to the standard procedure **C** for the synthesis of ProTide allylphosphonate using dimethyl allylphosphonate (500 mg, 3.33 mmol), 2,6-Lutidine (1.55 ml, 13.32 mmol), TMSBr (2.20 ml, 16.65 mmol) in anhydrous acetonitrile (25 ml). For the second step we used dry isopropyloxy-*L*-Alanine hydrochloride (558 mg, 3.33 mmol), dry 5,6,7,8-tetrahydro-1-naphthol (2.96 g, 19.98 mmol), dry triethylamine (6.9 ml, 49.96 mmol) in dry pyridine (10 ml) and a solution of Aldrithiol-2 (4.40 g, 19.98 mmol) and triphenylphosphine (5.24 g, 19.98 mmol) in dry pyridine (10 ml). After evaporation, the mixture was purified by Biotage Isolera One (100 g SNAP cartridge ULTRA, 100 ml/min, gradient eluent system EtOAc/Hexane 10% 1CV, 10-100% 12CV, 100% 2CV), to afford the title compound as a yellow foamy solid (750 mg, 55%). R_f = 0.51 (EtOAc/Hexane - 4:6). **^{31}P NMR (202 MHz, CD_3OD) δ_P**: 29.03, 28.43. **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 7.17-7.12 (m, 1H, Ar*H*), 7.05-7.00 (m, 1H, Ar*H*), 6.89-6.84 (m, 1H, Ar*H*), 6.09-5.85 (m, 1H, *CH*=), 5.32-5.24 (m, 2H, CH_2=), 5.01-4.88 (m, 1H, *CH*$(CH_3)_2$), 3.98-3.89 (m, 1H, *CH*CH_3 L-Ala), 2.85 (dt, $^2J_{H\text{-}P}$ = $^2J_{H\text{-}H}$ = 20.0 Hz, $^3J_{H\text{-}H}$ = 7.1 Hz, 2H, CH_2P), 2.77-2.74 (m, 4H, Ar*H*), 1.85-1.74 (m, 4H, Ar*H*), 1.30 (d, *J* = 7.1 Hz, 1.5H, CHCH_3 L-Ala), 1.25-1.23 (m, 4.5H, CHCH_3 L-Ala, CH$(CH_3)_2$), 1.19 (d, *J* = 6.05 Hz, 3H, CH$(CH_3)_2$). **^{13}C NMR (125 MHz, CD_3OD) δ_C**: 173.6 (d, $^3J_{C\text{-}P}$ = 4.0 Hz, *C*=O, ester), 173.2 (d, $^3J_{C\text{-}P}$ = 4.0 Hz, *C*=O, ester), 148.7 (d, $^2J_{C\text{-}P}$ = 10.2 Hz, *C*-O, Ph), 148.6 (d, $^2J_{C\text{-}P}$ = 10.6 Hz, *C*-O, Ph), 139.1 (*C*-Ar), 131.3 (*CH*-Ar), 131.2 (*CH*-Ar), 128.6 (d, $^3J_{C\text{-}P}$ = 7.0 Hz *C*-Ar), 128.5 (d, $^3J_{C\text{-}P}$ = 7.5 Hz *C*-Ar), 127.5 ($^2J_{C\text{-}P}$ = 11.0 Hz, *CH*=), 127.4 ($^2J_{C\text{-}P}$ = 11.0 Hz, *CH*=), 125.3 (*CH*-Ar), 125.1 (*CH*-Ar), 119.4 (d, $^3J_{C\text{-}P}$ = 14.6 Hz CH_2=), 119.3 (d, $^3J_{C\text{-}P}$ = 14.6 Hz CH_2=), 116.9 (d, $^3J_{C\text{-}P}$ = 3.1 Hz *CH*-Ar), 116.8 (d, $^3J_{C\text{-}P}$ = 3.5 Hz *CH*-Ar), 68.6 (*CH*$(CH_3)_2$), 68.5 (*CH*$(CH_3)_2$), 49.7 (*CH*CH_3 L-Ala), 49.4 (*CH*CH_3 L-Ala), 33.8 (d, $^1J_{C\text{-}P}$ = 129.5 Hz CH_2P), 33.6 (d, $^1J_{C\text{-}P}$ = 130.1 Hz CH_2P), 29.1 (CH_2-Ar), 23.3 (CH_2-Ar), 22.48 (CH_2-Ar), 22.46 (CH_2-Ar), 22.41 (CH_2-Ar), 20.59 (CH$(CH_3)_2$), 20.56 (CH$(CH_3)_2$), 20.54 (CH$(CH_3)_2$), 20.4 (CH$(CH_3)_2$), 19.9 (d, $^3J_{C\text{-}P}$ = 4.9 Hz, CHCH_3 L-Ala), 19.1 (d, $^3J_{C\text{-}P}$ = 5.4 Hz, CHCH_3 L-Ala).

Benzyl (allyl((5,6,7,8-tetrahydronaphthalen-1-yl)oxy)phosphoryl)-*L*-alaninate 147f



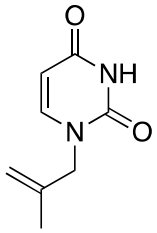
Prepared according to the standard procedure **C** for the synthesis of ProTide allylphosphonate using dimethyl allylphosphonate (500 mg, 3.33 mmol), 2,6-Lutidine (1.55 ml, 13.32 mmol), TMSBr (2.20 ml, 16.65 mmol) in anhydrous acetonitrile (25 ml). For the second step we used dry benzyloxy-*L*-Alanine hydrochloride (718 mg, 3.33 mmol), dry 5,6,7,8-tetrahydro-1-naphthol (2.96 g, 19.98 mmol), dry triethylamine (6.9 ml, 49.96 mmol) in dry pyridine (10 ml) and a solution of Aldrithiol-2 (4.40 g, 19.98 mmol) and triphenylphosphine (5.24 g, 19.98 mmol) in dry pyridine (10 ml). After evaporation, the mixture was purified by Biotage Isolera One (100 g SNAP cartridge ULTRA, 100 ml/min, gradient eluent system EtOAc/Hexane 10% 1CV, 10-100% 12CV, 100% 2CV), to afford the title compound as a yellow foamy solid (750 mg, 55%). R_f = 0.51 (EtOAc/Hexane - 4:6). **^{31}P NMR (202 MHz, CD_3OD) δ_P**: 28.94, 28.33. **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 7.35-7.24 (m, 5H, Ar*H*), 7.21-7.14 (m, 1H, Ar*H*), 7.06-7.94 (m, 1H, Ar*H*), 6.87-6.83 (m, 1H, Ar*H*), 5.98-5.82 (m, 1H, *CH*=), 5.30-5.17 (m, 2H, CH_2=), 5.13, 5.10 (ABq, J_{AB} = 12.2 Hz, 1H, CH_2Ph), 5.04 (AB app t, J_{AB} = 12.8 Hz, 1H, CH_2Ph), 4.12-4.01 (m, 1H, *CH*CH_3 L-Ala), 2.87-2.77 (m, 2H, CH_2P), 2.72-2.67 (m, 4H, Ar*H*), 1.79-1.71 (m, 4H, Ar*H*), 1.32 (d, *J* = 7.1 Hz, 1.5H, CHCH_3 L-Ala), 1.26 (d, *J* = 7.1 Hz, 1.5H, CHCH_3 L-Ala). **^{13}C NMR (125 MHz, CD_3OD) δ_C**: 173.8 (d, $^3J_{C\text{-}P}$ = 3.7 Hz, *C*=O, ester), 173.4 (d, $^3J_{C\text{-}P}$ = 4.1 Hz, *C*=O, ester), 148.8 (d, $^2J_{C\text{-}P}$ = 9.7 Hz, *C*-O, Ph), 148.7 (d, $^2J_{C\text{-}P}$ = 9.4 Hz, *C*-O, Ph), 139.13 (*C*-Ar), 139.11(*C*-Ar), 135.88 (*C*-Ar), 135.84 (*C*-Ar), 128.6 (d, $^3J_{C\text{-}P}$ = 5.5 Hz *C*-Ar), 128.5 (d, $^3J_{C\text{-}P}$ = 5.8 Hz *C*-Ar), 128.23 (*CH*-Ar), 128.20 (*CH*-Ar), 127.99 (*CH*-Ar), 127.94 (*CH*-Ar), 127.87 (*CH*-Ar), 127.5 ($^2J_{C\text{-}P}$ = 11.3 Hz, *CH*=), 127.4 ($^2J_{C\text{-}P}$ = 11.0 Hz, *CH*=), 125.4 (*CH*-Ar), 125.3 (*CH*-Ar), 125.17 (*CH*-Ar), 125.13 (*CH*-Ar), 119.5 (d, $^3J_{C\text{-}P}$ = 14.6 Hz CH_2=), 119.4 (d, $^3J_{C\text{-}P}$ = 14.8 Hz CH_2=), 117.0 (d, $^3J_{C\text{-}P}$ = 3.4 Hz *CH*-Ar), 116.9 (d, $^3J_{C\text{-}P}$ = 3.1 Hz *CH*-Ar), 66.5 (CH_2Ph), 66.4 (CH_2Ph), 49.7 (*CH*CH_3 L-Ala), 49.4 (*CH*CH_3 L-Ala), 33.8 (d, $^1J_{C\text{-}P}$ = 129.4 Hz CH_2P), 33.7 (d, $^1J_{C\text{-}P}$ = 130.2 Hz CH_2P), 29.18 (CH_2-Ar), 23.38 (CH_2-Ar), 22.5 (CH_2-Ar), 22.48 (CH_2-Ar), 22.43 (CH_2-Ar), 19.8 (d, $^3J_{C\text{-}P}$ = 5.3 Hz, CHCH_3 L-Ala), 19.1 (d, $^3J_{C\text{-}P}$ = 5.3 Hz, CHCH_3 L-Ala).

5-Methyl-1-(2-methylallyl)pyrimidine-2,4(1H,3H)-dione 152[10]



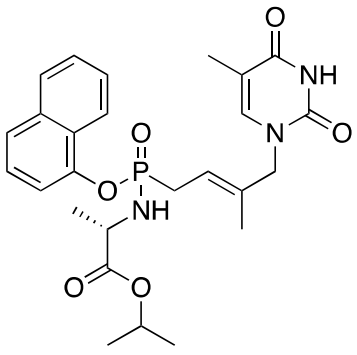
Prepared according to the standard procedure **D** for the synthesis of N^1-2'-methylallylpyrimidine using Thymine (1.5 g, 11.89 mmol), BSA (7.2 ml, 29.73 mmol), 3-bromo-2-methylpropene (2.40 ml, 23.79 mmol), NaI (1.96 g, 13.08 mmol) and TMSCl (1.51 ml, 11.89 mmol) in anhydrous acetonitrile (25 ml). After work up and evaporation, the compound was obtained as a pale yellow solid in quantitative yield (2.1 g). R_f = 0.45 (EtOAc/Hexane - 7:3). **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 7.34 (s, 1H, *H*-6), 4.98 (s, 1H, CH_2=), 4.80 (s, 1H, CH_2=), 4.30 (s, 2H, CH_2-N), 1.89 (s, 3H, CH_3, base), 1.76 (s, 3H, CH_3, alkene).

1-(2-Methylallyl)pyrimidine-2,4(1H,3H)-dione 153[10]



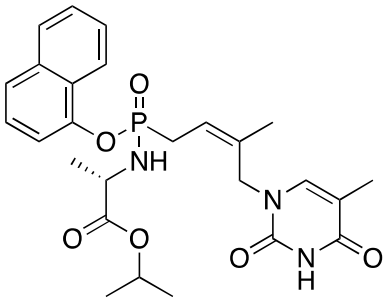
Prepared according to the standard procedure **D** for the synthesis of N^1-2'-methylallylpyrimidine using uracil (1.5 g, 13.38 mmol), BSA (8.18 ml, 33.46 mmol), 3-bromo-2-methylpropene (2.70 ml, 26.76 mmol), NaI (2.21 g, 14.72 mmol) and TMSCl (1.70 ml, 13.38 mmol) in anhydrous acetonitrile (25 ml). After work up and evaporation, the mixture was purified by Biotage Isolera One (50 g SNAP cartridge ULTRA, 100 ml/min, gradient eluent system EtOAc/Hexane 17% 1CV, 17-100% 10CV, 100% 3CV), to afford the title compound as a pale yellow solid (1.2 g, 51%). R_f = 0.25 (EtOAc/Hexane - 7:3). **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 7.50 (d, *J* = 7.8 Hz, 1H, *H*-6), 5.71 (d, *J* = 7.8 Hz, 1H, *H*-5), 4.98 (s, 1H, CH_2=), 4.81 (s, 1H, CH_2=), 4.33 (s, 2H, CH_2-N), 1.76 (s, 3H, CH_3, alkene).

Isopropyl (((*E*)-3-methyl-4-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)but-2-en-1-yl)(naphthalen-1-yloxy)phosphoryl)-*L*-alaninate *E*-154a and isopropyl (((*Z*)-3-methyl-4-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)but-2-en-1-yl)(naphthalen-1-yloxy)phosphoryl)-*L*-alaninate *Z*-154a

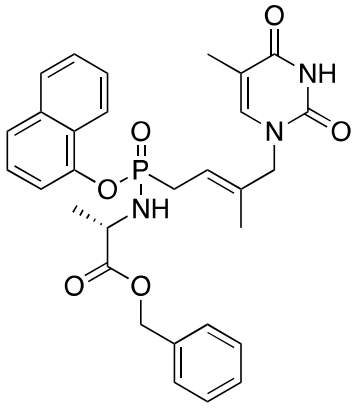


Prepared according to the standard procedure **E** for the synthesis of ANP ProTide using *O*-(1-naphthyl)-(isopropyloxy-*L*-Alanine)-allylphosphonate **147a** (150 mg, 415 µmol) and N^1-2'-methylallylthymine **152** (150 mg, 830.1 µmol) and Hoveyda-Grubbs 2nd generation catalyst (15 mol%) in dry CH_2Cl_2 (8 ml). After evaporation, the crude was purified by Biotage Isolera One (50 g SNAP cartridge ULTRA, 100 ml/min, gradient eluent system MeOH/CH_2Cl_2 1%

1CV, 1-10% 12CV, 10% 2CV), to afford a mixture of the E and Z isomer. The two isomers were then separated by reverse Biotage Isolera One (60 g SNAP cartridge KP-C18-HS, 100 ml/min, isocratic eluent system CH_3CN/H_2O 30-60% 12CV) to afford the title compound ***E*-154a** as pale yellow foamy solid (75 mg, 36%). R_f = 0.23 (CH_2Cl_2/MeOH - 95:5). **^{31}P NMR (202 MHz, CD_3OD) δ_P**: 30.32, 29.54. **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 8.13-8.12 (m, 1H, Ar*H*), 7.89-7.87 (m, 1H, Ar*H*), 7.71-7.68 (m, 1H, Ar*H*), 7.57-7.48 (m, 3H, Ar*H*), 7.45-7.39 (m, 1H, Ar*H*), 7.27 (s, 0.5H, *H*-6), 7.26 (s, 0.5H, *H*-6), 5.61-5.56 (m, 1H, *CH*=), 4.93-4.84 (m, 1H, *CH*(CH_3)$_2$), 4.32-4.26 (m, 2H, *CH$_2$*-N), 4.01-3.91 (m, 1H, *CH*CH_3 L-Ala), 3.08-2.86 (m, 2H, *CH$_2$*P), 1.75 (s, 3H, CH_3, base), 1.67 (s, 3H, *CH$_3$*, alkene), 1.27 (d, *J* = 6.9 Hz, 1.5H, CH*CH$_3$* L-Ala), 1.20-1.16 (m, 4.5H, CH*CH$_3$* L-Ala, CH(*CH$_3$*)$_2$), 1.13-1.10 (m, 3H, CH(*CH$_3$*)$_2$). **^{13}C NMR (125 MHz, CD_3OD) δ_C**: 173.5 (d, $^3J_{C\text{-}P}$ = 3.9 Hz, *C*=O, ester), 173.1 (d, $^3J_{C\text{-}P}$ = 3.5 Hz, *C*=O, ester), 165.34 (*C*-4), 165.32 (*C*-4), 151.69 (*C*-2), 151.61 (*C*-2), 146.5 (d, $^2J_{C\text{-}P}$ = 9.5 Hz, *C*-O, Ph), 146.3 (d, $^2J_{C\text{-}P}$ = 9.5 Hz, *C*-O, Ph), 140.94 (*C*-6), 140.92 (*C*-6), 135.5 (d, $^3J_{C\text{-}P}$ = 14.3 Hz, *C*=), 135.1 (d, $^3J_{C\text{-}P}$ = 14.7 Hz, *C*=), 134.9 (*C*-Ar), 127.48 (*CH*-Ar), 127.46 (*CH*-Ar), 126.7 (d, $^3J_{C\text{-}P}$ = 5.1 Hz *C*-Ar), 126.6 (d, $^3J_{C\text{-}P}$ = 5.1 Hz *C*-Ar), 126.3 (*CH*-Ar), 126.0 (*CH*-Ar), 125.16 (*CH*-Ar), 125.11 (*CH*-Ar), 124.3 (*CH*-Ar), 124.2 (*CH*-Ar), 121.4 (*CH*-Ar), 121.3 (*CH*-Ar), 117.1 ($^2J_{C\text{-}P}$ = 11.1 Hz, *CH*=), 116.6 ($^2J_{C\text{-}P}$ = 10.7 Hz, *CH*=), 115.3 (d, $^3J_{C\text{-}P}$ = 3.5 Hz *CH*-Ar), 115.1 (d, $^3J_{C\text{-}P}$ = 3.9 Hz *CH*-Ar), 110.1 (*C*-5), 68.69 (*CH*(CH_3)$_2$), 68.65 (*CH*(CH_3)$_2$), 53.5 (d, $^4J_{C\text{-}P}$ = 2.7 Hz, *CH$_2$*-N), 53.2 (d, $^4J_{C\text{-}P}$ = 2.3 Hz, *CH$_2$*-N), 49.7 (*CH*CH_3 L-Ala), 49.5 (*CH*CH_3 L-Ala), 28.3 (d, $^1J_{C\text{-}P}$ = 129.0 Hz *CH$_2$*P), 28.1 (d, $^1J_{C\text{-}P}$ = 130.0 Hz *CH$_2$*P), 20.55 (CH(*CH$_3$*)$_2$), 20.54 (CH(*CH$_3$*)$_2$), 20.48 (CH(*CH$_3$*)$_2$), 20.40 (CH(*CH$_3$*)$_2$), 19.8 (d, $^3J_{C\text{-}P}$ = 5.5 Hz, CH*CH$_3$* L-Ala), 19.1 (d, $^3J_{C\text{-}P}$ = 5.9 Hz, CH*CH$_3$* L-Ala), 13.3 (d, $^4J_{C\text{-}P}$ = 2.3 Hz, *CH$_3$*, alkene), 13.2 (d, $^4J_{C\text{-}P}$ = 2.7 Hz, *CH$_3$*, alkene), 10.8 (*C*H_3, base). **HPLC**: Reverse phase HPLC eluting with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 minutes, 1ml/min, λ = 254 nm and 263 nm, showed one peak with t_R 16.26 min. **HRMS (ESI):** m/z $[M+Na]^+$ calcd for $C_{26}H_{32}N_3O_6P$: 536.1926, found: 536.1921.

From PrepHPLC also the Z isomer ***Z*-154a** was isolated as pale yellow foamy solid (6 mg, 3%).  **^{31}P NMR (202 MHz, CD_3OD) δ_P**: 30.40, 29.66. **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 8.19-8.14 (m, 1H, Ar*H*), 7.90-7.85 (m, 1H, Ar*H*), 7.74-7.71 (m, 1H, Ar*H*), 7.57-7.39 (m, 5H, Ar*H*, *H*-6), 5.51-5.59 (m, 1H, *CH*=), 4.97-4.86 (m, 1H, *CH*(CH_3)$_2$), 4.32-4.26 (m, 2H, *CH*$_2$-N),4.04-3.98 (m, 1H, *CH*CH_3 L-Ala), 3.24-3.09 (m, 2H, *CH*$_2$P), 1.73-4.69 (m 6H, CH_3, base; *CH*$_3$, alkene), 1.27 (d, *J* = 7.0 Hz, 1.5H, CH*CH*$_3$ L-Ala), 1.21-1.12 (m, 7.5H, CH*CH*$_3$ L-Ala, CH(*CH*$_3$)$_2$, CH(*CH*$_3$)$_2$). **^{13}C NMR (125 MHz, CD_3OD) δ_C**: 173.5 (d, $^3J_{C\text{-}P}$ = 3.9 Hz, *C*=O, ester), 173.1 (d, $^3J_{C\text{-}P}$ = 3.5 Hz, *C*=O, ester), 165.3 (*C*-4), 151.8 (*C*-2), 151.7 (*C*-2), 146.4 (d, $^2J_{C\text{-}P}$ = 10.2 Hz, *C*-O, Ph), 146.2 (d, $^2J_{C\text{-}P}$ = 10.8 Hz, *C*-O, Ph), 1401.1 (*C*-6), 141.0 (*C*-6), 134.9 (*C*-Ar), 134.8 (d, $^3J_{C\text{-}P}$ = 14.6 Hz, *C*=), 134.5 (d, $^3J_{C\text{-}P}$ = 14.6 Hz, *C*=), 127.4 (*CH*-Ar), 126.9 (d, $^3J_{C\text{-}P}$ = 4.8 Hz *C*-Ar), 126.8 (d, $^3J_{C\text{-}P}$ = 5.3 Hz *C*-Ar), 126.3 (*CH*-Ar), 126.04 (*CH*-Ar), 126.01 (*CH*-Ar), 125.156 (*CH*-Ar), 125.12 (*CH*-Ar), 124.5 (*CH*-Ar), 124.4 (*CH*-Ar), 121.5 (*CH*-Ar), 121.4 (*CH*-Ar), 119.1 ($^2J_{C\text{-}P}$ = 11.1 Hz, *CH*=), 119.0 ($^2J_{C\text{-}P}$ = 10.4 Hz, *CH*=), 115.7 (d, $^3J_{C\text{-}P}$ = 3.4 Hz *CH*-Ar), 115.4 (d, $^3J_{C\text{-}P}$ = 3.4 Hz *CH*-Ar), 110.0 (*C*-5), 68.6 (*CH*(CH_3)$_2$), 49.7 (*CH*CH_3 L-Ala), 49.5 (*CH*CH_3 L-Ala), 47.1 (*CH*$_2$-N), 28.2 (d, $^1J_{C\text{-}P}$ = 129.0 Hz *CH*$_2$P), 28.0 (d, $^1J_{C\text{-}P}$ = 129.8 Hz *CH*$_2$P), 20.51 (CH(*CH*$_3$)$_2$), 20.50 (CH(*CH*$_3$)$_2$), 20.4 (CH(*CH*$_3$)$_2$), 20.3 (CH(*CH*$_3$)$_2$), 19.8 (d, $^3J_{C\text{-}P}$ = 5.5 Hz, CH*CH*$_3$ L-Ala), 19.0 (d, $^3J_{C\text{-}P}$ = 5.5 Hz, CH*CH*$_3$ L-Ala), 10.7 (d, $^4J_{C\text{-}P}$ = 3.0 Hz, *CH*$_3$, alkene), 10.6 (*C*H_3, base). **HPLC**: Reverse phase HPLC eluting with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 minutes, 1ml/min, λ = 254 nm and 263 nm, showed one peak with t_R 17.90 min.

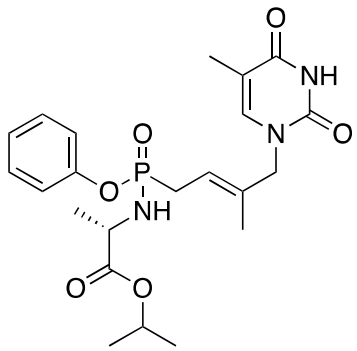
Benzyl (((*E*)-3-methyl-4-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)but-2-en-1-yl)(naphthalen-1-yloxy)phosphoryl)-*L*-alaninate *E*-154b



Prepared according to the standard procedure **E** for the synthesis of ANP ProTide using *O*-(1-naphthyl)-(benzyloxy-*L*-Alanine)-allylphosphonate **147b** (240 mg, 586.1 µmol) and *N*^{1}-2'-methylallylthymine **152** (211 mg, 1.17 mmol) and Hoveyda-Grubbs 2nd generation catalyst (15 mol%) in dry CH_2Cl_2 (10 ml). After evaporation, the crude was purified by Biotage Isolera One (120 g ZIP cartridge KP-SIL, 100 ml/min, gradient eluent system

MeOH/CH_2Cl_2 1% 1CV, 1-10% 12CV, 10% 2CV), to afford a mixture of the E and Z isomer. The two isomers were then separated by PrepHPLC (20 ml/min, isocratic eluting system CH_3CN/H_2O - 40/60, 30 minutes), to afford the title compound as pale yellow foamy solid (43 mg, 13%). R_f = 0.40 (CH_2Cl_2/MeOH - 95:5). **^{31}P NMR (202 MHz, CD_3OD) δ_P**: 30.28, 29.35. **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 8.12-8.10 (m, 1H, Ar*H*), 7.88-7.86 (m, 1H, Ar*H*), 7.70-7.66 (m, 1H, Ar*H*), 7.54-7.22 (m, 10H, Ar*H*), 5.53-5.45 (m, 1H, *CH*=), 5.12, 5.06 (ABq, J_{AB} = 12.2 Hz, 1H, *CH*$_2$Ph), 4.99, 4.95 (ABq, J_{AB} = 12.2 Hz, 1H, *CH*$_2$Ph), 4.25-4.20 (m, 2H, *CH*$_2$-N), 4.11-4.06 (m, 1H, *CH*CH_3 L-Ala), 3.02-2.82 (m, 2H, *CH*$_2$P), 1.74 (s, 3H, CH_3, base), 1.64 (d, *J* = 3.6 Hz 1.5H, *CH*$_3$, alkene), 1.61 (d, *J* = 3.5 Hz 1.5H, *CH*$_3$, alkene), 1.26 (d, *J* = 6.9 Hz, 1.5H, CH*CH*$_3$ L-Ala), 1.18 (d, *J* = 7.2 Hz, 1.5H, CH*CH*$_3$ L-Ala). **^{13}C NMR (125 MHz, CD_3OD) δ_C**: 173.7 (d, $^3J_{C\text{-}P}$ = 3.8 Hz, *C*=O, ester), 173.3 (d, $^3J_{C\text{-}P}$ = 3.7 Hz, *C*=O, ester), 165.35 (*C*-4), 165.32 (*C*-4), 151.67 (*C*-2), 151.60 (*C*-2), 146.5 (d, $^2J_{C\text{-}P}$ = 9.7 Hz, *C*-O, Ph), 146.3 (d, $^2J_{C\text{-}P}$ = 9.9 Hz, *C*-O, Ph), 140.9 (*C*-6), 135.8 (*C*-Ar), 135.7 (*C*-Ar), 135.4 (d, $^3J_{C\text{-}P}$ = 14.4 Hz, *C*=), 135.2 (d, $^3J_{C\text{-}P}$ = 14.7 Hz, *C*=), 134.9 (*C*-Ar), 128.19 (*CH*-Ar), 128.12 (*CH*-Ar), 127.9 (*CH*-Ar), 127.8 (*CH*-Ar), 127.5 (*CH*-Ar), 127.4 (*CH*-Ar), 126.7 (d, $^3J_{C\text{-}P}$ = 5.0 Hz *C*-Ar), 126.6 (d, $^3J_{C\text{-}P}$ = 5.3 Hz *C*-Ar), 126.3 (*CH*-Ar), 126.0 (*CH*-Ar), 125.19 (*CH*-Ar), 125.11 (*CH*-Ar), 124.3 (*CH*-Ar), 124.2 (*CH*-Ar), 121.4 (*CH*-Ar), 121.2 (*CH*-Ar), 117.1 ($^2J_{C\text{-}P}$ = 10.7 Hz, *CH*=), 116.6 ($^2J_{C\text{-}P}$ = 10.7 Hz, *CH*=), 115.4 (d, $^3J_{C\text{-}P}$ = 4.0 Hz *CH*-Ar), 115.1 (d, $^3J_{C\text{-}P}$ = 3.6 Hz *CH*-Ar), 110.1 (*C*-5), 66.5 (*CH*$_2$Ph), 66.4 (*CH*$_2$Ph), 53.4 (d, $^4J_{C\text{-}P}$ = 2.0 Hz, *CH*$_2$-N), 53.2 (d, $^4J_{C\text{-}P}$ = 2.2 Hz, *CH*$_2$-N), 49.6 (*CH*CH_3 L-Ala), 49.5 (*CH*CH_3 L-Ala), 28.3 (d, $^1J_{C\text{-}P}$ = 129.3 Hz *CH*$_2$P), 28.1 (d, $^1J_{C\text{-}P}$ = 130.2 Hz *CH*$_2$P), 19.6 (d, $^3J_{C\text{-}P}$ = 5.6 Hz, CH*CH*$_3$ L-Ala), 19.0 (d, $^3J_{C\text{-}P}$ = 5.3 Hz, CH*CH*$_3$ L-Ala), 13.3 (d, $^4J_{C\text{-}P}$ = 2.4 Hz, *CH*$_3$, alkene), 13.2 (d, $^4J_{C\text{-}P}$ = 2.4 Hz, *CH*$_3$, alkene), 10.8 (*C*H_3, base). **HPLC**: Reverse phase HPLC eluting with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 minutes, 1ml/min, λ = 254 nm and 263 nm, showed one peak with t_R 18.01 min. **HRMS (ESI):** m/z $[M+Na]^+$ calcd for $C_{30}H_{32}N_3O_6P$: 584.1926, found: 584.1921.

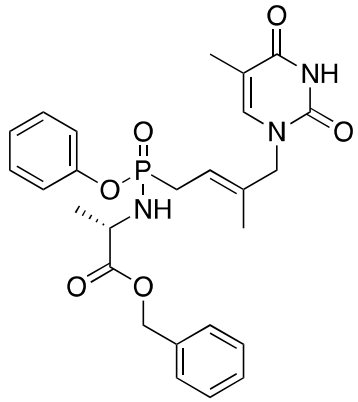
Isopropyl (((*E*)-3-methyl-4-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)but-2-en-1-yl)(phenoxy)phosphoryl)-*L*-alaninate *E*-154c



Prepared according to the standard procedure **E** for the synthesis of ANP ProTide using *O*-phenyl-(isopropyloxy-*L*-Alanine)-allylphosphonate **147c** (140 mg, 449.7 μmol) and N^1-2'-methylallylthymine **152** (162 mg, 899.4 μmol) and Hoveyda-Grubbs 2nd generation catalyst (15 mol%) in dry CH_2Cl_2 (8 ml). After evaporation, the crude was purified by Biotage Isolera One (25 g SNAP cartridge ULTRA, 75 ml/min, gradient eluent system MeOH/CH_2Cl_2 1% 1CV, 1-10% 12CV, 10% 2CV), to afford a mixture of the E and Z isomer. The two isomers were then separated by PrepHPLC (20 ml/min, gradient eluting system CH_3CN/H_2O from 10/90 to 100/0, 30 minutes), to afford the title compound as pale yellow foamy solid (20.4 mg, 10%). R_f = 0.27 (CH_2Cl_2/MeOH - 94:6). **^{31}P NMR (202 MHz, CD_3OD) δ_P**: 29.80, 29.03. **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 7.41-7.32 (m, 3H, *H*-6, Ar*H*), 7.22-7.16 (m, 3H, Ar*H*), 5,52 (q, *J* = 6.9 Hz, 0.4H, *CH*=), 5.43 (q, *J* = 6.9 Hz, 0.6H, *CH*=), 4.98 (sept, *J* = 6.2 Hz, 0.4H, *CH*$(CH_3)_2$), 4.92 (sept, *J* = 6.2 Hz, 0.6H, *CH*$(CH_3)_2$), 4.36-4.30 (m, 2H, CH_2-N), 3.97-3.91 (m, 1H, *CH*CH_3 L-Ala), 2.96-2.77 (m, 2H, CH_2P), 1.85 (s, 3H, CH_3, base), 1.72 (s, 1.2H, CH_3, alkene), 1.71 (s, 1.8H, CH_3, alkene),1.29 (d, *J* = 6.9 Hz, 1.8H, CH*CH*$_3$ L-Ala), 1.25 (d, *J* = 6.3 Hz, 1.2H, CH(*CH*$_3$)$_2$), 1.23 (d, *J* = 6.2 Hz, 1.2H, CH(*CH*$_3$)$_2$), 1.21-1.96 (m, 4.8H, CH*CH*$_3$ L-Ala, CH(*CH*$_3$)$_2$). **^{13}C NMR (125 MHz, CD_3OD) δ_C**: 173.6 (d, $^3J_{C\text{-}P}$ = 4.6 Hz, *C*=O, ester), 173.2 (d, $^3J_{C\text{-}P}$ = 4.1 Hz, *C*=O, ester), 165.3 (*C*-4), 151.7 (*C*-2), 151.6 (*C*-2), 150.5 (d, $^2J_{C\text{-}P}$ = 9.8 Hz, *C*-O, Ph), 150.3 (d, $^2J_{C\text{-}P}$ = 9.5 Hz, *C*-O, Ph), 141.0 (*C*-6), 135.4 (d, $^3J_{C\text{-}P}$ = 14.4 Hz, *C*=), 135.0 (d, $^3J_{C\text{-}P}$ = 14.4 Hz, *C*=), 129.32 (*CH*-Ar), 129.30 (*CH*-Ar), 124.5 (*CH*-Ar), 124.4 (*CH*-Ar), 120.6 (d, $^3J_{C\text{-}P}$ = 4.3 Hz *CH*-Ar), 120.4 (d, $^3J_{C\text{-}P}$ = 4.6 Hz *CH*-Ar), 117.2 (d, $^2J_{C\text{-}P}$ = 11.0 Hz, *CH*=), 116.6 (d, $^2J_{C\text{-}P}$ = 10.8 Hz, *CH*=), 110.1 (*C*-5), 68.67 (*CH*$(CH_3)_2$), 68.63 (*CH*$(CH_3)_2$), 53.5 (d, $^4J_{C\text{-}P}$ = 2.4 Hz, CH_2-N), 53.3 (d, $^4J_{C\text{-}P}$ = 2.4 Hz, CH_2-N), 49.6 (*CH*CH_3 L-Ala), 49.4 (*CH*CH_3 L-Ala), 28.2 (d, $^1J_{C\text{-}P}$ = 129.5 Hz, CH_2P), 28.0 (d, $^1J_{C\text{-}P}$ = 130.5 Hz, CH_2P), 20.58 (CH(*CH*$_3$)$_2$), 20.53 (CH(*CH*$_3$)$_2$), 20.4 (CH(*CH*$_3$)$_2$), 19.8 (d, $^3J_{C\text{-}P}$ = 5.4 Hz, CH*CH*$_3$ L-Ala), 19.1 (d, $^3J_{C\text{-}P}$ = 5.4 Hz, CH*CH*$_3$ L-Ala),13.2 (d, $^4J_{C\text{-}P}$ = 2.5 Hz, CH_3, alkene), 13.1 (d, $^4J_{C\text{-}P}$ = 2.2 Hz, CH_3, alkene), 10.8 (CH_3, base). **HPLC**: Reverse phase HPLC eluting with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 minutes, 1ml/min, λ = 254 nm and 263 nm,

showed one peak with t_R 13.94 min. **HRMS (ESI):** m/z $[M+Na]^+$ calcd for $C_{22}H_{30}N_3O_6P$: 486.1770, found: 486.1764.

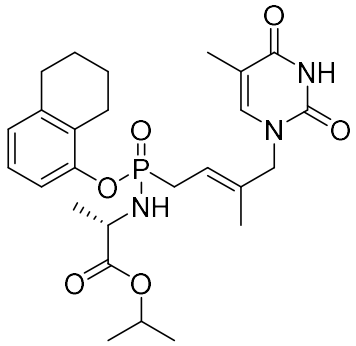
Benzyl (((*E*)-3-methyl-4-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)but-2-en-1-yl)(phenoxy)phosphoryl)-*L*-alaninate *E*-154d



Prepared according to the standard procedure **E** for the synthesis of ANP ProTide using *O*-phenyl-(benzyloxy-*L*-Alanine)-allylphosphonate **147d** (200 mg, 556.5 μmol) and N^1-2'-methylallylthymine **152** (200.6 mg, 1.11 mmol) and Hoveyda-Grubbs 2nd generation catalyst (15 mol%) in dry CH_2Cl_2 (8 ml). After evaporation, the crude was purified by Biotage Isolera One (25 g SNAP cartridge ULTRA, 75 ml/min, gradient eluent system 2-propanol/CH_2Cl_2 1% 1CV, 1-10% 12CV, 10% 2CV), to afford a mixture of the E and Z isomer. The two isomers were then separated by PrepHPLC (20 ml/min, isocratic eluting system CH_3CN/H_2O - 35/65, 30 minutes), to afford the title compound as pale yellow foamy solid (64 mg, 23%). R_f = 0.42 (CH_2Cl_2/2-propanol - 95:5). **^{31}P NMR (202 MHz, CD_3OD) δ_P**: 29.79, 28.99. **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 7.36-7.29 (m, 8H, *H*-6, Ar*H*), 7.20-7.14 (m, 3H, Ar*H*), 5.49-5.40 (m, 1H, *CH*=), 5.16, 5.13 (ABq, J_{AB} = 12.3 Hz, 1H, CH_2Ph), 5.08 (s app, 1H, CH_2Ph), 4.31-4.29 (m, 2H, CH_2-N), 4.08-4.01 (m, 1H, *CH*CH_3 L-Ala), 2.89-2.73 (m, 2H, CH_2P), 1.84 (s, 3H, CH_3, base), 1.67-1.64 (m, 3H, CH_3, alkene), 1.30 (d, *J* = 7.0 Hz, 1.5H, CHCH_3 L-Ala), 1.22 (d, *J* = 7.1 Hz, 1.5H, CHCH_3 L-Ala). **^{13}C NMR (125 MHz, CD_3OD) δ_C**: 173.8 (d, $^3J_{C\text{-}P}$ = 4.5 Hz, *C*=O, ester), 173.4 (d, $^3J_{C\text{-}P}$ = 3.9 Hz, *C*=O, ester), 165.3 (*C*-4), 151.7 (*C*-2), 151.6 (*C*-2), 150.5 (d, $^2J_{C\text{-}P}$ = 9.3 Hz, *C*-O, Ph), 150.4 (d, $^2J_{C\text{-}P}$ = 9.4 Hz, *C*-O, Ph), 140.98 (*C*-6), 140.97 (*C*-6), 135.9 (*C*-Ar), 135.8 (*C*-Ar), 135.3 (d, $^3J_{C\text{-}P}$ = 14.1 Hz, *C*=), 135.0 (d, $^3J_{C\text{-}P}$ = 14.0 Hz, *C*=), 129.35 (*CH*-Ar), 129.34 (*CH*-Ar), 128.23 (*CH*-Ar), 128.20 (*CH*-Ar), 128.01 (*CH*-Ar), 128.00 (*CH*-Ar), 127.96 (*CH*-Ar), 127.95 (*CH*-Ar), 124.6 (*CH*-Ar), 124.5 (*CH*-Ar), 120.6 (d, $^3J_{C\text{-}P}$ = 4.3 Hz *CH*-Ar), 120.4 (d, $^3J_{C\text{-}P}$ = 3.8 Hz *CH*-Ar), 117.2 (d, $^2J_{C\text{-}P}$ = 10.7 Hz, *CH*=), 116.6 (d, $^2J_{C\text{-}P}$ = 10.7 Hz, *CH*=), 110.13 (*C*-5), 110.11 (*C*-5), 65.5 (CH_2Ph), 66.4 (CH_2Ph), 53.5 (d, $^4J_{C\text{-}P}$ = 2.4 Hz, CH_2-N), 53.3 (d, $^4J_{C\text{-}P}$ = 2.3 Hz, CH_2-N), 49.6 (*CH*CH_3 L-Ala), 49.4 (*CH*CH_3 L-Ala), 28.2 (d, $^1J_{C\text{-}P}$ = 129.7 Hz, CH_2P), 28.0 (d, $^1J_{C\text{-}P}$ = 130.3 Hz, CH_2P), 19.7 (d, $^3J_{C\text{-}P}$ = 5.3 Hz, CHCH_3 L-Ala), 19.1 (d, $^3J_{C\text{-}P}$ = 5.3 Hz, CHCH_3 L-Ala), 13.3 (d, $^4J_{C\text{-}P}$ = 1.8 Hz, CH_3, alkene), 13.1 (d, $^4J_{C\text{-}P}$ = 2.2 Hz, CH_3, alkene), 10.9 (CH_3, base). **HPLC**:

Reverse phase HPLC eluting with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 minutes, 1ml/min, λ = 254 nm and 263 nm, showed one peak with t_R 15.21 min. **HRMS (ESI):** m/z $[M+Na]^+$ calcd for $C_{26}H_{30}N_3O_6P$: 534.1764, found: 534.1764.

Isopropyl (((*E*)-3-methyl-4-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)but-2-en-1-yl)((5,6,7,8-tetrahydronaphthalen-1-yl)oxy)phosphoryl)-*L*-alaninate *E*-154e and isopropyl (((*Z*)-3-methyl-4-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)but-2-en-1-yl)((5,6,7,8-tetrahydronaphthalen-1-yl)oxy)phosphoryl)-*L*-alaninate *Z*-154e



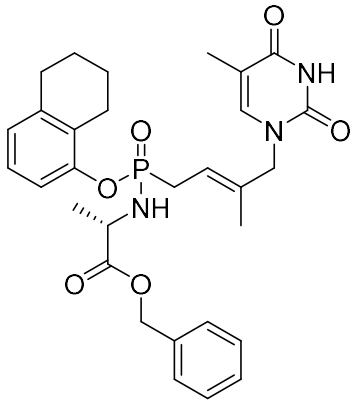
Prepared according to the standard procedure **E** for the synthesis of ANP ProTide using *O*-(5,6,7,8-tetrahydro-1-naphthyl)-(isopropyloxy-*L*-Alanine)-allylphosphonate **147e** (200 mg, 547.3 μmol) and N^1-2'-methylallylthymine **152** (197 mg, 1.09 mmol) and Hoveyda-Grubbs 2nd generation catalyst (15 mol%) in dry CH_2Cl_2 (10 ml). After evaporation, the crude was purified by Biotage Isolera One (25 g SNAP cartridge ULTRA, 75 ml/min, gradient eluent system 2-propanol/CH_2Cl_2 1% 1CV, 1-10% 12CV, 10% 2CV), to afford a mixture of the E and Z isomer. The two isomers were then separated by preparative RP-HPLC (20 ml/min, isocratic eluting system CH_3CN/H_2O - 35/65, 30 minutes), to afford the title compound ***E*-154e** as pale yellow foamy solid (72 mg, 26%). R_f = 0.26 (CH_2Cl_2/2-propanol - 95:5). **^{31}P NMR (202 MHz, CD_3OD) δ_P**: 29.38, 28.55. **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 7.34 (s, 0.5H, *H*-6), 7.33 (s, 0.5H, *H*-6), 7.17-7.12 (m, 1H, Ar*H*), 7.05-7.00 (m, 1H, Ar*H*), 6.89-6.86 (m, 1H, Ar*H*), 5.57-5.52 (m, 0.5H, *CH*=), 5.48-5.44 (m, 0.5H, *CH*=), 5.01-4.85 (m, 1H, *CH*$(CH_3)_2$), 4.36-4.29 (m, 2H, *CH*$_2$-N), 3.99-3.91 (m, 1H, *CH*CH_3 L-Ala), 2.94-2.79 (m, 2H, *CH*$_2$P), 2.77-2.74 (m, 2H, Ar*H*), 2.69-2.67 (m, 2H, Ar*H*), 1.84 (s, 3H, CH_3, base), 1.80-1.76 (m, 4H, Ar*H*), 1.70 (d, *J* = 2.9 Hz 3H, *CH*$_3$, alkene), 1.29 (d, *J* = 7.2 Hz, 1.5H, CH*CH*$_3$ L-Ala), 1.25-1.24 (m, 4.5H, CH*CH*$_3$ L-Ala, CH(*CH*$_3$)$_2$), 1.19 (d, *J* = 6.2 Hz, 3H, CH(*CH*$_3$)$_2$). **^{13}C NMR (125 MHz, CD_3OD) δ_C**: 173.7 (d, $^3J_{C\text{-}P}$ = 3.8 Hz, *C*=O, ester), 173.2 (d, $^3J_{C\text{-}P}$ = 4.1 Hz, *C*=O, ester), 165.3 (*C*-4), 151.7 (*C*-2), 151.6 (*C*-2), 148.8 (d, $^2J_{C\text{-}P}$ = 9.4 Hz, *C*-O, Ph), 148.7 (d, $^2J_{C\text{-}P}$ = 9.9 Hz, *C*-O, Ph), 141.05 (*C*-6), 141.02 (*C*-6), 139.19 (*C*-Ar), 139.16 (*C*-Ar), 135.1 (d, $^3J_{C\text{-}P}$ = 14.1 Hz, *C*=), 134.8 (d, $^3J_{C\text{-}P}$ = 14.3 Hz, *C*=), 128.4 (d, $^3J_{C\text{-}P}$ = 5.5 Hz *C*-Ar), 128.3 (d, $^3J_{C\text{-}P}$ = 5.8 Hz *C*-Ar), 125.4 (*CH*-Ar), 125.3 (*CH*-Ar), 125.1 (*CH*-Ar), 125.0 (*CH*-Ar), 117.4 ($^2J_{C\text{-}P}$ = 11.0 Hz, *CH*=), 116.9 ($^2J_{C\text{-}P}$ =

10.2 Hz, *CH*=), 116.8 (d, $^{3}J_{C\text{-}P}$ = 4.4 Hz *CH*-Ar), 116.7 (d, $^{3}J_{C\text{-}P}$ = 3.3 Hz *CH*-Ar), 110.1 (*C*-5), 110.1 (*C*-5), 68.66 (*CH*(CH_3)$_2$), 68.62 (*CH*(CH_3)$_2$), 53.6 (d, $^{4}J_{C\text{-}P}$ = 2.4 Hz, *CH*$_2$-N), 53.3 (d, $^{4}J_{C\text{-}P}$ = 2.4 Hz, *CH*$_2$-N), 49.7 (*CH*CH_3 L-Ala), 49.5 (*CH*CH_3 L-Ala), 29.1 (*CH*$_2$-Ar), 28.5 (d, $^{1}J_{C\text{-}P}$ = 129.8 Hz *CH*$_2$P), 28.2 (d, $^{1}J_{C\text{-}P}$ = 131.2 Hz *CH*$_2$P), 23.5 (*CH*$_2$-Ar), 22.47 (*CH*$_2$-Ar), 22.44 (*CH*$_2$-Ar), 22.42 (*CH*$_2$-Ar), 20.6 (CH(*CH*$_3$)$_2$), 20.56 (CH(*CH*$_3$)$_2$), 20.55 (CH(*CH*$_3$)$_2$), 20.4 (CH(*CH*$_3$)$_2$), 19.9 (d, $^{3}J_{C\text{-}P}$ = 5.2 Hz, CH*CH*$_3$ L-Ala), 19.1 (d, $^{3}J_{C\text{-}P}$ = 5.8 Hz, CH*CH*$_3$ L-Ala), 13.3 (d, $^{4}J_{C\text{-}P}$ = 2.4 Hz, *CH*$_3$, alkene), 13.2 (d, $^{4}J_{C\text{-}P}$ = 2.2 Hz, *CH*$_3$, alkene), 10.8 (*C*H_3, base). **HPLC**: Reverse phase HPLC eluting with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 minutes, 1ml/min, λ = 254 nm and 263 nm, showed one peak with t_R 16.85 min. **HRMS (ESI):** m/z $[M+Na]^+$ calcd for $C_{26}H_{36}N_3O_6P$: 540.2239, found: 540.2234.

From PrepHPLC also the Z isomer ***Z*-154e** was isolated as pale yellow foamy solid (7 mg, 3%). **^{31}P NMR (202 MHz, CD_3OD) δ_P**: 29.41, 28.57. **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 7.32 (s, 1H, *H*-6), 7.05-7.00 (m, 1H, Ar*H*), 6.95-6.89 (m, 1H, Ar*H*), 6.83-6.76 (m, 1H, Ar*H*), 5.53-5.48 (m, 1H, *CH*=), 4.88-4.77 (m, 1H, *CH*(CH_3)$_2$), 4.38-4.27 (m, 2H, *CH*$_2$-N), 3.85-3.81 (m, 1H, *CH*CH_3 L-Ala), 2.98-2.83 (m, 2H, *CH*$_2$P), 2.65-2.62 (m, 4H, Ar*H*), 1.71-1.68 (m, 7H, Ar*H*, CH_3, base), 1.60 (m, 3H, *CH*$_3$, alkene), 1.20-1.07 (m, 9H, CH*CH*$_3$ L-Ala, CH(*CH*$_3$)$_2$). **^{13}C NMR (125 MHz, CD_3OD) δ_C**: 173.7 (d, $^{3}J_{C\text{-}P}$ = 3.8 Hz, *C*=O, ester), 173.3 (d, $^{3}J_{C\text{-}P}$ = 3.8 Hz, *C*=O, ester), 165.3 (*C*-4), 151.7 (*C*-2), 151.6 (*C*-2), 148.8 (d, $^{2}J_{C\text{-}P}$ = 9.5 Hz, *C*-O, Ph), 148.7 (d, $^{2}J_{C\text{-}P}$ = 9.9 Hz, *C*-O, Ph), 141.05 (*C*-6), 141.02 (*C*-6), 139.19 (*C*-Ar), 139.16 (*C*-Ar), 135.1 (d, $^{3}J_{C\text{-}P}$ = 14.2 Hz, *C*=), 134.8 (d, $^{3}J_{C\text{-}P}$ = 14.2 Hz, *C*=), 128.4 (d, $^{3}J_{C\text{-}P}$ = 5.1 Hz *C*-Ar), 128.3 (d, $^{3}J_{C\text{-}P}$ = 5.6 Hz *C*-Ar), 125.4 (*CH*-Ar), 125.3 (*CH*-Ar), 125.1 (*CH*-Ar), 125.0 (*CH*-Ar), 119.3 ($^{2}J_{C\text{-}P}$ = 11.3 Hz, *CH*=), 119.2 ($^{2}J_{C\text{-}P}$ = 11.0 Hz, *CH*=), 117.2 (d, $^{3}J_{C\text{-}P}$ = 3.5 Hz *CH*-Ar), 117.0 (d, $^{3}J_{C\text{-}P}$ = 3.5 Hz *CH*-Ar), 110.0 (*C*-5), 68.66 (*CH*(CH_3)$_2$), 68.63 (*CH*(CH_3)$_2$), 49.7 (*CH*CH_3 L-Ala), 49.5 (*CH*CH_3 L-Ala), 47.3 (*CH*$_2$-N), 29.1 (*CH*$_2$-Ar), 28.5 (d, $^{1}J_{C\text{-}P}$ = 129.8 Hz *CH*$_2$P), 28.2 (d, $^{1}J_{C\text{-}P}$ = 131.2 Hz *CH*$_2$P), 26.4 (*CH*$_2$-Ar), 26.3 (*CH*$_2$-Ar), 25.8 (*CH*$_2$-Ar), 25.7 (*CH*$_2$-Ar), 20.5 (*CH*$_3$, alkene), 20.4 (*CH*$_3$, alkene), 19.97 (CH(*CH*$_3$)$_2$), 19.93 (CH(*CH*$_3$)$_2$), 19.7 (d, $^{3}J_{C\text{-}P}$ = 5.2 Hz, CH*CH*$_3$ L-Ala), 19.0 (d, $^{3}J_{C\text{-}P}$ = 5.8 Hz, CH*CH*$_3$ L-Ala), 10.7 (*C*H_3, base). **HPLC**: Reverse phase HPLC eluting with gradient method

CH_3CN/H_2O from 10/90 to 100/0 in 30 minutes, 1ml/min, λ = 254 nm and 263 nm, showed one peak with t_R 17.90 min.

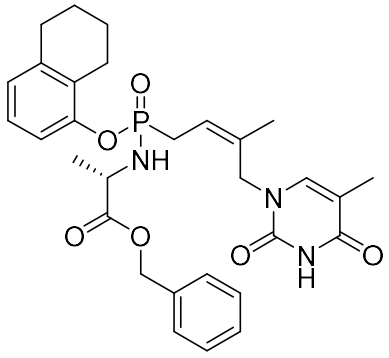
Benzyl (((*E*)-3-methyl-4-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)but-2-en-1-yl)((5,6,7,8-tetrahydronaphthalen-1-yl)oxy)phosphoryl)-*L*-alaninate *E*-154f and benzyl (((*Z*)-3-methyl-4-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)but-2-en-1-yl)((5,6,7,8-tetrahydronaphthalen-1-yl)oxy)phosphoryl)-*L*-alaninate *Z*-154f



Prepared according to the standard procedure **E** for the synthesis of ANP ProTide using *O*-(5,6,7,8-tetrahydro-1-naphthyl)-(benzyloxy-*L*-Alanine)-allylphosphonate **147f** (200 mg, 483.7 μmol) and N^1-2'-methylallylthymine **152** (174 mg, 967.4 μmol) and Hoveyda-Grubbs 2nd generation catalyst (15 mol%) in dry CH_2Cl_2 (8 ml). After evaporation, the crude was purified by Biotage Isolera One (25 g SNAP cartridge ULTRA, 75 ml/min, gradient eluent system 2-propanol/CH_2Cl_2 1% 1CV, 1-10% 12CV, 10% 2CV), to afford a mixture of the E and Z isomer. The two isomers were then separated by reverse Biotage Isolera One (60 g SNAP cartridge KP-C18-HS, 100 ml/min, isocratic eluent system CH_3CN/H_2O 30-60% 12CV) to afford the title compound ***E*-154f** as pale yellow foamy solid (36 mg, 14%). R_f = 0.23 (CH_2Cl_2/2-propanol - 95:5). **^{31}P NMR (202 MHz, CD_3OD) δ_P**: 29.36, 28.51. **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 7.36-7.28 (m, 6H, *H*-6, Ar*H*), 7.16-7.12 (m, 1H, Ar*H*), 7.04-6.95 (m, 1H, Ar*H*), 6.89-6.88 (m, 1H, Ar*H*), 5.49-5.42 (m, 1H, *CH*=), 5.15, 5.12 (ABq, J_{AB} = 12.2 Hz, 1H, CH_2Ph), 5.07, 5.05 (ABq, J_{AB} = 12.6 Hz, 1H, CH_2Ph), 4.31-4.22 (m, 2H, CH_2-N), 4.09-4.00 (m, 1H, $CHCH_3$ L-Ala), 2.90-2.77 (m, 2H, CH_2P), 2.74 (bs, 2H, Ar*H*), 2.66 (bs, 2H, Ar*H*), 1.83 (s, 3H, CH_3, base), 1.76-1.75 (m, 4H, Ar*H*), 1.66 (d, *J* = 2.9 Hz 1.8H, CH_3, alkene), 1.64 (d, *J* = 3.1 Hz 1.2H, CH_3, alkene), 1.31 (d, *J* = 7.0 Hz, 1.5H, CHCH_3 L-Ala), 1.26 (d, *J* = 7.1 Hz, 1.5H, CHCH_3 L-Ala). **^{13}C NMR (125 MHz, CD_3OD) δ_C**: 173.8 (d, $^3J_{C\text{-}P}$ = 3.8 Hz, *C*=O, ester), 173.4 (d, $^3J_{C\text{-}P}$ = 3.5 Hz, *C*=O, ester), 165.38 (*C*-4), 165.37 (*C*-4), 151.7 (*C*-2), 151.6 (*C*-2), 148.8 (d, $^2J_{C\text{-}P}$ = 9.8 Hz, *C*-O, Ph), 148.7 (d, $^2J_{C\text{-}P}$ = 9.5 Hz, *C*-O, Ph), 140.9 (*C*-6), 139.2 (*C*-Ar), 139.1 (*C*-Ar), 135.9 (*C*-Ar), 135.8 (*C*-Ar), 135.1 (d, $^3J_{C\text{-}P}$ = 14.5 Hz, *C*=), 134.8 (d, $^3J_{C\text{-}P}$ = 13.9 Hz, *C*=), 128.4 (d, $^3J_{C\text{-}P}$ = 5.4 Hz *C*-Ar), 128.3 (d, $^3J_{C\text{-}P}$ = 5.7 Hz *C*-Ar), 128.2 (*CH*-Ar), 128.1 (*CH*-Ar), 127.96 (*CH*-Ar), 127.92 (*CH*-Ar), 127.8 (*CH*-Ar), 125.4 (*CH*-Ar), 125.3 (*CH*-Ar), 125.1

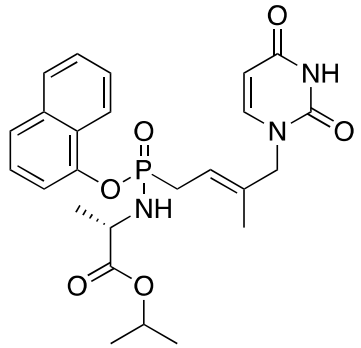
(*CH*-Ar), 125.0 (*CH*-Ar), 117.4 ($^{2}J_{C\text{-}P}$ = 10.9 Hz, *CH*=), 116.8 ($^{2}J_{C\text{-}P}$ = 10.4 Hz, *CH*=), 116.7 (d, $^{3}J_{C\text{-}P}$ = 3.2 Hz *CH*-Ar), 116.6 (d, $^{3}J_{C\text{-}P}$ = 3.2 Hz *CH*-Ar), 110.09 (*C*-5), 110.06 (*C*-5), 66.5 (*CH*$_2$Ph), 66.4 (*CH*$_2$Ph), 53.5 (d, $^{4}J_{C\text{-}P}$ = 2.1 Hz, *CH*$_2$-N), 53.3 (d, $^{4}J_{C\text{-}P}$ = 2.4 Hz, *CH*$_2$-N), 49.6 (*CH*CH$_3$ L-Ala), 49.5 (*CH*CH$_3$ L-Ala), 29.1 (*CH*$_2$-Ar), 28.2 (d, $^{1}J_{C\text{-}P}$ = 130.8 Hz *CH*$_2$P), 28.2 (d, $^{1}J_{C\text{-}P}$ = 130.8 Hz *CH*$_2$P), 23.3 (*CH*$_2$-Ar), 22.45 (*CH*$_2$-Ar), 22.43 (*CH*$_2$-Ar), 22.40 (*CH*$_2$-Ar), 19.7-19.6 (m, CH*CH*$_3$ L-Ala, *CH*$_3$, alkene), 19.0 (d, $^{3}J_{C\text{-}P}$ = 5.7 Hz, CH*CH*$_3$ L-Ala), 10.8 (*C*H$_3$, base). **HPLC**: Reverse phase HPLC eluting with gradient method CH$_3$CN/H$_2$O from 10/90 to 100/0 in 30 minutes, 1ml/min, λ = 254 nm and 263 nm, showed one peak with t_R 18.44 min. **HRMS (ESI):** m/z $[M+Na]^+$ calcd for $C_{30}H_{36}N_3O_6P$: 588.2239, found: 588.2234.

From PrepHPLC also the Z isomer ***Z*-154f** was isolated as pale yellow foamy solid (18 mg, 7%). **^{31}P NMR (202 MHz, CD$_3$OD) δ$_P$**: 29.38, 28.63. **^{1}H NMR (500 MHz, CD$_3$OD) δ$_H$**: 7.42-7.33 (m, 6H, *H*-6, Ar*H*), 7.15-7.12 (m, 1H, Ar*H*), 7.07-6.956(m, 1H, Ar*H*), 6.92-6.86 (m, 1H, Ar*H*), 5.60-5.55 (m, 1H, *CH*=), 5.15 (AB app s, 1H, *CH*$_2$Ph), 5.07 (AB app s, 1H, *CH*$_2$Ph), 4.46-4.26 (m, 2H, *CH*$_2$-N), 4.11-4.03 (m, 1H, *CH*CH$_3$ L-Ala), 3.07-2.90 (m, 2H, *CH*$_2$P), 2.76-2.70 (m, 4H, Ar*H*), 1.83-1.77 (m, 7H, Ar*H*, CH$_3$, base), 1.69 (d, *J* = 5.2 Hz 1.8H, *CH*$_3$, alkene), 1.66 (d, *J* = 5.2 Hz 1.2H, *CH*$_3$, alkene), 1.34 (d, *J* = 6.9 Hz, 1.5H, CH*CH*$_3$ L-Ala), 1.24 (d, *J* = 6.9 Hz, 1.5H, CH*CH*$_3$ L-Ala). **^{13}C NMR (125 MHz, CD$_3$OD) δ$_C$**: 173.8 (d, $^{3}J_{C\text{-}P}$ = 3.8 Hz, *C*=O, ester), 173.4 (d, $^{3}J_{C\text{-}P}$ = 3.5 Hz, *C*=O, ester), 165.3 (*C*-4), 151.7 (*C*-2), 148.8 (d, $^{2}J_{C\text{-}P}$ = 9.2 Hz, *C*-O, Ph), 148.7 (d, $^{2}J_{C\text{-}P}$ = 9.2 Hz, *C*-O, Ph), 141.1 (*C*-6), 141.0 (*C*-6), 139.25 (*C*-Ar), 139.21 (*C*-Ar), 135.9 (*C*-Ar), 135.8 (*C*-Ar), 134.4 (d, $^{3}J_{C\text{-}P}$ = 14.3 Hz, *C*=), 134.2 (d, $^{3}J_{C\text{-}P}$ = 13.5 Hz, *C*=), 128.7 (d, $^{3}J_{C\text{-}P}$ = 5.9 Hz *C*-Ar), 128.6 (d, $^{3}J_{C\text{-}P}$ = 5.0 Hz *C*-Ar), 128.18 (*CH*-Ar), 128.15 (*CH*-Ar), 127.94 (*CH*-Ar), 127.91 (*CH*-Ar), 127.87 (*CH*-Ar), 127.84 (*CH*-Ar), 125.4 (*CH*-Ar), 125.3 (*CH*-Ar), 125.2 (*CH*-Ar), 125.0 (*CH*-Ar), 119.2 ($^{2}J_{C\text{-}P}$ = 10.9 Hz, *CH*=), 119.0 ($^{2}J_{C\text{-}P}$ = 11.8 Hz, *CH*=), 117.2 (d, $^{3}J_{C\text{-}P}$ = 3.3 Hz *CH*-Ar), 116.9 (d, $^{3}J_{C\text{-}P}$ = 2.5 Hz *CH*-Ar), 110.05 (*C*-5), 110.02 (*C*-5), 66.5 (*CH*$_2$Ph), 66.4 (*CH*$_2$Ph), 49.7 (*CH*CH$_3$ L-Ala), 49.5 (*CH*CH$_3$ L-Ala), 47.2 (*CH*$_2$-N), 29.1 (*CH*$_2$-Ar), 28.3 (d, $^{1}J_{C\text{-}P}$ = 129.4 Hz *CH*$_2$P), 28.1 (d, $^{1}J_{C\text{-}P}$ = 130.2 Hz *CH*$_2$P), 26.4 (*CH*$_2$-Ar), 26.3 (*CH*$_2$-Ar), 25.8 (*CH*$_2$-Ar), 25.7 (*CH*$_2$-Ar), 19.7-19.6 (m, CH*CH*$_3$ L-Ala, *CH*$_3$, alkene), 18.8 (d, $^{3}J_{C\text{-}P}$ = 5.9 Hz, CH*CH*$_3$ L-Ala), 10.7 (*C*H$_3$, base). **HPLC**: Reverse phase



HPLC eluting with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 minutes, 1ml/min, λ = 254 nm and 263 nm, showed one peak with t_R 19.31 min.

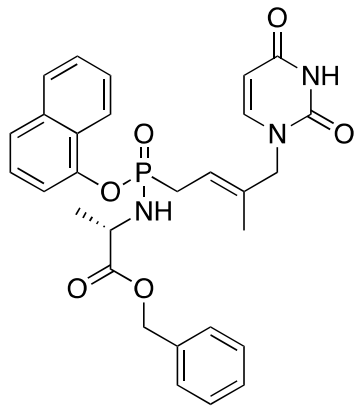
Isopropyl (((*E*)-4-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-3-methylbut-2-en-1-yl)(naphthalen-1-yloxy)phosphoryl)-*L*-alaninate *E*-156a



Prepared according to the standard procedure **E** for the synthesis of ANP ProTide using *O*-(1-naphthyl)-(isopropyloxy-*L*-Alanine)-allylphosphonate **147a** (150 mg, 415 μmol) and N^1-2'-methylallyluracyl **153** (137 mg, 830.1 μmol) and Hoveyda-Grubbs 2nd generation catalyst (15 mol%) in dry CH_2Cl_2 (8 ml). After evaporation, the crude was purified by Biotage Isolera One (50 g SNAP cartridge ULTRA, 100 ml/min, gradient eluent system MeOH/CH_2Cl_2 1% 1CV, 1-10% 12CV, 10% 2CV), to afford a mixture of the E and Z isomer. The two isomers were then separated by PrepHPLC (20 ml/min, isocratic eluting system CH_3CN/H_2O - 35/65, 30 minutes), to afford the title compound as pale yellow foamy solid (28 mg, 14%). R_f= 0.24 (CH_2Cl_2/MeOH - 96:4). **^{31}P NMR (202 MHz, CD_3OD) δ_P**: 30.28, 29.49. **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 8.14-8.13 (m, 1H, Ar*H*), 7.88-7.84 (m, 1H, Ar*H*), 7.70-7.65 (m, 1H, Ar*H*), 7.58-7.49 (m, 3H, Ar*H*), 7.46-7.38 (m, 2H, *H*-6, Ar*H*), 5.61-5.56 (m, 1.5H, *CH*=, *H*-5), 5.51-5.47 (m, 0.5H, *CH*=), 4.93 (sept, *J* = 6.5 Hz, 0.5H, *CH*$(CH_3)_2$), 4.88-4.84 (m, 0.5H, *CH*$(CH_3)_2$), 4.33-4.29 (m, 2H, *CH*$_2$-N), 4.04-3.97 (m, 1H, *CH*CH_3 L-Ala), 3.08-2.90 (m, 2H, *CH*$_2$P), 1.65 (bs, 3H, *CH*$_3$, alkene), 1.27 (d, *J* = 7.0 Hz, 1.5H, CH*CH*$_3$ L-Ala), 1.20 (d, *J* = 6.2 Hz, 1.5H, CH(*CH*$_3$)$_2$), 1.19 (d, *J* = 6.2 Hz, 1.5H, CH(*CH*$_3$)$_2$), 1.17 (d, *J* = 6.9 Hz, 1.5H, CH*CH*$_3$ L-Ala), 1.12 (d, *J* = 6.2 Hz, 1.5H, CH(*CH*$_3$)$_2$), 1.15 (d, *J* = 6.2 Hz, 1.5H, CH(*CH*$_3$)$_2$). **^{13}C NMR (125 MHz, CD_3OD) δ_C**: 173.6 (d, $^3J_{C\text{-}P}$ = 4.3 Hz, *C*=O, ester), 173.2 (d, $^3J_{C\text{-}P}$ = 4.1 Hz, *C*=O, ester), 165.17 (*C*-4), 165.15 (*C*-4), 151.5 (*C*-2), 151.4 (*C*-2), 146.5 (d, $^2J_{C\text{-}P}$ = 9.7 Hz, *C*-O, Ph), 146.3 (d, $^2J_{C\text{-}P}$ = 9.7 Hz, *C*-O, Ph), 145.2 (*C*-6), 145.1 (*C*-6), 135.2 (d, $^3J_{C\text{-}P}$ = 14.5 Hz, *C*=), 135.4 (d, $^3J_{C\text{-}P}$ = 14.5 Hz, *C*=), 134.9 (*C*-Ar), 127.5 (*CH*-Ar), 127.4 (*CH*-Ar), 126.8 (d, $^3J_{C\text{-}P}$ = 4.9 Hz *C*-Ar), 126.6 (d, $^3J_{C\text{-}P}$ = 5.1 Hz *C*-Ar), 126.3 (*CH*-Ar), 126.1 (*CH*-Ar), 125.2 (*CH*-Ar), 125.1 (*CH*-Ar), 124.3 (*CH*-Ar), 124.2 (*CH*-Ar), 121.5 (*CH*-Ar), 121.3 (*CH*-Ar), 117.4 ($^2J_{C\text{-}P}$ = 11.0 Hz, *CH*=), 116.9 ($^2J_{C\text{-}P}$ = 11.0 Hz, *CH*=), 115.4 (d, $^3J_{C\text{-}P}$ = 3.8 Hz *CH*-Ar), 115.1 (d, $^3J_{C\text{-}P}$ = 3.8 Hz *CH*-Ar), 101.2 (*C*-5), 68.69 (*CH*$(CH_3)_2$), 68.66 (*CH*$(CH_3)_2$), 53.7 (d, $^4J_{C\text{-}P}$ = 2.3 Hz, *CH*$_2$-N), 53.5 (d, $^4J_{C\text{-}P}$ = 2.3 Hz, *CH*$_2$-N), 49.7 (*CH*CH_3 L-Ala), 49.5 (*CH*CH_3 L-Ala),

28.3 (d, $^{1}J_{C\text{-}P}$ = 128.9 Hz *CH*$_2$P), 28.1 (d, $^{1}J_{C\text{-}P}$ = 129.8 Hz *CH*$_2$P), 20.6 (CH(*CH*$_3$)$_2$), 20.56 (CH(*CH*$_3$)$_2$), 20.52 (CH(*CH*$_3$)$_2$), 20.4 (CH(*CH*$_3$)$_2$), 19.8 (d, $^{3}J_{C\text{-}P}$ = 5.8 Hz, CH*CH*$_3$ L-Ala), 19.1 (d, $^{3}J_{C\text{-}P}$ = 5.5 Hz, CH*CH*$_3$ L-Ala), 13.3 (d, $^{4}J_{C\text{-}P}$ = 2.4 Hz, *CH*$_3$, alkene), 13.2 (d, $^{4}J_{C\text{-}P}$ = 2.2 Hz, *CH*$_3$, alkene). **HPLC**: Reverse phase HPLC eluting with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 minutes, 1ml/min, λ = 254 nm and 263 nm, showed one peak with t_R 15.57 min. **HRMS (ESI):** m/z $[M+Na]^+$ calcd for $C_{25}H_{30}N_3O_6P$: 522.1770, found: 522.1764.

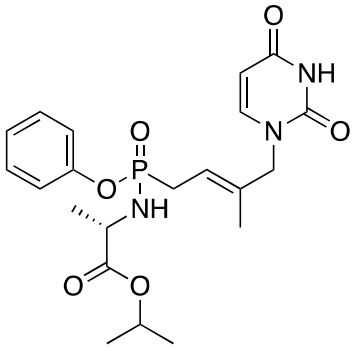
Benzyl (((*E*)-4-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-3-methylbut-2-en-1-yl)(naphthalen-1-yloxy)phosphoryl)-*L*-alaninate *E*-156b



Prepared according to the standard procedure **E** for the synthesis of ANP ProTide using *O*-(1-naphthyl)-(benzyloxy-*L*-Alanine)-allylphosphonate **147b** (240 mg, 586.1 μmol) and *N*^{1}-2'-methylallyluracyl **153** (195 mg, 1.17 mmol) and Hoveyda-Grubbs 2nd generation catalyst (15 mol%) in dry CH_2Cl_2 (10 ml). After evaporation, the crude was purified by Biotage Isolera One (120 g ZIP cartridge KP-SIL, 100 ml/min, gradient eluent system $MeOH/CH_2Cl_2$ 1% 1CV, 1-10% 12CV, 10% 2CV), to afford a mixture of the E and Z isomer. The two isomers were then separated by PrepHPLC (20 ml/min, isocratic eluting system CH_3CN/H_2O - 40/60, 30 minutes), to afford the title compound as pale yellow foamy solid (13 mg, 5%). R_f = 0.33 (CH_2Cl_2/MeOH - 95:5). **^{31}P NMR (202 MHz, CD_3OD) δ_P**: 30.33, 29.48. **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 8.08-8.06 (m, 1H, Ar*H*), 7.77-7.64 (m, 1H, Ar*H*), 7.59-7.55 (m, 1H, Ar*H*), 7.43-7.13 (m, 10H, , *H*-6, Ar*H*), 5.48 (d, *J* = 7.9 Hz, 1H, *H*-5), 5.42-5.34 (m, 1H, *CH*=), 5.01, 4.96 (ABq, J_{AB} = 12.2 Hz, 1H, *CH*$_2$Ph), 4.88, 4.84 (ABq, J_{AB} = 12.2 Hz, 1H, *CH*$_2$Ph), 4.16 (bs, 2H, *CH*$_2$-N), 4.00-3.94 (m, 1H, *CH*CH$_3$ L-Ala), 2.90-2.75 (m, 2H, *CH*$_2$P), 1.51 (d, *J* = 3.4 Hz 1.5H, *CH*$_3$, alkene), 1.49 (d, *J* = 3.5 Hz 1.5H, *CH*$_3$, alkene), 1.15 (d, *J* = 7.0 Hz, 1.5H, CH*CH*$_3$ L-Ala), 1.06 (d, *J* = 7.1 Hz, 1.5H, CH*CH*$_3$ L-Ala). **^{13}C NMR (125 MHz, CD_3OD) δ_C**: 173.7 (d, $^{3}J_{C\text{-}P}$ = 4.3 Hz, *C*=O, ester), 173.3 (d, $^{3}J_{C\text{-}P}$ = 4.1 Hz, *C*=O, ester), 163.5 (*C*-4), 151.5 (*C*-2), 151.4 (*C*-2), 146.5 (d, $^{2}J_{C\text{-}P}$ = 9.9 Hz, *C*-O, Ph), 146.3 (d, $^{2}J_{C\text{-}P}$ = 9.7 Hz, *C*-O, Ph), 145.2 (*C*-6), 145.1 (*C*-6), 135.8 (*C*-Ar), 135.7 (*C*-Ar), 135.3 (d, $^{3}J_{C\text{-}P}$ = 14.1 Hz, *C*=), 135.2 (d, $^{3}J_{C\text{-}P}$ = 14.8 Hz, *C*=), 134.9 (*C*-Ar), 128.18 (*CH*-Ar), 128.10 (*CH*-Ar), 127.9 (*CH*-Ar), 127.8 (*CH*-Ar), 126.7 (d, $^{3}J_{C\text{-}P}$ = 4.9 Hz *C*-Ar), 126.6 (d, $^{3}J_{C\text{-}P}$ = 4.7 Hz *C*-Ar), 126.3 (*CH*-

Ar), 126.08(*CH*-Ar), 126.06(*CH*-Ar), 125.17 (*CH*-Ar), 125.10 (*CH*-Ar), 124.3 (*CH*-Ar), 124.2 (*CH*-Ar), 121.4 (*CH*-Ar), 121.3 (*CH*-Ar), 117.3 ($^2J_{C\text{-}P}$ = 11.1 Hz, *CH*=), 116.8 ($^2J_{C\text{-}P}$ = 11.7 Hz, *CH*=), 115.17 (d, $^3J_{C\text{-}P}$ = 3.9 Hz *CH*-Ar), 115.10 (d, $^3J_{C\text{-}P}$ = 3.9 Hz *CH*-Ar), 101.2 (*C*-5), 66.5 (*CH*$_2$Ph), 66.4 (*CH*$_2$Ph), 53.7 (d, $^4J_{C\text{-}P}$ = 2.6 Hz, *CH*$_2$-N), 53.5 (d, $^4J_{C\text{-}P}$ = 2.6 Hz, *CH*$_2$-N), 49.6 (*CH*CH$_3$ L-Ala), 49.4 (*CH*CH$_3$ L-Ala), 28.2 (d, $^1J_{C\text{-}P}$ = 129.0 Hz *CH*$_2$P), 28.0 (d, $^1J_{C\text{-}P}$ = 129.9 Hz *CH*$_2$P), 19.6 (d, $^3J_{C\text{-}P}$ = 5.7 Hz, CH*CH*$_3$ L-Ala), 18.9 (d, $^3J_{C\text{-}P}$ = 5.7 Hz, CH*CH*$_3$ L-Ala), 13.2 (d, $^4J_{C\text{-}P}$ = 2.4 Hz, *CH*$_3$, alkene), 13.1 (d, $^4J_{C\text{-}P}$ = 2.4 Hz, *CH*$_3$, alkene). **HPLC**: Reverse phase HPLC eluting with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 minutes, 1ml/min, λ = 254 nm and 263 nm, showed one peak with t_R 15.87 min. **HRMS (ESI):** m/z $[M+Na]^+$ calcd for $C_{29}H_{30}N_3O_6P$: 570.1770, found: 570.1764.

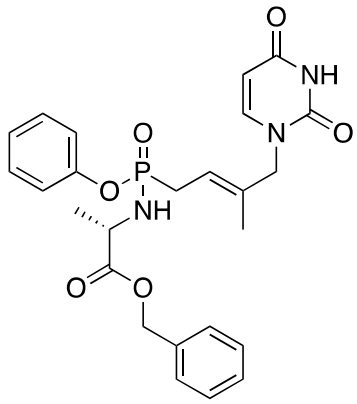
Isopropyl (((*E*)-4-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-3-methylbut-2-en-1-yl)(phenoxy)phosphoryl)-*L*-alaninate *E*-156c



Prepared according to the standard procedure **E** for the synthesis of ANP ProTide using *O*-phenyl-(isopropyloxy-*L*-Alanine)-allylphosphonate **147c** (140 mg, 449.7 μmol) and N^1-2'-methylallyluracyl **153** (150 mg, 1.11 mmol) and Hoveyda-Grubbs 2nd generation catalyst (15 mol%) in dry CH_2Cl_2 (8 ml). After evaporation, the crude was purified by Biotage Isolera One (25 g SNAP cartridge ULTRA, 75 ml/min, gradient eluent system MeOH/CH_2Cl_2 1% 1CV, 1-10% 12CV, 10% 2CV), to afford a mixture of the E and Z isomer. The two isomers were then separated by PrepHPLC (20 ml/min, gradient eluting system CH_3CN/H_2O from 10/90 to 100/0, 30 minutes), to afford the title compound as pale yellow foamy solid (20 mg, 10%). R_f = 0.42 (CH_2Cl_2/MeOH - 95:5). **^{31}P NMR (202 MHz, CD$_3$OD) δ$_P$**: 29.74, 28.97. **^{1}H NMR (500 MHz, CD$_3$OD) δ$_H$**: 7.53 (d, *J* = 7.8 Hz, 0.3H, *H*-6), 7.50 (d, *J* = 7.8 Hz, 0.7H, *H*-6), 7.38-7.33 (m, 2H, Ar*H*), 7.23-7.16 (m, 3H, Ar*H*), 5.67 (d, *J* = 7.9 Hz, 1H, *H*-5), 5.54 (q, *J* = 7.0 Hz, 0.3H, *CH*=), 5.46 (q, *J* = 7.0 Hz, 0.7H, *CH*=), 5.02-4.89 (m, 1H, *CH*(CH$_3$)$_2$), 4.36-4.33 (m, 2H, *CH*$_2$-N), 3.98-3.91 (m, 1H, *CH*CH$_3$ L-Ala), 2.95-2.77 (m, 2H, *CH*$_2$P), 1.72-1.71 (m, 3H, *CH*$_3$, alkene), 1.29 (d, *J* = 7.0 Hz, 2.1H, CH*CH*$_3$ L-Ala), 1.25 (d, *J* = 6.7 Hz, 0.9H, CH(*CH*$_3$)$_2$), 1.23 (d, *J* = 6.2 Hz, 0.9H, CH(*CH*$_3$)$_2$), 1.21-1.19 (m, 5.1H, CH*CH*$_3$ L-Ala, CH(*CH*$_3$)$_2$). **^{13}C NMR (125 MHz, CD$_3$OD) δ$_C$**: 173.5 (d, $^3J_{C\text{-}P}$ = 4.7 Hz, *C*=O, ester), 173.2 (d, $^3J_{C\text{-}P}$ = 4.1 Hz, *C*=O, ester), 165.2 (*C*-4), 151.5 (*C*-2), 151.4 (*C*-2), 150.6 (d, $^2J_{C\text{-}}$

$_P$ = 9.6 Hz, *C*-O, Ph), 150.4 (d, $^2J_{C\text{-}P}$ = 9.3 Hz, *C*-O, Ph), 145.32 (*C*-6), 145.30 (*C*-6), 135.2 (d, $^3J_{C\text{-}P}$ = 14.5 Hz, *C*=), 134.8 (d, $^3J_{C\text{-}P}$ = 14.2 Hz, *C*=), 129.3 (*CH*-Ar), 124.6 (*CH*-Ar), 124.4 (*CH*-Ar), 120.6 (d, $^3J_{C\text{-}P}$ = 4.6 Hz *CH*-Ar), 120.4 (d, $^3J_{C\text{-}P}$ = 4.3 Hz *CH*-Ar), 117.6 (d, $^2J_{C\text{-}P}$ = 11.2 Hz, *CH*=), 116.9 (d, $^2J_{C\text{-}P}$ = 10.7 Hz, *CH*=), 101.2 (*C*-5), 68.67 (*CH*$(CH_3)_2$), 68.64 (*CH*$(CH_3)_2$), 53.8 (d, $^4J_{C\text{-}P}$ = 2.4 Hz, CH_2-N), 53.5 (d, $^4J_{C\text{-}P}$ = 2.1 Hz, CH_2-N), 49.6 (*CH*CH_3 L-Ala), 49.4 (*CH*CH_3 L-Ala), 28.2 (d, $^1J_{C\text{-}P}$ = 129.7 Hz, CH_2P), 28.0 (d, $^1J_{C\text{-}P}$ = 130.3 Hz, CH_2P), 20.6 (CH(*CH*$_3$)$_2$), 20.5 (CH(*CH*$_3$)$_2$), 20.4 (CH(*CH*$_3$)$_2$), 19.8 (d, $^3J_{C\text{-}P}$ = 5.4 Hz, CH*CH*$_3$ L-Ala), 19.1 (d, $^3J_{C\text{-}P}$ = 5.4 Hz, CH*CH*$_3$ L-Ala),13.2 (d, $^4J_{C\text{-}P}$ = 2.4 Hz, *CH*$_3$, alkene), 13.1 (d, $^4J_{C\text{-}P}$ = 2.4 Hz, *CH*$_3$, alkene). **HPLC**: Reverse phase HPLC eluting with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 minutes, 1ml/min, λ = 254 nm and 263 nm, showed one peak with t_R 13.16 min. **HRMS (ESI):** m/z $[M+Na]^+$ calcd for $C_{21}H_{28}N_3O_6P$: 472.1613, found: 472.1608.

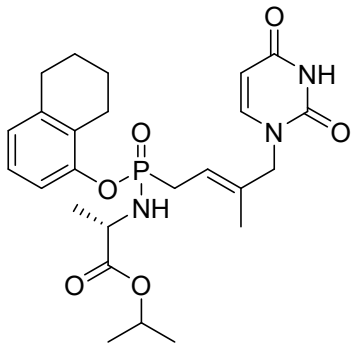
Benzyl (((*E*)-4-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-3-methylbut-2-en-1-yl)(phenoxy)phosphoryl)-*L*-alaninate *E*-156d



Prepared according to the standard procedure **E** for the synthesis of ANP ProTide using *O*-phenyl-(benzyloxy-*L*-Alanine)-allylphosphonate **147d** (200 mg, 556.5 μmol) and *N*^{1}-2'-methylallyluracyl **153** (184.9 mg, 1.11 mmol) and Hoveyda-Grubbs 2nd generation catalyst (15 mol%) in dry CH_2Cl_2 (8 ml). After evaporation, the crude was purified by Biotage Isolera One (25 g SNAP cartridge ULTRA, 75 ml/min, gradient eluent system 2-propanol/CH_2Cl_2 1% 1CV, 1-10% 12CV, 10% 2CV), to afford a mixture of the E and Z isomer. The two isomers were then separated by PrepHPLC (20 ml/min, isocratic eluting system CH_3CN/H_2O - 35/65, 30 minutes), to afford the title compound as pale yellow foamy solid (49 mg, 18%). R_f = 0.42 (CH_2Cl_2/2-propanol - 95:5). **^{31}P NMR (202 MHz, CD_3OD) δ_P**: 29.75, 28.94. **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 7.46 (d, *J* = 7.8 Hz, 1H, *H*-6), 7.37-7.29 (m, 7H, Ar*H*), 7.20-7.17 (m, 3H, Ar*H*), 5.67 (d, *J* = 7.8 Hz, 1H, *H*-5), 5.49-5.40 (m, 1H, *CH*=), 5.17, 5.14 (ABq, J_{AB} = 12.3 Hz, 1H, CH_2Ph), 5.08 (s app, 1H, CH_2Ph), 4.31-4.29 (m, 2H, CH_2-N), 4.08-4.04 (m, 1H, *CH*CH_3 L-Ala), 2.89-2.74 (m, 2H, CH_2P), 1.67-1.65 (m, 3H, *CH*$_3$, alkene), 1.30 (d, *J* = 6.9 Hz, 1.5H, CH*CH*$_3$ L-Ala), 1.22 (d, *J* = 7.2 Hz, 1.5H, CH*CH*$_3$ L-Ala). **^{13}C NMR (125 MHz, CD_3OD) δ_C**: 173.8 (d, $^3J_{C\text{-}P}$ = 4.4 Hz, *C*=O, ester), 173.4 (d, $^3J_{C\text{-}P}$ = 3.9 Hz, *C*=O, ester), 165.2 (*C*-4), 151.5 (*C*-2), 150.5 (d, $^2J_{C\text{-}P}$ =

9.2 Hz, *C*-O, Ph), 150.3 (d, $^{2}J_{C\text{-}P}$ = 10.0 Hz, *C*-O, Ph), 145.2 (*C*-6), 135.8 (*C*-Ar), 135.1 (d, $^{3}J_{C\text{-}P}$ = 14.4 Hz, *C*=), 134.8 (d, $^{3}J_{C\text{-}P}$ = 14.4 Hz, *C*=), 129.3 (*CH*-Ar), 128.23 (*CH*-Ar), 128.20 (*CH*-Ar), 128.0 (*CH*-Ar), 127.9 (*CH*-Ar), 124.6 (*CH*-Ar), 124.5 (*CH*-Ar), 120.6 (d, $^{3}J_{C\text{-}P}$ = 4.0 Hz *CH*-Ar), 120.4 (d, $^{3}J_{C\text{-}P}$ = 4.4 Hz *CH*-Ar), 117.5 (d, $^{2}J_{C\text{-}P}$ = 10.6 Hz, *CH*=), 116.9 (d, $^{2}J_{C\text{-}P}$ = 10.6 Hz, *CH*=), 101.2 (*C*-5), 65.5 (*CH*$_2$Ph), 66.4 (*CH*$_2$Ph), 53.8 (d, $^{4}J_{C\text{-}P}$ = 2.2 Hz, *CH*$_2$-N), 53.5 (d, $^{4}J_{C\text{-}P}$ = 2.4 Hz, *CH*$_2$-N), 49.5 (*CH*CH$_3$ L-Ala), 49.4 (*CH*CH$_3$ L-Ala), 28.2 (d, $^{1}J_{C\text{-}P}$ = 129.7 Hz, *CH*$_2$P), 28.0 (d, $^{1}J_{C\text{-}P}$ = 130.1 Hz, *CH*$_2$P), 19.7 (d, $^{3}J_{C\text{-}P}$ = 5.4 Hz, CH*CH*$_3$ L-Ala), 19.1 (d, $^{3}J_{C\text{-}P}$ = 5.2 Hz, CH*CH*$_3$ L-Ala), 13.2 (d, $^{4}J_{C\text{-}P}$ = 2.2 Hz, *CH*$_3$, alkene), 13.1 (d, $^{4}J_{C\text{-}P}$ = 2.2 Hz, *CH*$_3$, alkene). **HPLC**: Reverse phase HPLC eluting with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 minutes, 1ml/min, λ = 254 nm and 263 nm, showed one peak with t_R 14.56 min. **HRMS (ESI):** m/z $[M+Na]^+$ calcd for $C_{25}H_{28}N_3O_6P$: 520.1608, found: 520.1608.

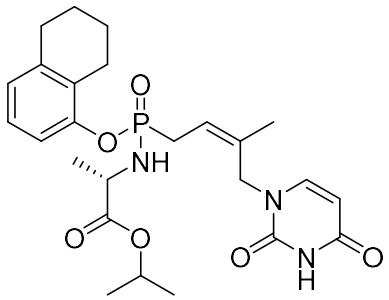
Isopropyl (((*E*)-4-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-3-methylbut-2-en-1-yl)((5,6,7,8-tetrahydronaphthalen-1-yl)oxy)phosphoryl)-*L*-alaninate *E*-156e and isopropyl (((*Z*)-4-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-3-methylbut-2-en-1-yl)((5,6,7,8-tetrahydronaphthalen-1-yl)oxy)phosphoryl)-*L*-alaninate *Z*-156e



Prepared according to the standard procedure **E** for the synthesis of ANP ProTide using *O*-(5,6,7,8-tetrahydro-1-naphthyl)-(isopropyloxy-*L*-Alanine)-allylphosphonate **147e** (200 mg, 547.3 μmol) and N^1-2'-methylallyluracyl **153** (181 mg, 1.09 mmol) and Hoveyda-Grubbs 2nd generation catalyst (15 mol%) in dry CH_2Cl_2 (10 ml). After evaporation, the crude was purified by Biotage Isolera One (25 g SNAP cartridge ULTRA, 75 ml/min, gradient eluent system 2-propanol/CH_2Cl_2 1% 1CV, 1-10% 12CV, 10% 2CV), to afford a mixture of the E and Z isomer. The two isomers were then separated by PrepHPLC (20 ml/min, isocratic eluting system CH_3CN/H_2O - 35/65, 30 minutes), to afford the title compound ***E*-156e** as pale yellow foamy solid (31 mg, 11%). R_f = 0.23 (CH_2Cl_2/2-propanol - 95:5). **^{31}P NMR (202 MHz, CD_3OD) δ_P**: 27.84, 27.00. **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 7.52-7.49 (m, 1H, *H*-6), 7.17-7.12 (m, 1H, Ar*H*), 7.06-7.00 (m, 1H, Ar*H*), 6.90-6.87 (m, 1H, Ar*H*), 5.67 (d, *J* = 7.9 Hz, 1H, *H*-5), 5.58-5.54 (m, 0.5H, *CH*=), 5.49-5.45 (m, 0.5H, *CH*=), 5.00-4.85 (m, 1H, *CH*$(CH_3)_2$), 4.35 (bs, 2H, *CH*$_2$-N), 3.99-3.91 (m, 1H, *CH*CH$_3$ L-Ala), 2.97-2.82 (m, 2H, *CH*$_2$P), 2.78-2.75 (m, 2H, Ar*H*), 2.71-2.68 (m, 2H, Ar*H*), 1.82-1.78 (m, 4H, Ar*H*),

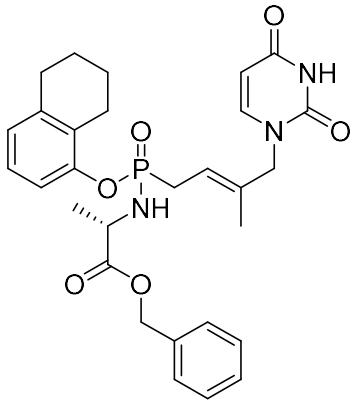
1.71 (d, *J* = 2.9 Hz 3H, *CH*$_3$, alkene), 1.30 (d, *J* = 7.0 Hz, 1.5H, CH*CH*$_3$ L-Ala), 1.25-1.24 (m, 4.5H, CH*CH*$_3$ L-Ala, CH(*CH*$_3$)$_2$), 1.19 (d, *J* = 6.3 Hz, 3H, CH(*CH*$_3$)$_2$). **^{13}C NMR (125 MHz, CD$_3$OD) δ$_C$**: 173.7 (d, $^3J_{C\text{-}P}$ = 3.9 Hz, *C*=O, ester), 173.2 (d, $^3J_{C\text{-}P}$ = 4.3 Hz, *C*=O, ester), 165.2 (*C*-4), 151.5 (*C*-2), 151.4 (*C*-2), 148.8 (d, $^2J_{C\text{-}P}$ = 9.5 Hz, *C*-O, Ph), 148.6 (d, $^2J_{C\text{-}P}$ = 9.7 Hz, *C*-O, Ph), 145.35 (*C*-6), 145.31 (*C*-6), 139.2 (*C*-Ar), 139.1 (*C*-Ar), 135.0 (d, $^3J_{C\text{-}P}$ = 14.5 Hz, *C*=), 134.6 (d, $^3J_{C\text{-}P}$ = 14.3 Hz, *C*=), 128.5 (d, $^3J_{C\text{-}P}$ = 5.4 Hz *C*-Ar), 128.3 (d, $^3J_{C\text{-}P}$ = 5.4 Hz *C*-Ar), 125.4 (*CH*-Ar), 125.3 (*CH*-Ar), 125.15 (*CH*-Ar), 125.10 (*CH*-Ar), 117.6 ($^2J_{C\text{-}P}$ = 11.0 Hz, *CH*=), 117.0 ($^2J_{C\text{-}P}$ = 10.9 Hz, *CH*=), 116.8 (d, $^3J_{C\text{-}P}$ = 3.3 Hz *CH*-Ar), 116.7 (d, $^3J_{C\text{-}P}$ = 3.3 Hz *CH*-Ar), 101.2 (*C*-5), 101.1 (*C*-5), 68.67 (*CH*(CH$_3$)$_2$), 68.63 (*CH*(CH$_3$)$_2$), 53.8 (d, $^4J_{C\text{-}P}$ = 2.4 Hz, *CH*$_2$-N), 53.5 (d, $^4J_{C\text{-}P}$ = 2.4 Hz, *CH*$_2$-N), 49.7 (*CH*CH$_3$ L-Ala), 49.5 (*CH*CH$_3$ L-Ala), 29.1 (*CH*$_2$-Ar), 28.5 (d, $^1J_{C\text{-}P}$ = 129.9 Hz *CH*$_2$P), 28.3 (d, $^1J_{C\text{-}P}$ = 130.9 Hz *CH*$_2$P), 23.3 (*CH*$_2$-Ar), 22.47 (*CH*$_2$-Ar), 22.44 (*CH*$_2$-Ar), 22.42 (*CH*$_2$-Ar), 20.6 (CH(*CH*$_3$)$_2$), 20.5 (CH(*CH*$_3$)$_2$), 20.4 (CH(*CH*$_3$)$_2$), 19.9 (d, $^3J_{C\text{-}P}$ = 5.0 Hz, CH*CH*$_3$ L-Ala), 19.1 (d, $^3J_{C\text{-}P}$ = 5.5 Hz, CH*CH*$_3$ L-Ala), 13.3 (d, $^4J_{C\text{-}P}$ = 2.3 Hz, *CH*$_3$, alkene), 13.2 (d, $^4J_{C\text{-}P}$ = 2.0 Hz, *CH*$_3$, alkene). **HPLC**: Reverse phase HPLC eluting with gradient method CH$_3$CN/H$_2$O from 10/90 to 100/0 in 30 minutes, 1ml/min, λ = 254 nm and 263 nm, showed one peak with t_R 16.14 min. **HRMS (ESI):** m/z [M+Na]$^+$ calcd for $C_{25}H_{34}N_3O_6P$: 526.2083, found: 526.2077.

From PrepHPLC also the Z isomer ***Z*-156e** was isolated as pale yellow foamy solid (2.5 mg, 1%). **^{31}P NMR (202 MHz, CD$_3$OD) δ$_P$**: 29.39, 28.63. **^{1}H NMR (500 MHz, CD$_3$OD) δ$_H$**: 7.50 (d, *J* = 7.6 Hz, 1H, *H*-6), 7.10-7.00 (m, 1H, Ar*H*), 6.95-6.88 (m, 1H, Ar*H*), 6.80-6.75 (m, 1H, Ar*H*), 5.54-5.38 (m 2H, *CH*=, *H*-5), 4.88-4.78 (m, 1H, *CH*(CH$_3$)$_2$), 4.38-4.29 (m, 2H, *CH*$_2$-N), 3.86-3.80 (m, 1H, *CH*CH$_3$ L-Ala), 2.97-2.84 (m, 2H, *CH*$_2$P), 2.66-2.58 (m, 4H, Ar*H*), 1.71-1.65 (m, 4H, Ar*H*), 1.61-1.54 (m, 3H, *CH*$_3$, alkene), 1.20-1.17 (m, 1.5H, CH*CH*$_3$ L-Ala), 1.13-1.07 (m, 7.5H, CH*CH*$_3$ L-Ala, CH(*CH*$_3$)$_2$), **^{13}C NMR (125 MHz, CD$_3$OD) δ$_C$**: 173.7 (d, $^3J_{C\text{-}P}$ = 3.9 Hz, *C*=O, ester), 173.2 (d, $^3J_{C\text{-}P}$ = 4.3 Hz, *C*=O, ester), 165.2 (*C*-4), 151.5 (*C*-2), 151.4 (*C*-2), 148.8 (d, $^2J_{C\text{-}P}$ = 9.5 Hz, *C*-O, Ph), 148.6 (d, $^2J_{C\text{-}P}$ = 9.7 Hz, *C*-O, Ph), 145.5 (*C*-6), 145.4 (*C*-6), 139.2 (*C*-Ar), 135.0 (d, $^3J_{C\text{-}P}$ = 14.5 Hz, *C*=), 134.6 (d, $^3J_{C\text{-}P}$ = 14.3 Hz, *C*=), 128.5 (d, $^3J_{C\text{-}P}$ = 5.4 Hz *C*-Ar), 128.3 (d, $^3J_{C\text{-}P}$ = 5.4 Hz *C*-Ar), 125.3 (*CH*-Ar), 125.2 (*CH*-Ar), 125.1 (*CH*-Ar), 125.0 (*CH*-Ar), 119.5 ($^2J_{C\text{-}P}$ = 10.1 Hz, *CH*=), 119.4 ($^2J_{C\text{-}P}$ = 10.8 Hz, *CH*=), 117.1



(d, $^{3}J_{C\text{-}P}$ = 3.3 Hz *CH*-Ar), 116.8 (d, $^{3}J_{C\text{-}P}$ = 3.3 Hz *CH*-Ar), 101.1 (*C*-5), 101.0 (*C*-5), 68.6 (*CH*(CH_3)$_2$), 49.7 (*CH*CH_3 L-Ala), 49.5 (*CH*CH_3 L-Ala), 47.0 (*CH_2*-N), 29.1 (*CH_2*-Ar), 28.2 (d, $^{1}J_{C\text{-}P}$ = 128.2 Hz *CH_2*P), 28.0 (d, $^{1}J_{C\text{-}P}$ = 130.5 Hz *CH_2*P), 23.4 (*CH_2*-Ar), 23.3 (*CH_2*-Ar), 22.47 (*CH_2*-Ar), 22.43 (*CH_2*-Ar), 20.57 (CH(*CH_3*)$_2$), 20.53 (CH(*CH_3*)$_2$), 20.4 (CH(*CH_3*)$_2$), 19.7 (d, $^{3}J_{C\text{-}P}$ = 4.7 Hz, CH*CH_3* L-Ala), 19.0 (d, $^{3}J_{C\text{-}P}$ = 5.4 Hz, CH*CH_3* L-Ala), 13.3 (d, $^{4}J_{C\text{-}P}$ = 2.7 Hz, *CH_3*, alkene).**HPLC**: Reverse phase HPLC eluting with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 minutes, 1ml/min, λ = 254 nm and 263 nm, showed one peak with t_R 16.82 min.

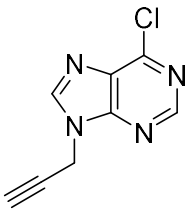
Benzyl (((*E*)-4-(2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-3-methylbut-2-en-1-yl)((5,6,7,8-tetrahydronaphthalen-1-yl)oxy)phosphoryl)-*L*-alaninate *E*-156f



Prepared according to the standard procedure **E** for the synthesis of ANP ProTide using *O*-(5,6,7,8-tetrahydro-1-naphthyl)-(benzyloxy-*L*-Alanine)-allylphosphonate **147f** (200 mg, 483.7 μmol) and *N*^{1}-2'-methylallyluracyl **153** (160 mg, 967.4 μmol) and Hoveyda-Grubbs 2nd generation catalyst (15 mol%) in dry CH_2Cl_2 (8 ml). After evaporation, the crude was purified by Biotage Isolera One (25 g SNAP cartridge ULTRA, 75 ml/min, gradient eluent system 2-propanol/CH_2Cl_2 1% 1CV, 1-10% 12CV, 10% 2CV), to afford a mixture of the E and Z isomer. The two isomers were then separated by PrepHPLC (20 ml/min, isocratic eluting system CH_3CN/H_2O - 40/60, 30 minutes), to afford the title compound as pale yellow foamy solid (14 mg, 5%). R_f = 0.25 (CH_2Cl_2/2-propanol - 95:5). **^{31}P NMR (202 MHz, CD_3OD) δ_P**: 29.33, 28.46. **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 7.34 (d, *J* = 7.8 Hz, 1H, *H*-6), 7.26-7.18 (m, 5H, Ar*H*), 7.03-6.99 (m, 1H, Ar*H*), 6.92-6.83 (m, 1H, Ar*H*), 6.77-6.73 (m, 1H, Ar*H*), 5.54 (d, *J* = 7.8 Hz, 0.6H, *H*-5), 5.53 (d, *J* = 7.9 Hz, 0.4H, *H*-5), 5.39-5.29 (m, 1H, *CH*=), 5.04, 5.01 (ABq, J_{AB} = 12.2 Hz, 1H, *CH_2*Ph), 4.95, 4.94 (ABq, J_{AB} = 12.2 Hz, 1H, *CH_2*Ph), 4.19-4.17 (m, 2H, *CH_2*-N), 3.97-3.88 (m, 1H, *CH*CH_3 L-Ala), 2.78-2.765 (m, 2H, *CH_2*P), 2.66-2.61 (m, 2H, Ar*H*), 2.56-2.53 (m, 2H, Ar*H*), 1.67-1.62 (m, 4H, Ar*H*), 1.54 (d, *J* = 3.8 Hz 1.8H, *CH_3*, alkene), 1.52 (d, *J* = 3.9 Hz 1.2H, *CH_3*, alkene), 1.20 (d, *J* = 6.9 Hz, 1.8H, CH*CH_3* L-Ala), 1.14 (d, *J* = 7.0 Hz, 1.2H, CH*CH_3* L-Ala). **^{13}C NMR (125 MHz, CD_3OD) δ_C**: 173.9 (d, $^{3}J_{C\text{-}P}$ = 4.0 Hz, *C*=O, ester), 173.4 (d, $^{3}J_{C\text{-}P}$ = 4.0 Hz, *C*=O, ester), 165.2 (*C*-4), 151.5 (*C*-2), 151.4 (*C*-2), 148.8 (d, $^{2}J_{C\text{-}P}$ = 9.1 Hz, *C*-O, Ph), 148.7 (d, $^{2}J_{C\text{-}P}$ = 9.7 Hz, *C*-O, Ph), 145.3 (*C*-6), 145.2 (*C*-6), 139.2 (*C*-Ar), 139.1 (*C*-Ar),

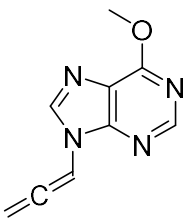
135.9 (*C*-Ar), 135.8 (*C*-Ar), 134.9 (d, $^{3}J_{C\text{-}P}$ = 14.7 Hz, *C*=), 134.7 (d, $^{3}J_{C\text{-}P}$ = 14.7 Hz, *C*=), 128.4 (d, $^{3}J_{C\text{-}P}$ = 4.7 Hz *C*-Ar), 128.3 (d, $^{3}J_{C\text{-}P}$ = 4.7 Hz *C*-Ar), 128.2 (*CH*-Ar), 128.1 (*CH*-Ar), 127.9 (*CH*-Ar), 127.8 (*CH*-Ar), 125.4 (*CH*-Ar), 125.3 (*CH*-Ar), 125.15 (*CH*-Ar), 125.08 (*CH*-Ar), 117.5 ($^{2}J_{C\text{-}P}$ = 10.9 Hz, *CH*=), 117.0 ($^{2}J_{C\text{-}P}$ = 10.9 Hz, *CH*=), 116.8 (d, $^{3}J_{C\text{-}P}$ = 3.2 Hz *CH*-Ar), 116.6 (d, $^{3}J_{C\text{-}P}$ = 3.2 Hz *CH*-Ar), 101.17 (*C*-5), 66.5 (*CH*$_2$Ph), 66.4 (*CH*$_2$Ph), 53.8 (d, $^{4}J_{C\text{-}P}$ = 2.5 Hz, *CH*$_2$-N), 53.5 (d, $^{4}J_{C\text{-}P}$ = 2.5 Hz, *CH*$_2$-N), 49.6 (*CH*CH$_3$ L-Ala), 49.5 (*CH*CH$_3$ L-Ala), 29.1 (*CH*$_2$-Ar), 28.4 (d, $^{1}J_{C\text{-}P}$ = 130.0 Hz *CH*$_2$P), 28.2 (d, $^{1}J_{C\text{-}P}$ = 130.8 Hz *CH*$_2$P), 23.3 (*CH*$_2$-Ar), 22.44 (*CH*$_2$-Ar), 22.42 (*CH*$_2$-Ar), 22.39 (*CH*$_2$-Ar), 19.7 (d, $^{3}J_{C\text{-}P}$ = 5.4 Hz, CH*CH*$_3$ L-Ala), 19.0 (d, $^{3}J_{C\text{-}P}$ = 5.6 Hz, CH*CH*$_3$ L-Ala), 13.2 (d, $^{4}J_{C\text{-}P}$ = 2.3 Hz, *CH*$_3$, alkene), 13.1 (d, $^{4}J_{C\text{-}P}$ = 2.4 Hz, *CH*$_3$, alkene). **HPLC**: Reverse phase HPLC eluting with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 minutes, 1ml/min, λ = 254 nm and 263 nm, showed one peak with t_R 17.66 min. **HRMS (ESI):** m/z $[M+Na]^+$ calcd for $C_{29}H_{34}N_3O_6P$: 574.2083, found: 574.2077.

6-Chloro-9-(prop-2-yn-1-yl)-9H-purine 165[11]



To a solution of 6-chloropurine (5g, 32.35 mmol) in dry DMF (150 ml) was added NaH (60% dispersion in mineral oil, 853.9 mg, 67.7 mmol) followed by propargyl bromide (6.7 ml, 35.59 mmol). The reaction was stirred at room temperature for 24 h, and the resulting mixture was separated between CH_2Cl_2 (200 ml) and water (3 x 200 ml). the organic layer was dried over $MgSO_4$, filtered and the solvent was removed under vacuum. The crude material was purified by Biotage Isolera One (100 g SNAP cartridge ULTRA, 100 ml/min, gradient eluent system EtOAc/CH_2Cl_2 20% 1CV, 20-100% 10CV, 100% 2CV), to afford the title compound as a yellow solid (2.5 g, 40%). R_f = 0.46 (CH_2Cl_2/EtOAc - 1:1). **^{1}H NMR (500 MHz, CDCl$_3$) δ$_H$**: 8.79 (s, 1H, *H*-2), 8.36 (s, 1H, *H*-8), 5.09 (d, *J* = 2.6 Hz, 2H, *CH*$_2$C), 2.61 (t, *J* = 2.6 Hz, 1H, C*CH*).

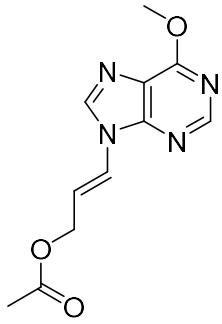
6-Methoxy-9-(propa-1,2-dien-1-yl)-9H-purine 166[11]



Under and argon atmosphere, 6-Chloro-9-propargyl purine **165** (3.2 g, 16.61 mmol) and K_2CO_3 (4.59 g, 33.23 mmol) were suspended in anhydrous MeOH (80 ml). The reaction mixture was refluxed for 15 minutes. After cooling to room temperature, the solvent was evaporated, and the reaction mixture extracted with EtOAc (100 ml) and water (3 x 80 ml). The organic phase was dried over $MgSO_4$, filtered, concentrated in vacuo and purified by

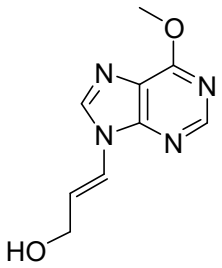
Biotage Isolera One (100 g SNAP cartridge ULTRA, 100 ml/min, gradient eluent system EtOAc/CH_2Cl_2 20% 1CV, 20-100% 10CV, 100% 2CV), to afford the title compound as a white solid (2.2 g, 71%). R_f = 0.57 (CH_2Cl_2/EtOAc - 1:1). **^{1}H NMR (500 MHz, $CDCl_3$) δ_H**: 8.49 (s, 1H, *H*-2), 7.99 (s, 1H, *H*-8), 7.30 (t, *J* = 6.5 Hz, 1H, =*CH*), 5.63 (d, *J* = 6.5 Hz, 2H, =CH_2), 4.13 (s, 3H, OCH_3).

(*E*)-3-(6-Methoxy-9H-purin-9-yl)allyl acetate 173[11]



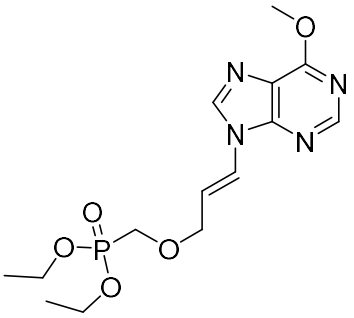
Under an argon atmosphere, compound **166** (1 g, 5.31 mmol) was dissolved in anhydrous acetonitrile (60 ml). Ag_2CO_3 (73.2 mg, 265.7 µmol) and glacial acetic acid (395.4 µl, 6.91 mmol) were added. The solution was refluxed for 16h. The resulting mixture was cooled down to room temperature, filtered through a pad of celite. Celite was rinsed with copious EtOAc. The filtrate was reduced under reduced pressure to give **173** as an orange solid in quantitative yield (1.5 g), used without further purification. R_f = 0.33 (CH_2Cl_2/EtOAc - 1:1). **^{1}H NMR (500 MHz, $CDCl_3$) δ_H**: 8.52 (s, 1H, *H*-2), 8.02 (s, 1H, *H*-8), 7.23 (dt, J_t = 14.4 Hz, *J* = 1.1 Hz, 1H, =*CH*N), 6.60 (dt, J_t = 14.4 Hz, *J* = 6.5 Hz, 1H, =*CH*CH_2), 4.71 (dd, *J* = 6.5 Hz, *J* = 1.1 Hz, 2H, CH_2), 4.13 (s, 3H, OCH_3), 2.05 (s, 3H, COCH_3).

(*E*)-3-(6-Methoxy-9H-purin-9-yl)prop-2-en-1-ol 176[11]



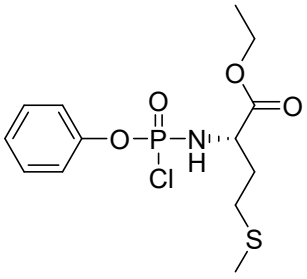
Compound **173** (1.9 g, 7.65 mmol) was solubilised in MeOH (80 ml), and KOH (858.9 mg, 15.31 mmol) was added. The solution was stirred for 16h at room temperature. Then, MeOH was removed under reduced pressure and the residue dissolved in CH_2Cl_2 (50 ml) and extracted with water (3 x 30 ml). The organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude product was purified by Biotage Isolera One (100 g SNAP cartridge ULTRA, 100 ml/min, gradient eluent system MeOH/CH_2Cl_2 2% 1CV, 2-20% 10CV, 20% 2CV), to afford the title compound as a yellow solid (1.6 g, quantitative). R_f = 0.42 (CH_2Cl_2/MeOH - 9:1). **^{1}H NMR (500 MHz, DMSO-d_6) δ_H**: 8.51 (s, 1H, *H*-2), 8.02 (s, 1H, *H*-8), 7.21 (dt, J_t = 14.4 Hz, *J* = 1.6 Hz, 1H, =*CH*N), 6.63 (dt, J_t = 14.4 Hz, *J* = 5.5 Hz, 1H, =*CH*CH_2), 4.36 (td, *J* = 5.5 Hz, *J* = 1.6 Hz, 2H, CH_2OH), 4.13 (s, 3H, OCH_3), 1.62 (t, *J* = 5.5 Hz, 1H, CH_2OH). **^{13}C NMR (125 MHz, DMSO-d_6) δ_C**: 161.2 (*C*-6), 152.7 (*C*-2), 151.4 (*C*-4), 139.9 (*C*-8), 121.7 (=*CH*N), 120.7 (*C*-5), 116.4 (=*CH*CH_2), 61.1 (CH_2OH), 54.4 (OCH_3).

Diethyl (*E*)-(((3-(6-methoxy-9H-purin-9-yl)allyl)oxy)methyl)phosphonate 177[11]



Under an argon atmosphere, NaH (60% dispersion in mineral oil, 105 mg, 4.36 mmol) was added to a solution of the hydroxyl derivative nucleoside **176** (600 mg, 2.91 mmol) in THF (60 ml). The mixture was cooled down to -20°C in a dry-ice/acetone bath, and trifluoromethanesulfonate **183** (961 mg, 3.20 mmol) was added dropwise. The resulting mixture was stirred at room temperature for 16h. The reaction was quenched with NH_4Cl (8 ml) and diluted with CH_2Cl_2 (60 ml). The organic layer was washed with water (3 x 50 ml) and dried over $MgSO_4$. After filtration, the solution was concentrated to dryness to give the pure product **177** (452 mg, 44%) as a yellow oil. R_f = 0.53 (CH_2Cl_2/MeOH - 9:1). **^{31}P NMR (202MHz, $CDCl_3$) δ_P**: 20.96. **^{1}H NMR (500 MHz, $CDCl_3$) δ_H**: 8.48 (s, 1H, *H*-2), 8.06 (s, 1H, *H*-8), 7.21 (dt, J_t = 14.4 Hz, J = 1.1 Hz, 1H, =*CH*N), 6.56 (dt, J_t = 14.4 Hz, J = 6.1 Hz, 1H, =*CH*CH_2), 4.27 (dd, J = 6.1 Hz, J = 1.1 Hz, 2H, =CH*CH_2*), 4.15-4.09 (m, 7H, 2 x O*CH_2*CH_3, O*CH_3*), 3.79 (d, $^2J_{H\text{-}P}$ = 8.5 Hz, 2H, *CH_2*P), 1.28 (t, J = 7.0 Hz, 6H, 2 x OCH$_2$*CH_3*).

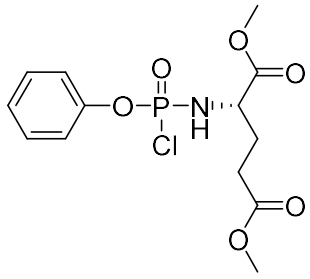
Ethyl (chloro(phenoxy)phosphoryl)-*L*-methioninate 179a



L-Glutamic acid dimethyl ester hydrochloride salt **178a** (3 g, 14.04 mmol) was dissolved in anhydrous CH_2Cl_2 (100 ml) under an argon atmosphere. To this solution the phenyl dichlorophosphate (2.10 ml, 14.04 mmol) was then added. The mixture was cooled to -78°C in a dry-ice/acetone bath. Et_3N (3.91 ml, 28.07 mmol) was added dropwise over 15 minutes and the reaction mixture was stirred at -78°C for 15 minutes. After this period the suspension was allowed to reach room temperature and stirred for further 1.5 h. When the reaction was judged completed (^{31}P NMR), the solvent was evaporated under reduced pressure. The resulting white residue was triturated with anhydrous Et_2O and the filtrate concentrated to give **179a** as a clear oil (4.95 g, quantitative). **^{31}P NMR (202MHz, $CDCl_3$) δ_P**: 8.53, 8.41. **^{1}H NMR (500 MHz, $CDCl_3$) δ_H**: 7.40-7.23 (m, 5H, Ar*H*), 4.68-4.64 (m, 1H, *NH* L-Met), 4.29-4.23 (m, 3H, *CH* L-Met, O*CH_2*CH_3), 2.65-2.60 (m, 2H, *CH_2*S L-Met), 2.21-1.97 (m, 5H, CH*CH_2*, S*CH_3* L-Met), 1.34-1.29 (m, 3H, OCH$_2$*CH_3*). **^{13}C NMR (125 MHz, $CDCl_3$) δ_C**: 171.8 (d, $^3J_{C\text{-}P}$ = 6.1 Hz, *C*=O), 171.7 (d, $^3J_{C\text{-}P}$ = 6.2

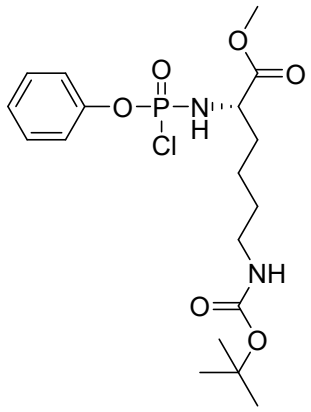
Hz, *C*=O), 149.8 (d, $^{2}J_{C\text{-}P}$ = 8.0 Hz, *C*-O, Ph), 149.7 (d, $^{2}J_{C\text{-}P}$ = 8.2 Hz, *C*-O, Ph), 129.9 (*CH*-Ar), 126.0 (*CH*-Ar), 120.5 (d, $^{3}J_{C\text{-}P}$ = 5.7 Hz, *CH*-Ar), 120.4 (d, $^{3}J_{C\text{-}P}$ = 5.9 Hz, *CH*-Ar), 62.1 (O*CH*$_2$CH$_3$), 62.0 (O*CH*$_2$CH$_3$), 53.9 (*CH* L-Met), 53.7 (*CH* L-Met), 33.2 (d, $^{3}J_{C\text{-}P}$ = 5.9 Hz, CH*CH*$_2$ L-Met), 33.1 (d, $^{3}J_{C\text{-}P}$ = 6.1 Hz, CH*CH*$_2$ L-Met), 29.6 (*CH*$_2$S L-Met), 29.5 (*CH*$_2$S L-Met), 15.38 (S*CH*$_3$ L-Met), 15.33 (S*CH*$_3$ L-Met), 14.1 (OCH$_2$*CH*$_3$).

Dimethyl (chloro(phenoxy)phosphoryl)-*L*-glutamate 179b



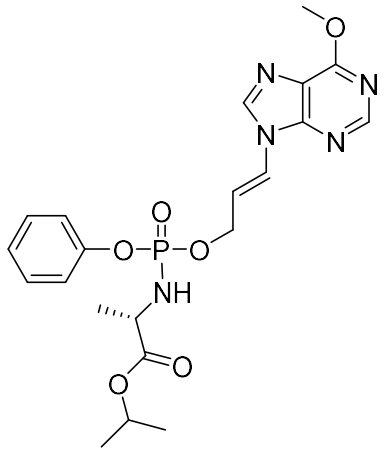
L-Glutamic acid dimethyl ester hydrochloride salt **178b** (3 g, 14.17 mmol) was dissolved in anhydrous CH_2Cl_2 (100 ml) under an argon atmosphere. To this solution the phenyl dichlorophosphate (2.12 ml, 14.17 mmol) was then added. The mixture was cooled to -78˚C in a dry-ice/acetone bath. Et_3N (3.95 ml, 28.35 mmol) was added dropwise over 15 minutes and the reaction mixture was stirred at -78˚C for 15 minutes. After this period the suspension was allowed to reach room temperature and stirred for further 1.5 h. When the reaction was judged completed (^{31}P NMR), the solvent was evaporated under reduced pressure. The resulting white residue was triturated with anhydrous Et_2O and the filtrate concentrated to give **179b** as a clear oil (5.0 g, quantitative).**^{31}P NMR (202MHz, CDCl$_3$) δ$_P$**: 8.33, 8.23. **^{1}H NMR (500 MHz, CDCl$_3$) δ$_H$**: 7.41-7.38 (m, 2H, Ar*H*), 7.29-7.26 (m, 3H, Ar*H*), 4.45-4.39 (m, 1H, *NH* L-Glu), 4.27-4.19 (m, 1H, *CH* L-Glu), 3.82 (s, 1.5H, COO*CH*$_3$), 3.80 (s, 1.5H, COO*CH*$_3$), 3.70 (s, 1.5H, COO*CH*$_3$), 3.66 (s, 1.5H, COO*CH*$_3$), 2.63-2.43 (m, 2H, CHCH$_2$*CH*$_2$ L-Glu), 2.30-2.22 (m, 1H, CH*CH*$_2$CH$_2$ L-Glu), 2.10-2.02 (m, 1H, CH*CH*$_2$CH$_2$ L-Glu). **^{13}C NMR (125 MHz, CDCl$_3$) δ$_C$**: 172.9 (*C*=O), 172.8 (*C*=O), 172.2 (*C*=O), 172.1 (*C*=O), 149.7 (d, $^{2}J_{C\text{-}P}$ = 6.6 Hz, C-O, Ph), 149.6 (d, $^{2}J_{C\text{-}P}$ = 6.7 Hz, C-O, Ph), 129.9 (*CH*-Ar), 126.1 (*CH*-Ar), 126.0 (*CH*-Ar), 120.55 (*CH*-Ar), 120.51 (*CH*-Ar), 54.1 (*CH* L-Glu), 53.8 (*CH* L-Glu), 52.9 (COO*CH*$_3$), 52.8 (COO*CH*$_3$), 51.8 (COO*CH*$_3$), 51.7 (COO*CH*$_3$), 29.5 (CHCH$_2$*CH*$_2$ L-Glu), 29.2 (CHCH$_2$*CH*$_2$ L-Glu), 28.9 (d, $^{3}J_{C\text{-}P}$ = 7.4 Hz, CH*CH*$_2$CH$_2$ L-Glu), 28.8 (d, $^{3}J_{C\text{-}P}$ = 7.4 Hz, CH*CH*$_2$CH$_2$ L-Glu).

Methyl N^6-(tert-butoxycarbonyl)-N^2-(chloro(phenoxy)phosphoryl)-*L*-lysinate 179c



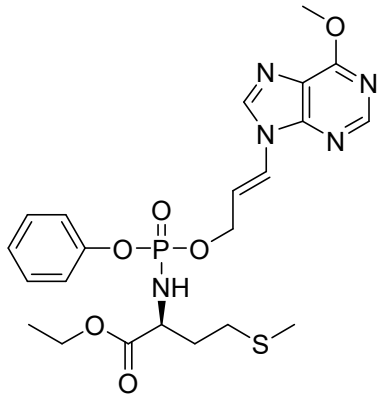
Nε-Boc-L-lysine methyl ester hydrochloride **178c** (500 mg, 1.68 mmol) was dissolved in anhydrous CH_2Cl_2 (16 ml) under an argon atmosphere. To this solution the phenyl dichlorophosphate (252.0 µl, 1.68 mmol) was then added. The mixture was cooled to -78˚C in a dry-ice/acetone bath. Et_3N (469.6 µl, 3.37 mmol) was added dropwise over 15 minutes and the reaction mixture was stirred at -78˚C for 15 minutes. After this period the suspension was allowed to reach room temperature and stirred for further 1.5 h. When the reaction was judged completed (^{31}P NMR), the solvent was evaporated under reduced pressure. The resulting white residue was triturated with anhydrous Et_2O and the filtrate concentrated to give **179c** as a clear oil (732 mg, quantitative).**^{31}P NMR (202MHz, $CDCl_3$) δ_P**: 8.48, 8.32. **^{1}H NMR (500 MHz, $CDCl_3$) δ_H**: 7.31-7.28 (m, 2H, Ar*H*), 7.20-7.15 (m, 3H, Ar*H*), 4.41-4.35 (m, 1H, *NH* L-Lys), 4.09-3.98 (m, 1H, *CH* L-Lys), 3.71 (s, 1.5H, COO*CH*$_3$), 3.69 (s, 1.5H, COO*CH*$_3$), 3.03-3.01 (m, 2H, CHCH$_2$CH$_2$CH$_2$*CH*$_2$NH L-Lys), 1.83-1.64 (m, 2H, CH*CH*$_2$CH$_2$CH$_2$CH$_2$NH L-Lys), 1.45-1.31 (m, 13H, CHCH$_2$*CH*$_2$*CH*$_2$CH$_2$NH L-Lys, *t*Bu). **^{13}C NMR (125 MHz, $CDCl_3$) δ_C**: 172.6 (d, $^3J_{C\text{-}P}$ = 6.2 Hz, CH*C*=O), 171.7 (d, $^3J_{C\text{-}P}$ = 6.2 Hz, CH*C*=O), 156.15 (*C*=O, Boc), 156.10 (*C*=O, Boc), 149.8 (d, $^2J_{C\text{-}P}$ = 8.0 Hz, *C*-O, Ph), 149.7 (d, $^2J_{C\text{-}P}$ = 8.0 Hz, *C*-O, Ph), 129.94 (*CH*-Ar), 129.92 (*CH*-Ar), 126.0 (*CH*-Ar), 120.5 (d, $^3J_{C\text{-}P}$ = 5.9 Hz, *CH*-Ar), 120.4 (d, $^3J_{C\text{-}P}$ = 5.7 Hz, *CH*-Ar), 84.1 (*C*(CH$_3$)$_3$), 84.0 (*C*(CH$_3$)$_3$), 54.8 (*CH* L-Lys), 54.3 (*CH* L-Lys), 52.7 (COO*CH*$_3$), 52.6 (COO*CH*$_3$), 45.8 (CHCH$_2$CH$_2$CH$_2$*CH*$_2$NH L-Lys), 33.5 (CH*CH*$_2$CH$_2$CH$_2$CH$_2$NH L-Lys), 33.4 (CH*CH*$_2$CH$_2$CH$_2$CH$_2$NH L-Lys), 29.5 (CHCH$_2$CH$_2$*CH*$_2$CH$_2$NH L-Lys), 29.3 (CHCH$_2$*CH*$_2$*CH*$_2$CH$_2$NH L-Lys), 28.4 (C(*CH*$_3$)$_3$), 22.0 (CHCH$_2$*CH*$_2$CH$_2$CH$_2$NH L-Lys), 21.9 (CHCH$_2$*CH*$_2$CH$_2$CH$_2$NH L-Lys).

Isopropyl ((((*E*)-3-(6-methoxy-9H-purin-9-yl)allyl)oxy)(phenoxy)phosphoryl)-*L*-alaninate 180a



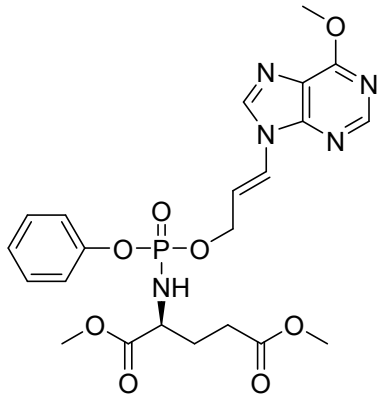
Hydroxyl nuceloside derivative **176** (200 mg, 969.9 µmol) was solubilised in THF (8 ml) and pyridine (4 ml), and NMI (386.5 µl, 4.85 µmol) was added dropwise at room temperature. The mixture was left to stir 10 minutes and then a solution of the phosphorochloridate **22** (355.8 mg, 1.16 mmol) in THF (1 ml) was added dropwise. After 16 h at room temperature, the reaction was concentrated to dryness and purified by Biotage Isolera One (50 g SNAP cartridge ULTRA, 100 ml/min, gradient eluent system MeOH/CH_2Cl_2 2% 1CV, 2-20% 15CV, 20% 2CV), to afford the title compound as a yellow solid (54 mg, 12%). R_f= 0.23 (CH_2Cl_2/MeOH - 95:0.5). **^{31}P NMR (202MHz, $CDCl_3$) δ_P**: 2.80, 2.63. **^{1}H NMR (500 MHz, $CDCl_3$) δ_H**: 8.49 (s, 0.5H, *H*-2), 8.48 (s, 0.5H, *H*-2), 7.98 (s, 0.5H, *H*-8), 7.97 (s, 0.5H, *H*-8), 7.24-7.21 (m, 2H, Ar*H*), 7.19-7.14 (m, 3H, Ar*H*, =*CH*N), 7.06-7.04 (m, 1H, Ar*H*), 6.69-6.62 (m, 1H, =*CH*CH_2), 4.96-4.88 (m, 1H, *CH*$(CH_3)_2$), 4.76-4.71 (m, 2H, *CH_2*CH=), 4.11 (s, 3H, O*CH_3*, base) 4.00-3.88 (m, 1H, *CH* L-Ala), 3.85-3.74 (m, 1H, *NH* L-Ala), 1.31 (d, *J* = 2.8 Hz, 1.5H, *CH_3* L-Ala), 1.30 (d, *J* = 3.0 Hz, 1.5H, *CH_3* L-Ala), 1.14 (d, *J* = 2.4 Hz, 1.5H, CH(*CH_3*)$_2$), 1.13 (d, *J* = 2.4 Hz, 3H, CH(*CH_3*)$_2$), 1.12 (d, *J* = 2.4 Hz, 1.5H, CH(*CH_3*)$_2$). **^{13}C NMR (125 MHz, $CDCl_3$) δ_C**: 173.0 (d, $^3J_{C\text{-}P}$ = 3.6 Hz, *C*=O), 172.9 (d, $^3J_{C\text{-}P}$ = 2.7 Hz, *C*=O), 161.1 (*C*-6), 152.6 (*C*-2), 151.1 (*C*-4), 150.7 (d, $^2J_{C\text{-}P}$ = 6.5 Hz, *C*-O, Ph), 150.6 (d, $^2J_{C\text{-}P}$ = 6.5 Hz, *C*-O, Ph), 140.0 (*C*-8), 129.67 (*CH*-Ar), 129.65 (*CH*-Ar), 124.9 (*CH*-Ar), 123.97 (=*CH*N), 123.91 (=*CH*N), 122.0 (*C*-5), 120.29 (d, $^3J_{C\text{-}P}$ = 4.6 Hz, *CH*-Ar), 120.21 (d, $^3J_{C\text{-}P}$ = 4.8 Hz, *CH*-Ar), 115.68 (d, $^3J_{C\text{-}P}$ = 6.4 Hz, =*CH*CH_2), 115.67 (d, $^3J_{C\text{-}P}$ = 7.1 Hz, =*CH*CH_2), 69.23 (*CH*$(CH_3)_2$), 69.21 (*CH*$(CH_3)_2$), 64.8 (d, $^2J_{C\text{-}P}$ = 4.7 Hz, =CH*CH_2*), 64.7 (d, $^2J_{C\text{-}P}$ = 4.7 Hz, =CH*CH_2*), 54.3 (O*CH_3*, base), 50.4 (*CH* L-Ala), 50.3 (*CH* L-Ala), 21.66 (CH(*CH_3*)$_2$), 21.60 (CH(*CH_3*)$_2$), 21.5 (CH(*CH_3*)$_2$), 20.99 (d, $^3J_{C\text{-}P}$ = 4.5 Hz, *CH_3* L-Ala), 20.90 (d, $^3J_{C\text{-}P}$ = 5.0 Hz, *CH_3* L-Ala). **HRMS (ESI):** m/z $[M+H]^+$ calcd for $C_{21}H_{27}N_5O_6P$: 476.1699, found: 476.1693. **HPLC**: Reverse phase HPLC eluting with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 minutes, 1ml/min, λ = 254 nm and 263 nm, showed one peak with t_R 16.27 min.

Ethyl ((((*E*)-3-(6-methoxy-9H-purin-9-yl)allyl)oxy)(phenoxy)phosphoryl)-L-methioninate 180b



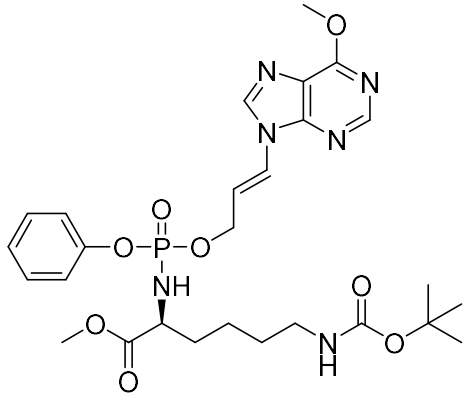
Hydroxyl nuceloside derivative **176** (200 mg, 969.9 µmol) was solubilised in THF (8 ml) and pyridine (4 ml), and NMI (386.5 µl, 4.85 µmol) was added dropwise at room temperature. The mixture was left to stir 10 minutes and then a solution of the phosphorochloridate **179a** (409.4 mg, 1.16 mmol) in THF (1 ml) was added dropwise. After 16 h at room temperature, the reaction was concentrated to dryness and purified by Biotage Isolera One (50 g SNAP cartridge ULTRA, 100 ml/min, gradient eluent system MeOH/CH_2Cl_2 2% 1CV, 2-20% 15CV, 20% 2CV), to afford the title compound as a yellow oil (110 mg, 22%). R_f = 0.25 (CH_2Cl_2/MeOH - 95:0.5). **^{31}P NMR (202MHz, $CDCl_3$) δ_P**: 3.00, 2.79. **^{1}H NMR (500 MHz, $CDCl_3$) δ_H**: 8.48 (s, 0.5H, *H*-2), 8.47 (s, 0.5H, *H*-2), 7.99 (s, 0.5H, *H*-8), 7.98 (s, 0.5H, *H*-8), 7.24-7.12 (m, 5H, Ar*H*, =*CH*N), 7.07-7.04 (m, 1H, Ar*H*), 6.70-6.61 (m, 1H, =*CH*CH_2), 4.77-4.71 (m, 2H, *CH_2*CH=), 4.12-3.95 (m, 7H, O*CH_3* base, O*CH_2*CH_3, *NHCH* L-Met), 2.47-2.38 (m, 2H, *CH_2*S L-Met), 2.01-1.92 (m, 4H, CH*CH_2*, S*CH_3* L-Met), 1.88-1.80 (m, 1H, CH*CH_2* L-Met), 1.16 (t, 1.5H, *J* = 7.1 Hz, OCH_2*CH_3*), 1.15 (t, 1.5H, *J* = 7.1 Hz, OCH_2*CH_3*). **^{13}C NMR (125 MHz, $CDCl_3$) δ_C**: 172.7 (d, $^3J_{C\text{-}P}$ = 5.5 Hz, *C*=O), 172.6 (d, $^3J_{C\text{-}P}$ = 5.2 Hz, *C*=O), 161.1 (*C*-6), 152.67 (*C*-2), 152.65 (*C*-2), 151.1 (*C*-4), 150.7 (d, $^2J_{C\text{-}P}$ = 6.6 Hz, *C*-O, Ph), 150.6 (d, $^2J_{C\text{-}P}$ = 7.0 Hz, *C*-O, Ph), 140.1 (*C*-8), 129.6 (*CH*-Ar), 124.98 (*CH*-Ar), 124.92 (*CH*-Ar), 124.0 (=*CH*N), 123.9 (=*CH*N), 121.9 (*C*-5), 120.2 (d, $^3J_{C\text{-}P}$ = 4.8 Hz, *CH*-Ar), 120.1 (d, $^3J_{C\text{-}P}$ = 5.0 Hz, *CH*-Ar), 115.6 (d, $^3J_{C\text{-}P}$ = 4.5 Hz, =*CH*CH_2), 115.5 (d, $^3J_{C\text{-}P}$ = 4.0 Hz, =*CH*CH_2), 64.9 (d, $^2J_{C\text{-}P}$ = 4.8 Hz, =CH*CH_2*), 64.8 (d, $^2J_{C\text{-}P}$ = 4.9 Hz, =CH*CH_2*), 61.6 (O*CH_2*CH_3), 54.3 (O*CH_3*, base), 53.6 (*CH* L-Met), 53.5 (*CH* L-Met), 33.5 (d, $^3J_{C\text{-}P}$ = 3.3 Hz, CH*CH_2*CH_2 L-Met), 33.4 (d, $^3J_{C\text{-}P}$ = 3.6 Hz, CH*CH_2*CH_2 L-Met), 29.6 (*CH_2*S L-Met), 29.5 (*CH_2*S L-Met), 15.27 (S*CH_3* L-Met), 15.26 (S*CH_3* L-Met), 14.13 (OCH_2*CH_3*), 14.10 (OCH_2*CH_3*). **HRMS (ESI):** m/z $[M+H]^+$ calcd for $C_{22}H_{29}N_5O_6PS$: 522.1576, found: 522.1571. **HPLC**: Reverse phase HPLC eluting with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 minutes, 1ml/min, λ = 254 nm and 263 nm, showed one peak with t_R 17.73 min.

Dimethyl ((((*E*)-3-(6-methoxy-9H-purin-9-yl)allyl)oxy)(phenoxy)phosphoryl)-*L*-glutamate 180c



Hydroxyl nuceloside derivative **176** (200 mg, 969.9 µmol) was solubilised in THF (8 ml) and pyridine (4 ml), and NMI (387.1 µl, 4.85 µmol) was added dropwise at room temperature. The mixture was left to stir 10 minutes and then a solution of the phosphorochloridate **179b** (407.0 mg, 1.16 mmol) in THF (1 ml) was added dropwise. After 16 h at room temperature, the reaction was concentrated to dryness and purified by Biotage Isolera One (50 g SNAP cartridge ULTRA, 100 ml/min, gradient eluent system MeOH/CH_2Cl_2 2% 1CV, 2-20% 15CV, 20% 2CV), to afford the title compound as a yellow oil (96 mg, 19%). R_f = 0.29 (CH_2Cl_2/MeOH - 95:0.5). **^{31}P NMR (202MHz, $CDCl_3$) δ_P**: 2.96, 2.86. **^{1}H NMR (500 MHz, $CDCl_3$) δ_H**: 8.47 (s, 0.5H, *H*-2), 8.46 (s, 0.5H, *H*-2), 8.05 (s, 0.5H, *H*-8), 8.02 (s, 0.5H, *H*-8), 7.23-7.13 (m, 5H, Ar*H*, =*CH*N), 7.05-7.02 (m, 1H, Ar*H*), 6.71-6.61 (m, 1H, =*CH*CH_2), 4.77-4.70 (m, 2H, *CH_2*CH=), 4.41 (t, $^{2}J_{H\text{-}P}$ = 10.7 Hz, $J_{NH\text{-}CH}$ = 10.7 Hz, 0.5H, *NH* L-Glu), 4.30 (t, $^{2}J_{H\text{-}P}$ = 10.5 Hz, $J_{NH\text{-}CH}$ = 10.5 Hz, 0.5H, *NH* L-Glu), 4.10 (s, 3H, O*CH₃* base), 4.03-3.95 (m, 1H, *CH* L-Glu), 3.60 (s, 1.5H, COO*CH₃*), 3.58 (s, 1.5H, COO*CH₃*), 3.53 (s, 1.5H, COO*CH₃*), 3.52 (s, 1.5H, COO*CH₃*), 2.41-2.20 (m, 2H, CHCH$_2$*CH₂* L-Glu), 2.15-1.99 (m, 1H, CH*CH₂*CH$_2$ L-Glu), 1.92-1.83 (m, 1H, CH*CH₂*CH$_2$ L-Glu). **^{13}C NMR (125 MHz, $CDCl_3$) δ_C**: 173.08 (d, $^{3}J_{C\text{-}P}$ = 4.7 Hz, *C*=O), 173.03 (*C*=O), 173.02 (d, $^{3}J_{C\text{-}P}$ = 3.3 Hz, *C*=O), 172.9 (*C*=O), 161.0 (*C*-6), 152.5 (*C*-2), 151.07 (*C*-4), 151.06 (*C*-4), 150.6 (d, $^{2}J_{C\text{-}P}$ = 6.9 Hz, *C*-O, Ph), 150.5 (d, $^{2}J_{C\text{-}P}$ = 6.5 Hz, *C*-O, Ph), 140.36 (*C*-8), 140.30 (*C*-8), 129.6 (*CH*-Ar), 124.9 (*CH*-Ar), 124.0 (=*CH*N), 123.9 (=*CH*N), 121.9 (*C*-5), 120.2 (d, $^{3}J_{C\text{-}P}$ = 5.3 Hz, *CH*-Ar), 120.1 (d, $^{3}J_{C\text{-}P}$ = 5.2 Hz, *CH*-Ar), 115.5 (d, $^{3}J_{C\text{-}P}$ = 4.9 Hz, =*CH*CH_2), 115.4 (d, $^{3}J_{C\text{-}P}$ = 5.5 Hz, =*CH*CH_2), 64.9 (d, $^{2}J_{C\text{-}P}$ = 5.0 Hz, =CH*CH_2*), 64.8 (d, $^{2}J_{C\text{-}P}$ = 4.9 Hz, =CH*CH_2*), 54.2 (O*CH₃*, base), 53.8 (*CH* L-Glu), 53.6 (*CH* L-Glu), 52.44 (COO*CH₃*), 52.42 (COO*CH₃*), 51.68 (COO*CH₃*), 51.64 (COO*CH₃*), 29.47 (CHCH$_2$*CH₂* L-Glu), 29.44 (CHCH$_2$*CH₂* L-Glu) 29.1 (d, $^{3}J_{C\text{-}P}$ = 6.0 Hz, CH*CH₂*CH$_2$ L-Glu), 29.0 (d, $^{3}J_{C\text{-}P}$ = 6.1 Hz, CH*CH₂*CH$_2$ L-Glu). **HRMS (ESI):** m/z $[M+H]^+$ calcd for $C_{22}H_{27}N_5O_8P$: 520.1597, found: 520.1592. **HPLC**: Reverse phase HPLC eluting with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 minutes, 1ml/min, λ = 254 nm and 263 nm, showed one peak with t_R 14.11 min.

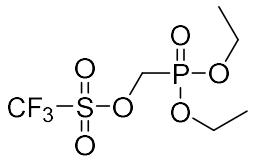
Methyl N^6-(tert-butoxycarbonyl)-N^2-((((*E*)-3-(6-methoxy-9H-purin-9-yl)allyl)oxy)(phenoxy)phosphoryl)-L-lysinate 180d



Hydroxyl nuceloside derivative **176** (150 mg, 727.4 µmol) was solubilised in THF (6 ml) and pyridine (3 ml), and NMI (289.9 µl, 3.64 µmol) was added dropwise at room temperature. The mixture was left to stir 10 minutes and then a solution of the phosphorochloridate **179c** (379.6 mg, 872.9 µmol) in THF (1 ml) was added dropwise. After 16 h at room temperature, the reaction was concentrated to dryness and purified by Biotage Isolera One (25 g SNAP cartridge ULTRA, 50 ml/min, gradient eluent system MeOH/CH_2Cl_2 2% 1CV, 2-20% 15CV, 20% 2CV), to afford the title compound as a yellow oil (33 mg, 8%). R_f = 0.32 (CH_2Cl_2/MeOH - 95:0.5).**^{31}P NMR (202MHz, CDCl$_3$) δ$_P$**: 2.98, 2.74. **^{1}H NMR (500 MHz, CDCl$_3$) δ$_H$**: 8.51 (s, 0.5H, *H*-2), 8.50 (s, 0.5H, *H*-2), 7.99 (s, 0.5H, *H*-8), 7.98 (s, 0.5H, *H*-8), 7.26-7.14 (m, 5H, Ar*H*, =*CH*N), 7.09-7.06 (m, 1H, Ar*H*), 6.69-6.61 (m, 1H, =*CH*CH_2), 4.76-4.71 (m, 2H, *CH_2*CH=), 4.49 (bs, 1H, *NH*C=O), 4.13 (s, 3H, O*CH_3* base), 4.00-3.86 (m, 1H, *CH* L-Lys), 3.562(s, 1.5H, COO*CH_3*), 3.61 (s, 1.5H, COO*CH_3*), 3.53-3.46 (m, 1H, *NH* L-Lys), 2.99-2.95 (m, 2H, CHCH$_2$CH$_2$CH$_2$*CH_2*NH L-Lys), 1.72-1.64 (m, 1H, CH*CH_2*CH$_2$CH$_2$CH$_2$NH L-Lys), 1.61-1.53 (m, 1H, CH*CH_2*CH$_2$CH$_2$CH$_2$NH L-Lys), 1.38-1.17 (m, 13H, CHCH$_2$*CH_2CH$_2$*CH$_2$NH L-Lys, *t*Bu). **^{13}C NMR (125 MHz, CDCl$_3$) δ$_C$**: 173.5 (d, $^3J_{C\text{-}P}$ = 6.0 Hz, *C*=O, ester), 173.4 (d, $^3J_{C\text{-}P}$ = 5.3 Hz, *C*=O, ester), 161.2 (*C*-6), 156.0 (*C*=O, Boc), 152.7 (*C*-2), 151.1 (*C*-4), 150.6 (d, $^2J_{C\text{-}P}$ = 6.6 Hz, *C*-O, Ph), 140.0 (*C*-8), 129.7 (*CH*-Ar), 125.06 (*CH*-Ar), 1245.00 (*CH*-Ar), 124.0 (=*CH*N), 123.9 (=*CH*N), 122.0 (*C*-5), 120.3 (d, $^3J_{C\text{-}P}$ = 4.7 Hz, *CH*-Ar), 120.1 (d, $^3J_{C\text{-}P}$ = 5.1 Hz, *CH*-Ar), 115.6 (d, $^3J_{C\text{-}P}$ = 6.4 Hz, =*CH*CH_2), 115.5 (d, $^3J_{C\text{-}P}$ = 6.9 Hz, =*CH*CH_2), 79.1 (*C*$(CH_3)_3$), 64.98 (d, $^2J_{C\text{-}P}$ = 4.8 Hz, =CH*CH_2*), 64.92 (d, $^2J_{C\text{-}P}$ = 4.3 Hz, =CH*CH_2*), 54.4 (*CH* L-Lys), 54.37 (O*CH_3*, base), 54.31 (*CH* L-Lys), 52.4 (COO*CH_3*), 40.1 (CHCH$_2$CH$_2$CH$_2$*CH_2*NH L-Lys), 34.0 (d, $^3J_{C\text{-}P}$ = 5.9 Hz, CH*CH_2*CH$_2$CH$_2$CH$_2$NH L-Lys), 33.9 (d, $^3J_{C\text{-}P}$ = 6.1 Hz, CH*CH_2*CH$_2$CH$_2$CH$_2$NH L-Lys), 29.49 (CHCH$_2$CH$_2$*CH_2*CH$_2$NH L-Lys), 29.42 (CHCH$_2$CH$_2$*CH_2*CH$_2$NH L-Lys), 28.4 (C(*CH_3*)$_3$), 22.1 (CHCH$_2$*CH_2*CH$_2$CH$_2$NH L-Lys), 22.0 (CHCH$_2$*CH_2*CH$_2$CH$_2$NH L-Lys). **HRMS (ESI):** m/z $[M+Na]^+$ calcd for $C_{27}H_{37}N_6NaO_8P$: 627.2308, found: 627.2303. **HPLC**: Reverse

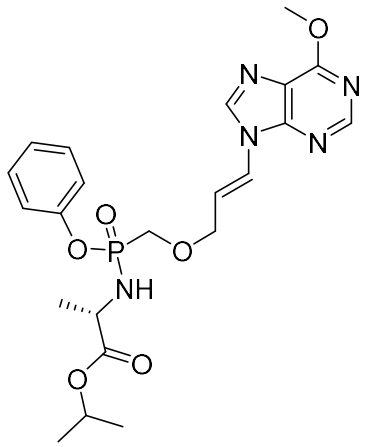
phase HPLC eluting with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 minutes, 1ml/min, λ = 254 nm and 263 nm, showed one peak with t_R 17.68 min.

(Diethoxyphosphoryl)methyl trifluoromethanesulfonate 183[11]



Under an argon atmosphere, to a stirred solution of diethyl (hydroxymethyl)phosphonate (877 µl, 5.95 mmol) and 2,6-lutidine (865 µl, 7.34 mmol) in anhydrous CH_2Cl_2 at -50°C, trifluoromethanesulfonic anhydride (1.15 ml, 6.84 mmol) was added dropwise. The resulting mixture was stirred at that temperature for 45 minutes and the at 0°C for further 30 minutes. The dark brown solution obtained was diluted with Et_2O (100ml) and washed with water (50 ml), HCl 1M solution (50 ml) and brine. The organic phase was dried over $MgSO_4$, filtered and concentrated to dryness to give **183** as a brown oil (1.35 g, 76%) used without further purification. **^{31}P NMR (202MHz, $CDCl_3$) δ_P**: 12.20. **^{19}F NMR (450 MHz, $CDCl_3$) δ_F**: -73.90. **^{1}H NMR (500 MHz, $CDCl_3$) δ_H**: 4.63 (d, *J* = 8.8 Hz, 2H, O*CH*$_2$P), 4.30-4.24 (m, 4H, 2 x O*CH*$_2$CH$_3$), 1.41 (t, *J* = 7.0 Hz, 6H, 2 x OCH$_2$*CH*$_3$).

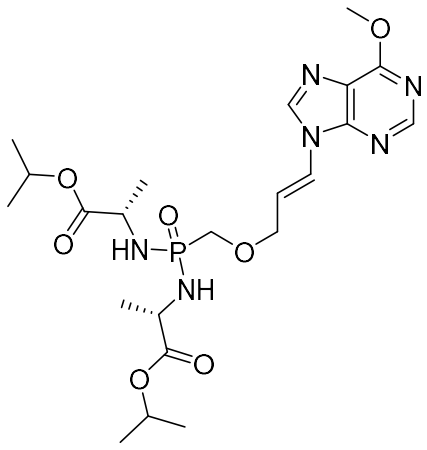
Isopropyl (((((*E*)-3-(6-methoxy-9H-purin-9-yl)allyl)oxy)methyl)(phenoxy)phosphoryl)-*L*-alaninate 184a



Under an argon atmosphere, 2,6-Lutidine (294.2 µl, 2.53 mmol) and TMSBr (416.6 µl, 3.16 mmol), were added to a solution of the phosphonate **177** (225 mg, 631.4 µmol) in anhydrous acetonitrile (15 ml). The mixture was stirred 16h at room temperature and then the volatiles evaporated without any contact with air. Then the flask was charged with dry amino acid ester hydrochloride **56** (136.2 mg, 631.4 µl), phenol (356.5 mg, 3.79 mmol), dry triethylamine (1.32 ml, 9.47 mmol) and dry pyridine (5 ml) and heated to 50˚C to obtain a homogenous solution. To this mixture was then added a solution of Aldrithiol-2 (834.6 mg, 3.79 mmol) and triphenylphosphine (993.76 mg, 3.79 mmol) in dry pyridine (5 ml) under argon atmosphere. The resulting mixture was stirred at 50˚C for 16 h. After evaporating all the volatiles, the residue was purified by two Biotage Isolera One (50 g SNAP cartridge ULTRA, 100 ml/min, gradient eluent system MeOH/CH_2Cl_2 2% 1CV, 2-20% 10CV, 20% 2CV and 10 g SNAP cartridge ULTRA, 36 ml/min, gradient eluent system MeOH/CH_2Cl_2 2% 1CV, 2-20% 15CV, 20% 2CV) to

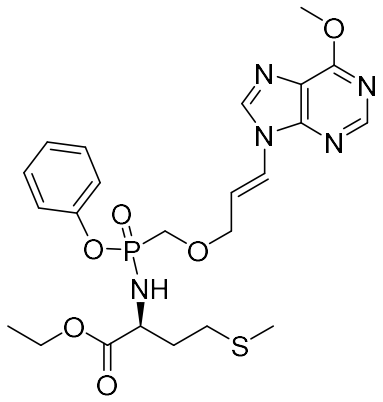
afford the title compound **184a** as a brown solid (60 mg, 22%). R_f = 0.46 (CH_2Cl_2/MeOH - 9:1). **^{31}P NMR (202MHz, $CDCl_3$) δ_P**: 22.07, 21.16. **^{1}H NMR (500 MHz, $CDCl_3$) δ_H**: 8.53 (s, 1H, *H*-2), 8.11 (s, 0.5H, *H*-8), 8.07 (s, 0.5H, *H*-8), 7.28-7.20 (m, 5H, Ar*H*, =*CH*N), 7.11-7.08 (m, 1H, Ar*H*), 6.63-6.54 (m, 1H, =*CH*CH_2), 4.98-4.90 (m, 1H, *CH*$(CH_3)_2$), 4.32-4.22 (m, 2H, *CH_2*CH=), 4.16 (s, 3H, O*CH_3*, base), 4.14-4.06 (m, 1H, *CH* L-Ala), 3.96-3.89 (m, 2H, *CH_2*P), 3.83-3.72 (m, 1H, *NH* L-Ala), 1.31-1.28 (m, 3H, *CH_3* L-Ala), 1.18-1.16 (m, 6H, CH(*CH_3*)$_2$). **^{13}C NMR (125 MHz, $CDCl_3$) δ_C**: 173.5 (d, $^3J_{C\text{-}P}$ = 5.8 Hz, *C*=O), 173.2 (d, $^3J_{C\text{-}P}$ = 5.6 Hz, *C*=O), 161.1 (*C*-6), 152.63 (*C*-2), 152.60 (*C*-2), 151.0 (*C*-4), 150.2 (d, $^2J_{C\text{-}P}$ = 8.9 Hz, *C*-O, Ph), 150.1 (d, $^2J_{C\text{-}P}$ = 8.3 Hz, *C*-O, Ph), 140.09 (*C*-8), 140.01 (*C*-8), 129.67 (*CH*-Ar), 129.62 (*CH*-Ar), 124.9 (*CH*-Ar), 123.45 (=*CH*N), 123.40 (=*CH*N), 121.1 (*C*-5), 120.7 (d, $^3J_{C\text{-}P}$ = 4.6 Hz, *CH*-Ar), 120.6 (d, $^3J_{C\text{-}P}$ = 4.5 Hz, *CH*-Ar), 116.6 (=*CH*CH_2), 116.4 (=*CH*CH_2), 70.9 (d, $^3J_{C\text{-}P}$ = 13.8 Hz, =CH*CH_2*), 70.8 (d, $^3J_{C\text{-}P}$ = 13.8 Hz, =CH*CH_2*), 69.1 (*CH*$(CH_3)_2$), 69.0 (*CH*$(CH_3)_2$), 65.7 (d, $^1J_{C\text{-}P}$ = 154.6 Hz, *CH_2*P), 65.4 (d, $^1J_{C\text{-}P}$ = 154.6 Hz, *CH_2*P), 54.2 (O*CH_3*, base), 49.7 (*CH* L-Ala), 49.6 (*CH* L-Ala), 21.6 (CH(*CH_3*)$_2$), 21.5 (CH(*CH_3*)$_2$), 21.4 (d, $^3J_{C\text{-}P}$ = 4.2 Hz, *CH_3* L-Ala), 21.3 (d, $^3J_{C\text{-}P}$ = 3.8 Hz, *CH_3* L-Ala). **HRMS (ESI):** m/z $[M+H]^+$ calcd for $C_{22}H_{29}N_5O_6P$: 490.1855, found: 490.1850. **HPLC**: Reverse phase HPLC eluting with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 minutes, 1ml/min, λ = 254 nm and 263 nm, showed one peak with t_R 15.67 min.

From the flash chromatography purification, a mixture of ProTide and Bis-amidate was isolated. Therefore, the mixture was purified by preparative TLC (CH_2Cl_2/MeOH – 9.5:0.5), to afford **diisopropyl 2,2'-((((((*E*)-3-(6-methoxy-9H-purin-9-yl)allyl)oxy)methyl)phosphoryl)bis(azanediyl))(2*S*,2'*S*)-dipropionate 185** (12 mg, 4%) as a brown solid. R_f = 0.37 (CH_2Cl_2/MeOH – 9:1). **^{31}P NMR (202MHz, $CDCl_3$) δ_P**: 20.84. **^{1}H NMR (500 MHz, $CDCl_3$) δ_H**: 8.50 (s, 1H, *H*-2), 8.10 (s, 1H, *H*-8), 7.25 (dt, J_t = 14.4 Hz, J = 1.5 Hz, 1H, =*CH*N), 6.62 (dt, J_t = 14.4 Hz, J = 5.8 Hz, 1H, =*CH*CH_2), 4.99-4.88 (m, 2H, 2 x *CH*$(CH_3)_2$), 4.30-4.22 (m, 2H, *CH_2*CH=), 4.13 (s, 3H, O*CH_3*, base) 4.01-3.93 (m, 2H, 2 x *CH* L-Ala), 3.72 (dd, J_G = 9.2 Hz, J = 3.9 Hz, 2H, *CH_2*P), 3.36-3.22 (m, 2H, 2 x *NH* L-Ala), 1.35 (d, J = 7.1 Hz, 3H, *CH_3* L-Ala), 1.32 (d, J = 7.1 Hz, 3H, *CH_3* L-Ala), 1.19-



1.14 (m, 12H, 2 x CH(*CH*$_3$)$_2$). **^{13}C NMR (125 MHz, CDCl$_3$) δ$_C$**: 174.1 (d, $^3J_{C\text{-}P}$ = 5.3 Hz, *C*=O), 174.0 (d, $^3J_{C\text{-}P}$ = 4.3 Hz, *C*=O), 161.2 (*C*-6), 152.6 (*C*-2), 151.1 (*C*-4), 140.2 (*C*-8), 123.4 (=*CH*N), 122.0 (*C*-5), 116.8 (=*CH*CH$_2$), 70.8 (d, $^3J_{C\text{-}P}$ = 13.3 Hz, =CH*CH$_2$*), 69.09 (*CH*(CH$_3$)$_2$), 69.01 (*CH*(CH$_3$)$_2$), 67.2 (d, $^1J_{C\text{-}P}$ = 134.3 Hz, *CH$_2$*P), 54.3 (O*CH$_3$*, base), 49.0 (*CH* L-Ala), 48.4 (*CH* L-Ala), 21.74 (CH(*CH$_3$*)$_2$), 21.71 (CH(*CH$_3$*)$_2$), 21.6 (CH(*CH$_3$*)$_2$), 21.5 (*CH$_3$* L-Ala), 21.4 (*CH$_3$* L-Ala). **HRMS (ESI):** m/z [M+H]$^+$ calcd for $C_{22}H_{36}N_6O_7P$: 527.2383, found: 527.2378. **HPLC**: Reverse phase HPLC eluting with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 minutes, 1ml/min, λ = 254 nm and 263 nm, showed one peak with t_R 14.53 min.

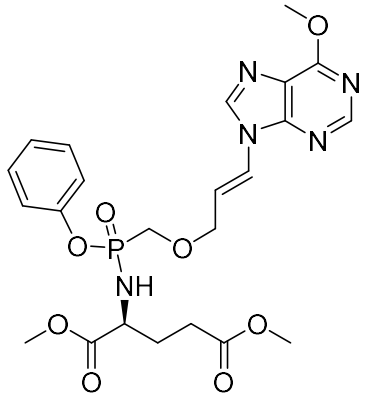
Ethyl ((((((*E*)-3-(6-methoxy-9H-purin-9-yl)allyl)oxy)methyl)(phenoxy)phosphoryl)-*L*-methioninate 184b



Under an argon atmosphere, 2,6-Lutidine (300.7µl, 2.58 mmol) and TMSBr (425.9 µl, 3.23 mmol), were added to a solution of the phosphonate **177** (230 mg, 645.5 µmol) in anhydrous acetonitrile (25 ml). The mixture was stirred 16h at room temperature and then the volatiles evaporated without any contact with air. Then the flask was charged with dry amino acid ester hydrochloride **178a** (137.9 mg, 645.5 µl), phenol (364.5 mg, 3.87 mmol), dry triethylamine (1.35 ml, 9.68 mmol) and dry pyridine (8 ml) and heated to 50˚C to obtain a homogenous solution. To this mixture was then added a solution of Aldrithiol-2 (853.0 mg, 3.87 mmol) and triphenylphosphine (1.02 g, 3.87 mmol) in dry pyridine (8 ml) under argon atmosphere. The resulting mixture was stirred at 50˚C for 16 h. After evaporating all the volatiles, the residue was purified by Biotage Isolera One (50 g SNAP cartridge ULTRA, 100 ml/min, gradient eluent system MeOH/CH_2Cl_2 2% 1CV, 2-20% 10CV, 20% 2CV) to afford the title compound **184b** as a yellow oil (194 mg, 56%). R_f = 0.64 (CH_2Cl_2/MeOH - 9:1).**^{31}P NMR (202MHz, CDCl$_3$) δ$_P$**: 22.48, 21.37. **^{1}H NMR (500 MHz, CDCl$_3$) δ$_H$**: 8.49 (s, 0.5H, *H*-2), 8.48 (s, 0.5H, *H*-2), 8.08 (s, 0.5H, *H*-8), 8.04 (s, 0.5H, *H*-8), 7.25-7.14 (m, 5H, Ar*H*, =*CH*N), 7.08-7.01 (m, 1H, Ar*H*), 6.59-6.49 (m, 1H, =*CH*CH$_2$), 4.28-4.00 (m, 8H, *CH$_2$*CH=, *CH* L-Met, O*CH$_3$* base, O*CH$_2$*CH$_3$), 3.91-3.81 (m, 3H, *NH* L-Met, *CH$_2$*P), 2.47-2.25 (m, 2H, *CH$_2$*S L-Met), 1.98-1.90 (m, 4H, CH*CH$_2$*, S*CH$_3$* L-Met), 1.82-1.73 (m, 1H, CH*CH$_2$* L-Met), 1.17-1.11 (m, 3H, OCH$_2$*CH$_3$*). **^{13}C NMR (125 MHz, CDCl$_3$) δ$_C$**: 173.1 (d, $^3J_{C\text{-}P}$ = 4.3 Hz, *C*=O), 172.8 (d, $^3J_{C\text{-}P}$ = 3.6 Hz,

C=O), 161.09 (*C*-6), 161.08 (*C*-6), 152.6 (*C*-2), 152.5 (*C*-2), 151.0 (*C*-4), 150.2 (d, $^{2}J_{C\text{-}P}$ = 8.2 Hz, *C*-O, Ph), 150.1 (d, $^{2}J_{C\text{-}P}$ = 8.5 Hz, *C*-O, Ph), 140.14 (*C*-8), 140.11 (*C*-8), 129.67 (*CH*-Ar), 129.64 (*CH*-Ar), 124.99 (*CH*-Ar), 124.90 (*CH*-Ar), 123.45 (=*CH*N), 123.44 (=*CH*N), 121.9 (*C*-5), 121.8 (*C*-5), 120.8 (d, $^{3}J_{C\text{-}P}$ = 4.2 Hz, *CH*-Ar), 120.5 (d, $^{3}J_{C\text{-}P}$ = 4.5 Hz, *CH*-Ar), 116.5 (=*CH*CH_2), 116.4 (=*CH*CH_2), 70.9 (=CH*CH_2*), 70.8 (=CH*CH_2*), 65.4 (d, $^{1}J_{C\text{-}P}$ = 154.0 Hz, *CH_2*P), 65.3 (d, $^{1}J_{C\text{-}P}$ = 154.0 Hz, *CH_2*P), 61.5 (O*CH_2*CH_3), 61.4 (O*CH_2*CH_3), 54.2 (O*CH_3*, base), 53.08 (*CH* L-Met), 53.05 (*CH* L-Met), 33.9 (d, $^{3}J_{C\text{-}P}$ = 4.6 Hz, CH*CH_2*CH_2 L-Met), 33.7 (d, $^{3}J_{C\text{-}P}$ = 4.4 Hz, CH*CH_2*CH_2 L-Met), 29.7 (*CH_2*S L-Met), 29.5 (*CH_2*S L-Met), 15.23 (S*CH_3* L-Met), 15.20 (S*CH_3* L-Met), 14.09 (OCH_2*CH_3*), 14.07 (OCH_2*CH_3*). **HRMS (ESI):** m/z $[M+H]^+$ calcd for $C_{23}H_{31}N_5O_6PS$: 536.1733, found: 536.1727. **HPLC**: Reverse phase HPLC eluting with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 minutes, 1ml/min, λ = 254 nm and 263 nm, showed one peak with t_R 16.06 min.

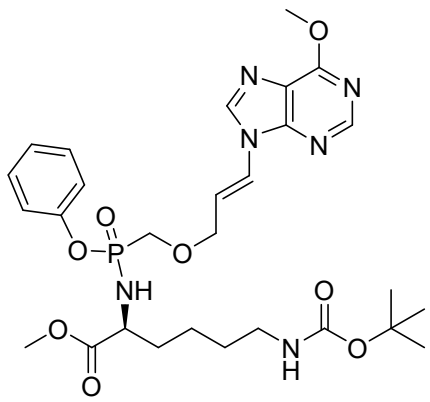
Dimethyl ((((*(E)*-3-(6-methoxy-9H-purin-9-yl)allyl)oxy)methyl)(phenoxy)phosphoryl)-*L*-glutamate 184c



Under an argon atmosphere, 2,6-Lutidine (351.0 µl, 2.16 mmol) and TMSBr (355.6 µl, 2.69 mmol), were added to a solution of the phosphonate **177** (192 mg, 538.8 µmol) in anhydrous acetonitrile (25 ml). The mixture was stirred 16 h at room temperature and then the volatiles evaporated without any contact with air. Then the flask was charged with dry amino acid ester hydrochloride **178b** (114.0 mg, 538.8 µl), phenol (304.0 mg, 3.23 mmol), dry triethylamine (1.13 ml, 8.08 mmol) and dry pyridine (6 ml) and heated to 50˚C to obtain a homogenous solution. To this mixture was then added a solution of Aldrithiol-2 (712.2 mg, 3.23 mmol) and triphenylphosphine (948.0 mg, 3.23 mmol) in dry pyridine (6 ml) under argon atmosphere. The resulting mixture was stirred at 50˚C for 16 h. After evaporating all the volatiles, the residue was purified by two Biotage Isolera One (50 g SNAP cartridge ULTRA, 100 ml/min, gradient eluent system MeOH/CH_2Cl_2 2% 1CV, 2-20% 10CV, 20% 2CV and 10 g SNAP cartridge ULTRA, 36 ml/min, gradient eluent system MeOH/CH_2Cl_2 2% 1CV, 2-20% 15CV, 20% 2CV) to afford the title compound **184c** as a yellow oil (157 mg, 55%). R_f= 0.39 (CH_2Cl_2/MeOH - 9:1). **^{31}P NMR (202MHz, $CDCl_3$) δ_P**: 22.44, 21.35. **^{1}H NMR (500 MHz, $CDCl_3$) δ_H**: 8.48 (s, 0.5H, *H*-2), 8.47 (s,

0.5H, *H*-2), 8.10 (s, 0.5H, *H*-8), 8.07 (s, 0.5H, *H*-8), 7.25-7.14 (m, 5H, Ar*H*, =*CH*N), 7.06-7.03 (m, 1H, Ar*H*), 6.59-6.50 (m, 1H, =*CH*CH_2), 4.26-4.06 (m, 6H, *CH_2*CH=, *CH* L-Glu, O*CH_3* base), 3.96-3.83 (m, 3H, *NH* L-Glu, *CH_2*P), 3.59 (s, 1.5H, COO*CH_3*), 3.58 (s, 1.5H, COO*CH_3*), 3.58 (s, 1.5H, COO*CH_3*), 3.51 (s, 1.5H, COO*CH_3*), 2.37-2.14 (m, 2H, CHCH_2*CH_2* L-Glu), 2.07-1.97 (m, 1H, CH*CH_2*CH_2 L-Glu), 1.86-1.77 (m, 1H, CH*CH_2*CH_2 L-Glu). **^{13}C NMR (125 MHz, $CDCl_3$) δC**: 173.4 (d, $^3J_{C\text{-}P}$ = 4.2 Hz, *C*=O), 172.8 (d, $^3J_{C\text{-}P}$ = 3.3 Hz, *C*=O), 173.1 (*C*=O), 173.0 (*C*=O), 161.1 (*C*-6), 161.0 (*C*-6), 152.59 (*C*-2), 152.58 (*C*-2), 151.07 (*C*-4), 151.05 (*C*-4), 150.1 (d, $^2J_{C\text{-}P}$ = 8.4 Hz, *C*-O, Ph), 150.0 (d, $^2J_{C\text{-}P}$ = 9.4 Hz, *C*-O, Ph), 140.19 (*C*-8), 140.17 (*C*-8), 129.67 (*CH*-Ar), 129.64 (*CH*-Ar), 124.9 (*CH*-Ar), 123.46 (=*CH*N), 123.45 (=*CH*N), 121.94 (*C*-5), 121.90 (*C*-5), 120.7 (d, $^3J_{C\text{-}P}$ = 4.5 Hz, *CH*-Ar), 120.6 (d, $^3J_{C\text{-}P}$ = 4.5 Hz, *CH*-Ar), 116.5 (=*CH*CH_2), 116.3 (=*CH*CH_2), 70.9 (d, $^3J_{C\text{-}P}$ = 14.2 Hz, =CH*CH_2*), 65.4 (d, $^1J_{C\text{-}P}$ = 153.6 Hz, *CH_2*P), 65.2 (d, $^1J_{C\text{-}P}$ = 154.1 Hz, *CH_2*P), 54.2 (*CH* L-Glu), 53.2 (O*CH_3*, base), 53.1 (O*CH_3*, base), 52.4 (COO*CH_3*), 52.3 (COO*CH_3*), 51.6 (COO*CH_3*), 29.58 (d, $^3J_{C\text{-}P}$ = 4.2 Hz, CH*CH_2*CH_2 L-Glu), 29.56 (CHCH_2*CH_2* L-Glu), 29.51 (CHCH_2*CH_2* L-Glu), 29.3 (d, $^3J_{C\text{-}P}$ = 4.5 Hz, CH*CH_2*CH_2 L-Glu). **HRMS (ESI):** m/z $[M+H]^+$ calcd for $C_{23}H_{29}N_5O_8P$: 534.1754, found: 534.1748. **HPLC**: Reverse phase HPLC eluting with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 minutes, 1ml/min, λ = 254 nm and 263 nm, showed one peak with t_R 16.05 min.

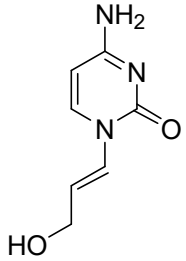
Methyl *N*^{6}-(tert-butoxycarbonyl)-*N*^{2}-(((((*E*)-3-(6-methoxy-9H-purin-9-yl)allyl)oxy)methyl)(phenoxy)phosphoryl)-*L*-lysinate 184d



Under an argon atmosphere, 2,6-Lutidine (300.7 µl, 2.58 mmol) and TMSBr (425.9 µl, 3.23 mmol), were added to a solution of the phosphonate **177** (230 mg, 645.4 µmol) in anhydrous acetonitrile (25 ml). The mixture was stirred 16 h at room temperature and then the volatiles evaporated without any contact with air. Then the flask was charged with dry amino acid ester hydrochloride **178c** (191.5 mg, 645.4 µl), phenol (364.4 mg, 3.87 mmol), dry triethylamine (1.35 ml, 9.68 mmol) and dry pyridine (8 ml) and heated to 50˚C to obtain a homogenous solution. To this mixture was then added a solution of Aldrithiol-2 (853.2 mg, 3.87 mmol) and triphenylphosphine (1.02 mg, 3.87 mmol) in dry pyridine (8 ml) under argon atmosphere. The resulting mixture was stirred

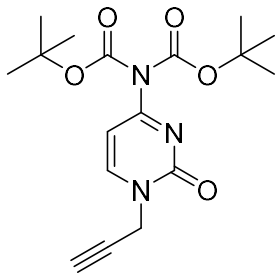
at 50˚C for 16 h. After evaporating all the volatiles, the residue was purified by Biotage Isolera One (50 g SNAP cartridge ULTRA, 100 ml/min, gradient eluent system MeOH/CH_2Cl_2 2% 1CV, 2-20% 10CV, 20% 2CV) to afford the title compound **184d** as a yellow oil (197 mg, 49%). R_f= 0.58 (CH_2Cl_2/MeOH - 9:1).**^{31}P NMR (202MHz, $CDCl_3$) δ_P**: 22.48, 21.44. **^{1}H NMR (500 MHz, $CDCl_3$) δ_H**: 8.49 (s, 0.5H, *H*-2), 8.48 (s, 0.5H, *H*-2), 8.10 (s, 0.5H, *H*-8), 8.06 (s, 0.5H, *H*-8), 7.25-7.16 (m, 5H, Ar*H*, =*CH*N), 7.12-7.04 (m, 1H, Ar*H*), 6.59-6.50 (m, 1H, =*CH*CH_2), 4.64 (bs, 0.5H, *NH*C=O), 4.59 (bs, 0.5H, *NH*C=O), 4.30-4.15 (m, 2H, *CH_2*CH=), 4.11 (s, 3H, O*CH_3* base), 4.07-3.96 (m, 1H, *CH* L-Lys), 3.91-3.81 (m, 2H, *CH_2*P), 3.78-3.65 (m, 1H, *NH* L-Lys), 3.59 (s, 1.5H, COO*CH_3*), 3.57 (s, 1.5H, COO*CH_3*), 2.95-2.89 (m, 2H, CHCH$_2$CH$_2$CH$_2$*CH_2*NH L-Lys), 1.65-1.60 (m, 1H, CH*CH_2*CH$_2$CH$_2$CH$_2$NH L-Lys), 1.54-1.46 (m, 1H, CH*CH_2*CH$_2$CH$_2$CH$_2$NH L-Lys), 1.34-1.06 (m, 13H, CHCH$_2$*CH_2CH$_2$*CH$_2$NH L-Lys, *t*Bu). **^{13}C NMR (125 MHz, $CDCl_3$) δ_C**: 174.0 (d, $^3J_{C\text{-}P}$ = 4.3 Hz, *C*=O, ester), 173.6 (d, $^3J_{C\text{-}P}$ = 3.4 Hz, *C*=O, ester), 161.13 (*C*-6), 161.11 (*C*-6), 155.9 (*C*=O, Boc), 152.63 (*C*-2), 152.60 (*C*-2), 151.0 (*C*-4), 150.2 (d, $^2J_{C\text{-}P}$ = 8.5 Hz, *C*-O, Ph), 150.0 (d, $^2J_{C\text{-}P}$ = 8.9 Hz, *C*-O, Ph), 140.1 (*C*-8), 140.0 (*C*-8), 129.67 (*CH*-Ar), 129.63 (*CH*-Ar), 125.0 (*CH*-Ar), 124.9 (*CH*-Ar), 123.5 (=*CH*N), 123.4 (=*CH*N), 121.96 (*C*-5), 121.93 (*C*-5), 120.9 (d, $^3J_{C\text{-}P}$ = 4.1 Hz, *CH*-Ar), 120.6 (d, $^3J_{C\text{-}P}$ = 4.6 Hz, *CH*-Ar), 116.6 (=*CH*CH_2), 116.4 (=*CH*CH_2), 78.9 (*C*$(CH_3)_3$), 71.0 (d, $^3J_{C\text{-}P}$ = 10.0 Hz, =CH*CH_2*), 70.9 (d, $^3J_{C\text{-}P}$ = 9.7 Hz, =CH*CH_2*), 65.5 (d, $^1J_{C\text{-}P}$ = 154.7 Hz, *CH_2*P), 65.4 (d, $^1J_{C\text{-}P}$ = 154.4 Hz, *CH_2*P), 54.3 (O*CH_3*, base), 53.7 (*CH* L-Lys), 53.5 (*CH* L-Lys), 52.3 (COO*CH_3*), 52.2 (COO*CH_3*), 40.1 (CHCH$_2$CH$_2$CH$_2$*CH_2*NH L-Lys), 34.3 (d, $^3J_{C\text{-}P}$ = 4.6 Hz, CH*CH_2*CH$_2$CH$_2$CH$_2$NH L-Lys), 34.1 (d, $^3J_{C\text{-}P}$ = 3.9 Hz, CH*CH_2*CH$_2$CH$_2$CH$_2$NH L-Lys), 29.3 (CHCH$_2$CH$_2$*CH_2*CH$_2$NH L-Lys), 29.2 (CHCH$_2$CH$_2$*CH_2*CH$_2$NH L-Lys), 28.3 (C(*CH_3*)$_3$), 22.2 (CHCH$_2$*CH_2*CH$_2$CH$_2$NH L-Lys), 22.0 (CHCH$_2$*CH_2*CH$_2$CH$_2$NH L-Lys). **HRMS (ESI):** m/z $[M+Na]^+$ calcd for $C_{28}H_{39}N_6NaO_8P$: 641.2465, found: 641.2459. **HPLC**: Reverse phase HPLC eluting with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 minutes, 1ml/min, λ = 254 nm and 263 nm, showed one peak with t_R 16.75 min.

(*E*)-4-amino-1-(3-hydroxyprop-1-en-1-yl)pyrimidin-2(1H)-one 186



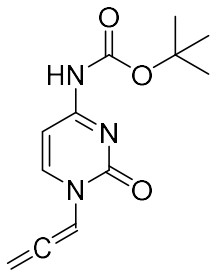
Under an argon atmosphere, to a suspension of compound **194** (2 g, 10.25 mmol) in anhydrous THF (60 ml) at -78°C, DIBAL-H (1 M solution in THF) (40.99 ml, 40.99 mmol) was added dropwise. The mixture was left stirring at room temperature. After 20 minutes the TLC showed the disappearance of the starting material, and the excess of the hydride was quenched by a slow addition of water. After 30 minutes stirring, the mixture was filtered on a small pad of celite which was rinsed with copious MeOH. The filtrate was then evaporated and triturated with MeOH to give the pure title compound **186** (1.5 g, 87%) as a glue solid. R_f = 0.15 (CH_2Cl_2/MeOH - 85:15). **^{1}H NMR (500 MHz, DMSO-d$_6$) δ_H**: 7.91 (d, *J* = 7.6 Hz, 1H, *H*-6), 7.42 (bs, 1H, NH_{2a}), 7.38 (bs, 1H, NH_{2b}), 7.18 (dt, J_t = 14.5 Hz, *J* = 1.3 Hz, 1H, N-*CH*=), 5.87 (dt, J_t = 14.5 Hz, *J* = 5.6 Hz, 1H, CH_2*CH*=), 5.84 (d, *J* = 7.6 Hz, 1H, *H*-5), 4.94 (bs, 1H, *OH*), 4.10 (d, *J* = 5.6 Hz, 2H, CH_2). **^{13}C NMR (125 MHz, DMSO-d$_6$) δ_C**: 165.9 (*C*-4), 154.4 (*C*-2), 141.1 (*C*-6), 126.6 (N-*CH*=), 117.1 (CH_2*CH*=), 95.6 (*C*-5), 59.9 (CH_2).

***tert*-Butyl (*tert*-butoxycarbonyl)(2-oxo-1-(prop-2-yn-1-yl)-1,2-dihydropyrimidin-4-yl)carbamate 190**



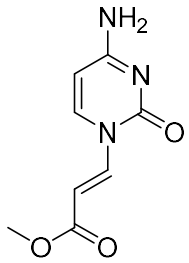
To a solution of the protected pyrimidine **119** (4.9 g, 15.74 mmol) in dry DMF (100 ml) was added NaH (60% dispersion in mineral oil, 415.4 mg, 17.31 mmol) followed by propargyl bromide (2.04 ml, 23.61 mmol). The reaction was stirred at room temperature for 24 h, and the resulting mixture was separated between CH_2Cl_2 (100 ml) and water (3 x 100 ml). the organic layer was dried over $MgSO_4$, filtered and the solvent was removed under vacuum. The crude material was crystallised from MeOH to afford the title compound as a white solid (2.6 g, 47%). R_f = 0.46 (CH_2Cl_2/EtOAc - 1:1). **^{1}H NMR (500 MHz, CD$_3$OD) δ_H**: 8.22 (d, *J* = 7.4 Hz, 1H *H*-6), 7.13 (d, *J* = 7.4 Hz, 1H, *H*-5), 4.74 (d, *J* = 2.6 Hz, 2H, CH_2C), 3.02 (t, *J* = 2.6 Hz, 1H, ≡*CH*), 1.57 (s, 18H,2 x *t*Bu).

tert-Butyl (2-oxo-1-(propa-1,2-dien-1-yl)-1,2-dihydropyrimidin-4-yl)carbamate 191



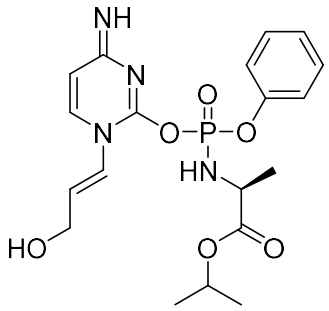
Under and argon atmosphere, *N*-propargyl pyrimidine **190** (2.6 g, 7.44 mmol) and K_2CO_3 (2.06 g, 14.88 mmol) were suspended in anhydrous MeOH (50 ml). The reaction mixture was refluxed for 15 minutes. After cooling to room temperature, the solvent was evaporated, and the reaction mixture extracted with EtOAc (50 ml) and water (3 x 50 ml). The organic phase was dried over $MgSO_4$, filtered, concentrated in vacuo and purified by Biotage Isolera One (100 g SNAP cartridge KP-SIL, 100 ml/min, gradient eluent system MeOH/CH_2Cl_2 20% 1CV, 20-100% 10CV, 100% 2CV), to afford the title compound as a white solid (690 mg, 37%). R_f = 0.42 (CH_2Cl_2/MeOH - 1:1). **^{1}H NMR (500 MHz, $CDCl_3$) δ_H**: 7.71 (d, *J* = 7.4 Hz, 1H, *H*-6), 7.54 (t, *J* = 6.6 Hz, 1H, =*CH*), 7.52 (bs, 1H, *NH*), 7.26 (d, *J* = 7.4 Hz, 1H, *H*-5), 5.66 (d, *J* = 6.6 Hz, 2H, =CH_2), 1.52 (s, 9H, *t*Bu).

Methyl (*E*)-3-(4-amino-2-oxopyrimidin-1(2H)-yl)acrylate 194[12]



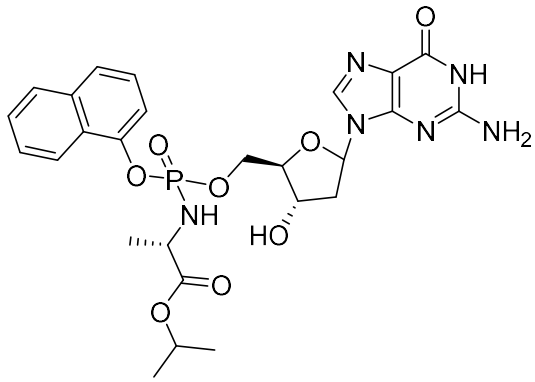
Under an argon atmosphere, cytosine **118** (3 g, 27.0 mmol) and Cs_2CO_3 (4.40 g, 13.50 mmol) were suspended in anhydrous DMF (200 ml) and left stirring 15 minutes at room temperature. Methyl propiolate (2.88 ml,32.40 mmol) was then added dropwise. The reaction was stirred for 30 minutes and a yellow suspension was observed. TLC confirmed the disappearance of the starting material and the suspension was evaporated. Water was then added to the residue and the solid filtered and washed with CH_2Cl_2 and hexane to give the titled compound **194** as light brown solid (5.13 g, 97%), used without further purification. R_f = 0.23 (CH_2Cl_2/MeOH - 9:1). **^{1}H NMR (500 MHz, DMSO-d_6) δ_H**: 8.21 (d, J_t = 14.6 Hz, 1H, N-*CH*=), 8.04 (d, *J* = 7.6 Hz, 1H, *H*-6), 7.78 (bs, 1H, NH_{2a}), 7.75 (bs, 1H, NH_{2b}), 6.17 (d, J_t = 14.6 Hz, 1H, CO*CH*=), 5.90 (d, *J* = 7.6 Hz, 1H, *H*-5), 3.69 (s, 3H, CH_3). **^{13}C NMR (125 MHz, DMSO-d_6) δ_C**: 167.2 (*C*=O, ester), 165.9 (*C*-4), 153.5 (*C*-2), 139.8 (*C*-6), 139.7 (N-*CH*=), 103.0 (CO*CH*=), 98.1 (*C*-5), 51.8 (CH_3).

Isopropyl (((1-((*E*)-3-hydroxyprop-1-en-1-yl)-4-imino-1,4-dihydropyrimidin-2-yl)oxy)(phenoxy)phosphoryl)-*L*-alaninate 196



Hydroxyl nuceloside derivative **186** (150 mg, 897.3 µmol) was suspended in anhydrous DMF (6 ml), and NMI (214.5 µl, 2.69 mmol) was added dropwise at room temperature. The mixture was left to stir 10 minutes and then a solution of the phosphorochloridate **22** (329.0 mg, 1.08 mmol) in anhydrous DMF (1 ml) was added dropwise. After 16 h at 50°C, the reaction was concentrated to dryness and purified by Biotage Isolera One (10 g SNAP cartridge ULTRA, 36 ml/min, gradient eluent system MeOH/CH_2Cl_2 2% 1CV, 2-20% 15CV, 20% 2CV), to afford the title compound **196** as a yellow oil (5 mg, 1.3%). R_f = 0.21 (CH_2Cl_2/MeOH - 9:1). **^{31}P NMR (202MHz, $CDCl_3$) δ_P**: 8.02 (bs), 6.81 (bs). **^{1}H NMR (500 MHz, DMSO-d_6) δ_H**: 7.73 (d, *J* = 7.4 Hz, 1H, *H*-6), 7.20-7.17 (m, 2H, Ar*H*), 7.11-7.08 (m, 2H, Ar*H*), 7.02-6.98 (m, 2H, Ar*H*, N-*CH*=), 6.08 (d, *J* = 7.4 Hz, 1H, *H*-5), 5.88 (dt, J_t = 14.3 Hz, *J* = 5.2 Hz, 1H, CH_2*CH*=),4.81-4.76 (m, 1H, *CH*(CH_3)$_2$), 4.08 (dd, *J* = 5.2 Hz, *J* = 1.2 Hz, 2H, *CH_2*), 3.95-3.85 (m, 1H, *CH* L-Ala), 1.18 (d, *J* = 6.8 Hz, 3H, *CH_3* L-Ala), 1.04-1.03 (m, 6H, CH(*CH_3*)$_2$). **^{13}C NMR (125 MHz, DMSO-d_6) δ_C**: 173.3 (*C*=O, ester), 150.7 (*C*-Ar), 129.2 (*CH*-Ar), 129.6 (*CH*-Ar), 124.6 (N-*CH*=), 120.5 (*CH*-Ar), 120.4 (*CH*-Ar), 68.5 (*CH*(CH_3)$_2$), 59.6 (*CH_2*), 50.1 (d, $^2J_{C\text{-}P}$ = 1.8 Hz, *CH* L-Ala), 20.4 (d, $^3J_{C\text{-}P}$ = 3.6 Hz, *CH_3* L-Ala), 19.1 (CH(*CH_3*)$_2$), 19.0 (CH(*CH_3*)$_2$). **(ES+) m/z**, found: 437.1 [M+H^+] and 459.1 [M+Na^+], $C_{19}H_{25}N_4O_6P$ required: 436.15 [M].

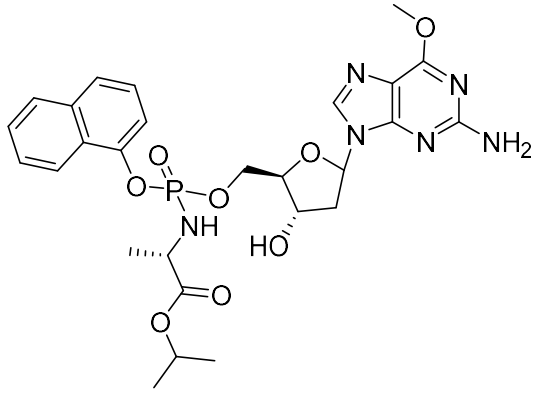
Isopropyl ((((2*R*,3*S*)-5-(2-amino-6-oxo-1,6-dihydro-9H-purin-9-yl)-3-hydroxytetrahydrofuran-2-yl)methoxy)(naphthalen-1-yloxy)phosphoryl)-*L*-alaninate 197



2'-Deoxyguanosine (300 mg, 1.12 mmol) was suspended in anhydrous DMF (8 mL). *t*BuMgCl (1 M in THF) (3.37 m, 3.37 mmol) was added dropwise and the resulting mixture was stirred for 30 minutes. A solution of the *p*-nitrophenolate **200** (514.5 mg, 1.12 mmol) in anhydrous DMF (2 mL) was added dropwise over a period of 15 minutes and the reaction mixture was stirred for 16 hours at room temperature. Afterwards, the solvent was evaporated under reduced

pressure on rotary evaporator and the residue was purified by two Biotage Isolera One (50 g SNAP cartridge ULTRA, 100 ml/min, gradient eluent system MeOH/CH_2Cl_2 2% 1CV, 2-20% 15CV, 20% 2CV and reverse phase 60 g SNAP cartridge KP-C18-HS, 100 ml/min, isocratic eluent system CH_3CN/H_2O 30-60% 12CV) to afford the title compound **197** as a yellow oil (98 mg, 15%). R_f = 0.25 (CH_2Cl_2/MeOH - 9:1). **^{31}P NMR (202 MHz, DMSO-d$_6$) δ$_P$**: 4.32, 4.17. **^{1}H NMR (500 MHz, DMSO-d$_6$) δ$_H$**: 10.63 (bs, 1H, *NH*, base), 8.14-8.11 (m, 1H, Ar*H*), 7.98-7.93 (m, 1H, Ar*H*), 7.82 (s, 1H, *H*-8), 7.77-7.72 (m, 1H, Ar*H*), 7.59-7.55 (m, 2H, Ar*H*), 7.49-7.41 (m, 2H, Ar*H*), 6.47 (bs, 2H, NH_2), 6.20-6.13 (m, 2H, *H*-1', *NH* L-Ala), 5.44-5.42 (m, 1H, *OH*-3'), 4.88-4.77 (m, 1H, $CH(CH_3)_2$), 4.41-4.34 (m, 1H, *H*-3'), 4.30-4.19 (m, 1H, *H*-5'$_a$), 4.16-4.08 (m, 1H, *H*-5'$_b$), 4.06-4.00 (m, 1H, *H*-4'), 3.92-3.80 (m, 1H, $CHCH_3$ L-Ala), 2.53-2.48 (m, 0.5H, *H*-2'$_a$), 2.43-2.37 (m, 1H, *H*-2'$_a$), 2.24-2.16 (m, 1H, *H*-2'$_b$), 1.22 (d, *J* = 6.7 Hz, 3H, CHCH_3 L-Ala), 1.13-1.08 (m, 6H, CH(CH_3)$_2$). **^{13}C NMR (125 MHz, DMSO-d$_6$) δ$_C$**: 173.2 (d, $^3J_{C\text{-}P}$ = 4.0 Hz, *C*=O, ester), 173.1 (d, $^3J_{C\text{-}P}$ = 4.9 Hz, *C*=O, ester), 157.1 (*C*-6), 154.1 (*C*-2), 151.4 (*C*-4), 146.98 (d, $^2J_{C\text{-}P}$ = 7.2 Hz, *C*-O, Naph), 146.95 (d, $^2J_{C\text{-}P}$ = 6.8 Hz, *C*-O, Naph), 135.5 (*C*-8), 135.3 (*C*-8), 1134.7 (*C*-Ar), 128.17 (*CH*-Ar), 128.13 (*CH*-Ar), 127.1 (*CH*-Ar), 126.75 (*CH*-Ar), 126.71 (*CH*-Ar), 126.5 (d, $^3J_{C\text{-}P}$ = 5.8 Hz, *C*-Ar), 126.4 (d, $^3J_{C\text{-}P}$ = 4.8 Hz, *C*-Ar), 126.1 (*CH*-Ar), 124.68 (*CH*-Ar), 124.64 (*CH*-Ar), 122.03 (*CH*-Ar), 122.00 (*CH*-Ar), 117.1 (*C*-5), 115.3 (d, $^3J_{C\text{-}P}$ = 2.6 Hz, *CH*-Ar), 115.2 (d, $^3J_{C\text{-}P}$ = 2.7 Hz, *CH*-Ar), 85.3 (d, $^3J_{C\text{-}P}$ = 8.0 Hz, *C*-4'), 85.2 (d, $^3J_{C\text{-}P}$ = 85.2 Hz, *C*-4'), 83.0 (*C*-1'), 82.9 (*C*-1'), 71.0 (*C*-3'), 70.9 (*C*-3'), 68.5 ($CH(CH_3)_2$), 68.4 ($CH(CH_3)_2$), 66.7 (d, $^2J_{C\text{-}P}$ = 5.2 Hz, *C*-5'), 66.2 (d, $^2J_{C\text{-}P}$ = 5.1 Hz, *C*-5'), 50.49 ($CHCH_3$ L-Ala), 50.41 ($CHCH_3$ L-Ala), 39.49 (*C*-2'), 39.43 (*C*-2'), 21.8 (CH(CH_3)$_2$), 21.7 (CH(CH_3)$_2$), 20.2 (d, $^3J_{C\text{-}P}$ = 6.5 Hz, CHCH_3 L-Ala), 20.1 (d, $^3J_{C\text{-}P}$ = 7.2 Hz, CHCH_3 L-Ala). **HPLC**: Reverse phase HPLC eluting with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 minutes, 1 mL/min, λ = 254 nm and 263 nm, showed two peaks with t_R 11.51 min. and t_R 11.92 min. **(ES+) m/z**, found: 587.1 [M+H$^+$] and 609.1 [M+Na$^+$], $C_{26}H_{31}N_6O_8P$ required: 586.19 [M].

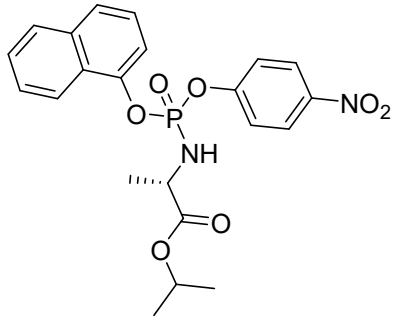
Isopropyl ((((2*R*,3*S*)-5-(2-amino-6-methoxy-9H-purin-9-yl)-3-hydroxytetrahydrofuran-2-yl)methoxy)(naphthalen-1-yloxy)phosphoryl)-*L*-alaninate 198



Compound **202** (3.00 g, 10.67 mmol) was suspended in anhydrous DMF (80 ml) under argon atmosphere, *t*BuMgCl (1 M in THF) (32.0 ml, 32.00 mmol) was added dropwise and the resulting mixture was stirred for 30 minutes. A solution of **200** (5.38 g, 11.73 mmol) in anhydrous DMF (20 mL) was added dropwise over a period of 15 minutes and the reaction mixture was stirred for 16 hours at room temperature. Afterwards, the solvent was evaporated under reduced pressure on rotary evaporator and the residue was diluted with water resulting in formation of a solid that was filtered off. The water phase was extracted two times with dichloromethane and two times with ethyl acetate. Organic layers were combined, dried over $MgSO_4$ and concentrated under reduced pressure. The crude was purified by two Biotage Isolera One (50 g SNAP cartridge ULTRA, 100 ml/min, gradient eluent system MeOH/CH_2Cl_2 2% 1CV, 2-20% 15CV, 20% 2CV and reverse phase 60 g SNAP cartridge KP-C18-HS, 100 ml/min, isocratic eluent system CH_3CN/H_2O 30-60% 12CV) to afford the title compound **198** as a white solid (101 mg, 2%). R_f = 0.33 (CH_2Cl_2/MeOH - 9:1). **^{31}P NMR (202 MHz, CD_3OD) δ_P**: 4.46, 4.32. **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 8.18- 7.33 (m, 8H, *H*-8, Ar*H*), 6.33-6.28 (m, 1H, *H*-1'), 4.95-4.89 (m, 1H, *CH*(CH_3)$_2$), 4.62-4.54 (m, 1H, *H*-3'), 4.49-4.32 (m, 2H, *H*-5'), 4.21-4.14 (m, 1H, *H*-4'), 4.03 (s, 3H, O*CH$_3$*), 4.05 (s, 1.5H, O*CH$_3$*), 4.00-3.91 (m, 1H, *CH*CH_3 L-Ala), 2.75-2.52 (m, 1H, *H*-2'$_a$), 2.38-2.26 (m, 1H, *H*-2'$_b$), 1.31-1.27 (m, 3H, CH*CH$_3$* L-Ala), 1.23-1.17 (m, 6H, CH(*CH$_3$*)$_2$). **^{13}C NMR (125 MHz, CD_3OD) δ_C**: 173.2 (d, $^3J_{C\text{-}P}$ = 4.5 Hz, *C*=O, ester), 173.0 (d, $^3J_{C\text{-}P}$ = 5.2 Hz, *C*=O, ester), 161.1 (*C*-6), 161.0 (*C*-6), 160.3 (*C*-2), 153.1 (*C*-4), 146.5 (d, $^2J_{C\text{-}P}$ = 6.8 Hz, *C*-O, Naph), 134.9 (*C*-8), 129.8 (*CH*-Ar), 129.2 (*CH*-Ar), 125.0 (*CH*-Ar), 120.21 (*CH*-Ar), 120.15 (*CH*-Ar), 116.2 (*C*-5), 85.2 (d, $^3J_{C\text{-}P}$ = 7.0 Hz, *C*-4'), 84.8 (d, $^3J_{C\text{-}P}$ = 7.1 Hz, *C*-4'), 84.8 (*C*-1'), 71.5 (*C*-3'), 71.3 (*C*-3'), 69.4 (*CH*(CH_3)$_2$), 69.2 (*CH*(CH_3)$_2$), 66.2 (d, $^2J_{C\text{-}P}$ = 5.3 Hz, *C*-5'), 66.1 (d, $^2J_{C\text{-}P}$ = 5.0 Hz, *C*-5'), 53.8 (O*CH$_3$*), 50.2 (*CH*CH_3 L-Ala), 50.0 (*CH*CH_3 L-Ala), 39.3 (*C*-2'), 38.8 (*C*-2'), 21.6 (CH(*CH$_3$*)$_2$), 21.5 (CH(*CH$_3$*)$_2$), 20.7 (d, $^3J_{C\text{-}P}$ = 5.3 Hz, CH*CH$_3$* L-Ala), 20.5 (d, $^3J_{C\text{-}P}$ = 5.2 Hz, CH*CH$_3$* L-Ala). **HPLC**: Reverse phase HPLC eluting with gradient method

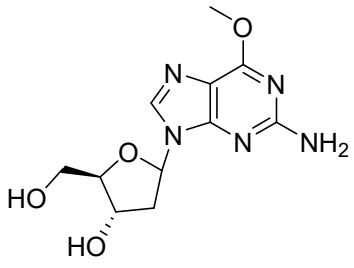
CH_3CN/H_2O from 10/90 to 100/0 in 30 minutes, 1 mL/min, λ = 254 nm and 263 nm, showed two peaks with t_R 15.15 min. and t_R 15.32 min. **(ES+) m/z**, found: 601.1 [$M+H^+$], $C_{27}H_{33}N_6O_8P$ required: 600.21 [M].

Isopropyl ((naphthalen-1-yloxy)(4-nitrophenoxy)phosphoryl)-*L*-alaninate 200[13]



A solution of 1-naphthol (1.69 g, 11.72 mmol) and anhydrous triethylamine (1.80 mL, 12.89 mmol) in anhydrous dichloromethane (25 ml) was added dropwise to a solution of *p*-nitrophenyl phosphorodichloridate (3.00 g, 11.72 mmol) in anhydrous dichloromethane (25 ml) under argon atmosphere at -78 °C. The resulting mixture was stirred at that temperature for 30 minutes . After that period, when ^{31}P NMR confirmed completion of the reaction ($CDCl_3$, a singlet at -5.63 ppm corresponding to desired phosphorochloridate was observed), the reaction mixture was added to a cold solution (0°C) of *L*-alanine isopropyl ester hydrochloride (1.96 g, 11.72 mmol) in anhydrous dichloromethane (25 ml). Subsequently, anhydrous triethylamine (3.43 ml, 24.61 mmol) was added dropwise and the mixture was stirred at 0 °C for further 30 minutes. Once ^{31}P NMR confirmed completion of the reaction dichloromethane was evaporated under reduced pressure without any contact with air. The residue was suspended in diethyl ether and stirred at 0 °C for 30 minutes. The white solid was filtered off and the filtrate was concentrated under reduced pressure on rotary evaporator without any contact with air to obtain **200** as yellow oil (5.1 g, 95%). **^{31}P NMR (202 MHz, $CDCl_3$) δ_P**: -2.79, -2.85. **^{1}H-NMR ($CDCl_3$, 500 MHz) δ_H**: 8.13-8.10 (m, 2H, Ar*H*) 7.98-7.96 (m, 1H, Ar*H*), 7.78-7.77 (m, 1H, Ar*H*), 7.62-7.60 (m, 1H, Ar*H*), 7.49-7.43 (m, 3H, Ar*H*), 7.34-7.29 (m, 3H, Ar*H*), 4.96-4.82 (m, 1H, *CH*$(CH_3)_2$), 4.15-3.96 (m, 2H, *CH*CH_3 L-Ala, -*NH*), 1.31-1.28 (m, 3H, CH*CH₃* L-Ala), 1.15-1.07 (m, 6H, CH(*CH₃*)$_2$).

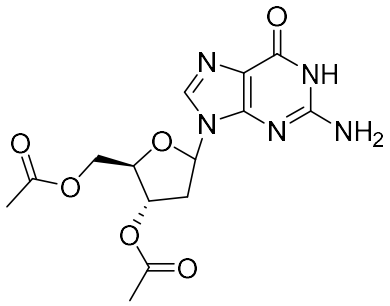
(2*R*,3*S*)-5-(2-Amino-6-methoxy-9H-purin-9-yl)-2(hydroxymethyl)tetrahydrofuran-3-ol 202[14]



Freshly prepared 1M solution of $NaOCH_3$ (5.84 g, 108.18 mmol) in anhydrous MeOH (108.2 ml) was added dropwise to a solution of **206** (8.00 g, 21.64 mmol) in anhydrous methanol (50 ml) under argon atmosphere in a 500 ml round-bottom flask, cooled to 0°C. The reaction mixture

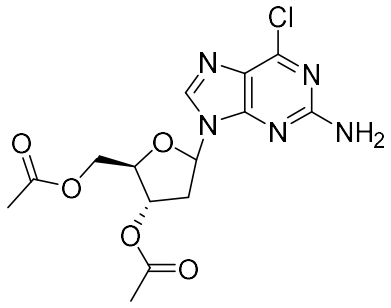
was stirred at room temperature for 6 hours, until no more starting material could be observed at TLC plate. The mixture was concentrated to dryness under reduced pressure; the residue was dissolved in a small amount of water to obtain a transparent yellow solution. Acetic acid was used to adjust pH of the solution to pH 7, resulting in formation of a white solid. The solid was removed by filtration and the solution extracted with EtOAc (4x 100 ml). The solid was washed with ethyl acetate (50 ml x 5) and with dichloromethane (50 ml x 5) and then dissolved in as small amount of water and extracted ethyl acetate (50 ml x 2) and with dichloromethane (50 ml x 2). All organic layers were combined, dried over $MgSO_4$ and concentrated under reduced pressure on rotary evaporator yielding pure **202** (4.21 g, 69 %). R_f = 0.37 (DCM/MeOH, 9:1). **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 8.05 (s, 1H, *H*-8), 6.34 (dd, *J* = 8.3, *J* = 6.1 Hz, 1H, *H*-1'), 4.59-4.57 (m, 1H, *H*-3'), 4.09-4.04 (m, 4H, *H*-4', O*CH*$_3$), 3.86 (dd, *J* = 12.2, *J* = 3.1 Hz, 1H, *H*-5'$_a$), 3.76 (dd, *J* = 12.2, *J* = 3.4 Hz, 1H, *H*-5'$_b$), 2.84-2.76 (m, 1H, *H*-2'$_a$), 2.36 (ddd, *J* = 13.4, *J* = 6.0, *J* = 2.6 Hz, 1H, *H*-2'$_b$).

((2*R*,3*S*)-3-Acetoxy-5-(2-amino-6-oxo-1,6-dihydro-9H-purin-9-yl)tetrahydrofuran-2-yl)methyl acetate 205[15]



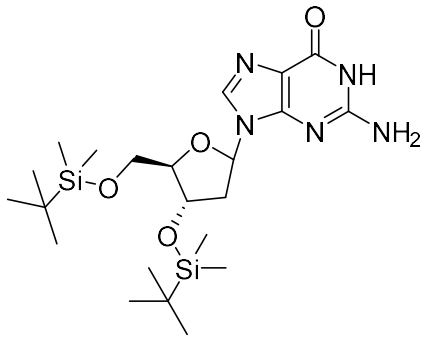
2′-deoxyguanosine **201** (10.00 g, 37.42 mmol), 4-(dimethylamino)pyridine (0.46 g, 3.74 mmol) and triethylamine (13.6 ml, 97.29 mmol) were dissolved in anhydrous acetonitrile (500 ml) under argon atmosphere in a 1000 mL round-bottom flask and the solution was cooled to 0 °C. Acetic anhydride (8.5 ml, 89.81 mmol) was added dropwise and the resulting reaction mixture was stirred overnight at room temperature. After addition of methanol (200 ml), the formed solid was filtered using a Büchner funnel and washed with methanol and hexane to obtain **205** as white solid (12.60 g, 96 %). R_f = 0.47 (DCM/MeOH, 9:1). **^{1}H NMR (500 MHz, DMSO-d_6) δ_H**: 10.67 (s, 1H, *NH*), 7.92 (s, 1H, *H*-8), 6.50 (s, 2H, -*NH*$_2$), 6.14 (dd, *J* = 8.8, *J* = 5.9 Hz, 1H, *H*-1'), 5.30 (dt, *J* = 6.2, 1.9 Hz, 1H, *H*-3'), 4.31-4.25 (m, 1H, *H*-5'$_a$), 4.22-4.17 (m, 2H, *H*-4', *H*-5'$_b$), 2.96-2.88 (m, 1H, *H*-2'$_a$), 2.46 (ddd, J_G = 14.2, *J* = 6.0, *J* = 2.1 Hz, 1H, *H*-2'$_b$), 2.09 (s, 3H, *CH*$_3$), 2.05 (s, 3H, *CH*$_3$).

((2*R*,3*S*)-3-Acetoxy-5-(2-amino-6-chloro-9H-purin-9-yl)tetrahydrofuran-2-yl)methyl acetate 206[16]



Compound **205** (6.00 g, 17.08 mmol.) was suspended in anhydrous acetonitrile (100 ml) under argon atmosphere together with benzyltriethylammonium chloride (5.83 g, 25.62 mmol) and *N,N*-dimethylaniline (13.0 ml, 102.47 mmol). The resulting mixture was cooled to 0°C and phosphoryl chloride (9.6 ml, 102.47 mmol) was added dropwise. The mixture was stirred for 10 minutes at room temperature and then heated to reflux in preheated oil bath. The reaction was monitored every 10 minutes by TLC, and after 1 hour, when there were no further changes observed on TLC plate, the reaction mixture was cooled with an ice bath and concentrated to dryness under reduced pressure. Ice water (20 ml) was added under cooling in order to hydrolyse the remaining phosphoryl chloride, the mixture was stirred for 20 minutes and subsequently extracted with ethyl acetate. The organic layers were joined, dried over $MgSO_4$ and the solvent was evaporated under reduced pressure on rotary evaporator. The crude residue was purified by column chromatography on silica using DCM/MeOH, 95:5 as the eluting system yielding **206** as a white foam (2.90 g, 46 %). R_f: 0.43 (DCM/MeOH, 95:5). **^{1}H NMR (500 MHz, $CDCl_3$) δ_H**: 7.94 (s, 1H, *H*-8), 6.31 (dd, *J* = 7.9, *J* = 6.2 Hz, 1H, *H*-1'), 5.45 (dt, *J* = 6.3, *J* = 2.5 Hz, 1H, *H*-3'), 5.21 (s, 2H, -*NH*$_2$), 4.48 (dd, *J* = 14.5, *J* = 6.01 Hz, 1H, *H*-5'$_a$), 4.41-4.36 (m, 2H, *H*-4' and *H*-5'$_b$), 3.00 (ddd, *J* = 14.2, *J* = 7.9, *J* = 6.4 Hz, 1H, *H*-2'$_a$), 2.59 (ddd, *J* = 14.2, *J* = 6.2, *J* = 2.6 Hz, 1H, *H*-2'$_b$), 2.16 (s, 3H, *CH*$_3$), 2.11 (s, 3H, *CH*$_3$).

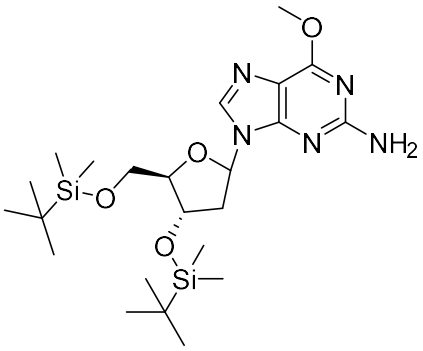
2-Amino-9-((4*S*,5*R*)-4-((*tert*-butyldimethylsilyl)oxy)-5-(((tert-butyldimethylsilyl)oxy)methyl)tetrahydrofuran-2-yl)-1,9-dihydro-6H-purin-6-one 210



TBDMSCl (7.0 g, 0.046 mol) was added to a mixture of **201** (5 mg, 0.018 mol) and imidazole (6.3 g, 0.093 mol) in 50 ml of dry pyridine at room temperature under nitrogen and the resulting mixture stirred for 12 h. The solvent was removed under reduced pressure, and the solid taken up with EtOAc, washed with 5% $NaHCO_3$, water and brine. The organic phase was dried over $MgSO_4$, filtered and the solvent removed under vacuum to obtain **210** (7.3g, 82%).**^{1}H-NMR (500 MHz, CD_3OD) δ_H**: 7.84 (s, 1H,

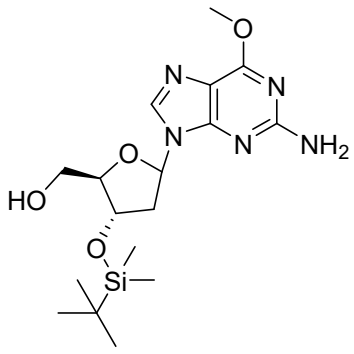
H-8), 6.17 (t, *J* = 6.4, 1H, *H*-1'), 4.43-4.42 (m, 1H, *H*-3'), 3.89-3.80 (m, 1H, *H*-4'), 3.79-3.72 (m, 2H, *H*-5'), 2.52-2.47 (m, 1H, *H*-2'$_a$), 2.31-2.30 (m, 1H, *H*-2'$_b$), 0.81 (s, 18H, 2 x *t*Bu), 0.0 (s, 12H, 4 x CH_3).

9-((4*S*,5*R*)-4-((*tert*-Butyldimethylsilyl)oxy)-5-(((tert-butyldimethylsilyl)oxy)methyl)tetrahydrofuran-2-yl)-6-methoxy-9H-purin-2-amine 211



To a solution of **210** (0.810 g, 1.63 mmol) in 16 ml of dry pyridine (15 ml) at 0ºC, trifluoroacetic anhydride (1.21g, 0.81 ml) was added dropwise and the mixture stirred for 15-20 minutes at 0ºC. After this period, a solution of 0.17M sodium methoxide (1.55g, 28.76 mmol) in dry methanol was added slowly via cannula to the previous solution. The mixture was stirred at room temperature for 20 h. After this period the reaction mixture was poured into 50 mL of water. The mixture was partitioned using petroleum ether (4 x 60 ml). The combined organic layers were evaporated, traces of pyridine were removed by co-evaporation of toluene (5 x 20 ml) to obtain crude **211**, which after crystallization with petroleum ether (50ml) afford pure **211** as pale-yellow solid. (0.681 g, 82%). **^{1}H-NMR (500 MHz, $CDCl_3$) δ_H**: 8.02 (s, 1H, *H*-8), 6.21 (t, *J* = 6.5, 1H, *H*-1'), 4.72 (s, 2H, NH_2), 4.50-4.47 (m, 1H, *H*-3'), 3.88-3.86 (m, 1H, *H*-4'), 3.71 (dd, *J* = 11.2 Hz, *J* = 4.2 Hz, 1H, *H*-5'$_a$), 3.95 (s, 3H, OCH_3), 3.71 (dd, *J* = 11.2 Hz, *J* = 3.5 Hz, 1H, *H*-5'$_b$), 2.49-2.44 (m, 1H, *H*-2'$_a$), 2.20 (ddd, *J* = 13.1 Hz, *J* = 6.4 Hz, *J* = 3.9 Hz, 1H, *H*-2'$_b$), 0.83 (s, 18H, 2 x *t*Bu), 0.0 (s, 6H, 2 x CH_3), -0.01 (s, 3H, CH_3), -0.02 (s, 3H, CH_3).

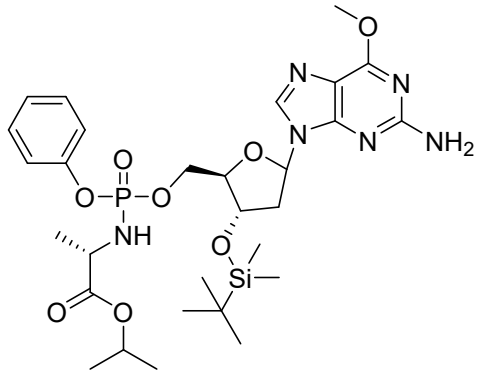
((2*R*,3*S*)-5-(2-Amino-6-methoxy-9H-purin-9-yl)-3-((tert-butyldimethylsilyl)oxy)tetrahydrofuran-2-yl)methanol 212



The bis-silylated nucleoside **211** (1.07 g, 2.09 mmol) was dissolved in THF (20.7 ml) and the solution was cooled at 0 ºC in an ice/water bath. A solution of TFA/H_2O (10 ml, 1:1, v/v) was added in a dropwise fashion to the flask and allowed to stir for 30 minutes at 0 ºC. The solution was carefully neutralised by addition of $NaHCO_3$ (aqueous saturated solution) and then extracted with EtOAc (3 x 50 ml). The organic layers were combined, dried over $MgSO_4$ and concentrated under reduced pressure to give the title compound as a white solid. (0.680 g, 82%). R_f: 0.20 (CH_2Cl_2/MeOH-9:1).**^{1}H-NMR (500 MHz, DMSO-d_6) δ_H**: 8.09

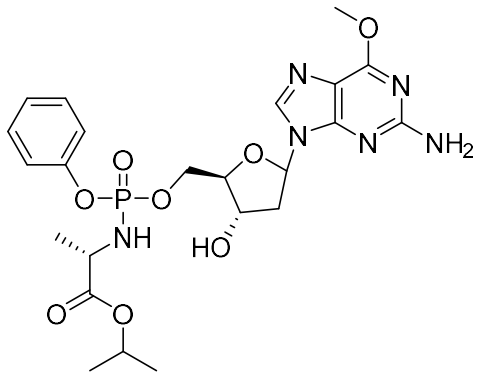
(s, 1H, *H*-8), 6.44 (s, 2H, *NH*$_2$), 6.20 (dd, *J* = 7.9 Hz, 6.0 Hz, 1H, *H*-1'), 5.04 (t, *J* = 5.6 Hz, 1H, 5'-*OH*), 4.53-4.51 (m, 1H, *H*-3'), 3.95 (s, 3H, O*CH*$_3$), 3.82-3.80 (m, 1H, *H*-4'), 3.57-3.52 (m, 1H, *H*-5'$_a$), 3.51-3.46 (m, 1H, *H*-5'$_b$), 2.72-2.66 (m, 1H, *H*-2'$_a$), 2.20 (ddd, *J* = 13.1 Hz, *J* = 5.8 Hz, *J* = 2.4 Hz, 1H, *H*-2'$_b$), 0.83 (s, 9H, *t*Bu), 0.10 (s, 6H, 2 x *CH*$_3$). **(ES+) m/z**, found: 396.23 [M+H$^+$], $C_{17}H_{29}N_5O_4Si$ required: 395.54 [M].

Isopropyl ((((2*R*,3*S*)-5-(2-amino-6-methoxy-9H-purin-9-yl)-3-((*tert*-butyldimethylsilyl)oxy)tetrahydrofuran-2-yl)methoxy)(phenoxy)phosphoryl)-*L*-alaninate 213



Under an argon atmosphere, the mono-silylated nucleoside **212** (300 mg, 758.47 µmol) was dissolved in THF (10 ml) and the suspension was cooled at 0 ºC in an ice/water bath. *t*BuMgCl (1 M in THF solution) (2.28 ml, 2.28 mmol) was then added and the mixture allowed to stir for 10 minutes. A solution of the phosphorochloridate **22** (463.72 mg, 1.52 mmol) in THF (1 ml) was added slowly dropwise and the resulting mixture was stirred at room temperature for 16 hours. After this period, the solvent was evaporated to dryness and the crude product was purified by Biotage Isolera One (100 g SNAP cartridge ULTRA, 100 ml/min, gradient eluent system MeOH/CH_2Cl_2 1% 1CV, 1-10% 15CV, 10% 2CV) to give the title compound as a solid (200 mg, 40%). R_f = 0.45 (CH_2Cl_2/MeOH – 96/4).**^{31}P NMR (202 MHz, DMSO-d$_6$) δ$_P$**: 3.78, 3.71. **^{1}H NMR (500 MHz, DMSO-d$_6$) δ$_H$**: 7.95 (s, 0.5H, *H*-8), 7.92 (s, 0.5H, *H*-8), 7.27-7.21 (m, 2H, Ar*H*), 7.10-7.04 (m, 3H, Ar*H*), 6.37 (s, 2H, *NH*$_2$), 6.15-6.11 (m, 1H, *H*-1'), 4.76-4.71 (m, 1H, *CH*(CH_3)$_2$), 4.47-4.44 (m, 1H, *H*-3'), 4.18-4.09 (m, 1H, *H*-5'$_a$), 4.04-4.00 (m, 1H, *H*-5'$_b$), 3.94-3.88 (m, 1H, *H*-4'), 3.86 (s, 3H, O*CH*$_3$), 3.66-3.61 (m, 1H, *CH*CH_3 L-Ala), 2.73-2.60 (m, 1H, *H*-2'$_a$), 2.17-2.11 (m, 1H, *H*-2'$_b$), 1.10 (d, *J* = 7.6 Hz, 1.5H, CH*CH*$_3$ L-Ala), 1.08 (d, *J* = 7.0 Hz, 1.5H, CH*CH*$_3$ L-Ala), 1.04-1.02 (m, 6H, CH(*CH*$_3$)$_2$), 0.78 (s, 4.5H, *t*Bu), 0.77 (s, 4.5H, *t*Bu), 0.00 (s, 3H, *CH*$_3$), -0.01 (s, 3H, *CH*$_3$). **(ES+) m/z, found**: 665.29 [M+H$^+$], $C_{29}H_{45}N_6O_8PSi$ required: 664.77 [M].

Isopropyl ((((2*R*,3*S*)-5-(2-amino-6-methoxy-9H-purin-9-yl)-3-hydroxytetrahydrofuran-2-yl)methoxy)(phenoxy)phosphoryl)-*L*-alaninate 214



Under an argon atmosphere, the 3'-silylated ProTide **213** (0.120 g, 0.189 mmol) was dissolved in anhydrous CH_2Cl_2 (8 ml) and the solution was cooled at 0 ºC in an ice/water bath. Me_3SiOTf (0.171 ml, 0.948 mmol) was then added dropwise at 0 ºC and the mixture allowed to stir 5 minutes followed by addition of an excess of neutral alumina (grade I, 1g). The mixture was then filtrated through a small pad of alumina with CH_2Cl_2/MeOH – 9/1 as eluent. The filtrate was concentrated under reduced pressure and purified by Biotage Isolera One (25 g SNAP cartridge ULTRA, 75 ml/min, gradient eluent system MeOH/CH_2Cl_2 1% 1CV, 1-10% 12CV, 1-20% 5CV) to give the title compound as a solid (50 mg, 48%). R_f 0.50 (CH_2Cl_2/MeOH-9:1). **^{31}P NMR (202 MHz, CD_3OD) δ_P**: 4.08, 3.88. **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 7.98 (s, 1H, *H*-8), 7.38-7.14 (m, 5H, Ar*H*), 6.37-6.32 (m, 1H, *H*-1'), 4.99-4.90 (m, 1H, *CH*(CH_3)$_2$), 4.64-4.60 (m, 1H, *H*-3'), 4.44-4.25 (m, 2H, *H*-5'), 4.20-4.12 (m, 1H, *H*-4'), 4.05 (s, 1.5H, O*CH$_3$*), 4.07 (s, 1.5H, O*CH$_3$*), 3.93-3.83 (m, 1H, *CH*CH_3 L-Ala), 2.86-2.71 (m, 1H, *H*-2'$_a$), 2.44-2.37 (m, 1H, *H*-2'$_b$), 1.32-1.26 (m, 3H, CH*CH$_3$* L-Ala), 1.23-1.18 (m, 6H, CH(*CH$_3$*)$_2$). **^{13}C NMR (125 MHz, CD_3OD) δ_C**: 173.2 (d, $^3J_{C\text{-}P}$ = 4.6 Hz, *C*=O, ester), 172.9 (d, $^3J_{C\text{-}P}$ = 5.4 Hz, *C*=O, ester), 161.58 (*C*-6), 161.56 (*C*-6), 159.46 (*C*-2), 159.43 (*C*-2), 153.2 (*C*-4), 153.1 (*C*-4), 150.55 (d, $^2J_{C\text{-}P}$ = 6.9 Hz, *C*-O, Ph), 150.50 (d, $^2J_{C\text{-}P}$ = 6.8 Hz, *C*-O, Ph), 137.9 (*C*-8), 137.7 (*C*-8), 129.7 (*CH*-Ar), 129.6 (*CH*-Ar), 125.1 (*CH*-Ar), 120.19 (*CH*-Ar), 120.15 (*CH*-Ar), 116.1 (*C*-5), 116.1 (*C*-5), 85.0 (d, $^3J_{C\text{-}P}$ = 7.2 Hz, *C*-4), 84.9 (d, $^3J_{C\text{-}P}$ = 7.3 Hz, *C*-4), 84.3 (*C*-1'), 83.9 (*C*-1'), 71.7 (*C*-3'), 71.4 (*C*-3'), 69.4 (*CH*(CH_3)$_2$), 69.3 (*CH*(CH_3)$_2$), 66.2 (d, $^2J_{C\text{-}P}$ = 5.5 Hz, *C*-5'), 66.0 (d, $^2J_{C\text{-}P}$ = 5.2 Hz, *C*-5'), 53.8 (O*CH$_3$*), 50.4 (*CH*CH_3 L-Ala), 50.3 (*CH*CH_3 L-Ala), 39.3 (*C*-2'), 38.9 (*C*-2'), 21.6 (CH(*CH$_3$*)$_2$), 21.5 (CH(*CH$_3$*)$_2$), 20.8 (d, $^3J_{C\text{-}P}$ = 5.3 Hz, CH*CH$_3$* L-Ala), 20.7 (d, $^3J_{C\text{-}P}$ = 5.2 Hz, CH*CH$_3$* L-Ala). **HPLC**: Reverse phase HPLC eluting with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 minutes, 1 mL/min, λ = 254 nm and 263 nm, showed two peaks with t_R 13.84 min. and t_R 14.05 min. **(ES+) m/z**, found: 551.20 [M+H$^+$], $C_{23}H_{31}N_6O_8P$ required: 550.19 [M].

5.5 References

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Appendix: Publications

Elisa Pileggi, Michaela Serpi, Graciela Andrei, Dominique Schols, Robert Snoeck, and Fabrizio Pertusati. Expedient synthesis and biological evaluation of alkenyl acyclic nucleoside phosphonate prodrugs. *Bioorganic & Medicinal Chemistry*, 2018, 26, 3596-3609. https://doi.org/10.1016/j.bmc.2018.05.034

Elisa Pileggi, Michaela Serpi, Fabrizio Pertusati. Preparation of pyrimidine alkenyl acyclic nucleoside phosphonoamidates. *Current Protocols in Nucleic Acid Chemistry*, 2018, 74(1), e56. https://doi.org/10.1002/cpnc.56

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Expedient synthesis and biological evaluation of alkenyl acyclic nucleoside phosphonate prodrugs

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ABSTRACT

The importance of phosphonoamidate prodrugs (ProTides) of acyclic nucleoside phosphonate (ANPs) is highlighted by the approval of Tenofovir Alafenamide Fumarate for the treatment of HIV and HBV infections. In the present paper we are reporting an expedient, one-pot, two-steps synthesis of allyl phosphonoamidates and diamidates that offers a time saving strategy when compared to literature methods. The use of these substrates in the cross metathesis reactions with alkenyl functionalised thymine and uracil nucleobases is reported. ANPs prodrugs synthesized via this methodology were evaluated for their antiviral activities against DNA and RNA viruses. It is anticipated that the use of 5,6,7,8-tetrahydro-1-napthyl as aryloxy moiety is capable to confer antiviral activity among a series of otherwise inactive uracil ProTides.

1. Introduction

The ProTide approach, pioneered by Chris Mcguigan's group,[1,2] is a powerful technology aimed to optimize intracellular drug delivery and circumvent metabolic bottlenecks in the activation of nucleoside-based antiviral and anticancer drugs. In the last years this technology has displayed a great deal of success in the antiviral field with two compounds in the market: the phosphoramidate Sofosbuvir [3,4] (Sovaldi®) approved in 2013 against HCV infections and the phosphonoamidate tenofovir alafenamide fumarate[5] (TAF, Vemlidy®) approved in 2015 for the treatment of HIV[6,7] and later in 2016 for HBV infections[8,9] (Fig. 1).

Several other ProTides have entered in clinical trials while many others are in preclinical evaluation either as antiviral or anticancer drugs.[2,10,11] Given the tremendous importance of phosphor(n)oamidate prodrugs in the antiviral arena and beyond, after the approval of Sofosbuvir and TAF, the application of the ProTide technology has grown dramatically and it has started to show very promising results in other therapeutic areas as well.[12–14] While there are several efficient procedures to synthesize phosphoroamidate nucleosides, the phosphonoamidate cognate class especially of acyclic nucleoside phosphonates (ANPs) lacks of such plethora of synthetic methodologies.[15]

ANPs play a key role in the treatment of viral infections, and this class of compounds can be regarded as one of the most significant group of drugs in the antiviral field.[16,17] Discovered almost 30 years ago, a great wealth of research has been dedicated to the development of efficient synthetic methodologies that resulted in a great variety of ANPs.[18–22] These new structures offer a potential for the discovery of more effective drugs against a variety of infectious diseases including antiparasitic,[23–29] antimicrobial,[30–33] and antitubercolous[34,35] medicines. Among these synthetic strategies, quite recently, Agrofoglio's group has elaborated a novel, efficient and straightforward synthesis of C5-alkenyl substituted ANPs via olefin cross-metathesis.[36–42] Although structure-activity relationship (SAR) studies on acyclic nucleosides have not clarified their pharmacophore model, the introduction of a rigid structural element such as the double bond has proved to be extremely important for their antiviral activity.[43,44] Precisely, the *trans*-alkene skeleton is able to mimic the three-dimensional geometry of the ribose ring maintaining also an electronic contribution similar to the one provided by the oxygen.[45] There are considerable evidences that the *trans*-alkenyl acyclic nucleotide motif has a strong affinity with recombinant human thymidylate kinase (hTMPK) active site, responsible for the nucleotide phosphorylation and consequently correlated to its antiviral activity.[41]Interestingly, Agrofoglio's group employed the olefin cross-metathesis methodology also for the direct synthesis of a vast array of unsaturated ANPs analogues including *bis*-POM, *bis*-POC, and alkoxyesters prodrugs.[36,38–41,46,47] Although adopting a different procedure, our group extended the range of prodrugs of (*E*)-but-2-enyl-pyrimidine, by synthesising their ProTide and bisamidate derivatives.[48] In this study we showed that the ProTide technology was able to broaden the spectrum of antiviral activity when compared to other

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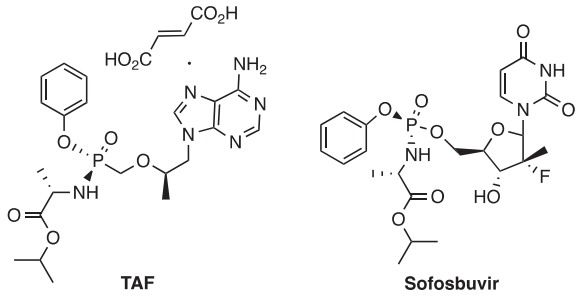


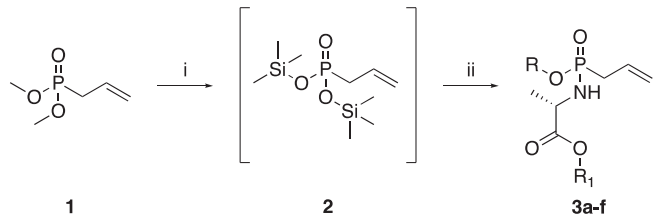
Figure 1. Structures of Sofosbuvir and TAF.

phosphate prodrug approaches. However, we discovered that this methodology suffers from the limitation that only linear olefin must be employed, as with trisubstituted alkenyl derivatives we observed only formation of traces of the desired ProTides. This finding prompted us to investigate the possibility of using the cross-metathesis for the direct synthesis of unsaturated branched ANP phosphonoamidates. At the time we started this investigation, no application of such procedure for the synthesis of ProTides was yet reported. However, during the preparation of this manuscript, a paper reporting the use of the cross metathesis for the synthesis of ProTide derivatives of linear (*E*)-but-2-enyl nucleoside scaffold, was published.[49] The prodrugs described in this work belong to the same family of compounds previously reported by us,[48] and indeed their antiviral profile was in agreement with our published results. In the present article, we would like to report an effective and improved methodology for the synthesis of allyl phosphonoamidate and their further application in olefin cross-metathesis for the synthesis of ANP ProTides. We also anticipate that our two-steps, one-pot methodology can also be applied to the synthesis of symmetrical allyl phosphonodiamidates. Compared with the recently published procedure,[49] our synthetic strategy presents some advantages which we believe, merit consideration.

2. Results and discussion

2.1. Chemistry

Our research began with the synthesis of the aryloxy allylphosphonoamidate synthon **3a**, for which the only literature procedure available is a long and tedious multistep sequence.[50,51] Based on our experience in the application of Holy's one-pot procedure for the direct synthesis of phosphonodiamidates,[52] we envisaged that this protocol could be used to get access to the desired synthon starting from the commercially available dimethyl allylphosphonate **1** (Scheme 1). This methodology was already adapted in our laboratory for the synthesis of adefovir and tenofovir phosphonoamidate prodrugs[53] and more recently for the preparation of (*E*)-but-2-enyl pyrimidine ProTides.[48] Briefly, commercial dimethyl allylphosphonate **1** was converted into the corresponding silyl ester **2**, by reaction with an excess of bromotrimethylsilane (5.0 equivalents). Due to the hydrolytically instability of this ester, **2** was not isolated but immediately dissolved in a mixture



Scheme 1. Synthesis of *O*-Aryl-(L-alanine-ester)-allylphosphonate. *Reagents and conditions*: *i.* TMSBr (5.0 equiv), 2,6-Lutidine (4.0 equiv), CH_3CN, rt, 16 h; *ii.* Amino acid ester hydrochloride (1.0 equiv), aryl-alcohol (6.0 equiv), Et_3N (15.0 equiv), aldrithiol-2 (6.0 equiv), PPh_3 (6.0 equiv), pyridine, 50 °C, 16 h.

Table 1
Substitution pattern and isolated yields of allyl phosphonoamidates **3a–f**.

Entry	Cpds	Aryl	Amino acid	Ester	Yield[a]
1	**3a**	1-Naph	L-Ala	*i*-Pr	79%
2	**3b**	1-Naph	L-Ala	Bz	78%
3	**3c**	Ph	L-Ala	*i*-Pr	65%
4	**3d**	Ph	L-Ala	Bz	42%
5	**3e**	TH-1-Naph	L-Ala	*i*-Pr	55%
6	**3f**	TH-1-Naph	L-Ala	Bz	55%

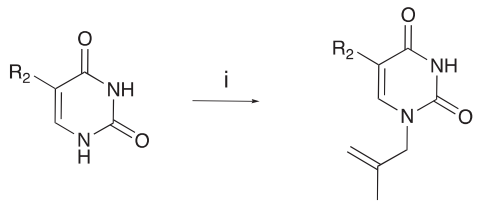
[a] Yield are determined for isolated, purified compounds; see experimental part for details.

of pyridine/Et_3N and treated with the L-alanine isopropyl ester hydrochloride (1.0 equivalents), an excess of 1-naphthol (6.0 equivalents), and a premade solution of PPh_3 (6.0 equivalents) and aldrithiol-2 (6.0 equivalents) in pyridine. After 16 h, the crude mixture did not show the presence of either the desired product or phosphonodiamidate compound (which, based on our experience, is almost invariably formed). We attributed this lack of reactivity to the decomposition of the disilyl ester **2** caused by the release of hydrobromic acid, generated by the hydrolysis of the excess of TMSBr used. Pleasingly, when we attempted the reaction in the presence of 2,6-lutidine (4.0 equivalents) as acid scavenger, the formation of the desired product **3a** was observed (^{31}P NMR and LC-MS analysis of the crude mixture). **3a** was isolated by flash chromatography in excellent yield (79%) (Table 1, Entry 1). Quite surprisingly, no evidence of side reactions[48] (bromination of the double bond and formation of the phosphonodiamidate) have been observed.

With the above methodology, we prepared six different allyl phosphonate analogues **3a-f** in which a variety of aryloxy groups were introduced in combination with two different amino acid esters (L-alanine isopropyl or benzyl esters). From Table 1 it can be appreciated that our method worked well with aryl alcohols with different steric requirements. In particular, we were able to prepare the allyl phosphonoamidates bearing the 5,6,7,8-tetrahydro-1-napthol **3e** and **3f** (Entries 5 and 6, Table 1), which have shown to impart remarkable antiviral activities in compounds of previous series.[48,53]

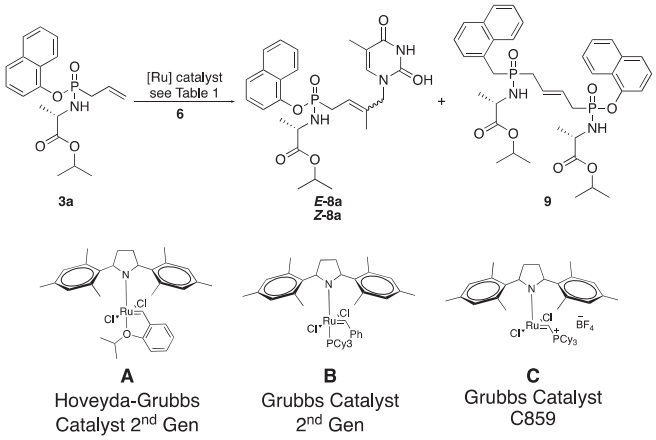
This procedure is short and efficient, representing an improvement of the literature method, which accounts for a 29% overall yield in four steps.[49]

With these allyl phosphonoamidates in hand we began the synthesis of (*E*)-methylbut-2-enyl pyrimidine **6** and **7**, selected as the other partner for the cross-metathesis reaction. These nucleosides and their *bis*-POM prodrugs were originally prepared by Agrofoglio and colleagues,[38] which found the latest to have moderate activities against feline herpes virus (FHV) and feline corona virus (FCoV). Considering that ProTides of alkenyl pyrimidine with "linear" (*E*)-but-2-enyl double bond have shown improved antiviral activities and a broad antiviral spectrum when compared to the corresponding *bis*-POM derivatives, we were now interested in investigating whether ProTide of branched alkenyl pyrimidine might have the same effect. We therefore synthesised a thymine and uracil derivative **6** and **7** as reported in Scheme 2.



Scheme 2. Synthesis of N^1-2′methylallylpyrimidine. *Reagents and conditions*: *i.* 3-Bromo-2-methylpropene (2.0 equiv), BSA (2.5 equivalents), NaI (1.1 equiv), TMSCl (1 equiv), CH_3CN, reflux temperature, 16 h.

Table 2
Screened conditions for CM.[a]



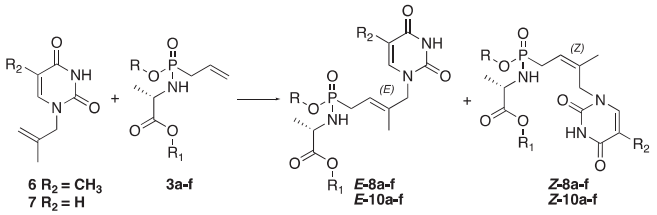
Entry	cat	E-8a/9	E-8a/Z-8a	8a (%)
1[b]	A	1:0.4	1:0.2	24%
2[b]	B	1:1.4	1:0.1	11%
3[b]	C	1:9	1:0.7	3%
4[c]	A	1:0.3	1:0.2	26%
5[c,d]	A	1:0.3	1:0.2	26%

[a] **Reaction conditions:** allyl phosphonoamidate **3a** (1.0 equiv), olefin **6** (2.0 equiv) in CH_2Cl_2 at reflux temperature. Catalyst (5 mol%) added at t = 0, 2, 4 h. Ratio Het/Homo and *E/Z* determined by HPLC.
[b] Reactions sonicated for 24 h.
[c] Reactions sonicated for 36 h.
[d] further addition of the catalyst (5 mol%) after 24 h.

With both alkenyl derivatives in hand we were in the position to investigate the cross-metathesis conditions between the aryloxy allyl-phosphonoamidate synthon **3a** and the olefin **6** as model reaction. First we employed the same CM conditions developed and used by Agrofoglio for the synthesis of the corresponding *bis*-POM alkenyl derivatives[38]. As expected we obtained a mixture of *E/Z* isomers of which the desired compound ***E*-8a** was afforded in 24% yield (Entry 1, Table 2). Both ***E*-8a** and ***Z*-8a** isomers were isolated by preparative reverse phase-HPLC and their configurations were confirmed by NOESY experiments. The homodimer **9** was formed along with the *E/Z* derivatives. Any attempt to improve the reaction outcome using different catalysts (Hoveyda-Grubbs 2nd generation catalyst (**A**), Grubbs 2nd generation catalyst (**B**) and Grubbs catalyst C859 (**C**) failed providing **8a** in similar or lower yield and almost identical *E/Z* ratio (Entries 2–3, Table 2). Since catalyst **A** resulted the best in terms of product/homodimer ratio further screening was conducted keeping **A** as catalyst. Prolonged reaction time (Entry 4, Table 2) resulted in a slightly increased yield that however, was not further improved with addition of more catalyst (Entry 5, Table 2,). These conditions are different from those reported by Agrofoglio in his recent paper,[49] where (*E*)-but-2-enyl pyrimidine ProTides were formed via cross metathesis only when water was used as solvent.

Using these conditions, we prepared different aryloxy phosphonoamidates of both thymine and uracil derivatives. The desired compounds ***E*-8a–f** and ***E*-10a–f** were isolated in moderate yields (Scheme 3, Table 3). In few cases *Z*-isomers (***Z*-8a**, ***Z*-8e**, ***Z*-8f**, ***Z*-10e**) were also isolated in 1 to 7% yield (Scheme 3).

Pleased by the outcome of the above procedure, and to expand the versatility of this methodology, we decided to use the same reaction conditions to prepare the symmetrical phosphonodiamidate **12**. Briefly, the desired *bis*-amidate intermediate **11** was obtained in 52% yield by treating the allyl phosphonate **1** with an excess of TMSBr (in presence of 4.0 equivalents of lutidine) and the resulting silyl diester reacted with an excess (5.0 equivalents) of L-alanine isopropyl hydrochloride (Scheme 4). Compound **11** was then subjected to olefin cross-metathesis reaction with compound **7** under the conditions reported in Scheme 4. Phosphonodiamidate **12** was obtained as a mixture of the *E* and *Z* isomers. The *E*-isomer was isolated in 2% yield, after purification by preparative reverse phase-HPLC.



Scheme 3. ProTide synthesis via cross-metathesis. *Reagents and conditions*: allyl phosphonoamidates **3a–f** (1.0 equiv), olefin **6** or **7** (2.0 equiv) in CH_2Cl_2 at reflux temperature; Hoveyda-Grubbs 2nd generation catalyst (5 mol%) added after 0, 2 and 4 h; reactions sonicated for 24 h.

Since ruthenium catalyst was used during the synthesis, we were interested in measuring its residual amount in the final sample. ICP-MS experiment on compound ***E*-10e** showed ruthenium content of

Table 3
Substitution pattern and isolated yields of phosphonoamidates ***E*-8a–f** and ***E*-10a–f.**

Cpds	R	R_1	R_2	Yield[a]
***E*-8a**	1-Naph	i-Pr	CH_3	36%
***E*-8b**	1-Naph	Bz	CH_3	13%
***E*-8c**	Ph	i-Pr	CH_3	10%
***E*-8d**	Ph	Bz	CH_3	23%
***E*-8e**	TH-1-Naph	i-Pr	CH_3	26%
***E*-8f**	TH-1-Naph	Bz	CH_3	14%
***E*-10a**	1-Naph	i-Pr	H	14%
***E*-10b**	1-Naph	Bz	H	5%
***E*-10c**	Ph	i-Pr	H	10%
***E*-10d**	Ph	Bz	H	18%
***E*-10e**	TH-1-Naph	i-Pr	H	11%
***E*-10f**	TH-1-Naph	Bz	H	5%

[a] Yields were determined for isolated, purified compounds; see experimental part for details.

0.116 mg/g. Further purification[54] will have to be considered if this methodology will be used for preparing compounds progressing to preclinical and clinical evaluation in order to comply the FDA recommended limits for residual metal catalyst in a drug.[55]

2.2. Antiviral activity and serum stability

All the ProTide derivatives synthesised were evaluated against a panel of DNA and RNA viruses as previously described.[48] None of the compounds were active against herpes simplex virus-1 (KOS) (HVS-1), herpes simplex virus-2 (G) (HVS-2), thymidine kinase deficient herpes simplex virus-1 (KOS Acyclovir-resistant strain) (TK^- HSV-1), vaccinia virus (VV), adenovirus-2 (AV-2), human coronavirus (HCoV-229E) in HEL cells, parainfluenza-3 virus (HPIV-3), reovirus-1 (REO-1), vesicular stomatitis virus (VSV), respiratory syncytial virus (RSV) in HeLa cells, influenza A/H1N1, influenza A/H3N2 and influenza B in MDCK cells.

As shown in Table 4, thymine derivatives ***E*-8a–f** showed weak antiviral activity against varicella-zoster virus (VZV TK^+ and TK^-) and human cytomegalovirus (HCMV AD-169 strain and Davis strain) with EC_{50} ranging from 20 to 76 μM, whereas uracil derivatives ***E*-10a-c** were mostly inactive against these viruses with the exception of ***E*-10a** (EC_{50} = 20 μM VZV TK^+) and ***E*-10b** (EC_{50} = 58 μM VZV TK^-). Interestingly uracil derivatives ***E*-10e-f**, bearing the 5,6,7,8-tetrahydro-1-napthol as aryl moiety, resulted slightly active against VZV both TK^+ and TK^- strains, confirming once again the biological potential of this promoiety. No specific information about the 5,6,7,8-tetrahydro-1-naphtol LD_{50} is reported in the literature as for phenol and 1-napthol. However, in previous studies [48,53] we have shown that in an *in vitro* assay the CC_{50} values of ANP ProTides bearing the 5,6,7,8 tehydro-1-napthyl moiety have a comparable CC_{50} values to those bearing phenol and 1-napthol. This is also observed in the presented studies. Remarkably, all the *Z* isomers isolated (***Z*-8a**,**e**,**f** and ***Z*-10e**) showed to some extent antiviral activity against both AD-169 and Davis HCMV strains. Furthermore, compound ***Z*-8e** was found weakly active against Sindbis Virus (SINV), coxsackie virus B4, Punta Toro virus (PTV) and yellow fever virus (YFV) in Vero cells with EC_{50} values in the range of 20–58 μM.

None of the compounds showed significant cytotoxicity. Being able to inhibit VZV, ProTides of allylphosphonate pyrimidine showed a broader antiviral activity than the corresponding *bis*-POM prodrugs, previously reported by Agrofoglio.[41] On the contrary linear alkenyl derivatives showing higher EC_{50} against VZV perform better than those branched, suggesting that a more substituted double bond is detrimental for the antiviral activity.

The metabolic activation of phosphonoamidates follows the same two-enzymatic steps involved in the activation of the phosphoroamidates.[11] Although the use of 5,6,7,8-tetrahydro-1-naphthol as aryloxy group in the ProTides is quite recent we have shown its metabolic activation by carboxypeptidase Y in previous studies.[53] To prove the stability of this class of compound we have performed stability assays of compound ***E*-8e**, in rat and human sera, which indicate a suitable pharmacokinetic profile of the tested phosphonoamidate with a half-life higher than 12 h (Fig. 2).

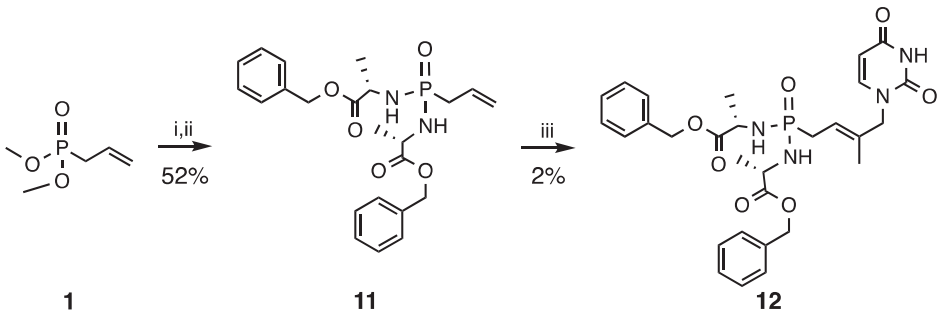
3. Conclusion

In conclusion, we have successfully reported the one pot-two steps synthesis of a family of allyl phosphonoamidates. Our methodology is an important improvement of a recently reported strategy[49] that allows the synthesis of these substrate in a shorter synthetic sequence and with an overall higher yield. We also extended this protocol to the synthesis of hitherto unknown allyl phosphonodiamidate. We also proved that both synthons are capable to undergo alkene cross-metathesis with alkenyl functionalized uracil and thymine nucleobases although the yields need to be further optimized, especially in the case of phosphonodiamidates. These phosphonoamidate prodrugs were evaluated for their biological activity against a panel of DNA and RNA viruses. None of the compounds prepared, showed significant cytotoxicity. ProTides of allylphosphonate pyrimidine showed a broader antiviral activity than the corresponding *bis*-POM prodrugs against VZV infected cells. We have also demonstrated, once again, that the introduction of 5,6,7,8-tetrahydro-1-naphthyl moiety into the ProTide scaffold is capable to increase the antiviral activity of the prodrug. Finally, not only the *E*-isomers showed some biological activity, but also all the *Z* isomers isolated (***Z*-8a**,**e**,**f** and ***Z*-10e**) showed to some extent antiviral activity against both AD-169 and Davis HCMV strains. Further studies directed to the optimization of the cross metathesis procedure especially for the allyl phosphonoamidate, are currently in progress in our laboratory.

4. Experimental section

4.1. Chemistry

All solvents used were anhydrous and used as supplied by Sigma-Aldrich. All commercially available reagents were supplied by either



Scheme 4. Synthesis of symmetrical allyl phosphonodiamidate 12. *Reagents and conditions: i.* TMSBr (5.0 equiv), 2,6-Lutidine (4.0 equiv), CH_3CN, rt, 16 h; *ii.* benzyloxy-L-alanine hydrochloride (5.0 equiv), Et_3N (15.0 equiv), aldrithiol-2 (6.0 equivalents), PPh_3 (6.0 equiv), pyridine, 50 °C, 16 h; iii. N^1-2′-methylallyl-uracil **7** (2 equiv), Hoveyda-Grubbs 2nd generation catalyst (15 mol%), CH_2Cl_2, sonicated for 24 h, at reflux temperature.

Table 4
Antiviral activity of alkenyl ANP ProTides.

Cpds	EC_{50} (HEL cells) (μM)				MCC (HEL cells) (μM)	EC_{50}(Vero cells) (μM)				MCC (Vero cells)(μM)
	VZV		HCMV			SINV	Coxsackie Virus B4	PTV	YFV	
	TK^+	TK^-	AD-169	Davis						
***E*-8a**	44.72	>100	>100	>100	>100	>100	>100	>100	>100	≥20
***E*-8b**	34.2	55.27	>100	>100	>100	>100	>100	>100	>100	>100
***E*-8c**	76.47	>100	>100	>100	>100	>100	>100	>100	>100	≥20
***E*-8d**	55.7	46.66	>100	>100	>100	>100	>100	>100	>100	>100
***E*-8e**	58.48	53.48	>100	>20	>100	>100	>100	>100	>100	≥20
***E*-8f**	50.17	47.19	>100	>100	>100	>100	>100	>100	>100	≥100
***E*-10a**	20	>100	>100	>100	>100	>100	>100	>100	>100	>100
***E*-10b**	100	58.48	>100	>100	>100	>100	>100	>100	>100	>100
***E*-10c**	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
***E*-10d**	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
***E*-10e**	29.91	71.52	>100	>100	>100	>100	>100	>100	>100	>100
***E*-10f**	55.7	52.53	>100	>100	>100	>100	>100	>100	>100	≥100
***Z*-8a**	39.86	41.57	>20	44.72	100	>100	>100	>100	>100	≥20
***Z*-8e**	>20	>20	44.72	>20	100	45	58	45	58	>100
***Z*-8f**	17.03	65.1	76.47	76.47	>100	>100	>100	>100	>100	≥20
***Z*-10e**	58.48	100	>20	54.69	100	>100	>100	>100	>100	>100
Acyclovir	3.55	14.87	–	–	>440	–	–	–	–	–
Brivudin	0.012	0.57	–	–	>300	–	–	–	–	–
Ganciclovir	–	–	11.43	2.29	–	–	–	–	–	–
Cidofovir	–	–	1.24	0.76	–			–	–	–
DS-10.000	–	–	–	–	–	20	7.6	7.6	34	>100
Ribavirin	–	–	–	–	–	>250	>250	126	>250	>250
Mycophenolic acid	–	–	–	–	–	4	>100	6.1	4	>100

EC_{50}: 50% effective concentration or concentration required inhibiting viral induced cytopathic effect (HCMV, SINV, coxsackie virus B4, PTV and YFV) or plaque formation (VZV) by 50%.
MCC: minimal cytotoxic concentration that causes a microscopically alteration of cell morphology.

Sigma-Aldrich or Fisher and used without further purification. All nucleosides and solid reagents were dried for several hours under high vacuum prior to use. For analytical thin-layer chromatography (TLC), precoated aluminium-backed plates (60F-54, 0.2 mm thickness; supplied by E. Merck AG, Darmstadt, Germany) were used and developed by an ascending elution method. For preparative thin-layer chromatography (prep TLC), preparative TLC plates (20 cm × 20 cm, 500–2000 μm) were purchased from Merck. After solvent evaporation, compounds were detected by quenching of the fluorescence, at 254 nm upon irradiation with a UV lamp. Column chromatography purifications were carried out by means of automatic Biotage Isolera One. Fractions containing the product were identified by TLC and pooled,

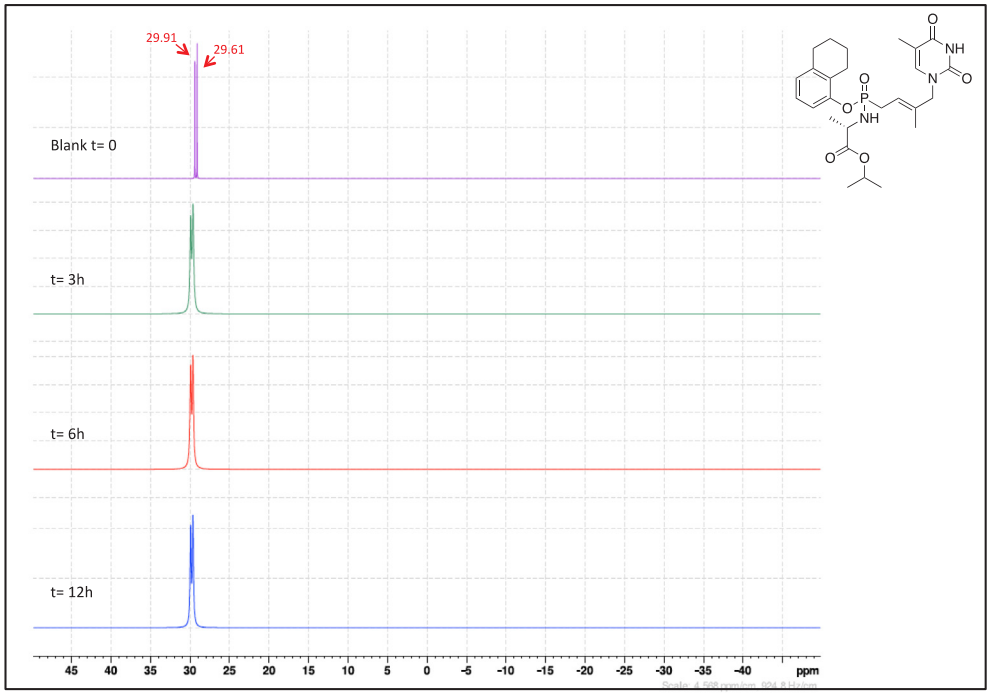


Figure 2. Stability assay of **E-8e** in Human Serum at 37 °C monitored by ^{31}P NMR (202 MHz, DMSO-d_6/H_2O).

and the solvent was removed in vacuo. ^{1}H, ^{31}P and ^{13}C NMR spectra were recorded in a Bruker Avance 500 spectrometer at 500 MHz, 202 MHz and 125 MHz respectively and auto-calibrated to the deuterated solvent reference peak in case of ^{1}H and ^{13}C NMR and 85% H_3PO_4 for ^{31}P NMR experiments. All ^{31}P and ^{13}C NMR spectra were proton-decoupled. Chemical shifts are given in parts per million (ppm) and coupling constants (J) are measured in Hertz (Hz). The following abbreviations are used in the assignment of NMR signals: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), bs (broad singlet), dd (doublet of doublet), ddd (doublet of doublet of doublet), dt (doublet of triplet). The assignment of the signals in ^{1}H NMR and ^{13}C NMR was done based on the analysis of coupling constants and additional two-dimensional experiments (COSY, HSQC). Analytical High-Performance Liquid Chromatography (HPLC) analysis was performed using both Spectra System SCM (with X-select-C18, 5 mm, 4.8 × 150 mm column) and Varian Prostar system (LCWorkstation-Varian Prostar 335 LC detector). Preparative HPLC was performed with Varian Prostar (with pursuit XRs C18 150 × 21.2 mm column). Low and high-resolution mass spectrometry was performed on a Bruker Daltonics MicroTof-LC system (atmospheric pressure ionization, electron spray mass spectroscopy) in positive mode.

The ≥95% purity of the final compounds (***E*-8a–f**, ***E*-10a–f**, ***Z*-8a,e,f** and ***Z*-10e**) was confirmed by HPLC analysis.

4.1.1. General procedure A for the preparation of O-Aryl-(L-alanine-ester)-allylphosphonate (3a–f)

In a round bottom flask, under an argon atmosphere, 2,6-Lutidine (4 eq) and trimethylsilyl bromide (TMSBr, 5 eq) were added to a solution of dimethyl allylphosphonate (1 eq) in anhydrous acetonitrile (8 ml/mmol of allylphosphonate). The mixture was stirred 16 h at room temperature and then the volatiles evaporated without any contact with air. Then the flask was charged with dry aminoacid ester hydrochloride (1 eq), dry aryl-alcohol (6 eq), dry triethylamine (15 eq) and dry pyridine (3 ml/mmol of allylphosphonate) and heated to 50 °C to obtain a homogenous solution. To this mixture was then added a solution of aldrithiol-2 (6 eq) and triphenylphosphine (6 eq) in dry pyridine (3 ml/mmol of allylphosphonate) under argon atmosphere. The resulting mixture was stirred at 50 °C for 16 h. After evaporating all the volatiles, the residue was purified by Biotage Isolera One.

4.1.1.1. O-(1-Naphthyl)-(isopropyloxy-L-alanine)-allylphosphonate (3a).

Prepared according to the standard procedure **A** for the synthesis of allylphosphonoamidate using dimethyl allylphosphonate (500 mg, 3.33 mmol), 2,6-Lutidine (1.55 ml, 13.32 mmol), TMSBr (2.20 ml, 16.65 mmol) in anhydrous acetonitrile (25 ml). For the second step we used dry isopropyloxy-L-alanine hydrochloride (558 mg, 3.33 mmol), dry 1-Naphthol (2.88 g, 19.98 mmol), dry triethylamine (6.9 ml, 49.96 mmol) in dry pyridine (10 ml) and a solution of aldrithiol-2 (4.40 g, 19.98 mmol) and triphenylphosphine (5.24 g, 19.98 mmol) in dry pyridine (10 ml). After evaporation, the mixture was purified by Biotage Isolera One (100 g SNAP cartridge ULTRA, 100 ml/min, gradient eluent system EtOAc/Hexane 10% 1CV, 10–100% 12CV, 100% 2CV), to afford the title compound as a yellow oil (940 mg, 79%). R_f = 0.58 (EtOAc/Hexane – 4:6). **^{31}P NMR (202 MHz, CD_3OD) δ_P**: 30.01, 29.43. **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 8.19 (d, J = 7.2 Hz, 1H, Ar*H*), 7.89 (d, J = 7.9 Hz 1H, Ar*H*), 7.71–7.69 (m, 1H, Ar*H*), 7.58–7.40 (m, 4H, Ar*H*), 6.07–5.91 (m, 1H, *CH*═), 5.38–5.28 (m, 2H, CH_2═), 5.95–4.82 (m, 1H, *CH*$(CH_3)_2$), 3.99–3.97 (m, 1H, *CH*CH_3 l-Ala), 3.03–2.93 (m, 2H, CH_2P), 1.25 (d, J = 7.8 Hz, 1.5H, CHCH_3 l-Ala), 1.21–1.10 (m, 7.5H, CHCH_3 l-Ala, CH$(CH_3)_2$). **^{13}C NMR (125 MHz, CD_3OD) δ_C**: 173.5 (d, $^{3}J_{C\text{-}P}$ = 4.2 Hz, *C*═O, ester), 173.1 (d, $^{3}J_{C\text{-}P}$ = 4.2 Hz, *C*═O, ester), 146.4 (d, $^{2}J_{C\text{-}P}$ = 8.5 Hz, *C*—O, Ph), 146.3 (d, $^{2}J_{C\text{-}P}$ = 8.5 Hz, *C*—O, Ph), 134.9 (*C*-Ar), 127.4 $^{2}J_{C\text{-}P}$ = 9.3 Hz, *CH*═), 123.3 ($^{2}J_{C\text{-}P}$ = 10.9 Hz, *CH*═), 126.9 (d, $^{3}J_{C\text{-}P}$ = 5.6 Hz *C*-Ar), 126.8 (d, $^{3}J_{C\text{-}P}$ = 4.9 Hz *C*-Ar), 126.3 (*CH*-Ar), 125.95 (*CH*-Ar), 125.90 (*CH*-Ar), 125.1 (*CH*-Ar), 125.0 (*CH*-Ar), 124.3 (*CH*-Ar), 124.2 (*CH*-Ar), 121.6 (*CH*-Ar), 121.4 (*CH*-Ar), 119.7 (d, $^{3}J_{C\text{-}P}$ = 14.2 Hz CH_2═), 119.6 (d, $^{3}J_{C\text{-}P}$ = 13.8 Hz CH_2═), 115.4 (d, $^{3}J_{C\text{-}P}$ = 4.1 Hz *CH*-Ar), 115.2 (d, $^{3}J_{C\text{-}P}$ = 3.4 Hz *CH*-Ar), 68.6 (*CH*$(CH_3)_2$), 68.5 (*CH*$(CH_3)_2$), 49.6 (*CH*CH_3 l-Ala), 49.4 (*CH*CH_3 l-Ala), 33.7 (d, $^{1}J_{C\text{-}P}$ = 129.0 Hz CH_2P), 33.5 (d, $^{1}J_{C\text{-}P}$ = 129.6 Hz CH_2P), 20.5 (CH$(CH_3)_2$), 20.4 (CH$(CH_3)_2$), 20.3 (CH$(CH_3)_2$), 19.7 (d, $^{3}J_{C\text{-}P}$ = 5.4 Hz, CHCH_3 l-Ala), 19.1 (d, $^{3}J_{C\text{-}P}$ = 5.4 Hz, CHCH_3 l-Ala).

4.1.1.2. O-(1-Naphthyl)-(benzyloxy-L-alanine)-allylphosphonate (3b).

Prepared according to the standard procedure **A** for the synthesis of allylphosphonoamidate using dimethyl allylphosphonate (500 mg, 3.33 mmol), 2,6-Lutidine (1.55 ml, 13.32 mmol), TMSBr (2.20 ml, 16.65 mmol) in anhydrous acetonitrile (25 ml). For the second step we used dry benzyloxy-L-alanine hydrochloride (718 mg, 3.33 mmol), dry 1-Naphthol (2.88 g, 19.98 mmol), dry triethylamine (6.9 ml, 49.96 mmol) in dry pyridine (10 ml) and a solution of aldrithiol-2 (4.40 g, 19.98 mmol) and triphenylphosphine (5.24 g, 19.98 mmol) in dry pyridine (10 ml). After evaporation, the mixture was purified by Biotage Isolera One (100 g SNAP cartridge ULTRA, 100 ml/min, gradient eluent system EtOAc/Hexane 10% 1CV, 10–100% 12CV, 100% 2CV), to afford the title compound as a yellow oil (1.1 g, 78%). R_f = 0.58 (EtOAc/Hexane – 4:6). **^{31}P NMR (202 MHz, CD_3OD) δ_P**: 30.09, 29.48. **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 8.17 (s, 1H, Ar*H*), 7.86 (s, 1H, Ar*H*), 7.69–7.65 (m, 1H, Ar*H*), 7.52–7.22 (m, 9H, Ar*H*), 5.99–5.89 (m, 1H, *CH*═), 5.30–5.24 (m, 2H, CH_2═), 5.09, 5.03 (ABq, J_{AB} = 12.1 Hz, 1H, CH_2Ph), 4.97, 4.93 (ABq, J_{AB} = 12.1 Hz, 1H, CH_2Ph), 4.09–4.07 (m, 1H, *CH*CH_3 l-Ala), 2.95–2.91 (m, 2H, CH_2P), 1.26 (d, J = 6.8 Hz, 1.5H, CHCH_3 l-Ala), 1.16 (d, J = 6.8 Hz, 1.5H, CHCH_3 l-Ala). **^{13}C NMR (125 MHz, CD_3OD) δ_C**: 173.7 (d, $^{3}J_{C\text{-}P}$ = 3.9 Hz, *C*═O, ester), 173.2 (d, $^{3}J_{C\text{-}P}$ = 4.0 Hz, *C*═O, ester), 146.4 (d, $^{2}J_{C\text{-}P}$ = 9.7 Hz, *C*—O, Ph), 146.3 (d, $^{2}J_{C\text{-}P}$ = 10.0 Hz, *C*—O, Ph), 135.8 (*C*-Ar), 135.7 (*C*-Ar), 134.9 (*C*-Ar), 128.17 (*CH*-Ar), 128.12 (*CH*-Ar), 127.9 (*CH*-Ar), 127.8 (*CH*-Ar), 127.48 (*CH*-Ar), 127.42 (*CH*-Ar), 127.3 ($^{2}J_{C\text{-}P}$ = 11.3 Hz, *CH*═), 127.2 ($^{2}J_{C\text{-}P}$ = 11.0 Hz, *CH*═), 126.8 (d, $^{3}J_{C\text{-}P}$ = 5.0 Hz *C*-Ar), 126.7 (d, $^{3}J_{C\text{-}P}$ = 5.3 Hz *C*-Ar), 126.3 (*CH*-Ar), 125.98 (*CH*-Ar), 125.93 (*CH*-Ar), 125.18 (*CH*-Ar), 125.10 (*CH*-Ar), 124.3 (*CH*-Ar), 124.2 (*CH*-Ar), 121.6 (*CH*-Ar), 121.4 (*CH*-Ar), 119.7 (d, $^{3}J_{C\text{-}P}$ = 15.2 Hz CH_2═), 119.6 (d, $^{3}J_{C\text{-}P}$ = 14.9 Hz CH_2═), 115.4 (d, $^{3}J_{C\text{-}P}$ = 3.9 Hz *CH*-Ar), 115.2 (d, $^{3}J_{C\text{-}P}$ = 3.9 Hz *CH*-Ar), 66.5 (CH_2Ph), 66.3 (CH_2Ph), 49.6 (*CH*CH_3 l-Ala), 49.4 (*CH*CH_3 l-Ala), 33.7 (d, $^{1}J_{C\text{-}P}$ = 129.2 Hz CH_2P), 33.5 (d, $^{1}J_{C\text{-}P}$ = 129.7 Hz CH_2P), 19.6 (d, $^{3}J_{C\text{-}P}$ = 5.3 Hz, CHCH_3 l-Ala), 19.0 (d, $^{3}J_{C\text{-}P}$ = 5.8 Hz, CHCH_3 l-Ala).

4.1.1.3. O-Phenyl-(isopropyloxy-L-alanine)-allylphosphonate (3c).

Prepared according to the standard procedure **A** for the synthesis of allylphosphonoamidate using dimethyl allylphosphonate (500 mg, 3.33 mmol), 2,6-Lutidine (1.55 ml, 13.32 mmol), TMSBr (2.20 ml, 16.65 mmol) in anhydrous acetonitrile (25 ml). For the second step we used dry isopropyloxy-L-alanine hydrochloride (558.3 mg, 3.33 mmol), dry phenol (1.88 g, 19.98 mmol), dry triethylamine (6.9 ml, 49.96 mmol) in dry pyridine (10 ml) and a solution of aldrithiol-2 (4.40 g, 19.98 mmol) and triphenylphosphine (5.24 g, 19.98 mmol) in dry pyridine (10 ml). After evaporation, the mixture was purified by Biotage Isolera One (100 g SNAP cartridge ULTRA, 100 ml/min, gradient eluent system EtOAc/Hexane 10% 1CV, 10–100% 12CV, 100% 2CV), to afford the title compound as a yellow oil (670 mg, 65%). R_f = 0.37 (EtOAc/Hexane – 6:4). **^{31}P NMR (202 MHz, $CDCl_3$) δ_P**: 26.77, 26.35. **^{1}H NMR (500 MHz, $CDCl_3$) δ_H**: 7.32–7.28 (m, 2H, Ar*H*), 7.22–7.20 (m, 2H, Ar*H*), 7.14–7.13 (m, 1H, Ar*H*), 5.95–5.82 (m, 1H, *CH*═), 5.32–5.25 (m, 2H, CH_2═), 5.00–4.94 (m, 1H, *CH*$(CH_3)_2$), 4.14–3.96 (m, 1H, *CH*CH_3 l-Ala), 3.51 (dd, $^{2}J_{H\text{-}P}$, $^{3}J_{NH,CH}$ = 10.3 Hz, 0.5H, *NH* l-Ala), 3.41 (dd, $^{2}J_{H\text{-}P}$, $^{3}J_{NH,CH}$ = 10.7 Hz, 0.5H, *NH* l-Ala), 2.81–2.72 (m, 2H, CH_2P), 1.29 (d, J = 7.2 Hz, 1.5H, CHCH_3 l-Ala), 1.23–1.20 (m, 7.5H, CHCH_3 l-Ala, CH$(CH_3)_2$). **^{13}C NMR (125 MHz, $CDCl_3$) δ_C**: 173.5 (d, $^{3}J_{C\text{-}P}$ = 4.7 Hz, *C*═O, ester), 173.1 (d, $^{3}J_{C\text{-}P}$ = 4.7 Hz, *C*═O, ester), 150.6 (d, $^{2}J_{C\text{-}P}$ = 9.1 Hz, *C*—O, Ph), 150.5

(d, $^2J_{C\text{-}P}$ = 9.4 Hz, *C*—O, Ph), 129.4 (*CH*-Ar), 129.3 (*CH*-Ar), 127.5 ($^2J_{C\text{-}P}$ = 11.3 Hz, *CH*═), 127.4 ($^2J_{C\text{-}P}$ = 11.3 Hz, *CH*═), 124.6 (*CH*-Ar), 124.5 (*CH*-Ar), 120.8 (d, $^3J_{C\text{-}P}$ = 4.0 Hz *CH*-Ar), 120.6 (d, $^3J_{C\text{-}P}$ = 4.0 Hz *CH*-Ar), 119.6 (d, $^3J_{C\text{-}P}$ = 14.6 Hz CH_2═), 119.6 (d, $^3J_{C\text{-}P}$ = 14.6 Hz CH_2═), 68.59 (*CH*$(CH_3)_2$), 68.57 (*CH*$(CH_3)_2$), 49.6 (*CH*CH_3 l-Ala), 49.7 (*CH*CH_3 l-Ala), 33.8 (d, $^1J_{C\text{-}P}$ = 129.3 Hz CH_2P), 33.6 (d, $^1J_{C\text{-}P}$ = 129.7 Hz CH_2P), 20.86 (CH(CH_3)$_2$), 20.82 (CH(CH_3)$_2$), 20.81 (CH(CH_3)$_2$), 20.75 (CH(CH_3)$_2$), 20.0 (d, $^3J_{C\text{-}P}$ = 5.3 Hz, CHCH_3 l-Ala), 19.5 (d, $^3J_{C\text{-}P}$ = 5.1 Hz, CHCH_3 l-Ala).

*4.1.1.4. O-Phenyl-(benzyloxy-L-alanine)-allylphosphonate (**3d**)*. Prepared according to the standard procedure **A** for the synthesis of allylphosphonoamidate using dimethyl allylphosphonate (500 mg, 3.33 mmol), 2,6-Lutidine (1.55 ml, 13.32 mmol), TMSBr (2.20 ml, 16.65 mmol) in anhydrous acetonitrile (25 ml). For the second step we used dry benzyloxy-*L*-alanine hydrochloride (718 mg, 3.33 mmol), dry phenol (1.88 g, 19.98 mmol), dry triethylamine (6.9 ml, 49.96 mmol) in dry pyridine (10 ml) and a solution of aldrithiol-2 (4.40 g, 19.98 mmol) and triphenylphosphine (5.24 g, 19.98 mmol) in dry pyridine (10 ml). After evaporation, the mixture was purified by Biotage Isolera One (100 g SNAP cartridge ULTRA, 100 ml/min, gradient eluent system EtOAc/Hexane 10% 1CV, 10–100% 12CV, 100% 2CV), to afford the title compound as a yellow oil (500 mg, 42%). R_f = 0.22 (EtOAc/Hexane – 4:6). **^{31}P NMR (202 MHz, CD_3OD) δ_P**: 29.64, 28.99. **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 7.35–7.28 (m, 7H, Ar*H*), 7.22–7.14 (m, 3H, Ar*H*), 5.91–5.81 (m, 1H, *CH*═), 5.27–5.18 (m, 2H, CH_2═), 5.14, 5.12 (ABq, J_{AB} = 12.5 Hz, 1H, CH_2Ph), 5.06 (s app, 1H, CH_2Ph), 4.10–4.01 (m, 1H, *CH*CH_3 l-Ala), 2.82–2.75 (m, 2H, CH_2P), 1.31 (d, *J* = 7.2 Hz, 1.5H, CHCH_3 l-Ala), 1.22 (d, *J* = 7.5 Hz, 1.5H, CHCH_3 l-Ala). **^{13}C NMR (125 MHz, CD_3OD) δ_C**: 172.3 (d, $^3J_{C\text{-}P}$ = 4.1 Hz, *C*═O, ester), 171.9 (d, $^3J_{C\text{-}P}$ = 3.9 Hz, *C*═O, ester), 149.0 (d, $^2J_{C\text{-}P}$ = 9.5 Hz, *C*—O, Ph), 148.9 (d, $^2J_{C\text{-}P}$ = 9.5 Hz, *C*—O, Ph), 134.37 (*C*-Ar), 134.34 (*C*-Ar), 127.84 (*CH*-Ar), 127.81 (*CH*-Ar), 126.75 (*CH*-Ar), 126.73 (*CH*-Ar), 126.5 (*CH*-Ar), 126.49 (*CH*-Ar), 126.46 (*CH*-Ar), 125.8 ($^2J_{C\text{-}P}$ = 10.1 Hz, *CH*═), 125.7 ($^2J_{C\text{-}P}$ = 10.1 Hz, *CH*═), 123.1 (*CH*-Ar), 123.0 (*CH*-Ar), 119.2 (d, $^3J_{C\text{-}P}$ = 4.3 Hz *CH*-Ar), 119.0 (d, $^3J_{C\text{-}P}$ = 4.3 Hz *CH*-Ar), 118.2 (d, $^3J_{C\text{-}P}$ = 14.5 Hz CH_2═), 118.0 (d, $^3J_{C\text{-}P}$ = 14.6 Hz CH_2═), 65.0 (CH_2Ph), 64.9 (CH_2Ph), 48.09 (*CH*CH_3 l-Ala), 47.9 (*CH*CH_3 l-Ala), 32.1 (d, $^1J_{C\text{-}P}$ = 129.7 Hz CH_2P), 31.9 (d, $^1J_{C\text{-}P}$ = 129.7 Hz CH_2P), 18.2 (d, $^3J_{C\text{-}P}$ = 5.3 Hz, CHCH_3 l-Ala), 17.7 (d, $^3J_{C\text{-}P}$ = 5.3 Hz, CHCH_3 l-Ala).

*4.1.1.5. O-(5,6,7,8-Tetrahydro-1-naphthyl)-(isopropyloxy-L-alanine)-allylphosphonate (**3e**)*. Prepared according to the standard procedure **A** for the synthesis of allylphosphonoamidate using dimethyl allylphosphonate (500 mg, 3.33 mmol), 2,6-Lutidine (1.55 ml, 13.32 mmol), TMSBr (2.20 ml, 16.65 mmol) in anhydrous acetonitrile (25 ml). For the second step we used dry isopropyloxy-*L*-alanine hydrochloride (558 mg, 3.33 mmol), dry 5,6,7,8-tetrahydro-1-naphthol (2.96 g, 19.98 mmol), dry triethylamine (6.9 ml, 49.96 mmol) in dry pyridine (10 ml) and a solution of aldrithiol-2 (4.40 g, 19.98 mmol) and triphenylphosphine (5.24 g, 19.98 mmol) in dry pyridine (10 ml). After evaporation, the mixture was purified by Biotage Isolera One (100 g SNAP cartridge ULTRA, 100 ml/min, gradient eluent system EtOAc/Hexane 10% 1CV, 10–100% 12CV, 100% 2CV), to afford the title compound as a yellow foamy solid (750 mg, 55%). R_f = 0.51 (EtOAc/Hexane – 4:6). **^{31}P NMR (202 MHz, CD_3OD) δ_P**: 29.04, 28.43. **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 7.17–7.12 (m, 1H, Ar*H*), 7.05–7.00 (m, 1H, Ar*H*), 6.89–6.87 (m, 1H, Ar*H*), 5.99–5.85 (m, 1H, *CH*═), 5.32–5.24 (m, 2H, CH_2═), 5.01–4.88 (m, 1H, *CH*$(CH_3)_2$), 3.98–3.89 (m, 1H, *CH*CH_3 l-Ala), 2.85 (dt, $^2J_{H\text{-}P}$ = $^2J_{H\text{-}H}$ = 20.0 Hz, $^3J_{H\text{-}H}$ = 7.1 Hz, 2H, CH_2P), 2.77–2.74 (m, 4H, Ar*H*), 1.80–1.79 (m, 4H, Ar*H*), 1.30 (d, *J* = 7.1 Hz, 1.5H, CHCH_3 l-Ala), 1.25–1.23 (m, 4.5H, CHCH_3 l-Ala, CH(CH_3)$_2$), 1.19 (d, *J* = 6.05 Hz, 3H, CH(CH_3)$_2$). **^{13}C NMR (125 MHz, CD_3OD) δ_C**: 173.6 (d, $^3J_{C\text{-}P}$ = 4.0 Hz, *C*═O, ester), 173.2 (d, $^3J_{C\text{-}P}$ = 4.0 Hz, *C*═O, ester), 148.7 (d, $^2J_{C\text{-}P}$ = 10.2 Hz, *C*—O, Ph), 148.6 (d, $^2J_{C\text{-}P}$ = 10.6 Hz, *C*—O, Ph), 139.1 (*C*-Ar), 131.3 (*CH*-Ar), 131.2 (*CH*-Ar), 128.6 (d, $^3J_{C\text{-}P}$ = 7.0 Hz *C*-Ar), 128.5 (d, $^3J_{C\text{-}P}$ = 7.5 Hz *C*-Ar), 127.5 ($^2J_{C\text{-}P}$ = 11.0 Hz, *CH*═), 127.4 ($^2J_{C\text{-}P}$ = 11.0 Hz, *CH*═), 125.3 (*CH*-Ar), 125.1 (*CH*-Ar), 119.4 (d, $^3J_{C\text{-}P}$ = 14.6 Hz CH_2═), 119.3 (d, $^3J_{C\text{-}P}$ = 14.6 Hz CH_2═), 116.9 (d, $^3J_{C\text{-}P}$ = 3.1 Hz *CH*-Ar), 116.8 (d, $^3J_{C\text{-}P}$ = 3.5 Hz *CH*-Ar), 68.6 (*CH*$(CH_3)_2$), 68.5 (*CH*$(CH_3)_2$), 49.7 (*CH*CH_3 l-Ala), 49.4 (*CH*CH_3 l-Ala), 33.8 (d, $^1J_{C\text{-}P}$ = 129.5 Hz CH_2P), 33.6 (d, $^1J_{C\text{-}P}$ = 130.1 Hz CH_2P), 29.1 (CH_2-Ar), 23.3 (CH_2-Ar), 22.48 (CH_2-Ar), 22.46 (CH_2-Ar), 22.41 (CH_2-Ar), 20.59 (CH(CH_3)$_2$), 20.56 (CH(CH_3)$_2$), 20.54 (CH(CH_3)$_2$), 20.4 (CH(CH_3)$_2$), 19.9 (d, $^3J_{C\text{-}P}$ = 4.9 Hz, CHCH_3 l-Ala), 19.1 (d, $^3J_{C\text{-}P}$ = 5.4 Hz, CHCH_3 l-Ala).

*4.1.1.6. O-(5,6,7,8-Tetrahydro-1-naphthyl)-(benzyloxy-L-alanine)-allylphosphonate (**3f**)*. Prepared according to the standard procedure **A** for the synthesis of allylphosphonoamidate using dimethyl allylphosphonate (500 mg, 3.33 mmol), 2,6-Lutidine (1.55 ml, 13.32 mmol), TMSBr (2.20 ml, 16.65 mmol) in anhydrous acetonitrile (25 ml). For the second step we used dry benzyloxy-*L*-alanine hydrochloride (718 mg, 3.33 mmol), dry 5,6,7,8-tetrahydro-1-naphthol (2.96 g, 19.98 mmol), dry triethylamine (6.9 ml, 49.96 mmol) in dry pyridine (10 ml) and a solution of aldrithiol-2 (4.40 g, 19.98 mmol) and triphenylphosphine (5.24 g, 19.98 mmol) in dry pyridine (10 ml). After evaporation, the mixture was purified by Biotage Isolera One (100 g SNAP cartridge ULTRA, 100 ml/min, gradient eluent system EtOAc/Hexane 10% 1CV, 10–100% 12CV, 100% 2CV), to afford the title compound as a yellow foamy solid (750 mg, 55%). R_f = 0.51 (EtOAc/Hexane – 4:6). **^{31}P NMR (202 MHz, CD_3OD) δ_P**: 28.81, 28.20. **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 7.35–7.31 (m, 5H, Ar*H*), 7.17–7.14 (m, 1H, Ar*H*), 7.06–7.94 (m, 1H, Ar*H*), 6.87–6.83 (m, 1H, Ar*H*), 5.94–5.82 (m, 1H, *CH*═), 5.27–5.20 (m, 2H, CH_2═), 5.13, 5.10 (ABq, J_{AB} = 12.2 Hz, 1H, CH_2Ph), 5.04 (AB app t, J_{AB} = 12.8 Hz, 1H, CH_2Ph), 4.12–4.01 (m, 1H, *CH*CH_3 l-Ala), 2.86–2.77 (m, 2H, CH_2P), 2.72–2.67 (m, 4H, Ar*H*), 1.79–1.71 (m, 4H, Ar*H*), 1.32 (d, *J* = 7.1 Hz, 1.5H, CHCH_3 l-Ala), 1.26 (d, *J* = 7.1 Hz, 1.5H, CHCH_3 l-Ala). **^{13}C NMR (125 MHz, CD_3OD) δ_C**: 173.8 (d, $^3J_{C\text{-}P}$ = 3.7 Hz, *C*═O, ester), 173.4 (d, $^3J_{C\text{-}P}$ = 4.1 Hz, *C*═O, ester), 148.8 (d, $^2J_{C\text{-}P}$ = 9.7 Hz, *C*—O, Ph), 148.7 (d, $^2J_{C\text{-}P}$ = 9.4 Hz, *C*—O, Ph), 139.13 (*C*-Ar), 139.11 (*C*-Ar), 135.88 (*C*-Ar), 135.84 (*C*-Ar), 128.6 (d, $^3J_{C\text{-}P}$ = 5.5 Hz *C*-Ar), 128.5 (d, $^3J_{C\text{-}P}$ = 5.8 Hz *C*-Ar), 128.23 (*CH*-Ar), 128.20 (*CH*-Ar), 127.99 (*CH*-Ar), 127.94 (*CH*-Ar), 127.87 (*CH*-Ar), 127.5 ($^2J_{C\text{-}P}$ = 11.3 Hz, *CH*═), 127.4 ($^2J_{C\text{-}P}$ = 11.0 Hz, *CH*═), 125.4 (*CH*-Ar), 125.3 (*CH*-Ar), 125.17 (*CH*-Ar), 125.13 (*CH*-Ar), 119.5 (d, $^3J_{C\text{-}P}$ = 14.6 Hz CH_2═), 119.4 (d, $^3J_{C\text{-}P}$ = 14.8 Hz CH_2═), 117.0 (d, $^3J_{C\text{-}P}$ = 3.4 Hz *CH*-Ar), 116.9 (d, $^3J_{C\text{-}P}$ = 3.1 Hz *CH*-Ar), 66.5 (CH_2Ph), 66.4 (CH_2Ph), 49.7 (*CH*CH_3 l-Ala), 49.4 (*CH*CH_3 l-Ala), 33.8 (d, $^1J_{C\text{-}P}$ = 129.4 Hz CH_2P), 33.7 (d, $^1J_{C\text{-}P}$ = 130.2 Hz CH_2P), 29.18 (CH_2-Ar), 23.38 (CH_2-Ar), 22.5 (CH_2-Ar), 22.48 (CH_2-Ar), 22.43 (CH_2-Ar), 19.8 (d, $^3J_{C\text{-}P}$ = 5.3 Hz, CHCH_3 l-Ala), 19.1 (d, $^3J_{C\text{-}P}$ = 5.3 Hz, CHCH_3 l-Ala).

*4.1.2. General procedure B for the preparation of N^1-2′-methylallylpyrimidine (**6, 7**)*

In a round bottom flask, under an argon atmosphere, to a solution of the nucleobase (1 eq) in anhydrous acetonitrile (2 ml/mmol of nucleobase) was added BSA (2.5 eq). The mixture was refluxed until clear solution was observed (usually 5 min). 3-bromo-2-methylpropene (2.0 eq), NaI (1.1 eq) and TMSCl (1 eq) were then added to the reaction mixture. The solution was refluxed 16 h and then evaporated under reduced pressure. The residue was dissolved in EtOAc, washed with $NaHCO_3$ (aqueous saturated solution), Na_2SO_4 (aqueous saturated solution), H_2O, brine and dried over $MgSO_4$. The resulting mixture was evaporated and the residue was purified by Biotage Isolera One.

*4.1.2.1. N^1-2′-Methylallyl-thymine (**6**)*. Prepared according to the standard procedure **B** for the synthesis of N^1-2′-methylallylpyrimidine using thymine (1.5 g, 11.89 mmol), BSA (7.2 ml, 29.73 mmol), 3-

bromo-2-methylpropene (2.40 ml, 23.79 mmol), NaI (1.96 g, 13.08 mmol) and TMSCl (1.51 ml, 11.89 mmol) in anhydrous acetonitrile (25 ml). After work up and evaporation, the compound was obtained as a pale yellow solid in quantitative yield (2.1 g). R_f = 0.45 (EtOAc/Hexane – 7:3).**^{1}H NMR (500 MHz, CD_3OD) δ_H**: 7.34 (s, 1H, *H*-6), 4.98 (s, 1H, CH_2═), 4.80 (s, 1H, CH_2═), 4.30 (s, 2H, CH_2-N), 1.89 (s, 3H, CH_3, base), 1.76 (s, 3H, CH_3, alkene).

4.1.2.2. N^1-2′-Methylallyl-uracil (7). Prepared according to the standard procedure **B** for the synthesis of N^1-2′-methylallylpyrimidine using uracil (1.5 g, 13.38 mmol), BSA (8.18 ml, 33.46 mmol), 3-bromo-2-methylpropene (2.70 ml, 26.76 mmol), NaI (2.21 g, 14.72 mmol) and TMSCl (1.70 ml, 13.38 mmol) in anhydrous acetonitrile (25 ml). After work up and evaporation, the mixture was purified by Biotage Isolera One (50 g SNAP cartridge ULTRA, 100 ml/min, gradient eluent system EtOAc/Hexane 17% 1CV, 17–100% 10CV, 100% 3CV), to afford the title compound as a pale yellow solid (1.2 g, 51%). R_f = 0.25 (EtOAc/Hexane – 7:3).**^{1}H NMR (500 MHz, CD_3OD) δ_H**: 7.50 (d, *J* = 7.8 Hz, 1H, *H*-6), 5.71 (d, *J* = 7.8 Hz, 1H, *H*-5), 4.98 (s, 1H, CH_2═), 4.81 (s, 1H, CH_2═), 4.33 (s, 2H, CH_2-N), 1.76 (s, 3H, CH_3, alkene).

*4.1.3. General procedure C for the preparation of (E)-N^1-(4′-O-Aryl-(L-alanine-ester)-phosphinyl-2′-methyl-but-2′-enyl)pyrimidine (**E-8a–f**, **E-10a–f**)*

To a solution of *O*-Aryl-(*L*-alanine-ester)-allylphosphonate (1 eq) and N^1-2′-methylallylpyrimidine (2 eq) in dry CH_2Cl_2 (20 ml/mmol allylphosphonate), was added Hoveyda-Grubbs 2nd generation catalyst (15 mol%). The catalyst was added in three equal portion of 5 mol% at t = 0, 2, 4 h over the course of the reaction. The solution was sonicated under argon atmosphere for 24 h. Volatiles were then evaporated, and the residue was purified by Biotage Isolera One. Also a reverse phase chromatography was necessary to gain pure final products.

*4.1.3.1. (E)-N^1-(4′-O-(1-Naphthyl)-(isopropyloxy-L-alanine)-phosphinyl-2′-methyl-but-2′-enyl)thymine (**E-8a**) and (Z)-N^1-(4′-O-(1-naphthyl)-(isopropyloxy-L-alanine)-phosphinyl-2′-methyl-but-2′-enyl)thymine (**Z-8a**)*. Prepared according to the standard procedure **C** for the synthesis of ANP ProTide using *O*-(1-naphthyl)-(isopropyloxy-*L*-alanine)-allylphosphonate **3a** (150 mg, 415 μmol) and N^1-2′-methylallylthymine (150 mg, 830.1 μmol) and Hoveyda-Grubbs 2nd generation catalyst (15 mol%) in dry CH_2Cl_2 (8 ml). After evaporation, the crude was purified by Biotage Isolera One (50 g SNAP cartridge ULTRA, 100 ml/min, gradient eluent system MeOH/CH_2Cl_2 1% 1CV, 1–10% 12CV, 10% 2CV), to afford a mixture of the *E* and *Z* isomers. The two isomers were then separated by reverse Biotage Isolera One (60 g SNAP cartridge KP-C18-HS, 100 ml/min, isocratic eluent system CH_3CN/H_2O 30–60% 12CV) to afford the title compound *E* as pale yellow foamy solid (75 mg, 36%). R_f = 0.23 (CH_2Cl_2/MeOH – 95:5).**^{31}P NMR (202 MHz, CD_3OD) δ_P**: 30.32, 29.54. **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 8.13–8.12 (m, 1H, Ar*H*), 7.89–7.87 (m, 1H, Ar*H*), 7.71–7.68 (m, 1H, Ar*H*), 7.57–7.48 (m, 3H, Ar*H*), 7.45–7.39 (m, 1H, Ar*H*), 7.27 (s, 0.5H, *H*-6), 7.26 (s, 0.5H, *H*-6), 5.61–5.56 (m, 1H, *CH*═), 4.93–4.84 (m, 1H, *CH*$(CH_3)_2$), 4.32–4.26 (m, 2H, CH_2-N), 4.01–3.91 (m, 1H, *CH*CH_3 l-Ala), 3.08–2.86 (m, 2H, CH_2P), 1.75 (s, 3H, CH_3, base), 1.67 (s, 3H, CH_3, alkene), 1.27 (d, *J* = 6.9 Hz, 1.5H, CHCH_3 l-Ala), 1.20–1.16 (m, 4.5H, CHCH_3 l-Ala, CH$(CH_3)_2$), 1.13–1.10 (m, 3H, CH$(CH_3)_2$). **^{13}C NMR (125 MHz, CD_3OD) δ_C**: 173.5 (d, $^3J_{C\text{-}P}$ = 3.9 Hz, *C*═O, ester), 173.1 (d, $^3J_{C\text{-}P}$ = 3.5 Hz, *C*═O, ester), 165.34 (*C*-4), 165.32 (*C*-4), 151.69 (*C*-2), 151.61 (*C*-2), 146.5 (d, $^2J_{C\text{-}P}$ = 9.5 Hz, *C*—O, Ph), 146.3 (d, $^2J_{C\text{-}P}$ = 9.5 Hz, *C*—O, Ph), 140.94 (*C*-6), 140.92 (*C*-6), 135.5 (d, $^3J_{C\text{-}P}$ = 14.3 Hz, *C*═), 135.1 (d, $^3J_{C\text{-}P}$ = 14.7 Hz, *C*═), 134.9 (*C*-Ar), 127.48 (*CH*-Ar), 127.46 (*CH*-Ar), 126.7 (d, $^3J_{C\text{-}P}$ = 5.1 Hz *C*-Ar), 126.6 (d, $^3J_{C\text{-}P}$ = 5.1 Hz *C*-Ar), 126.3 (*CH*-Ar), 126.0 (*CH*-Ar), 125.16 (*CH*-Ar), 125.11 (*CH*-Ar), 124.3 (*CH*-Ar), 124.2 (*CH*-Ar), 121.4 (*CH*-Ar), 121.3 (*CH*-Ar), 117.1 ($^2J_{C\text{-}P}$ = 11.1 Hz, *CH*═), 116.6 ($^2J_{C\text{-}P}$ = 10.7 Hz, *CH*═), 115.3 (d, $^3J_{C\text{-}P}$ = 3.5 Hz *CH*-Ar), 115.1 (d, $^3J_{C\text{-}P}$ = 3.9 Hz *CH*-Ar), 110.1 (*C*-5), 68.69 (*CH*$(CH_3)_2$), 68.65 (*CH*$(CH_3)_2$), 53.5 (d, $^4J_{C\text{-}P}$ = 2.7 Hz, CH_2-N), 53.2 (d, $^4J_{C\text{-}P}$ = 2.3 Hz, CH_2-N), 49.7 (*CH*CH_3 l-Ala), 49.5 (*CH*CH_3 l-Ala), 28.3 (d, $^1J_{C\text{-}P}$ = 129.0 Hz CH_2P), 28.1 (d, $^1J_{C\text{-}P}$ = 130.0 Hz CH_2P), 20.55 (CH$(CH_3)_2$), 20.54 (CH$(CH_3)_2$), 20.48 (CH$(CH_3)_2$), 20.40 (CH$(CH_3)_2$), 19.8 (d, $^3J_{C\text{-}P}$ = 5.5 Hz, CHCH_3 l-Ala), 19.1 (d, $^3J_{C\text{-}P}$ = 5.9 Hz, CHCH_3 l-Ala), 13.3 (d, $^4J_{C\text{-}P}$ = 2.3 Hz, CH_3, alkene), 13.2 (d, $^4J_{C\text{-}P}$ = 2.7 Hz, CH_3, alkene), 10.8 (CH_3, base). **HPLC**: Reverse phase HPLC eluting with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 min, 1 ml/min, λ = 254 nm and 263 nm, showed one peak with Rt 16.26 min. **HRMS (ESI):** *m/z* $[M+Na]^+$ calcd for $C_{26}H_{32}N_3O_6P$: 536.1926, found: 536.1921.

From PrepHPLC also the *Z* isomer ***Z*-8a** was isolated as pale yellow foamy solid (6 mg, 3%).**^{31}P NMR (202 MHz, CD_3OD) δ_P**: 30.40, 29.66. **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 8.19–8.13 (m, 1H, Ar*H*), 7.90–7.85 (m, 1H, Ar*H*), 7.78–7.67 (m, 1H, Ar*H*), 7.57–7.43 (m, 5H, Ar*H*, *H*-6), 5.73–5.65 (m, 1H, *CH*═), 4.97–4.86 (m, 1H, *CH*$(CH_3)_2$), 4.49 (bs, 2H, CH_2-N), 4.04–3.98 (m, 1H, *CH*CH_3 l-Ala), 3.24–3.07 (m, 2H, CH_2P), 1.76–1.70 (m 6H, CH_3, base; CH_3, alkene), 1.27 (d, *J* = 7.0 Hz, 1.5H, CHCH_3 l-Ala), 1.21–1.12 (m, 7.5H, CHCH_3 l-Ala, CH$(CH_3)_2$, CH$(CH_3)_2$). **^{13}C NMR (125 MHz, CD_3OD) δ_C**: 173.5 (d, $^3J_{C\text{-}P}$ = 3.9 Hz, *C*═O, ester), 173.1 (d, $^3J_{C\text{-}P}$ = 3.5 Hz, *C*═O, ester), 165.3 (*C*-4), 151.8 (*C*-2), 151.7 (*C*-2), 146.4 (d, $^2J_{C\text{-}P}$ = 10.2 Hz, *C*—O, Ph), 146.2 (d, $^2J_{C\text{-}P}$ = 10.8 Hz, *C*—O, Ph), 1401.1 (*C*-6), 141.0 (*C*-6), 134.9 (*C*-Ar), 134.8 (d, $^3J_{C\text{-}P}$ = 14.6 Hz, *C*═), 134.5 (d, $^3J_{C\text{-}P}$ = 14.6 Hz, *C*═), 127.4 (*CH*-Ar), 126.9 (d, $^3J_{C\text{-}P}$ = 4.8 Hz *C*-Ar), 126.8 (d, $^3J_{C\text{-}P}$ = 5.3 Hz *C*-Ar), 126.3 (*CH*-Ar), 126.04 (*CH*-Ar), 126.01 (*CH*-Ar), 125.156 (*CH*-Ar), 125.12 (*CH*-Ar), 124.5 (*CH*-Ar), 124.4 (*CH*-Ar), 121.5 (*CH*-Ar), 121.4 (*CH*-Ar), 119.1 ($^2J_{C\text{-}P}$ = 11.1 Hz, *CH*═), 119.0 ($^2J_{C\text{-}P}$ = 10.4 Hz, *CH*═), 115.7 (d, $^3J_{C\text{-}P}$ = 3.4 Hz *CH*-Ar), 115.4 (d, $^3J_{C\text{-}P}$ = 3.4 Hz *CH*-Ar), 110.0 (*C*-5), 68.6 (*CH*$(CH_3)_2$), 49.7 (*CH*CH_3 l-Ala), 49.5 (*CH*CH_3 l-Ala), 47.1 (CH_2-N), 28.2 (d, $^1J_{C\text{-}P}$ = 129.0 Hz CH_2P), 28.0 (d, $^1J_{C\text{-}P}$ = 129.8 Hz CH_2P), 20.51 (CH$(CH_3)_2$), 20.50 (CH$(CH_3)_2$), 20.4 (CH$(CH_3)_2$), 20.3 (CH$(CH_3)_2$), 19.8 (d, $^3J_{C\text{-}P}$ = 5.5 Hz, CHCH_3 l-Ala), 19.0 (d, $^3J_{C\text{-}P}$ = 5.5 Hz, CHCH_3 l-Ala), 10.7 (d, $^4J_{C\text{-}P}$ = 3.0 Hz, CH_3, alkene), 10.6 (CH_3, base). **HPLC**: Reverse phase HPLC eluting with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 min, 1 ml/min, λ = 254 nm and 263 nm, showed one peak with Rt 17.90 min.

*4.1.3.2. (E)-N^1-(4′-O-(1-naphthyl)-(benzyloxy-L-alanine)-phosphinyl-2′-methyl-but-2′-enyl)thymine (**E-8b**)*. Prepared according to the standard procedure **C** for the synthesis of ANP |ProTide using *O*-(1-naphthyl)-(benzyloxy-*L*-alanine)-allylphosphonate **3b** (240 mg, 586.1 μmol) and N^1-2′-methylallylthymine (211 mg, 1.17 mmol) and Hoveyda-Grubbs 2nd generation catalyst (15 mol%) in dry CH_2Cl_2 (10 ml). After evaporation, the crude was purified by Biotage Isolera One (120 g ZIP cartridge KP-SIL, 100 ml/min, gradient eluent system MeOH/CH_2Cl_2 1% 1CV, 1–10% 12CV, 10% 2CV), to afford a mixture of the *E* and *Z* isomers. The two isomers were then separated by PrepHPLC (20 ml/min, isocratic eluting system CH_3CN/H_2O – 40/60, 30 min), to afford the title compound as pale yellow foamy solid (43 mg, 13%). R_f = 0.40 (CH_2Cl_2/MeOH – 95:5).**^{31}P NMR (202 MHz, CD_3OD) δ_P**: 30.35, 29.51. **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 8.12–8.10 (m, 1H, Ar*H*), 7.88–7.87 (m, 1H, Ar*H*), 7.70–7.66 (m, 1H, Ar*H*), 7.54–7.22 (m, 10*H*, Ar*H*), 5.53–5.45 (m, 1H, *CH*═), 5.12, 5.06 (ABq, J_{AB} = 12.2 Hz, 1H, CH_2Ph), 4.99, 4.95 (ABq, J_{AB} = 12.2 Hz, 1H, CH_2Ph), 4.26–4.20 (m, 2H, CH_2-N), 4.11–4.06 (m, 1H, *CH*CH_3 l-Ala), 3.02–2.86 (m, 2H, CH_2P), 1.74 (s, 3H, CH_3, base), 1.64 (d, *J* = 3.6 Hz 1.5H, CH_3, alkene), 1.61 (d, *J* = 3.5 Hz 1.5H, CH_3, alkene), 1.26 (d, *J* = 6.9 Hz, 1.5H, CHCH_3 l-Ala), 1.18 (d, *J* = 7.2 Hz, 1.5H, CHCH_3 l-Ala). **^{13}C NMR (125 MHz, CD_3OD) δ_C**: 173.7 (d, $^3J_{C\text{-}P}$ = 3.8 Hz, *C*═O, ester), 173.3 (d, $^3J_{C\text{-}P}$ = 3.7 Hz, *C*═O, ester), 165.35 (*C*-4), 165.32 (*C*-4), 151.67 (*C*-2), 151.60 (*C*-2), 146.5 (d, $^2J_{C\text{-}P}$ = 9.7 Hz, *C*—O, Ph), 146.3 (d, $^2J_{C\text{-}P}$ = 9.9 Hz, *C*—O, Ph), 140.9 (*C*-6), 135.8 (*C*-Ar), 135.7 (*C*-Ar), 135.4 (d, $^3J_{C\text{-}P}$ = 14.4 Hz, *C*═), 135.2 (d, $^3J_{C\text{-}P}$ = 14.7 Hz, *C*═), 134.9 (*C*-Ar), 128.19 (*CH*-Ar), 128.12 (*CH*-Ar), 127.9 (*CH*-Ar), 127.8 (*CH*-Ar), 127.5 (*CH*-Ar), 127.4 (*CH*-Ar), 126.7 (d, $^3J_{C\text{-}P}$ = 5.0 Hz *C*-Ar), 126.6 (d, $^3J_{C\text{-}P}$ = 5.3 Hz *C*-Ar), 126.3

(*CH*-Ar), 126.0 (*CH*-Ar), 125.19 (*CH*-Ar), 125.11 (*CH*-Ar), 124.3 (*CH*-Ar), 124.2 (*CH*-Ar), 121.4 (*CH*-Ar), 121.2 (*CH*-Ar), 117.1 ($^2J_{C-P}$ = 10.7 Hz, *CH*═), 116.6 ($^2J_{C-P}$ = 10.7 Hz, *CH*═), 115.4 (d, $^3J_{C-P}$ = 4.0 Hz *CH*-Ar), 115.1 (d, $^3J_{C-P}$ = 3.6 Hz *CH*-Ar), 110.1 (*C*-5), 66.5 (CH_2Ph), 66.4 (CH_2Ph), 53.4 (d, $^4J_{C-P}$ = 2.0 Hz, CH_2-N), 53.2 (d, $^4J_{C-P}$ = 2.2 Hz, CH_2-N), 49.6 (*CH*CH_3 l-Ala), 49.5 (*CH*CH_3 l-Ala), 28.3 (d, $^1J_{C-P}$ = 129.3 Hz CH_2P), 28.1 (d, $^1J_{C-P}$ = 130.2 Hz CH_2P), 19.6 (d, $^3J_{C-P}$ = 5.6 Hz, CHCH_3 l-Ala), 19.0 (d, $^3J_{C-P}$ = 5.3 Hz, CHCH_3 l-Ala), 13.3 (d, $^4J_{C-P}$ = 2.4 Hz, CH_3, alkene), 13.2 (d, $^4J_{C-P}$ = 2.4 Hz, CH_3, alkene), 10.8 (CH_3, base). **HPLC**: Reverse phase HPLC eluting with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 min, 1 ml/min, λ = 254 nm and 263 nm, showed one peak with Rt 18.01 min. **HRMS (ESI):** *m/z* $[M+Na]^+$ calcd for $C_{30}H_{32}N_3O_6P$: 584.1926, found: 584.1921.

*4.1.3.3. (E)-N^1-(4′-O-Phenyl-(isopropyloxy-L-alanine)-phosphinyl-2′-methyl-but-2′-enyl)thymine (**E-8c**)*. Prepared according to the standard procedure **C** for the synthesis of ANP ProTide using *O*-phenyl-(isopropyloxy-*L*-alanine)-allylphosphonate **3c** (140 mg, 449.7 μmol) and N^1-2′-methylallylthymine (162 mg, 899.4 μmol) and Hoveyda-Grubbs 2nd generation catalyst (15 mol%) in dry CH_2Cl_2 (8 ml). After evaporation, the crude was purified by Biotage Isolera One (25 g SNAP cartridge ULTRA, 75 ml/min, gradient eluent system MeOH/CH_2Cl_2 1% 1CV, 1–10% 12CV, 10% 2CV), to afford a mixture of the *E* and *Z* isomers. The two isomers were then separated by PrepHPLC (20 ml/min, gradient eluting system CH_3CN/H_2O from 10/90 to 100/0, 30 min), to afford the title compound as pale yellow foamy solid (20.4 mg, 10%). R_f = 0.27 (CH_2Cl_2/MeOH – 94:6). **^{31}P NMR (202 MHz, CD_3OD) δ_P**: 29.80, 29.03. **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 7.38–7.33 (m, 3H, *H*-6, Ar*H*), 7.22–7.16 (m, 3H, Ar*H*), 5.52 (q, *J* = 6.9 Hz, 0.4H, *CH*═), 5.43 (q, *J* = 6.9 Hz, 0.6H, *CH*═), 4.98 (sept, *J* = 6.2 Hz, 0.4H, *CH*$(CH_3)_2$), 4.92 (sept, *J* = 6.2 Hz, 0.6H, *CH*$(CH_3)_2$), 4.36–4.30 (m, 2H, CH_2-N), 3.97–3.91 (m, 1H, *CH*CH_3 l-Ala), 2.96–2.77 (m, 2H, CH_2P), 1.85 (s, 3H, CH_3, base), 1.72 (s, 1.2H, CH_3, alkene), 1.71 (s, 1.8H, CH_3, alkene), 1.29 (d, *J* = 6.9 Hz, 1.8H, CHCH_3 l-Ala), 1.25 (d, *J* = 6.3 Hz, 1.2H, CH$(CH_3)_2$), 1.23 (d, *J* = 6.2 Hz, 1.2H, CH$(CH_3)_2$), 1.21–1.96 (m, 4.8H, CHCH_3 l-Ala, CH$(CH_3)_2$). **^{13}C NMR (125 MHz, CD_3OD) δ_C**: 173.6 (d, $^3J_{C-P}$ = 4.6 Hz, *C*═O, ester), 173.2 (d, $^3J_{C-P}$ = 4.1 Hz, *C*═O, ester), 165.3 (*C*-4), 151.7 (*C*-2), 151.6 (*C*-2), 150.5 (d, $^2J_{C-P}$ = 9.8 Hz, *C*—O, Ph), 150.3 (d, $^2J_{C-P}$ = 9.5 Hz, *C*—O, Ph), 141.0 (*C*-6), 135.4 (d, $^3J_{C-P}$ = 14.4 Hz, *C*═), 135.0 (d, $^3J_{C-P}$ = 14.4 Hz, *C*═), 129.32 (*CH*-Ar), 129.30 (*CH*-Ar), 124.5 (*CH*-Ar), 124.4 (*CH*-Ar), 120.6 (d, $^3J_{C-P}$ = 4.3 Hz *CH*-Ar), 120.4 (d, $^3J_{C-P}$ = 4.6 Hz *CH*-Ar), 117.2 (d, $^2J_{C-P}$ = 11.0 Hz, *CH*═), 116.6 (d, $^2J_{C-P}$ = 10.8 Hz, *CH*═), 110.1 (*C*-5), 68.67 (*CH*$(CH_3)_2$), 68.63 (*CH*$(CH_3)_2$), 53.5 (d, $^4J_{C-P}$ = 2.4 Hz, CH_2-N), 53.3 (d, $^4J_{C-P}$ = 2.4 Hz, CH_2-N), 49.6 (*CH*CH_3 l-Ala), 49.4 (*CH*CH_3 l-Ala), 28.2 (d, $^1J_{C-P}$ = 129.5 Hz, CH_2P), 28.0 (d, $^1J_{C-P}$ = 130.5 Hz, CH_2P), 20.58 (CH$(CH_3)_2$), 20.53 (CH$(CH_3)_2$), 20.4 (CH$(CH_3)_2$), 19.8 (d, $^3J_{C-P}$ = 5.4 Hz, CHCH_3 l-Ala), 19.1 (d, $^3J_{C-P}$ = 5.4 Hz, CHCH_3 l-Ala), 13.2 (d, $^4J_{C-P}$ = 2.5 Hz, CH_3, alkene), 13.1 (d, $^4J_{C-P}$ = 2.2 Hz, CH_3, alkene), 10.8 (CH_3, base). **HPLC**: Reverse phase HPLC eluting with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 min, 1 ml/min, λ = 254 nm and 263 nm, showed one peak with Rt 13.94 min. **HRMS (ESI):** *m/z* $[M+Na]^+$ calcd for $C_{22}H_{30}N_3O_6P$: 486.1770, found: 486.1764.

*4.1.3.4. (E)-N^1-(4′-O-Phenyl-(benzyloxy-L-alanine)-phosphinyl-2′-methyl-but-2′-enyl)thymine (**E-8d**)*. Prepared according to the standard procedure **C** for the synthesis of ANP ProTide using *O*-phenyl-(benzyloxy-*L*-alanine)-allylphosphonate **3d** (200 mg, 556.5 μmol) and N^1-2′-methylallylthymine (200.6 mg, 1.11 mmol) and Hoveyda-Grubbs 2nd generation catalyst (15 mol%) in dry CH_2Cl_2 (8 ml). After evaporation, the crude was purified by Biotage Isolera One (25 g SNAP cartridge ULTRA, 75 ml/min, gradient eluent system 2-propanol/CH_2Cl_2 1% 1CV, 1–10% 12CV, 10% 2CV), to afford a mixture of the *E* and *Z* isomers. The two isomers were then separated by PrepHPLC (20 ml/min, isocratic eluting system CH_3CN/H_2O – 35/65, 30 min), to afford the title compound as pale yellow foamy solid (64 mg, 23%). R_f = 0.42 (CH_2Cl_2/2-propanol – 95:5). **^{31}P NMR (202 MHz, CD_3OD) δ_P**: 29.79, 28.99. **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 7.36–7.29 (m, 8H, *H*-6, Ar*H*), 7.20–7.14 (m, 3H, Ar*H*), 5.49–5.40 (m, 1H, *CH*═), 5.16, 5.13 (ABq, J_{AB} = 12.3 Hz, 1H, CH_2Ph), 5.08 (s app, 1H, CH_2Ph), 4.28–4.23 (m, 2H, CH_2-N), 4.07–4.01 (m, 1H, *CH*CH_3 l-Ala), 2.89–2.73 (m, 2H, CH_2P), 1.84 (s, 3H, CH_3, base), 1.67–1.64 (m, 3H, CH_3, alkene), 1.30 (d, *J* = 7.0 Hz, 1.5H, CHCH_3 l-Ala), 1.22 (d, *J* = 7.1 Hz, 1.5H, CHCH_3 l-Ala). **^{13}C NMR (125 MHz, CD_3OD) δ_C**: 173.8 (d, $^3J_{C-P}$ = 4.5 Hz, *C*═O, ester), 173.4 (d, $^3J_{C-P}$ = 3.9 Hz, *C*═O, ester), 165.3 (*C*-4), 151.7 (*C*-2), 151.6 (*C*-2), 150.5 (d, $^2J_{C-P}$ = 9.3 Hz, *C*—O, Ph), 150.4 (d, $^2J_{C-P}$ = 9.4 Hz, *C*—O, Ph), 140.98 (*C*-6), 140.97 (*C*-6), 135.9 (*C*-Ar), 135.8 (*C*-Ar), 135.3 (d, $^3J_{C-P}$ = 14.1 Hz, *C*═), 135.0 (d, $^3J_{C-P}$ = 14.0 Hz, *C*═), 129.35 (*CH*-Ar), 129.34 (*CH*-Ar), 128.23 (*CH*-Ar), 128.20 (*CH*-Ar), 128.01 (*CH*-Ar), 128.00 (*CH*-Ar), 127.96 (*CH*-Ar), 127.95 (*CH*-Ar), 124.6 (*CH*-Ar), 124.5 (*CH*-Ar), 120.6 (d, $^3J_{C-P}$ = 4.3 Hz *CH*-Ar), 120.4 (d, $^3J_{C-P}$ = 3.8 Hz *CH*-Ar), 117.2 (d, $^2J_{C-P}$ = 10.7 Hz, *CH*═), 116.6 (d, $^2J_{C-P}$ = 10.7 Hz, *CH*═), 110.13 (*C*-5), 110.11 (*C*-5), 65.5 (CH_2Ph), 66.4 (CH_2Ph), 53.5 (d, $^4J_{C-P}$ = 2.4 Hz, CH_2-N), 53.3 (d, $^4J_{C-P}$ = 2.3 Hz, CH_2-N), 49.6 (*CH*CH_3 l-Ala), 49.4 (*CH*CH_3 l-Ala), 28.2 (d, $^1J_{C-P}$ = 129.7 Hz, CH_2P), 28.0 (d, $^1J_{C-P}$ = 130.3 Hz, CH_2P), 19.7 (d, $^3J_{C-P}$ = 5.3 Hz, CHCH_3 l-Ala), 19.1 (d, $^3J_{C-P}$ = 5.3 Hz, CHCH_3 l-Ala), 13.3 (d, $^4J_{C-P}$ = 1.8 Hz, CH_3, alkene), 13.1 (d, $^4J_{C-P}$ = 2.2 Hz, CH_3, alkene), 10.9 (CH_3, base). **HPLC**: Reverse phase HPLC eluting with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 min, 1 ml/min, λ = 254 nm and 263 nm, showed one peak with Rt 15.21 min. **HRMS (ESI):** *m/z* $[M+Na]^+$ calcd for $C_{26}H_{30}N_3O_6P$: 534.1764, found: 534.1764.

*4.1.3.5. (E)-N^1-(4′-O-(5,6,7,8-Tetrahydro-1-naphthyl)-(isopropyloxy-L-alanine)-phosphinyl-2′-methyl-but-2′-enyl)thymine (**E-8e**) and (Z)-N^1-(4′-O-(5,6,7,8-tetrahydro-1-naphthyl)-(isopropyloxy-L-alanine)-phosphinyl-2′-methyl-but-2′-enyl)thymine (**Z-8e**)*. Prepared according to the standard procedure **C** for the synthesis of ANP ProTide using *O*-(5,6,7,8-tetrahydro-1-naphthyl)-(isopropyloxy-*L*-alanine)-allylphosphonate **3e** (200 mg, 547.3 μmol) and N^1-2′-methylallylthymine (197 mg, 1.09 mmol) and Hoveyda-Grubbs 2nd generation catalyst (15 mol%) in dry CH_2Cl_2 (10 ml). After evaporation, the crude was purified by Biotage Isolera One (25 g SNAP cartridge ULTRA, 75 ml/min, gradient eluent system 2-propanol/CH_2Cl_2 1% 1CV, 1–10% 12CV, 10% 2CV), to afford a mixture of the *E* and *Z* isomers. The two isomers were then separated by PrepHPLC (20 ml/min, isocratic eluting system CH_3CN/H_2O – 35/65, 30 min), to afford the title compound *E* as pale yellow foamy solid (72 mg, 26%). R_f = 0.26 (CH_2Cl_2/2-propanol – 95:5). **^{31}P NMR (202 MHz, CD_3OD) δ_P**: 29.35, 28.55. **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 7.34 (s, 0.5H, *H*-6), 7.33 (s, 0.5H, *H*-6), 7.17–7.12 (m, 1H, Ar*H*), 7.05–7.00 (m, 1H, Ar*H*), 6.89–6.86 (m, 1H, Ar*H*), 5.57–5.52 (m, 0.5H, *CH*═), 5.50–5.44 (m, 0.5H, *CH*═), 5.01–4.88 (m, 1H, *CH*$(CH_3)_2$), 4.36–4.29 (m, 2H, CH_2-N), 3.99–3.91 (m, 1H, *CH*CH_3 l-Ala), 2.94–2.82 (m, 2H, CH_2P), 2.77–2.74 (m, 2H, Ar*H*), 2.69–2.67 (m, 2H, Ar*H*), 1.84 (s, 3H, CH_3, base), 1.80–1.76 (m, 4H, Ar*H*), 1.70 (d, *J* = 2.9 Hz 3H, CH_3, alkene), 1.29 (d, *J* = 7.2 Hz, 1.5H, CHCH_3 l-Ala), 1.25–1.24 (m, 4.5H, CHCH_3 l-Ala, CH$(CH_3)_2$), 1.19 (d, *J* = 6.2 Hz, 3H, CH$(CH_3)_2$). **^{13}C NMR (125 MHz, CD_3OD) δ_C**: 173.7 (d, $^3J_{C-P}$ = 3.8 Hz, *C*═O, ester), 173.2 (d, $^3J_{C-P}$ = 4.1 Hz, *C*═O, ester), 165.3 (*C*-4), 151.7 (*C*-2), 151.6 (*C*-2), 148.8 (d, $^2J_{C-P}$ = 9.4 Hz, *C*—O, Ph), 148.7 (d, $^2J_{C-P}$ = 9.9 Hz, *C*—O, Ph), 141.05 (*C*-6), 141.02 (*C*-6), 139.19 (*C*-Ar), 139.16 (*C*-Ar), 135.1 (d, $^3J_{C-P}$ = 14.1 Hz, *C*═), 134.8 (d, $^3J_{C-P}$ = 14.3 Hz, *C*═), 128.4 (d, $^3J_{C-P}$ = 5.5 Hz *C*-Ar), 128.3 (d, $^3J_{C-P}$ = 5.8 Hz *C*-Ar), 125.4 (*CH*-Ar), 125.3 (*CH*-Ar), 125.1 (*CH*-Ar), 125.0 (*CH*-Ar), 117.4 ($^2J_{C-P}$ = 11.0 Hz, *CH*═), 116.9 ($^2J_{C-P}$ = 10.2 Hz, *CH*═), 116.8 (d, $^3J_{C-P}$ = 4.4 Hz *CH*-Ar), 116.7 (d, $^3J_{C-P}$ = 3.3 Hz *CH*-Ar), 110.1 (*C*-5), 110.1 (*C*-5), 68.66 (*CH*$(CH_3)_2$), 68.62 (*CH*$(CH_3)_2$), 53.6 (d, $^4J_{C-P}$ = 2.4 Hz, CH_2-N), 53.3 (d, $^4J_{C-P}$ = 2.4 Hz, CH_2-N), 49.7 (*CH*CH_3 l-Ala), 49.5 (*CH*CH_3 l-Ala), 29.1 (CH_2-Ar), 28.5 (d, $^1J_{C-P}$ = 129.8 Hz

CH$_2$P), 28.2 (d, $^1J_{C\text{-}P}$ = 131.2 Hz *CH*$_2$P), 23.5 (*CH*$_2$-Ar), 22.47 (*CH*$_2$-Ar), 22.44 (*CH*$_2$-Ar), 22.42 (*CH*$_2$-Ar), 20.6 (CH(*CH*$_3$)$_2$), 20.56 (CH(*CH*$_3$)$_2$), 20.55 (CH(*CH*$_3$)$_2$), 20.4 (CH(*CH*$_3$)$_2$), 19.9 (d, $^3J_{C\text{-}P}$ = 5.2 Hz, CH*CH*$_3$ l-Ala), 19.1 (d, $^3J_{C\text{-}P}$ = 5.8 Hz, CH*CH*$_3$ l-Ala), 13.3 (d, $^4J_{C\text{-}P}$ = 2.4 Hz, *CH*$_3$, alkene), 13.2 (d, $^4J_{C\text{-}P}$ = 2.2 Hz, *CH*$_3$, alkene), 10.8 (*CH*$_3$, base). **HPLC**: Reverse phase HPLC eluting with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 min, 1 ml/min, λ = 254 nm and 263 nm, showed one peak with Rt 16.85 min. **HRMS (ESI):** *m/z* $[M+Na]^+$ calcd for $C_{26}H_{36}N_3O_6P$: 540.2239, found: 540.2234.

From PrepHPLC also the *Z* isomer ***Z*-8e** was isolated as pale yellow foamy solid (7 mg, 3%). **^{31}P NMR (202 MHz, CD_3OD) δ_P**: 29.41, 28.64. **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 7.33 (s, 1H, *H*-6), 7.05–7.00 (m, 1H, Ar*H*), 6.95–6.89 (m, 1H, Ar*H*), 6.80–6.76 (m, 1H, Ar*H*), 5.53–5.48 (m, 1H, *CH*═), 4.88–4.77 (m, 1H, *CH*(CH$_3$)$_2$), 4.38–4.27 (m, 2H, *CH*$_2$-N), 3.86–3.81 (m, 1H, *CH*CH$_3$ l-Ala), 2.98–2.82 (m, 2H, *CH*$_2$P), 2.65–2.62 (m, 4H, Ar*H*), 1.71–1.68 (m, 7H, Ar*H*, CH$_3$, base), 1.61–1.60 (m, 3H, *CH*$_3$, alkene), 1.20–1.07 (m, 9H, CH*CH*$_3$ l-Ala, CH(*CH*$_3$)$_2$). **^{13}C NMR (125 MHz, CD_3OD) δ_C**: 173.7 (d, $^3J_{C\text{-}P}$ = 3.8 Hz, *C*═O, ester), 173.3 (d, $^3J_{C\text{-}P}$ = 3.8 Hz, *C*═O, ester), 165.3 (*C*-4), 151.7 (*C*-2), 151.6 (*C*-2), 148.8 (d, $^2J_{C\text{-}P}$ = 9.5 Hz, *C*—O, Ph), 148.7 (d, $^2J_{C\text{-}P}$ = 9.9 Hz, *C*—O, Ph), 141.05 (*C*-6), 141.02 (*C*-6), 139.19 (*C*-Ar), 139.16 (*C*-Ar), 135.1 (d, $^3J_{C\text{-}P}$ = 14.2 Hz, *C*═), 134.8 (d, $^3J_{C\text{-}P}$ = 14.2 Hz, *C*═), 128.4 (d, $^3J_{C\text{-}P}$ = 5.1 Hz *C*-Ar), 128.3 (d, $^3J_{C\text{-}P}$ = 5.6 Hz *C*-Ar), 125.4 (*CH*-Ar), 125.3 (*CH*-Ar), 125.1 (*CH*-Ar), 125.0 (*CH*-Ar), 119.3 ($^2J_{C\text{-}P}$ = 11.3 Hz, *CH*═), 119.2 ($^2J_{C\text{-}P}$ = 11.0 Hz, *CH*═), 117.2 (d, $^3J_{C\text{-}P}$ = 3.5 Hz *CH*-Ar), 117.0 (d, $^3J_{C\text{-}P}$ = 3.5 Hz *CH*-Ar), 110.0 (*C*-5), 68.66 (*CH*(CH$_3$)$_2$), 68.63 (*CH*(CH$_3$)$_2$), 49.7 (*CH*CH$_3$ l-Ala), 49.5 (*CH*CH$_3$ l-Ala), 47.3 (*CH*$_2$-N), 29.1 (*CH*$_2$-Ar), 28.5 (d, $^1J_{C\text{-}P}$ = 129.8 Hz *CH*$_2$P), 28.2 (d, $^1J_{C\text{-}P}$ = 131.2 Hz *CH*$_2$P), 26.4 (*CH*$_2$-Ar), 26.3 (*CH*$_2$-Ar), 25.8 (*CH*$_2$-Ar), 25.7 (*CH*$_2$-Ar), 20.5 (*CH*$_3$, alkene), 20.4 (*CH*$_3$, alkene), 19.97 (CH(*CH*$_3$)$_2$), 19.93 (CH(*CH*$_3$)$_2$), 19.7 (d, $^3J_{C\text{-}P}$ = 5.2 Hz, CH*CH*$_3$ l-Ala), 19.0 (d, $^3J_{C\text{-}P}$ = 5.8 Hz, CH*CH*$_3$ l-Ala), 10.7 (*CH*$_3$, base). **HPLC**: Reverse phase HPLC eluting with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 min, 1 ml/min, λ = 254 nm and 263 nm, showed one peak with Rt 17.90 min.

*4.1.3.6. (E)-N^1-(4′-O-(5,6,7,8-Tetrahydro-1-naphthyl)-(benzyloxy-L-alanine)-phosphinyl-2′-methyl-but-2′-enyl)thymine (**E-8f**) and (Z)-N^1-(4′-O-(5,6,7,8-tetrahydro-1-naphthyl)-(benzyloxy-L-alanine)-phosphinyl-2′-methyl-but-2′-enyl)thymine (**Z-8f**)*. Prepared according to the standard procedure **C** for the synthesis of ANP ProTide using *O*-(5,6,7,8-tetrahydro-1-naphthyl)-(benzyloxy-L-alanine)-allylphosphonate **3f** (200 mg, 483.7 μmol) and *N*^{1}-2′-methylallylthymine (174 mg, 967.4 μmol) and Hoveyda-Grubbs 2nd generation catalyst (15 mol%) in dry CH_2Cl_2 (8 ml). After evaporation, the crude was purified by Biotage Isolera One (25 g SNAP cartridge ULTRA, 75 ml/min, gradient eluent system 2-propanol/CH_2Cl_2 1% 1CV, 1–10% 12CV, 10% 2CV), to afford a mixture of the *E* and *Z* isomers. The two isomers were then separated by reverse Biotage Isolera One (60 g SNAP cartridge KP-C18-HS, 100 ml/min, isocratic eluent system CH_3CN/H_2O 30–60% 12CV) to afford the title compound *E* as pale yellow foamy solid (36 mg, 14%). R_f = 0.23 (CH_2Cl_2/2-propanol – 95:5). **^{31}P NMR (202 MHz, CD_3OD) δ_P**: 29.36, 28.51. **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 7.36–7.28 (m, 6H, *H*-6, Ar*H*), 7.16–7.12 (m, 1H, Ar*H*), 7.04–6.95 (m, 1H, Ar*H*), 6.89–6.85 (m, 1H, Ar*H*), 5.49–5.42 (m, 1H, *CH*═), 5.15, 5.12 (ABq, J_{AB} = 12.2 Hz, 1H, *CH*$_2$Ph), 5.07, 5.05 (ABq, J_{AB} = 12.6 Hz, 1H, *CH*$_2$Ph), 4.31–4.22 (m, 2H, *CH*$_2$-N), 4.09–4.00 (m, 1H, *CH*CH$_3$ l-Ala), 2.90–2.77 (m, 2H, *CH*$_2$P), 2.74 (bs, 2H, Ar*H*), 2.66 (bs, 2H, Ar*H*), 1.83 (s, 3H, CH$_3$, base), 1.76–1.75 (m, 4H, Ar*H*), 1.66 (d, *J* = 2.9 Hz 1.8H, *CH*$_3$, alkene), 1.64 (d, *J* = 3.1 Hz 1.2H, *CH*$_3$, alkene), 1.31 (d, *J* = 7.0 Hz, 1.5H, CH*CH*$_3$ l-Ala), 1.26 (d, *J* = 7.1 Hz, 1.5H, CH*CH*$_3$ l-Ala). **^{13}C NMR (125 MHz, CD_3OD) δ_C**: 173.8 (d, $^3J_{C\text{-}P}$ = 3.8 Hz, *C*═O, ester), 173.4 (d, $^3J_{C\text{-}P}$ = 3.5 Hz, *C*═O, ester), 165.38 (*C*-4), 165.37 (*C*-4), 151.7 (*C*-2), 151.6 (*C*-2), 148.8 (d, $^2J_{C\text{-}P}$ = 9.8 Hz, *C*—O, Ph), 148.7 (d, $^2J_{C\text{-}P}$ = 9.5 Hz, *C*—O, Ph), 140.9 (*C*-6), 139.2 (*C*-Ar), 139.1 (*C*-Ar), 135.9 (*C*-Ar), 135.8 (*C*-Ar), 135.1 (d, $^3J_{C\text{-}P}$ = 14.5 Hz, *C*═), 134.8 (d, $^3J_{C\text{-}P}$ = 13.9 Hz, *C*═), 128.4 (d, $^3J_{C\text{-}P}$ = 5.4 Hz *C*-Ar), 128.3 (d, $^3J_{C\text{-}P}$ = 5.7 Hz *C*-Ar), 128.2 (*CH*-Ar), 128.1 (*CH*-Ar), 127.96 (*CH*-Ar), 127.92 (*CH*-Ar), 127.8 (*CH*-Ar), 125.4 (*CH*-Ar), 125.3 (*CH*-Ar), 125.1 (*CH*-Ar), 125.0 (*CH*-Ar), 117.4 ($^2J_{C\text{-}P}$ = 10.9 Hz, *CH*═), 116.8 ($^2J_{C\text{-}P}$ = 10.4 Hz, *CH*═), 116.7 (d, $^3J_{C\text{-}P}$ = 3.2 Hz *CH*-Ar), 116.6 (d, $^3J_{C\text{-}P}$ = 3.2 Hz *CH*-Ar), 110.09 (*C*-5), 110.06 (*C*-5), 66.5 (*CH*$_2$Ph), 66.4 (*CH*$_2$Ph), 53.5 (d, $^4J_{C\text{-}P}$ = 2.1 Hz, *CH*$_2$-N), 53.3 (d, $^4J_{C\text{-}P}$ = 2.4 Hz, *CH*$_2$-N), 49.6 (*CH*CH$_3$ l-Ala), 49.5 (*CH*CH$_3$ l-Ala), 29.1 (*CH*$_2$-Ar), 28.2 (d, $^1J_{C\text{-}P}$ = 130.8 Hz *CH*$_2$P), 28.2 (d, $^1J_{C\text{-}P}$ = 130.8 Hz *CH*$_2$P), 23.3 (*CH*$_2$-Ar), 22.45 (*CH*$_2$-Ar), 22.43 (*CH*$_2$-Ar), 22.40 (*CH*$_2$-Ar), 19.7–19.6 (m, CH*CH*$_3$ l-Ala, *CH*$_3$, alkene), 19.0 (d, $^3J_{C\text{-}P}$ = 5.7 Hz, CH*CH*$_3$ l-Ala), 10.8 (*CH*$_3$, base). **HPLC**: Reverse phase HPLC eluting with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 min, 1 ml/min, λ = 254 nm and 263 nm, showed one peak with Rt 18.44 min. **HRMS (ESI):** *m/z* $[M+Na]^+$ calcd for $C_{30}H_{36}N_3O_6P$: 588.2239, found: 588.2234.

From PrepHPLC also the *Z* isomer ***Z*-8f** was isolated as pale yellow foamy solid (18 mg, 7%). **^{31}P NMR (202 MHz, CD_3OD) δ_P**: 29.38, 28.63. **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 7.42–7.33 (m, 6H, *H*-6, Ar*H*), 7.15–7.12 (m, 1H, Ar*H*), 7.07–6.95 (m, 1H, Ar*H*), 6.92–6.86 (m, 1H, Ar*H*), 5.60–5.55 (m, 1H, *CH*═), 5.15 (AB app s, 1H, *CH*$_2$Ph), 5.07 (AB app s, 1H, *CH*$_2$Ph), 4.46–4.26 (m, 2H, *CH*$_2$-N), 4.11–4.03 (m, 1H, *CH*CH$_3$ l-Ala), 3.07–2.90 (m, 2H, *CH*$_2$P), 2.76–2.70 (m, 4H, Ar*H*), 1.83–1.77 (m, 7H, Ar*H*, CH$_3$, base), 1.69 (d, *J* = 5.2 Hz 1.8H, *CH*$_3$, alkene), 1.66 (d, *J* = 5.2 Hz 1.2H, *CH*$_3$, alkene), 1.34 (d, *J* = 6.9 Hz, 1.5H, CH*CH*$_3$ l-Ala), 1.24 (d, *J* = 6.9 Hz, 1.5H, CH*CH*$_3$ l-Ala). **^{13}C NMR (125 MHz, CD_3OD) δ_C**: 173.8 (d, $^3J_{C\text{-}P}$ = 3.8 Hz, *C*═O, ester), 173.4 (d, $^3J_{C\text{-}P}$ = 3.5 Hz, *C*═O, ester), 165.3 (*C*-4), 151.7 (*C*-2), 148.8 (d, $^2J_{C\text{-}P}$ = 9.2 Hz, *C*—O, Ph), 148.7 (d, $^2J_{C\text{-}P}$ = 9.2 Hz, *C*—O, Ph), 141.1 (*C*-6), 141.0 (*C*-6), 139.25 (*C*-Ar), 139.21 (*C*-Ar), 135.9 (*C*-Ar), 135.8 (*C*-Ar), 134.4 (d, $^3J_{C\text{-}P}$ = 14.3 Hz, *C*═), 134.2 (d, $^3J_{C\text{-}P}$ = 13.5 Hz, *C*═), 128.7 (d, $^3J_{C\text{-}P}$ = 5.9 Hz *C*-Ar), 128.6 (d, $^3J_{C\text{-}P}$ = 5.0 Hz *C*-Ar), 128.18 (*CH*-Ar), 128.15 (*CH*-Ar), 127.94 (*CH*-Ar), 127.91 (*CH*-Ar), 127.87 (*CH*-Ar), 127.84 (*CH*-Ar), 125.4 (*CH*-Ar), 125.3 (*CH*-Ar), 125.2 (*CH*-Ar), 125.0 (*CH*-Ar), 119.2 ($^2J_{C\text{-}P}$ = 10.9 Hz, *CH*═), 119.0 ($^2J_{C\text{-}P}$ = 11.8 Hz, *CH*═), 117.2 (d, $^3J_{C\text{-}P}$ = 3.3 Hz *CH*-Ar), 116.9 (d, $^3J_{C\text{-}P}$ = 2.5 Hz *CH*-Ar), 110.05 (*C*-5), 110.02 (*C*-5), 66.5 (*CH*$_2$Ph), 66.4 (*CH*$_2$Ph), 49.7 (*CH*CH$_3$ l-Ala), 49.5 (*CH*CH$_3$ l-Ala), 47.2 (*CH*$_2$-N), 29.1 (*CH*$_2$-Ar), 28.3 (d, $^1J_{C\text{-}P}$ = 129.4 Hz *CH*$_2$P), 28.1 (d, $^1J_{C\text{-}P}$ = 130.2 Hz *CH*$_2$P), 26.4 (*CH*$_2$-Ar), 26.3 (*CH*$_2$-Ar), 25.8 (*CH*$_2$-Ar), 25.7 (*CH*$_2$-Ar), 19.7–19.6 (m, CH*CH*$_3$ l-Ala, *CH*$_3$, alkene), 18.8 (d, $^3J_{C\text{-}P}$ = 5.9 Hz, CH*CH*$_3$ l-Ala), 10.7 (*CH*$_3$, base). **HPLC**: Reverse phase HPLC eluting with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 min, 1 ml/min, λ = 254 nm and 263 nm, showed one peak with Rt 19.31 min.

*4.1.3.7. (E)-N^1-(4′-O-(1-Naphthyl)-(isopropyloxy-L-alanine)-phosphinyl-2′-methyl-but-2′-enyl)uracil (**E-10a**)*. Prepared according to the standard procedure **C** for the synthesis of ANP ProTide using *O*-(1-naphthyl)-(isopropyloxy-L-alanine)-allylphosphonate **3a** (150 mg, 415 μmol) and *N*^{1}-2′-methylallyluracil (137 mg, 830.1 μmol) and Hoveyda-Grubbs 2nd generation catalyst (15 mol%) in dry CH_2Cl_2 (8 ml). After evaporation, the crude was purified by Biotage Isolera One (50 g SNAP cartridge ULTRA, 100 ml/min, gradient eluent system MeOH/CH_2Cl_2 1% 1CV, 1–10% 12CV, 10% 2CV), to afford a mixture of the *E* and *Z* isomers. The two isomers were then separated by PrepHPLC (20 ml/min, isocratic eluting system CH_3CN/H_2O – 35/65, 30 min), to afford the title compound as pale yellow foamy solid (28 mg, 14%). R_f = 0.24 (CH_2Cl_2/MeOH – 96:4). **^{31}P NMR (202 MHz, CD_3OD) δ_P**: 30.28, 29.49. **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 8.14–8.13 (m, 1H, Ar*H*), 7.88–7.84 (m, 1H, Ar*H*), 7.70–7.67 (m, 1H, Ar*H*), 7.58–7.49 (m, 3H, Ar*H*), 7.44–7.38 (m, 2H, *H*-6, Ar*H*), 5.61–5.57 (m, 1.5H, *CH*═, *H*-5), 5.51–5.47 (m, 0.5H, *CH*═), 4.93 (sept, *J* = 6.5 Hz, 0.5H, *CH*(CH$_3$)$_2$), 4.88–4.84 (m, 0.5H, *CH*(CH$_3$)$_2$), 4.33–4.25 (m, 2H, *CH*$_2$-N), 4.04–3.97 (m, 1H, *CH*CH$_3$ l-Ala), 3.08–2.90 (m, 2H, *CH*$_2$P), 1.65 (bs, 3H, *CH*$_3$, alkene), 1.27 (d, *J* = 7.0 Hz, 1.5H, CH*CH*$_3$ l-Ala), 1.20 (d, *J* = 6.2 Hz, 1.5H, CH(*CH*$_3$)$_2$), 1.19 (d, *J* = 6.2 Hz, 1.5H, CH(*CH*$_3$)$_2$), 1.17 (d, *J* = 6.9 Hz, 1.5H, CH*CH*$_3$ l-Ala), 1.12 (d, *J* = 6.2 Hz, 1.5H, CH(*CH*$_3$)$_2$),

1.15 (d, J = 6.2 Hz, 1.5H, CH(CH_3)$_2$). **^{13}C NMR (125 MHz, CD_3OD) δ_C**: 173.6 (d, $^3J_{C\text{-}P}$ = 4.3 Hz, *C*═O, ester), 173.2 (d, $^3J_{C\text{-}P}$ = 4.1 Hz, *C*═O, ester), 165.17 (*C*-4), 165.15 (*C*-4), 151.5 (*C*-2), 151.4 (*C*-2), 146.5 (d, $^2J_{C\text{-}P}$ = 9.7 Hz, *C*—O, Ph), 146.3 (d, $^2J_{C\text{-}P}$ = 9.7 Hz, *C*—O, Ph), 145.2 (*C*-6), 145.1 (*C*-6), 135.2 (d, $^3J_{C\text{-}P}$ = 14.5 Hz, *C*═), 135.4 (d, $^3J_{C\text{-}P}$ = 14.5 Hz, *C*═), 134.9 (*C*-Ar), 127.5 (*CH*-Ar), 127.4 (*CH*-Ar), 126.8 (d, $^3J_{C\text{-}P}$ = 4.9 Hz *C*-Ar), 126.6 (d, $^3J_{C\text{-}P}$ = 5.1 Hz *C*-Ar), 126.3 (*CH*-Ar), 126.1 (*CH*-Ar), 125.2 (*CH*-Ar), 125.1 (*CH*-Ar), 124.3 (*CH*-Ar), 124.2 (*CH*-Ar), 121.5 (*CH*-Ar), 121.3 (*CH*-Ar), 117.4 ($^2J_{C\text{-}P}$ = 11.0 Hz, *CH*═), 116.9 ($^2J_{C\text{-}P}$ = 11.0 Hz, *CH*═), 115.4 (d, $^3J_{C\text{-}P}$ = 3.8 Hz *CH*-Ar), 115.1 (d, $^3J_{C\text{-}P}$ = 3.8 Hz *CH*-Ar), 101.2 (*C*-5), 68.69 (*CH*(CH$_3$)$_2$), 68.66 (*CH*(CH$_3$)$_2$), 53.7 (d, $^4J_{C\text{-}P}$ = 2.3 Hz, CH_2-N), 53.5 (d, $^4J_{C\text{-}P}$ = 2.3 Hz, CH_2-N), 49.7 (*CH*CH$_3$ l-Ala), 49.5 (*CH*CH$_3$ l-Ala), 28.3 (d, $^1J_{C\text{-}P}$ = 128.9 Hz CH_2P), 28.1 (d, $^1J_{C\text{-}P}$ = 129.8 Hz CH_2P), 20.6 (CH(*CH*$_3$)$_2$), 20.56 (CH(*CH*$_3$)$_2$), 20.52 (CH(*CH*$_3$)$_2$), 20.4 (CH(*CH*$_3$)$_2$), 19.8 (d, $^3J_{C\text{-}P}$ = 5.8 Hz, CH*CH*$_3$ l-Ala), 19.1 (d, $^3J_{C\text{-}P}$ = 5.5 Hz, CH*CH*$_3$ l-Ala), 13.3 (d, $^4J_{C\text{-}P}$ = 2.4 Hz, CH_3, alkene), 13.2 (d, $^4J_{C\text{-}P}$ = 2.2 Hz, CH_3, alkene). **HPLC**: Reverse phase HPLC eluting with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 min, 1 ml/min, λ = 254 nm and 263 nm, showed one peak with Rt 15.57 min. **HRMS (ESI):** *m/z* [M + Na]$^+$ calcd for $C_{25}H_{30}N_3O_6P$: 522.1770, found: 522.1764.

*4.1.3.8. (E)-N^1-(4′-O-(1-naphthyl)-(benzyloxy-L-alanine)-phosphinyl-2′-methyl-but-2′-enyl)uracil (**E-10b**)*. Prepared according to the standard procedure **C** for the synthesis of ANP ProTide using *O*-(1-naphthyl)-(benzyloxy-*L*-alanine)-allylphosphonate **3b** (240 mg, 586.1 μmol) and *N*^{1}-2′-methylallyluracil (195 mg, 1.17 mmol) and Hoveyda-Grubbs 2nd generation catalyst (15 mol%) in dry CH_2Cl_2 (10 ml). After evaporation, the crude was purified by Biotage Isolera One (120 g ZIP cartridge KP-SIL, 100 ml/min, gradient eluent system MeOH/CH_2Cl_2 1% 1CV, 1–10% 12CV, 10% 2CV), to afford a mixture of the *E* and *Z* isomers. The two isomers were then separated by PrepHPLC (20 ml/min, isocratic eluting system CH_3CN/H_2O – 40/60, 30 min), to afford the title compound as pale yellow foamy solid (13 mg, 5%). R_f = 0.33 (CH_2Cl_2/MeOH – 95:5). **^{31}P NMR (202 MHz, CD_3OD) δ_P**: 30.33, 29.48. **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 8.01–7.99 (m, 1H, Ar*H*), 7.78–7.64 (m, 1H, Ar*H*), 7.59–7.55 (m, 1H, Ar*H*), 7.44–7.13 (m, 10*H*, *H*-6, Ar*H*), 5.48 (d, J = 7.9 Hz, 1H, *H*-5), 5.42–5.34 (m, 1H, *CH*═), 5.01, 4.96 (ABq, J_{AB} = 12.2 Hz, 1H, CH_2Ph), 4.88, 4.84 (ABq, J_{AB} = 12.2 Hz, 1H, CH_2Ph), 4.16 (bs, 2H, CH_2-N), 4.00–3.94 (m, 1H, *CH*CH$_3$ l-Ala), 2.90–2.75 (m, 2H, CH_2P), 1.51 (d, J = 3.4 Hz 1.5H, CH_3, alkene), 1.49 (d, J = 3.5 Hz 1.5H, CH_3, alkene), 1.15 (d, J = 7.0 Hz, 1.5H, CH*CH*$_3$ l-Ala), 1.06 (d, J = 7.1 Hz, 1.5H, CH*CH*$_3$ l-Ala). **^{13}C NMR (125 MHz, CD_3OD) δ_C**: 173.7 (d, $^3J_{C\text{-}P}$ = 4.3 Hz, *C*═O, ester), 173.3 (d, $^3J_{C\text{-}P}$ = 4.1 Hz, *C*═O, ester), 163.5 (*C*-4), 151.5 (*C*-2), 151.4 (*C*-2), 146.5 (d, $^2J_{C\text{-}P}$ = 9.9 Hz, *C*—O, Ph), 146.3 (d, $^2J_{C\text{-}P}$ = 9.7 Hz, *C*—O, Ph), 145.2 (*C*-6), 145.1 (*C*-6), 135.8 (*C*-Ar), 135.7 (*C*-Ar), 135.3 (d, $^3J_{C\text{-}P}$ = 14.1 Hz, *C*═), 135.2 (d, $^3J_{C\text{-}P}$ = 14.8 Hz, *C*═), 134.9 (*C*-Ar), 128.18 (*CH*-Ar), 128.10 (*CH*-Ar), 127.9 (*CH*-Ar), 127.8 (*CH*-Ar), 126.7 (d, $^3J_{C\text{-}P}$ = 4.9 Hz *C*-Ar), 126.6 (d, $^3J_{C\text{-}P}$ = 4.7 Hz *C*-Ar), 126.3 (*CH*-Ar), 126.08 (*CH*-Ar), 126.06 (*CH*-Ar), 125.17 (*CH*-Ar), 125.10 (*CH*-Ar), 124.3 (*CH*-Ar), 124.2 (*CH*-Ar), 121.4 (*CH*-Ar), 121.3 (*CH*-Ar), 117.3 ($^2J_{C\text{-}P}$ = 11.1 Hz, *CH*═), 116.8 ($^2J_{C\text{-}P}$ = 11.7 Hz, *CH*═), 115.17 (d, $^3J_{C\text{-}P}$ = 3.9 Hz *CH*-Ar), 115.10 (d, $^3J_{C\text{-}P}$ = 3.9 Hz *CH*-Ar), 101.2 (*C*-5), 66.5 (CH_2Ph), 66.4 (CH_2Ph), 53.7 (d, $^4J_{C\text{-}P}$ = 2.6 Hz, CH_2-N), 53.5 (d, $^4J_{C\text{-}P}$ = 2.6 Hz, CH_2-N), 49.6 (*CH*CH$_3$ l-Ala), 49.4 (*CH*CH$_3$ l-Ala), 28.2 (d, $^1J_{C\text{-}P}$ = 129.0 Hz CH_2P), 28.0 (d, $^1J_{C\text{-}P}$ = 129.9 Hz CH_2P), 19.6 (d, $^3J_{C\text{-}P}$ = 5.7 Hz, CH*CH*$_3$ l-Ala), 18.9 (d, $^3J_{C\text{-}P}$ = 5.7 Hz, CH*CH*$_3$ l-Ala), 13.2 (d, $^4J_{C\text{-}P}$ = 2.4 Hz, CH_3, alkene), 13.1 (d, $^4J_{C\text{-}P}$ = 2.4 Hz, CH_3, alkene). **HPLC**: Reverse phase HPLC eluting with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 min, 1 ml/min, λ = 254 nm and 263 nm, showed one peak with Rt 15.87 min. **HRMS (ESI):** *m/z* [M + Na]$^+$ calcd for $C_{29}H_{30}N_3O_6P$: 570.1770, found: 570.1764.

*4.1.3.9. (E)-N^1-(4′-O-Phenyl-(isopropyloxy-L-alanine)-phosphinyl-2′-methyl-but-2′-enyl)uracil (**E-10c**)*. Prepared according to the standard procedure **C** for the synthesis of ANP ProTide using *O*-phenyl-(isopropyloxy-*L*-alanine)-allylphosphonate **3c** (140 mg, 449.7 μmol) and *N*^{1}-2′-methylallyluracil (150 mg, 1.11 mmol) and Hoveyda-Grubbs 2nd generation catalyst (15 mol%) in dry CH_2Cl_2 (8 ml). After evaporation, the crude was purified by Biotage Isolera One (25 g SNAP cartridge ULTRA, 75 ml/min, gradient eluent system MeOH/CH_2Cl_2 1% 1CV, 1–10% 12CV, 10% 2CV), to afford a mixture of the *E* and *Z* isomers. The two isomers were then separated by PrepHPLC (20 ml/min, gradient eluting system CH_3CN/H_2O from 10/90 to 100/0, 30 min), to afford the title compound as pale yellow foamy solid (20 mg, 10%). R_f = 0.42 (CH_2Cl_2/MeOH – 95:5). **^{31}P NMR (202 MHz, CD_3OD) δ_P**: 29.74, 28.97. **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 7.53 (d, J = 7.8 Hz, 0.3H, *H*-6), 7.50 (d, J = 7.8 Hz, 0.7H, *H*-6), 7.38–7.33 (m, 2H, Ar*H*), 7.23–7.16 (m, 3H, Ar*H*), 5.67 (d, J = 7.9 Hz, 1H, *H*-5), 5.54 (q, J = 7.0 Hz, 0.3H, *CH*═), 5.46 (q, J = 7.0 Hz, 0.7H, *CH*═), 5.02–4.89 (m, 1H, *CH*(CH$_3$)$_2$), 4.36–4.35 (m, 2H, CH_2-N), 3.98–3.91 (m, 1H, *CH*CH$_3$ l-Ala), 2.94–2.77 (m, 2H, CH_2P), 1.72–1.71 (m, 3H, CH_3, alkene), 1.29 (d, J = 7.0 Hz, 2.1H, CH*CH*$_3$ l-Ala), 1.25 (d, J = 6.7 Hz, 0.9H, CH(*CH*$_3$)$_2$), 1.23 (d, J = 6.2 Hz, 0.9H, CH(*CH*$_3$)$_2$), 1.21–1.19 (m, 5.1H, CH*CH*$_3$ l-Ala, CH(*CH*$_3$)$_2$). **^{13}C NMR (125 MHz, CD_3OD) δ_C**: 173.5 (d, $^3J_{C\text{-}P}$ = 4.7 Hz, *C*═O, ester), 173.2 (d, $^3J_{C\text{-}P}$ = 4.1 Hz, *C*═O, ester), 165.2 (*C*-4), 151.5 (*C*-2), 151.4 (*C*-2), 150.6 (d, $^2J_{C\text{-}P}$ = 9.6 Hz, *C*—O, Ph), 150.4 (d, $^2J_{C\text{-}P}$ = 9.3 Hz, *C*—O, Ph), 145.32 (*C*-6), 145.30 (*C*-6), 135.2 (d, $^3J_{C\text{-}P}$ = 14.5 Hz, *C*═), 134.8 (d, $^3J_{C\text{-}P}$ = 14.2 Hz, *C*═), 129.3 (*CH*-Ar), 124.6 (*CH*-Ar), 124.4 (*CH*-Ar), 120.6 (d, $^3J_{C\text{-}P}$ = 4.6 Hz *CH*-Ar), 120.4 (d, $^3J_{C\text{-}P}$ = 4.3 Hz *CH*-Ar), 117.6 (d, $^2J_{C\text{-}P}$ = 11.2 Hz, *CH*═), 116.9 (d, $^2J_{C\text{-}P}$ = 10.7 Hz, *CH*═), 101.2 (*C*-5), 68.67 (*CH*(CH$_3$)$_2$), 68.64 (*CH*(CH$_3$)$_2$), 53.8 (d, $^4J_{C\text{-}P}$ = 2.4 Hz, CH_2-N), 53.5 (d, $^4J_{C\text{-}P}$ = 2.1 Hz, CH_2-N), 49.6 (*CH*CH$_3$ l-Ala), 49.4 (*CH*CH$_3$ l-Ala), 28.2 (d, $^1J_{C\text{-}P}$ = 129.7 Hz, CH_2P), 28.0 (d, $^1J_{C\text{-}P}$ = 130.3 Hz, CH_2P), 20.6 (CH(*CH*$_3$)$_2$), 20.5 (CH(*CH*$_3$)$_2$), 20.4 (CH(*CH*$_3$)$_2$), 19.8 (d, $^3J_{C\text{-}P}$ = 5.4 Hz, CH*CH*$_3$ l-Ala), 19.1 (d, $^3J_{C\text{-}P}$ = 5.4 Hz, CH*CH*$_3$ l-Ala), 13.2 (d, $^4J_{C\text{-}P}$ = 2.4 Hz, CH_3, alkene), 13.1 (d, $^4J_{C\text{-}P}$ = 2.4 Hz, CH_3, alkene). **HPLC**: Reverse phase HPLC eluting with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 min, 1 ml/min, λ = 254 nm and 263 nm, showed one peak with Rt 13.16 min. **HRMS (ESI):** *m/z* [M + Na]$^+$ calcd for $C_{21}H_{28}N_3O_6P$: 472.1613, found: 472.1608.

*4.1.3.10. (E)-N^1-(4′-O-Phenyl-(benzyloxy-L-alanine)-phosphinyl-2′-methyl-but-2′-enyl)uracil (**E-10d**)*. Prepared according to the standard procedure **C** for the synthesis of ANP ProTide using *O*-phenyl-(benzyloxy-*L*-alanine)-allylphosphonate **3d** (200 mg, 556.5 μmol) and *N*^{1}-2′-methylallyluracil (184.9 mg, 1.11 mmol) and Hoveyda-Grubbs 2nd generation catalyst (15 mol%) in dry CH_2Cl_2 (8 ml). After evaporation, the crude was purified by Biotage Isolera One (25 g SNAP cartridge ULTRA, 75 ml/min, gradient eluent system 2-propanol/CH_2Cl_2 1% 1CV, 1–10% 12CV, 10% 2CV), to afford a mixture of the *E* and *Z* isomers. The two isomers were then separated by PrepHPLC (20 ml/min, isocratic eluting system CH_3CN/H_2O – 35/65, 30 min), to afford the title compound as pale yellow foamy solid (49 mg, 18%). R_f = 0.42 (CH_2Cl_2/2-propanol – 95:5). **^{31}P NMR (202 MHz, CD_3OD) δ_P**: 29.75, 28.94. **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 7.46 (d, J = 7.8 Hz, 1H, *H*-6), 7.37–7.29 (m, 7H, Ar*H*), 7.20–7.14 (m, 3H, Ar*H*), 5.67 (d, J = 7.8 Hz, 1H, *H*-5), 5.50–5.40 (m, 1H, *CH*═), 5.17, 5.14 (ABq, J_{AB} = 12.3 Hz, 1H, CH_2Ph), 5.08 (s app, 1H, CH_2Ph), 4.31–4.29 (m, 2H, CH_2-N), 4.08–4.04 (m, 1H, *CH*CH$_3$ l-Ala), 2.89–2.74 (m, 2H, CH_2P), 1.67–1.65 (m, 3H, CH_3, alkene), 1.30 (d, J = 6.9 Hz, 1.5H, CH*CH*$_3$ l-Ala), 1.22 (d, J = 7.2 Hz, 1.5H, CH*CH*$_3$ l-Ala). **^{13}C NMR (125 MHz, CD_3OD) δ_C**: 173.8 (d, $^3J_{C\text{-}P}$ = 4.4 Hz, *C*═O, ester), 173.4 (d, $^3J_{C\text{-}P}$ = 3.9 Hz, *C*═O, ester), 165.2 (*C*-4), 151.5 (*C*-2), 150.5 (d, $^2J_{C\text{-}P}$ = 9.2 Hz, *C*—O, Ph), 150.3 (d, $^2J_{C\text{-}P}$ = 10.0 Hz, *C*—O, Ph), 145.2 (*C*-6), 135.8 (*C*-Ar), 135.1 (d, $^3J_{C\text{-}P}$ = 14.4 Hz, *C*═), 134.8 (d, $^3J_{C\text{-}P}$ = 14.4 Hz, *C*═), 129.3 (*CH*-Ar), 128.23 (*CH*-Ar), 128.20 (*CH*-Ar), 128.0 (*CH*-Ar), 127.9 (*CH*-Ar), 124.6 (*CH*-Ar), 124.5 (*CH*-Ar), 120.6 (d, $^3J_{C\text{-}P}$ = 4.0 Hz *CH*-Ar), 120.4 (d, $^3J_{C\text{-}P}$ = 4.4 Hz *CH*-Ar), 117.5 (d, $^2J_{C\text{-}P}$ = 10.6 Hz, *CH*═), 116.9 (d, $^2J_{C\text{-}P}$ = 10.6 Hz, *CH*═), 101.2 (*C*-5), 65.5 (CH_2Ph),

66.4 (*CH*$_2$Ph), 53.8 (d, $^4J_{C\text{-}P}$ = 2.2 Hz, *CH*$_2$-N), 53.5 (d, $^4J_{C\text{-}P}$ = 2.4 Hz, *CH*$_2$-N), 49.5 (*CH*CH$_3$ l-Ala), 49.4 (*CH*CH$_3$ l-Ala), 28.2 (d, $^1J_{C\text{-}P}$ = 129.7 Hz, *CH*$_2$P), 28.0 (d, $^1J_{C\text{-}P}$ = 130.1 Hz, *CH*$_2$P), 19.7 (d, $^3J_{C\text{-}P}$ = 5.4 Hz, CH*CH*$_3$ l-Ala), 19.1 (d, $^3J_{C\text{-}P}$ = 5.2 Hz, CH*CH*$_3$ l-Ala), 13.2 (d, $^4J_{C\text{-}P}$ = 2.2 Hz, *CH*$_3$, alkene), 13.1 (d, $^4J_{C\text{-}P}$ = 2.2 Hz, *CH*$_3$, alkene). **HPLC**: Reverse phase HPLC eluting with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 min, 1 ml/min, λ = 254 nm and 263 nm, showed one peak with Rt 14.56 min. **HRMS (ESI):** *m/z* $[M+Na]^+$ calcd for $C_{25}H_{28}N_3O_6P$: 520.1608, found: 520.1608.

*4.1.3.11. (E)-N^1-(4′-O-(5,6,7,8-Tetrahydro-1-naphthyl)-(isopropyloxy-L-alanine)-phosphinyl-2′-methyl-but-2′-enyl)uracil (**E-10e**) and (Z)-N^1-(4′-O-(5,6,7,8-tetrahydro-1-naphthyl)-(isopropyloxy-L-alanine)-phosphinyl-2′-methyl-but-2′-enyl)uracil (**Z-10e**)*. Prepared according to the standard procedure **C** for the synthesis of ANP ProTide using *O*-(5,6,7,8-tetrahydro-1-naphthyl)-(isopropyloxy-*L*-alanine)-allylphosphonate **3e** (200 mg, 547.3 μmol) and *N*^{1}-2′-methylallyluracil (181 mg, 1.09 mmol) and Hoveyda-Grubbs 2nd generation catalyst (15 mol%) in dry CH_2Cl_2 (10 ml). After evaporation, the crude was purified by Biotage Isolera One (25 g SNAP cartridge ULTRA, 75 ml/min, gradient eluent system 2-propanol/CH_2Cl_2 1% 1CV, 1–10% 12CV, 10% 2CV), to afford a mixture of the *E* and *Z* isomers. The two isomers were then separated by PrepHPLC (20 ml/min, isocratic eluting system CH_3CN/H_2O – 35/65, 30 min), to afford the title compound *E* as pale yellow foamy solid (31 mg, 11%). R_f = 0.23 (CH_2Cl_2/2-propanol – 95:5). **^{31}P NMR (202 MHz, CD_3OD) δ_P**: 27.84, 27.00. **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 7.52–7.49 (m, 1H, *H*-6), 7.17–7.12 (m, 1H, Ar*H*), 7.06–7.00 (m, 1H, Ar*H*), 6.90–6.87 (m, 1H, Ar*H*), 5.67 (d, *J* = 7.9 Hz, 1H, *H*-5), 5.58–5.54 (m, 0.5H, *CH*═), 5.49–5.45 (m, 0.5H, *CH*═), 5.02–4.85 (m, 1H, *CH*(CH$_3$)$_2$), 4.35 (bs, 2H, *CH*$_2$-N), 3.99–3.91 (m, 1H, *CH*CH$_3$ l-Ala), 2.97–2.82 (m, 2H, *CH*$_2$P), 2.76 (bs, 2H, Ar*H*), 2.69 (bs, 2H, Ar*H*), 1.80–1.78 (m, 4H, Ar*H*), 1.71 (d, *J* = 2.9 Hz 3H, *CH*$_3$, alkene), 1.30 (d, *J* = 7.0 Hz, 1.5H, CH*CH*$_3$ l-Ala), 1.25–1.24 (m, 4.5H, CH*CH*$_3$ l-Ala, CH(*CH*$_3$)$_2$), 1.19 (d, *J* = 6.3 Hz, 3H, CH(*CH*$_3$)$_2$). **^{13}C NMR (125 MHz, CD_3OD) δ_C**: 173.7 (d, $^3J_{C\text{-}P}$ = 3.9 Hz, *C*═O, ester), 173.2 (d, $^3J_{C\text{-}P}$ = 4.3 Hz, *C*═O, ester), 165.2 (*C*-4), 151.5 (*C*-2), 151.4 (*C*-2), 148.8 (d, $^2J_{C\text{-}P}$ = 9.5 Hz, *C*—O, Ph), 148.6 (d, $^2J_{C\text{-}P}$ = 9.7 Hz, *C*—O, Ph), 145.35 (*C*-6), 145.31 (*C*-6), 139.2 (*C*-Ar), 139.1 (*C*-Ar), 135.0 (d, $^3J_{C\text{-}P}$ = 14.5 Hz, *C*═), 134.6 (d, $^3J_{C\text{-}P}$ = 14.3 Hz, *C*═), 128.5 (d, $^3J_{C\text{-}P}$ = 5.4 Hz *C*-Ar), 128.3 (d, $^3J_{C\text{-}P}$ = 5.4 Hz *C*-Ar), 125.4 (*CH*-Ar), 125.3 (*CH*-Ar), 125.15 (*CH*-Ar), 125.10 (*CH*-Ar), 117.6 ($^2J_{C\text{-}P}$ = 11.0 Hz, *CH*═), 117.0 ($^2J_{C\text{-}P}$ = 10.9 Hz, *CH*═), 116.8 (d, $^3J_{C\text{-}P}$ = 3.3 Hz *CH*-Ar), 116.7 (d, $^3J_{C\text{-}P}$ = 3.3 Hz *CH*-Ar), 101.2 (*C*-5), 101.1 (*C*-5), 68.67 (*CH*(CH$_3$)$_2$), 68.63 (*CH*(CH$_3$)$_2$), 53.8 (d, $^4J_{C\text{-}P}$ = 2.4 Hz, *CH*$_2$-N), 53.5 (d, $^4J_{C\text{-}P}$ = 2.4 Hz, *CH*$_2$-N), 49.7 (*CH*CH$_3$ l-Ala), 49.5 (*CH*CH$_3$ l-Ala), 29.1 (*CH*$_2$-Ar), 28.5 (d, $^1J_{C\text{-}P}$ = 129.9 Hz *CH*$_2$P), 28.3 (d, $^1J_{C\text{-}P}$ = 130.9 Hz *CH*$_2$P), 23.3 (*CH*$_2$-Ar), 22.47 (*CH*$_2$-Ar), 22.44 (*CH*$_2$-Ar), 22.42 (*CH*$_2$-Ar), 20.6 (CH(*CH*$_3$)$_2$), 20.5 (CH(*CH*$_3$)$_2$), 20.4 (CH(*CH*$_3$)$_2$), 19.9 (d, $^3J_{C\text{-}P}$ = 5.0 Hz, CH*CH*$_3$ l-Ala), 19.1 (d, $^3J_{C\text{-}P}$ = 5.5 Hz, CH*CH*$_3$ l-Ala), 13.3 (d, $^4J_{C\text{-}P}$ = 2.3 Hz, *CH*$_3$, alkene), 13.2 (d, $^4J_{C\text{-}P}$ = 2.0 Hz, *CH*$_3$, alkene). **HPLC**: Reverse phase HPLC eluting with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 min, 1 ml/min, λ = 254 nm and 263 nm, showed one peak with Rt 16.14 min. **HRMS (ESI):** *m/z* $[M+Na]^+$ calcd for $C_{25}H_{34}N_3O_6P$: 526.2083, found: 526.2077.

From PrepHPLC also the *Z* isomer ***Z*-10e** was isolated as pale yellow foamy solid (2.5 mg, 1%). **^{31}P NMR (202 MHz, CD_3OD) δ_P**: 29.39, 28.62. **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 7.50 (d, *J* = 7.6 Hz, 1H, *H*-6), 7.10–7.00 (m, 1H, Ar*H*), 6.95–6.88 (m, 1H, Ar*H*), 6.80–6.75 (m, 1H, Ar*H*), 5.54–5.38 (m 2H, *CH*═, *H*-5), 4.88–4.78 (m, 1H, *CH*(CH$_3$)$_2$), 4.38–4.29 (m, 2H, *CH*$_2$-N), 3.86–3.80 (m, 1H, *CH*CH$_3$ l-Ala), 2.97–2.84 (m, 2H, *CH*$_2$P), 2.66–2.58 (m, 4H, Ar*H*), 1.71–1.65 (m, 4H, Ar*H*), 1.61–1.54 (m, 3H, *CH*$_3$, alkene), 1.20–1.17 (m, 1.5H, CH*CH*$_3$ l-Ala), 1.13–1.07 (m, 7.5H, CH*CH*$_3$ l-Ala, CH(*CH*$_3$)$_2$), **^{13}C NMR (125 MHz, CD_3OD) δ_C**: 173.7 (d, $^3J_{C\text{-}P}$ = 3.9 Hz, *C*═O, ester), 173.2 (d, $^3J_{C\text{-}P}$ = 4.3 Hz, *C*═O, ester), 165.2 (*C*-4), 151.5 (*C*-2), 151.4 (*C*-2), 148.8 (d, $^2J_{C\text{-}P}$ = 9.5 Hz, *C*—O, Ph), 148.6 (d, $^2J_{C\text{-}P}$ = 9.7 Hz, *C*—O, Ph), 145.5 (*C*-6), 145.4 (*C*-6), 139.2 (*C*-Ar), 135.0 (d, $^3J_{C\text{-}P}$ = 14.5 Hz, *C*═), 134.6 (d, $^3J_{C\text{-}P}$ = 14.3 Hz, *C*═), 128.5 (d, $^3J_{C\text{-}P}$ = 5.4 Hz *C*-Ar), 128.3 (d, $^3J_{C\text{-}P}$ = 5.4 Hz *C*-Ar), 125.3 (*CH*-Ar), 125.2 (*CH*-Ar), 125.1 (*CH*-Ar), 125.0 (*CH*-Ar), 119.5 ($^2J_{C\text{-}P}$ = 10.1 Hz, *CH*═), 119.4 ($^2J_{C\text{-}P}$ = 10.8 Hz, *CH*═), 117.1 (d, $^3J_{C\text{-}P}$ = 3.3 Hz *CH*-Ar), 116.8 (d, $^3J_{C\text{-}P}$ = 3.3 Hz *CH*-Ar), 101.1 (*C*-5), 101.0 (*C*-5), 68.6 (*CH*(CH$_3$)$_2$), 49.7 (*CH*CH$_3$ l-Ala), 49.5 (*CH*CH$_3$ l-Ala), 47.0 (*CH*$_2$-N), 29.1 (*CH*$_2$-Ar), 28.2 (d, $^1J_{C\text{-}P}$ = 128.2 Hz *CH*$_2$P), 28.0 (d, $^1J_{C\text{-}P}$ = 130.5 Hz *CH*$_2$P), 23.4 (*CH*$_2$-Ar), 23.3 (*CH*$_2$-Ar), 22.47 (*CH*$_2$-Ar), 22.43 (*CH*$_2$-Ar), 20.57 (CH(*CH*$_3$)$_2$), 20.53 (CH(*CH*$_3$)$_2$), 20.4 (CH(*CH*$_3$)$_2$), 19.7 (d, $^3J_{C\text{-}P}$ = 4.7 Hz, CH*CH*$_3$ l-Ala), 19.0 (d, $^3J_{C\text{-}P}$ = 5.4 Hz, CH*CH*$_3$ l-Ala), 13.3 (d, $^4J_{C\text{-}P}$ = 2.7 Hz, *CH*$_3$, alkene). **HPLC**: Reverse phase HPLC eluting with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 min, 1 ml/min, λ = 254 nm and 263 nm, showed one peak with Rt 16.82 min.

*4.1.3.12. (E)-N^1-(4′-O-(5,6,7,8-Tetrahydro-1-naphthyl)-(benzyloxy-L-alanine)-phosphinyl-2′-methyl-but-2′-enyl)uracil (**E-10f**)*. Prepared according to the standard procedure **C** for the synthesis of ANP ProTide using *O*-(5,6,7,8-tetrahydro-1-naphthyl)-(benzyloxy-*L*-alanine)-allylphosphonate **3f** (200 mg, 483.7 μmol) and *N*^{1}-2′-methylallyluracil (160 mg, 967.4 μmol) and Hoveyda-Grubbs 2nd generation catalyst (15 mol%) in dry CH_2Cl_2 (8 ml). After evaporation, the crude was purified by Biotage Isolera One (25 g SNAP cartridge ULTRA, 75 ml/min, gradient eluent system 2-propanol/CH_2Cl_2 1% 1CV, 1–10% 12CV, 10% 2CV), to afford a mixture of the *E* and *Z* isomers. The two isomers were then separated by PrepHPLC (20 ml/min, isocratic eluting system CH_3CN/H_2O – 40/60, 30 min), to afford the title compound as pale yellow foamy solid (14 mg, 5%). R_f = 0.25 (CH_2Cl_2/2-propanol – 95:5). **^{31}P NMR (202 MHz, CD_3OD) δ_P**: 29.33, 28.46. **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 7.34 (d, *J* = 7.8 Hz, 1H, *H*-6), 7.26–7.18 (m, 5H, Ar*H*), 7.03–6.99 (m, 1H, Ar*H*), 6.93–6.83 (m, 1H, Ar*H*), 6.77–6.73 (m, 1H, Ar*H*), 5.54 (d, *J* = 7.8 Hz, 0.6H, *H*-5), 5.53 (d, *J* = 7.9 Hz, 0.4H, *H*-5), 5.39–5.29 (m, 1H, *CH*═), 5.04, 5.01 (ABq, J_{AB} = 12.2 Hz, 1H, *CH*$_2$Ph), 4.95, 4.94 (ABq, J_{AB} = 12.2 Hz, 1H, *CH*$_2$Ph), 4.19–4.17 (m, 2H, *CH*$_2$-N), 3.97–3.88 (m, 1H, *CH*CH$_3$ l-Ala), 2.78–2.765 (m, 2H, *CH*$_2$P), 2.63 (bs, 2H, Ar*H*), 2.56 (bs, 2H, Ar*H*), 1.67–1.62 (m, 4H, Ar*H*), 1.54 (d, *J* = 3.8 Hz 1.8H, *CH*$_3$, alkene), 1.52 (d, *J* = 3.9 Hz 1.2H, *CH*$_3$, alkene), 1.20 (d, *J* = 6.9 Hz, 1.8H, CH*CH*$_3$ l-Ala), 1.14 (d, *J* = 7.0 Hz, 1.2H, CH*CH*$_3$ l-Ala). **^{13}C NMR (125 MHz, CD_3OD) δ_C**: 173.9 (d, $^3J_{C\text{-}P}$ = 4.0 Hz, *C*═O, ester), 173.4 (d, $^3J_{C\text{-}P}$ = 4.0 Hz, *C*═O, ester), 165.2 (*C*-4), 151.5 (*C*-2), 151.4 (*C*-2), 148.8 (d, $^2J_{C\text{-}P}$ = 9.1 Hz, *C*—O, Ph), 148.7 (d, $^2J_{C\text{-}P}$ = 9.7 Hz, *C*—O, Ph), 145.3 (*C*-6), 145.2 (*C*-6), 139.2 (*C*-Ar), 139.1 (*C*-Ar), 135.9 (*C*-Ar), 135.8 (*C*-Ar), 134.9 (d, $^3J_{C\text{-}P}$ = 14.7 Hz, *C*═), 134.7 (d, $^3J_{C\text{-}P}$ = 14.7 Hz, *C*═), 128.4 (d, $^3J_{C\text{-}P}$ = 4.7 Hz *C*-Ar), 128.3 (d, $^3J_{C\text{-}P}$ = 4.7 Hz *C*-Ar), 128.2 (*CH*-Ar), 128.1 (*CH*-Ar), 127.9 (*CH*-Ar), 127.8 (*CH*-Ar), 125.4 (*CH*-Ar), 125.3 (*CH*-Ar), 125.15 (*CH*-Ar), 125.08 (*CH*-Ar), 117.5 ($^2J_{C\text{-}P}$ = 10.9 Hz, *CH*═), 117.0 ($^2J_{C\text{-}P}$ = 10.9 Hz, *CH*═), 116.8 (d, $^3J_{C\text{-}P}$ = 3.2 Hz *CH*-Ar), 116.6 (d, $^3J_{C\text{-}P}$ = 3.2 Hz *CH*-Ar), 101.17 (*C*-5), 66.5 (*CH*$_2$Ph), 66.4 (*CH*$_2$Ph), 53.8 (d, $^4J_{C\text{-}P}$ = 2.5 Hz, *CH*$_2$-N), 53.5 (d, $^4J_{C\text{-}P}$ = 2.5 Hz, *CH*$_2$-N), 49.6 (*CH*CH$_3$ l-Ala), 49.5 (*CH*CH$_3$ l-Ala), 29.1 (*CH*$_2$-Ar), 28.4 (d, $^1J_{C\text{-}P}$ = 130.0 Hz *CH*$_2$P), 28.2 (d, $^1J_{C\text{-}P}$ = 130.8 Hz *CH*$_2$P), 23.3 (*CH*$_2$-Ar), 22.44 (*CH*$_2$-Ar), 22.42 (*CH*$_2$-Ar), 22.39 (*CH*$_2$-Ar), 19.7 (d, $^3J_{C\text{-}P}$ = 5.4 Hz, CH*CH*$_3$ l-Ala), 19.0 (d, $^3J_{C\text{-}P}$ = 5.6 Hz, CH*CH*$_3$ l-Ala), 13.2 (d, $^4J_{C\text{-}P}$ = 2.3 Hz, *CH*$_3$, alkene), 13.1 (d, $^4J_{C\text{-}P}$ = 2.4 Hz, *CH*$_3$, alkene). **HPLC**: Reverse phase HPLC eluting with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 min, 1 ml/min, λ = 254 nm and 263 nm, showed one peak with Rt 17.66 min. **HRMS (ESI):** *m/z* $[M+Na]^+$ calcd for $C_{29}H_{34}N_3O_6P$: 574.2083, found: 574.2077.

*4.1.4. bis(Benzyloxy-L-alanine)-allylphosphonate (**11**)*

In a round bottom flask, under an argon atmosphere, 2,6-Lutidine (1.55 ml. 13.22 mmol) and TMSBr, (2.20 ml, 16.65 mmol) were added to a solution of dimethyl allylphosphonate (500 mg, 3.33 mmol), in anhydrous acetonitrile (25 ml). The mixture was stirred 16 h at room temperature and then the volatiles evaporated without any contact with air. Then the flask was charged with dry aminoacid ester hydrochloride

(3.6 g, 16.65 mmol), dry triethylamine (6.9 ml, 49.96 mmol) and dry pyridine (10 ml) and heated to 50 °C to obtain a homogenous solution. To this mixture was then added a solution of aldrithiol-2 (4.40 g, 19.98 mmol) and triphenylphosphine (5.24 g, 19.98 mmol) in dry pyridine (10 ml) under argon atmosphere. The resulting mixture was stirred at 50 °C for 16 h. After evaporating all the volatiles, the residue was purified by Biotage Isolera One (100 g SNAP cartridge ULTRA, 100 ml/min, gradient eluent system EtOAc/Hexane 10% 1CV, 10–100% 12CV, 100% 2CV and 50 g SNAP cartridge ULTRA, 100 ml/min, gradient eluent system MeOH/EtOAc 0% 1CV, 0–20% 15CV, 20% 3CV), to afford the title compound as a yellow oil (770 mg, 52%). R_f = 0.44 (EtOAc/MeOH – 98:2). **^{31}P NMR (202 MHz, CD_3OD) δ_P**: 27.47. **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 7.39–7.29 (m, 10*H*, Ar*H*), 5.88–5.79 (m, 1H, *CH*═), 5.19–5.09 (m, 6H, CH_2═, 2xCH_2Ph), 4.07–4.01 (m, 2H, 2x*CH*CH_3 l-Ala), 21.36 (dd, J_G = 19.5 Hz, J = 7.2 Hz, 2H, CH_2P), 1.41 (d, J = 7.0 Hz, 3H, CHCH_3 l-Ala), 1.31 (d, J = 7.2 Hz, 3H, CHCH_3 l-Ala). **^{13}C NMR (125 MHz, CD_3OD) δ_C**: 174.28 (d, $^3J_{C\text{-}P}$ = 4.3 Hz, *C*═O, ester), 174.23 (d, $^3J_{C\text{-}P}$ = 4.3 Hz, *C*═O, ester), 135.95 (*C*-Ar), 135.91 (*C*-Ar), 128.5 ($^2J_{C\text{-}P}$ = 10.9 Hz, *CH*═), 128.36 (*CH*-Ar), 128.33 (*CH*-Ar), 128.1 (*CH*-Ar), 128.0 (*CH*-Ar), 119.0 (d, $^3J_{C\text{-}P}$ = 13.0 Hz CH_2═), 66.6 (CH_2Ph), 66.5 (CH_2Ph), 48.9 (*CH*CH_3 l-Ala), 48.5 (*CH*CH_3 l-Ala), 34.7 (d, $^1J_{C\text{-}P}$ = 111.4 Hz CH_2P), 19.9 (d, $^3J_{C\text{-}P}$ = 5.4 Hz, CHCH_3 l-Ala), 19.8 (d, $^3J_{C\text{-}P}$ = 4.3 Hz, CHCH_3 l-Ala).

4.1.5. (*E*)-N^1-(bis(Benzyloxy-L-alanine)-phosphinyl-2′-methyl-but-2′-enyl) uracil (12)

Prepared according to the standard procedure **C** using *bis*(benzyloxy-L-alanine)-allylphosphonate **11** (200 mg, 854.9 μmol) and N^1-2′-methylallyluracil (150 mg, 1.71 mmol) and Hoveyda-Grubbs 2nd generation catalyst (15 mol%) in dry CH_2Cl_2 (10 ml). Volatiles were then evaporated and the residue was purified by Biotage Isolera One (25 g SNAP cartridge ULTRA, 75 ml/min, gradient eluent system 2-propanol/CH_2Cl_2 1% 1CV, 1–10% 12CV, 10% 2CV), to afford a mixture of the *E* and *Z* isomers. The two isomers were then separated by Preparative HPLC (20 ml/min, gradient eluting system CH_3CN/H_2O from 5/95 to 100/0, 30 min), to afford the title compound as pale yellow foamy solid (5 mg, 2%). R_f = 0.30 (CH_2Cl_2/2-propanol – 95:5). **^{31}P NMR (202 MHz, CD_3OD) δ_P**: 27.88. **^{1}H NMR (500 MHz, CD_3OD) δ_H**: 7.36 (d, J = 7.9 Hz, 1H, *H*-6), 7.28–7.17 (m, 10*H*, Ar*H*), 5.56 (d, J = 7.9 Hz, 1H, *H*-5), 5.32–5.28 (m, 1H, *CH*═), 5.06, 4.99 (m, 4H, 2xCH_2Ph), 4.14 (s, 2H, CH_2-N), 3.91–3.84 (m, 2H, 2x*CH*CH_3 l-Ala), 2.55–2.41 (m, 2H, CH_2P), 1.50 (d, J = 3.1 Hz, 3H, CH_3, alkene), 1.26 (d, J = 7.1 Hz, 3H, CHCH_3 l-Ala), 1.18 (d, J = 7.1 Hz, 3H, CHCH_3 l-Ala). **^{13}C NMR (125 MHz, CD_3OD) δ_C**: 174.3 (d, $^3J_{C\text{-}P}$ = 4.6 Hz, *C*═O, ester), 174.1 (d, $^3J_{C\text{-}P}$ = 3.7 Hz, *C*═O, ester), 165.2 (*C*-4), 151.5 (*C*-2), 145.2 (*C*-6), 135.95 (*C*-Ar), 135.91 (*C*-Ar), 133.9 (d, $^3J_{C\text{-}P}$ = 13.8 Hz, *C*═), 128.22 (*CH*-Ar), 128.21 (*CH*-Ar), 128.04 (*CH*-Ar), 128.01 (*CH*-Ar), 127.98 (*CH*-Ar), 127.96 (*CH*-Ar), 118.7 (d, $^2J_{C\text{-}P}$ = 9.7 Hz, *CH*═), 101.2 (*C*-5), 66.58 (CH_2Ph), 66.53 (CH_2Ph), 53.7 (d, $^4J_{C\text{-}P}$ = 2.4 Hz, CH_2-N), 48.8 (*CH*CH_3 l-Ala), 48.5 (*CH*CH_3 l-Ala), 29.0 (d, $^1J_{C\text{-}P}$ = 112.5 Hz, CH_2P), 19.8 (d, $^3J_{C\text{-}P}$ = 5.4 Hz, CHCH_3 l-Ala), 19.6 (d, $^3J_{C\text{-}P}$ = 4.8 Hz, CHCH_3 l-Ala), 13.1 (d, $^4J_{C\text{-}P}$ = 2.0 Hz, CH_3, alkene). **HPLC**: Reverse phase HPLC eluting with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 min, 1 ml/min, λ = 254 nm and 263 nm, showed one peak with Rt 15.79 min. **HRMS (ESI):** *m/z* $[M+Na]^+$ calcd for $C_{29}H_{35}N_4O_7P$: 605.2141, found: 605.2136.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2018.05.034.

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Preparation of Pyrimidine Alkenyl Acyclic Nucleoside Phosphonoamidates

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This synthetic protocol describes two strategies for the preparation of pyrimidine alkenyl acyclic nucleoside phosphonoamidates (ANPs), including linear and trisubstituted alkenyl derivatives. For the first procedure, a bis-trimethylsilyl ester of the parent alkenyl ANPs is the key intermediate that reacts with the desired amino acid ester and aryl alcohol. For the second procedure, an allyl phosphonoamidate bearing the ProTide promoieties is the key synthon employed as olefin partner for a cross-metathesis reaction with an alkylated nucleobase. © 2018 by John Wiley & Sons, Inc.

Keywords: acyclic nucleoside phosphonate • allylphosphonoamidate • antiviral • cross-metathesis • ProTide • prodrug

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INTRODUCTION

This unit presents two different synthetic strategies for the synthesis of alkenyl acyclic nucleoside phosphonoamidate prodrugs. The first methodology (Basic Protocol 1) consists in the preparation of linear (*E*)-but-2-enyl pyrimidine ProTide via the bis-trimethylsilyl ester of the parent alkenyl dimethylphosphonate nucleoside, synthetized following the procedure reported in Basic Protocol 1 of Bessières et al. (2001), another unit of *Current Protocols in Nucleic Acid Chemistry*. This intermediate, obtained by treatment of the parent nucleoside with an excess of trimethylsilyl bromide (TMSBr), is reacted, without purification, with the desired amino acid ester and an excess of phenol in pyridine in the presence of triethylamine, aldrithiol-2, and triphenylphosphine.

The procedure reported in Basic Protocol 2 involves, in the first instance, the preparation of the allylphosphonoamidate intermediate obtained in the same way as in Basic Protocol 1. This derivative is then reacted with an alkylated nucleobase via olefin cross-metathesis using Hoveyda-Grubbs second generation catalyst to obtain the branched (*E*)-2-methyl-but-2-enyl pyrimidine ProTide.

NOTE: All glassware should be oven dried, and all reactions should be performed under anhydrous conditions.

CAUTION: All reactions must be run in a suitable fume hood with efficient ventilation. Safety glasses and reagent-impermeable protective gloves should be worn at all time.

Compound characterization. Chemical characterization data are provided for all compounds. ^{1}H, ^{31}P, and ^{13}C NMR spectra were recorded in a Bruker Avance 500

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spectrometer at 500 MHz, 202 MHz, and 125 MHz, respectively, and auto-calibrated to the deuterated solvent reference peak in the case of ^{1}H and ^{13}C NMR and 85% H_3PO_4 for ^{31}P NMR experiments. All ^{31}P and ^{13}C NMR spectra were proton-decoupled. Chemical shifts are given in parts per million (ppm), and coupling constants (J) are measured in Hertz (Hz) and related to multiplicities. Analytical High Performance Liquid Chromatography (HPLC) analysis was performed using Varian Prostar system (LC-Workstation-Varian Prostar 335 LC detector). High-resolution mass spectrometry was performed on a Bruker Daltonics MicroTof-LC system (atmospheric pressure ionization, electron spray mass spectroscopy) in positive mode.

BASIC PROTOCOL 1

PREPARATION OF (*E*)-BUT-2-ENYL PHOSPHONOAMIDATE PYRIMIDINE

The synthesis of phosphonodiamidate prodrugs of ANPs via bis-trimethylsilyl ester was reported by Holy and colleagues (Jansa et al., 2011) and then successfully adapted by us for the synthesis of adefovir and tenofovir phosphonoamidate prodrugs (Pertusati et al., 2014). In this protocol, we are reporting a modification of this methodology (Pertusati, Serafini, Albadry, Snoeck, & Andrei, 2017) for the synthesis of S_P and R_P isomers of (*E*)-N^1-(4′-*O*-phenyl-(neopentyloxy-*L*-alanine)-phosphinyl-but-2-enyl)thymine (**3a** and **3b**; see Figure 1). (*E*)-N^1-(4′-dimethoxyphosphinyl-2′-butenyl) thymine (**1**), prepared according to literature procedures (Topalis et al., 2011), is reacted overnight with TMSBr at room temperature to afford intermediate **2**, which, after removal of the volatile compounds, is used in the next step without further purification. The mixture of diastereoisomers **3a** and **3b** is obtained by stirring **2** with the desired amino acid ester salt and an excess of phenol in presence of aldrithiol-2 and triphenylphosphine at 50°C for 16 hr. Purification by flash chromatography, followed by preparative HPLC, allows the separation of the two diastereoisomers (**3a** and **3b**).

Materials

(*E*)-N^1-(4′-dimethoxyphosphinyl-2′-butenyl) thymine (see Basic Protocol 1 of Bessières et al., 2001)
Dry argon (Ar)
Anhydrous acetonitrile (CH_3CN, Sigma-Aldrich)
Bromotrimethylsilane (TMSBr) (Sigma-Aldrich)
Anhydrous pyridine (Py, Sigma-Aldrich)
L-Alanine neopentyl ester tosylate (see Support Protocol in Serpi, Madela, Pertusati, & Slusarczyk, 2013)
Phenol (PhOH, Sigma-Aldrich)
Triethylamine (Et_3N, Sigma-Aldrich)
Aldrithiol-2 (Sigma-Aldrich)
Triphenylphosphine (PPh_3, Sigma-Aldrich)

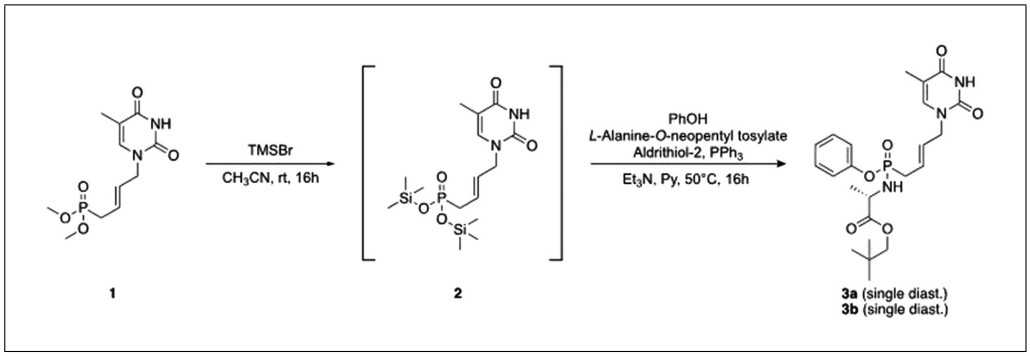

Figure 1 Synthesis of S_P and R_P isomer of (*E*)-N^1-(4′-*O*-phenyl-(neopentyloxy-*L*-alanine)-phosphinyl-but-2-enyl)thymine (**3a** and **3b**).

Methanol (MeOH, VWR Scientific)
Toluene (VWR Scientific)
Hexane (VWR Scientific)
Dichloromethane (CH_2Cl_2, VWR Scientific)
Anhydrous $MgSO_4$ (Sigma-Aldrich)
Silica gel (35 to 70 μ, 60A; Fisher)
Acetonitrile, HPLC-grade (CH_3CN, VWR Scientific)
Water, HPLC grade (VWR Scientific)

100-mL round-bottom flask
Magnetic stir bar
Magnetic stirring and heating plate
Oil vacuum pump
Vacuum desiccator
Oil bath
100-mL separatory funnel
Glass flash chromatography column
Glass funnel
Analytical TLC plate (aluminum-backed TLC plates, precoated with silica gel 60 F_{254}, 0.2 mm; Merck Kieselgel)
Preparative TLC plate (aluminum-backed TLC plates, precoated with silica gel 60 F_{254}, 20 × 20, 500-2000 μm; Merck Kieselgel)
Preparative TLC chamber
UV light source
Preparative HPLC system (Varian Prostar; LC Workstation-Varian Prostar 335 LC detector; Varian Pursuit XRs 5 C18 150 × 21.2 mm reversed-phase column)

Additional reagents and equipment for thin-layer chromatography (Meyers, 2001)

Prepare phosponoamidate 3

1. Place 0.087 g (0.275 mmol) of (*E*)-N^1-(4′-dimethoxyphosphinyl-2′-butenyl)thymine (**1**) in a 100-mL round bottom flask containing a magnetic stir bar, and apply an argon atmosphere.
2. Add 10 mL of anhydrous acetonitrile.
3. While stirring, add 0.182 mL (1.38 mmol) of TMSBr at room temperature and continue stirring for 16 hr under an argon atmosphere to obtain a brown solution.
4. After this period, evaporate the solvent under reduced pressure on a rotary evaporator without any contact with air, to afford **2** as a brown foamy solid crude mixture.
5. Dry the crude residue under vacuum for 1 hr (oil pump).
6. Dissolve the solid in 5 mL of anhydrous pyridine under an argon atmosphere.
7. Add 0.091 g (0.275 mmol) of dry *L*-alanine neopentyl ester tosylate, 0.432 g (1.65 mmol) of phenol, and 3.4 mL (24.9 mmol) of triethylamine.
8. Place the reaction mixture in an oil bath, heat at 50°C, and stir for 10 min to obtain a yellow solution.
9. In a separate flask, prepare a solution with 0.155 g (1.65 mmol) of aldrithiol-2 and 0.363 g (1.65 mmol) of triphenylphosphine in 5 mL of anhydrous pyridine under an argon atmosphere.
10. Add the aldrithiol/triphenylphosphine solution to the stirring reaction mixture and keep at 50°C for 4 hr.

11. Allow mixture to cool down to room temperature.

12. Evaporate the reaction mixture to dryness using a rotary evaporator under reduced pressure.

13. Add a 1:1:1:1 (v/v) mixture of methanol, water, toluene, and hexane (10/10/10/10 mL) to the residue and transfer the mixture into a 100-mL separatory funnel.

14. Remove the upper layer (hexane/toluene) and wash the lower phase with a 1:1 (v/v) mixture of toluene and hexane 1:1 (v/v) three times, each time with 10 mL.

15. Remove the upper layer and extract the lower layer ($MeOH/H_2O$) three times, each time with 20 mL CH_2Cl_2.

16. Combine the CH_2Cl_2 phases, dry over $MgSO_4$, filter by gravity filtration, and then evaporate using a rotary evaporator under reduced pressure.

17. Dissolve the crude product in the minimum amount of CH_2Cl_2 and carefully place the solution on top of a glass flash chromatography column packed with silica gel in CH_2Cl_2. Elute using a gradient solution of CH_2Cl_2/MeOH (99:1 to 93:7 v/v).

18. Monitor the fractions by TLC (see Meyers, 2001) and visualize by UV light, combine the fractions containing the products and evaporate to dryness using a rotary evaporator under reduced pressure.

19. Complete the purification of products by preparative thin layer chromatography on silica gel as follows. Dissolve the crude product in the minimum amount of CH_2Cl_2, apply the sample on a TLC plate about 1.5 cm from the bottom edge, and allow the solvent to evaporate. Place the TLC plate in a separation chamber containing 200 mL of 95:5 (v/v) CH_2Cl_2/MeOH. When the solvent reaches 1.5 cm from the upper edge, remove the TLC plate and allow the solvents to evaporate.

20. Scrape off the backing material of the desired band, visualized using UV light, and extract it with minimal 90:10 (v/v) CH_2Cl_2/MeOH solution. Filter the silica off using a glass filter funnel, wash it using a small amount of 90:10 (v/v) CH_2Cl_2/MeOH solution, and concentrate the filtrate using a rotary evaporator under reduced pressure.

21. Dissolve the isomer mixture in MeOH (HPLC-grade) and separate isomers by preparative HPLC (20 mL/min, gradient eluting system CH_3CN/H_2O—from 10/90 to 100/0, 30 min) to afford compounds as foamy solids.

22. Characterize the compound by ^{31}P NMR, ^{1}H NMR, ^{13}C NMR, and MS.

^{31}P NMR spectra documented below were obtained with proton decoupling

*(E)-N^1-(4′-O-phenyl-(neopentyloxy-L-alanine)-phosphinyl-but-2-enyl)thymine (**3a** and **3b**).*

***3a**: Yield 0.021 g (16%). $R_f = 0.32$ (CH_2Cl_2/MeOH - 95:5).*

^{31}P-NMR (202 MHz, CD_3OD) δ_P: 29.23.

^{1}H-NMR (500 MHz, CD_3OD) δ_H: 7.41 (1H, d, J = 1.1 Hz, H-6), 7.38-7.34 (2H, m, CH-Ph), 7.21-7.18 (3H, m, CH-Ph), 5.83-5.79 (2H, m, $NCH_2CH=$ and $=CHCH_2P$), 4.36 (2H, t, J = 4.8 Hz, CH_2N), 4.05-3.99 (1H, m, $CHCH_3$), 3.87, 3.77 (2H, AB, $J_{AB} = 10.5$ Hz, $CH_2C(CH_3)_3$), 2.87 (2H, ddd, J = 6.4 and 4.6 Hz, $^2J_{PH} = 20.5$ Hz, CH_2P), 1.87 (3H, d, J =1.2 Hz, CH_3), 1.26 (3H, d, J = 7.3 Hz, $CHCH_3$), 0.96 (9H, s, $C(CH_3)_3$).

^{31}C-NMR (125 MHz, $CDCl_3$) δ_C: 174.05 (d, $^3J_{PC} = 4.9$ Hz, COO), 163.9 (C-4), 150.67 (C-2), 150.38 (d $^2J_{CP} = 9.1$ Hz, C-ipso Ph), 139.74 (CH-6), 129.77 (CH-Ph), 129.47 (d, $^2J_{PC} = 10.7$ Hz, $= CHCH_2P$), 129.21 (d, $^3J_{PC} = 14.7$ Hz, $NCH_2CH=$), 124.64 (CH-Ph), 120.68 (d, $^3J_{PC} = 4.5$ Hz, CH-Ph), 111.00 (C-5), 74.71 ($CH_2C(CH_3)_3$), 49.73 ($CHCH_3$),

49.49 (CH_2N), 32.44 (d, $^1J_{PC}$ = 127.2 Hz, CH_2P), 29.69 ($C(CH_3)_3$), 26.32 ($C(CH_3)_3$), 21.53 (d $^3J_{PC}$ = 6.3 Hz, $CHCH_3$), 10.87 (CH_3).

HPLC: Reversed-phase HPLC eluting with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 min, 1 mL/min, λ = 254 nm and 263 nm, showed one peak with t_R 16.06 min.

MS(ESI+) m/z = 500.2 [M + Na^+] (100%).

***3b**: Yield 0.013 g (10%). R_f = 0.29 (CH_2Cl_2/MeOH - 95:5).*

^{31}P-NMR (202 MHz, $CDCl_3$) δ_P: 28.51.

^{1}H-NMR (500 MHz, CD_3OD) δ_H: 7.37 (1H, d, J = 1.1 Hz, H-6), 7.35-7.32 (2H, m, CH-Ph), 7.22-7.17 (3H, m, CH-Ph), 5.79-5.76 (2H, m, NCH_2CH= and =$CHCH_2P$), 4.35 (2H, m, CH_2N), 3.91, 3.82 (2H, AB, J_{AB} = 10.5 Hz, $CH_2C(CH_3)_3$), 3.67-3.60 (1H, m, $CHCH_3$), 2.86-2.80 (2H, m, CH_2P), 1.87 (3H, s, CH_3), 1.38 (3H, d, J = 7.2 Hz, $CHCH_3$), 0.99 (9H, s, $C(CH_3)_3$).

^{31}C-NMR (125 MHz, $CDCl_3$) δ_C: 173.79 (d, $^3J_{PC}$ = 4.9 Hz, COO), 164.01 (C-4), 150.72 (C-2), 150.43 (d, $^2J_{CP}$ = 7.84 Hz, C-ipso Ph), 139.66 (CH-6), 129.77 (CH-Ph), 129.24 (d, $^2J_{PC}$ = 14.7 Hz, = $CHCH_2P$), 125.16 (d, $^2J_{PC}$ = 11.0 Hz, NCH_2CH=), 124.86 (CH-Ph), 120.47 (d, $^3J_{PC}$ = 4.9 Hz, CH-Ph), 111.05 (C-5), 74.78 ($CH_2C(CH_3)$), 49.62 ($CHCH_3$), 49.23 (CH_2N), 32.79 (d, $^1J_{PC}$ = 130.8 Hz, CH_2P), 29.70 ($C(CH_3)_3$), 29.36 ($C(CH_3)_3$), 21.79 (d, $^3J_{PC}$ = 2.5 Hz, $CHCH_3$), 12.30 (CH_3).

HPLC: Reversed-phase HPLC eluting with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 min, 1 mL/min, λ = 254 nm and 263 nm, showed one peak with t_R 16.14 min.

MS(ESI+) m/z = 500.2 [M + Na^+] (100%).

PREPARATION OF (*E*)-2-METHYL-BUT-2-ENYL PHOSPHONOAMIDATE PYRIMIDINE

BASIC PROTOCOL 2

This protocol describes the preparation of phosphonoamidate prodrugs of trisubstituted alkenyl acyclonucleoside using a cross-metathesis reaction. Olefin cross-metathesis methodology has been used for the direct synthesis of a vast array of unsaturated ANPs analogs including bis-POM, bis-POC, and alkoxyester prodrugs (Hamada et al., 2013; Pradère, Clavier, Roy, Nolan, & Agrofoglio, 2011). Only a very recent application of such a procedure for the preparation of ProTides has been reported (Bessières et al., 2018). Despite some similarities, the synthetic strategy we are reporting here differs from that published by Agrofoglio and colleagues.

This methodology first involves the synthesis of the aryloxy allylphosphonoamidate **6** as the key synthon. Briefly, the commercial dimethyl allylphosphonate **4** is converted into the corresponding silyl ester **5** in the presence of an excess of TMSBr and 2,6-lutidine as an acid scavenger. After removal of the volatiles, **5** is used without further purification, and treated with the amino acid ester hydrochloride and an excess of aryl alcohol in presence of aldrithiol-2 and triphenylphosphine at 50°C for 16 hr to obtain the desired allylphosphonoamidate **6**.

The second olefin partner for the cross-metathesis reaction (**9** and **10**) is synthesized by N^1-substitution using 3-bromo-2-methylpropene (Bessieres et al., 2016).

As illustrated in Figure 2, the allylphosphonoamidate intermediate **6** is then sonicated with 2-methylallyl pyrimidines **9** and **10** in presence of Hoveyda-Grubbs second-generation catalyst in CH_2Cl_2 at reflux for 24 hr, to obtain the final ProTides **11** and **12**.

Materials

Dimethyl allylphosphonate (**4**) (Alfa Aesar)
Dry argon (Ar)
Anhydrous acetonitrile (CH_3CN, Sigma-Aldrich)
2,6-Lutidine (Sigma-Aldrich)

Bromotrimethylsilane (TMSBr; Sigma-Aldrich)
Anhydrous pyridine (Py, Sigma-Aldrich)
L-Alanine isopropyl ester hydrochloride (Sigma-Aldrich)
1-Naphthol (1-NaphOH, Sigma-Aldrich), dry
Triethylamine (Et_3N, Sigma-Aldrich)
Aldrithiol-2 (Sigma-Aldrich)
Triphenylphosphine (PPh_3, Sigma-Aldrich)
Ethyl acetate (EtOAc, VWR Scientific)
Hexane (VWR Scientific)
Anhydrous dichloromethane (CH_2Cl_2, Sigma-Aldrich)
Anhydrous acetonitrile (CH_3CN, Sigma-Aldrich)
Uracil (**7**) (Sigma-Aldrich)
Thymine (**8**) (Sigma-Aldrich)
N,*O*-Bis(trimethylsilyl)acetamide (BSA, Sigma-Aldrich)
3-Bromo-2-methylpropene (Sigma-Aldrich)
Sodium iodide (NaI, Sigma-Aldrich)
Chlorotrimethylsilane (TMSCl, Sigma-Aldrich)
$NaHCO_3$
Na_2SO_4
Anhydrous $MgSO_4$ (Sigma-Aldrich)
Hoveyda-Grubbs Catalyst, 2nd Generation (Sigma-Aldrich)
Methanol (MeOH, VWR Scientific)
Acetonitrile HPLC grade (CH_3CN, VWR chemicals)
Water, HPLC-grade (VWR chemicals)
Deuterated methanol (CD_3OD), 99.8% pure (Goss Scientific, used for NMR characterization)

50- and 100-mL round-bottom flask
Magnetic stir bar
Magnetic stirring and heating plate
Rotary evaporator equipped with vacuum pump
Oil vacuum pump
Vacuum desiccator
Glass funnel
Filter paper
Oil bath
UV light source
Analytical TLC plate (aluminum-backed TLC plates, precoated with silica gel 60 F_{254}, 0.2 mm; Merck Kieselgel)
Automatic flash chromatography system (Biotage Isolera One)
100-g and 50-g SNAP cartridge ULTRA (Biotage)
Reflux condenser
250-mL separatory funnel
Fisherbrand 11203 Ultrasonic Cleaner
60 g SNAP cartridge KP-C18-HS (Biotage)
Preparative HPLC (Varian Prostar; LC Workstation-Varian Prostar 335 LC detector; Varian Pursuit XRs 5 C18 150 × 21.2 mm reversed-phase column)

Preparation of allylphosphonoamidate derivative

1. Place 0.500 g (3.3 mmol) of **4** in a 100-mL round bottom flask containing a magnetic stir bar and apply an argon atmosphere.
2. Add 25 mL anhydrous acetonitrile and 1.55 mL (13.3 mmol) of 2,6-lutidine.

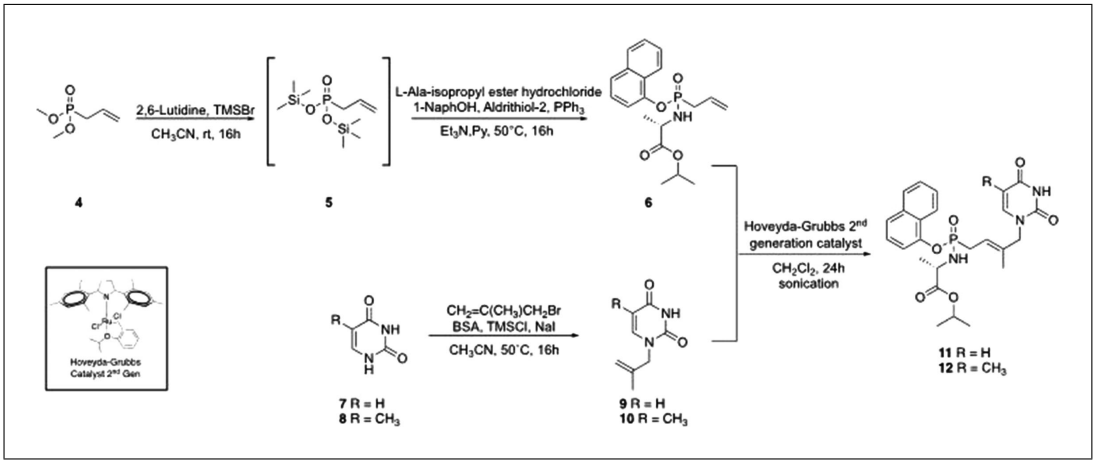

Figure 2 Preparation of (*E*)-N^1-(4′-*O*-(1-Naphthyl)-(isopropyloxy-*L*-Alanine)-phosphinyl-2′-methyl-but-2′-enyl) pyrimidines (**11** and **12**) via cross metathesis using *O*-(1-naphthyl)-(isopropyloxy-*L*-Alanine)-allylphosphonate (**6**) as key synthon.

3. While stirring, add 2.20 mL (16.6 mmol) of TMSBr at room temperature and continue stirring for 16 hr under an argon atmosphere to obtain a brown solution.
4. After this period, evaporate the solvent under reduced pressure on a rotary evaporator without any contact with air to afford **5** as a brown foamy solid crude mixture.
5. Dry the crude residue under vacuum for 1 hr (oil pump).
6. Dissolve the solid in 10 mL of anhydrous pyridine under an argon atmosphere.
7. Add 0.558 g (3.3 mmol) of dry *L*-alanine isopropyl ester hydrochloride, 2.88 g (19.9 mmol) of dry 1-naphthol, and 6.9 mL (49.9 mmol) of triethylamine.
8. Place the reaction mixture in an oil bath, heat at 50°C, and stir for 10 min to obtain a yellow solution.
9. Prepare a solution with 4.40 g (19.9 mmol) of aldrithiol-2 and 5.24 g (19.9 mmol) of triphenylphosphine in 10 mL of anhydrous pyridine under an argon atmosphere.
10. Add the aforementioned solution to the reaction mixture while stirring and keep at 50°C for 16 hr.
11. Allow the mixture to cool down to room temperature.
12. Monitor the reaction by TLC (Meyers, 2001) using 4:6 (v/v) EtOAc/hexane and visualize by UV light (**6**, $R_f = 0.58$).
13. Evaporate the reaction mixture to dryness using a rotary evaporator under reduced pressure.
14. Purify the residue by Biotage Isolera One as follows. Dissolve the crude product in the minimum amount of CH_2Cl_2 and carefully place into a 100 g SNAP cartridge ULTRA. Purify using a 100 mL/min gradient eluent system of EtOAc/hexane 10%, 1 column volume (1CV) 10 to 100% 12CV, 100% 2CV.
15. Monitor the fractions by TLC (Meyers, 2001) and visualize by UV light, combine the fractions containing the pure product, and evaporate to dryness using a rotary evaporator under reduced pressure to afford compound **6** as a yellow oil.

16. Characterize the compounds by ^{31}P NMR, ^{1}H NMR, and ^{13}C NMR.

^{31}P NMR spectra documented below were obtained with proton decoupling.

*O-(1-naphthyl)-(isopropyloxy-L-Alanine)-allylphosphonate (**6**). Yield 0.940 g (79%). R_f: 0.58 (EtOAc/Hexane - 4:6).*

^{31}P NMR (202 MHz, CD_3OD) δ_P: 30.01, 29.43.

^{1}H NMR (500 MHz, CD_3OD) δ_H: 8.19 (d, J = 7.2 Hz, 1H, ArH), 7.89 (d, J = 7.9 Hz 1H, ArH), 7.71-7.69 (m, 1H, ArH), 7.58-7.40 (m, 4H, ArH), 6.07-5.91 (m, 1H, CH=), 5.38-5.28 (m, 2H, CH_2=), 5.95-4.82 (m, 1H, $CH(CH_3)_2$), 3.99-3.97 (m, 1H, $CHCH_3$ L-Ala), 3.03-2.93 (m, 2H, CH_2P), 1.25 (d, J = 7.8 Hz, 1.5H, $CHCH_3$ L-Ala), 1.21-1.10 (m, 7.5H, $CHCH_3$ L-Ala, $CH(CH_3)_2$).

^{13}C NMR (125 MHz, CD_3OD) δ_C: 173.5 (d, $^3J_{C\text{-}P}$ = 4.2 Hz, C = O, ester), 173.1 (d, $^3J_{C\text{-}P}$ = 4.2 Hz, C = O, ester), 146.4 (d, $^2J_{C\text{-}P}$ = 8.5 Hz, C-O, Ph), 146.3 (d, $^2J_{C\text{-}P}$ = 8.5 Hz, C-O, Ph), 134.9 (C-Ar), 127.4 ($^2J_{C\text{-}P}$ = 9.3 Hz, CH=), 123.3 ($^2J_{C\text{-}P}$ = 10.9 Hz, CH=), 126.9 (d, $^3J_{C\text{-}P}$ = 5.6 Hz C-Ar), 126.8 (d, $^3J_{C\text{-}P}$ = 4.9 Hz C-Ar), 126.3 (CH-Ar), 125.95 (CH-Ar), 125.90 (CH-Ar), 125.1 (CH-Ar), 125.0 (CH-Ar), 124.3 (CH-Ar), 124.2 (CH-Ar), 121.6 (CH-Ar), 121.4 (CH-Ar), 119.7 (d, $^3J_{C\text{-}P}$ = 14.2 Hz CH_2=), 119.6 (d, $^3J_{C\text{-}P}$ = 13.8 Hz CH_2=), 115.4 (d, $^3J_{C\text{-}P}$ = 4.1 Hz CH-Ar), 115.2 (d, $^3J_{C\text{-}P}$ = 3.4 Hz CH-Ar), 68.6 ($CH(CH_3)_2$), 68.5 ($CH(CH_3)_2$), 49.6 ($CHCH_3$ L-Ala), 49.4 ($CHCH_3$ L-Ala), 33.7 (d, $^1J_{C\text{-}P}$ = 129.0 Hz CH_2P), 33.5 (d, $^1J_{C\text{-}P}$ = 129.6 Hz CH_2P), 20.5 ($CH(CH_3)_2$), 20.4 ($CH(CH_3)_2$), 20.3 ($CH(CH_3)_2$), 19.7 (d, $^3J_{C\text{-}P}$ = 5.4 Hz, $CHCH_3$ L-Ala), 19.1 (d, $^3J_{C\text{-}P}$ = 5.4 Hz, $CHCH_3$ L-Ala).

Prepare N^1-2′-methylallyl-pyrimidines

17. Dissolve 1.5 g of nucleobase (13.3 mmol of **7**, 11.8 mmol of **8**) in 25 mL of anhydrous acetonitrile in a 100-mL round bottom flask containing a magnetic stir bar, and apply an argon atmosphere.

18. While stirring add *N*,*O*-Bis(trimethylsilyl)acetamide (BSA).

    For **9**: 8.18 mL (33.4 mmol) of BSA
    For **10**: 7.20 mL (29.7 mmol) of BSA

19. Place the reaction mixture in an oil bath, heat at reflux temperature using a reflux apparatus, and stir until a clear solution is observed (usually 10 min).

20. Add, under an argon atmosphere, 3-bromo-2-methylpropene, NaI, and chlorotrimethylsilane.

    For **9**: 2.40 mL (23.7 mmol) of 3-bromo-2-methylpropene, 1.96 g (13.1 mmol) of NaI, 1.51 mL (11.8 mmol) of chlorotrimethylsilane
    For **10**: 2.70 mL (26.7 mmol) of 3-bromo-2-methylpropene, 2.21 g (14.7 mmol) of NaI, 1.70 mL (13.3 mmol) of chlorotrimethylsilane

21. Stir under reflux under an argon atmosphere for 16 hr.

22. Monitor the reaction by TLC (Meyers, 2001) and visualize with UV light using 7:3 (v/v) EtOAc/hexane and visualize by UV light (**9**, R_f: 0.25; **10**, R_f: 0.45).

23. Evaporate the solvent to dryness under reduced pressure on a rotary evaporator.

24. Dissolve the residue in 50 mL of EtOAc and wash the mixture in sequence with 20 mL of $NaHCO_3$ aqueous saturated solution, 20 mL of Na_2SO_4 aqueous saturated solution, and 20 mL of H_2O using a 250-mL separatory funnel.

25. Dry the organic phase over anhydrous $MgSO_4$, filter by gravity filtration, and evaporate the solution to dryness using a rotary evaporator under reduced pressure.

26. Purify the residue by Biotage Isolera One as follows. Dissolve the crude product in the minimum amount of CH_2Cl_2 and carefully place into a 50-g SNAP cartridge ULTRA. Purify using a 100 mL/min gradient eluent system of EtOAc/hexane 17% 1CV, 17% to 100% 10CV, 100% 3CV.

27. Monitor the fractions by TLC (Meyers, 2001) and visualize by UV light, combine the fractions containing the pure product, and evaporate to dryness using a rotary evaporator under reduced pressure to afford compounds **9** and **10** as pale-yellow solids.

28. Characterize the compounds by ^{1}H NMR.

*N^1-2′-methylallyl-uracil (**9**). Yield 1.2 g (51%). R_f: 0.25 (EtOAc/Hexane - 7:3).*

^{1}H NMR (500 MHz, CD_3OD) δ_H: 7.50 (d, J = 7.8 Hz, 1H, H-6), 5.71 (d, J = 7.8 Hz, 1H, H-5), 4.98 (s, 1H, CH_2=), 4.81 (s, 1H, CH_2=), 4.33 (s, 2H, CH_2-N), 1.76 (s, 3H, CH_3, alkene).

*N^1-2′-methylallyl-thymine (**10**). Yield 2.1 g (98%). R_f: 0.45 (EtOAc/Hexane - 7:3).*

^{1}H NMR (500 MHz, CD_3OD) δ_H: 7.34 (s, 1H, H-6), 4.98 (s, 1H, CH_2=), 4.80 (s, 1H, CH_2=), 4.30 (s, 2H, CH_2-N), 1.89 (s, 3H, CH_3, base), 1.76 (s, 3H, CH_3, alkene).

Olefin cross-metathesis

29. Dissolve 0.150 g of the allylphosphonoamidate (**6**, from step 15, 415.0 µmol) in 10 mL of anhydrous dichloromethane in a 50-mL round-bottom flask containing a magnetic stir bar, and apply an argon atmosphere.

30. Add N^1-2′-methylallyl-pyrimidine (from step 27).

For **11**: 0.137 g (830.1 µmol) of **9**.
For **12**: 0.150 g (830.1 µmol) of **10**.

31. Add 0.039 g (62.2 µmol, 15 mol%) of Hoveyda-Grubbs second-generation catalyst in three equal portions of 5 mol% at $t = 0$, 2, and 4 hr over the course of the reaction.

32. Sonicate the reaction mixture at 37 MHz for 24 hr.

33. Monitor the reaction by TLC (Meyers, 2001) using 95:5 (v/v) CH_2Cl_2/MeOH and visualize by UV light (**11**, R_f: 0.22; **12**, R_f: 0.24).

34. Evaporate the reaction mixture to dryness using a rotary evaporator. Dissolve the crude product in the minimum amount of 99:1 (v/v) CH_2Cl_2/MeOH and carefully place into a 50 g SNAP cartridge ULTRA. Purify using a 100 mL/min gradient eluent system: MeOH/CH_2Cl_2 1% 1CV, 1 to 10% 12CV, 10% 2CV.

35. Monitor the fractions by TLC (Meyers, 2001) and visualize by UV light, combine the fractions containing a mixture of *E* and *Z* isomers of the compound, and evaporate to dryness using a rotary evaporator under reduced pressure.

36. Separate the two isomers by reversed-phase chromatography.

For **11**: Dissolve the product in MeOH (HPLC gradient) and purify by preparative HPLC (20 mL/min, isocratic eluting system CH_3CN/H_2O—35/65, 30 min) to afford the compound as pale yellow foamy solid.

For **12**: Dissolve the product in MeOH, carefully place into a 60 g SNAP cartridge KP-C18-HS, and purify by reversed-phase flash chromatography using a 100 mL/min, isocratic eluent system of CH_3CN/H_2O 40/60 12CV, to afford the compound as pale yellow foamy solid.

37. Characterize the compounds by ^{31}P NMR, ^{1}H NMR and ^{13}C NMR, HRMS, and HPLC.

^{31}P NMR spectra documented below were obtained with proton decoupling.

*(E)-N^{1}-(4′-O-(1-naphthyl)-(isopropyloxy-L-Alanine)-phosphinyl-2′-methyl-but-2′-enyl)uracil (**11**). Yield 0.028 g (14%). $R_f = 0.22$ (CH_2Cl_2/MeOH - 95:5).*

^{31}P NMR (202 MHz, CD_3OD) δ_P: 30.28, 29.49.

^{1}H NMR (500 MHz, CD_3OD) δ_H: 8.14-8.13 (m, 1H, ArH), 7.88-7.84 (m, 1H, ArH), 7.70-7.67 (m, 1H, ArH), 7.58-7.49 (m, 3H, ArH), 7.44-7.38 (m, 2H, H-6, ArH), 5.61-5.57 (m, 1.5H, CH=, H-5), 5.51-5.47 (m, 0.5H, CH=), 4.93 (sept, J = 6.5 Hz, 0.5H, $CH(CH_3)_2$), 4.88-4.84 (m, 0.5H, $CH(CH_3)_2$), 4.33-4.25 (m, 2H, CH_2-N), 4.04-3.97 (m, 1H, $CHCH_3$ L-Ala), 3.08-2.90 (m, 2H, CH_2P), 1.65 (bs, 3H, CH_3, alkene), 1.27 (d, J = 7.0 Hz, 1.5H, $CHCH_3$ L-Ala), 1.20 (d, J = 6.2 Hz, 1.5H, $CH(CH_3)_2$), 1.19 (d, J = 6.2 Hz, 1.5H, $CH(CH_3)_2$), 1.17 (d, J = 6.9 Hz, 1.5H, $CHCH_3$ L-Ala), 1.12 (d, J = 6.2 Hz, 1.5H, $CH(CH_3)_2$), 1.15 (d, J = 6.2 Hz, 1.5H, $CH(CH_3)_2$).

^{13}C NMR (125 MHz, CD_3OD) δ_C: 173.6 (d, $^{3}J_{C\text{-}P} = 4.3$ Hz, C = O, ester), 173.2 (d, $^{3}J_{C\text{-}P} = 4.1$ Hz, C=O, ester), 165.17 (C-4), 165.15 (C-4), 151.5 (C-2), 151.4 (C-2), 146.5 (d, $^{2}J_{C\text{-}P} = 9.7$ Hz, C-O, Ph), 146.3 (d, $^{2}J_{C\text{-}P} = 9.7$ Hz, C-O, Ph), 145.2 (C-6), 145.1 (C-6), 135.2 (d, $^{3}J_{C\text{-}P} = 14.5$ Hz, C=), 135.4 (d, $^{3}J_{C\text{-}P} = 14.5$ Hz, C=), 134.9 (C-Ar), 127.5 (CH-Ar), 127.4 (CH-Ar), 126.8 (d, $^{3}J_{C\text{-}P} = 4.9$ Hz C-Ar), 126.6 (d, $^{3}J_{C\text{-}P} = 5.1$ Hz C-Ar), 126.3 (CH-Ar), 126.1 (CH-Ar), 125.2 (CH-Ar), 125.1 (CH-Ar), 124.3 (CH-Ar), 124.2 (CH-Ar), 121.5 (CH-Ar), 121.3 (CH-Ar), 117.4 ($^{2}J_{C\text{-}P} = 11.0$ Hz, CH=), 116.9 ($^{2}J_{C\text{-}P} = 11.0$ Hz, CH=), 115.4 (d, $^{3}J_{C\text{-}P} = 3.8$ Hz CH-Ar), 115.1 (d, $^{3}J_{C\text{-}P} = 3.8$ Hz CH-Ar), 101.2 (C-5), 68.69 ($CH(CH_3)_2$), 68.66 ($CH(CH_3)_2$), 53.7 (d, $^{4}J_{C\text{-}P} = 2.3$ Hz, CH_2-N), 53.5 (d, $^{4}J_{C\text{-}P} = 2.3$ Hz, CH_2-N), 49.7 ($CHCH_3$ L-Ala), 49.5 ($CHCH_3$ L-Ala), 28.3 (d, $^{1}J_{C\text{-}P} = 128.9$ Hz CH_2P), 28.1 (d, $^{1}J_{C\text{-}P} = 129.8$ Hz CH_2P), 20.6 ($CH(CH_3)_2$), 20.56 ($CH(CH_3)_2$), 20.52 ($CH(CH_3)_2$), 20.4 ($CH(CH_3)_2$), 19.8 (d, $^{3}J_{C\text{-}P} = 5.8$ Hz, $CHCH_3$ L-Ala), 19.1 (d, $^{3}J_{C\text{-}P} = 5.5$ Hz, $CHCH_3$ L-Ala), 13.3 (d, $^{4}J_{C\text{-}P} = 2.4$ Hz, CH_3, alkene), 13.2 (d, $^{4}J_{C\text{-}P} = 2.2$ Hz, CH_3, alkene).

HPLC: Reversed-phase HPLC eluting with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 min, 1 mL/min, $\lambda = 254$ nm and 263 nm, showed one peak with t_R 15.57 min.

HRMS (ESI): m/z $[M+Na]^+$ calcd for $C_{25}H_{30}N_3O_6P$: 522.1770, found: 522.1764.

*(E)-N^{1}-(4′-O-(1-naphthyl)-(isopropyloxy-L-Alanine)-phosphinyl-2′-methyl-but-2′-enyl)thymine (**12**). Yield 0.075 g (36%). $R_f = 0.24$ (CH_2Cl_2/MeOH - 95:5).*

^{31}P NMR (202 MHz, CD_3OD) δ_P: 30.32, 29.54.

^{1}H NMR (500 MHz, CD_3OD) δ_H: 8.13-8.12 (m, 1H, ArH), 7.89-7.87 (m, 1H, ArH), 7.71-7.68 (m, 1H, ArH), 7.57-7.48 (m, 3H, ArH), 7.45-7.39 (m, 1H, ArH), 7.27 (s, 0.5H, H-6), 7.26 (s, 0.5H, H-6), 5.61-5.56 (m, 1H, CH=), 4.93-4.84 (m, 1H, $CH(CH_3)_2$), 4.32-4.26 (m, 2H, CH_2-N), 4.01-3.91 (m, 1H, $CHCH_3$ L-Ala), 3.08-2.86 (m, 2H, CH_2P), 1.75 (s, 3H, CH_3, base), 1.67 (s, 3H, CH_3, alkene), 1.27 (d, J = 6.9 Hz, 1.5H, $CHCH_3$ L-Ala), 1.20-1.16 (m, 4.5H, $CHCH_3$ L-Ala, $CH(CH_3)_2$), 1.13-1.10 (m, 3H, $CH(CH_3)_2$).

^{13}C NMR (125 MHz, CD_3OD) δ_C: 173.5 (d, $^{3}J_{C\text{-}P} = 3.9$ Hz, C=O, ester), 173.1 (d, $^{3}J_{C\text{-}P} = 3.5$ Hz, C = O, ester), 165.34 (C-4), 165.32 (C-4), 151.69 (C-2), 151.61 (C-2), 146.5 (d, $^{2}J_{C\text{-}P} = 9.5$ Hz, C-O, Ph), 146.3 (d, $^{2}J_{C\text{-}P} = 9.5$ Hz, C-O, Ph), 140.94 (C-6), 140.92 (C-6), 135.5 (d, $^{3}J_{C\text{-}P} = 14.3$ Hz, C=), 135.1 (d, $^{3}J_{C\text{-}P} = 14.7$ Hz, C=), 134.9 (C-Ar), 127.48 (CH-Ar), 127.46 (CH-Ar), 126.7 (d, $^{3}J_{C\text{-}P} = 5.1$ Hz C-Ar), 126.6 (d, $^{3}J_{C\text{-}P} = 5.1$ Hz C-Ar), 126.3 (CH-Ar), 126.0 (CH-Ar), 125.16 (CH-Ar), 125.11 (CH-Ar), 124.3 (CH-Ar), 124.2 (CH-Ar), 121.4 (CH-Ar), 121.3 (CH-Ar), 117.1 ($^{2}J_{C\text{-}P} = 11.1$ Hz, CH=), 116.6 ($^{2}J_{C\text{-}P} = 10.7$ Hz, CH=), 115.3 (d, $^{3}J_{C\text{-}P} = 3.5$ Hz CH-Ar), 115.1 (d, $^{3}J_{C\text{-}P} = 3.9$ Hz CH-Ar), 110.1 (C-5), 68.69 ($CH(CH_3)_2$), 68.65 ($CH(CH_3)_2$), 53.5 (d, $^{4}J_{C\text{-}P} = 2.7$ Hz, CH_2-N), 53.2 (d, $^{4}J_{C\text{-}P} = 2.3$ Hz, CH_2-N), 49.7 ($CHCH_3$ L-Ala), 49.5 ($CHCH_3$ L-Ala), 28.3 (d, $^{1}J_{C\text{-}P} = 129.0$ Hz CH_2P), 28.1 (d, $^{1}J_{C\text{-}P} = 130.0$ Hz CH_2P), 20.55 ($CH(CH_3)_2$), 20.54 ($CH(CH_3)_2$), 20.48 ($CH(CH_3)_2$), 20.40 ($CH(CH_3)_2$), 19.8 (d, $^{3}J_{C\text{-}P} = 5.5$ Hz, $CHCH_3$

L-Ala), 19.1 (d, $^{3}J_{C\text{-}P} = 5.9$ Hz, $CHCH_3$ L-Ala), 13.3 (d, $^{4}J_{C\text{-}P} = 2.3$ Hz, CH_3, alkene), 13.2 (d, $^{4}J_{C\text{-}P} = 2.7$ Hz, CH_3, alkene), 10.8 (CH_3, base).

HPLC: Reversed-phase HPLC eluting with gradient method CH_3CN/H_2O from 10/90 to 100/0 in 30 min, 1 mL/min, $\lambda = 254$ nm and 263 nm, showed one peak with t_R 16.26 min.

HRMS (ESI): m/z $[M+Na]^+$ calcd for $C_{26}H_{32}N_3O_6P$: 536.1926, found: 536.1921.

COMMENTARY

Background Information

In recent years, the ProTide approach, pioneered by Chris McGuigan's group, has displayed a great deal of success in the development of nucleoside-based antivirals and anticancer drugs (Pertusati, McGuigan, & Serpi, 2015; Serpi et al., 2013). Sofosbuvir (Nakamura et al., 2016) and TAF (Abdul Basit, Dawood, Ryan, & Gish, 2017; Ray, Fordyce, & Hitchcock, 2016), on the market for viral infections, and Acelerin (Slusarczyk et al., 2014) and NUC 3373 (McGuigan et al., 2011), in clinical trials (Phase III and Phase I) for patients with advanced solid tumors, are the undeniable proof of how powerful this technology is.

While there are several efficient procedures to synthesize phosphoroamidate nucleosides, the phosphonoamidate cognate class, especially of acyclic nucleoside phosphonates (ANPs), lacks such a plethora of synthetic methodologies (Pradere, Garnier-Amblard, Coats, Amblard, & Schinazi, 2014).

We were able to synthetize prodrugs of adefovir and tenofovir in moderate yield (Pertusati et al., 2014) by adaption of the one-pot procedure for preparing phosphonodiamidate, reported by Jansa et al. (2011). Unfortunately, when these conditions were applied on the alkenyl-pyrimidine substrate, only traces of the desired phosphonoamidate product were detected, with the phosphonodiamidate being the major product. Increasing the equivalents of the aryl-alcohol (6 equivalents) with respect to the amino acid (1 equivalent) proved necessary to obtain the desired phosphonoamidate in moderate yield, as reported in Basic Protocol 1 for the preparation of linear (*E*)-4-phosphonoamidate-but-2′en-1′-yl pyrimidine (Pertusati et al., 2017). However, we discovered that this methodology suffers from the limitation that only linear olefin must be employed, as with trisubstituted alkenyl derivatives no formation of the desired ProTide was observed in our hands. This finding prompted us to investigate and then develop the methodology reported in Basic Protocol 2, using a cross-metathesis reaction for the direct synthesis of branched unsaturated ANP phosphonoamidates. At the time we started this investigation, no application of such a procedure for the synthesis of ProTides was yet reported. However, recently, a paper reporting on the use of cross-metathesis for the synthesis of ProTide derivatives of linear (*E*)-but-2-enyl nucleoside scaffold was published by Agrofoglio and colleagues (Bessières et al., 2018).

Both Agrofoglio's procedure and our own involves the preparation of the aryl allylphosphonoamidate intermediate to be reacted with the alkylated nucleobase in the CM reaction. However, our synthetic pathway using the one-pot procedure reported by Holi (Jansa et al., 2011) has proven to be a shorter and efficient approach for the synthesis of the allylphosphonoamidate synthon. Moreover, cross-metathesis conditions appear to be different. Dichloromethane was the solvent of choice in our case, with branched alkenyl nucleosides, whereas for Agrofoglio's linear olefin only water was effective.

Critical Parameters and Troubleshooting

The successful preparation of the silylated intermediates **2** and **5** in both Basic Protocol 1 and 2 is critical for the outcome of the two synthetic procedures. In particular, timing (16 hr) is a crucial parameter for this step, as in the case of too short a reaction time only partial dealkylation can be observed. The silyl esters **2** and **5** are air and moisture sensitive compounds, and therefore must be kept under strictly dry atmosphere at all times.

For Basic Protocol 2, the presence of 2,6-lutidine was revealed to be essential for the first step, as only degradation of the silyl ester intermediate **5** is detected when this acid scavenger is not present.

For the preparation of the aryl phosphonoamidate moiety (ProTide approach) in both Basic Protocols 1 and 2, the aryl alcohol to amino acid ester ratio needs to be 6 to 1 in favor of the aryloxy reagent to reduce the formation of the bisamidate derivative as byproduct. For the cross-metathesis reaction, sequential catalyst loading is a crucial parameter to

afford the desired transalkylidenation product in good yield.

The synthetic procedures described in this unit are intended for use only by persons with prior training in experimental organic chemistry and thus with knowledge of the common chemical laboratory techniques, such as extraction, solvent evaporation, column chromatography, TLC, and HPLC. Characterization of the products demands knowledge of monodimensional (^{1}H, ^{13}C and ^{31}P) and bidimensional (COSY, HSQC, HMBC and NOESY) NMR experiments, as well as mass spectroscopy. Careful attention to details of basic organic synthesis is required. General laboratory safety is also of primary concern when hazardous materials are involved. Strict adherence to the reported procedures is therefore highly recommended.

Understanding Results

The approaches applied in both Basic Protocols 1 and 2 can be applied to prepare numerous alkenyl acyclic pyrimidine ProTide derivatives with different aryloxy and amino acid ester moieties. Moreover, both the protocols can be adapted to obtain the bis-amidate derivatives when, in the first step of the two methodologies, only the amino acid ester is employed.

The cross-metathesis reaction can be significantly influenced by the length of the acyclic side chain. When the CM procedure reported in Basic Protocol 2 is employed for the preparation of aryl vinylphosphonoamidate derivatives, no formation of the final product is observed.

Time Considerations

According to Basic Protocol 1, 2 weeks are required for the nucleoside preparation, including purification and characterization of the final ProTide. In case of Basic Protocol 2, only 1 week is needed for the synthesis, characterization of the phosphonoamidate prodrug including the preparation of the two olefins, and the cross-metathesis reaction.

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