Synthesis of 1-deoxy-D-xylulose 5-phosphate reductoisomerase inhibitors as antimalarials

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List of Abbreviations

Ac	Acetyl
ACT	Artemisinin-based Combination Therapy
Ar	Aryl
Asn	Asparagine
Asp	Aspartate
ATP	Adenosine Triphosphate
BAIB	(Diacetoxyiodo)benzene
Bn	Benzyl
Boc	*tert*-Butoxycarbonyl
BOM	Benzyloxymethyl
BOMCl	Benzyl Chloromethyl ether
br	Broad
BSTFA	*N,O*-Bis(trimethylsilyl)trifluoroacetamide
CBz	Benzyloxycarbonyl
CDI	1,1'-Carbonyldiimidazole
CDP-ME	4-diphosphocytidyl-2C-methyl-D-erythritol
CoA	Coenzyme A
d	Doublet
Da	Dalton
DAST	Diethylaminosulfur trifluoride
DCE	1,2-Dichloroethane
DIAD	Diisopropyl azodicarboxylate
DIPEA	*N*,*N*-Diisopropylethylamine
DMAP	4-(Dimethylamino)-pyridine
DMAPP	Dimethylallyl Diphosphate
DME	1,2-Dimethoxyethane
DMF	Dimethylformamide
DMPU	1,3-Dimethyl-3,4,5,6-tetrahydro-2(1*H*)-pyrimidinone
DMSO	Dimethyl sulfoxide
DOXP	1-Deoxy-D-xylulose-5-phosphate
dpi	days after infection
DXR	1-Deoxy-D-xylulose-5-phosphate reducto-isomerase
EcDXR	*Escherichia coli* 1-Deoxy-D-xylulose-5-phosphate reducto-isomerase
E. coli	*Escherichia coli*
EDC	*N*-(3-Dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride
ESI	Electrospray Ionization
et al.	Et Alii (and others)
Glu	Glutamine
h	Hour
His	Histidine
HMG	3-Hydroxy-3-Methylglutarate
HMPA	Hexamethylphosphoric triamide
(HP)LC	High Pressure/Performance Liquid Chromatography

Hz	Hertz
i.p.	Intraperitoneal
IPP	Isopentenyl Diphosphate
*i*Pr	Isopropyl
J	Coupling Constant
KDA	Potassium Diisopropylamide
KHMDS	Potassium bis(trimethylsilyl)amide
LDA	Lithium Diisopropylamide
LICA	Lithium *N*-cyclohexyl-*N*-isopropylamide
LiHMDS	Lithium bis(trimethylsilyl)amide
Lys	Lysine
m	Multiplet
Met	Methionine
MS	Mass Spectrometry
MST	Mean Survival Time
MVA	Mevalonic Acid
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (reduced form)
NaHMDS	Sodium bis(trimethylsilyl)amide
nd	not determined
NFBS,NFSI	*N*-Fluorobenzenesulfonimide
NFOBS	*N*-Fluoro-*o*-benzenedisulfonimide
NFPMS	*N*-Fluoroperfluoromethanesulfonimide
NHA	*N*-hydroxyazetidin-2-one
NMR	Nuclear Magnetic Resonance Spectroscopy
NMMO,NMO	4-Methylmorpholine *N*-oxide
Nosyl, Ns	2-Nitrobenzenesulfonyl
on.	overnight
P. falciparum	*Plasmodium falciparum*
P. malariae	*Plasmodium malariae*
P. ovale	*Plasmodium ovale*
P. vivax	*Plasmodium vivax*
PfDXR	*Plasmodium falciparum* 1-Deoxy-D-xylulose-5-phosphate reducto-isomerase
PK/PD	Pharmacokinetics/Pharmacodynamics
PMB	*para*-Methoxybenzyl
POM	Pivaloyloxymethyl
ppm	Parts Per Million
q	Quadruplet
(Q)SAR	(Quantitative) Structure-Activity Relationship
Rf	Ratio to front
RP	Reversed Phase
rt	room temperature
s	Singlet
Selectfluor™, F-TEDA-BF_4	1-(chloromethyl)-4-fluoro-1,4-diazabicyclo[2.2.2]octane bis(tetrafluoroborate)

Ser	Serine
t	Triplet
TBAF	Tetra(*n*-butyl)ammonium fluoride
TBDMS	*tert*-Butyldimethylsilyl
TEMPO	2,2,6,6-Tetramethyl-1-piperidinyloxy, free radical
TES	Triethylsilyl
Tf, Triflyl	Trifluoromethanesulfonyl
TFA	Trifluoroacetic Acid
THF	Tetrahydrofuran
TLC	Thin Layer Chromatography
TMEDA	*N,N,N',N'*-Tetramethylethylenediamine
TMS	Tetramethylsilane (in NMR) / Trimethylsilyl (as protecting group)
TMSBr	Bromotrimethylsilane
Trp	Tryptophane
Ts, Tosyl	*p*-Toluenesulfonyl
TsCl	*p*-Toluenesulfonyl chloride
UV	Ultraviolet
WHO	World Health Organization
wt.	weight
δ	Chemical Shift

Chapter I

Introduction

I.Introduction

I.A. Malaria

I.A.1. Prevalence and Progress

In 2010, 3.3 billion people worldwide were at risk of malaria according to the World Health Organization's (WHO) estimations. There were 216 million cases of malaria, 81% of them were found in Africa. An estimated 655 000 people, of which 86% were children under 5 years old, died of malaria in 2010. Generally, 91% of malaria deaths occurred in Africa[1].

Table I.1: Estimated malaria cases and deaths by WHO Region, 2010[1]

Region	Estimated Cases	Estimated Deaths
Africa	174 million	596 000
Americas	1 million	1 000
Eastern Mediterranean	10 million	15 000
Europe	2 000	0
South-East Asia	28 million	38 000
Western Pacific	2 million	5 000

Still according to the WHO, malaria mortality rates are declining: a 5% reduction was seen between 2010 (655 000 malaria deaths) and 2009 (691 000 malaria deaths). During the last decade, malaria mortality rates have fallen by more than 25%, with the largest reductions seen in the European (99%), American (55%) and Western Pacific (42%) and African Regions (33%)[1].
Important factors that mediated this trend are the increased availability of longlasting insecticidal nets, indoor residual spraying, and better access to diagnostic testing and effective treatment with artemisinin-based combination therapies (ACTs). In sub-Saharan Africa, improvements in the socio-economic situation in many countries have contributed to the decreased number of deaths among children under 5 from all causes, including malaria[1]. Although this represents a nice progress,

mortality numbers are still disturbingly high bearing in mind that malaria is entirely preventable and treatable.

I.A.2. Plasmodium

Malaria is a life-threatening tropical disease caused in humans by four species of the genus *Plasmodium*, namely:

P. falciparum which causes malaria tropica

P. malariae which causes malaria tertiana

P. ovale which causes malaria quartana

P. vivax which causes malaria tertiana

Of highest relevance are *P. falciparum* for it causes the highest mortality rates, and *P. vivax* for its ability to form dormant liver stages (hypnozoites) in infected patients. *Plasmodium* species belong to the phylum *apicomplexa*: eukaryotic unicellular organisms (protists) named after their *apical* complex or apicoplast, an intracellular structure required for invasion of host cells which is composed of cytoskeletal elements combined with specific secretory organelles. Almost all apicomplexan parasites bear a unique apicomplexan plastid that has no photosynthetic function. This apicoplast was most probably acquired through secondary endosymbiosis of a plastid-containing red alga and is consequently surrounded by four membranes: two 'inner' membranes from the initial plastid envelope, a periplastid membrane originating from the plasma membrane of the engulfed alga and an outer membrane formed by the initial phagotrophic membrane. This is important because a number of targets for antimalarial drugs are located inside the apicoplast, drugs that therefore have to cross many membrane barriers before reaching their target[2].

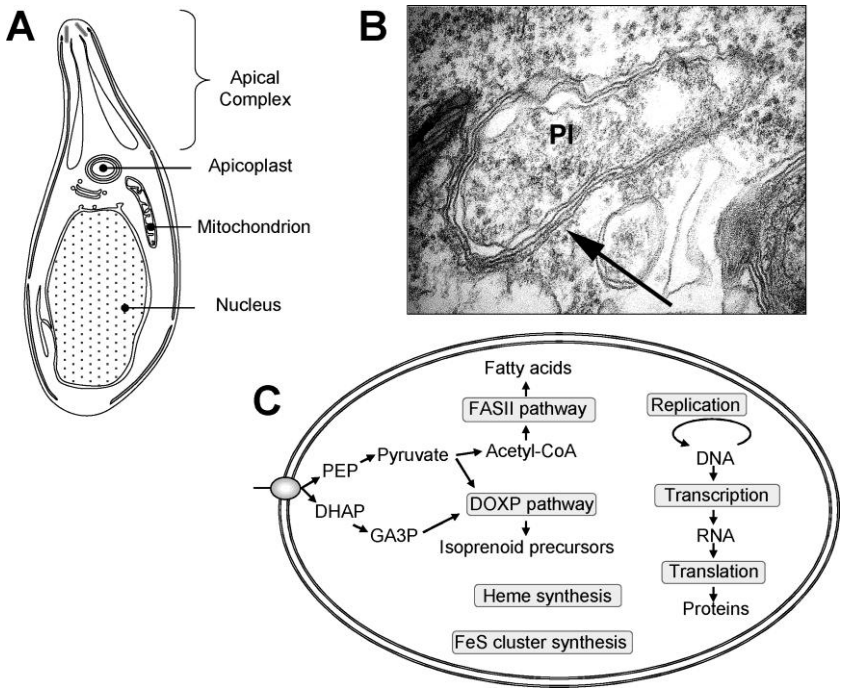


Illustration I.1: Apicoplast of *P. falciparum*: a relict nonphotosynthetic plastid involved in essential metabolic functions. (A) Schematic drawing of *P. falciparum*, a unicellular eukaryote belonging to the Apicomplexa phylum, showing the three DNA-containing organelles, i.e., the nucleus, the mitochondrion, and the apicoplast. (B) Electron microscopic image showing the location of the apicoplast in *P. falciparum*. The apicoplast is basically a plastid (Pl) surrounded by four membranes (arrow). (C) Four major biosynthetic pathways located in the apicoplast: a fatty acid synthesis pathway (FASII), the nonmevalonate pathway or DOXP pathway (see below), a heme synthesis pathway, and an iron/sulfur (FeS) cluster synthesis pathway. The apicoplast possesses a 35 kilobase genomic DNA which is replicated, transcribed into RNA, and translated into proteins via its own machineries of prokaryotic origin. Figure from: *Chemical Reviews* (2012), 112, 1269–1283

The vector for malaria transmission is the female *Anopheles* mosquito, which transmits the parasites by biting humans in search of a blood meal. In the gut of the mosquito, *Plasmodium* undergoes its sexual reproductive cycle, whereas in the mammalian host it undergoes cycles of asexual divisions known as schizogony. These cycles, which take place first in the liver cells but in a later stage continue in the red blood cells, cause the often deadly symptoms of malaria[3].

I.A.3. Current therapies

In general, for malaria prophylaxis established drugs from the quinine family (quinine, chloroquine, mefloquine, halofantrine) are still of frequent use. Also atovaquone (an inhibitor of the mitochondrial electron transport) combined with proguanil (an antifolate), or the antibiotic doxycylin are mainstream malaria prophylactics[4].

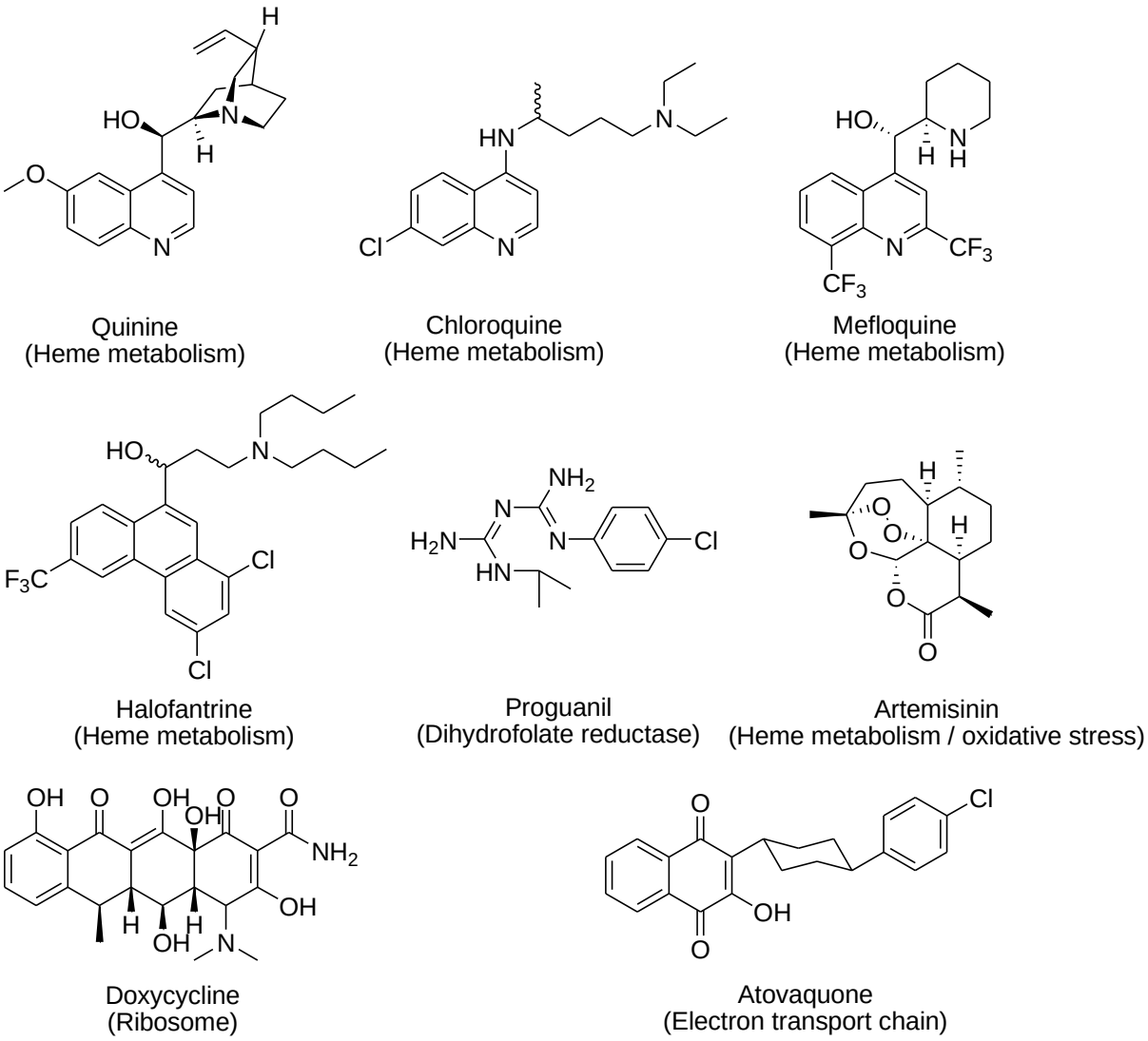


Figure I.1: Mainstream antimalarials (and their target, in parentheses) used for prophylaxis and therapy

Concerning the treatment of malaria infections, on the other hand, chemotherapy should never be based on the same drugs the patient used for prophylaxis before he got infected. Nowadays,

artemisinin-based combination therapies (ACTs) are recommended as the first-line treatment for malaria caused by *P. falciparum*, but they are also in use for infections by other *Plasmodium* species. For uncomplicated *falciparum* malaria, the following combinations are used: artemether-lumefantrine, atovaquone-proguanil or quinine plus doxycycline or clindamycin. For *vivax* malaria, radical cure (treatment of both blood and liver stages, preventing recrudescence and relapse) is obtained with chloroquine-primaquine. In case of chloroquine resistance, amodiaquine-primaquine or lumefantrine plus quinine or artemether are used. In mixed infections (*falciparum*-*vivax*), treatment of the *P. falciparum* malaria will usually also cure the *vivax* part. Primaquine is added to achieve radical cure (see above)[4]. In 2010, 181 million courses of ACTs were procured worldwide in the public sector, up from 158 million in 2009, and just 11 million in 2005[1].

I.A.4. Resistance

Continuous exposure of a *Plasmodium* parasite population to a drug may lead to the development of resistance against that drug through natural selection, but it may also cause the selection of genetic traits that favor initiation of resistance to novel, unrelated antimalarials[5]. Several factors are of importance in the acquisition of drug resistance:

- Mechanism of action: resistance can be generated by a single mutation in the case of drugs which act against a single target, such as an enzyme (monogenetic resistance);

- Pharmacokinetics: when low plasma concentrations of an antimalarial are present, parasites are not killed, although selective pressure will result in the selection of resistant parasite strains[6];

- Drug efficiency: if a drug is not sufficiently effective, it will leave a surviving part of the parasite population behind, thus stimulating the development of resistance;

- Population immunity: antimalarial chemotherapy may be more efficient in populations with a high immune response, because the opportunity for the parasite to spread and infect the host is decreased.

Of all *Plasmodium* species, P. falciparum shows the highest potential to develop (multi)drug resistance[7]. The first case of resistance was discovered around 1910 for quinine, and resistance against almost all mainstream antimalarials has emerged since then with an increasing speed: whereas the quinine resistance took 280 years to develop, resistance against artemisinin was already reported a few years after its introduction.

Table I.2: Chronology of resistance against common antimalarials

Antimalarial	Introduced in	First case of resistance
Quinine	1632	1910
Chloroquine	1945	1957
Proguanil	1948	1949
Sulfadoxine-Pyrimethamine	1967	1967
Mefloquine	1977	1982
Atovaquone	1996	1996

Because of the importance of ACTs as first-line therapy for uncomplicated *P. falciparum* malaria[8, 9], development of resistance against artemisinin is a major concern. It was first confirmed on the Cambodia-Thailand border in 2009 but is now suspected in parts of Myanmar and Vietnam as well. Fortunately, ACTs remain effective in most settings as long as the combination partner drug is effective.

Ongoing efforts by the WHO to limit the spread of artemisinin-resistant parasites by means of containment zones has resulted in a strongly diminished *falciparum* malaria burden within these zones. However, the proportion of patients with resistant parasites has increased. Therefore, it is necessary to eliminate all parasites in areas with documented resistance, an endeavor for which the WHO started the Global Plan for Artemisinin Resistance Containment (GPARC) in 2010. Additionally, the marketing of oral artemisinin-based monotherapies, which has been one of the major causes of emerging drug resistance, is discouraged[1].

I.A.5. Vaccine

An effective vaccine against malaria has long been envisaged as a valuable addition to the available tools for malaria control, although there are some important issues concerning the development of such a vaccine. First of all, it takes about 5 years for children in endemic areas to develop full immunity against malaria. Hence the high mortality rate for malaria in children under 5. Furthermore, this immunity seems to disappear over time, suggesting that a continuous exposure to the parasite is necessary in order to keep the immunity at a high level. Immunity against *Plasmodium* can thus be considered incomplete, making lifelong protection hard to realize. Nevertheless, malaria vaccines have been the subject of intensive research over the past years[10-17]. The WHO declared that, if the development of a vaccine that prevents malaria infection would prove unrealistic, the concept of a vaccine that could interrupt malaria transmission is also of interest[1].

There are currently no licensed malaria vaccines but over 20 research projects are in clinical trials. The most advanced of these is a vaccine called RTS,S/AS01 developed by a GlaxoSmithKline partnership, which is currently in Phase 3 clinical trials and at least 5-10 years ahead of other candidate malaria vaccines[18-20]. It is a *P. falciparum* vaccine, with no protection expected against *P. vivax*. Phase 3 trial results are still being collected, but in the first interim report an efficacy of 55% reduction in frequency of malaria episodes during the 12 months follow-up was communicated.

I.A.6. The Need for New Antimalarials Acting onto New Targets

Several reasons for the development of new antimalarials can be recited.

First of all there is the issue of resistance against virtually all antimalarials currently in clinical use as described above. Secondly, most –if not all- of nowadays antimalarials show a significant toxicity, translating in sometimes severe adverse effects while they often have to be used in pregnant women and young children. Furthermore, with ACTs being todays number one treatment choice, the price of antimalarial treatment has also become an issue as artemisinin, a natural product of limited supply, is quite expensive. As also stated above, a vaccine offering broad spectrum and long term protection against malaria is not to be expected soon. Besides all this, there is already a need to extend the

portfolio of antimalarial drugs for the treatment of pregnant women and in regions of multidrug resistance[21]. All these reasons underscore the importance of antimalarial chemotherapy and the continuing need of research for new classes of antimalarial agents.

New drug candidates that are based on line-extension of already used antimalarials risk the fast induction of resistance through their lack of structural diversity with their predecessors, or by targeting the same enzyme or pathway.[21] Hence the need for new drugs that are structurally unrelated to drugs used before, in order to avoid fast development of resistance. This might imply also drugs acting against new, unsourced, targets. In general an ideal antimalarial should have the following PK/PD properties:

- good bioavailability to cure sufficient parasite exposure, preferably also after oral administration in order to facilitate use in non-hospital settings;
- low inter-individual pharmacokinetic variability;
- well tolerated by the patient, low toxicity;
- a pharmacokinetic profile which allows for shorter duration of treatment with fewer doses (a 3-day maximum therapy for cure with once or twice a day dosing is desirable for concern about patient compliance);
- predictable pharmacokinetic profile such that the drug is retained for sufficient length of time (adequately long half-life) to ensure that it kills the parasites, but does not accumulate to any extent to cause damage to the host[22].

Concerning the half-life of antimalarials, two issues are important. On the one hand, antimalarials with a long half-life are useful for chemoprophylaxis since longer maintained plasma levels allow for longer dosage intervals, and for directly observed chemotherapy (with observed compliance). However, they are also particularly vulnerable to the development of resistance as they

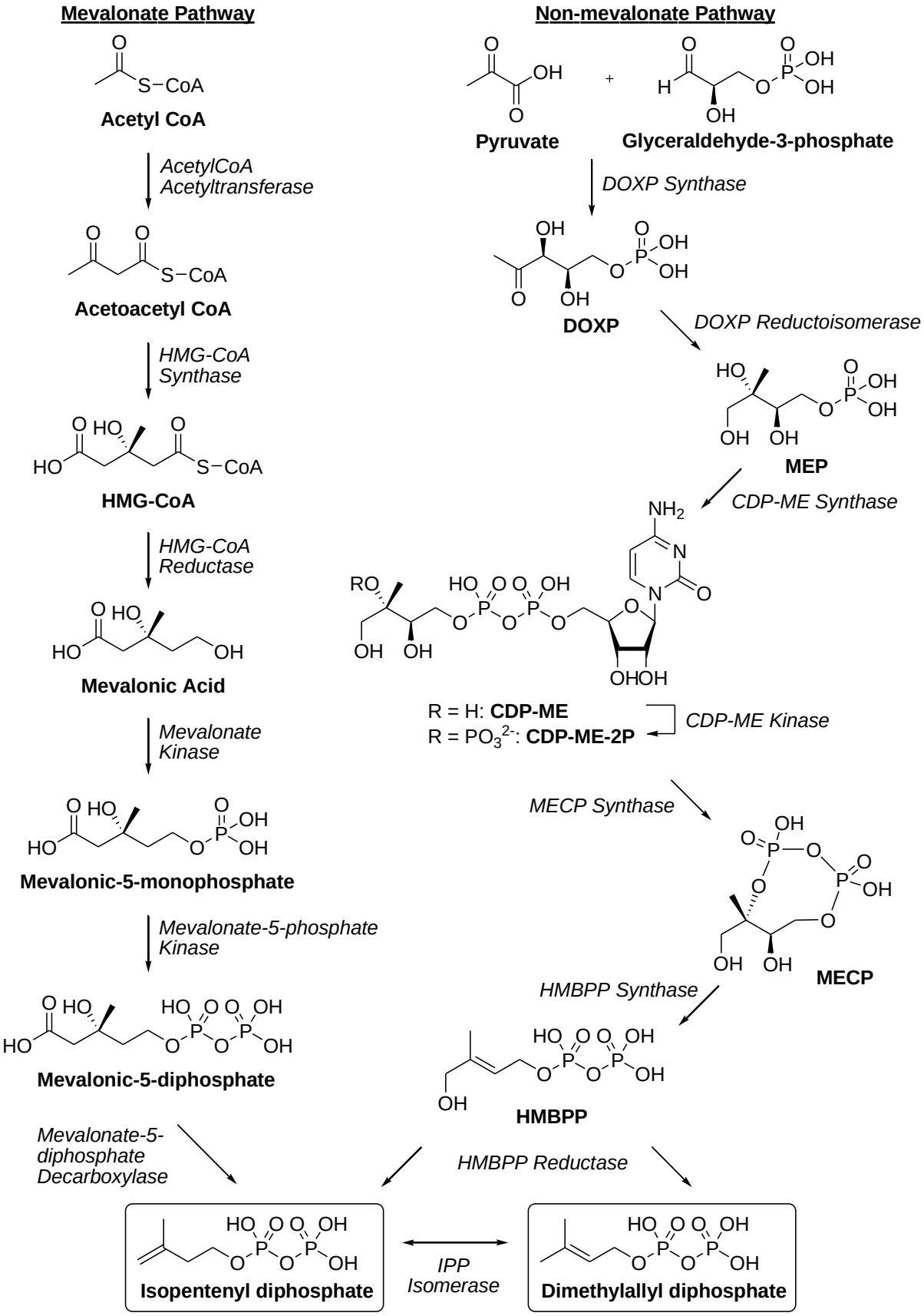
may produce subtherapeutic plasma levels for significant periods of time, thus providing selection pressure. Drugs with a short half-life, on the other hand, will not pose such problems of inducing resistance but these need frequent administration, thus creating the risk of low compliance[23-30]. In general, antimalarials for treatment in endemic areas are preferred to have a short plasma half-life[26, 31].

Over the past few years, considerable efforts have also been made through public-private partnerships, resulting in the progression of several drugs and drug combinations to various stages of clinical development[3]. The development of new antimalarials has unfortunately been of very low interest to the pharmaceutical industry due to the increased costs of developing and registering of pharmaceuticals, combined with the prospect of low commercial returns.[21] Currently, mainly academic groups have identified a large number of potentially new targets, and target validation, drug discovery and lead optimization are in progress[3].

I.B. The Non-Mevalonate Pathway

I.B.1. Isoprenoids

Isoprenoids form the largest class of natural products with more than 35000 compounds known so far, comprising a heterogeneous group of biologically important primary and secondary metabolites[32]. They are found in all living organisms[33, 34] where they carry out vital biological functions such as: modulation of membrane properties in archaebacteria[35], eubacteria and eukaryotes[36], electron transport carriers (e.g. ubiquinone)[37], light harvesting and photoprotection (carotenoids, chlorophyll side chains), growth and development regulation (steroid hormones, cytokinins)[38], signal transduction (prenylation of proteins)[39], but also pollination, seed dispersion and repelling of herbivores (toxins) in plants[40, 41]. In contrast to their huge diversity, all isoprenoids are formed from the same two common five-carbon building blocks isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) as described by Ruzicka's isoprene rule[42, 43]. Until the late 1980's it was believed that in all organisms IPP and DMAPP were formed exclusively by the mevalonate pathway that was known since the early 1950s[44-46]. The mevalonate pathway, as depicted in scheme I.1, starts with the condensation of two molecules of acetyl-CoA to form acetoacetyl-CoA, which is then converted to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). In the next step, HMG-CoA is reduced to mevalonic acid (MVA) by HMG-CoA reductase, an extensively studied key enzyme in this pathway[47] and also the target of the statins, a class of human cholesterol-reducing drugs. Subsequently, MVA undergoes two phosphorylation steps, yielding mevalonic-5-diphosphate (MVA-PP), which is finally converted by an ATP-dependent decarboxylation into IPP. IPP and its isomer DMAPP are interconverted by IPP isomerase[48].



Scheme I.1: Mevalonate versus Non-mevalonate Pathway

I.B.2. The Non-Mevalonate Pathway as a Drug Target

In the early 1990's the groups of Arigoni and Rohmer independently established the existence of a second pathway for the biosynthesis of isoprenoid building blocks, starting from the triose pool of intermediary metabolism[49]. The history of the discovery of this so-called non-mevalonate pathway has been reviewed[50]. The pathway is also known as the 1-deoxy-D-xylulose-5-phosphate (DOXP) pathway, the Rohmer pathway or the 2C-methyl-D-erythritol-4-phosphate (MEP) pathway[48] and comprises seven enzymatic steps as depicted in Scheme I.1. First, glyceraldehyde-3-phosphate is condensed with pyruvate in a transketolase-type reaction catalyzed by DOXP synthase (DXS) to produce DOXP[51]. Next, DOXP is transformed into MEP by DOXP-reductoisomerase (DXR, IspC) in a single step comprising an intramolecular rearrangement and an NADPH-mediated reduction[52]. The mechanism of this step is still a matter of debate and will be handled in more detail below, but nevertheless it is also the most relevant and most studied step of the non-mevalonate pathway. In the following step, MEP is transformed into 4-diphosphocytidyl-2C-methyl-D-erythritol or CDP-ME by the CTP-dependent action of CDP-ME synthase (YgbP, IspD)[53]. CDP-ME is then phosphorylated at the 2-hydroxy position by CDP-ME kinase (YchB, IspE), using ATP as a cofactor with the formation of 4-diphosphocytidyl-2C-methyl-D-erythritol-2-phosphate (CDP-ME2P)[54]. This CDP-ME2P is converted by MECP synthase (YgbB, IspF) into 2-C-methyl-D-erythritol-2,4-cyclodiphosphate (MECP) using CTP as a cofactor[55], and then transformed into 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate (HMBPP) by HMBPP synthase (GcpE, IspG)[56, 57]. Finally, HMBPP reductase (IPP/DMAPP synthase, LytB, IspH) catalyzes the conversion of HMBPP into a mixture of IPP and DMAPP[58]. HMBPP synthase seemingly replicates the activity of IPP isomerase from the mevalonate pathway[59].

The mevalonate pathway is the only pathway for isoprenoid biosynthesis in mammals. It is also present in the cytoplasm of higher plants and in some bacteria. The non-mevalonate pathway, on the other hand, is present in the chloroplast of plants and is found widespread in bacteria, sometimes together with the mevalonate pathway[60, 61]. In some important pathogenic bacteria, and also in *Mycobacterium tuberculosis* and in *Plasmodium* (where it is located in the apicoplast), it is the only

biosynthetic route to vital isoprenoids[48]. Together with the fact that all enzymes of the non-mevalonate pathway are absent in humans (as well as in yeasts and funghi), this makes it an interesting target for antimalarial drugs[48].

I.B.3. DXR

I.B.3.a. Structure

As stated above, DXR is currently the most studied[62] and also clinically the most relevant enzyme of the non-mevalonate pathway. Several NMR- and X-ray studies have been conducted to unravel its structure and function[63-70]. Most of these studies are performed on the *E. coli* isozyme[67, 69-71] for it is easier to handle than, for instance, the *Plasmodium* isozyme. Fortunately, X-ray structures of both the *M. tuberculosis*[65, 66] as well as the *P. falciparum*[72] isozymes have been published, the latter both in the absence and in the presence of fosmidomycin, the lead structure for DXR inhibitor development (see below). PfDXR, in its active form (that is: Lys75 to Ser488), is a homodimer with an approximate mass of 47 kDa consisting of two subunits that each contain an NADPH molecule and a divalent metal cation (usually Mg^{2+}, Mn^{2+} or Co^{2+}). The overall structure of PfDXR is essentially similar to those of DXRs from other species[63, 66-73]. Each DXR subunit consists of two large domains separated by a cleft containing a deep pocket, a linker region and a small C-terminal domain. One of the large domains holds the groups necessary for catalysis (metal and substrate binding, residues 231 to 369) while the other one (residues 77 to 230) is involved in the binding of NADPH. The catalytic domain is an α/β-type structure consisting of five α-helices and four β-strands in which a crevice is found with the bound divalent metal cation at the bottom. A phosphate binding site is also present, relying mostly on H-bridge interactions with serine, asparagine and lysine residues. Upon binding of the substrate or an inhibitor, the enzyme undergoes an induced fit and the active site is covered by a flexible loop. This flexible loop complicates the acquisition of representative X-ray structures and thus DXR ligand design.

I.B.3.B. Function

DXR catalyzes the second step in the non-mevalonate pathway: the conversion of DOXP into MEP, consisting of an isomerization followed by an NADPH-mediated reduction. Two mechanisms have been proposed for the isomerization step: a stepwise fragmentation-reassembly via retro-aldol/aldol-mechanism or a concerted α-ketol rearrangement (Figure I.2)[74-78].

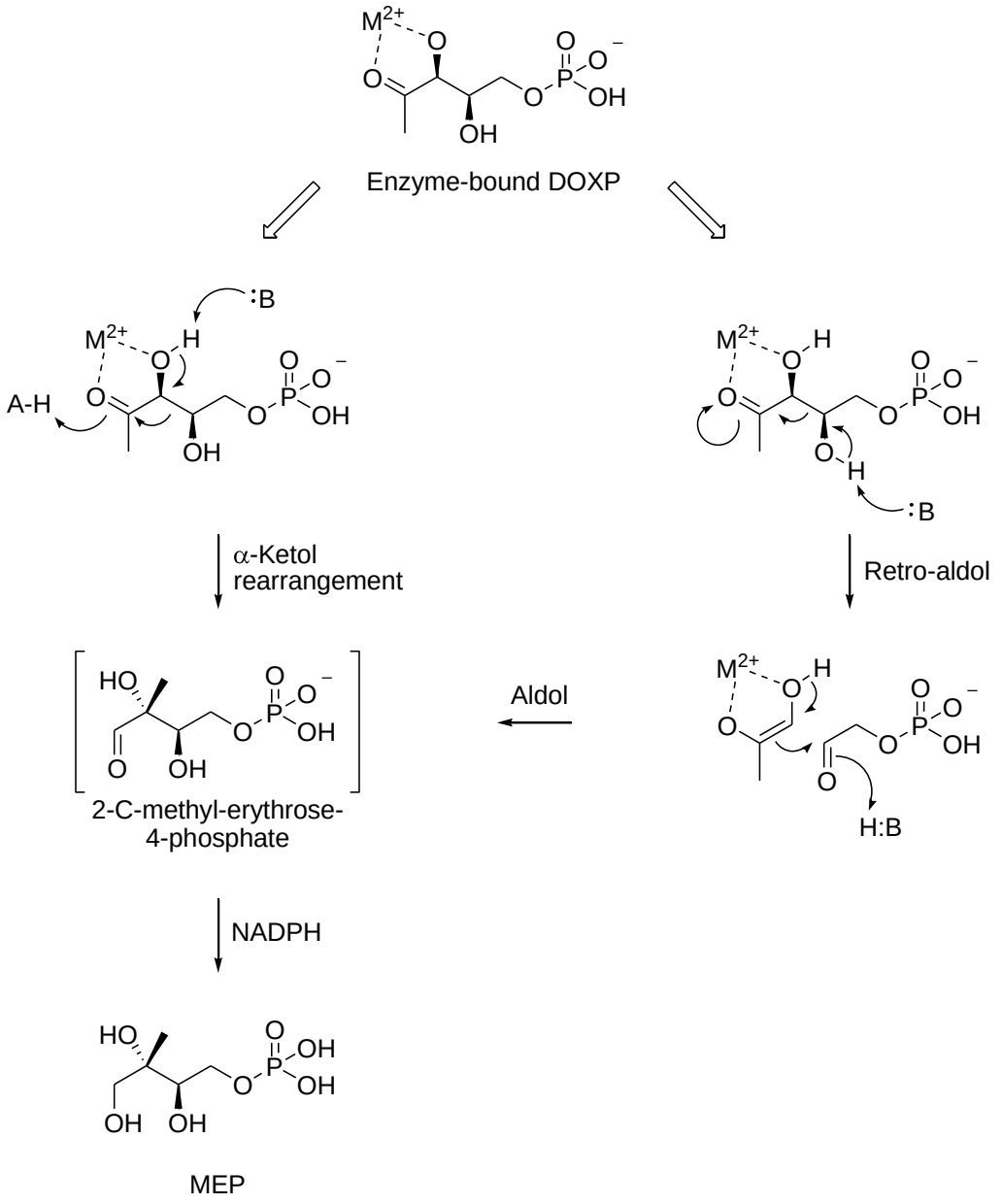


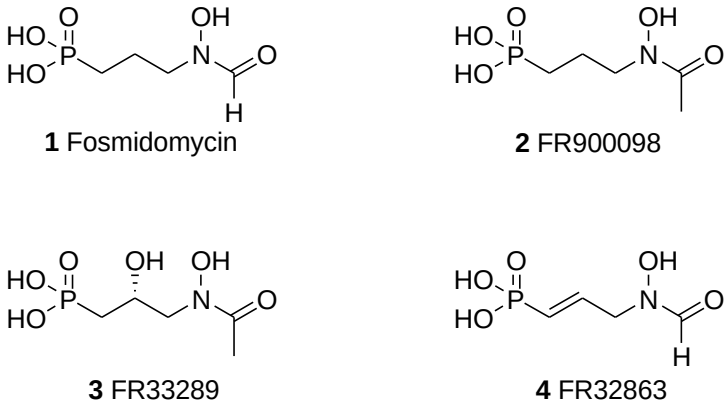
Figure I.2: Two alternative mechanisms for the isomerization of DOXP in DXR

The retro-aldol/aldol mechanism involves the cleavage of the C3-C4 bond in DOXP with the formation of two intermediates: glycolaldehyde and hydroxyacetone. So far, the involvement of neither of these intermediates in the reaction could be proven, so the logical conclusion would be that the isomerization goes via the α-ketol rearrangement[75]. On the other hand, experiments based on the kinetic isotope effect support the retro-aldol/aldol route[76, 77]. Support for this mechanism has also been gathered by Fox and Poulter[79] and by the group of Liu[80] by using fluorinated substrate analogues. After the aldehyde intermediate is formed by isomerization, it is reduced by transfer of the pro-*S* hydride ion of NADPH to its *RE* face.

I.C. Fosmidomycin

I.C.1. Fosmidomycin as a DXR Inhibitor

Fosmidomycin, also known as FR31564 or 3-(*N*-formyl-*N*-hydroxyamino)propylphosphonic acid, was discovered in the late 1970s by Fujisawa Company as a natural antibiotic from *Streptomyces lavendulae* together with its congeners FR900098 (the *N*-acetyl congener of fosmidomycin), FR33289 (the β-OH congener of FR900098) and FR32863 or α,β-unsaturated fosmidomycin[81-84]. In many assays, FR900098 has shown to be more potent than fosmidomycin and these two analogues are the most studied ones of this series.



1 Fosmidomycin **2** FR900098

3 FR33289 **4** FR32863

Figure I.3: Structures of fosmidomycin congeners originally discovered by Fujisawa Company

Fosmidomycin was also found to show herbicidal activity. After its discovery it was tested in a Phase II clinical trial for the treatment of urinary tract infections but due to the availability of more potent antibiotics at that time, further development of fosmidomycin and its congeners was discontinued. In 1998, Kuzuyama et al. identified fosmidomycin as a putative pathway-specific inhibitor that was active against most Gram-negative and some Gram-positive bacteria through a database search to identify specific inhibitors of the non-mevalonate pathway. Further work then showed that fosmidomycin was an astonishingly non-toxic specific inhibitor of DXR[78, 85]. In an *in vitro* experiment, fosmidomycin inhibited purified recombinant EcDXR in a dose-dependent manner with

an IC_{50} of 8.2 nM[78]. Furthermore, the inhibitory effect of fosmidomycin in an *E. coli* growth assay was overcome by the addition of 2-C-methylerythritol (the free alcohol of MEP). These results, together with studies of isoprenoid biosynthesis in plants, led to the conclusion that fosmidomycin is a specific inhibitor of DXR activity[56, 85].

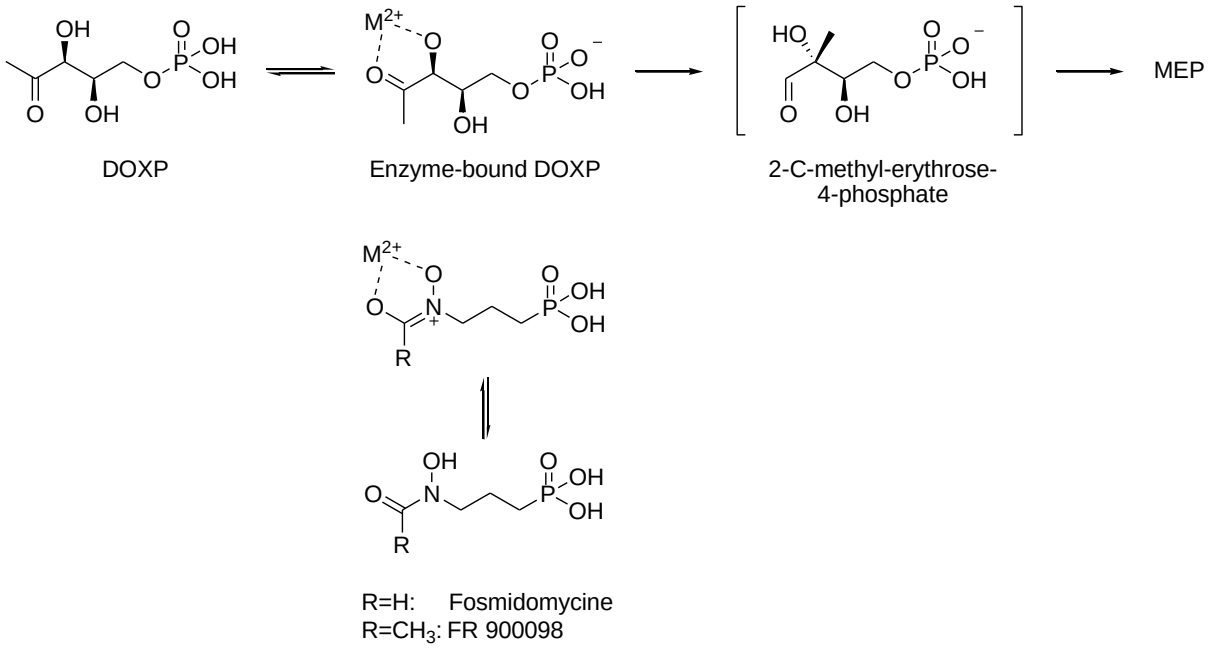


Figure I.4: Structure and binding analogy between DOXP and Fosmidomycin/FR900098

X-ray crystal structures of DXR-metal-fosmidomycin/FR900098 complexes with both EcDXR[73] and PfDXR[72] as well as MtDXR[65] reveal a substrate-like binding mode of the inhibitor: the phosphonate functionality occupies the phosphate binding site, while the hydroxamate chelates the catalytic divalent cation. The three-carbon spacer interacts with a methionine residue and is covered by the indole ring of a tryptophane residue of the flexible loop. In EcDXR, the phosphonate forms hydrogen bonds to Ser185, Ser221 and Lys227. The three-carbon spacer interacts with Met213, while the hydroxamate chelates the catalytic divalent cation that is bound by residues Asp149, Glu151 and Glu230 (Diagram I.1).

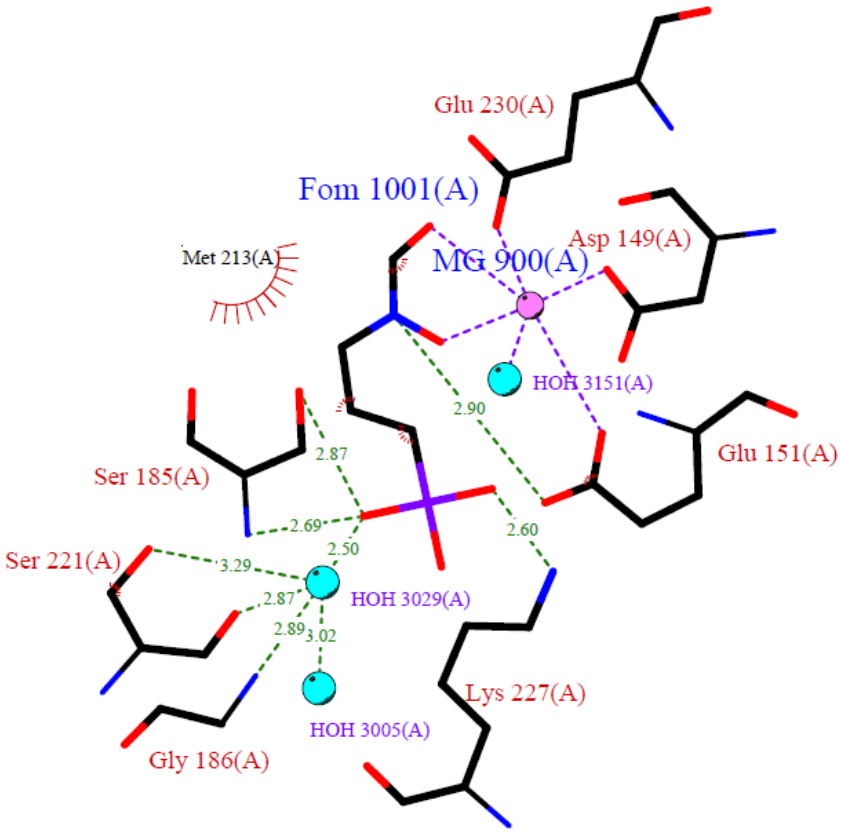


Diagram I.1: Simplified diagram of Fosmidomycin ("Fom 1001") binding to EcDXR[86]

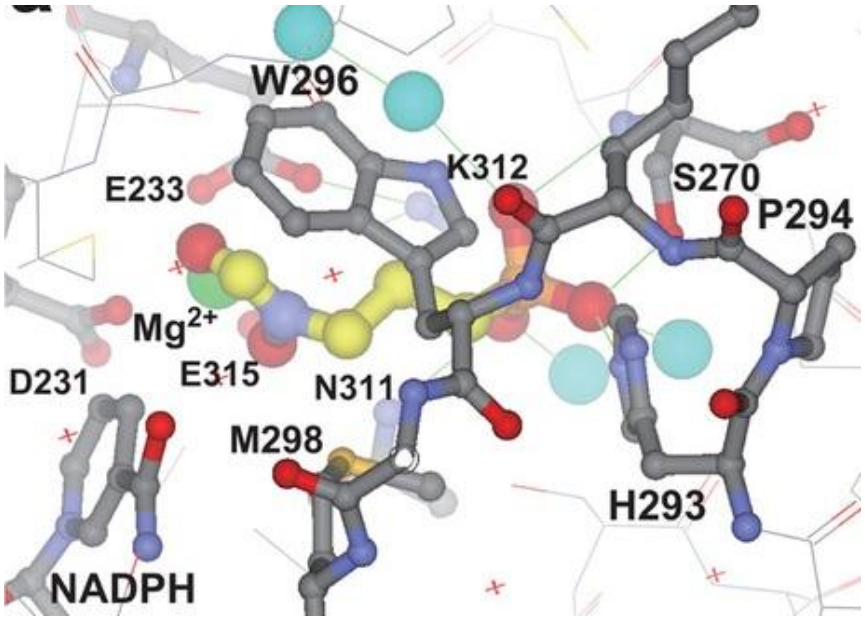


Diagram I.2: Fosmidomycin complex with PfDXR. The carbon atoms of fosmidomycin, the four buried water molecules, and the bound Mg^{2+} ion are shown in yellow, cyan, and green, respectively. Figure from *Scientific Reports* **2011,** *1*.

In PfDXR, the phosphonate is anchored in the phosphate binding site by a hydrogen bond network with Ser270, Asn311, two water molecules and His293. The three-carbon spacer lies parallel to the indole ring of Trp296 and also interacts with Met298, while the hydroxamate chelates the catalytic divalent cation that is bound by residues Asp231, Glu233 and Glu315[72](Diagram I.2). It is necessary that both oxygen atoms of the retrohydroxamate functionality adopt a *cis* (Z) conformation. Fosmidomycin has been described both as a mixed-type inhibitor (both competitive and non-competitive)[56] and as a slow, tight-binding competitive inhibitor of DXR[67].

I.C.2. Fosmidomycin as an Antimalarial

In 1999, Jomaa et al. showed that fosmidomycin and its acetyl congener FR900098 inhibit PfDXR in a dose-dependent manner. They also revealed its *in vitro* antimalarial effect on *P. falciparum* infected erythrocytes as well as its *in vivo* effect in *P. berghei* infected mice. Furthermore, they provided evidence for the presence of the non-mevalonate pathway in the apicoplast of *P. falciparum*, thus opening the way for the use and development of fosmidomycin as an antimalarial[87]. Shortly after, fosmidomycin was tested in humans and cured uncomplicated *P. falciparum* malaria[88, 89] , albeit with a high rate of recrudescence. This precluded its use in monotherapy, leading to the successful combination with clindamycin[90, 91] or artesunate[92].

The major advantage of fosmidomycin/FR900098 is its very low toxicity, and also its low serum half-life[93] (see I.A.6.). The latter can be seen both as an advantage and as a disadvantage as described above. Unfortunately, both compounds also show poor oral bioavailability of around 20-40%, probably due to the very polar nature of both the phosphonic acid and the retrohydroxamate structure[94].

Although fosmidomycin shows a very attractive antimalarial potential, acting on a new, hitherto unsourced target, it certainly also has some shortcomings. Optimization efforts directed at improving fosmidomycin's potency as well as reducing its polarity could lead to new, promising antimalarials.

I.D. SAR of Fosmidomycin Analogues

Since the potential of inhibitors of the non-mevalonate pathway as new antimicrobial or antiparasitic drugs became clear[61, 95], and Kuzuyama et al. identified fosmidomycin as a DXR inhibitor[78] a lot of research has been dedicated to the structural modification of this lead in search of more potent antibacterial and herbicidal[96] analogues. The establishment of fosmidomycin as an antimalarial by Jomaa et al.[87] gave this research a new twist and since then, many fosmidomycin analogues have been synthesized and tested specifically for their antiplasmodial properties. Here, we will give an overview of the structural modifications of fosmidomycin that have been published and try to derive some structure-(antimalarial) activity relationship. Generally, fosmidomycines are evaluated in three different ways: *in vitro* enzyme kinetics, *in vitro Plasmodium* growth inhibition and *in vivo* experiments in mice infected with *P.berghei*. The former method is applied on different enzymes, usually EcDXR or (recombinant) PfDXR but also *Synechocystis*-DXR. Moreover, inhibitory activities are reported in various ways: as percentual inhibition for a given concentration of inhibitor, as a K_i-value or as an IC_{50}-value. Because fosmidomycin, and expectedly also its structural analogues, are slow tight-binding inhibitors of DXR, measured DXR inhibition values (K_i's) may vary strongly depending on whether the inhibitor was added to an enzyme-substrate mixture or rather was pre-equilibrated with DXR before addition of substrate and activity measurement. Often it is not clearly mentioned which conditions were applied. Moreover, also the concentration of enzyme, substrate and inhibitor used in testing can significantly influence measured K_i values, thus complicating the comparison of K_i values measured by different groups or in different experiments[97]. Concerning the *in vitro Plasmodium* growth inhibition: large variability in measured results is often seen, due to the use of different strains of *P. falciparum* for this test. Depending on the origin of the strain used, its sensitivity for different antimalarials, hence also the tested ones, may vary significantly. Finally, also the communication of *in vivo* results is not unambiguous: percentage survival after certain time intervals, relative reduction in infected red blood cells or graphic representations of mice survival have all been used.

These facts make the strict comparison of activity among different groups of published fosmidomycin analogues a very ambiguous task. In the best case reported results include the activity of a reference substance (usually fosmidomycin and/or FR900098), but often they don't. Therefore, we will restrict the next part to a description of trends in fosmidomycin SAR rather than trying to elaborate numerical data. More recently, also the search for antimycobacterial fosmidomycine analogues has received considerable interest[98-102], although it seems that penetration of the mycobacterial cell wall by such polar compounds is still a major hurdle[103]. Note that in *Plasmodium* and some other species, so-called parasite-induced new permeability pathways appear to facilitate the uptake of these indeed very polar molecules[104]. Some trends in fosmidomycin SAR, albeit not strictly for antimalarial activity, can also be deduced from these data.

Fosmidomycin is composed of three main structural parts: a phosphonic acid group, the three-carbon spacer and the retrohydroxamate. The following review will divide modifications according to this structural division, and will focus mainly on modifications from a medicinal point of view. We will not include all substrate-, transition-state- and product-analogues that have been mainly synthesized as tools to unravel DXR's mode of action. Although some of these analogues showed a moderate DXR inhibitory effect, most featured a phosphate functionality, precluding their use as drugs because of chemical and enzymatic instability. Therefore, these analogues will not separately be discussed but may be mentioned among other modifications where appropriate.

I.D.1. Modifications of the phosphonate functionality

The phosphonate functionality in fosmidomycin acts as an isostere for the phosphate group of DOXP (Phosphate isosteres are discussed in more detail in Chapter II), thus securing its binding in the phosphate binding site of DXR[72]. In many cases, enzyme phosphate binding sites are mainly composed of glycine residues without specific recognition motifs for the charged phosphate group[105, 106]. Such binding sites can usually be targeted by neutral heterocyclic groups bearing suitable H-bond

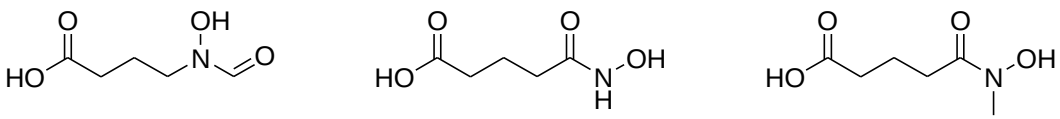
acceptors. Unfortunately, as is described above, the phosphate binding site of DXR is formed by polar amino acid side chains (for EcDXR: Ser 186, Ser222, Asn227 and Lys228[73]) acting as H-bond donors and relies heavily on an H-bond network with the charged phosphate group acting as an acceptor. The presence of a phosphate or phosphonate group has thus turned out to be necessary for efficient binding to DXR. Nevertheless, numerous attempts have been made to omit the use of such an extremely polar group. Woo et al.[107] synthesized the phosphate analogues of fosmidomycin and of FR900098, resp. **5** and **6** (Figure I.5). Both showed a significantly stronger DXR inhibition than fosmidomycin (K_i of 19nM (**5**) and 2nM (**6**) on *Synechocystis* DXR vs. 57nM for fosmidomycin). Fosmidomycin phosphate analogue **5** was known before as fosfoxacin[108] and has antibiotic properties.

5
Fosfoxacin

6

Figure I.5: Phosphate analogues of fosmidomycin and FR900098

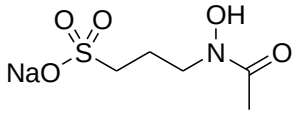
While the phosphonate group provides a chemically and enzymatically stable alternative for the labile phosphate[109], it is still a diprotic acidic group that makes the molecule extremely polar. Several research groups have therefore tried to exchange the phosphonate for less polar groups. Woo et al.[107] switched the phosphonate in fosmidomycin for a carboxylic acid group (**7**), and the Rohmer group[110] published the hydroxamate-carboxylic acid analogues **8** and **9**.



7 **8** **9**

Figure I.6: Carboxylic acid analogues of fosmidomycin

All three analogues showed at least a 3000-fold decrease in DXR inhibition. This loss of effect was ascribed to the planar nature of the carboxylic acid group, which contrasts with the tetrahedral shape of the phosphonic acid group. In order to mimic this tetrahedral head group, three sulfonate analogues[110, 111] (**10**-**12**), a series of (aryl)alkylsulfones[111] (**13a**-**f**) and a series of (aryl)alkylsulfonamides[111] (**15a**-**e**) were synthesized by the groups of Schlitzer, Rohmer and Proteau. Again, none of these analogues showed inhibitory potential when tested on EcDXR.



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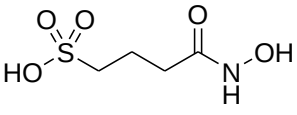

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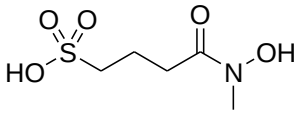

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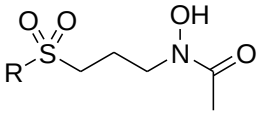
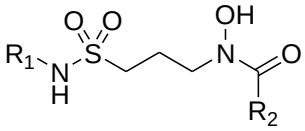


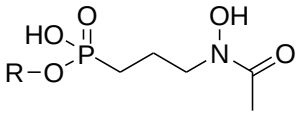
13

a R = Me
b R = Et
c R = nPr
d R = nBu
e R = Bn
f R = Phenethyl



14 $R_1 = R_2 = H$

15a R_1 = H ; R_2 = Me
15b R_1 = Me ; R_2 = Me
15c R_1 = Et ; R_2 = Me
15d R_1 = nPr ; R_2 = Me
15e R_1 = nBu ; R_2 = Me



16

a R = Me
b R = Et
c R = nPr
d R = nBu
e R = isopentyl
f R = phenethyl
g R = 1-naphthylethyl
h R = 2-naphthylethyl

Figure I.7: Sulfonate-, sulfone-, sulfonamide- and mono-(aryl)alkylphosphonate analogues of fosmidomycin/FR900098

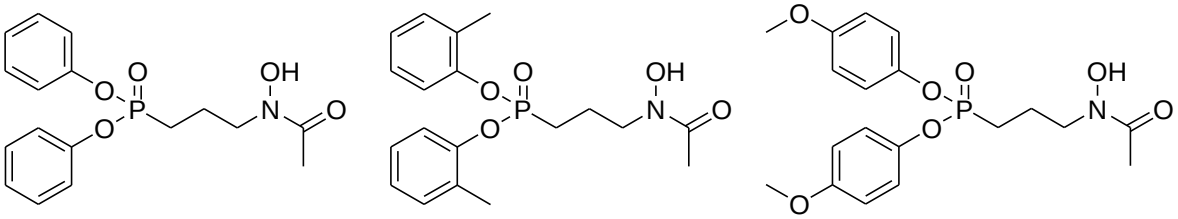
In the same paper of the Schlitzer group[111], a series of FR900098 analogues featuring a phosphonate mono(aryl)alkyl-ester is described (**16a**-**h**). Although these also showed a decrease in potency, it was less pronounced than in the former series and especially the phenethyl and naphthylethyl derivatives **16f**-**h** showed *in vitro* growth inhibition of *P. falciparum* with an IC_{50} only about ten times higher than that of fosmidomycin. Uh et al.[98] tested a number of FR900098 dialkylphosphonate analogues, albeit only for their antibacterial and antimycobacterial potency, and found out that these showed hardly any activity at all. From these data we can conclude that the

diprotic phosphate or phosphonate functionality is essential for fosmidomycin analogues in order to bind to DXR. The introduction of phosphonate mono-esters yields a decrease in the otherwise high polarity of these analogues, but it comes at a price of losing potency[111], that may be partly compensated by improved cellular uptake.

I.D.1.a. Phosphonate Prodrugs

An obvious way to overcome the high polarity of phosphonates, and the consequent low cell permeability, is to convert them into prodrugs[112]. This is done by attaching lipophilic, chemically and/or enzymatically labile groups to the phosphonate. The lipophilic prodrug can then more easily cross membrane barriers before these groups are cleaved, preferably inside the target organism, by spontaneous or enzymatic hydrolysis.

A first implementation of this strategy to FR900098 was published by Reichenberg et al.[113], who synthesized the three different phenolic phosphonate esters depicted in Figure I.8.

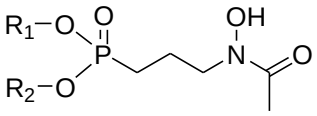


17 **18** **19**

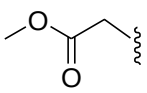
Figure I.8: Diaryl phosphonate prodrugs

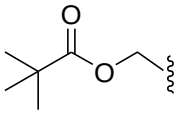
Their activity was tested in *P.vinckei* infected mice. Diphenylphosphonate **17** proved to be more active than FR900098, whereas the bis(*o*-tolyl)-ester **18** was less active. These prodrugs are expected to be hydrolyzed by non-specific esterases and probably the *o*-methyl substituents caused too much sterical hindrance for this to occur. Bis-(*p*-methoxyphenyl)-ester **19** showed the highest activity of the three analogues, equaling the potency of i.p. FR900098. Because of the high doses needed in fosmidomycin therapies and the toxicity of phenol, this strategy was not pursued.

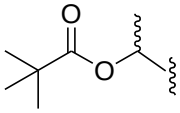
A different kind of prodrugs was proposed by Ortmann et al.[114, 115], who published a series of acyloxyalkyl and alkyloxycarbonyloxyalkyl-esters of FR900098, including the two 'mono-prodrugs' **20k** and **20l** (Figure I.9).

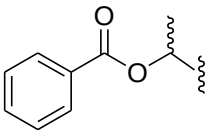


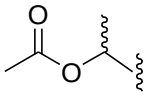
20

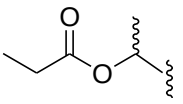
a $R_1 = R_2 =$ 

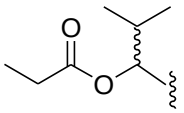
b $R_1 = R_2 =$ 

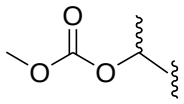
c $R_1 = R_2 =$ 

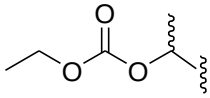
d $R_1 = R_2 =$ 

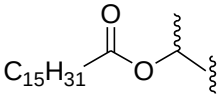
e $R_1 = R_2 =$ 

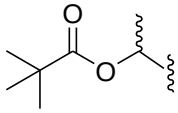
f $R_1 = R_2 =$ 

g $R_1 = R_2 =$ 

h $R_1 = R_2 =$ 

i $R_1 = R_2 =$ 

j $R_1 = R_2 =$ 

k $R_1 = H$; $R_2 =$ 

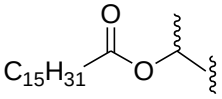
l $R_1 = H$; $R_2 =$ 

Figure I.9: Acyloxyalkyl- and alkyloxycarbonyloxyalkyl-ester prodrugs of FR900098

Except for these 'mono-prodrugs', all compounds showed good antimalarial activity when tested in *P.berghei* infected mice, surpassing FR900098's activity. Since this discovery the group of Kurz has often reported bis(pivaloyloxymethyl)-ester ("bis-POM") prodrugs of fosmidomycin analogues[116-119] (in later papers, the free phosphonates are also evaluated[120, 121]). While this strategy usually results in

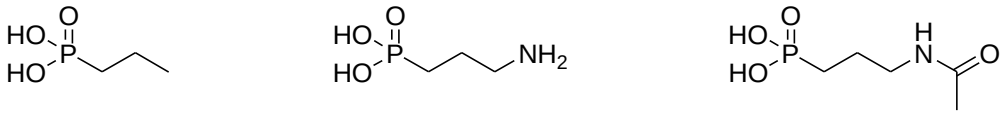
an enhanced potency, it also precludes the *in vitro* testing of such analogues for their DXR inhibitory potency. Other groups prefer to first assess the enzyme kinetics of fosmidomycin analogues in their free acid form and only synthesize prodrugs of the most promising inhibitors afterwards. Anyhow, both lines of thought have their pro's and con's, but the presence of both strategies in literature often complicates the deduction of trends in fosmidomycin SAR.

I.D.2. Modifications of the retrohydroxamate functionality

Being an extremely efficient metal chelator, the retrohydroxamate coordinates the divalent metal cation (Mg^{2+}, Mn^{2+} or Co^{2+}) in the active site of the enzyme similarly as the β-hydroxyketone moiety in DOXP. This ion is bound in the *Plasmodium* isozyme by residues Asp231, Glu233 and Glu315 and has a distorted trigonal bipyramidal geometry[72] (for the *E. coli* isozyme, Behrendt et al.[121] report an octahedral coordination). The equatorial ligands are: the side chains of Asp231 and Glu233 together with the *N*-hydroxyl oxygen of the retrohydroxamate, while the axial ligands are the side chain of Glu315 and the carbonyl oxygen of the retrohydroxamate[72]. It is thus necessary that the carbonyl oxygen and the N-OH of the retrohydroxamate adopt a *cis*-conformation, as is discussed in detail in Chapter III.

I.D.2.a. Necessity of the N-OH group

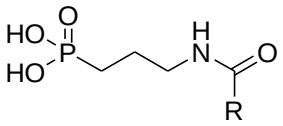
From the above, one could infer that an intact retrohydroxamate is necessary for a strong interaction of fosmidomycin analogues with DXR. This statement has been corroborated by a vast amount of experimental data. Mercklé et al.[97] tested the inhibition of EcDXR by propylphosphonate **21**, aminopropylphosphonate **22** and 3-acetamidopropylphosphonate **23** (Figure I.10), which can be seen as three fosmidomycin analogues in which the retrohydroxamate has been deconstructed to a decreasing extent.



21 **22** **23**

Figure I.10: Fosmidomycin analogues with absent or partial retrohydroxamate

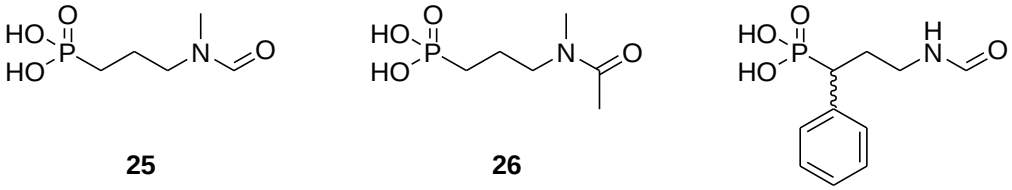
Although all three molecules inhibited EcDXR by at least a factor 10^6 weaker than fosmidomycin, surprisingly the primary amine analogue showed the strongest potency of the three. A series of 3-amidopropylphosphonates (**24**, Figure I.11) bearing different *N*-acylsubstituents was synthesized by Giessmann et al.[105], but none of those showed a detectable DXR inhibition when tested up to 30µM, indicating the importance of the N-OH group for DXR inhibition.



24
R = alkyl, aryl, indole-3-alkyl,...

Figure I.11: Amide analogues of fosmidomycin

This assumption was further proven by Woo et al.[107] who synthesized molecules **25** and **26**, the analogues of fosmidomycin resp. FR900098 in which the N-OH is replaced by an *N*-Me group, again at the cost of all potency.



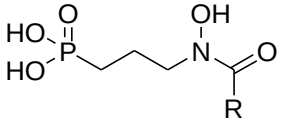
25 **26** **27**

Figure I.12: Dialkylamide fosmidomycin analogues and α-phenyl-substituted amide analogue

One exception to this "N-OH necessity" was seen in α-phenyl-amidopropylphosphonate **27,** which was formed as a by-product in the synthesis of α-phenylfosmidomycin by Timothy Haemers[122]. This byproduct was tested for EcDXR-inhibition together with the succeeded products and showed an IC_{50} of 2.39µM, compared to 0.030µM for fosmidomycin and 0.31µM for the corresponding *N*-acetyl retrohydroxamate (**51a**). More importantly though, when tested for growth inhibition of the chloroquine-sensitive *P. falciparum* 3D7-strain, it showed an IC_{50} of only 2.0µM compared to 1.1µM for fosmidomycin. Possibly the potency displayed by **27** is an indication that amides may replace the retrohydroxamate functionality. Possibly, the lower polarity of the amide compared to the retrohydroxamate counterpart leads to a better uptake into the parasite.

I.D.2.b. Influence of the acyl residue

Besides the N-OH function, also the *N*-acyl residue of the retrohydroxamate has been extensively varied. The most evident example in this series is FR900098, the acetyl congener of fosmidomycin. Reported (relative) activities vary strongly between different publications, research groups and used test condition[97, 105, 110, 111, 120, 123, 124]. Generally, FR900098 has been reported as equipotent to or up to twice as potent as fosmidomycin concerning EcDXR inhibition. In the *in vitro P. falciparum* growth inhibition assay FR900098 usually performs twice as well as fosmidomycin[105, 120]. Therefore, and also because of its higher stability, the *N*-acetyl moiety is usually preferred over the *N*-formyl residue. Giessmann et al.[105] and Ortmann et al.[123] investigated the further elongation of the *N*-acyl residue for the exploration of additional, undefined binding sites and in search of more lipophilic molecules (**28**, Figure I.13).

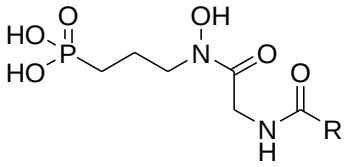


28

R = alkyl, arylalkyl, pentafluorophenyl, n-ketoalkyl...

Figure I.13: Fosmidomycin analogues with varied acyl chain

Only an *N*-benzoyl analogue showed a submicromolar inhibition and was about half as potent as fosmidomycin both on EcDXR and against *P. falciparum in vitro*. All other acyl residues caused a significant loss of DXR inhibitory potency. Via flexible docking it was observed that these larger acyl residues prevent the retrohydroxamate from adopting the necessary conformation for efficient chelation of the divalent metal cation. A comparable series of molecules bearing sterically even more demanding analogues is found in the aforementioned paper by Giessmann et al.[105] (**29**, Figure I.14)



29

R = alkyl, arylalkyl, indole-3-alkyl, phenoxyalkyl...

Figure I.14: Varied acyl moieties connected via a glycine linker to the retrohydroxamate

Here, a glycine linker is used to connect various acyl groups to the nitrogen of a fosmidomycin backbone. Again, introduction of such large acyl groups causes a drop in activity for up to three orders of magnitude compared to parent fosmidomycin and FR900098, except for a phenylether analogue. The authors suggest that the phenylether chain occupies an additional binding site at relatively large distance from the actual fosmidomycin binding site, causing a slightly different orientation for the retrohydroxamate group and the three-carbon spacer of this molecule relative to fosmidomycins bound conformation.

I.D.2.c. Reversal of the retrohydroxamate

The essence of the retrohydroxamate in fosmidomycin is a carbonyl function vicinal to an N-OH group, forming a bidentate chelator when both groups are in a *cis*-conformation, thereby mimicking the chelating β-hydroxyketone functionality of DOXP. Kuntz et al.[125] were the first to infer that the vicinal carbonyl and N-OH groups could switch places, thus changing the retrohydroxamate into a

hydroxamate, while essentially retaining their chelating properties as depicted in Figure I.15. They subsequently synthesized hydroxamates **30** and **31**, the reversed analogues of resp. fosmidomycin and FR900098.

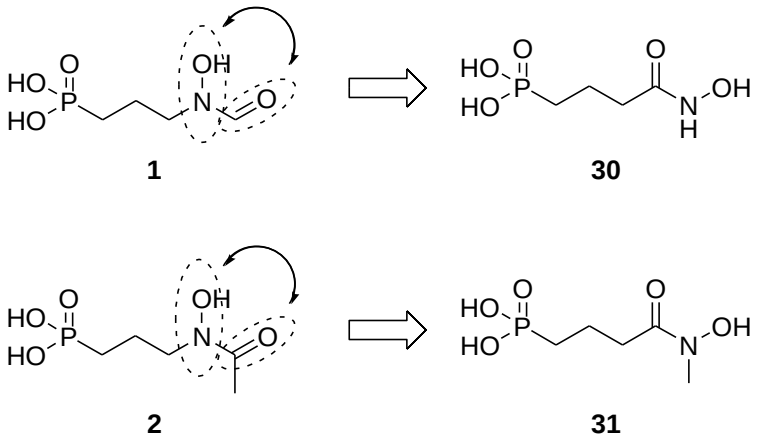
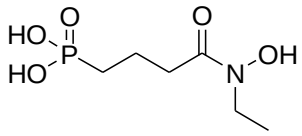


Figure I.15: Reversal of the retrohydroxamate function in fosmidomycin and FR900098

Both analogues were tested for their inhibitory activity on EcDXR and were shown to be slow tight-binding inhibitors like fosmidomycin. The *N*-methylated analogue **31** was a stronger EcDXR inhibitor than the N-H analogue **30,** which performed equally well as fosmidomycin. Analogue **30** was later resynthesized by Woo et al.[107] and tested on *Synechocystis* DXR, indicating that it is a slower binder than fosmidomycin, needing more time to form the tightly bound DXR-inhibitor complex. The better performance of the *N*-methylated hydroxamate was ascribed to a hydrophobic interaction between the methyl group and the indole ring of Trp212 by Zingle et al.[110], analogous to the methyl of the acetyl residue of FR900098 or the terminal methyl group of DOXP. They also tested the *N*-ethylated analogue **32**, but found out that this substituent is too bulky to fit in the apparently narrow active site around the metal ion. Phaosiri and Proteau[126] had come to the same conclusion earlier after testing the substrate analogue 1-methyl-DOXP (**33**).



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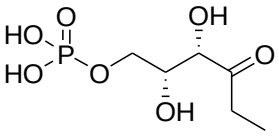
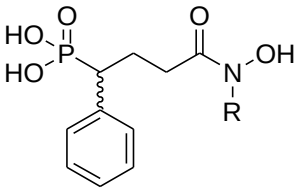


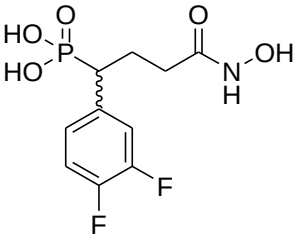
33

Figure I.16: *N*-Ethylated reversed hydroxamate and analogous 1-Me-DOXP

Recently, Behrendt et al.[121] combined the reversed hydroxamate with the favourable α-aryl substitution (see below), yielding a series of analogues as those depicted in Figure I.17.



34 R = H
35 R = Me
36 R = Et
37 R = iPr



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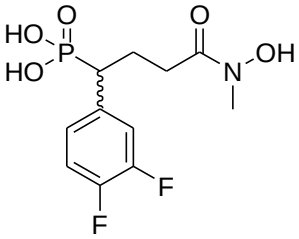


39

Figure I.17: α-Aryl reverse hydroxamate analogues of fosmidomycin

These analogues were the first hydroxamate analogues to be tested on both EcDXR and PfDXR and for *P. falciparum* growth inhibition *in vitro*. As before, *N*-unsubstituted analogue **34** caused less inhibition of EcDXR than *N*-methylated **35**. When tested on PfDXR, **35** was about twice as potent as FR900098 and **34**. Concerning *in vitro P. falciparum* growth, *N*-methylated **35** outperformed FR900098, whereas **34** showed comparable activity as fosmidomycin. Once more, *N*-ethylation as in **36** caused a severe loss of potency while *N*-isopropyl analogue **37** had no activity at all. The α-difluorophenyl analogues **38** and **39** showed the highest potency of this series and were therefore also tested *in vivo*. Both showed an equal activity on PfDXR, and *N*-methylated **39** was slightly better than *N*-unsubstituted **38** in inhibiting PfK1 growth. When tested in mice infected with *P.berghei* though, an inverse trend was seen as **38** performed better than **39** (89% vs. 78% mean suppression of infected RBC; 60% survival rate on day 13 for **38** vs. 25% for **39**).

Table 1.3: Biological activities of α-substituted reversed hydroxamate analogues

	Enzyme inhibition		*In vitro* growth inhibition
	EcDXR	PfDXR	*P. falciparum* K1
Analogue	IC_{50} (μM)	IC_{50} (μM)	IC_{50} (μM)
Fosmidomycin (**1**)	221.3±14.4	143.7±15.5	3.71±2.47
FR 900098 (**2**)	131.3±2.9	15.3±1.2	1.48±0.84
34	592±25	11.6±3.1	3.87±1.81
35	243±29.6	3.1±0.3	0.59±0.2
36	15±0.4	0.015±0.002	1.3±1.5
37	Inactive	Inactive	inactive
38	0.21±0.02	0.003±0.001	0.38±0.17
39	0.12±0.07	0.003±0.001	0.29±0.20

I.D.2.d. Substitution with other metal chelators

As the (retro)hydroxamate moiety of fosmidomycin (and its analogues discussed so far) "only" serves to chelate the divalent metal cation in the active center of DXR, it should theoretically be possible to substitute this group with other metal chelators while retaining or improving DXR affinity (and perhaps reduce polarity of resultant molecules). Typically, hard metal ions like Mg^{2+} with a small ionic radius, high electronegativity and low polarizability are found in the DXR active site. Stable complexation with such ions is achieved by dioxygen based hard ligands such as the retrohydroxamate found in fosmidomycin but also catechols[127]. Catechols **40a** and **40b** (Figure I.18) were synthesized by Deng et al.[127] and tested for their inhibitory potency of EcDXR, showing IC_{50} values of 24.8μM (**40a**) and 4.5μM (**40b**) indicating a strong preference for the 1,3,4-orientation on the catechol over the 1,2,3-orientation. In search of lipophilic fosmidomycin analogues against *M. tuberculosis*, Andaloussi et al.[101] resynthesized **40b**, together with *N*-hydroxypyridinone **40c**, oxazolopyridinones **40d-f**[96], 5-hydantoin **40g** and cyclic carbamate **40h**. Only catechol **40b** and *N*-hydroxypyridinone **40c** showed more than 50% inhibition of MtDXR at 100μM. Although these data

are based on the *Mycobacterium* isozyme, they confirm that the DXR active site is very narrow around the metal cation.

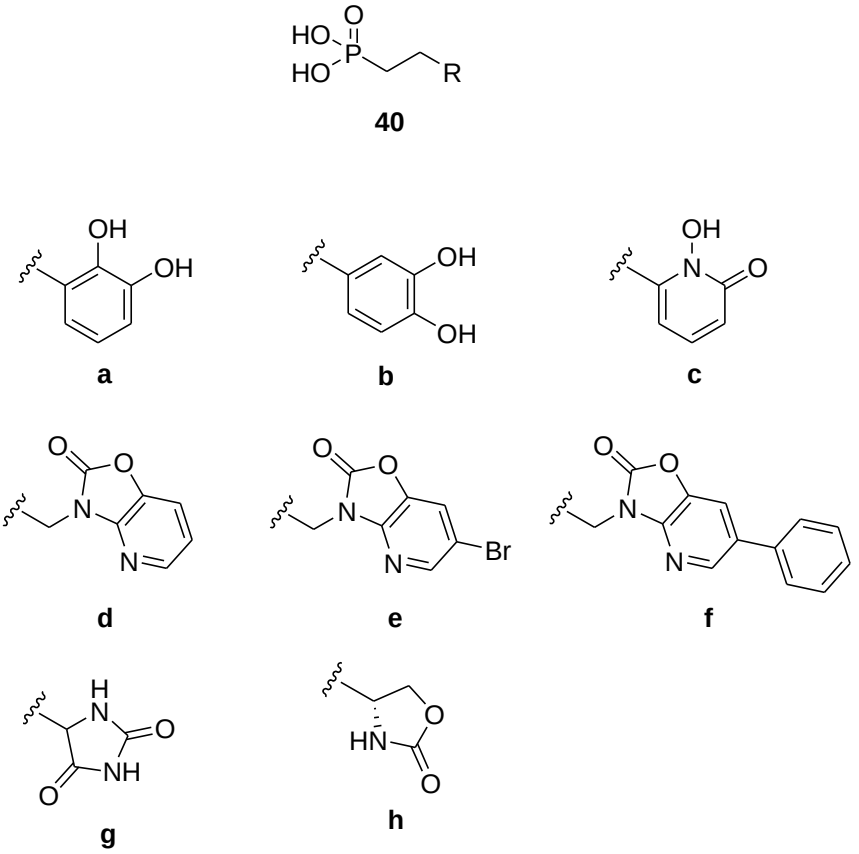


Figure I.18: Fosmidomycin analogues based on non-hydroxamate metal chelators

Oxazolidinone **40h** had been tested before[97] as its phosphate analogue (**41c**) together with its enantiomer (**41b**), its regioisomers (**41d-e**) and *N*-hydroxy-oxazolidinone **41a** depicted in Figure I.19.

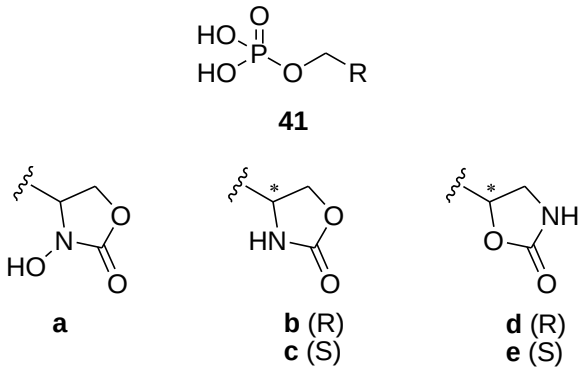


Figure I.19: Fosmidomycin analogues with rigidified chelating moiety

These molecules resulted from an attempt to rigidify the fosmidomycin structure by incorporation of the retrohydroxamate into a cyclic carbamate. Rigidification of the enzyme inhibitor should increase its potency by ameliorating the entropy loss of binding[128, 129]. All five molecules turned out to be weak EcDXR inhibitors in the high-µM to low-mM range.

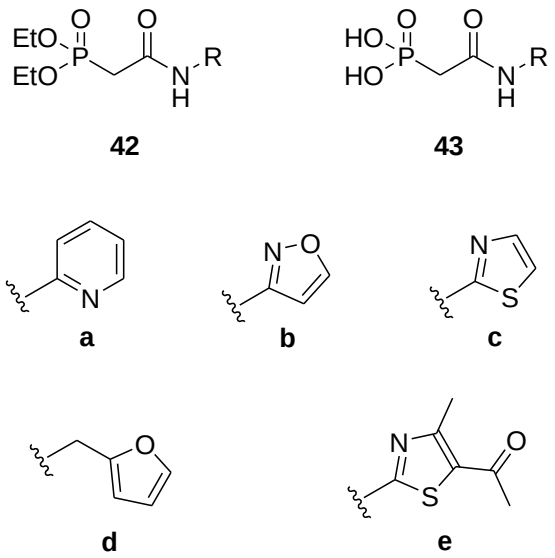


Figure I.20: Phosphonated N-heteroarylcarboxamides as fosmidomycin mimics

In search of alternative metal chelating groups, Bodill et al.[130] synthesized phosphonate esters **42a-e** and the corresponding phosphonic acids **43a-e** depicted in Figure I.20. Although evidence for the binding of these molecules to DXR was found, they probably do so in a different orientation from fosmidomycin. When tested *in vitro* for their EcDXR inhibitory activity, all analogues showed IC_{50}s of at least 5 orders of magnitude higher than that of fosmidomycin, with the most bulky analogue **43e** showing no inhibition at all at 500µM. Overall, substitution of the (retro)hydroxamate function in fosmidomycin analogues with more 'exotic' metal chelators has up till now not been very successful. Apparently the active site of DXR is very tight especially around the bound metal cation and introduction of sterically demanding metal-chelating groups is detrimental for inhibitory activity.

I.D.3. Modifications of the three-carbon spacer

In contrast to the phosphonate and hydroxamate groups that turned out to be essential for fosmidomycin's activity and tolerate only little modification, the three-carbon spacer is more amenable to synthetic modification, as outlined below.

I.D.3.a. Spacer length

An obvious modification is to change the length or the nature of the carbon spacer in fosmidomycin. Already at the time of fosmidomycin's discovery the influence of shortening this spacer on the (antibiotic) activity was tested. Ethylene analogues **44** and **45** (Figure I.21) were synthesized and tested on a panel of bacteria by Hemmi et al.[83] but showed no antibiotic activity at all. The influence of both shortening and lengthening with one methylene unit was also tested in the reversed hydroxamate series with analogues **46**-**49**[110] (Figure I.21). The 'normal-length' counterparts of these molecules (**30** and **31**) were described under I.D.2.c. Especially shortening of the molecule led to a dramatic decrease in EcDXR affinity (Table I.4). The authors inferred that, by shortening the spacer, these molecules probably cannot simultaneously occupy both the phosphonate and metal binding sites of DXR anymore leading to the decreased affinity. Lengthening of the spacer by one methylene unit in **48** and **49** also led to a decreased activity, although to a lesser extent. Although these molecules are theoretically long enough to occupy both binding sites, the longer spacer probably prevents adopting the optimal conformation for enzyme binding, or is maybe forced to adopt a conformation leading to energetically unfavorable intramolecular interactions.

Recently, Behrendt et al. synthesized the analogue of **46** bearing a phenyl substituent in α-position, because addition of an aromatic group in α-position has led to successful DXR inhibitors before (see below). Unfortunately, this substituent did not compensate for the detrimental effect of the shortened spacer as **50** showed no EcDXR nor PfDXR inhibitory activity at all[120].

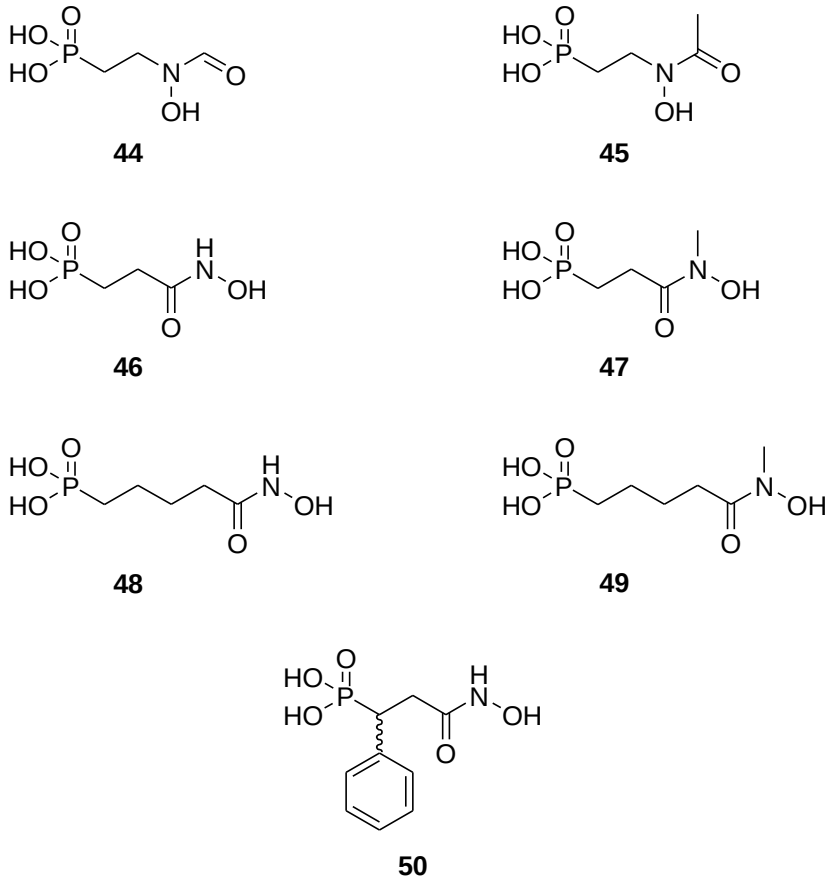


Figure I.21: Fosmidomycin analogues with shortened and lengthened spacer

Table 1.4: Influence of spacer length on the biological activity of fosmidomycin analogues

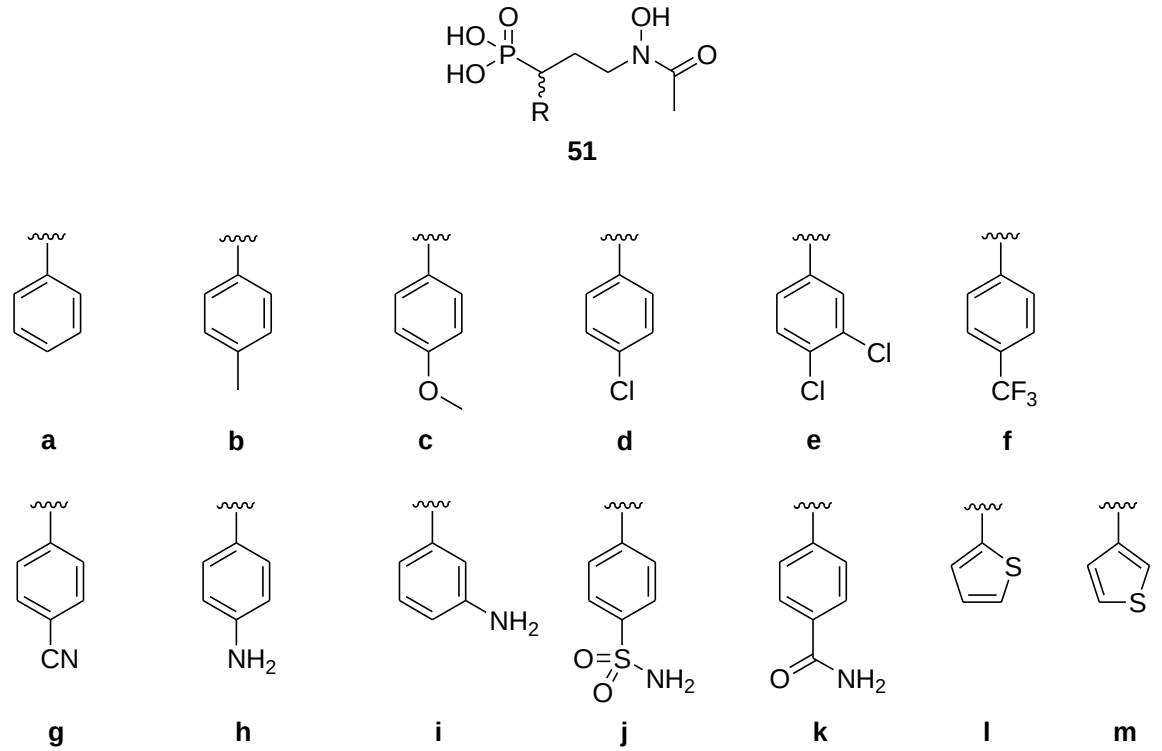
	EcDXR inhibition	
	IC_{50} (µM)	IC_{50} (µM)
Analogue	Without preincubation	With preincubation
Fosmidomycin (**1**)	0.25	0.032
FR 900098 (**2**)	-	0.032
46	1000	1000
48	77	19
47	2.8	0.27
49	0.9	0.11
50	-	inactive

From the above, one can conclude that a spacer of exactly three (carbon) atoms length between the phosphonate and chelator (hydroxamate or retrohydroxamate) is mandatory for good DXR inhibitory activity.

I.D.3.b. α-Aryl modifications

Together with the invention of phosphonate prodrugs (see above), the introduction of aromatic substituents in α-position of the phosphonate of fosmidomycin/FR900098 has afforded the most promising fosmidomycin analogues to date. So far, all α-substituted analogues in this overview have been synthesized and tested as racemates. Separation of their enantiomers and testing of those has been suggested as a way of finding the preferred binding mode of these analogues. On the other hand, *in vivo* racemization of such molecules is not unthinkable as the benzylic α-proton is quite acidic. Crystallographic work on MtDXR with α-aryl FR900098 analogues by Andaloussi et al. indicated preferential binding of the *S*-enantiomers to the enzyme.

A series of α-aryl analogues of FR900098, substituted according to Topliss' scheme (up to level 3), was synthesized in our group by Haemers et al.[122, 124] (Figure I.22, **51a-e**) and expanded by Devreux et al.[131, 132] with some electron-withdrawing substituents (**51f, g, j, k**), as well as two aniline analogues (**51i, j**) and two α-thienyl analogues (**51l, m**). Of the most promising FR900098-analogues, also the formylated versions (fosmidomycin analogues) were synthesized. When tested for their EcDXR inhibitory activity, all α-aryl analogues showed a weaker activity than fosmidomycin and FR900098, except for *N*-formyl analogue **52a** which was equipotent to fosmidomycin. Generally, activity of these analogues seems to be Hammett σ^+-controlled (4-Cl ≈ 3,4-diCl >H > 4-Me > 4-MeO). When tested for their *in vitro P. falciparum* growth inhibition, analogues **51a-e** as well as 4-CN analogues **51g** and **52a** all surpassed fosmidomycin's activity. Both 4-CN analogues (**51g**, **52a**) were about four times as active as fosmidomycin, and about twice as active as FR900098. Contrary to the activity trend observed with unsubstituted fosmidomycin and FR900098, the *N*-formyl analogues (**52a-c**) were consistently more potent than their *N*-acetyl counterparts (**51c, 51e, 51g**).



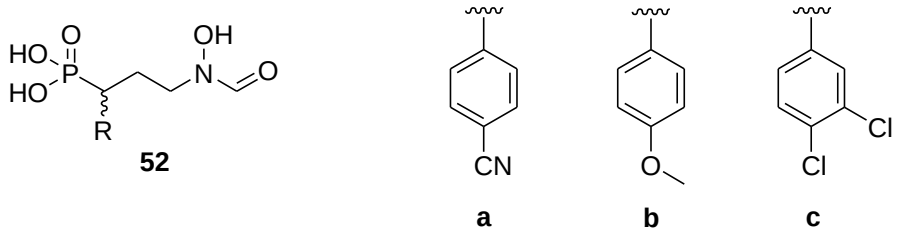


Figure I.22: Alpha-aryl substituted analogues of FR900098 and fosmidomycin

Kurz et al. combined the α-aryl modification with the use of phosphonate prodrugs (**53a-b** and **54a-d** in Figure I.23)[116]. Again, the *N*-formyl analogues showed a stronger *in vitro P. falciparum* growth inhibition than their acetyl counterparts, with α-phenyl analogue **53a** being about as active as the analogous (bis-POM) prodrug of FR900098. Addition of electron-withdrawing substituents to the phenyl ring as in **53b** and **54b**-**c** slightly improved the antimalarial activity while the electron-donating *ortho*-methyl groups in **54d** were detrimental.

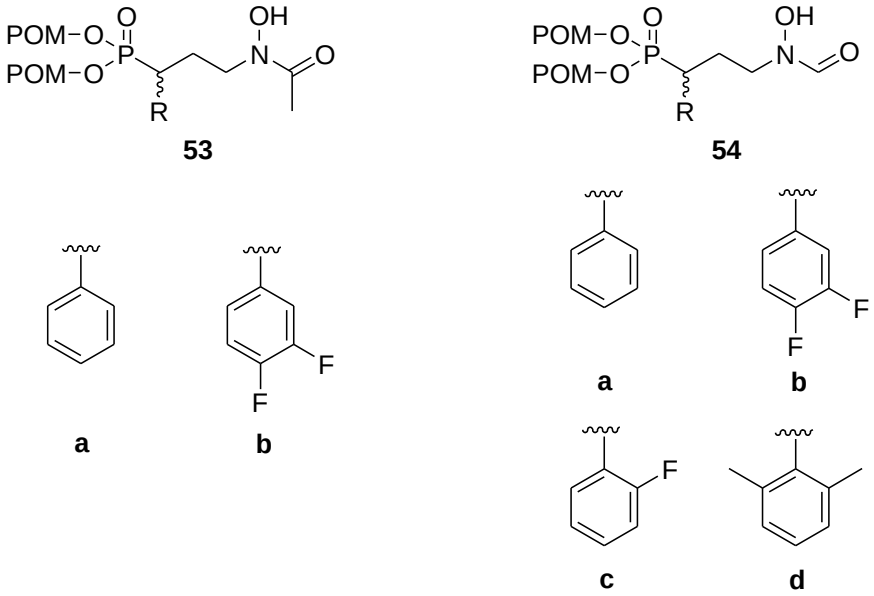


Figure I.23: Prodrugs of α-aryl fosmidomycin/FR900098 analogues

Remarkably, these prodrugs gave comparable IC_{50} values in the *in vitro P. falciparum* 3D7 growth inhibition assay as the free acids (**51** – **52**) described above. This may indicate that cellular uptake of the phosphonic acids is not a limiting factor for activity.

The combination of α-aryl substituents with a reversed hydroxamate group was explored by Behrendt et al.[120, 121], resulting in a series of α-aryl,N-H analogues (**55a**,**55c** and **55e**) and their *N*-methylated counterparts (**55b**, **55d**, **55f** and **55g**) depicted in Figure I.24.

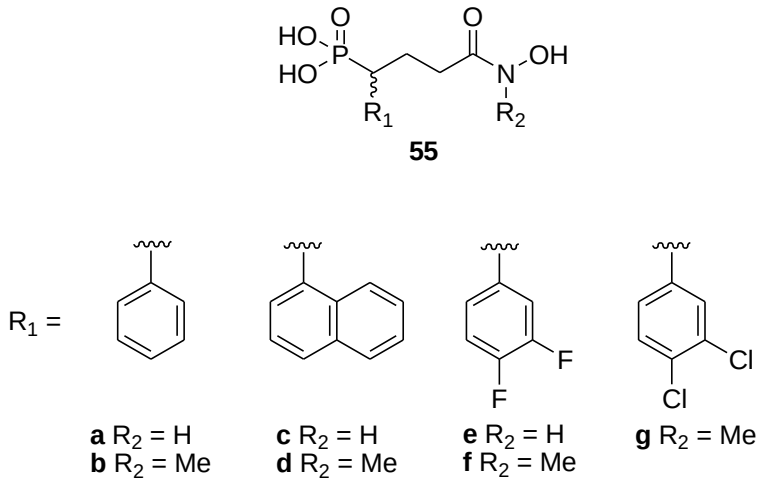


Figure I.24: α-Aryl fosmidomycin analogues bearing a reversed hydroxamate

Table 1.5: Biological activities of α-substituted reversed hydroxamate analogues

	Enzyme inhibition		*In vitro* growth inhibition
	EcDXR	PfDXR	*P. falciparum* K1
Analogue	IC_{50} (μM)	IC_{50} (μM)	IC_{50} (μM)
Fosmidomycin (**1**)	0.2213	0.1437	3.71
FR 900098 (**2**)	0.1313	0.0153	1.48
55a	0.592	0.0116	3.87
55b	0.243	0.0031	0.59
55c	7.4	0.0037	2.40
55d	3.8	0.0090	0.97
55e	0.21	0.0030	0.38
55f	0.12	0.0030	0.29
55g	0.18	0.0040	0.41

Here again, these molecules were not very promising compared to fosmidomycin when tested for EcDXR inhibition but (see Table 1.5), interestingly, surpassed fosmidomycin's activity when tested on PfDXR, up to two orders of magnitude. Contrary to the retrohydroxamates described above, in this series the *N*-methylated analogues were strongly favored over their N-H counterparts. When tested for *in vitro P. falciparum* (K1 strain) growth inhibition, these molecules appeared to be very potent with an IC_{50} as low as 0.29μM for **55f**. To assess the binding mode of these molecules, an X-ray crystallographic study of the EcDXR-complex of the most potent analogue (**55f**) was undertaken. The overall structure of the EcDXR-**55f** complex was identical with other published EcDXR structures, except for the flexible loop, which was distorted. This could be caused by the absence of NADPH in the complex, or by the protruding difluorophenyl substituent. From an overlay of the EcDXR-fosmidomycin complex with the EcDXR-**55f** complex a consistent binding mode for the metal chelating moiety is seen: both give an octahedral coordination of the divalent metal cation, as can also be seen in Diagram I.3.

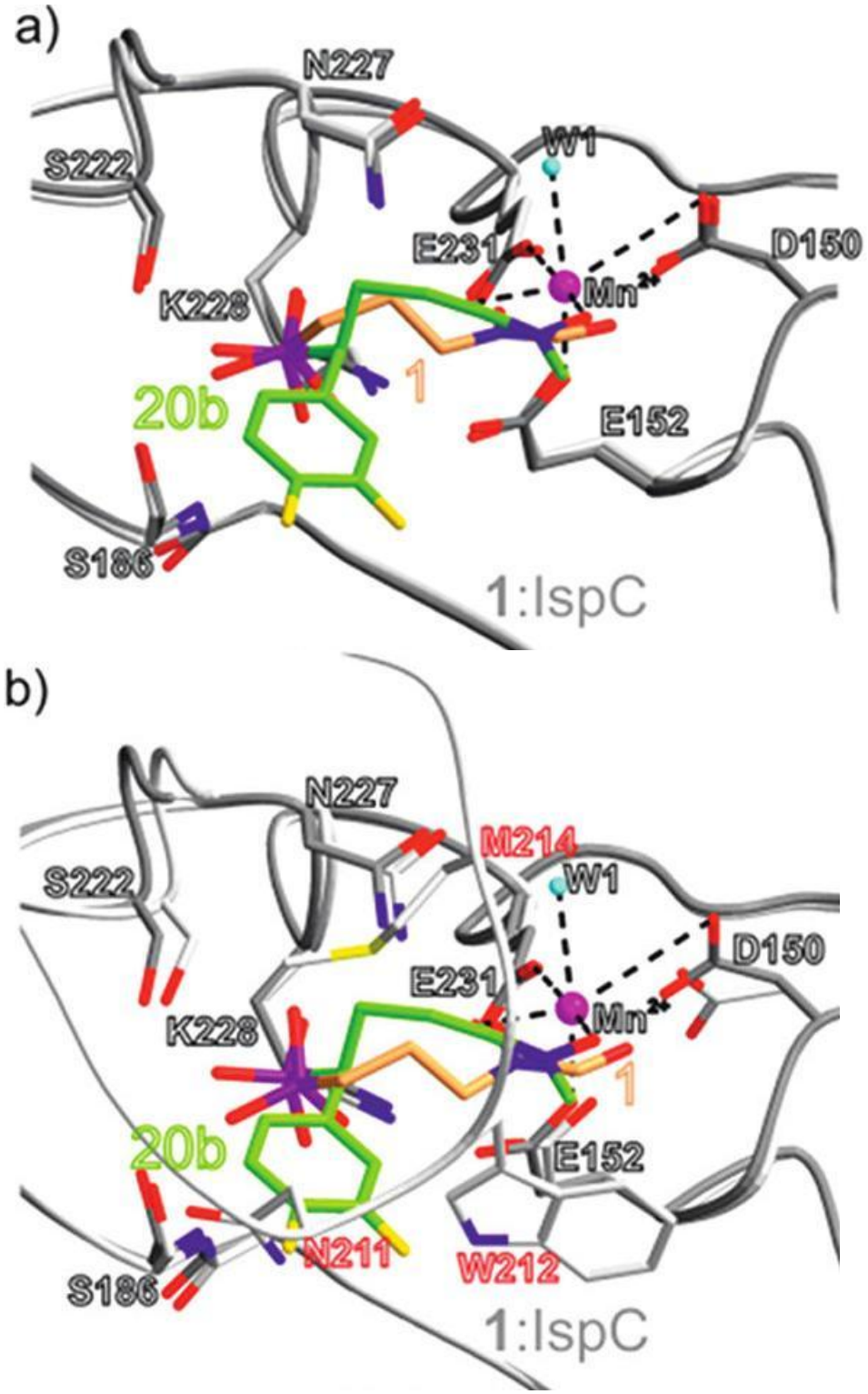


Diagram I.3: (a) Structural superposition of **55f** and **1** at the active site of EcDXR (labeled IspC) in the open conformation (PDB codes 3R0I (20b) and 1ONP16).(b) Superposition of **55f** and 1/EcDXR (IspC)/NADPH (closed conformation) complex (PDB codes 3R0I and 1Q0L17). The steric clash between the α-difluorophenylsubstituent (green) and three aminoacid residues (Asn 211, Trp 212 and Met 214) of the flexible loop can be seen. Figure from: *Journal of Medicinal Chemistry* 2011, 54 (19), 6796-6802.

This overlay also suggests a steric clash between the α-aryl substituent with amino acid side chains from the flexible loop (especially Asn211, Trp212, Met214), preventing the loop from closing, which could explain the relatively low affinity of **55f** for EcDXR compared to PfDXR. In PfDXR, Deng et al. (see below) describe the possibility of Trp212 to undergo a rotation, leading to a favorable π-π-stacking interaction with the α-arylsubstituent[133]. Furthermore, the X-ray structure shows intramolecular Van der Waals interactions between the aryl ring, the carbon backbone and the *N*-methyl. The latter has apparently no interactions with the enzyme. A further reason for the difference in affinity of **55f** for EcDXR versus PfDXR could be that, although the residues forming the active sites are conserved, the overall sequence homology between these two enzymes is very low.

Another variation on the 'α-aryl theme' consists of the combination with an α,β-unsaturation, resulting in two series of resp. *cis*-analogues **56a-e** and *trans*-analogues **57a-d** as depicted in Figure I.25[131, 134].

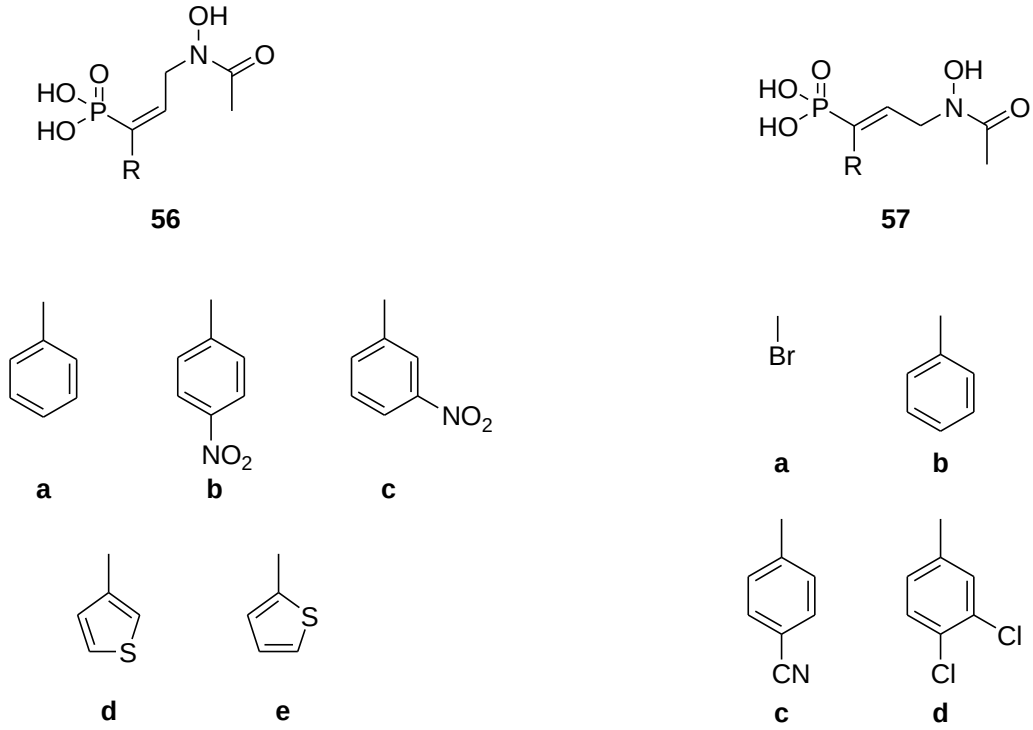
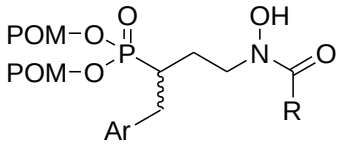


Figure I.25: α,β-Unsaturated,α-aryl analogues of fosmidomycin

Of these molecules, only the *trans*-analogues **57a-d** showed some inhibition of EcDXR, albeit much weaker than fosmidomycin and FR900098. *cis*-Analogues **56a-e** showed IC_{50} values (EcDXR) that are at least three orders of magnitude higher than those for fosmidomycin and FR900098. One exception is the unsaturated α-bromo analogue **57a** which showed an acceptable EcDXR inhibition (IC_{50} = 0.45µM, compared to 0.034µM for fosmidomycin). Apparently, the unsaturation combined with the α-aryl substituents constrains the molecule too much for optimal binding to the enzyme.

I.D.3.c. α-Alkyl based modifications

Two kinds of α-alkylated fosmidomycin analogues have been published so far: analogues bearing only (hydroxy)alkyl substituents in α-position[116] and a series of α-arylmethyl analogues[118]. Both were synthesized and tested as bis-POM prodrugs. The latter series, as depicted in FigureI.26, can be seen as analogues of the α-aryl-fosmidomycines described in I.D.3.b. with a methyl tether inserted between the aryl group and the fosmidomycin backbone.

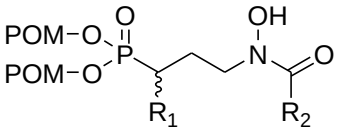


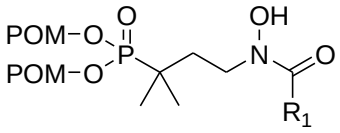
58
R = H or Me

Figure I.26: α-Arylmethyl substituted fosmidomycin analogues

All of these analogues were synthesized and tested both as formyl (fosmidomycin derived) and as acetyl (FR900098 derived) hydroxamates. In line with previous results, formyl derivatives were consistently more active than their acetyl counterparts, and electron-withdrawing substituents on the phenyl ring improved the antiplasmodial activity in a *P. falciparum*3D7 growth inhibition assay. Electron-donating substituents led to a decreased activity, while also tetrahydronaphthyl-analogues showed a decreased activity probably due to steric factors. The most potent molecule of this series showed an intermediate activity between bis-POM fosmidomycin and bis-POM FR900098.

A series of analogues with alkyl groups in α-position was also synthesized both as formyl- and acetylretrohydroxamates and evaluated as bis-POM prodrugs for their *P. falciparum*3D7 growth inhibition potency[116] (**59**-**64** in Figure I.27).





59a R_1 = Me; R_2 = H
59b R_1 = Me; R_2 = Me
60a R_1 = Et; R_2 = H
60b R_1 = Et; R_2 = Me
61a R_1 = nPr; R_2 = H
61b R_1 = nPr; R_2 = Me
62a R_1 = iPr; R_2 = H
62b R_1 = iPr; R_2 = Me
63a R_1 = CH_2OH; R_2 = H
63b R_1 = CH_2OH; R_2 = Me

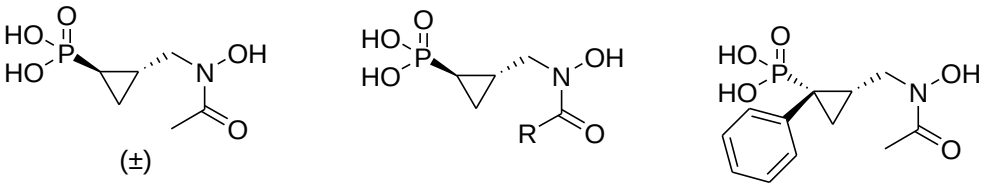
64a R_1 = H
64b R_1 = Me

Figure I.27: α-Alkyl and α-hydroxymethyl analogues of fosmidomycin and FR900098

Except for α-monomethyl analogues **59a** and **59b**, which showed a potency equal to that of the α-phenylfosmidomycin and FR900098 prodrugs, all other α-alkyl-, α,α-dimethyl- and α-hydroxymethyl analogues showed a strongly diminished antiplasmodial activity. Generally, formyl analogues **59**-**64a** showed better activities than their acetyl counterparts **59**-**64b**.

I.D.3.d. Conformationally restricted analogues

Vincent Devreux from our group incorporated the α,β-bond of fosmidomycin into a cyclopropane structure[131, 135], resulting in molecules **65** – **67** depicted in Figure I.28. First, the racemic *trans* analogue **65** bearing an *N*-acetyl moiety was synthesized, and due to its promising activity, enantiopure 1*R*,2*S*-analogues bearing three different acyl moieties were subsequently synthesized. Furthermore, α-phenyl analogue **67**, which is reminiscent of the α-aryl-α,β-unsaturated fosmidomycin analogues described above (that were synthesized later!) was also included.



(±)

65

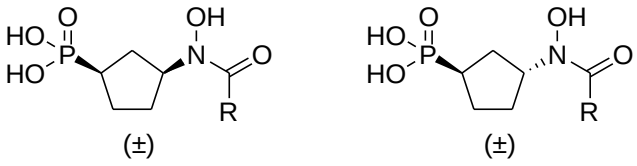
66a R = H
66b R = Me
66c R = Et

67

Figure I.28: Cyclopropyl analogues of fosmidomycin

An EcDXR inhibition assay showed that racemic **65** was about three times less potent than fosmidomycin and FR900098. Its enantiopure 1*R*,2*S* counterpart **66b**, on the other hand, showed a comparable activity to FR900098, indicating that the 1*R*,2*S*-configuration is the favored one for DXR binding. Replacing the acetyl group for a formyl resulted in an 8-fold drop in potency, while a propionyl group caused an even more drastic loss of activity. Conformationally restricted α-phenyl analogue **67** did not inhibit EcDXR to an appreciable extent. When tested for *P. falciparum* growth inhibition, enantiopure **66b** was equipotent with fosmidomycin on the Dd2 strain and surpassed its activity against the 3D7 strain. Again, the formyl analogue **66a** was less potent (about 5-fold) than the acetyl analogue.

The entire three-carbon spacer of fosmidomycin has been conformationally locked as well, by incorporation into a cyclopentane structure[122, 136]. Timothy Haemers from our group synthesized both the racemic *cis*(**68**) and *trans*(**69**) analogues (Figure I.29).



(±)

68a R = H
68b R = Me

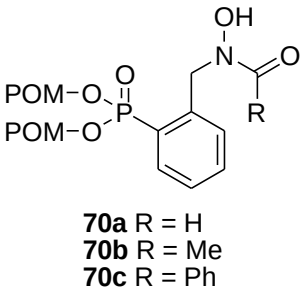
(±)

69a R = H
69b R = Me

Figure I.29: Cyclopentyl analogues of fosmidomycin

From EcDXR inhibition experiments the following was concluded: 1) *trans* isomers showed a higher potency than *cis* isomers (in accordance with the results of the cyclopropyl analogues); 2) formyl analogues performed better than acetyl analogues (cfr. α-aryl analogues). Consequently, formylated *trans*-analogue **69a** showed the highest EcDXR inhibition of this series (albeit 1 order of magnitude weaker than fosmidomycin), acetylated *cis*-analogue **68b** performed worst (3 orders of magnitude weaker than fosmidomycin).

The modifications described above both retain the sp^3-character of the three-carbon spacer. In contrast to this is the modification tested by Kurz et al.[117] depicted in Figure I.30, which results in partially planar fosmidomycin analogues.



70a R = H
70b R = Me
70c R = Ph

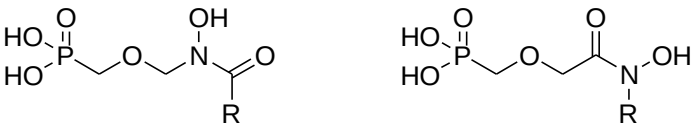
Figure I.30: Conformationally restrained aromatic fosmidomycin analogues

These molecules were tested as their bis-POM prodrugs and with three different acyl moieties: formyl, acetyl and benzoyl. Unfortunately, all of them showed only moderate *P. falciparum*3D7 growth inhibition, with IC_{50} values one to two orders of magnitude higher than those of bis-POM fosmidomycin and bis-POM FR900098. Concerning acyl residues, benzoyl was favoured over acetyl, which itself performed better than formyl.

I.D.3.e. β-Position modifications

There are only two groups of fosmidomycin analogues featuring a modification of the three-carbon spacer in β-position reported to date. The first is (*R*)-β-hydroxyfosmidomycin or FR33289 (**3**), which was characterized by Hemmi et al. at the discovery of fosmidomycin and its analogues[83].

Although this compound was never tested for its DXR inhibitory potency or *Plasmodium* growth inhibition, a low antibacterial effect was reported. The second modification consists of the replacement of the β-methylene unit by an oxygen atom[122, 137], resulting in ethers **71a**-**b** and **72a**-**b** depicted in Figure I.31.



71a R = H
71b R = Me

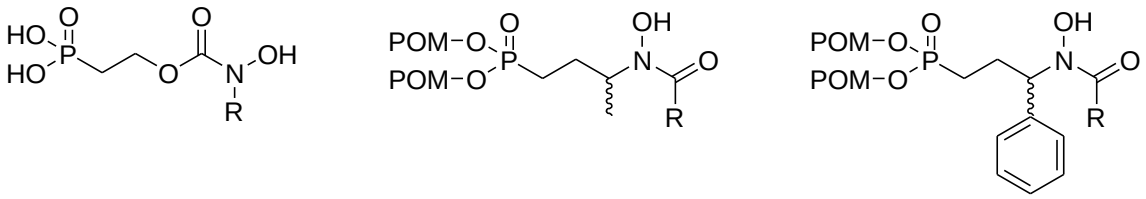
72a R = H
72b R = Me

Figure I.31: β-oxa analogues of fosmidomycin

In the EcDXR inhibition assay, the *N*-acetyl analogue **71b** and *N*-methyl analogue **72b** performed better than their N-formyl and N-H counterparts. Both **71b** and **72b** were almost as potent as fosmidomycin and FR900098, with reversed hydroxamate **72b** being slightly more potent than retrohydroxamate **71b**. Due to stability issues, **72b** was converted to its bis-POM prodrug for *in vitro* *P. falciparum* growth inhibition testing, where it outperformed the bis-POM prodrug of FR900098.

I.D.3.f. γ-Position modifications

Fosmidomycin analogues modified in γ-position are scarcely found in literature, just like the β-analogues described above. Compounds **73a** and **73b**[122, 137] depicted in Figure I.32 can be seen as γ-oxa-derivatives analogous to the β-oxa-analogues described above. Chemically though, these are N-hydroxycarbamates and apparently this change to a different functional group has had a major influence on the DXR inhibitory activity of these molecules as none of both was found to inhibit EcDXR.



73a R = H
73b R = Me

74a R = H
74b R = Me

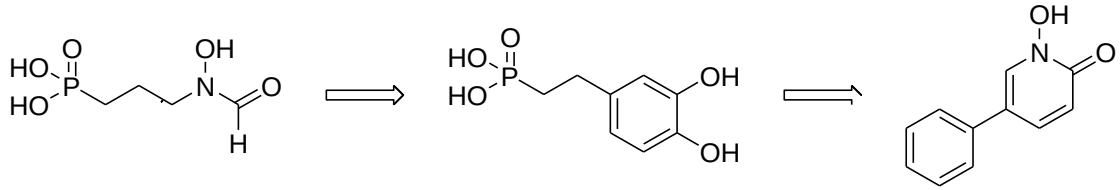
75a R = H
75b R = Me

Figure I.32: γ-modified fosmidomycin analogues

Kurz et al. reported the synthesis of racemic γ-methyl and γ-phenyl analogues **74a**-**b** and **75a**-**b**, as bis-POM prodrugs. Only incomplete inhibition of *in vitro P. falciparum* growth by these analogues at 100µM was seen. This is once more an indication that increasing the steric bulk near the metal chelator part of fosmidomycin analogues is detrimental to their activity.

I.D.4. Modifications with omission of phosphonate or (retro)hydroxamate moiety

Recently, Deng et al. tried a new strategy in their search for more lipophilic DXR inhibitors, by creating fosmidomycin analogues that lack either the phosphonate or the (retro)hydroxamate part. First, they tested new metal chelating groups based on the catechol structure[127], leading to the aforementioned analogues **40a** and **40b**. In order to enhance the lipophilicity of these molecules, and thus increase their antibacterial activity, the ethylphosphonate moiety was replaced for a phenyl or a benzyl group. Variations of the catechol metal chelator such as *N*-hydroxypyridinone were also tested, resulting in a series of lipophilic DXR inhibitors of which **76** (Figure I.33) showed the strongest EcDXR inhibition as well as a broad antibacterial activity.



1 Fosmidomycin

40b

76

Figure I.33: Development of lipophilic DXR inhibitors lacking the phosphonate group

Having proven that lipophilic molecules lacking the phosphonate group are valuable leads for DXR inhibitor design, the group then adopted a rational approach starting from α-(3,4-dichlorophenyl)fosmidomycin **52c**[133]. Two 'halves' of this molecule were synthesized and tested on EcDXR: the benzylphosphonate **77** and phenylalkyl-retrohydroxamate **78** (Figure I.34). Of these, only the phosphonate showed DXR inhibition. Because electron-withdrawing substituents on the aryl ring caused an increase in activity in the α-aryl series, it was decided to synthesize pyridyl analogues **79** and **80**. Again, the retrohydroxamate showed no considerable DXR inhibition while the phosphonate showed an increased inhibition of EcDXR.

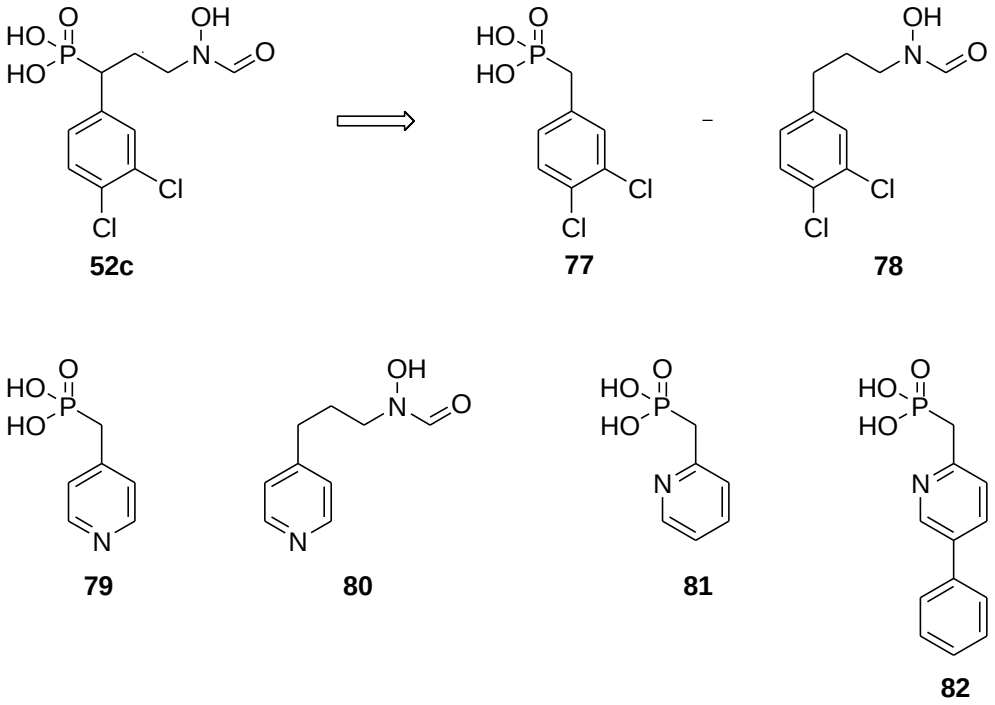


Figure I.34: Fragment-based approach toward lipophilic DXR inhibitors starting from α-(3,4-dichlorophenyl)fosmidomycin

In order to gain insight in the binding mode of these new structures, X-ray crystallographic studies were conducted on the complex of EcDXR with these new benzylphosphonates. Crystal structure data illuminate that the pyridyl ring in **81** undergoes a π-π-stacking interaction with the indole ring of the highly flexible Trp211 of the flexible loop part of DXR. Possible charge transfer makes this interaction particularly strong, which declares the better affinity of **81** for DXR than that

of **77**. When fosmidomycin itself is bound to DXR, the flexible loop shuts off the active site with Trp211 covering the apolar carbon spacer of fosmidomycin in an apolar interaction. Binding of the α-aryl derivatives like **77** leads to an intermediate case with also a π-π-interaction between the aryl group and Trp211 which is then flipped 180°, leading to a weaker interaction than with the above pyridyls. This study confirms the very important role of the flexible loop of DXR and especially Trp211, which is conserved across the species using the non-mevalonate pathway. In the same crystallographic study, two hydrophobic pockets in the DXR active site are also revealed. By means of docking experiments, one of these pockets is assigned as the binding pocket for the aryl groups of the α-aryl fosmidomycins as well as for the pyridyl group of **79**. In order to further explore the pyridylmethylphosphonate scaffold of **79** as a lead for DXR inhibitor design, Deng et al. synthesized a vast amount of analogues of **79** featuring several substituted aryl groups, different acidic groups and various linker lengths[99]. From the DXR inhibition data of these series, it was concluded that the phosphonate group is essential as well as one methylene unit as a linker. Furthermore, a 5-pyridyn-2-yl-substituent proved optimal, resulting in **82** as the most potent DXR inhibitor of this series. With their work, Deng et al. are the first to prove that it is possible to develop potent DXR inhibitors starting from the fosmidomycin scaffold by removing one of the major structural motifs: the retrohydroxamate. The gain in lipophilicity (leading to more favorable pharmacokinetics) and/or the extra interaction of the added lipophilic part with the newly defined hydrophobic pocket of DXR clearly makes up for the loss of the strong interaction of the metal chelator with the enzyme's catalytic metal cation.

I.D.5. Conclusions regarding fosmidomycin SAR

The vast amount of experimental data described in the previous sections allows us to postulate a few conclusions on the SAR of fosmidomycin analogues as antimalarial DXR inhibitors.
The importance of the retrohydroxamate in DXR inhibitors has recently become very questionable due to the work of Deng et al.[99, 133]: they show that a (retro)hydroxamate group is not necessary for a strong DXR ligand if other functional groups (in this case the phenylpyridyl moiety) cause new favorable interactions with the enzyme. In all other, 'classic' fosmidomycin analogues, the retrohydroxamate moiety proved to be necessary. Furthermore, apart from reversing it into a hydroxamate or substituting it for a catechol, all other changes to this group have led to a strong decrease in DXR inhibition.

So far, the diprotic phosphonate group with its tetrahedral configuration has proven essential for DXR inhibitory activity. Neither its exchange for other acids (e.g., carboxylic and sulfonic) nor the introduction of heterocycle-based groups have led to strong DXR inhibitors so far. Nevertheless, finding a more lipophilic substituent for this extremely polar functionality is expected to ameliorate the pharmacokinetic properties of such analogues. In order to decrease the polarity of fosmidomycin analogues, and thus increase their gastrointestinal uptake, several prodrugs of the phosphonate group have been developed. Remarkably, while the use of acyloxyalkyl ester prodrug strategies does increase the efficacy of FR900098 after oral administration (see I.D.1.a.), the use of the same strategy (in the α-aryl series) did not lead to a significantly higher antimalarial effect in the blood-based *in vitro* assay (see I.D.3.b.). Apparently, the use of phosphonate prodrugs does increase the uptake of such analogues after oral administration, but has little influence on the cellular and/or parasitic uptake. Probably there is a specific active uptake mechanism involved in the transport of fosmidomycin analogues into the Plasmodium parasite. The uptake of highly charged compounds such as fosmidomycin analogues into *Plasmodium*-infected erythrocytes is believed to be facilitated by so-called parasite-induced new permeability pathways (NPPs)[104].

Fosmidomycin's three-carbon spacer has so far been the most successfully modified part of the molecule. A few modifications, such as rigidification of the spacer, led to only a modest increase in DXR inhibition but also provided important information on the conformational preferences of DXR inhibitors. Except for the β-oxa-modification, which showed a strong DXR inhibition but suffered from a low stability, no derivatizations of the β-position have been published so far but studies are undubitably underway to explore these. The γ-position of the spacer is apparently too close to the (retro)hydroxamate group to allow succesfull modification: any steric bulk that is added in this position invariably leads to a loss of activity. α-Substitution, particularly with aryl groups is the most promising strategy so far, both for 'classic' fosmidomycin analogues as for 'truncated' phosphonates lacking a metal chelator. Generally, the addition of lipophilic/aromatic groups to an active molecule is frowned upon in medicinal chemistry for it does not enhance the ligand efficiency of the molecule, but only leads to nonspecific Van der Waals interactions with the apolar surface of the target protein. Then again, fosmidomycin with its two acidic functional groups for only a propylene chain is probably one of the most polar drugs. Therefore, one can question whether adding lipophilic groups to this molecule is also disfavored. Furthermore, besides increasing the molecules lipophilicity and thereby improving its pharmacokinetics, the added aryl substituents lead to an extra interaction with the enzyme under the form of a π-π-stacking with a Trp from the loop. We could thus conclude that these α-aryl substituents do increase the ligand efficiency in an active manner and do not categorize under the 'adding grease-strategy'.

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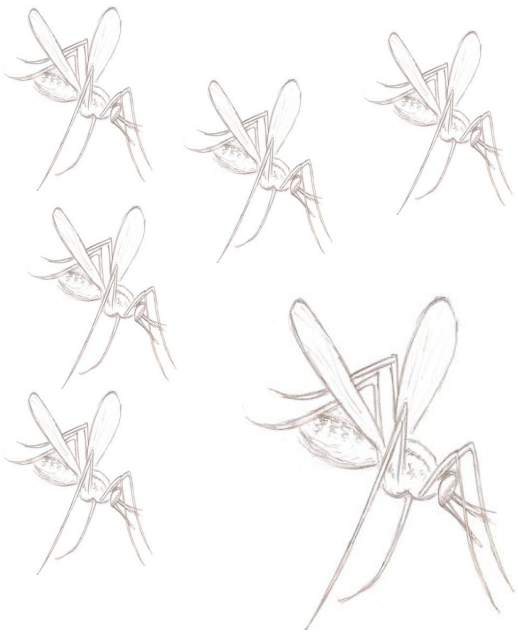


Chapter II

Objectives

II.Objectives

As described in the general introduction, one way to reduce the high daily dose of fosmidomycin required in clinical studies is to structurallyoptimize it in order to improve its potency as an antimalarial. Many efforts towards this goal have already been made, and from these we learned that:

-The phosphonate group is essential for good binding to DXR. A phosphate functionality may yield a better interaction with the enzyme, but is too unstable to be used in potential drugs.

-According to the most recent findings, the retrohydroxamate group can be omitted, provided that well-chosen aromatic groups in α-position to the phosphonate enhance the binding efficiency by forming π-π-stacking interactions with a Trp residue in DXR. If DXR inhibitors are to be designed with a metal chelating group, a retrohydroxamate (acetyl or formyl) or a hydroxamate (preferably *N*-methylated) are preferred.

-Derivatization of the three-carbon spacer of fosmidomycin may lead to analogues that show enhanced DXR inhibition. Among these, α-aryl analogues are the most promising, especially if the aryl moiety bears electron-withdrawing substituents.

The objective of this work is the synthesis of new and potent DXR inhibitors as antimalarials. We chose FR900098 as the lead structure, because of the higher stability of the acetylhydroxamic acid compared to the formylhydroxamic acid in fosmidomycin, and its higher potency (except in the α-aryl series). Formylhydroxamic acid analogues of selected promising derivatives can be synthesized in a second phase.

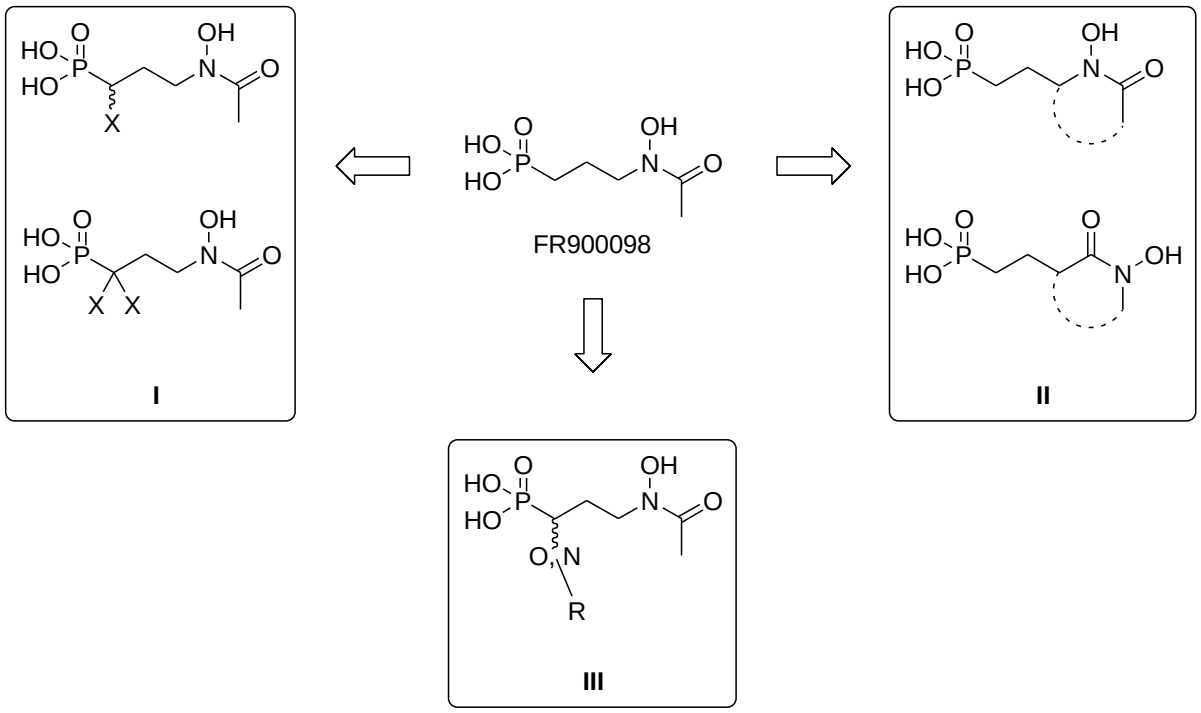


Figure II.1: Overview of planned modifications of FR900098

Four main strategies toward new FR900098 analogues are proposed (Figure II.1):

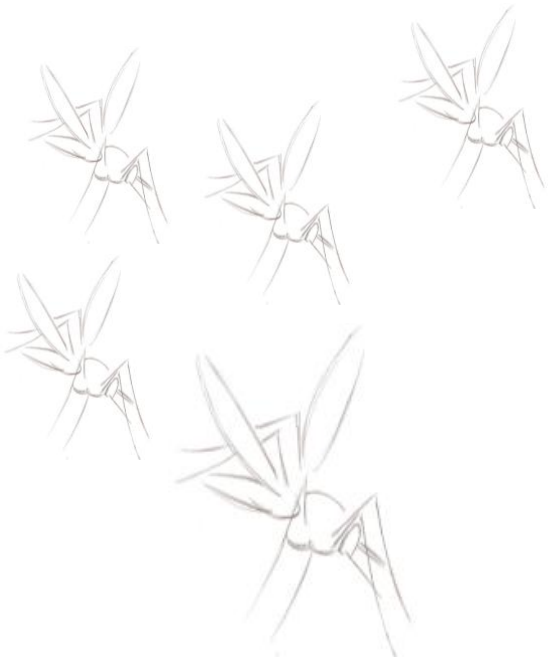
(I) **The introduction of (a) halogen atom(s) in α-position of the phosphonate.** In the α-aryl series of fosmidomycin analogues, the strongest DXR inhibition was detected with electron withdrawing substituents. We inferred that these might increase the acidity of the phosphonic acid and thereby lead to a stronger interaction with the phosphate binding site of the enzyme (apart from possible interactions between the aryl moiety and the enzyme which became clear only later during this work). A similar effect could possibly be achieved by introducing halogen atoms on the α-carbon of FR900098, hopefully also resulting in an increased affinity for DXR. Since α-halogenated phosphonates are known as excellent phosphate mimics, the envisaged molecules represent stable analogues of fosfoxacin, a potent but unstable DXR inhibitor.

(II) **Restriction of the preferred binding conformation of the (retro)hydroxamate by cyclization.** The retrohydroxamate in fosmidomycin or FR900098 can adopt two

conformations: E and Z, forming an equilibrium mixture. Of these, only the Z conformation is favorable for binding the divalent metal cation in the enzyme. By incorporating the (retro)hydroxamate into a cyclic structure, we aim at restricting it in a conformation that is favorable for binding to the enzyme, which should result in an increased affinity.

(III) **α-Heteroatom analogues.** Introduction of a heteroatom (*N* or *O*) in α-position of FR900098 will lead to new analogues that can serve as starting points for extensive derivatisation via *N*- or *O*-based functional groups. This strategy may lead to new DXR inhibitors and allows for exploration of a broad range of substituents.

(IV) **Addressing the polarity issue by means of phosphonate prodrugs.** As described in the general introduction, the high polarity of the phosphonic acid in fosmidomycin analogues significantly limits their gastrointestinal uptake. To address this issue, synthesized analogues exhibiting good *in vitro* activity will be converted to their corresponding phosphonate prodrugs for *in vivo* evaluation.



Chapter III

Alpha-halogenated analogues of FR900098

III.Alpha-halogenated analogues of FR900098

III.A Introduction

III.A.1. Fluorinated phosphonates

The organic phosphate group (R-OPO_3^{2-}) is omnipresent in a wide range of biologically active natural products[1], but is considered impractical in drug design due to its low stability towards hydrolysis, either spontaneous or enzymatic by digestive phosphatases. Therefore, stable isosteric analogues of this group have been heavily sought-after. A popular surrogate for the phosphate group is the phosphonate functionality (R-$CH_2PO_3^{2-}$), in which the bridging oxygen is replaced by a carbon directly linked to the phosphorus atom resulting in a chemically very stable phosphate isostere. The chemistry of phosphonates has been thoroughly studied and well reviewed[2]. The phosphonate group is also present in many natural products[3], of which fosmidomycin and FR900098 are the most relevant in the context of this work. In these compounds the phosphonate acts as an isostere for the phosphate group of DOXP.

In the 1980's the groups of Blackburn[4, 5] and McKenna and Shen[6] suggested that introduction of one or two halogen atoms, and in particular fluorine, in α-position of the phosphonate should lead to superior bioisosteres more accurately mimicking the steric and polar character of the phosphate moiety. It is a well known principle in medicinal chemistry that the CHF and CF2 groups can sterically and electronically mimic an oxygen[7], and in this case decrease the second pKa of the phosphonic acid (Figure III.1).

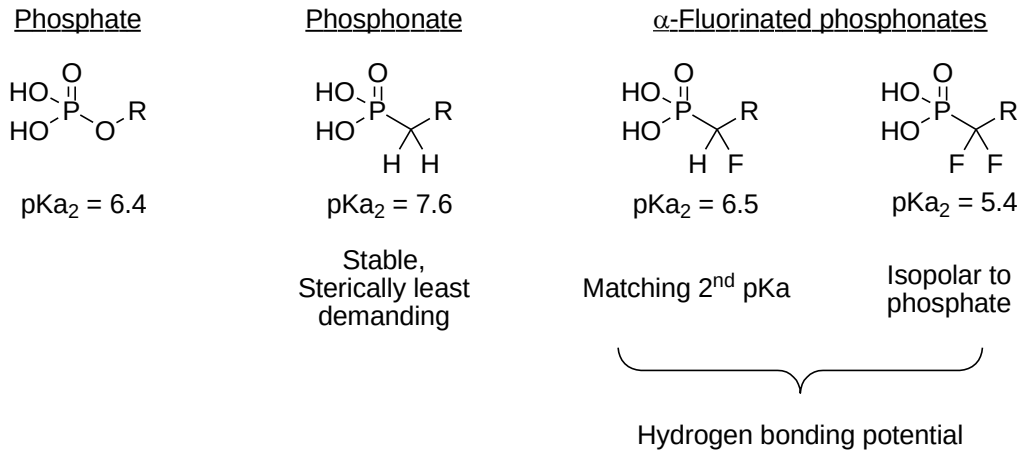


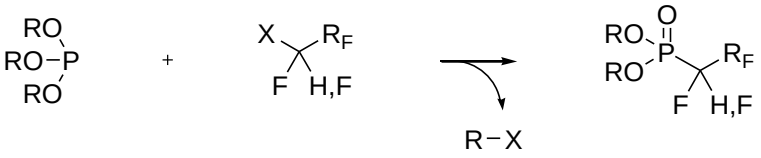
Figure III.1: Comparison of the phosphate group to its different isosteres[8]

In fact, the α-monofluorophosphonate is the best phosphate mimic in terms of pKa_2[9], whereas the α,α-difluorophosphonate more closely resembles the polarity profile of the phosphate group[10, 11]. Other parameters that potentially favor α-fluorinated phosphonates over their nonhalogenated congeners are: (a) an increased P-CF_2-C or P-CHF-C dihedral angle, (b) the possibility for C-F•••H-R hydrogen (halogen) bonding, (c) an increased hydrolytic and oxidative stability[8].

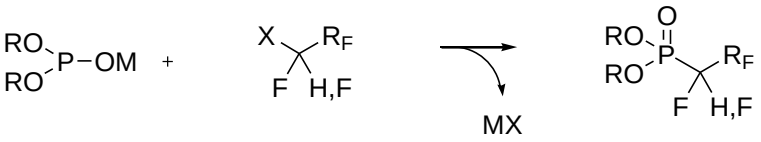
The chemistry of α-fluorinated phosphonates forms a much younger field of research than that of the ordinary phosphonates, but good reviews on this topic have already been published[8, 12]. Six major synthetic methodologies for the construction of α-fluorinated (and sometimes α-chlorinated) phosphonates can be distinguished:

1. **Arbuzov or Michaelis-Becker reaction.** This reaction between a trivalent phosphorus derivative (di- or trialkyl phosphite) and a fluorohaloalkane[13-16] suffers from incompatibility with functional groups and has only been successful with relatively short, unfunctionalized and polyhalogenated alkanes or alkenes.

Arbuzov reaction:



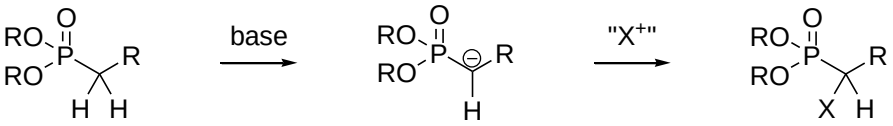
Michaelis-Becker reaction:



R = alkyl, aryl; X = Cl, Br or I; R_F = F-containing alk(en)yl;
M = Li, Na or K

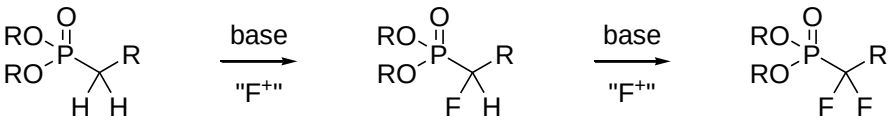
2. **Electrophilic halogenation of a phosphonate carbanion**[17]. This reaction involves the abstraction of a phosphonate α-proton with a strong base, followed by attack of the formed carbanion at low temperature on a suitable source of electrophilic halogen[18-21], for instance *N*-fluorobenzenesulfonimide (NFBS), hexachloroethane, 1,2-dibromotetrachloroethane or I_2. The α,α-difluoromethylenephosphonate moiety can be constructed in this fashion in two consecutive fluorination steps.

Electrophilic monohalogenation



X^+ = electrophilic halogen donor, e.g. $(PhSO_2)_2NF$, $Cl_3C\text{-}CCl_3$, $BrCl_2C\text{-}CCl_2Br$, I_2

Two-step electrophilic difluorination

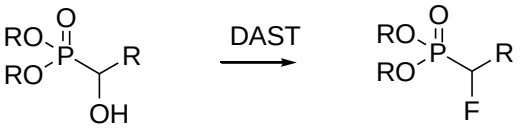


F^+ = electrophilic fluorine donor, e.g. NFBS, NFOBS, NFPMS, Selectfluor™

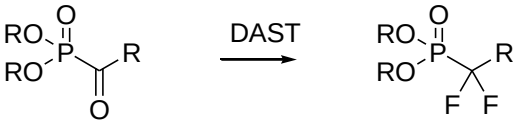
3. **Nucleophilic fluorination of a functionalized phosphonate substrate.** Due to the generally poor nucleophilic character of fluoride-containing reagents, only phosphonate substrates containing leaving groups with high nucleofugicity meet the requirements for

useful transformations. In practice, the most successful procedures for mono- and difluorination of alkylphosphonate derivatives are the C-OH → C-F resp. C=O → CF_2 transformations of resp. α-hydroxy- and α-ketophosphonates with DAST[22-24]. This strategy implies that functional groups that could also react with DAST should be appropriately protected or avoided.

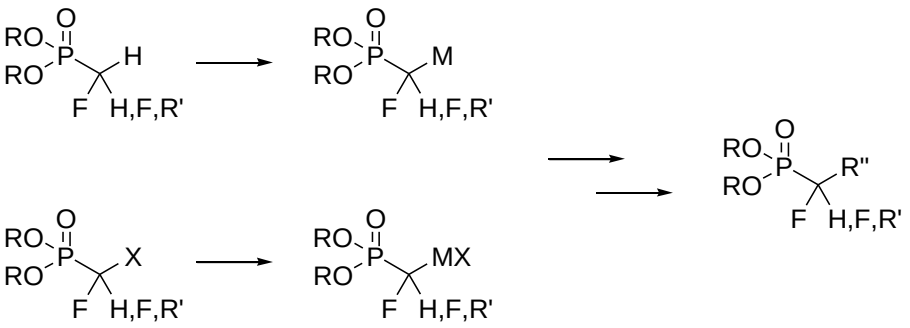
Nucleophilic monofluorination



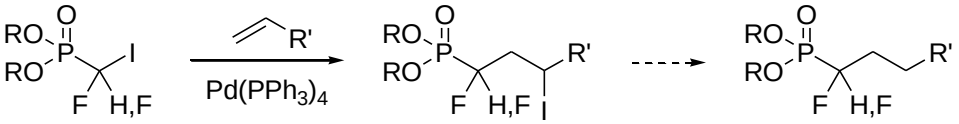
Nucleophilic difluorination



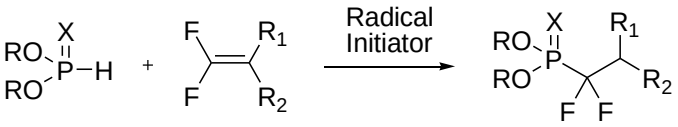
4. **Syntheses via (alkylation of) fluorinated phosphonate carbanions.** This is a quite broadly applicable and hence popular strategy that may give access to both α-monofluorophosphonate and α,α-difluorophosphonate derivatives[25, 26]. The carbanions can be formed either by metalation of a dialkyl difluoromethylphosphonate with common alkali metal bases[15, 27-30] or by insertion of a metal (Mg, Zn, Cd,…) into the C-Br bond of a dialkyl bromodifluoromethylphosphonate[31-33], followed by attack of the carbanion on a whole range of electrophiles[32-36] (alkyl halides, alkyl triflates, aldehydes, acyl halides, activated olefins, epoxides…).



5. **Transition metal catalysis.** This approach is mostly restricted to the palladium-catalyzed addition of dialkyl iodo(di- or mono)fluoromethylphosphonate to terminal alkenes resulting in α-fluoro,γ-iodoalkylphosphonates[37]. The latter can be selectively reduced to remove the γ-iodine[38].



6. **Radical reactions.** These form a totally different field of chemistry and suffer from low tolerability towards functional groups. Perhaps of greatest importance is the radical addition of dialkyl (thio)phosphites to gem-difluoroenol ethers[39, 40] or 2,2-alkyl,1,1-difluoroalkenes[41] to form the corresponding α,α-difluorophosphonates, although also the addition of dialkyl difluoromethyl(thio)phosphonate radicals to alkenes and alkynes has been reported[42].



R = alkyl; X = O, S; R_1 = O-alkyl, alkyl; R_2 = alkyl

III.A.2. Halogenated fosmidomycin analogues

Fosmidomycin and FR900098 are potent DXR inhibitors showing structural analogy to DOXP, the phosphate-bearing substrate of the enzyme. As stated above, the phosphonate structure in fosmidomycin is a useful mimic of the DOXP phosphate moiety in terms of stability, though it does not perfectly mimic its polarity and pKa_2. Woo et al. synthesized analogues **2** and **3** (Figure III.2) of fosmidomycin and FR900098 in which the phosphonate functionality was switched back to a phosphate, and found out that both analogues surpassed fosmidomycin in inhibition of *Synechocystis* DXR activity[43]. The *N*-formyl-analogue **2** was actually described before and known as fosfoxacin[44], but its activity against DXR had not been tested. Following the above reasoning, α-fluorinated analogues

of fosmidomycin were expected to be good DXR inhibitors. Therefore we undertook the synthesis of α-fluoro- and α,α-difluoro-FR900098 analogues **4a** and **6** as well as α-chloro-analogue **4b** (which could be synthesized in an analogues manner, see below), as well as the hydroxamate congener **5**.

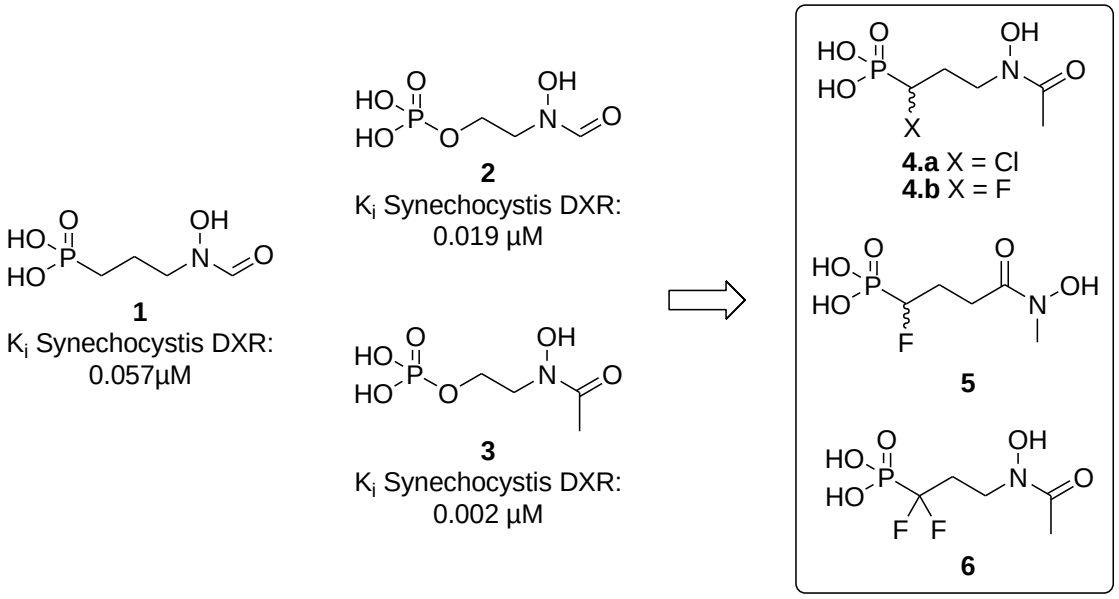
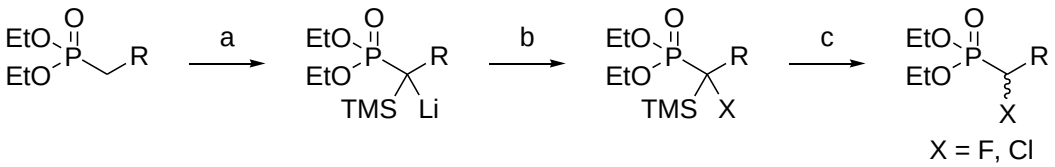


Figure III.2: Inhibitory effect of fosmidomycin, fosfoxacin and its acetyl congener on *Synechocystis* DXR[43] as a rationale for the synthesis of halogenated phosphonate analogues.

III.B Mono-halogenated analogues

III.B.1. α-Halogenated retrohydroxamates

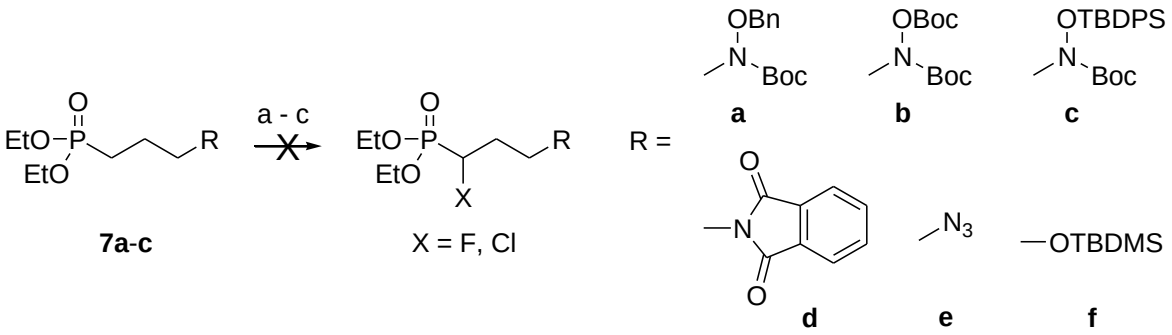
For the introduction of the desired halogens into FR900098, we adopted the strategy of Iorga et al., who described a straightforward one-pot method for the electrophilic halogenation of diethyl alkylphosphonates[45] (Scheme III.1).



Reagents and Conditions: (a) LDA (2eq.), TMSCl, THF, -78 °C; (b) $(PhSO_2)_2NF$ or C_2Cl_6, -78 to 0 °C; (c) EtOLi/EtOH, 0 °C

Scheme III.1: Method for the α-monohalogenation of alkylphosphonates[45]

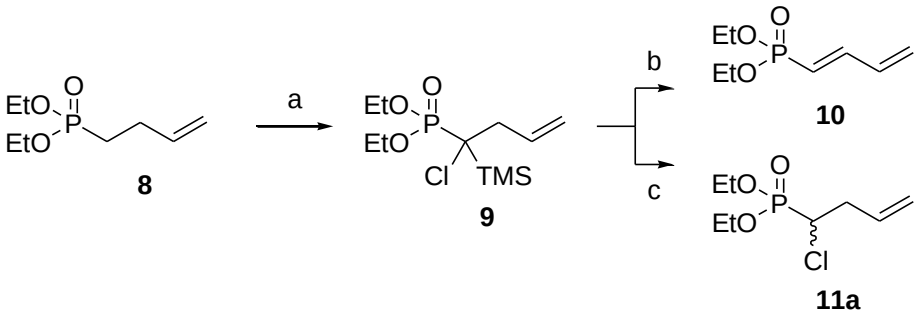
First, the alkylphosphonate is deprotonated with LDA followed by a monosilylation in order to prevent overhalogenation. A second equivalent of LDA then again forms an α-carbanion, which may attack an electrophilic halogen source, in this case *N*-fluorobenzenesulfonimide[46] or hexachloroethane, resulting in an α-TMS,α-halogenated alkylphosphonate. Finally, the silyl group is removed *in situ* by lithium ethoxide – ethanol. This strategy has several advantages such as high yield, high speed, ready availability of electrophilic halogenating reagents and easy elimination of byproducts. Hence it seemed obvious to apply it for the α-halogenation of a suitably protected FR900098 derivative. Apart from the obvious necessity to protect the retrohydroxamate N-OH, we anticipated enolization of the *N*-acetyl might cause additional problems. Iorgas halogenation conditions were tried on both an *N*-Boc,*O*-benzyl- and an *N*-Boc,*O*-Boc-protected FR900098 precursor (**7a** and **7b**, SchemeIII.2), unfortunately without success: reaction mixtures immediately turned black and no product nor any desired intermediate could be isolated. Knowing the susceptibility of some benzyl ethers towards LDA and nucleophiles, we also tested the reaction on *N*-Boc,*O*-silyl-protected precursor **7c**, alas without success.



Reagents and Conditions: (a) LDA (2eq.), TMSCl, THF, -78 °C; (b) $(PhSO_2)_2NF$ or C_2Cl_6, -78 to 0 °C; (c) EtOLi/EtOH, 0 °C

Scheme III.2: Substrates failed in the α-monohalogenation reaction

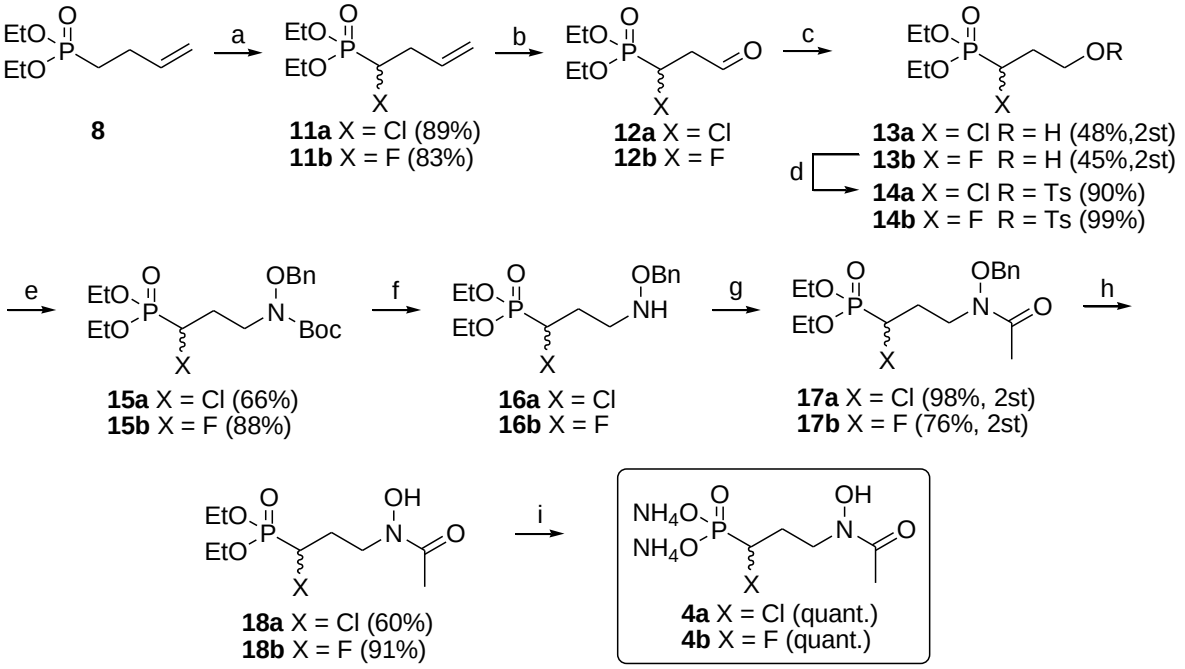
Apparently, the retrohydroxamate functionality –and even its protected precursors- are too sensitive to the reaction conditions used. It has to be mentioned here that Iorga only describes the halogenation of unfunctionalized alkylphosphonates and also elsewhere in literature this procedure is only applied to structurally robust/simple molecules[47]. Therefore, we retreated a step from the protected FR900098 toward a more robust / structurally simpler precursor from which the retrohydroxamate could be synthesized in a later stage. Protected amines or alcohols were a logical choice, but unfortunately, neither azide **7e**, nor phthalimide **7d** or silylated alcohol **7f** could be halogenated using this protocol. Trying to cut down even more on functionality in our precursor, or move more towards the alkanes described in the original paper, we ended up with diethyl but-3-enylphosphonate **8** which could cleanly be fluorinated. However, upon applying the described conditions for α-chlorination, we noticed the formation of a side product that could be identified as diene **10** (Scheme III.3). This originated most probably from elimination of HCl from the desired product with the formation of a conjugated diene as the driving force. Later we also noticed this reaction to take place during fluorination, although to a lesser extent. We circumvented this side reaction by switching from the basic lithium ethoxide – ethanol conditions to TBAF in acetic acid for the removal of the α-TMS group.



Reagents and Conditions: (a) (i) LDA (2eq.), TMSCl, THF, -78 °C; (ii) C_2Cl_6, -78 to 0 °C; (b) EtOLi/EtOH, 0 °C; (c) AcOH, TBAF, THF

Scheme III.3: Desilylation conditions

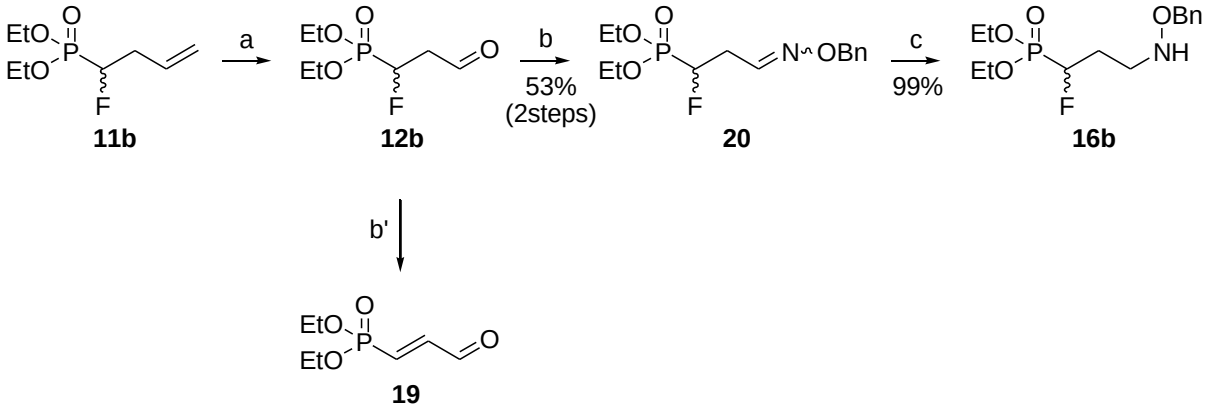
With the α-halogenated precursors **11a-b** in hand, we then assembled the hydroxamate head group as depicted in Scheme III. 4. First the double bond was oxidized with NMO and osmium tetroxide as a catalyst.



Reagents and Conditions: (a) (i) LDA (2eq.), TMSCl, $(PhSO_2)_2NF$ / C_2Cl_6, THF, -78 °C, (ii) TBAF, AcOH, THF; (b) (i) OsO_4, dioxane, (ii) $NaIO_4$; (c) $NaBH_4$, MeOH; (d) TsCl, Et_3N, CH_2Cl_2; (e) BocNH(OBn), NaH, DMF; (f) TFA, CH_2Cl_2; (g) Ac_2O, Et_3N, DMAP, CH_2Cl_2; (h) H_2, Pd/C, EtOAc; (i) (i) TMSBr, CH_2Cl_2, (ii) NH_4OH, THF

Scheme III.4: General synthesis of α-monohalogenated FR900098 analogues

The resulting vicinal diol was then cleaved oxidatively with sodium periodate and the resulting aldehyde reduced with sodium borohydride to give alcohols **13a-b**. Subsequently, the alcohols were converted into tosylates **14a-b**, which were substituted with *N*-Boc,*O*-benzylhydroxylamine. Treatment of **15a-b** with trifluoroacetic acid in dichloromethane gave oxyamines **16a-b**, which were acetylated with acetic anhydride. For the upscaling of the fluorinated analogue, the procedure was shortened both in reaction time and number of steps (Scheme III.5). The olefin was cleaved in a one-pot dihydroxylation – oxidative cleavage using catalytic potassium osmate and potassium periodate both as a cooxidant in the dihydroxylation and as main oxidant in the vicinal diol oxidative cleavage. Not only did we win a step this way, the dihydroxylation also proceeded much smoother with periodate as the oxidant instead of NMO. Furthermore, instead of reducing the aldehyde, it was reacted with *O*-benzylhydroxylamine in acetic acid to form oxime **20**. A comparable oxime formation[48] was successfully accomplished before in pyridine, but according to literature, it can also be realized in acidic media[49] with an optimal reaction rate around pH = 4. We deliberately chose for an acidic reaction medium in order to prevent elimination of HF from aldehyde **12b** and formation of phosphonoacrylate **19**. The resulting oxime was then reduced with sodium cyanoborohydride in hydrochloric acid - methanol affording oxyamine **16b** in a shorter, more convenient and more atom-economic way. Other, more benign reductants ($NaBH_4$, sodium triacetoxyborohydride, borane) for the reduction of this kind of oximes, as well as one-pot reductive oxyaminations have been explored before in our lab and proved unsuccessful. Clearly, a two-step strategy, involving oximation, isolation of the oxime and then reduction with cyanoborohydride in acidic methanol is preferred.



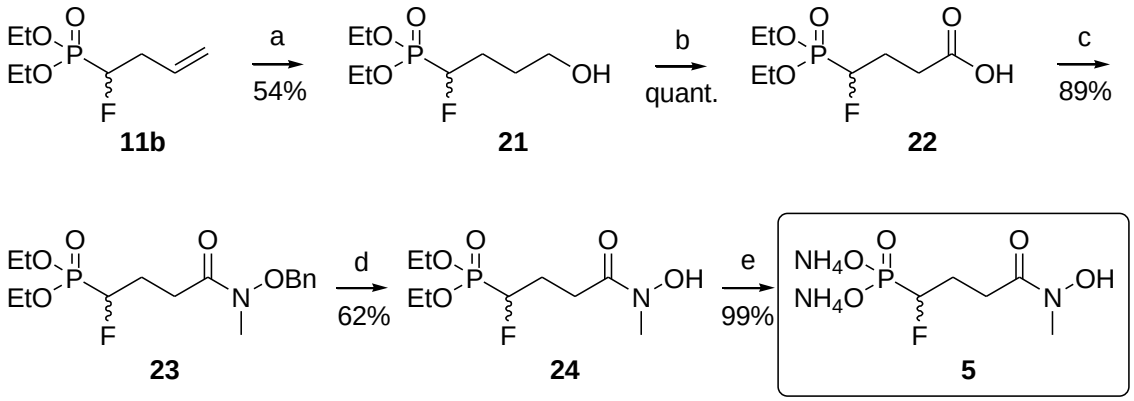
Reagents and Conditions: (a) $NaIO_4$, $K_2OsO_4.2H_2O$, H_2O, THF; (b) $BnONH_2$, AcOH, THF; (b') $BnONH_2$, base; (c) $NaBH_3CN$, HCl, MeOH

Scheme III.5: Shorter synthesis of α-fluoro-oxyamine precursor **16b**

Finally, debenzylation of the retrohydroxamate by hydrogenation over palladium on carbon, followed by TMSBr-deprotection of the phosphonate esters and basic workup gave **4a** and **4b** as bisammonium salts.

III.B.2. α-Fluoro hydroxamate analogue

One advantage of executing the electrophilic halogenation on the butenylphosphonate is that we ended up with a more versatile synthon. Having α-fluorophosphonate **11b** in hands, we decided to also explore its conversion to hydroxamate **5** (Scheme III.6). As was described in the introduction, replacing the native retrohydroxamate in fosmidomycin or FR900098 for a *N*-methylated hydroxamate (*N*-methyl,*N*-hydroxyamide) has often resulted in potent DXR ligands. Hydroboration of **11b** gave rise to alcohol **21** which was oxidized with TEMPO-BAIB to carboxylic acid **22**. This acid was activated with 1,1'-carbonyldiimidazole (CDI) and coupled with *N*-methyl,*O*-benzylhydroxylamine to give **23**, which was then deprotected in the same way as **17a-b** to give **5** as the bisammonium salt.



Reagents and conditions: (a) (i) BH_3.THF (ii) NaOH, H_2O_2; (b) TEMPO, BAIB, MeCN/H_2O; (c) 1,1'-carbonyldiimidazole, Me-NH-OBn; (d) H_2, Pd/C, EtOAc; (e) (i) TMSBr, CH_2Cl_2, (ii) NH_4OH, THF

Scheme III.6: Synthesis of α-fluoro hydroxamate analogue **5**

III.B.3. Influence of α-halogen substitution on pKa

In order to assess the influence of the introduced halogens on the ionization of these phosphonates, the pK_a's of compounds **4a** and **4b,** as well as the FR900098 reference were estimated from the pH dependence of their ^{31}P chemical shift. Towards this end, the ^{31}P chemical shift of each compound was measured at different pH and plotted as a function of these pH values. The pKa of each compound is estimated to be at the pH of the inflection point of its titration curve.

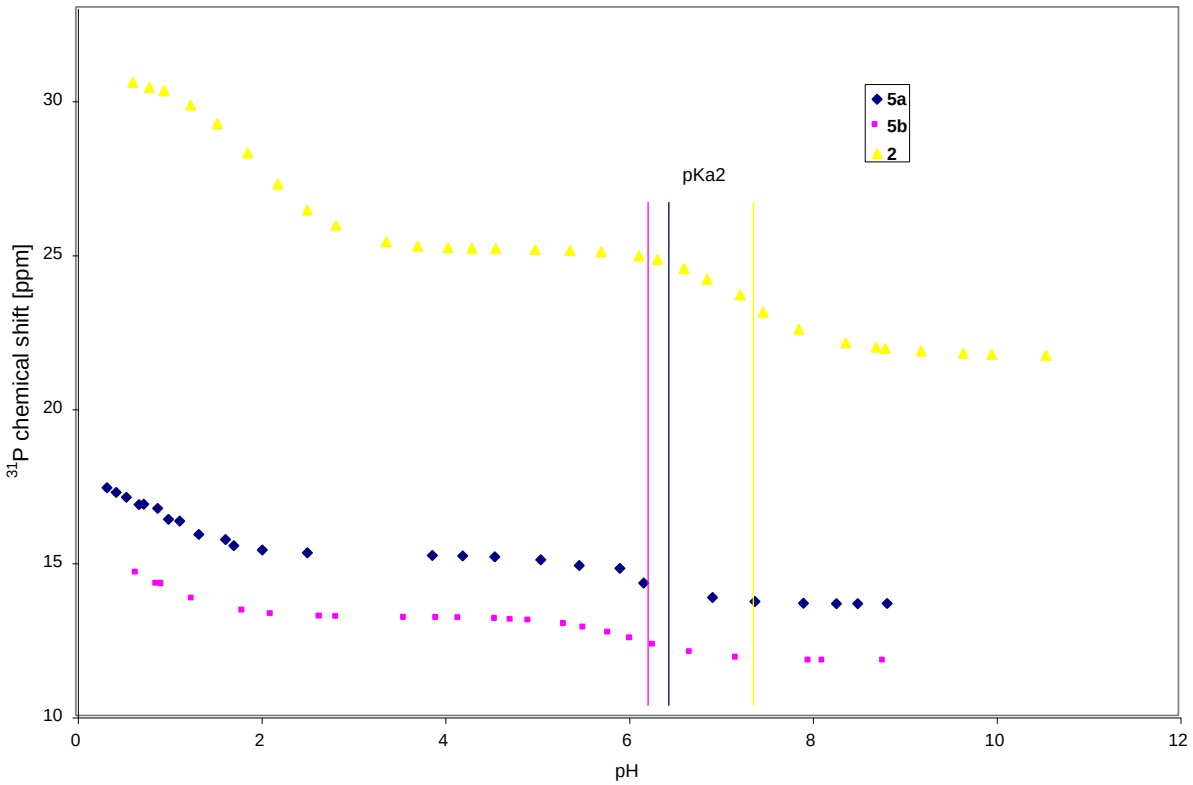


Figure III.3: Overall titration curves for α-chloro- and α-fluoro-FR900098 and FR900098 itself

Of special interest here is the second pKa of each molecule, as the value of this pKa determines whether the phosphonate will be in its single- or double-ionized form at physiological pH. Therefore a cutout of the titration curves is displayed from which the pKa_2 can be estimated.

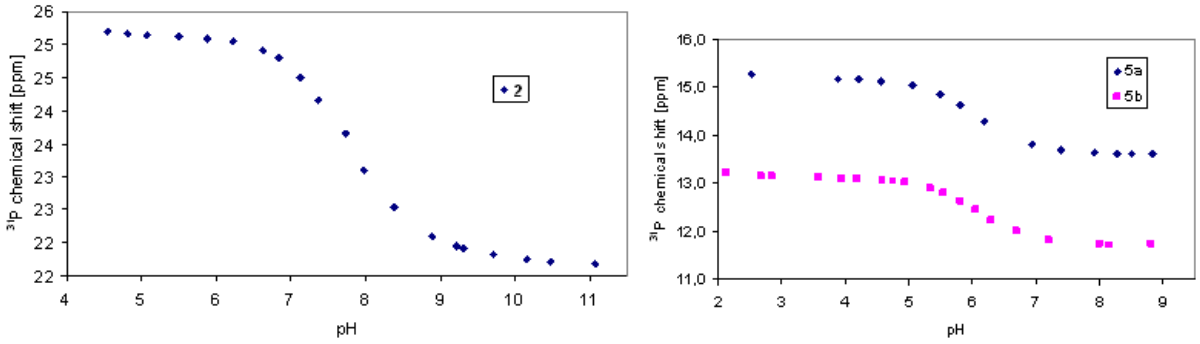


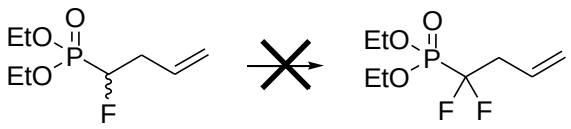
Figure III.4: Detail of titration curves around their second inflection point

From these figures, a pKa_2 of 7.35 can be estimated for FR900098, whereas both the α-chlorinated analogue **4a** and the α-fluorinated analogue **4b** show a pKa_2 of around 6. It can thus be concluded that introduction of a halogen in α-position of the phosphonate moiety indeed lowered the pKa_2 of those compounds thereby more closely resembling that of a phosphate moiety[9].

III.C α,α-Difluoro-FR900098

III.C.1. Electrophilic fluorination of an α-fluorophosphonate

Having successfully performed the electrophilic monohalogenation as described above, we decided to explore a similar strategy for the synthesis of α,α-difluoro-FR900098 by performing a second fluorination of α-monofluoro-phosphonate **11b** under the same conditions (Scheme III.7). A first attempt, using the same conditions as for the first fluorination without the silylation step (LDA, NFBS, THF, -78 °C) resulted in a deep brown coloration of the reaction mixture indicating breakdown, and indeed no product nor any starting material could be recovered. In their paper on the discovery and use of NFBS, Differding et al.[47] documented the sensitivity of this type of reaction to temperature and the base involved, and recommended the use of KDA (from nBuLi and KOtBu) and to work below -90 °C. Unfortunately, in our hands these conditions (KDA, NFBS, THF, -95 °C) also resulted in total decomposition of the α-fluoro butenylphosphonate **11b**.



11b **25**

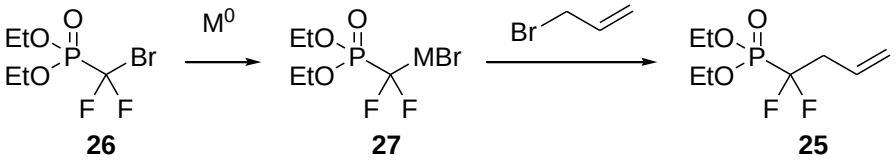
Reagents and Conditions: LDA / KDA, NFBS, THF, -78 or -90 °C

Scheme III.7: Attempted electrophilic second fluorination

Therefore, we switched to method 4 outlined in the introduction: alkylation of a fluorinated phosphonate carbanion.

III.C.2. Alkylation of diethyl bromodifluoromethylphosphonate

First we tried to reproduce an experiment by the Burton group[32] comprising the insertion of cadmium into the bromine-carbon bond of diethyl bromodifluoromethylphosphonate followed by the attack of this organometal reagent onto allyl bromide, resulting in the difluoro-analogue of **11b** (Scheme III.8).

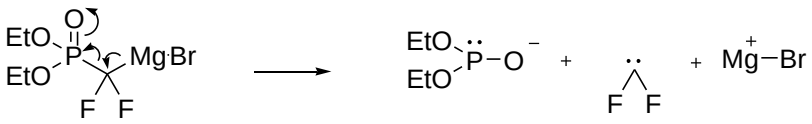


M = Cd, Mg, Zn

Reagents and Conditions: DME/THF or Et_2O; ultrasound; 0° C/ rt / 70° C; up to 48h

Scheme III.8: Metal insertion into bromodifluoromethylphosphonate and subsequent alkylation

The metal insertion proved to be quite hard and not reproducible, resulting in low yields for the coupling with allyl bromide. It has to be noted here that the monitoring of this reaction is not trivial as diethyl bromodifluoromethylphosphonate **26** is slightly volatile, does not quench UV light on TLC and also does not stain with common reagents such as permanganate, phosphomolibdic acid or cerium-ammonium-molybdate. The bad results obtained when using cadmium, together with its toxicity, urged us to try out other metals such as magnesium and zinc. The reaction of **26** with magnesium turnings resulted in a red-black solution, pointing to breakdown probably via carbene formation (Scheme III.9).



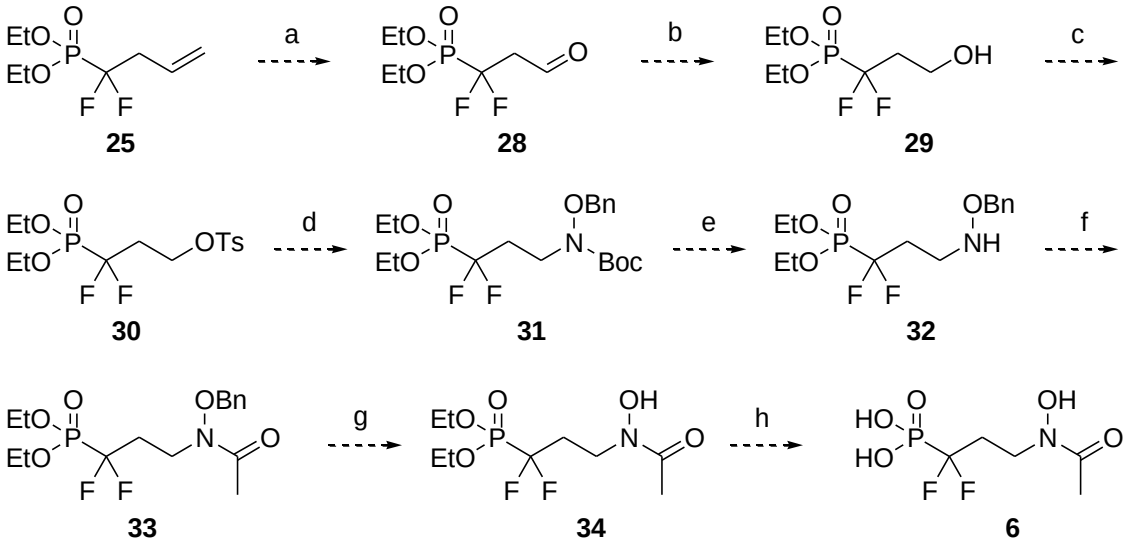
Reagents and Conditions: $(EtO)_2P(O)CH_2Br$, Mg powder, THF, 1,2-dibromoethane, ultrasound, 0° C to 50° C

Scheme III.9: Postulated carbene formation upon magnesium insertion

According to literature data[50, 51], Mg insertion into **26** can only be accomplished through the use of Grignard reagents such as *i*PrMgBr. Finally, we achieved nearly quantitative metal insertion using activated zinc dust in DME[32] though only upon prolonged sonication. The formation of the metalated species, which is described as quite stable at room temperature, can be followed by ^{31}P-NMR: the starting bromide shows up as a triplet at δ=5.29ppm which upon Zn insertion shifts downfield to

δ=13.85ppm. On ^{19}F-NMR a shift is seen from δ=-136.5 to δ=-128ppm. The use of Rieke-zinc[52-55] (preformed from lithium naphthalenide and anhydrous zinc chloride), in order to speed up the metal insertion and improve the yield, only resulted in debromination of the starting phosphonate resulting in diethyl difluoromethylphosphonate. The copper(I)-catalyzed addition of the phosphonate-zinc reagent proceeded rather slowly (which is in accordance to literature findings[32]), typically giving yields between 50% and 70% after two days of reaction at room temperature. Here again, difficulties in monitoring the volatile and hard-to-stain reagents complicated the procedure. In the end, the use of ordinary zinc dust (activated by simply washing with dilute hydrochloric acid) and the copper(I)-catalyzed allyl bromide coupling allowed us to synthesize a sufficient amount of precursor **25**.

With the α,α-difluorobutenylphosphonate **25** in hand, we envisaged the construction of the retrohydroxamate as accomplished for the monohalogenated analogues (Scheme II.10).

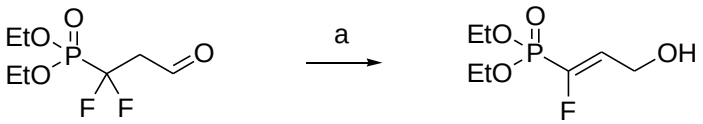


Reagents and Conditions: (a) (i) OsO_4, dioxane, (ii) $NaIO_4$, H_2O, THF; (b) $NaBH_4$, MeOH; (c) TsCl, Et_3N, CH_2Cl_2; (d) BocNH(OBn), NaH, DMF; (e) TFA, CH_2Cl_2; (f) Ac_2O, Et_3N, DMAP, CH_2Cl_2; (g) H_2, Pd/C, MeOH; (h) (i) TMSBr, CH_2Cl_2, (ii) NH_4OH, THF, quantitative

Scheme III.10: Anticipated general synthetic route toward α,α-difluoro-FR900098

Osmium tetroxide-catalyzed NMO oxidation of the double bond in **25** followed by cleavage with sodium periodate gave aldehyde **28** together with undefined polar products. These 'polar products' were visible as a significant baseline spot on TLC and their formation, with concomitant reduction of

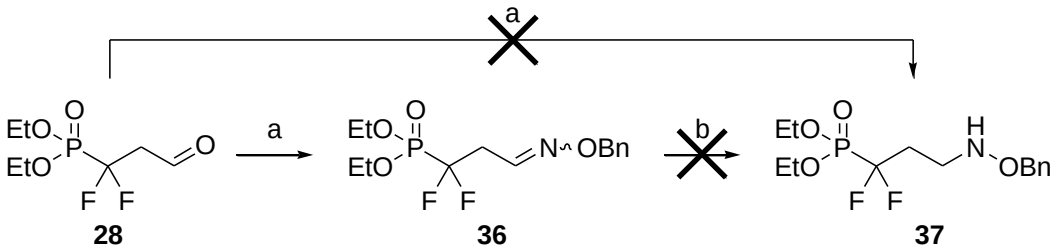
the yield, was a problem with most reactions involving the difluorophosphonate. Only much later in this work their origin became clear, as will be described further in this thesis. Attempted reduction of aldehyde **28** with sodium borohydride also suffered heavily from this breakdown to polar products and on top of that, the only product that could be effectively isolated was allylic alcohol **35** (Scheme III.11), probably formed by E_2 elimination.



Reagents and Conditions: $NaBH_4$ (4 eq.), EtOH, rt, on.

Scheme III.11: Elimination upon attempted borohydride reduction

To circumvent the use of basic reaction conditions, we explored the reductive amination of aldehyde **28** with *O*-benzylhydroxylamine and triacetoxyborohydride in acidic conditions, but unfortunately this reaction got stuck at the oxime stage and also suffered from fluoride elimination (Scheme III.12).

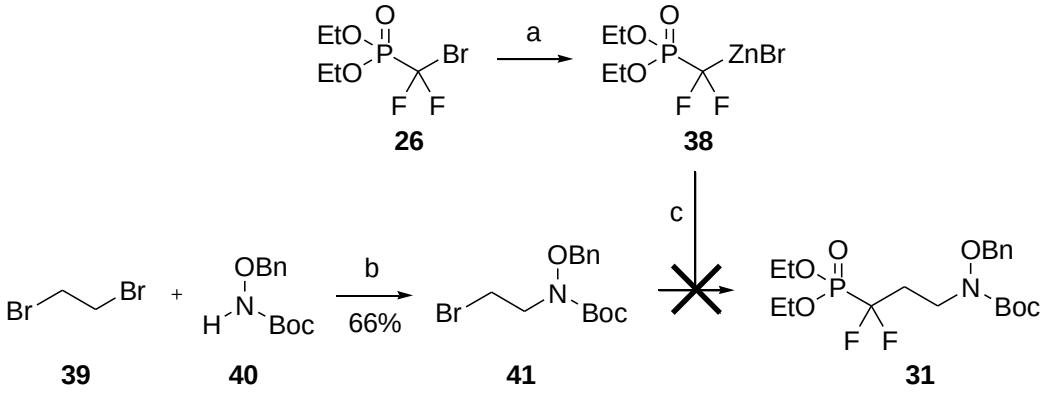


Reagents and Conditions: (a) $BnONH_2$, $NaBH(OAc)_3$, AcOH, DCE, rt, on.; (b) $NaBH_3CN$, HCl, MeOH

Scheme III.12: Attempted reductive (oxy)amination of aldehyde **28**

Attempted reduction of oxime **36** with sodium cyanoborohydride in hydrochloric acid – methanol led to total decomposition of the oxime. These findings indicated that an α,α-difluorophosphonate bearing molecule is not amenable to many synthetic manipulations. Therefore, we considered a

more convergent synthesis based on the coupling of the difluoromethylphosphonate with a 'tail part' that already features a (protected) retrohydroxamate (Scheme III.13).



Reagents and Conditions: (a) Zn, DME, ultrasound, rt, 48h; (b) NaH (1.1eq.), DMF, rt, on.; (c) DME, CuBr, rt, on.

Scheme III.13: Attempted convergent route toward protected α,α-difluoro-precursor **31**

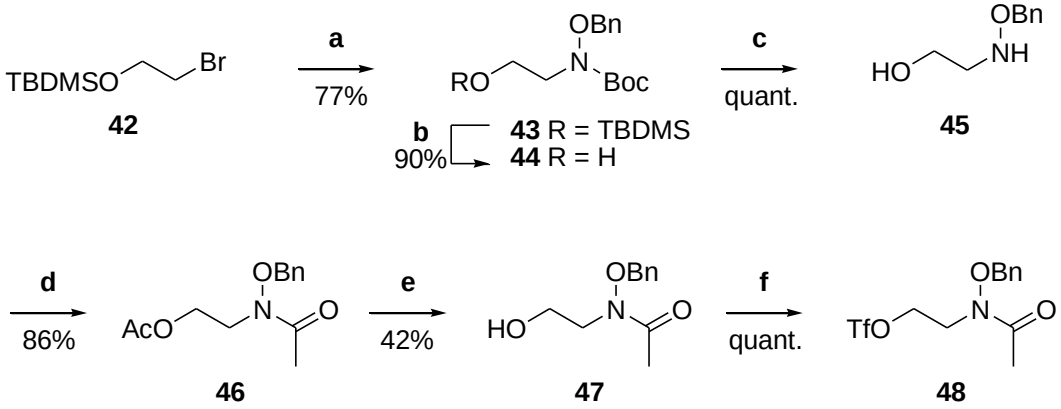
N-Boc,*O*-benzylhydroxylamine (**40**) was coupled with 1,2-dibromoethane (**39**) resulting in the alkyl bromide **41**. Attempts to couple this 'tail' with zinc halide **38,** analogous to the coupling with allyl bromide in the presence of copper(I)bromide, were unsuccessful. Either the protected retrohydroxamate degrades under the reaction conditions used, or the coupling reaction is very limited in terms of substrates. This assumption, together with the difficulties associated with the zinc insertion into the bromodifluorophosphonate building block, led us to invstigate another strategy: the attack of a lithiated difluoromethylphosphonate onto a primary alkyl triflate[29, 30].

III.C.3. Alkylation of diethyl difluoromethylphosphonate

Retaining the idea of convergence, a hydroxyethylated hydroxamate tail **47** was constructed first, using two different strategies as depicted in Scheme III.14. Method A is based on the alkylation of *N*-Boc,*O*-benzylhydroxylamine with silylated 2-bromoethanol **42**, followed by sequential deprotection, bis-acetylation and ester hydrolysis. Method B is more straightforwardand involves a two-step reductive oxyamination of silylated glycolaldehyde followed by acetylation and final desilylation.

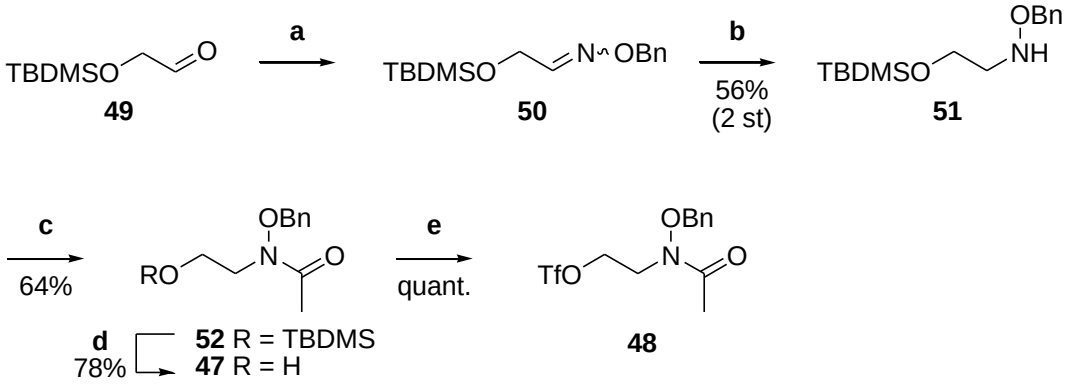
Alcohol **47** was then quantitatively converted to its triflate using triflic anhydride and DIPEA in dichloromethane, followed by extraction at 0 °C and removal of the volatiles *in vacuo*.

Method A (overall yield of alcohol **47** = 25%)**:**



Reagents and Conditions: (a) BocNHOBn, NaH, DMF; (b) NH_4F, MeOH; (c) TFA, CH_2Cl_2; (d) Ac_2O, DMAP, Et_3N, CH_2Cl_2; (e) NaOMe, MeOH; (f) Tf_2O, DIPEA, CH_2Cl_2

Method B (overall yield of alcohol **47** = 28%)**:**

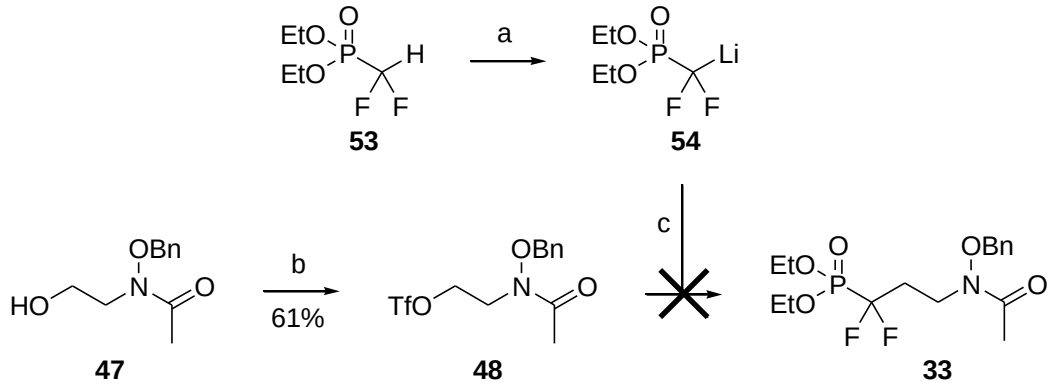


Reagents and Conditions: (a) BocNHOBn, NaH, DMF; (b) NH_4F, MeOH; (c) TFA, CH_2Cl_2; (d) Ac_2O, DMAP, Et_3N, CH_2Cl_2; (e) NaOMe, MeOH; (f) Tf_2O, DIPEA, CH_2Cl_2

Scheme III.14: Synthesis of 'tail' alcohol **47** via two different routes

In their original paper[29], Berkowitz et al. propose two basic strategies for the displacement of triflates by difluoromethylphosphonates. The first encompasses the *in situ* formation of the anion, by mixing diethyl difluoromethyl phosphonate with the triflate and a lithium complexant (HMPA or TMEDA) in THF followed by addition of LDA. The second method consists of the lithiation of diethyl

difluoromethyl phosphonate with LDA in the presence of HMPA or TMEDA in THF followed by the addition of a solution of the triflate. Because of the risk of elimination reactions we chose the latter method (Scheme III.15). A solution of LDA in THF was prepared, HMPA or DMPU (as a non-carcinogenic alternative) was added followed by diethyl difluoromethylphosphonate (**53**). After a few minutes, this mixture was added to a solution of triflate **48** in THF at -78 °C and stirred for five minutes. The reaction mixture was quenched by the addition of saturated aqueous ammonium chloride. Analysis of the reaction mixture revealed degradation of the starting material to very polar products.

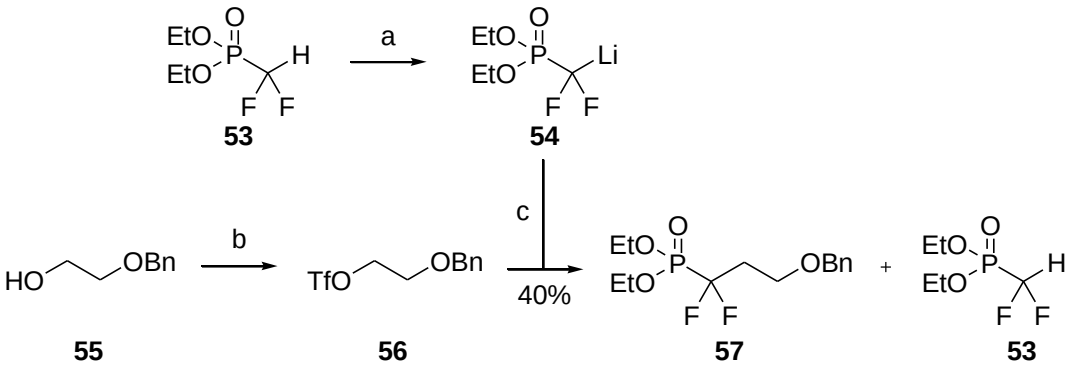


Reagents and Conditions: (a) LDA, DMPU, THF, -78 °C; (b) Tf_2O, DIPEA, CH_2Cl_2, -60 °C; (c) THF, DMPU, -78 °C

Scheme III.15: Attempted convergent synthesis via triflate displacement with difluoromethylenephosphonate anion

Apparently the protected retrohydroxamate moiety of **48**, which resembles a Weinreb amide, did not withstand the harsh reaction conditions. According to the literature[28-30] though, the triflate-displacing strategy has given satisfying yields with a whole range of differently protected (benzyl, allyl, TES, TBDMS, BOM, PMB, isopropylidene) sugar primary triflates as well as an (1*H*-pyrrol-1-yl)alkyl triflate and silyloxyalkyl- or naphthylalkyltriflates. Therefore, we wanted to give this strategy another try, this time with a less elaborated 'tail'. Having already ruled out the use of an alkene, we set our hopes on a simple alcohol as a handle for the construction of the retrohydroxamate,

protected with the robust benzyl group (Scheme III.16). Thus, 2-benzyloxyethanol (**55**) was triflated under the same conditions as **47** in good yield, followed by lithium diethyl difluoromethylphosphonate attack in THF and DMPU at -78 °C resulting in α,α-difluoro benzyloxypropylphosphonate **57** in acceptable yield. According to NMR, however, this product was contaminated with a considerable amount of the starting phosphonate (**53**) that coeluted upon purification and which, as stated before, is not visible on TLC.

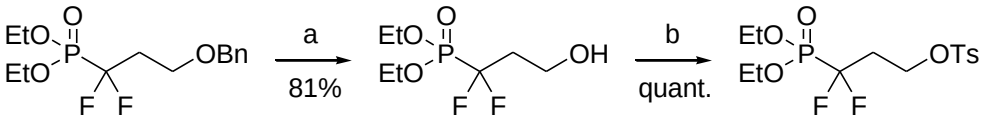


Reagents and Conditions: (a) LDA, DMPU, THF, -78 °C; (b) Tf_2O, DIPEA, Et_2O; (c) THF, DMPU, -78 °C

Scheme III.16: Triflate displacement by diethyl difluoromethylphosphonate anion

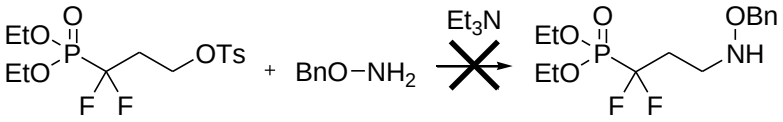
To get rid of this contaminant, we figured that the reductive debenzylation of **57** might shift the product to a lower Rf, thus enabling its chromatographic purification. To our amazement, the debenzylation of **57** under 'standard' conditions (atmospheric pressure hydrogen, 10% palladium on charcoal or Pd black in methanol or THF) gave no result at all. Probably, the diethyl difluoromethylphosphonate in the starting material inhibited the reduction by poisoning of the palladium catalyst. This problem was overcome by switching to catalytic hydrogen transfer debenzylation[56, 57] using formic acid as a hydrogen donor on the one hand, and recycling the difluoromethyl phosphonate contaminant by means of distillation upon resynthesis of **57** on big scale on the other hand. Alcohol **58** was subsequently converted to its tosylate **59,** which could then be substituted by a suitable oxyamine (Scheme III.17). Because of the ease with which these molecules

undergo elimination, we were reluctant to use strong bases and therefore tried to substitute the tosylate with simple *O*-benzylhydroxylamine (**60**) in the presence of triethylamine.

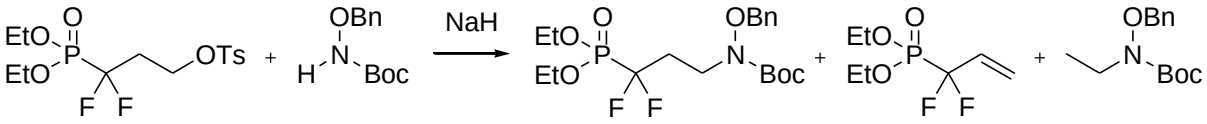


57 **58** **59**

Reagents and Conditions: (a) HCOOH, Pd/C, MeOH, on., rt; (b) TsCl, DMAP, Et_3N, CH_2Cl_2, 1h



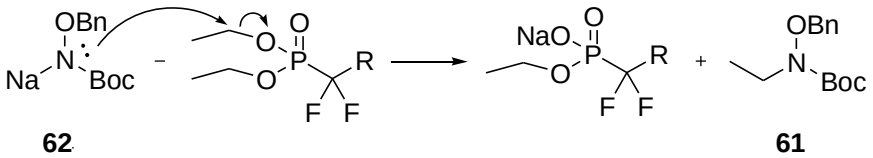
59 **60** **32**



59 **40** **31** (10%) **60** (50%) **61** (20%)

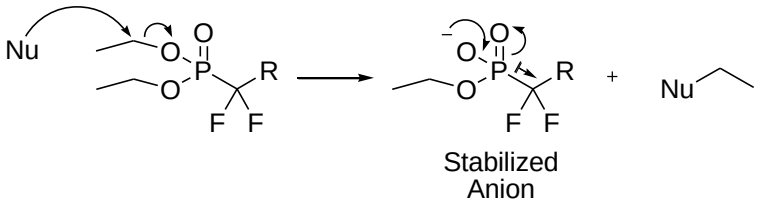
Scheme III.17: Synthesis of tosylate **59** and attempts toward substitution by a suitably protected hydroxylamine.

This proved to be unsuccessful, forcing us to try the more established route using *N*-Boc,*O*-benzylhydroxylamine (**40**) and sodium hydride. This wasn't a big success either as the desired product **31** was only formed in a very low yield, due to formation of two major products: propylenephosphonate **60,** resulting from E2-elimination of the tosylate, and ethylated oxyamine **61**, possibly resulting from the attack of deprotonated *N*-Boc,*O*-benzylhydroxylamine onto one of the ethyl esters of the phosphonate as depicted in Scheme III.18. The elimination product could not be entirely separated from the desired product.



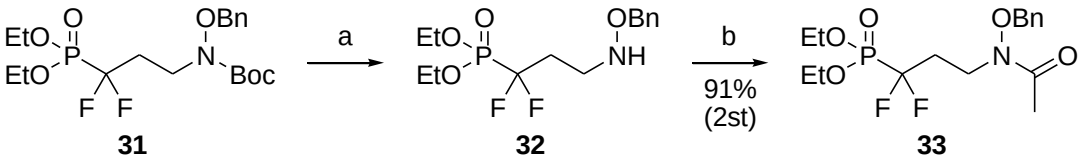
Scheme III.18: Attack of deprotonated BocN(Na)OBn onto an ethyl ester of the difluorophosphonate

Indeed, the introduction of two fluorine atoms onto the α-carbon increases the acidity of the phosphonate and thus also its leaving group capacity, thereby decreasing the stability of the phosphonate ethyl esters as protecting groups (Scheme III.19). This also explains the generally low yields and the presence of polar (breakdown) products in most reactions involving the difluoromethylene moiety: whenever basic/nucleophilic conditions are present, a (partial) breakdown ensues resulting in ethylated nucleophile (probably volatile and/or non-chromophoric hence unnoticed on TLC in the case of small nucleophiles) and a monoethyl α,α-difluoroalkylphosphonic mono-acid. It also explains that reactions involving acidic conditions usually gave better yields and cleaner reaction mixtures.



Scheme III.19: Formation of a stabilized anion by loss of an ester from a α,α-difluoroalkylphosphonate

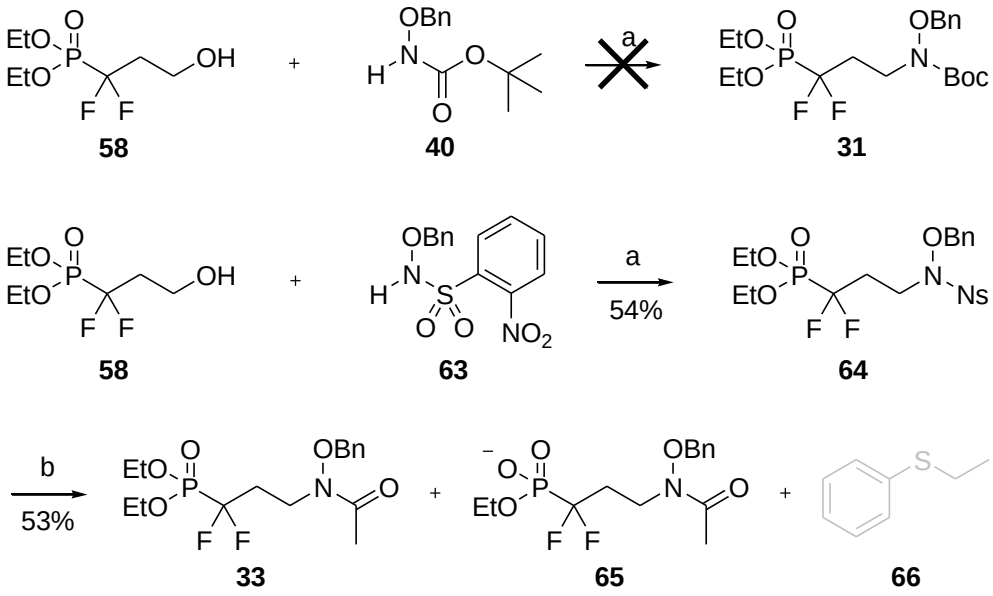
In line with these assumptions, the subsequent Boc removal from **31** with TFA proceeded in good yield without any problems (Scheme III.20). Also the acetylation of oxyamine **32** with acetic anhydride in the presence of triethylamine proceeded smoothly to yield protected α,α-difluoro-FR900098, albeit in a minute quantity.



Reagents and Conditions: (a) TFA, CH_2Cl_2; (b) Ac_2O, Et_3N, DMAP, CH_2Cl_2

Scheme III.20: Boc removal and acetylation

Because of the poor yield of the tosylate displacement, we set out for another synthesis of the retrohydroxamate functionality using gentler reaction conditions (Scheme III.21). Our first choice was the Mitsunobu reaction for it provides both the required mild conditions and allows direct conversion of the free alcohol to a protected retrohydroxamate. Trying to introduce *N*-Boc,*O*-benzylhydroxylamine under these conditions proved unsuccessful, due to the insufficient acidity of this carbamate on the one hand and the inactivated alcohol **58** on the other hand. Previously, Vincent Devreux from our group tackled a similar problem by replacing the Boc-group for a nosyl (2-nitrobenzenesulfonyl) group, thereby lowering the pKa of the N-H and increasing its reactiveity[58-60]. When we applied this strategy, *N*-nosyl,*O*-benzylhydroxylamine **64** was formed in 54% yield, without any elimination occurring.

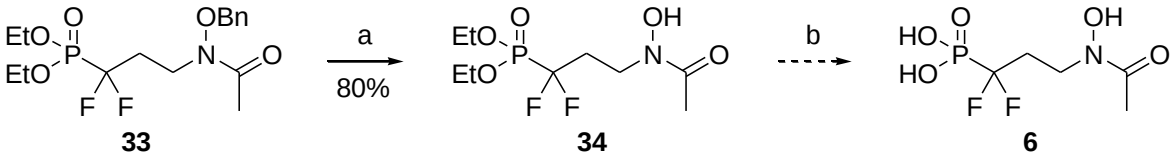


Reagents and Conditions: (a) Ph_3P, DIAD, THF; (b) (i) PhSH, K_2CO_3, MeCN, DMSO, (ii) Ac_2O

Scheme III.21: Mitsunobu approach toward **33**

For the subsequent nosyl deprotection and acetylation, we borrowed the practical one-pot strategy also developed by Vincent Devreux: first the nosyl group is removed by nucleophilic attack with thiophenol under basic conditions (K_2CO_3), followed by the addition of acetic anhydride to acetylate the formed oxyamine and any remaining thiophenol, thus avoiding problems of thiophenol smell and toxicity. Applied to nosylate **64**, this strategy yielded once again protected α,α-difluoro-FR900098 **33** in 53% yield. Phosphonate ethyl ester cleavage by thiophenol was also noticed by negative mode MS of the reaction mixture (signal of phosphonate mono-ethyl ester).

Finally, having synthesized a sufficient amount of protected product **33**, attempts toward final deprotection were made (Scheme III.22). First the benzyl group was removed using catalytic transfer hydrogenation with formic acid as the hydrogen donor. It is known that the solvent can have a dramatic effect on the hydrogenation rate, and therefore it was carried out in ethyl acetate resulting in a total deprotection in only one hour with a yield of 80% after column chromatography.

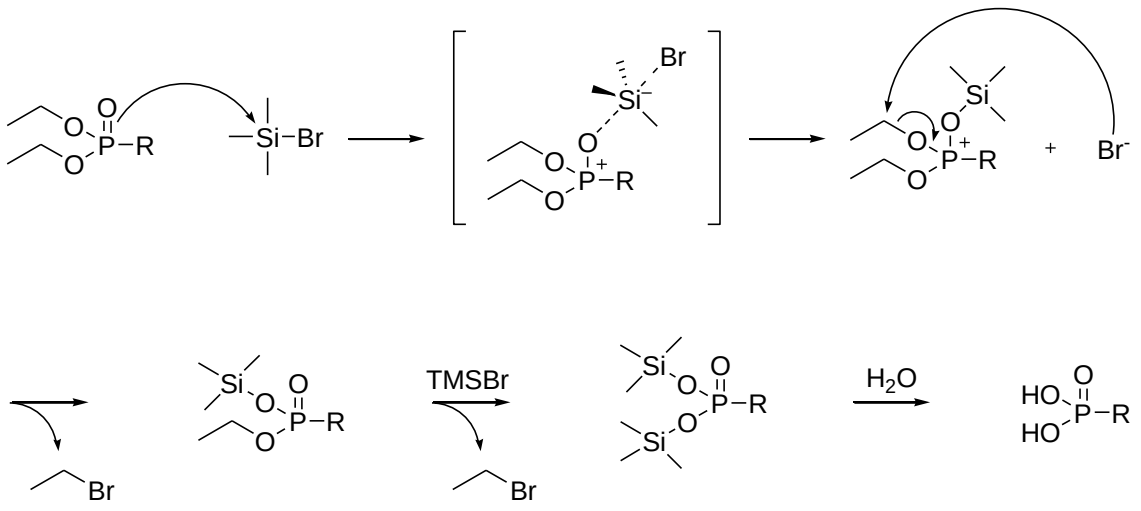


33 **34** **6**

Reagents and Conditions: (a) HCOOH, Pd/C, EtOAc, 1h, rt; (b) TMSBr, CH_2Cl_2

Scheme III.22: Final deprotection of protected α,α-difluoro-FR900098 (**33**)

Deprotection of phosphonate esters with TMSBr, despite being a frequently used technique, still remains a bit of a black box for several reasons. To better understand these, we should first take a closer look on the (postulated) mechanism of TMSBr mediated phosphonate ester deprotection[61] depicted in Scheme III.23. The reaction follows a mechanism similar to that of the Arbuzov reaction for phosphonate synthesis, going via a $\sigma^4\lambda^4$ intermediate: it begins with the attack of an electron pair of the phosphoryl functionality onto TMSBr with expulsion of bromide, forming the $\sigma^4\lambda^4$ phosphonium intermediate. The bromide then attacks one of the esters of the phosphonium intermediate, resulting in an alkyl bromide and restoration of the $\sigma^4\lambda^5$ configuration of the mixed alkyl-silyl phosphonate ester.

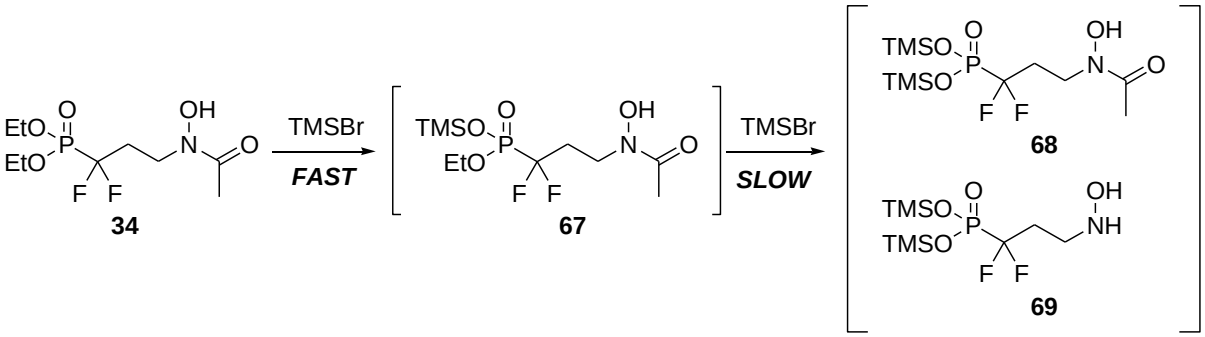


Scheme III.23: Postulated mechanism for the TMSBr-mediated phosphonate de-esterification

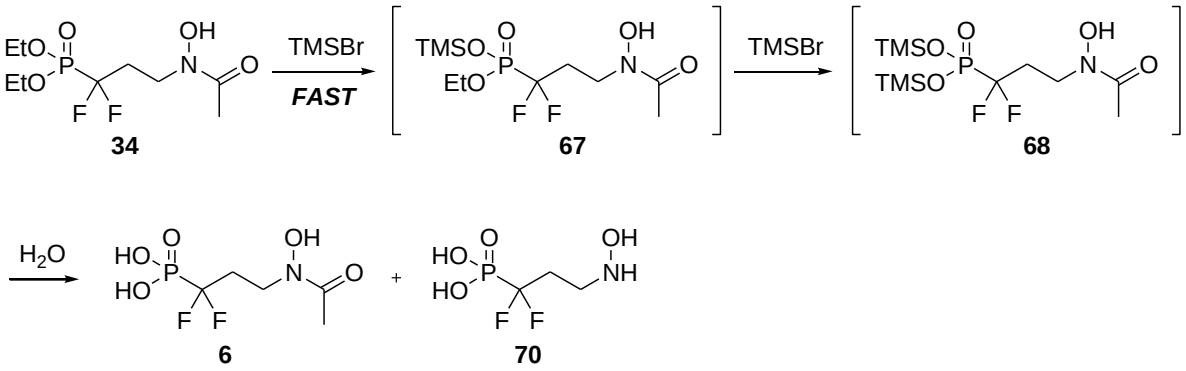
This mechanism is repeated for the second alkyl ester, finally resulting in a bis-silylated phosphonate and two equivalents of alkyl bromide. Generally, all volatiles (excess TMSBr, solvent and alkyl bromide) are then removed *in vacuo* before water is added to hydrolyze the silyl phosphonate. In this manner, a clean and uncontaminated product can be obtained. In practice, however, the monitoring of this reaction poses a few problems. As the introduction of moisture leads to formation of harmful HBr and may prematurely hydrolyze the (mono)silylated phosphonate leading to precipitation from the reaction mixture thus rendering it unavailable for further reaction, reaction sampling should be restricted to a minimum. Moreover, as long as the reaction is not finished, only unstable silyl(hemi)esters are present, which should be hydrolyzed to their corresponding mono-esters or free phosphonates before TLC or MS analysis, thus calling for tedious sample preparation. Good monitoring of the reaction mixture by (LC)MS is further complicated by the fact that the starting material (= protected phosphonate) is best ionized in positive mode, whereas intermediate and final products exclusively ionize in negative mode. Followup by TLC is impractical as the silylether intermediates are not stable enough whereas the free phosphonic acid and the monoester are far too polar for straight-phase chromatography. Again, the absence of any chromophore at all in these molecules as well as their problematic staining hamper their detection via chromatography, both TLC and HPLC. Fortunately, ^{31}P-NMR can be successfully applied to monitor phosphonate deprotections. Because the deprotection of a phosphonate goes via intermediates with a pretty large difference in chemical environment around the phosphorus atom, all these intermediates have a different chemical shift and can usually be easily differentiated in a ^{31}P-NMR spectrum of the reaction mixture. Generally the fully alkylated phosphonate will be most downfield (δ = 5 to 30 ppm) and the deprotected phosphonate most upfield (δ = -10 to 5 ppm) with the intermediates in between these values. Since follow-up of a bigger-scale phosphonate deprotection via sample prelevation poses the same problems of moisture sensitivity, the reaction rate of a particular deprotection can elegantly be estimated by performing a test deprotection in a flame-sealed NMR tube as used for the deprotection of the difluoro-molecule. A small amount of **33** was

dissolved in deuterochloroform in a standard NMR tube, 10 equivalents of TMSBr was added and the tube was flame-sealed. The tube was vortexed regularly to ensure proper mixing of the reaction mixture and the reaction was followed by ^{31}P-NMR ($\delta_{starting\ material}$ = 7 ppm). As we noticed by MS that the first ethyl ester was quickly removed, the peak appearing at δ = -1.5 ppm was attributed to the formation of the mixed ethyl-silylester **67** (Scheme III.24). Knowing the reactivity of the difluorophosphonate and the loss of ethyl groups we encountered during almost all reactions this is not surprising. Opposed to the ease with which the first ester was removed, removal of the second ethyl ester proved problematic[62]. This reaction proceeded so slowly that, before it was finished, a by-product started forming, which was visible as a second triplet on the ^{31}P-NMR spectrum, intertwined with the product triplet around δ = -10 ppm. Judging from its chemical shift that was so close to that of the free phosphonate, this side-product must originate from breakdown remote from the phosphorous atom, probably de-acetylation of the retrohydroxamate (**69**). In an attempt to speed up the (second) ester removal, we decided to use a higher concentration of TMSBr (using a 1:1 mixture of CDCl3 and TMSBr): this time the second ester could be removed before noticeable formation of any side product.

Low TMSBr concentration (10eq.):



High TMSBr concentration (reaction in 1:1 TMSBr - $CDCl_3$):

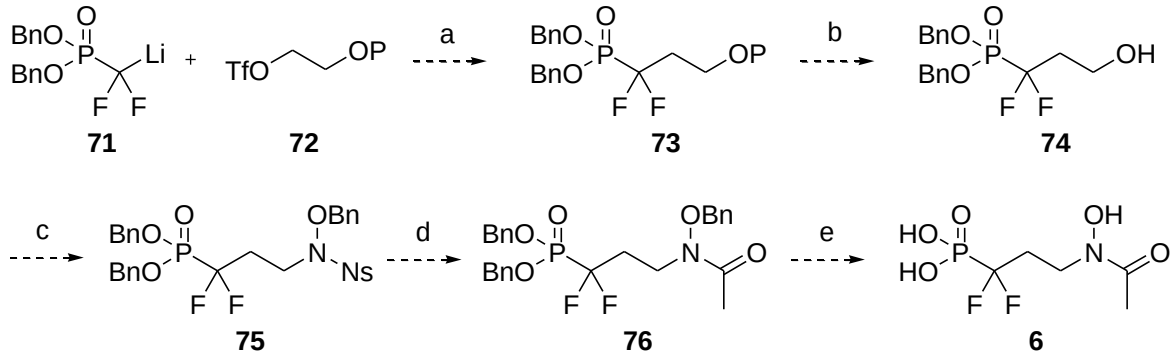


Scheme III.24: Phosphonate deprotection of **34** at low (up) and high (down) TMSBr concentration

Unfortunately, upon workup of the reaction mixture containing bis-TMS intermediate **68** (removal of all volatiles *in vacuo* at 25 °C, followed by hydrolysis of the silyl esters with water) a quick breakdown of the retrohydroxamate (loss of acetyl) was noticed, giving rise to an inseparable mixture of desired product **6** and its *N*-deacetyl analogue **70**. Even upon using ammonium hydroxide to neutralize the acid in a second attempt, with formation of the phosphonate ammonium salt, loss of the *N*-acetyl was noticed. Acid mediated breakdown is thus apparently already taking place during the removal of the reaction volatiles, which indicates the high acid sensitivity of the retrohydroxamate moiety.

III.C.4. Benzylphosphonate approach

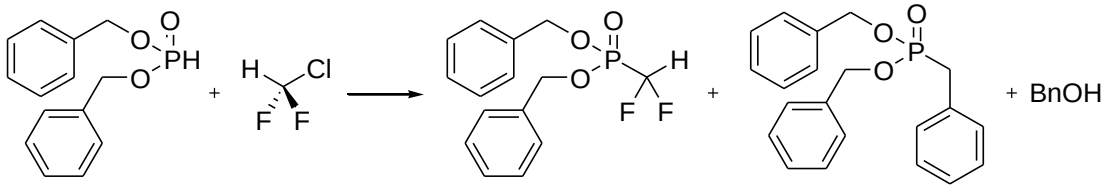
The problems encountered with the workup of the TMSBr-deprotection forced us to try a different protecting group strategy for the phosphonate that would allow for a gentler deprotection, preferably not involving the generation of acid (apart from the phosphonic acid itself). In (fluoroalkyl)phosphonate chemistry, two such groups are commonly used: the di-allylphosphonate and the dibenzylphosphonate[30]. The former is deprotected using catalytic palladium ($Pd(PPh_3)_4$) and a suitable allyl acceptor (e.g. potassium 2-methylhexanoate), whereas the latter can be debenzylated by reduction on palladium metal (Pd black or Pd/C), followed by filtration to remove the catalyst. The latter method is preferred since it allows for an easy deprotection under gentle conditions and an easy workup. Unfortunately, benzyl phosphonate esters are less stable and more prone to nucleophilic attack than their alkyl counterparts. Together with the experienced increased electrophilicity of α,α-difluoromethylphosphonate esters, this could lead to (too) labile protecting groups. Nevertheless, this strategy was tried as a last resort in our attempts to synthesize α,α-difluoro-FR900098 (**6**).



Reagents and Conditions: (a) DMPU, THF, -78 °C; (b) protecting group removal; (c) BnONHNs, DIAD, PPh_3, THF; (d) (i) PhSH, K_2CO_3, MeCN, DMSO, (ii) Ac_2O; (e) H_2, Pd/C, MeOH

Scheme III.25: Anticipated synthetic route toward α,α-difluoro-FR900098 using phosphonate benzyl esters

Essentially, we planned to repeat the triflate displacement strategy onto a suitably protected ethylene glycol triflate **72** analogous to the ethylphosphonate route[28], followed by introduction of the retrohydroxamate by a Mitsunobu reaction with *N*-nosyl,*O*-benzylhydroxylamine as depicted in Scheme III.25. Evidently, 2-benzyloxyethanol could not be used this time due to its incompatibility with the benzyl protection of the phosphonate.Contrary to its ethyl counterpart, dibenzyl difluoromethylphosphonate **79** is not commercially available so it had to be synthesized from chlorodifluoromethane (**78**) and sodium dibenzylphosphite at low temperature[30, 63], resulting in the desired product (**79**) together with an amount of benzyl alcohol (**81**) and dibenzyl benzylphosphonate (**80**) (Scheme III.26).

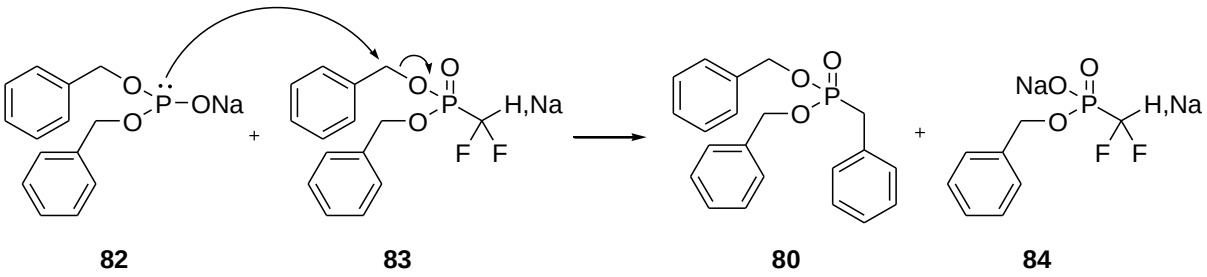


77 **78** **79** (68%) **80** **81**

Reagents and Conditions: NaHMDS, THF, -55 to -40 °C, on.

Scheme III.26: Synthesis of dibenzyl difluoromethylphosphonate

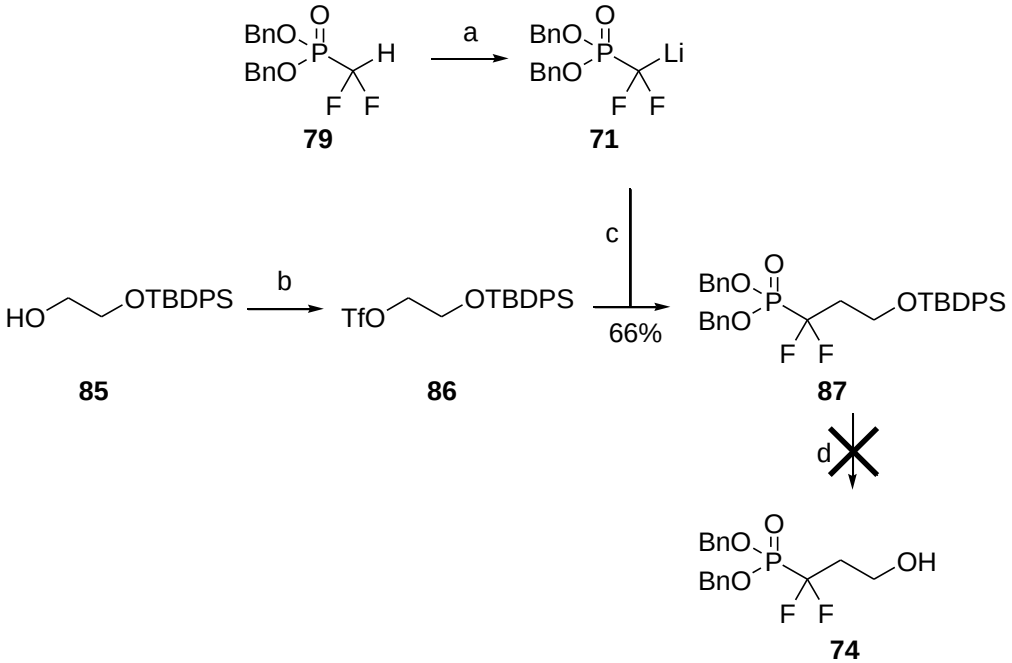
The latter is the result of an attack of deprotonated dibenzylphosphite **82** onto a benzyl ester of the already formed dibenzyl difluoromethylphosphonate **83** (Scheme III.27), illustrating the increased electrophilicity mentioned above.



82 **83** **80** **84**

Scheme III.27: Formation of dibenzyl benzylphosphonate from dibenzylphosphite and a benzylphosphonate

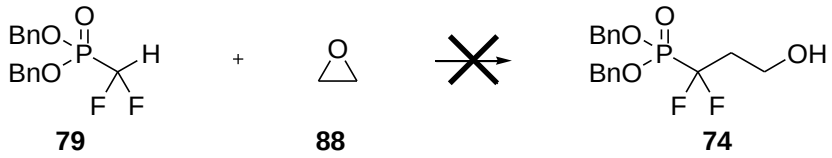
Next, ethyleneglycol was mono-protected with a *tert*-butyldiphenylsilyl-group, triflated and subjected to triflate displacement with lithium dibenzyl difluoromethylphosphonate **71** in DMPU/THF resulting in silylether phosphonate **87** in 66% yield. Unfortunately, all attempts to desilylate ether **87** resulted in extensive breakdown of the benzylphosphonate (Scheme III.28).



Reagents and Conditions: (a) LDA, DMPU, THF, -78 °C; (b) Tf_2O, DIPEA, Et_2O; (c) THF, DMPU, -78 °C; (d) TBAF (1.1 eq.)or HF-pyridine, THF

Scheme III.28: Attempted synthesis of alcohol precursor **74**

In a final attempt we tried to circumvent the need for an alcohol deprotection step (step b in Scheme III.25) by synthesizing alcohol **74** from dibenzyl lithiodifluoromethylphosphonate **71** and ethylene oxide as depicted in Scheme III.29.



79 **88** **74**

Reagents and Conditions: nBuLi (1 eq.), $BF_3.OEt_2$ (1.3 eq.), THF, -78 °C, 3h

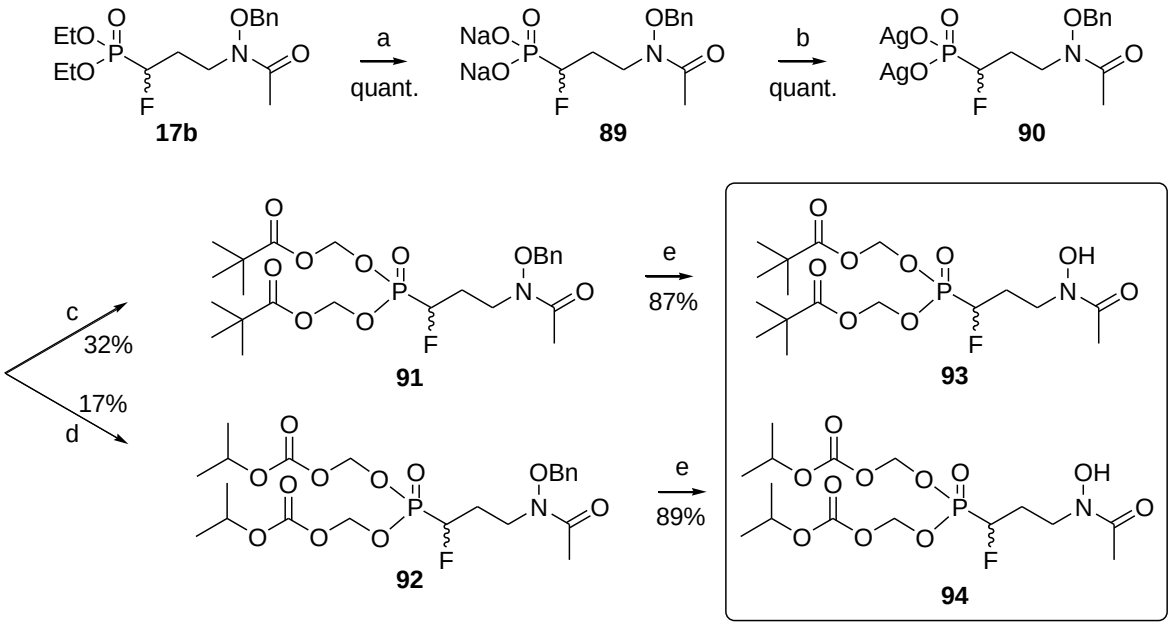
Scheme III.29: Attempted synthesis of alcohol **74** from ethylene oxide

Also this attempt was unsuccessful, yielding mainly benzyl alcohol and phosphonate breakdown products and no desired alcohol **74**.

Apparently, benzyl esters are too weak as protecting groups for this synthesis, and together with the knowledge of the acid sensitivity of the retrohydroxamate combined with a quite strong α,α-difluorophosphonic acid this made us cease our attempts towards α,α-difluoro-FR900098.

III.D. Prodrugs

As mentioned above, the α-halogenated FR900098-analogues showed the expected decreased pKa which should lead to a better affinity for DXR. On the other hand, the increased polarity of these analogues might also hamper their pharmacokinetic properties. Therefore, we decided to synthesize two phosphonate prodrug forms of α-fluoro-FR900098 (**4b**): its bis(pivaloyloxymethyl)-ester (**93**) and its bis(isopropyloxycarbonyloxymethyl)-ester (**94**)(Scheme III.30).



Reagents and conditions: (a) (i) BSTFA, TMSBr, MeCN, (ii) NaOH, H_2O; (b) $AgNO_3$, H_2O; (c) $tBuC(O)OCH_2I$, PhME; (d) $iPrOC(O)OCH_2I$, PhMe; (e) H_2, Pd/C, EtOAc

Scheme III.30: Synthesis of prodrugs of α-fluoro-FR900098

Prodrugs of the acyloxymethyl-phosphonate ester type are generally synthesized in three steps from fully protected fosmidomycin precursors[64-70]: first, the phosphonate is deprotected while maintaining the (retro)hydroxamate protecting benzyl group. Then the free phosphonic acid is treated with an appropriate chloromethyl ester or –carbonate in the presence of a base, often at elevated temperatures and with addition of sodium iodide to improve the yield. Finally, the (retro)hydroxamate is debenzylated, resulting in the desired phosphonate prodrug. When the free

phosphonate of α-fluoro-analogue **17b** was reacted with chloromethyl pivalate (POMCl), the desired protected bis-POM ester **91** was only obtained in 18% yield.

To increase this low yield, two adaptations to the above strategy were made. First, commercial chloromethyl pivalate was transformed into iodomethyl pivalate under Finkelstein conditions[71]. Secondly, after phosphonate deprotection of **17b** the phosphonic acid was neutralized with a stoichiometric amount of sodium hydroxide to convert it into its disodium salt **89**. This disodium salt was then converted into the disilver salt **90** by aqueous metathesis with a silver nitrate solution. Reaction of **90** with iodomethyl pivalate in toluene gave the protected bis-POM analogue **91** in 32% yield.

For the synthesis of carbonate **92**, chloromethyl isopropyl carbonate was first synthesized according to a literature procedure[72] and then transformed into iodomethyl isopropyl carbonate under Finkelstein conditions[71]. Reaction of disilver phosphonate **90** with iodomethyl isopropyl carbonate in toluene gave bis-carbonate analogue **92** in 17% yield. Both protected hydroxamates were then debenzylated in good yields, resulting in α-fluoro prodrugs **93** and **94**.

III.E. Biological Results

The three α-halogenated phosphonic acids **4a**, **4b** and **5** were tested for their capacity to inhibit EcDXR using a spectrophotometric assay i.e. by monitoring the decrease in NADPH absorption at 340 nm. EcDXR inhibition caused by these analogues was compared with fosmidomycin, FR900098 and α-(3,4-dichlorophenyl)fosmidomycin, all incubated at 100nM (Table III.1). Both α-fluorinated analogues **4b** and **5** outperformed the three reference compounds, whereas the α-chloro analogue **4a** exhibited stronger EcDXR inhibition than both fosmidomycin and α-(3,4-dichlorophenyl) fosmidomycin, but slightly weaker than FR900098.

The free phosphonic acids and the prodrugs **93** and **94** were also tested in duplicate for their inhibitory effect against intraerythrocytic forms of *P. falciparum* (strains GHA and K1) using a microdilution assay[73]. Phosphonic acids **4a**, **4b** and **5** showed submicromolar activity on both strains and appeared to be 5- to 6-fold more active than fosmidomycin and slightly superior to FR900098 on the K1 strain. More spectacular, though, is the submicromolar activity displayed by both prodrugs of α-fluoro FR900098. Remarkably, the bis-carbonate based prodrug (**94**) was twice as active as the more 'classic' bis-POM prodrug (**93**) . To the best of our knowledge, these are the lowest IC_{50} values for a fosmidomycin based DXR inhibitor encountered so far (the former being Behrendt's α-aryl reversed hydroxamates[74]), and further *in vivo* testing for these compounds is warranted. All α-halogenated compounds and their prodrugs were also tested for their cytotoxicity against MRC-5 SV2 cells, human fetal lung fibroblast, where they showed IC_{50} values of more than 64 μM. Furthermore, the title compounds were screened for their activity against *T. cruzei, L. infantum, T. brucei, T. rhodesiense, E. coli, S. aureus, C. albicans, T. rubrum* and *A. fumigatus*. No activity (IC_{50} >64 μM) was seen for all analogues against any of these organisms, confirming their selectivity for *Plasmodium* species.

Chart III.1: EcDXR inhibition of α-halogenated FR900098 analogues incubated at 100 nM

(α-(Cl_2Ph)-Fos = α-(3,4-dichlorophenyl) fosmidomycin)

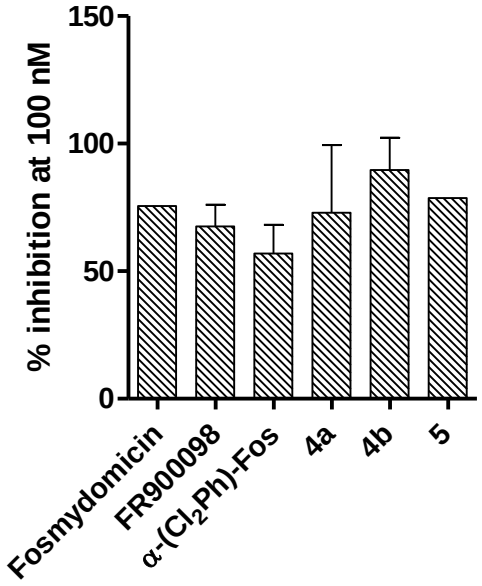


Table III.1: *In vitro* EcDXR inhibition and *P. falciparum* growth inhibition by α-halogenated FR900098 analogues and their prodrugs.

Compound	EcDXR Enzyme Inhibition			*In vitro* *P. falciparum* growth inhibition (IC_{50} in µM)	
	% inhibition at 100 nM	K_i (nM)	IC_{50} (nM)	*Pf* GHA	*Pf* K1
Fosmidomycin	75.57	-	-	-	1.73
FR900098	67.60	15.11	30.23	-	0.42
α-(3,4-dichlorophenyl) fosmidomycin	56.92	-	-	0.60	0.16
4a	72.94	14.2	28.3	0.82	0.30
4b	89.75	11.3	22.7	0.70	0.29
5	78.73	-	-	0.73	0.31
93	-	-	-	-	0.07
94	-	-	-	-	0.03

The reference α-(3,4-dichlorophenyl)fosmidomycin and the fluorinated analogues **4b** and **5** were also evaluated *in vivo* in the *P. berghei* (GFP ANKAstrain) acute mouse model after intraperitoneal dosing at 50 mg/kg for 5 consecutive days. Chloroquine (10 mg/kg for 5 days) was included as reference treatment. The animals were observed for the occurrence/presence of clinical or adverse effects during the course of the experiment. In case of very severe clinical signs, either due to toxicity or malaria, animals were euthanized for welfare reasons. Parasitemia was determined on days 4, 7, and 14 on surviving animals using flow cytometry (10 µL of blood in 2000 µL of PBS). Percentage reduction of parasitemia compared to vehicle-treated infected controls is used as a measure for drug activity, and the mean survival time (MST) was calculated (Table III.2). Strangely enough, α-(3,4-dichlorophenyl)fosmidomycin did not show any relevant activity. On the other hand, **5** resulted in 85% suppression of parasitemia at 4 dpi, which dropped to 42% at 7 dpi and 41% at 14 dpi. The mean survival time was 11.7 days. Compound **4b** resulted in 88% suppression of parasitemia at 4 dpi, which after ending the treatment also dropped to 62% at 7 dpi and 32% at 14 dpi. The overall MST was 15.8 days.

Table III.2: In vivo testing of α-fluoro FR900098 analogues: Survivors and Mean Survival Time (MST in Days) in the *P. berghei* (GFP ANKA Strain) Acute Mouse Model

Treatment (ip for 5 consecutive days)	Parasitemia suppression (day 4)	Survivors day 7	Survivors day 11	Survivors day 14	Survivors day 25	MST
Vehicle	0	1/6	1/6	1/6	0/6	8.5
Chloroquine (10 mg/kg)	100	6/6	6/6	6/6	3/6	20.7
Fosmidomycin (50 mg/kg)	82	6/6	4/6	3/6	nd	11.5
FR900098 (50 mg/kg)	93	6/6	3/6	2/6	nd	10.8
α-(3,4-dichlorophenyl) fosmidomycin (50 mg/kg)	46	4/5	0/5	0/5	0/5	7.0
4b (50 mg/kg)	88	4/4	2/4	2/4	0/4	15.8
5 (50 mg/kg)	85	2/3	2/3	1/3	0/3	11.7

These data clearly demonstrate that the three synthesized phosphonic acids have promising *in vitro* activity and that **4b** and **5** surpass the antimalarial activity of FR900098 *in vivo*. Both prodrugs **93** and **94** evoke high expectations and *in vivo* testing of these compounds is awaited.

III.F. Experimental Details

General Methods and Materials. ^{1}H, ^{13}C, ^{19}F and ^{31}PNMR spectra were recorded in $CDCl_3$, acetone-d_6, DMSO-d_6 or D_2O on a Varian Mercury 300 spectrometer. Chemical shifts are given in parts per million (ppm) (δ relative to residual solvent peak for ^{1}H and ^{13}C and to external D_3PO_4 for ^{31}P). All solvents and chemicals were used as purchased unless otherwise stated. Purity of the final compounds was deduced from clean ^{1}H, ^{13}C and ^{31}P NMR spectra, high resolution mass spectra and assessed by LC-DAD-MS. Reversed phase chromatograms were recorded on a Phenomenex Luna C-18 2.5 µm particle (100 x 2.00 mm) column in a Waters Alliance 2695 XE HPLC system spectrometer with quaternary pump, DAD detector and coupled to a Waters LCT Premier XE orthogonal time-of-flight spectrometer with API-ES source. High resolution mass spectroscopy spectra for all compounds were also recorded on a Waters LCT Premier XE orthogonal time-of-flight spectrometer with API-ES source.

Spectrophotometric DXR inhibition assay. This assay was performed by the Unit of theoretical and structural physico-chemistry, Department of Chemistry, University of Namur (FUNDP).

***In vitro P. falciparum* growth inhibition assay and microbial screening.** Performed by the Laboratory for Microbiology, Parasitology and Hygiene, Faculty of Pharmaceutical, Biomedical and Veterinary Sciences, University of Antwerp. Two strains of P.falciparum are used: 1/ GHA strain (P. falciparum GHA), derived from a Ghanese patient and chloroquine sensitive and 2/ K1 strain (P.falciparum K1) resistant to chloroquine. The strains are maintained in RPMI-1640 medium supplemented with 0.37 mM hypoxanthine, 25 mM Hepes, 25 mM NaHC03, and 10% O+ human serum together with 2-4% washed human O+ erythrocytes. All cultures and assays are conducted under an atmosphere of 4% C02, 3% 02 and 93% N2. Assays are performed in 96-well microtiter plates, each well containing 10 µl of the watery compound dilutions together with 190 µl of the malaria parasite inoculum (1% parasitaemia, 2% HCT). After 72h incubation, plates are frozen and

stored at –20°C. After thawing, 20 µl of each well is transferred into another plate together with 100 µl Malstat reagent and 20 µl of a 1/1 mixture of PES (phenazine ethosulfate, 0.1 mg/ml) and NBT (Nitro Blue Tetrazolium Grade III, 2 mg/ml). Change in colour is measured spectrophotometrically at 655 nm. Biological screening tests: The integrated panel of microbial screens used in this study and the standard screening methodologies were adopted from those described by Cos et al.

Cytotoxicity. Performed by the Laboratory for Microbiology, Parasitology and Hygiene, Faculty of Pharmaceutical, Biomedical and Veterinary Sciences, University of Antwerp. MRC-5_{SV2} cells are cultured in Earl's MEM + 5% FCSi. Assays are performed in 96-well microtiter plates, each well containing about 10^4 cells/well. After 3 days incubation, cell viability is assessed fluorimetrically after addition of resazurin and fluorescence is measured (l_{ex} 550 nm, l_{em} 590 nm). The results are expressed as % reduction in cell growth/viability compared to untreated control wells and a CC_{50} is determined. Cytotoxic reference compounds include tamoxifen.

Diethyl 1-chlorobut-3-enylphosphonate (11a). To a solution of diisopropylamine (4.42 mL, 31 mmol) in dry THF (90 mL) was added nBuLi (20 mL of a 1.5 M solution in THF) while stirring at -80 °C. After 15 min, diethyl but-3-enylphosphonate (2.89 g, 15 mmol) was slowly added to this solution, maintaining the temperature at -80 °C. Stirring was continued for 10 min at this temperature and then TMSCl (2.11 mL, 16.5 mmol) was added. The mixture was allowed to warm slowly to 0 °C, cooled again to -80 °C, and hexacloroethane (3.91 g, 16 mmol) in THF (60 mL) was added. The reaction mixture was allowed to warm to room temperature again and quenched by addition of aqueous hydrochloric acid. The mixture was poured into 2 M hydrochloric acid and ice and extracted with dichloromethane. The combined organic layers were washed with brine, dried over anhydrous $MgSO_4$ and the solvents were removed by rotoevaporation. The residue was then dissolved in THF (150 mL) and acetic acid (50 mL) was added, followed by TBAF in THF (20 mL of a 1 M solution). After 7 hours of stirring at room temperature, ^{31}P NMR revealed that the reaction was complete (shift from δ 24.39 to 19.89 ppm) and an aqueous solution of $NaHCO_3$ was added. The mixture was extracted

three times with dichloromethane, the combined organic layers were shaken against brine and dried over $MgSO_4$ and the solvent was removed *in vacuo*. Purification by column chromatography (hexanes/ethyl acetate: 25/75) gave 3.01g of the title compound as a light yellow oil (89%). ^{1}H-NMR (300.01 MHz, $CDCl_3$) δ 1.36 (6H, t, J = 7.2 Hz), 2.52-2.63 (1H, m), 2.79-2.87 (1H, m), 3.87 (1H, dt, J = 10.8 and 3.0 Hz), 4.22 (4H, dq, app. sx, J = 7.3 Hz), 5.17 (2H, t, J = 8.6 Hz), 5.81-5.94 (1H, m);^{13}C NMR (75 MHz, $CDCl_3$) δ 16.6 (CH_3, $^{3}J_{PC}$ = 2.8 Hz), 16.7 (CH_3, $^{3}J_{PC}$ = 2.8 Hz), 36.8 (CH_2), 51.6 (CHCl, $^{1}J_{PC}$ = 159.6 Hz), 63.7 (OCH_2, $^{2}J_{PC}$ = 6.9 Hz), 64.0 (OCH_2, $^{2}J_{PC}$ = 6.8 Hz), 118.9 (=CH_2), 133.2 (=CH, $^{3}J_{PC}$= 13.6 Hz); ^{31}P NMR (121.45 MHz, $CDCl_3$) δ 19.86; HRMS (ESI) *m/z* 227,0601 [$(M+H)^+$, calcd for $C_8H_{17}ClO_3P^+$ 227.0598].

Diethyl 1-fluorobut-3-enylphosphonate (11b). To a solution of diisopropylamine (5.05 mL, 36 mmol) in dry THF (60 mL) was added nBuLi (23 mL of a 1.5 M solution in THF) while stirring at -80 °C. After 15 min , diethyl but-3-enylphosphonate (2.88 g, 15 mmol) was slowly added to this solution, maintaining the temperature at -80 °C. Stirring was continued for 10 min at this temperature and then TMSCl (2.11 mL, 17 mmol) was added. The mixture was allowed to warm slowly to 0 °C, cooled again to -80 °C, and *N*-fluorobenzenesulfonimide (5.20 g, 16 mmol) in THF (60mL) was added. The reaction mixture was allowed to warm to room temperature again and quenched by addition of aqueous hydrochloric acid. The mixture was poured into 2 M hydrochloric acid and ice and extracted with dichloromethane. The combined organic layers were washed with saturated aqueous sodium bicarbonate, brine, dried over anhydrous $MgSO_4$ and the solvents were removed by rotoevaporation. The residue was then dissolved in dichloromethane (20 mL), hexane (200 mL) was added and the white precipitate was filtered off over a celite filter. The filtrate was evaporated *in vacuo* and the residue redissolved in THF (50 mL), followed by the addition of a 1 M solution of lithium ethoxide in ethanol (20 mL). After stirring at 0 °C for 30 min the reaction mixture was diluted with dichloromethane and washed with 2 M hydrochloric acid. The organic layer was shaken with brine, dried over anhydrous $MgSO_4$ and the solvents were removed *in vacuo*. Flash

column chromatography (hexanes/ethyl acetate: 25/75) gave 2.62 g (83%) of **11b** as a colorless liquid. ^{1}H NMR (300.01 MHz, $CDCl_3$) δ 1.35 (6H, t, *J* = 7.2 Hz), 2.56-2.74 (2H, m), 4.14-4.26, (4H, m), 4.63-4.84, (1H, m), 5.15-5.23, (2H, m), 5.80-5.93, (1H, m); ^{13}C NMR (75.44MHz, $CDCl_3$): δ 16.5 (CH_3, $^{3}J_{PC}$ = 2.5 Hz), 16.6 (CH_3, $^{3}J_{PC}$ = 2.5 Hz), 34.7 (CH_2, $^{2}J_{PC}$ =1.7 Hz, $^{2}J_{FC}$ = 20.5 Hz), 63.0 (OCH_2, $^{2}J_{PC}$ = 6.6 Hz), 63.4 (OCH_2, $^{2}J_{PC}$ = 6.9 Hz), 88.2 (CHF, $^{1}J_{PC}$ = 170.0 Hz, $^{1}J_{CF}$ = 182.2 Hz); 118.8 ($=CH_2$), 132.2 (=CH, $^{3}J_{CF}$ = 13.8 Hz, $^{3}J_{CP}$= 3.9 Hz); ^{31}P NMR (121.45 MHz, $CDCl_3$) δ 17.41 ($^{2}J_{PF}$ = 74.8 Hz); HRMS (ESI) *m/z* 211.0900 [$(M+H)^+$, calcd for $C_8H_{17}FO_3P^+$ 211.0899].

Diethyl 1-chloro-3-hydroxypropylphosphonate (13a). To a solution of **11a** (2.27 g, 10 mmol) and 4-methylmorpholine-*N*-oxide (1.41 g, 12 mmol) in dioxane (80 mL) was added 3.8 mL of a 4% aqueous solution of OsO_4. After 4 hours of stirring at room temperature and protected from light, the reaction was complete according to TLC. The reaction mixture was quenched by addition of an aqueous solution of $Na_2S_2O_3$ and extracted thrice with EtOAc. The combined organic fractions were washed with brine and dried over Na_2SO_4 after which the solvent was removed *in vacuo*. The resulting oil was dissolved in THF (25 mL) and sodium periodate (4.28 g, 20 mmol) in water (25 mL) was added. After 3 hours the reaction mixture was diluted with EtOAc and filtered over a celite pad. The filtrate was washed with brine, dried over Na_2SO_4 and evaporated to give crude diethyl 1-chloro-2-formylethylphosphonate **12a**. This residue was dissolved in ethanol (80 mL) and sodium borohydride (1.21 g, 32 mmol) was added. The mixture was stirred overnight at room temperature, diluted with EtOAc and washed with water. The aqueous layer was extracted twice with EtOAc. The combined organic phases were washed with brine and dried over Na_2SO_4. Removal of the solvent followed by column chromatography (hexanes/acetone: 25/75) yielded 1.11 g of the title compound as a colorless oil (48%). ^{1}H NMR (300.01 MHz, $CDCl_3$) δ 1.37 (6H, t, *J* = 7.2 Hz), 2.02-2.38, (2H, m), 2.43-2.46 (1H, m), 3.76-3.97 (2H, m), 4.11-4.3051 (5H, m); ^{31}P NMR (121.45MHz, $CDCl_3$) δ 21.24; HRMS (ESI) *m/z* 231.0540 [$(M+H)^+$, calcd for $C_7H_{17}ClO_4P^+$ 231.0547].

Diethyl 1-fluoro-3-hydroxypropylphosphonate (13b). To a solution of diethyl 1-fluorobut-3-enylphosphonate (**11b**) (918mg, 4.37 mmol) and 4-methylmorpholine-*N*-oxide (614 mg, 5.24 mmol) in dioxane (40 mL) was added 1.67 mL of a 4% aqueous solution of OsO_4. After 4 hours of stirring at room temperature and protected from light, the reaction was complete according to TLC. The reaction mixture was quenched by addition of an aqueous solution of $Na_2S_2O_3$ and extracted thrice with EtOAc. The combined organic fractions were washed with brine and dried over Na_2SO_4 after which the solvent was removed *in vacuo*. The resulting oil was dissolved in THF (15 mL) and sodium periodate (1.50 g, 6.99 mmol) in water (10 mL) was added. After 3 hours the reaction mixture was diluted with EtOAc and filtered over a celite pad. The filtrate was washed with brine, dried over Na_2SO_4 and evaporated to give crude diethyl 1-fluoro-2-formylethylphosphonate (**12b**). This was dissolved in ethanol (45 mL) and sodium borohydride (653 mg, 17 mmol) was added. The mixture was stirred overnight at room temperature, diluted with EtOAc and washed with water. The aqueous layer was extracted twice with EtOAc. The combined organic phases were washed with brine and dried over Na_2SO_4. Removal of the solvent followed by column chromatography (hexanes/acetone: 25/75) yielded 421 mg of diethyl 1-fluoro 3-hydroxypropylphosphonate (**13b**) as a colorless oil (45%). ^{1}H NMR (300.01 MHz, $CDCl_3$) δ 1.38 (6H, t, *J* = 7.0 Hz), 2.05-2.24 (2H, m), 2.71 (1H, br.s), 3.77-3.85 (2H, m), 4.21 (4H, dq, app. sx, *J* = 7.0 Hz), 4.96 (1H, dddd, *J* = 3.2, 5.6, 9.1 and 46.3 Hz); ^{13}C NMR (75.44 MHz, $CDCl_3$) δ 16.6 (CH_3, $^3J_{PC}$ = 3.0 Hz), 16.7 (CH_3, $^3J_{PC}$ = 3.0 Hz), 33.5 (CH_2, $^2J_{CF}$ = 19.6 Hz), 57.8 (CH_2, $^3J_{CF}$ = 5.0 Hz, $^3J_{PC}$ = 11.1 Hz), 63.4 (CH_2, $^3J_{CF}$ = 6.9 Hz, $^2J_{PC}$ = 34.0 Hz), 86.4 (CHF, $^1J_{CF}$ = 179.4 Hz, $^1J_{PC}$ = 171.4 Hz); ^{31}P NMR (121.43 MHz, $CDCl_3$) δ 18.63 (d, $^2J_{PF}$ = 74.7 Hz); HRMS (ESI) m/z 215.0845 [$(M+H)^+$, calcd for $C_7H_{17}FO_4P^+$ 215,0843].

3-(Ethoxyphosphono)-3-chloropropyl 4-methylbenzenesulfonate (14a). To a solution of **13a** (1.05 g, 4.6 mmol) in dichloromethane (9 mL) was added Et_3N (1.91 mL, 1.4 mmol), DMAP (167 mg, 0.14 mmol) and TsCl (1.3 g, 6.8 mmol). After one hour of stirring at room temperature the reaction was quenched with saturated aqueous NH_4Cl and extracted thrice with dichloromethane. The

combined organic phases were washed with brine and dried over anhydrous $MgSO_4$. Purification by flash column chromatography over two consecutive columns (dichloromethane/acetone: 9/1 then hexanes/dichloromethane/acetone: 6/2/2) yielded 1.64 g (90%) of a colorless oil. ^{1}H NMR (300.01 MHz, $CDCl_3$) δ 1.33 (6H, t, *J* = 7.0 Hz), 1.98-2.54 (2H, m), 2.44 (3H, s), 3.95 (1H, dt, *J* = 11.4 and 3.2 Hz), 4.13 – 4.27 (6H, m), 7.35 (2H, d, *J* = 8.4 Hz), 7.79 (2H, d, *J* = 8.1 Hz); ^{13}C NMR (75.44 MHz, $CDCl_3$): δ 16.5 (CH_3, $^3J_{PC}$ = 2.2 Hz), 16.6 (CH_3, $^3J_{PC}$ = 1.9 Hz), 21.8 (CH_3), 32.0 (CH_2), 47.9 (P-CHCl, $^1J_{PC}$ = 162.9 Hz), 63.7 (OCH_2, $^2J_{PC}$ = 6.9 Hz), 64.2 (OCH_2, $^2J_{PC}$ = 6.9 Hz), 66.0 (CH_2, $^3J_{PC}$ = 13.6 Hz); 128.1 (=CH_2), 130.1 (=CH_2), 132.7 (=CH_2), 145.2 (=CH_2); ^{31}P NMR (121.45 MHz, $CDCl_3$) δ 19.21; HRMS (ESI) *m/z* 385.0687 [$(M+H)^+$, calcd for $C_{14}H_{23}ClO_6PS^+$ 385.0636].

3-(Ethoxyphosphono)-3-fluoropropyl 4-methylbenzenesulfonate (14b). To a solution of diethyl 1-fluoro 3-hydroxypropylphosphonate (**13b**) (420 mg, 2.0 mmol) in dichloromethane (4 mL) was added Et_3N (0.82 mL, 5.9 mmol), DMAP (72mg, 0.59 mmol) and TsCl (561 mg, 2.9 mmol). After one hour of stirring at room temperature the reaction was quenched with saturated aqueous NH_4Cl and extracted thrice with dichloromethane. The combined organic phases were washed with brine and dried over $MgSO_4$. Purification by flash column chromatography (hexanes/dichloromethane/acetone: 6/2/2) yielded 720 mg (99%) of a colorless oil. ^{1}H NMR (300.01 MHz, $CDCl_3$) δ 1.33 (6H, 2t, *J* = 7.0 Hz), 2.14-2.33 (2H, m), 2.44 (3H, s), 4.11-4.24 (2 + 4H, m), 4.79 (1H, dddd, *J* = 3.9, 8.1, 14.1 and 47.1 Hz), 7.28-7.36 (2H, m), 7.77-7.81 (2H, m);^{13}C NMR (75.44 MHz, $CDCl_3$): δ 16.5 (CH_3), 16.6 (CH_3), 21.8 (CH_3), 30.2 (CH_2, $^2J_{CF}$ = 20.2 Hz, $^2J_{PC}$ = 0.6 Hz), 63.4 (OCH_2, $^4J_{CF}$ = 6.9 Hz, $^2J_{PC}$ = 27.9 Hz), 65.3 (SCH_2, $^3J_{CF}$ = 4.4 Hz, $^3J_{PC}$ = 14.1 Hz), 84.6 (CHF, $^1J_{CF}$ = 173.6 Hz, $^1J_{PC}$ = 180.8 Hz), 128.1 (=CH), 130.1 (=CH), 132.8 (=C_q), 145.2 (=C_q); ^{31}P NMR (121.45MHz, $CDCl_3$) δ 16.75 (d, $^2J_{PF}$ = 49.9 Hz); HRMS (ESI) m/z 369.0946 [$(M+H)^+$, calcd for $C_{14}H_{23}FO_6PS^+$ 369,0932].

***tert*-Butyl *N*-benzyloxy(3-(ethoxyphosphono)-3-chloropropyl)carbamate (15a).** To a solution of *t*-butyl *N*-benzyloxycarbamate (1.04 g, 4.7 mmol) in dry DMF (20 mL) cooled to 0 °C was added sodium hydride (186 mg of a 60% dispersion in mineral oil; 4.7 mmol) while stirring. After a few

minutes the ice bath was removed and the solution stirred for another half hour. Then, **14a** (1.63 g, 4.2 mmol) dissolved in 20 mL dry DMF was added and the mixture was heated to 50 °C. After 3 hours the mixture was allowed to cool down, quenched by addition of aqueous NH_4Cl and extracted three times with dichloromethane. The combined organic fractions were washed with brine and dried over $MgSO_4$. Purification of the crude product by flash column chromatography (hexanes/ethyl acetate: 50/50) yielded 1.22 g of **15a** as a colorless oil (66%). ^{1}H NMR (300.01 MHz, $CDCl_3$) δ 1.33 (6H, 2t, *J* = 7.3 Hz), 1.51 (9H, s), 1.96-2.50 (2H, m), 3.65-3.70 (2H, m), 3.97 (1H, dt, *J* = 11.1 and 2.9 Hz), 4.84 (2H, ABq, *J* = 9.7 Hz), 7.33-7.42 (5H, m); ^{13}C NMR (75.44 MHz, $CDCl_3$): δ 16.5 (CH_3, $^3J_{PC}$ = 2.5 Hz), 16.6 (CH_3, $^3J_{PC}$ = 2.5 Hz), 28.4 (3 x CH_3), 30.3 (CH_2), 46.8 (CH_2, $^3J_{PC}$ = 14.4 Hz), 50.0 (CClH, $^1J_{PC}$ = 161.7 Hz), 63.6 (OCH_2, $^2J_{PC}$ = 6.9 Hz), 63.9 (OCH_2, $^2J_{PC}$ = 7.2 Hz), 77.2 (CH_2), 81.9 ($C(CH_3)_3$), 128.6 ($=CH_2$), 128.8 ($=CH_2$), 129.6 ($=CH_2$), 135.5 ($CH_2\underline{C}(CH)_2$), 156.5 (C=O); ^{31}P NMR (121.45 MHz, $CDCl_3$) δ 19.89; HRMS (ESI) *m/z* 436.1645 [$(M+H)^+$, calcd for $C_{19}H_{32}ClNO_6P^+$ 436.1650].

***tert*-Butyl *N*-benzyloxy(3-(ethoxyphosphono)-3-fluoropropyl)carbamate (15b).** To a solution of *t*-butyl *N*-benzyloxycarbamate (473 mg, 2.1 mmol) in dry DMF (10mL) cooled to 0 °C was added sodium hydride (85 mg of a 60% dispersion in mineral oil, 2.1 mmol) while stirring. After a few minutes the icebath was removed and the solution stirred for another half hour. Then, 3-(ethoxyphosphono) 3-fluoropropyl 4-methylbenzenesulfonate (**14b**) (710 mg, 1.9 mmol) dissolved in 10 mL dry DMF was added and the mixture was heated to 50 °C. After 2.5 hours the mixture was allowed to cool down, quenched by addition of aqueous NH_4Cl and extracted three times with dichloromethane. The combined organic fractions were washed with brine and dried over $MgSO_4$. Purification of the crude product by flash column chromatography (hexanes/ethyl acetate: 50/50) yielded 711 mg of a colorless oil (88%).^{1}H NMR (300.01 MHz, $CDCl_3$) δ 1.34 (6H, 2t, *J* = 7.2 Hz), 1.50 (9H, s), 2.08-2.32 (2H, m), 3.63 (2H, t, *J* = 6.9 Hz), 4.18 (4H, dq, app. sx, *J* = 7.2 and 6.9 Hz), 4.80 (1H, ddt, *J* = 46.8, 13.5 and 3.9 Hz), 4.86 (2H, ABq, *J* = 10.2 Hz), 7.29-7.42 (5H, m);^{13}C NMR (75.44 MHz, $CDCl_3$): δ 16.6 (CH_3, $^3J_{PC}$ = 2.8 Hz), 16.7 (CH_3, $^3J_{PC}$ = 2.8 Hz), 28.5 (3 x CH_3), 45.9 (CH_2, $^3J_{CF}$ = 15.2 Hz, $^3J_{PC}$

= 3.6 Hz), 63.3 (OCH_2, $^4J_{CF}$ = 6.9 Hz, $^2J_{PC}$ = 23.8 Hz), 77.4 (OCH_2), 82.0 (C_q), 86.8 (CHF, $^1J_{CF}$ = 180.0 Hz, $^1J_{PC}$ = 171.9 Hz), 128.7 (=CH), 128.8 (=CH), 129.6 (=CH), 135.6 (C_q), 156.6 (C=O); ^{31}P NMR (121.45 MHz, $CDCl_3$) δ 17.45 (d, $^2J_{PF}$ = 74.8 Hz); HRMS (ESI) m/z 420.1904 [$(M+H)^+$, calcd for $C_{19}H_{32}FNO_6P^+$ 420.1946].

Diethyl 3-(*N*-(benzyloxy)acetamido)-1-chloropropylphosphonate (17a). 15a (1.2 g; 2.8 mmol) was dissolved in dichloromethane (27 mL), cooled to 0 °C and TFA (15 mL) was added. After stirring for 30 minutes, the ice bath was removed and 15 minutes later the reaction was complete according to TLC. Toluene (15 mL) was added to the mixture and the solvents were removed *in vacuo*. When the product approached dryness, another 7 mL of toluene was added and the solution rotoevaporated again. The residue was then redissolved in dry dichloromethane (20 mL) and acetic anhydride (1.75 mL; 18 mmol), Et_3N (0.8 mL; 5.5 mmol) and DMAP (67 mg; 0.55 mmol) were added. After 4 hours of stirring at room temperature, the reaction was quenched with 0.1 M HCl and extracted with dichloromethane. The combined organic fractions were washed with saturated aqueous sodium bicarbonate and dried over anhydrous Na_2SO_4. Flash column chromatography (dichloromethane/acetone: 8/2) yielded 669 mg of **17a** as a colorless oil (98%). ^{1}H NMR (300.01 MHz, $CDCl_3$) δ 1.31-1.36 (6H, 2t, *J* = 7.3 Hz), 2.02-2.54 (2H, m), 2.10 (3H, s), 3.89 (2H, t, *J* = 6.5 Hz), 3.96 (1H, dt, *J* = 11.1 and 3.2 Hz), 4.14-4.26 (m, 4H), 4.83 (2H, ABq, *J* = 10.3 Hz), 7.39 (5H, s); ^{13}C NMR (75.44 MHz, $CDCl_3$): δ 16.6 (CH_3, $^3J_{PC}$ = 2.2 Hz), 16.6 (CH_3, $^3J_{PC}$ = 2.2 Hz), 20.7 (CH_3), 30.1 (CH_2), 42.5 (CH_2), 50.1 (CClH, $^1J_{PC}$ = 162.0 Hz), 63.7 (OCH_2, $^2J_{PC}$ = 6.9 Hz), 64.0 (OCH_2, $^2J_{PC}$ = 6.9 Hz), 77.4 (CH_2), 128.9 (=CH_2), 129.3 (=CH_2), 129.5 (=CH_2), 134.3 (C=O); ^{31}P NMR (121.45 MHz, $CDCl_3$) δ 19.58; HRMS (ESI) *m/z* 378.1207 [$(M+H)^+$, calcd for $C_{16}H_{26}ClNO_5P^+$ 378.1232].

Diethyl 3-(*N*-(benzyloxy)acetamido)-1-fluoropropylphosphonate (17b). 15b (700 mg, 1.7 mmol) was dissolved in dichloromethane (20 mL), cooled to 0 °C and TFA (10 mL) was added. After stirring for 30 minutes, the icebath was removed and 20 minutes later the reaction was done, according to TLC. Toluene (15mL) was added to the mixture and the solvents were removed *in vacuo*. When the

product approached dryness, another 5 mL of toluene was added and the solution rotoevaporated again. The residue was then redissolved in dry dichloromethane (14 mL) and acetic anhydride (1.3 mL, 14 mmol), Et_3N (0.58 mL, 4.2 mmol) and DMAP (51 mg, 0.42 mmol) were added. After 2 hours of stirring at room temperature, the reaction was quenched with 0,1 M HCl and extracted with dichloromethane. The combined organic fractions were washed with saturated aqueous $NaHCO_3$ and dried over anhydrous Na_2SO_4. Flash column chromatography (dichloromethane/acetone: 8/2) yielded 384 mg of a colorless oil (63%).^{1}H NMR (300.01 MHz, $(CD_3)_2CO$) δ 1.39 (6H, td, *J* = 1.5 and 7.0 Hz), 2.15 (3H, s), 2.17-2.43 (2H, m), 3.97 (2H, t, *J* = 6.7 Hz), 4.24 (4H, app. sx t, *J* = 7.0 and 1.5 Hz), 4.97 (1H, ddt, *J* = 46.6, 9.4 and 4.1 Hz), 5.05 (2H, s), 7.48-7.60 (5H, m); ^{13}C NMR (75.44MHz, $(CD_3)_2CO$)): δ 16.8 (CH_3, $^3J_{PC}$ = 3.0 Hz), 16.8 (CH_3, $^3J_{PC}$ = 3.0 Hz), 20.7 (CH_3), 28.6 (CH_2, $^2J_{CF}$ = 21.6 Hz), 42.4 (CH_2), 63.4 (OCH_2, $^4J_{CF}$ = 6.6 Hz, $^2J_{PC}$ = 23.8 Hz), 76.8 (OCH_2), 87.6 (CHF, $^1J_{CF}$ = 178.9 Hz, $^1J_{PC}$ = 170.6 Hz), 129.4 (=CH_2), 129.6 (=CH_2), 130.4 (=CH_2), 136.1 (C_q), 172.0 (C=O); ^{31}P NMR (121.45MHz, $(CD_3)_2CO$) δ 17.97 (d, $^2J_{PF}$ = 72.6 Hz); ^{19}F NMR (282.29 MHz, $(CD_3)_2CO$) δ -211.14 (dddd, *J* = 91.4, 54.3, 36.2 and 19.0 Hz); HRMS (ESI) m/z 362.1561 [$(M+H)^+$, calcd for $C_{16}H_{26}FNO_5P^+$ 362.1527].

Diethyl 3-(*N*-hydroxyacetamido)-1-chloropropylphosphonate (18a). A mixture of **17a** (465 mg; 1.2 mmol) and Pd/C (10%; 90 mg) in THF (12 mL) was stirred under an H_2-atmosphere for 6 hours. The reaction mixture was then filtered over a celite pad and the filtrate was evaporated *in vacuo*. Purification of the residue over two consecutive flash columns (hexanes/acetone: 50/50, then dichloromethane/methanol: 98/2) yielded 207 mg (60%) of the title compound as a colorless oil. ^{1}H NMR (300.01 MHz, DMSO-d6) δ 1.26 (6H, t, *J* = 7.0 Hz), 1.83-2.31 (2H, m), 1.98 (3H, s), 3.67-3.72 (2H, m), 4.06-4.17 (4H, m), 4.34 (1H, dt, *J* = 2.9 and 10.5 Hz); ^{13}C NMR (75.44 MHz, DMSO-d6): δ 16.2 (CH_3, $^3J_{PC}$ = 1.7 Hz), 16.3 (CH_3, $^3J_{PC}$ = 1.7 Hz), 20.3 (CH_3), 29.3 (CH_2), 44.5 (CH_2, $^3J_{PC}$ = 14.4 Hz), 49.4 (CClH, $^1J_{PC}$ = 158.4 Hz), 62.8 (OCH_2, $^2J_{PC}$ = 6.6 Hz), 63.1 (OCH_2, $^2J_{PC}$ = 6.9 Hz), 170.7 (C=O); ^{31}P NMR (121.45 MHz, DMSO-d6) δ 20.57; HRMS (ESI) *m/z* 288.0762 [$(M+H)^+$, calcd for $C_9H_{20}ClNO_5P^+$ 288.0762].

Diethyl 3-(*N*-hydroxyacetamido)-1-fluoropropylphosphonate (18b). A mixture of **17b** (364 mg, 1.0 mmol) and Pd/C (10%, 73 mg) in THF (10 mL) was stirred under an H_2-atmosphere for 5 hours. The reaction mixture was then filtered over a celite pad and the filtrate was evaporated *in vacuo*. Purification of the residue by flash column chromatography (dichloromethane/methanol: 98/2 to 95/5) yielded 248 mg (91%) of a colorless oil. ^{1}H NMR (300.01 MHz, DMSO-d6) δ 1.26 (6H, t, *J* = 7.03 Hz), 1.92-2.20 (2H, m), 1.99 (s, 3H), 3.56-3.76 (2H, m), 4.05-4.16 (4H, m), 4.98 (1H, ddt, *J* = 46.3, 10.3 and 3.2 Hz), 9.84 (1H, s); ^{13}C NMR (75.44 MHz, DMSO-d6): δ 16.3 (CH_3, $^3J_{PC}$ = 2.8 Hz), 16.3 (CH_3, $^3J_{PC}$ = 3.0 Hz), 20.3 (CH_3), 27.4 (CH_2, $^2J_{CF}$ = 20.5 Hz), 43.4 (CH_2, $^3J_{PC}$ = 16.0 Hz), 62.6 (OCH_2, $^4J_{CF}$ = 6.6 Hz, $^2J_{PC}$ = 19.1 Hz), 86.1 (CHF, $^1J_{CF}$ = 177.5 Hz, $^1J_{PC}$ = 168.9 Hz), 170.7 (C=O); ^{31}P NMR (121.45 MHz, DMSO-d6) δ 18.15 (d, $^2J_{PF}$ = 72.1 Hz); ^{19}F NMR (282.29 MHz, DMSO-d6) δ -210.42 (dddd, *J* = 91.4, 56.0, 37.1 and 19.8 Hz) HRMS (ESI) m/z 272.1037 [$(M+H)^+$, calcd for $C_9H_{20}FNO_5P^+$ 272.1058].

3-(*N*-hydroxyacetamido)-1-chloropropylphosphonic acid, bisammonium salt (4a). To a solution of **18a** (150 mg; 0.52 mmol) in dry dichloromethane (5 mL) was added TMSBr (0.7 mL, 5.20 mmol) while stirring at 0 °C. After 45 minutes the ice bath was removed and stirring was continued at room temperature. After three days, ^{31}P-NMR revealed the presence of uncompletely deprotected material, so another 0.2 mL of TMSBr was added. After another three days of stirring at room temperature, the volatiles were removed *in vacuo*, the residue was redissolved in 5% aqueous ammonia and washed with diethyl ether. The aqueous phase was then lyophilized to give 138 mg of a very hygroscopic, off-white powder. ^{1}H NMR (300.01 MHz, D_2O) δ 1.92-2.57 (2H, m), 2.16 (3H, s), 3.71-3.92 (2H, m), 3.92-4.06 (1H, m); ^{13}C NMR (75.44 MHz, D_2O): δ 19.5 (CH_3), 30.7 (CH_2), 45.9 (CH_2, $^3J_{PC}$ = 13.0 Hz), 54.8 (CClH, $^1J_{PC}$ = 139.0 Hz), 174.0 (C=O); ^{31}P NMR (121.45 MHz, DMSO-d6) δ 11.85; HRMS (ESI) *m/z* 232.0135 [$(M+H)^+$, calcd for $C_5H_{12}ClNO_5P^+$ 232.0136].

3-(*N*-hydroxyacetamido)-1-fluoropropylphosphonic acid, bisammonium salt (4b). To a solution of **18b** (223 mg, 0.82 mmol) in dry dichloromethane (8 mL) was added TMSBr (1.1 mL, 8.2 mmol) while stirring at 0 °C. After 45 minutes the icebath was removed and stirring was continued at room

temperature. After 3 days, ^{31}P NMR revealed the presence of uncompletely deprotected material, so another 0.2mL of TMSBr was added. After another 4 days of stirring at room temperature, the volatiles were removed *in vacuo*, the residue was redissolved in 5% aqueous ammonia and washed with diethyl ether. The aqueous phase was then lyophilized to give 207 mg of **4b** as a very hygroscopic, off-white powder. ^{1}H NMR (300.01 MHz, D_2O) δ 1.96-2.22 (2H, m), 2.11 (3H, s), 3.77 (2H, app. d sx, *J* = 70.8 and 7.5 Hz), 4.33 (1H, ddt, *J* = 48.6, 10.3 and 4.1 Hz); ^{13}C NMR (75.44 MHz, D_2O): δ 19.5 (CH_3), 28.4 (CH_2, $^{2}J_{CF}$ = 20.2 Hz), 45.0 (CH_2, $^{3}J_{CF}$ = 13.6 Hz, $^{3}J_{PC}$ = 3.6 Hz), 90.7 (CHF, $^{1}J_{CF}$ = 171.0 Hz, $^{1}J_{PC}$ = 154.0 Hz), 174.0 (C=O); ^{31}P NMR (121.45 MHz, D_2O) δ 11.80 (d, $^{2}J_{PF}$ = 62.3 Hz); HRMS (ESI) m/z 216.0455 [$(M+H)^+$, calcd for $C_5H_{12}FNO_5P^+$ 216.0432].

Diethyl 3-(benzyloxy)imino-1-fluoropropylphosphonate (20). Diethyl 1-fluoro-2-formylethylphosphonate **12b** (4.24g, 20 mmol) was dissolved in THF (15 mL) and acetic acid (20 mL) and cooled to 0 °C with an icebath. Benzylhydroxylamine (free base, 1.82g, 14.74 mmol) was dissolved in 5 mL THF in a separate flask and this was transferred to the above mixture. The icebath was removed and the mixture was stirred for two hours at room temperature. Ethyl acetate was then added and the mixture was extracted two times with water. The combined water layers were back-extracted twice with ethyl acetate. The combined organic fractions were washed with brine, dried over anhydrous Na_2SO_4 and the solvents were removed *in vacuo*. Column chromatography (hexanes/ethyl acetate: 50/50) afforded 3.34g (53%) of the product as a colorless liquid. The product is formed as a 1:2.4 mixture of E and Z isomers of the oxime. 1H NMR (300 MHz, acetone) δ ppm 1.27 - 1.35 (m, 6 H) 2.78 - 3.08 (m, 2 H) 4.09 - 4.25 (m, 4 H) 4.92 - 5.09 (m, 1 H) 5.09 - 5.25 (m, 2 H) 6.93 (t, J=5.13 Hz,1 H) 7.25 - 7.43 (m, 5 H); HRMS (ESI) m/z 318.1243 [$(M+H)^+$, calcd for $C14H22FNO4P^+$ 318,1265].

Diethyl 3-(benzyloxyamino)-1-fluoropropylphosphonate (16b). A solution of **20** (3.34g, 10.53 mmol) in methanol (50 mL) was cooled to 0 °C, 5 drops of methyl orange solution were added followed by one drop of 6 M aqueous HCl, resulting in a red-pink coloring of the reaction mixture.

Sodium cyanoborohydride powder (2.65g, 42.11 mmol) was added over the course of a few minutes and more HCl was added in order to keep a milky pink solution. After 4.5 hours of stirring at room temperature, another 630 mg of sodium cyanoborohydride were added and the reaction mixture was stirred for 1 more hour. The reaction was then quenched by the addition of aqueous 2.5 M NaOH and extracted three times with ethyl acetate (**Caution! HCN is volatile and very toxic! The aqueous phase should be kept at pH > 12**). The combined organic fractions were washed with brine, dried over anhydrous Na_2SO_4 and the solvents were removed *in vacuo*, resulting in the crude oxyamine **16b** that was used as such in the next reaction.

pKa determination of phosphonates by ^{31}P NMR titration. A small amount (3 mg) of compound **4a**, **4b** or FR900098 was dissolved in acetate buffer (pH3, 0.025M) and D_2O (100 µL) was added. The mixture was further acidified by dropwise addition of aqueous HCl (2M), the pH was measured and the ^{31}P NMR was acquired. The pH was then increased stepwise by addition of aqueous NaOH (dropwise, 0.1 and 0.05M), followed by measurement of the ^{31}P chemical shift (D_3PO_4 in D_2O as an external standard). The measured ^{31}P shifts were plotted in function of their corresponding pH and the pK_a's could be estimated to be at the pH of the inflection points of each titration curve.

Diethyl 1-fluoro-4-hydroxybutylphosphonate (21). To a solution of **11b** (1.05 g, 5 mmol) in dry THF (50 mL) was added BH_3.THF (1 M in THF, 5.5 mL) while stirring at 0 °C. After 10 minutes the icebath was removed and the reaction mixture was stirred at room temperature for 2 hours. Water (0.1 mL) was added, then aqueous sodium hydroxide (0.5 mL of a 3 M solution) and aqueous hydrogen peroxide (0.5 mL of a 30% solution) and the mixture was stirred at 50 °C for 1 hour. The reaction was quenched by addition of aqueous $Na_2S_2O_3$ and extracted with ethyl acetate. The combined organic fractions were washed with brine, dried over anhydrous Na_2SO_4 and the solvents were removed *in vacuo*. Purification of the crude material by flash column chromatography (hexanes/acetone: 75/25 to 25/75) gave 506 mg of a colorless oil (44%).^{1}H NMR (300.01 MHz, $CDCl_3$) δ 1.34 (6H, s, *J* = 7.0 Hz), 1.69-2.12 (5H, m), 3.68 (2H, t, *J* = 5.9 Hz), 4.19 (4H, app sx, *J* = 7.03 Hz), 4.75

(1H, dddd, *J* = 47.2, 9.4, 4.4 and 3.2 Hz); ^{31}P NMR (121.45 MHz, $CDCl_3$) δ 18.00 (d, $^2J_{PF}$ = 75.3 Hz); HRMS (ESI) m/z 229.0994 [$(M+H)^+$, calcd for$C_8H_{19}FO_4P^+$ 229.1000].

Diethyl 1-fluoro-4-carboxybutylphosphonate (22). To **21** (465 mg, 2.04 mmol), TEMPO (65 mg, 0.41 mmol) and BAIB (1.446 g, 4.49 mmol) in a flask was added MeCN (5 mL) and the contents were sonicated in order to dissolve all solids. Subsequently, water (5 mL) was added and the reaction was stirred for 6 hours at room temperature and protected from light. The volatiles were then removed *in vacuo*, the resulting crude material was co-evaporated twice with THF and purified on a silica gel column (CH_2Cl_2/MeOH/HCOOH: 95/5/0.1) to yield 498 mg of a golden oil (quantitative). ^{1}H NMR (300.01 MHz, $CDCl_3$) δ 1.35 (6H, td, *J* = 2.6 and 7.3 Hz), 2.10-2.31 (2H, m), 2.51-2.65 (2H, m), 4.21 (4H, app sx d, *J* = 1.8 and 6.9 Hz), 4.73-4.95 (2H, m), 10.39 (1H, br s); ^{31}P NMR (121.43 MHz, $CDCl_3$) δ 17.38 (d, $^2J_{PF}$ = 75.18 Hz); HRMS (ESI) m/z 243.0793 [$(M+H)^+$, calcd for $C_8H_{17}FO_5P^+$ 243.0792].

Diethyl 3-(*N*-(benzyloxy)-*N*-methylcarbamoyl)-1-fluoropropylphosphonate (23). To a solution of **22** (372 mg, 1.54 mmol) in dry dichloromethane (5 mL) was added CDI (290 mg, 1.79 mmol) while stirring vigorously. In a separate flask, BnO-NH(Me).TFA (553 mg, 2.20 mmol) was dissolved in 3 mL dry dichloromethane and Et_3N (0.31 mL, 2.23 mmol) was added, resulting in the precipitation of Et_3N.TFA. After 90 minutes of stirring at room temperature, the BnO-NH(Me) solution was added to the phosphonate solution and the reaction was stirred overnight at room temperature. The reaction was then quenched with aqueous NH_4Cl and extracted thrice with dichloromethane. The combined organic fractions were washed with brine, dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. Purification of the crude material by silica gel column chromatography (hexanes/ethyl acetate: 25/75 to 0/100) resulted in 498 mg of a colorless oil (89%).^{1}H NMR (300.01 MHz, $(CD_3)_2CO$) δ 1.31 (6H, t, *J* = 7.0 Hz), 2.03-2.21 (2H, m), 2.65-2.78 (2H, m), 3.18 (3H, s), 4.10-4.21 (4H, m), 4.76-4.97 (1H, m), 4.95 (2H, s), 7.39-7.51 (5H, m); ^{31}P NMR (121.45 MHz, $(CD_3)_2CO$) δ 18.24 (d, $^2J_{PF}$ = 37.72 Hz); HRMS (ESI) m/z 362.1367 [$(M+H)^+$, calcd for $C_{16}H_{26}FNO_5P^+$ 362.1527].

D**iethyl 3-(*N*-hydroxy-*N*-methylcarbamoyl)-1-fluoropropylphosphonate (24).** A solution of **23** (297 mg, 0.82 mmol) in THF (8 mL) was hydrogenated at atmospheric pressure in the presence of Pd (10% wt. on activated carbon, 60 mg). After stirring for 2 hours, the reaction mixture was filtered through a celite pad. The solvent was removed under vacuum, and the crude mixture was purified by flash column chromatography on two consecutive columns (hexanes/acetone: 50/50 then dichloromethane/methanol: 95/5) to yield 138 mg (62%) of a golden brown oil. ^{1}H NMR (300.01 MHz, $(CD_3)_2CO$) δ 1.33 (6H, t, *J* = 7.3 Hz), 2.08-2.22 (4H, m), 2.57-2.79 (2H, m), 3.18 (3H, s), 4.18 (4H, qdd, *J* = 11.1, 7.0 and 4.1 Hz), 4.91 (1H, dddd, *J* = 46.6, 9.7, 4.1 and 2.9 Hz), 8.95 (1H, br s); ^{13}C NMR (75.44 MHz, $(CD_3)_2CO$) δ 16.7 (CH_3, $^{3}J_{PC}$ = 2.5 Hz), 16.8 (CH_3, $^{3}J_{PC}$ = 2.8 Hz), 26.3 (CH_2, $^{2}J_{CF}$ = 19.6 Hz), 27.8 (CH_2, $^{3}J_{PC}$ = 10.8 Hz), 36.2 (CH_3), 63.4 (OCH_2, $^{4}J_{CF}$ = 6.6 Hz, $^{2}J_{PC}$ = 22.1 Hz), 89.0 (CHF, $^{1}J_{CF}$ = 177.7 Hz, $^{1}J_{PC}$ = 169.5 Hz), 173.3 (C=O); ^{31}P NMR (121.45 MHz, $(CD_3)_2CO$) δ 18.40 (d, $^{2}J_{PF}$ = 74.2Hz); HRMS (ESI) m/z 272.1047 [$(M+H)^+$, calcd for $C_9H_{20}FNO_5P^+$ 272.1058].

3-(*N*-hydroxy-*N*-methylcarbamoyl)-1-fluoropropylphosphonic acid, bisammonium salt (5). 24 (119 mg, 0.44 mmol) was dissolved in dry dichloromethane under inert atmosphere and cooled to 0 °C. TMSBr (0.6 mL, 4.4 mmol) was added dropwise while stirring. The icebath was removed and the reaction was stirred at room temperature. After 24 hours another 0.3 mL of TMSBr were added and the reaction was stirred for another 4 days. The volatiles were removed *in vacuo*, the crude material was dissolved in 5% aqueous ammonia and washed with diethyl ether. Lyophilisation of the ammonia solution yielded the product as a brown solid in quantitative yield. ^{1}H NMR (300.01 MHz, D_2O) δ 1.82-2.00 (2H, m), 2.11-2.62 (2H, m), 3.07 (3H, s), 4.18-4.45 (1H, m); ^{13}C NMR (75.44 MHz, D_2O) δ 27.0 (CH_2, $^{2}J_{CF}$ = 19.6 Hz), 34.5 (CH_2, $^{3}J_{CF}$ = 12.2 Hz, $^{3}J_{PC}$ = 1.0 Hz),36.1 (CH_3), 92.9 (CHF, $^{1}J_{CF}$ = 171.1 Hz, $^{1}J_{PC}$ = 153.7 Hz), 175.5 (C=O); ^{31}P NMR (121.45 MHz, D_2O) δ 11.74 (d, $^{2}J_{PF}$ = 63.27 Hz); HRMS (ESI) m/z 216.0437 [$(M+H)^+$, calcd for $C_5H_{12}FNO_5P^+$ 216.0432].

Diethyl 1,1-difluorobut-3-enylphosphonate (25). Zinc dust (10 µm particle size) was activated by washing with the following sequence: 2% aq. HCl (2x) – H_2O (2x) – acetone – ether, and dried in

vacuo at 120 °C overnight. 1.1g (16.85 mmol) of this zinc dust was suspended in dry dimethoxyethane and diethyl bromodifluoromethylphosphonate (3.0 g, 11.24 mmol) was injected slowly in this suspension. After 24 hours of stirring at room temperature, no progress was seen in the formation of the metal insertion product so the reaction mixture was sonicated for four hours, resulting in the consumption of the zinc dust. The solution of phosphonodifluoromethyl zinc reagent was then transferred to a separate flamedried flask containing anhydrous CuBr (6.5 mg, 0.044 mmol). Allyl bromide (0.98 mL, 11.24 mmol) was added and the reaction mixture was stirred overnight at room temperature after which ^{31}P-NMR confirmed the formation of **25** (triplet at δ = 5.92 ppm). The reaction was quenched by addition of aqueous NH_4Cl and extracted three times with dichloromethane. The combined organic fractions were washed with brine and dried over anhydrous Na_2SO_4. Purification of the crude product by flash column chromatography (hexanes/ethyl acetate: 75/25) yielded 1.73 g of a colorless liquid (68%). 1H NMR (300 MHz, CHLOROFORM-d) δ ppm 1.36 (t, J=7.18 Hz, 6 H) 2.82 (tt, J=19.48, 6.59 Hz, 2 H) 4.25 (quin, J=7.25 Hz, 4 H) 5.21 - 5.26 (m, 1 H) 5.28 (s, 1 H) 5.72 - 5.95 (m, 1 H) ^{31}P NMR (121.45 MHz, CHLOROFORM-d) δ 6.889 (t, $^{2}J_{PF}$ = 107.48 Hz); HRMS (ESI) m/z 229.0788 [$(M+H)^+$, calcd for C8H16F2O3P+ 229.0800].

Diethyl 1,1-difluoro-2-formylethylphosphonate (28). The title compound was synthesized from diethyl 1,1-difluorobut-3-enylphosphonate **(25)** (955 mg, 4.19 mmol) using the same method as for **12b**, giving 560mg (58%) of crude **28** that was used as such in the next reactions. HRMS (ESI) m/z 231.0614 [$(M+H)^+$, calcd for $C7H14F2O4P^+$ 231,0592].

***tert*-Butyl *N*-(benzyloxy)-*N*-(2-bromoethyl)carbamate (41).** To a solution of *t*-butyl *N*-benzyloxycarbamate (447 mg, 2 mmol) in dry DMF (4 mL) was added sodium hydride (88 mg of a 60% dispersion in mineral oil, 2.2 mmol) under vigorous stirring at room temperature. After 30 minutes, neat 1,2-dibromoethane (1.7 mL, 3758 mg, 20 mmol) was added quickly via syringe to the clear solution. The reaction mixture was stirred overnight at room temperature, quenched by the addition of saturated aqueous NH_4Cl and partitioned between aqueous 0.1N HCl and diethyl ether. The

extraction with diethyl ether was repeated two times, the combined organic phases were washed with brine, dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. The resulting crude mixture was purified by flash column chromatography (hexanes/ethyl acetate: 90/10)to yield 433.6 mg (66 %) of **41** as a colourless oil. 1H NMR (300 MHz, acetone) δ ppm 1.34 - 1.62 (m, 9 H) 3.44 - 3.64 (m, 2 H) 3.70 - 3.92 (m, 2 H) 4.91 (s, 2 H) 7.23 - 7.61 (m, 5 H); HRMS (ESI) m/z 330.0705 $[(M+H)^+$, calcd for C14H21BrNO3+ 330,0699]

***tert*-Butyl benzyloxy(2-((tert-butyldimethylsilyl)oxy)ethyl)carbamate (43).** The title compound was synthesized from (2-bromoethoxy)(tert-butyl)dimethylsilane **(42)** (613.25 mg, 2.56 mmol) and *t*-butyl *N*-benzyloxycarbamate (520 mg, 2.33 mmol)using the same method as for **41**, giving 688 mg (77%) of pure **43** after column chromatography. 1H NMR (300 MHz, CHLOROFORM-d) d ppm 0.07 (s, 6 H) 0.90 (s, 9 H) 1.51 (s, 9 H) 3.55 (t, J=6.44 Hz, 2 H) 3.76 (t, J=6.44 Hz, 2 H) 4.87 (s, 2 H) 7.27 - 7.52 (m, 5 H) ; HRMS (ESI) m/z 382.2446 $[(M+H)^+$, calcd for C20H36NO4Si+ 382,2408]

***tert*-Butyl *N*-(benzyloxy)-*N*-(2-hydroxyethyl)carbamate (44).** To a solution of **43** (686 mg, 1.80 mmol) in methanol was added ammonium fluoride (133 mg, 3.60 mmol) and the reaction mixture was stirred at 50 °C for 3.5 hours. All volatiles were removed and the resulting crude material was purified by flash column chromatography (hexanes/ethyl acetate: 75/25)to yield 437 mg (90 %) of **44** as a colourless oil. 1H NMR (300 MHz, acetone) δ ppm 1.41 - 1.57 (m, 9 H) 3.50 - 3.62 (m, 2 H) 3.63 - 3.78 (m, 3 H) 4.89 (s, 2 H) 7.18 - 7.50 (m, 5 H); HRMS (ESI) m/z 268.1555 $[(M+H)^+$, calcd for C14H22NO4+ 268,1543]

2-(*N*-(benzyloxy)acetamido)ethyl acetate (46). 44 (408 mg; 1.53 mmol) was dissolved in dichloromethane (10 mL), cooled to 0 °C and TFA (6 mL) was added. After stirring for 30 minutes, the ice bath was removed and 15 minutes later the reaction was complete according to TLC. Toluene (10 mL) was added to the mixture and the solvents were removed *in vacuo*. When the product approached dryness, another 25 mL of toluene was added and the solution rotoevaporated again,

giving 454 mg of a yellow oil. This oil was dissolved in ethyl acetate and washed with aqueous K_2CO_3, water and aqueous NaOH, dried over anhydrous Na_2SO_4 and evaporated *in vacuo,* yielding 264 mg of **45** as a free base. The residue was then redissolved in dry dichloromethane (15 mL) and acetic anhydride (2.93 mL; 31 mmol), Et_3N (1.3 mL; 9.24 mmol) and DMAP (56 mg; 0.46 mmol) were added. After 6 hours of stirring at room temperature, the reaction mixture was reduced to half its volume *in vacuo*, 10 mL of toluene was added and the mixture was evaporated to dryness. The residue was taken up in dichloromethane and washed with saturated aqueous sodium bicarbonate, then brine and dried over anhydrous Na_2SO_4. Evaporation yielded 333 mg of crude **46** as a colorless oil (86 %) that was used as such in the next reaction. HRMS (ESI) m/z 252.1285 [$(M+H)^+$, calcd for $C13H18NO4^+$ 252,1230]

***N*-(Benzyloxy)-*N*-(2-hydroxyethyl)acetamide (47). 46** (341 mg, 1.4 mmol) and sodium methoxide (7 mg, 0.14 mmol) were dissolved in methanol (8 mL) and stirred at room temperature for 1.5 hour. All volatiles were removed *in vacuo*, the residue was taken up in ethyl acetate and washed with aqueous NH_4Cl, brine, dried over anhydrous Na_2SO_4 and evaporated. Purification by flash column chromatography (hexanes/ethyl acetate: 50/50 to 0/100)of the crude material gave 123 mg (42 %) of **47** as a white solid. 1H NMR (300 MHz, CHLOROFORM-d) δ ppm 2.14 (s, 3 H) 3.82 (s, 4 H) 4.88 (s, 2 H) 7.40 (s, 5 H); HRMS (ESI) m/z 210.1123 [$(M+H)^+$, calcd for $C11H16NO3^+$ 210,1125]

***tert*-Butyldimethyl((*N*-benzyloxy)iminoethoxy)silane (50).**

2-((*tert*-butyldimethylsilyl)oxy)acetaldehyde (**49**) (334 mg, 1.92 mmol) and benzylhydroxylamine hydrochloride (336 mg, 1.1 mmol) were suspended in methanol, Et_3N (0.29 m L, 212 mg, 2.1 mmol) was added and the mixture was stirred at room temperature for two hours. Water was added and the mixture was extracted with diethyl ether (2x). The combined organic fractions were washed with brine, dried over anhydrous $MgSO_4$ and evaporated, yielding 348 mg of crude imine **50** that was immediately reduced in the next reaction.

***tert*-Butyldimethyl(2-(*N*-benzyloxyamino)ethoxy)silane (51).** Crude imine **50** was reduced using the same method as for **16b**, resulting in 300 mg (56%, two steps) of crude **51** as a colourless oil that was used as such in the next step.

***N*-(Benzyloxy)-*N*-(2-((tert-butyldimethylsilyl)oxy)ethyl)acetamide (52).** Crude oxyamine **51** was dissolved in dry dichloromethane (10 mL) and acetic anhydride (1.13 mL, 12 mmol), Et_3N (0.50 mL, 3.6 mmol) and DMAP (44 mg, 0.36 mmol) were added. The reaction mixture was stirred overnight at room temperature, quenched with 0,1 M HCl and extracted with dichloromethane. The combined organic fractions were washed with saturated aqueous $NaHCO_3$ and dried over anhydrous Na_2SO_4. Flash column chromatography (hexanes/ethyl acetate: 75/25 to 50/50) afforded 222 mg of **52** as a colorless oil (64%).^{1}H NMR (300 MHz, CHLOROFORM-d) δ ppm 0.07 (s, 6 H) 0.89 (s, 9 H) 2.09 (s, 3 H) 3.80 (d, J=3.51 Hz, 4 H) 4.89 (s, 2 H) 7.39 (s, 5 H); HRMS (ESI) m/z 324.1972 [$(M+H)^+$, calcd for $C17H30NO3Si^+$ 324,1989]

***N*-(Benzyloxy)-*N*-(2-hydroxyethyl)acetamide (47).** The title compound was synthesized from *N*-(benzyloxy)-*N*-(2-((tert-butyldimethylsilyl)oxy)ethyl)acetamide **(52)** (220 mg, 0.68 mmol) using the same method as for **44**, giving 111 mg (78%) of **47** as a white solid. Spectral data: see above.

2-(*N*-(Benzyloxy)acetamido)ethyl trifluoromethanesulfonate (48). Triflic anhydride (162 mg, 0.57 mmol) was dissolved in dry dichloromethane cooled to -78 °C. To this, a cooled solution of alcohol **47** (100 mg, 0.48 mmol) and DIPEA (74 mg, 0.574 mmol) in dichloromethane was added and the mixture was allowed to warm to 0° C. The reaction mixture was then partitioned between ice-cold diethyl ether and saturated aqueous $NaHCO_3$, the aqueous phase was extracted two times with diethyl ether and once with dichloromethane. The combined organic fractions were washed with ice-cold brine and dried over anhydrous Na_2SO_4, followed by removal of the solvent *in vacuo* at 12°C, giving crude **48** as a slight yellow oil that was used as such in the next reaction.

2-(Benzyloxy)ethyl trifluoromethanesulfonate (56). The title compound was synthesized from 2-benzyloxyethanol **(55)** (761 mg, 0.71 mmol) and triflic anhydride (1552 mg, 5.5 mmol) using the same method as for **48**, giving crude **56** as a dark purple liquid that was used as such in the next reaction.

Diethyl 3-(benzyloxy)-1,1-difluoropropylphosphonate (57). Preparation of LDA – DMPU solution: Diisopropylamine (1.8 mL, 12.8 mmol) and DMPU (1.35 mL, 11.2 mmol) were mixed with THF (6 mL) and cooled to -78 °C. nBuLi (7.5 mL of a 1.6 M solution in hexanes) was added, the mixture was warmed up to room temperature and cooled again to -78 °C.
Diethyl difluoromethyl phosphonate (2107 mg, 11.2 mmol) and 2-(benzyloxy)ethyl triflate **56** were dissolved in THF (6 mL) and cooled to -78 °C, and the freshly prepared LDA – DMPU solution was added, followed by stirring of the reaction mixture at -78°C for 20 minutes. The reaction was quenched by the addition of saturated aqueous NH_4Cl and extracted three times with ethyl acetate. The combined organic phases were washed with brine, dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to give 3.101g of a brown liquid that contained a considerable amount of unreacted diethyl difluoromethyl phosphonate according to ^{31}P-NMR. This was removed by vacuum distillation (55 °C, 1.2 mbar), followed by purification of the product by flash column chromatography (hexanes/ethyl acetate: 75/25 to 25/75), yielding 561 mg (54%) of **57** as a colorless liquid. 1H NMR (300 MHz, CHLOROFORM-d) δ ppm 1.26 - 1.50 (m, 6 H) 2.26 - 2.62 (m, 2 H) 3.77 (t, J=7.18 Hz, 2 H) 4.27 (quin, J=7.32 Hz, 4 H) 4.54 (s, 2 H) 7.19 - 7.47 (m, 5 H); HRMS (ESI) m/z 323.1200 [$(M+H)^+$, calcd for $C14H22F2O4P^+$ 323,1218]

Diethyl 1,1-difluoro-3-hydroxypropylphosphonate (58). To a solution of **57** (1280 mg, 3.97 mmol) in methanol was added formic acid (2 mL) and Pd (10% wt. on carbon). The reaction mixture was stirred overnight at room temperature, filtered over a celite pad and evaporated to dryness. The resulting turbid yellow liquid was purified by flash column chromatography (hexanes/ethyl acetate: 50/50 to 0/100), yielding 746 mg (81%) of **58** as a colorless liquid. 1H NMR (300 MHz, acetone) δ ppm

1.25 - 1.45 (m, 6 H) 2.17 - 2.45 (m, 2 H) 3.80 (t, J=6.74 Hz, 2 H) 4.15 - 4.35 (m, 4 H) 4.46 (br. s., 1 H); HRMS (ESI) m/z 233.0756 [$(M+H)^+$, calcd for $C7H16F2O4P^+$ 233,0749]

3-(Ethoxyphosphono)-3,3-difluoropropyl 4-methylbenzenesulfonate (59). The title compound was synthesized from diethyl 1,1-difluoro-3-hydroxypropylphosphonate **(58)** (516 mg, 2.22 mmol), TsCl (636 mg, 3.33 mmol), Et_3N(0.93 mL, 6.66 mmol) and DMAP (82 mg, 0.67 mmol) using the same method as for **14b**, giving crude **59** as a brown oil that was used as such in the next reaction. 1H NMR (300 MHz, acetone) δ ppm 1.32 (t, J=6.88 Hz, 6 H) 2.31 - 2.65 (m, 6 H) 4.11 - 4.40 (m, 6 H) 7.37 - 7.60 (m, 2 H) 7.72 - 7.93 (m, 2 H); HRMS (ESI) m/z 233.0756 [$(M+H)^+$, calcd for $C14H22F2O6PS^+$ 387,0837]

***tert*-Butyl *N*-benzyloxy(3-(ethoxyphosphono)-3,3-difluoropropyl)carbamate (31).** To a solution of *t*-butyl *N*-benzyloxycarbamate (639 mg, 2.86 mmol) in dry DMF (7 mL) cooled to 0 °C was added sodium hydride (120 mg of a 60% dispersion in mineral oil, 2.9 mmol) while stirring. After a few minutes the icebath was removed and the solution stirred for another half hour. Then, 3-(ethoxyphosphono) 3,3-difluoropropyl 4-methylbenzenesulfonate (**59**) (866 mg, 2.2 mmol) dissolved in 4 mL dry DMF was added and the mixture was stirred overnight at room temperature. Then, another equivalent of *t*-butyl *N*-benzyloxycarbamate (491 mg, 2.2 mmol) in dry DMF (2 mL) was added and the reaction mixture was stirred for another 3 hours. The reaction was quenched by addition of aqueous NH_4Cl and extracted three times with ethyl acetate. The combined organic fractions were washed with brine and dried over $MgSO_4$ giving 1.58g of a yellow oil. Purification of the crude product by flash column chromatography (hexanes/ethyl acetate: 75/25 to 60/40) yielded 95 mg of **31** as a colorless oil (10%).^{1}H NMR (300 MHz, acetone) δ ppm 1.25 - 1.39 (m, 6 H) 1.50 (s, 9 H) 2.19 - 2.49 (m, 2 H) 3.59 - 3.81 (m, 2 H) 4.13 - 4.37 (m, 4 H) 4.89 (s, 2 H) 7.25 - 7.56 (m, 5 H); HRMS (ESI) m/z 438.1859 [$(M+H)^+$, calcd for $C19H31F2NO6P^+$ 438,1852]

Diethyl 3-(*N*-(benzyloxy)acetamido)-1,1-difluoropropylphosphonate (33). 31 (270 mg, 0.62 mmol) was dissolved in dichloromethane (6 mL), cooled to 0 °C and TFA (2.5 mL) was added. After

stirring for 10 minutes, the icebath was removed and 1hour later the reaction was done, according to TLC. Toluene (15mL) was added to the mixture and the solvents were removed *in vacuo*. When the product approached dryness, another 5 mL of toluene was added and the solution rotoevaporated again. The residue was then redissolved in dry dichloromethane (4 mL) and acetic anhydride (0.6 mL, 6.4 mmol), Et_3N (0.25 mL, 1.8 mmol) and DMAP (5 mg, 0.04 mmol) were added. After 2 hours of stirring at room temperature, the reaction was quenched with 0,1 M HCl and extracted with dichloromethane. The combined organic fractions were washed with saturated aqueous $NaHCO_3$, then with brine and dried over anhydrous Na_2SO_4. Flash column chromatography (hexanes/ethyl acetate: 50/50) yielded 136 mg of a colorless oil (91%).^{1}H NMR (300 MHz, acetone) δ ppm 1.22 - 1.45 (m, 6 H) 2.05 (s, 3 H) 2.20 - 2.55 (m, 2 H) 3.78 - 4.00 (m, 2 H) 4.13 - 4.37 (m, 4 H) 4.97 (s, 2 H) 7.24 - 7.63 (m, 5 H); HRMS (ESI) m/z 380.1426 [$(M+H)^+$, calcd for $C16H25F2NO5P^+$ 380,1433]

Diethyl (3-(*N*-(benzyloxy)-2-nitrophenylsulfonamido)-1,1-difluoropropyl)phosphonate (64). To a stirred solution of alcohol **58** (81 mg, 0.35 mmol), *N*-(benzyloxy)-2-nitrobenzenesulfonamide **63** (117 mg, 0.38 mmol) and triphenylphosphine (100 mg, 0.38 mmol) was added DIAD (0.08 mL, 0.38 mmol) at room temperature. After stirring overnight at room temperature, another 0.3 equivalents of each *N*-(benzyloxy)-2-nitrobenzenesulfonamide **63** (34 mg, 0.11 mmol), triphenylphosphine (29 mg, 0.11 mmol) and DIAD (0.02 mL, 0.11 mmol) were added and the mixture was stirred for another 3 hours. All volatiles were subsequently removed *in vacuo* and the resulting crude was purified by flash column chromatography (hexanes/ethyl acetate: 50/50) to yield 99 mg of **64** as a gold oil (54 %). 1H NMR (300 MHz, acetone) δ ppm 1.23 - 1.44 (m, 6 H) 2.12 - 2.45 (m, 2 H) 3.40 (br. s., 2 H) 4.22 (dqd, J=8.57, 7.01, 7.01, 7.01, 1.76 Hz, 4 H) 5.12 (s, 2 H) 7.29 - 7.63 (m, 5 H) 7.74 - 7.99 (m, 2 H) 7.99 - 8.12 (m, 1 H) 8.12 - 8.29 (m, 1 H); HRMS (ESI) m/z 523.1118 [$(M+H)^+$, calcd for $C20H26F2N2O8PS^+$ 523,1110]

Diethyl 3-(*N*-(benzyloxy)acetamido)-1,1-difluoropropylphosphonate (33). To a solution of **64** (22 mg, 0.042 mmol) and anhydrous K_2CO_3 (23 mg, 0.168 mmol) in acetonitrile (0.98 mL) and DMSO

(20µL) was added thiophenol (15µL, 0.126 mmol). After stirring at room temperature for 2.5 hours, acetic anhydride (45µL, 0.480 mmol) was added and stirring was continued overnight. Water and ethyl acetate were then added, resulting in the formation of a homogeneous emulsion which was broken up by the addition of aqueous HCl. The acidified aqueous phase was extracted two times with ethyl acetate and the combined organic fractions were dried over anhydrous Na_2SO_4. All volatiles were subsequently removed *in vacuo* and the resulting crude was purified by flash column chromatography (hexanes/ethyl acetate: 50/50) to yield 8.44 mg of **33** as a gold oil (53 %). Analytical data: see above.

Diethyl 3-(*N*-hydroxyacetamido)-1,1-difluoropropylphosphonate (34). To a solution of **33** (120 mg, 0.316 mmol) in ethyl acetate was added formic acid (0.15 mL) and Pd (10% wt. on carbon). The reaction mixture was stirred for 1 hour at room temperature, filtered over a celite pad and evaporated to dryness. The resulting brown liquid was purified by flash column chromatography (hexanes/ethyl acetate: 25/75), yielding 73 mg (80%) of **34** as a colorless oil. 1H NMR (300 MHz, acetone) d ppm 1.30 - 1.44 (m, 6 H) 1.98 - 2.10 (m, 3 H) 2.22 - 2.57 (m, 2 H) 3.74 - 3.94 (m, 2 H) 4.17 - 4.38 (m, 4 H); HRMS (ESI) m/z 290.0977 [$(M+H)^+$, calcd for $C9H19F2NO5P^+$ 290,0963]

Dibenzyl difluoromethyl phosphonate (79). To a solution of dibenzyl phosphite (14.61 g, 44.6 mmol) in THF (65 mL)cooled to 0 °C was added NaHMDS (25 mL of a 2M solution in THF, 50 mmol). The reaction mixture was then cooled to -55 °C and a dry-ice condenser was installed on the reaction flask. Chlorodifluoromethane (10 g, 116 mmol) from a pressure tin was pumped into the reaction mixture via a submerged needle (no bubbling was noticed!) and the mixture was stirred at -55 °C. After 4 hours, the cooling bath was removed, another 16g (185 mmol) of chlorodifluoromethane was added and the reaction mixture was stirred overnight at room temperature. The reaction was quenched by addition of saturated aqueous NH_4Cl and extracted three times with dichloromethane. The combined organic fractions were washed with saturated aqueous $NaHCO_3$, brine and dried over anhydrous Na_2SO_4giving 11 g of a brown oily liquid. Purification of the crude product by flash column

chromatography (toluene/ethyl acetate: 80/20) yielded 9.470 g of **79** as a colorless oil (68%).^{1}H 1H NMR (300 MHz, acetone) δ ppm 5.22 (d, J=8.49 Hz, 4 H) 6.29 (app dt, J=27.30 Hz, J = 48.30 Hz, 1H) 7.24 - 7.56 (m, 9 H); HRMS (ESI) m/z 313.0795 [(M+H)$^{+}$, calcd for C15H16F2O3P^{+} 313,0800]

2-((*tert*-butyldiphenylsilyl)oxy)ethyl trifluoromethanesulfonate (86). The title compound was synthesized from 2-((*tert*-butyldiphenylsilyl)oxy)ethanol **(85)** (300 mg, 1 mmol) and triflic anhydride (339 mg, 1.2 mmol) using the same method as for **48**, giving crude **86** as a brown oil that was used as such in the next reaction.

Dibenzyl (3-((tert-butyldiphenylsilyl)oxy)-1,1-difluoropropyl)phosphonate (87). The title compound was synthesized from dibenzyl difluoromethyl phosphonate **(79)** and 2-((*tert*-butyldiphenylsilyl)oxy)ethyl trifluoromethanesulfonate **(86)** using the same method as for compound **57**, giving 196 mg (66%) of **87** as a colourless oil. 1H NMR (300 MHz, acetone) δ ppm 0.93 - 1.15 (m, 9 H) 2.24 - 2.60 (m, 2 H) 3.98 (t, J=6.88 Hz, 2 H) 5.16 (dd, J=8.49, 1.17 Hz, 4 H) 7.24 - 7.56 (m, 15 H) 7.56 - 7.85 (m, 5 H); HRMS (ESI) m/z 595.2235 [(M+H)$^{+}$, calcd for C33H38F2O4PSi^{+} 595,2240]

(3-(*N*-(benzyloxy)acetamido)-1-fluoropropyl)phosphonic acid, disilver salt (90). Diethyl 3-(*N*-(benzyloxy)acetamido)-1-fluoropropylphosphonate **(17b)** (317 mg, 0.87 mmol) was coevaporated twice with dry toluene, dissolved in dry acetonitrile (1 mL) and the solution was cooled to 0 °C. BSTFA (0.7 mL, 1.74 mmol) was added, followed by TMSBr. The cooling bath was removed and the reaction mixture was stirred at room temperature for 3 hours after which ^{31}P-NMR of a reaction sample confirmed that the reaction was finished. Dry toluene (10 mL) was added to the reaction mixture and all volatiles were removed by rotoevaporation. The resulting oil was taken up in THF and water (1 mL) was added. After stirring for 10 minutes, volatiles were removed again and a little methanol was added which caused the product to form a white foam upon rotoevaporation.

The resulting white foam was dissolved in water and NaOH (1.75 mL of a 1 M aqueous solution) was added under vigorous stirring, followed by aqueous $AgNO_3$ (298 mg, dissolved in 1 mL H_2O), resulting

in a very fine white opaque suspension of **90**. The suspension was filtered over a glassfibre pad, washed consecutively with water, ethanol and diethyl ether and dried, shielded from light, under vacuum over P_2O_5 to give 448 mg of **90** as an off-white powder.

(((3-(N-(benzyloxy)acetamido)-1-fluoropropyl)phosphoryl)bis(oxy))bis(methylene) bis(2,2-dimethylpropanoate) (91). Disilver salt **90** (430.74 mg, 0.83 mmol) was dissolved in toluene (2 mL) and iodomethyl pivalate (703 mg, 2.91 mmol), dissolved in 1 mL toluene, was added. The reaction mixture was shielded from light and stirred at room temperature for 5 hours, after which TLC showed that all starting material was consumed. Water and ethyl acetate were added and the aqueous layer was extracted three times with ethyl acetate. The combined organic fractions were washed with brine and dried over anhydrous $MgSO_4$. Purification of the crude product by flash column chromatography (hexanes/ethyl acetate: 75/25 to 50/50) yielded 143 mg of **91** as a colorless oil (32%).^{1}H NMR (300 MHz, CHLOROFORM-d) δ ppm 1.09 - 1.35 (m, 18 H) 2.09 (s, 3 H) 2.11 - 2.24 (m, 1 H) 2.24 - 2.37 (m, 1 H) 3.68 - 3.98 (m, 2 H) 4.70 - 4.87 (m, 2 H) 4.95 (dt, J=9.81, 3.73 Hz, 1 H) 5.54 - 5.86 (m, 4 H) 7.28 - 7.51 (m, 5 H); ^{13}C NMR (75 MHz, CHLOROFORM-*d*) δ ppm 20.49, 26.78, 26.79, 27.52 (d, J= 18.80 Hz), 38.71, 38.72, 41.38, 76.54, 81.81 (dd, app. t, J=6.91 Hz), 86.16 (dd, J= 183.27Hz, J=171.40 Hz), 128.76, 129.08, 129.34, 134.16, 172.59, 176.69, 176.73; HRMS (ESI) m/z 534.2274 [$(M+H)^+$, calcd for $C24H38FNO9P^+$ 534,2263]

(((3-(N-(benzyloxy)acetamido)-1-fluoropropyl)phosphoryl)bis(oxy))bis(methylene) diisopropyl dicarbonate (92). The title compound was synthesized from disilver salt **90** (450 mg, 0.87 mmol) and iodomethyl isopropyl carbonate (743 mg, 3.05 mmol) using the same method as described for **91**, giving 80 mg (17%) of **92** as a colourless oil. 1H NMR (300 MHz, CHLOROFORM-d) δ ppm 1.30 (d, J=6.15 Hz, 12 H) 2.08 (s, 3 H) 2.12 - 2.41 (m, 2 H) 3.70 - 3.91 (m, 2 H) 4.74 - 4.86 (m, 2 H) 4.86 - 5.04 (m, 2 H) 5.70 (dd, J=12.45, 3.37 Hz, 4 H) 7.29 - 7.51 (m, 5 H); ^{13}C NMR (75 MHz, CHLOROFORM-*d*) δ ppm 20.43, 21.52, 27.35 (dd, J= 19.90 Hz, J= 1.38 Hz), 41.39, 73.44 (dd, J= 3.32Hz, J=0.55 Hz), 76.48,

84.38 (dt, app. t, J= 6.08 Hz), 86.17 (dd, J= 183.00 Hz, J=172.49 Hz), 128.68, 128.98, 129.26, 134.13, 152.90, 152.93, 172.52;HRMS (ESI) m/z 538.1822 [$(M+H)^+$, calcd for $C_{22}H_{34}FNO_{11}P^+$ 538,1848]

(((1-fluoro-3-(N-hydroxyacetamido)propyl)phosphoryl)bis(oxy))bis(methylene) bis(2,2-dimethylpropanoate) (93). 91 (145 mg, 0.27 mmol) was dissolved in freshly distilled THF (2.7 mL), Pd (10% wt. on activated carbon) was added and the mixture was stirred under a hydrogen atmosphere at room temperature. After 6 hours, the reaction mixture was filtered over a glassfibre pad and the filter was rinsed with ethyl acetate. The combined organic fractions were evaporated and the resulting oil was purified by flash column chromatography (hexanes/ethyl acetate: 50/50 to 25/75) to give 104 mg (87 %) of **93** as a colourless oil. ^{1}H NMR (300 MHz, CHLOROFORM-d) δ ppm 1.09 - 1.44 (m, 18 H) 2.15 (s, 3 H) 2.13 - 2.27 (m, 1 H) 2.30 - 2.39 (m, 1 H) 3.59 - 3.80 (m, 2 H) 4.95 – 5.18 (m, 1 H) 5.55 - 5.90 (m, 4 H); ^{13}C NMR (75 MHz, CHLOROFORM-*d*) δ ppm 20.50, 26.79, 26.80, 27.58 (d, J= 18.75 Hz), 38.66, 38.67, 41.55, 81.10 (dd, app. t, J=6.80 Hz), 86.70 (dd, J= 185.23Hz, J=171.92 Hz), 173.44, 177.00, 177.75; HRMS (ESI) m/z 444.1809 [$(M+H)^+$, calcd for $C_{17}H_{32}FNO_9P^+$ 444,1793]

(((1-fluoro-3-(N-hydroxyacetamido)propyl)phosphoryl)bis(oxy))bis(methylene) diisopropyl dicarbonate (94). The title compound was synthesized from **92** (80 mg, 0.149 mmol) using the same method as described for **93**, giving 58 mg (89%) of **94** as a colourless oil. 1H NMR (300 MHz, CHLOROFORM-d) d ppm 1.15 - 1.42 (m, 12 H) 1.95 - 2.33 (m, 3 H) 2.36 (br. s., 2 H) 3.53 - 4.21 (m, 2 H) 4.70 - 5.09 (m, 3 H) 5.52 - 5.89 (m, 4 H); ^{13}C NMR (75 MHz, CHLOROFORM-*d*) δ ppm 14.07, 21.54, 27.33, 27.55, 29.65, 44.03, 73.10, 73.63, 73.75, 84.57, 152.92, 152.95; ^{13}C NMR (75 MHz, CHLOROFORM-*d*) δ ppm 14.07, 21.54, 27.42 (dd, J= 19.50 Hz, J= 1.20 Hz), 41.39, 73.44 (dd, J= 3.32Hz, J=0.55 Hz), 84.57 (dt, app. t, J= 6.10 Hz), 86.17 (dd, J= 184.12 Hz, J=172.55 Hz), 152.90, 152.93, 172.52 ^{31}P NMR (121.45MHz, $CDCl_3$) δ 18.01 (d, $^2J_{PF}$ = 68.2 Hz);HRMS (ESI) m/z 448.1390 [$(M+H)^+$, calcd for $C_{15}H_{28}FNO_{11}P^+$ 448,1379]

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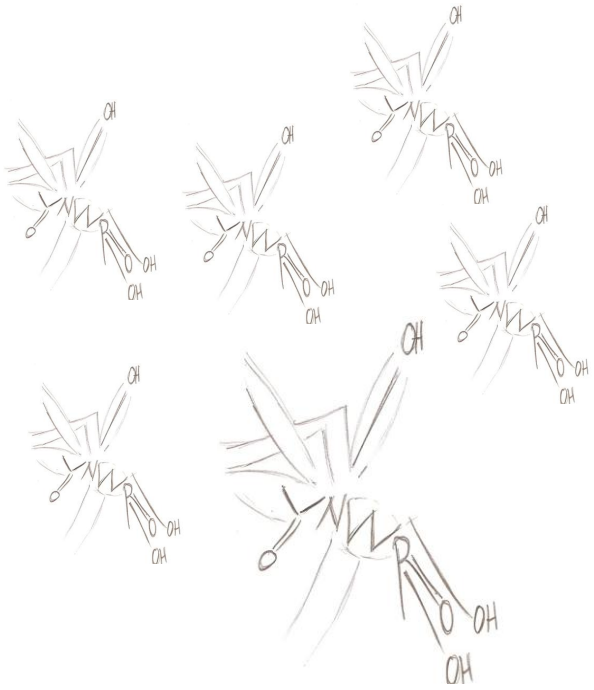


Chapter IV

Conformationally restricted (retro)hydroxamates

IV.Conformationally restricted (retro)hydroxamates

IV.A Rationale: Conformational Analysis of Hydroxamates

Judging from the bond lengths in hydroxamic acids and the planarity of their structure, a partial double-bond character is expected for the carbonyl-nitrogen bond[1]. This implies the existence of stable conformations with restricted rotation about this bond[2], with an average bond rotation energy barrier of 67 kJ/mol (compared to 50 kJ/mol for an ester and 75-95 kJ/mol for an amide). Kolasa[3] studied the influence of *N*-substituents, acyl groups and solvent polarity on the conformation of structurally small (mainly *N*-formyl and *N*-acetyl) hydroxamic acids and came to the following conclusions (summarized in Figure IV.1):

1. *N*-formyl and *N*-acetyl hydroxamates show Z-E-equilibrium in solution with a Z/E ratio dependent on solvent polarity: the higher the polarity of the solvent, the more E-conformer in the mixture.

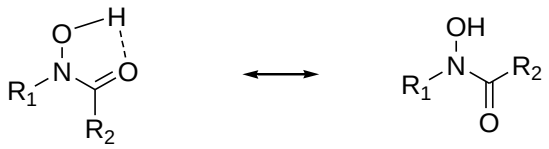
2. Bulky *N*-substituents in formyl- and acetylhydroxamic acids favor the presence of the Z-conformer.

3. Substitution of the *N*-formyl group for an *N*-acetyl group favours the formation of the E-conformer.

4. The presence of electron-donating groups in α-position of the *N*-substituent favours the formation of E-conformer.

5. Protonation of the hydroxamic acid group leads to a decreased amount of the E-conformer.

6. For N-unsubstituted hydroxamates, the Z-conformation is preferred (mainly in the solid state).



cis (Z)
apolar media
bulky R_1, R_2 = H
protonated form

trans (E)
polar media
R_2 = Me
R_1 = electron donors in α

FigureIV.1: Cis-trans (E-Z) equilibrium of *N*-alkylhydroxamic acids and the factors influencing it[3]

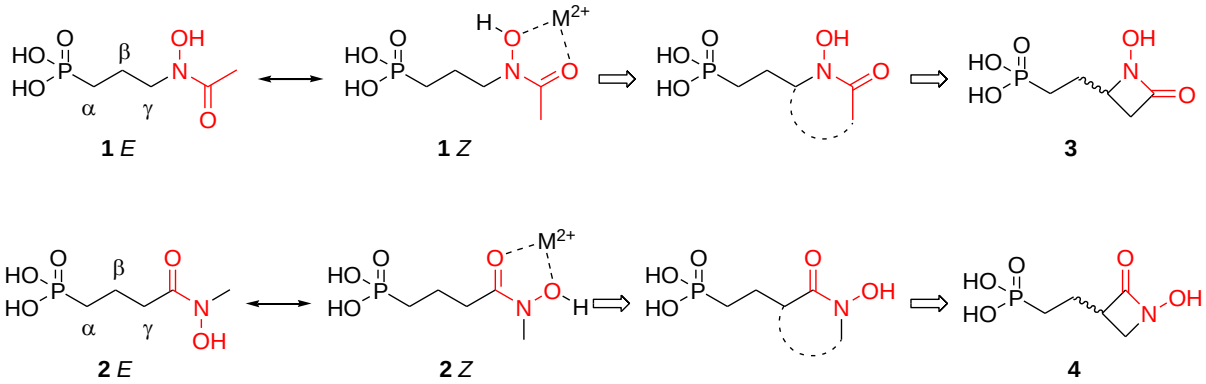
As stated in the general introduction, the retrohydroxamate moiety is one of the main features of fosmidomycin, FR900098 and their (bioactive) analogues. Apart from a few exceptions in the form of larger/more lipophilic acyl residues, only inverting the retrohydroxamate into a (preferentially *N*-methylated) hydroxamate is allowed in order not to lose all DXR inhibitory activity. Both the retrohydroxamate and the hydroxamate groups act as strong bidentate chelators for the bivalent metal cation (usually Mn^{2+}) in the active site of the enzyme[4], a function for which it is necessary to adopt the Z-conformation[5]. Under standard conditions, however, we can assume that the (retro)hydroxamate in fosmidomycin and its analogues will be present as a mixture of E and Z conformer and that, according to the above, *in vivo* the unwanted E-conformer will be favoured. Hence, better DXR ligands could be designed by stabilizing the Z conformer in fosmidomycin analogues. Towards this end, the (retro)hydroxamate may be locked in its metal-chelating (cis) conformation by incorporating it into a ring structure, which means (see also Figure 2):

-For the retrohydroxamate: interconnection of the acetyl-CH_3 with the γ-carbon of the propyl spacer.

-For the hydroxamate: interconnection of the *N*-methyl with the γ-carbon of the propyl spacer.

This principle has been applied before in the development of hydroxamic acid-containing molecules, usually by incorporation of the hydroxamate into a six-membered ring[6], a functionality that is also present in several natural products[7]. In the case of fosmidomycin analogues, literature data (see Chapter I: Introduction) indicate that the active site of DXR is very tight especially around the aforementioned divalent metal cation and any sterical bulk around the (retro)hydroxamate is

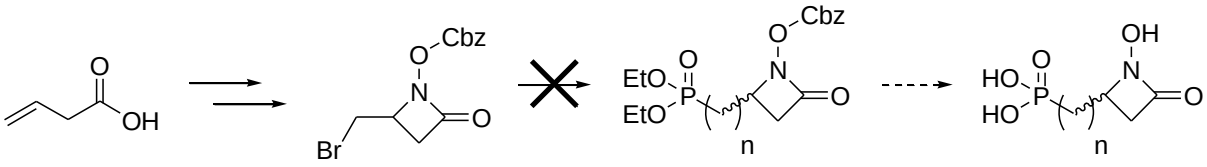
disfavoured. In the case of hydroxamate analogues, the only 'bulk' that is tolerated is the methyl group: N-methylation has resulted in a higher potency[8, 9]. For retrohydroxamate analogues exchanging the *N*-formyl hydrogen of fosmidomycin for a methyl group as in FR900098 tends to slightly improve the inhibitory activity. Larger *N*-alkyl (for hydroxamates) or *N*-acyl (for retrohydroxamates) substituents generally lead to a significant drop in inhibitory activity. With respect to the interconnection strategy outlined above, this means a direct connection of the mentioned methyl groups with the spacer γ-carbon, implying the synthesis of *N*-hydroxybetalactams or 3/4-phosphonoethyl *N*-hydroxyazetidin-2-ones ("NHAs") **3** and **4**.



FigureIV.2: (Retro)Hydroxamate E/Z-equilibrium in FR900098 (1) and its hydroxamate analogue (2), metal binding by the Z-conformers and restriction of the preferred conformation by cyclization

IV.B Previous Attempts

A first attempt toward the synthesis of the 4-(ω-phosphono**e**thyl)-NHA (**3**) and the 4-(ω-phosphono**m**ethyl)-NHA (**9**) analogues ("retrohydroxamate *N*-hydroxybetalactam" analogues with 1- resp. 2-carbon spacer) was made by Timothy Haemers from our group[10], based on a procedure by Rajendra and Miller[11].



5 **6** **7** n = 1 **8** n = 2 **9** n = 1 **3** n = 2

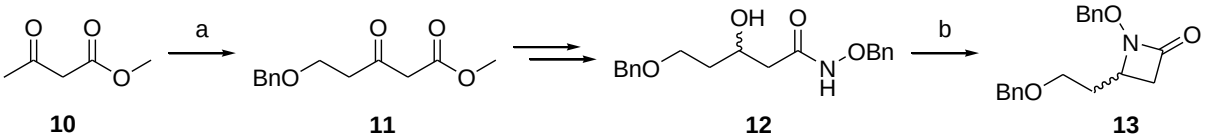
SchemeIV.1: Attempted synthesis of N-hydroxyazetidinone analogues by Haemers[10]

The key step in this procedure was the construction of benzyl (2-(bromomethyl)-4-oxoazetidin-1-yl) carbonate (or "CBz-protected bromomethyl *N*-hydroxybetalactam") **6** from but-3-enoic acid **5** (vinylacetic acid). The so formed alkyl bromide **6** would then be phosphonylated by either an Arbuzov reaction, leading to the 'short-chain' analogue **9** or by a substitution with a protected methylphosphonate carbanion, leading to the 'long-chain' analogue **3,** but unfortunately both attempts failed. Most probably this is the result of the inherent susceptibility of *N*-hydroxyazetidin-2-ones toward nucleophilic ring opening, which will be further illustrated throughout this chapter.

IV.C New Strategy

An elegant strategy for the synthesis of benzyl protected 4-alkyl-NHA **13** was developed by the group of Miller, based on the intramolecular ring closure of a β-hydroxy *O*-benzylated hydroxamate **12** under Mitsunobu conditions[12] as depicted in scheme 2. This β-OH-hydroxamate is derived from a β-keto-ester **11** through a ketone reduction, ester hydrolysis and amidation with *O*-benzylhydroxylamine. An interesting fact is that β-keto-ester **11** is synthesized by alkylation of the 4-

position of methyl acetoacetate **10** (via its dianion[13]), potentially allowing for variation in the alkyl chain of the azetidinone.

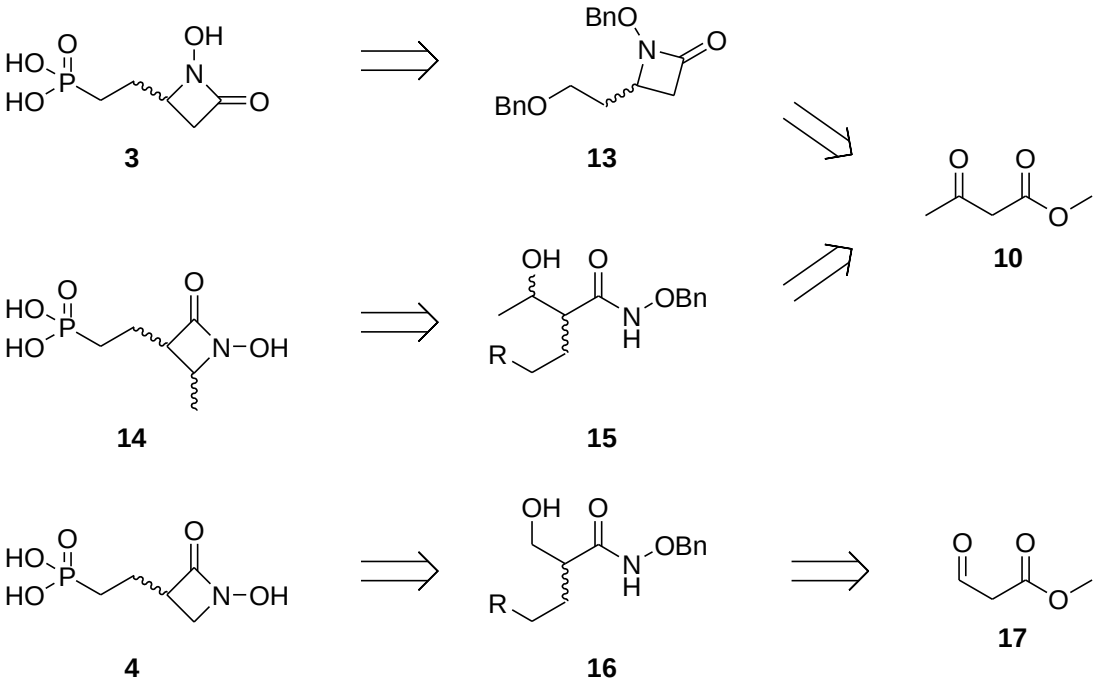


10 **11** **12** **13**

Reagents and Conditions: (a) NaH (1eq.), nBuLi (1eq.), BOMCl, THF; (b) Ph_3P, DIAD, THF

SchemeIV.2: Synthesis of protected *N*-hydroxyazetidin-2-ones from betaketo-esters via a Mitsunobu reaction according to Miller et al.[12]

Applying this strategy to the synthesis of retrohydroxamate analogue **3** is evident: only a formal exchange of the benzyl ether in **13** for a phosphonic acid group should give access to the desired product.



3 **13** **10**

14 **15**

4 **16** **17**

Figure IV.3: Retrosynthetic analysis towards N-hydroxyazetidinone FR900098 analogues

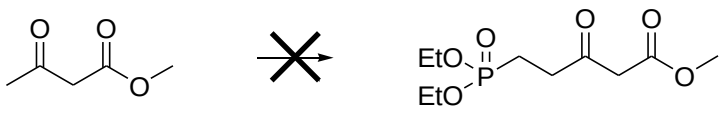
But also hydroxamate analogue **14** could probably be synthesized from the same starting compound **10.** Alkylation of methyl acetoacetate in 2-position (via its 'first' anion) would result in the 3-methylated 'inverted' *N*-hydroxybetalactam **14**. The non-methylated version of this molecule (**4**) should be synthesized starting from a formylacetate **17** instead of an acetoacetate, although (polymerization) problems are to be expected with such an aldehyde. Formylation of the γ-carbon of a γ-butyrylphosphonate could be a workaround here.

IV.D Chemistry

We directed our attention first toward 4-phosphonoethyl NHA **3** because its synthesis was expected to be highly analogous to the described procedure by Lee et al.[12]

IV.D.1. First Approach: phosphonomethylation of acetoacetate

As stated above, the formal exchange of the alkyl benzylether in **13** with a phosphonate group would result in desired analogue **3**. With the difficulties in mind experienced by Haemers when trying to introduce a phosphonate onto an NHA, we opted to introduce the phosphonate group as early as possible in the synthetic sequence, preferably before ring closure. Therefore, we tried to alkylate the methyl acetoacetate dianion with diethyl chloromethylphosphonate (instead of BOMCl used by Miller), unfortunately without success.



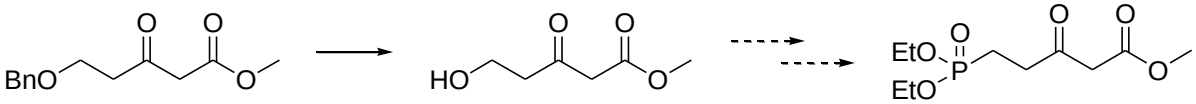
10 **18**

Reagents and Conditions: (i) NaH (1 eq.), nBuLi (1.1 eq.), THF, -40° C, (ii) $(EtO)_2P(O)CH_2Cl$ (1eq.)

SchemeIV.3: Attempted phosphonomethylation of methyl acetoacetate

IV.D.2. Second Approach: phosphonate substitution of benzyl ether

As an obvious alternative for the unsuccessful phosphonomethylation we repeated Lee's procedure for the synthesis of benzylether **11**. Subsequent debenzylation and turning the resulting alcohol into a leaving group should allow to install the phosphonate functionality.



11 **19** **18**

Reagents and conditions: see table

Scheme IV.4: Debenzylation and attempted phosphonylation of a β-keto ester

Table IV.1: Debenzylation conditions tested

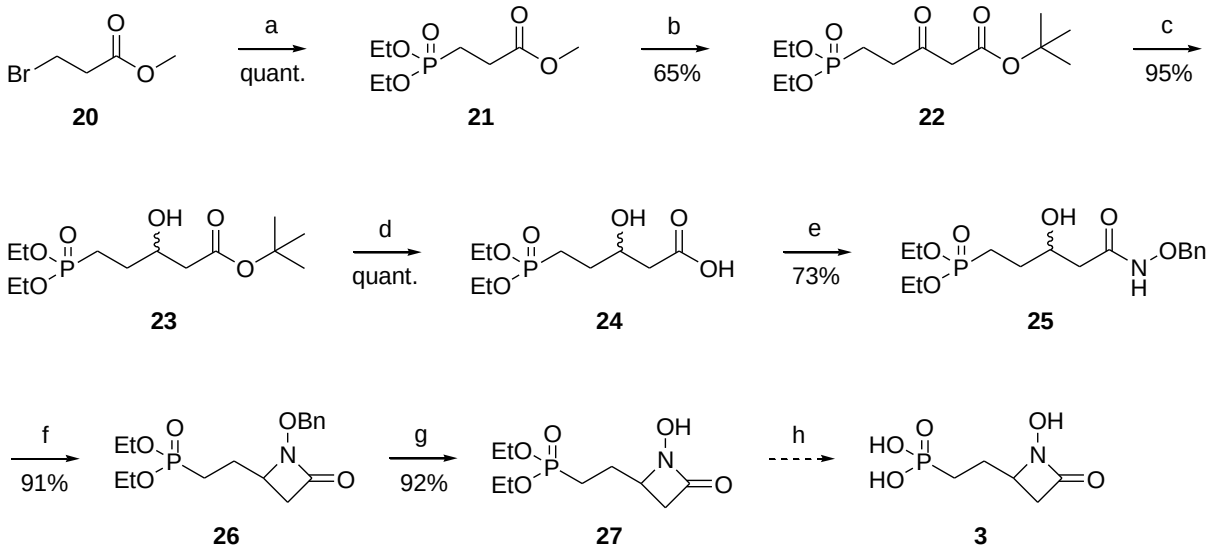
Entry	Method	Result
1	Pd/C, H_2 atmospheric pressure, EtOAc	No reaction
2	Pd black, H_2 bubbling, EtOAc	No reaction
3	Pd black, H_2 bubbling, THF	No reaction
4	Pd/C, formic acid, MeOH	Total conversion

To our surprise, **11** turned out to be very reluctant towards debenzylation. Probably this product, or a contaminant, poisoned the catalyst because only when we turned toward the more aggressive hydrogen transfer debenzylation with formic acid, good conversion into alcohol **19** was seen. Small scale test reactions also showed that the phosphonylation of **19** was problematic. Therefore, a new and more robust approach was developed:

IV.D.3. Third Approach: Claisen condensation of phosphonopropionate

Instead of trying to introduce the phosphonate functionality onto a β-keto-ester, it turned out to be much more convenient to synthesize the desired δ-phosphono-β-keto-ester from an alkyl phosphonopropionate and an alkyl acetate via a Claisen condensation. Thus, methyl (diethylphosphono)propionate **21** was synthesized first through an Arbuzov reaction of methyl 3-bromopropionate **20** and triethyl phosphite, and subsequently used in a Claisen condensation with

tert-butyl acetate using lithium *N*-isopropyl-*N*-cyclohexylamide (LICA) as a base[14] with good result. We chose for a *t*-butyl ester because it allows for an acidic hydrolysis (TFA) with simple removal of all volatiles *in vacuo*, obviating the need for the tedious extraction of a carboxylic acid.



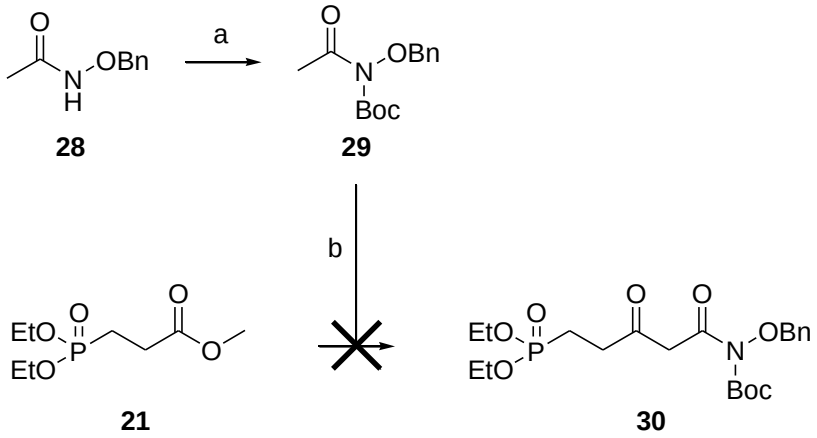
Reagents and Conditions: (a) $(EtO)_3P$, 100 °C; (b) LICA, tBuOAc, THF, -60 °C; (c) $NaBH_4$, MeOH, 0 °C; (d) TFA, Et_3SiH, CH_2Cl_2, 0 °C; (e) EDC.HCl, $BnONH_2$, THF, H_2O, rt; (f)DIAD, Ph_3P, THF; (g)H_2, Pd/C, MeOH, rt, 45 min; (h) TMSBr, CH_2Cl_2 or HCl, H_2O

SchemeIV.5: Attempted synthesis of the 4-phosphonoalkyl *N*-hydroxyazetidin-2-one analogue

With phosphonate **22** in hands, we envisaged to follow a synthetic route analogous to Lee's protocol, as depicted in Scheme 5: reduction of ketone **22** with sodium borohydride proceeded in excellent yield. The *t*-butyl ester was then hydrolyzed with TFA resulting in the free acid (**24**) which was activated using EDC and subsequently coupled with *O*-benzylhydroxylamine hydrochloride in the presence of triethylamine. The ring closure by intramolecular Mitsunobu reaction gave NHA **26** in a modest yield upon first attempt, with as a main side-product a dimeric species resulting from intermolecular Mitsunobu reaction. This side-reaction could be suppressed by diluting the reaction mixture from the original 0.1M to 0.05M (starting material concentration). The crude reaction mixture after Mitsunobu reaction also contained a considerable amount of triphenylphosphine oxide, which eluted very close to the desired product and complicated the purification. Three flash

chromatographic separations were necessary to obtain **26** in pure form. although we made two efforts to reduce the number of synthetic steps.

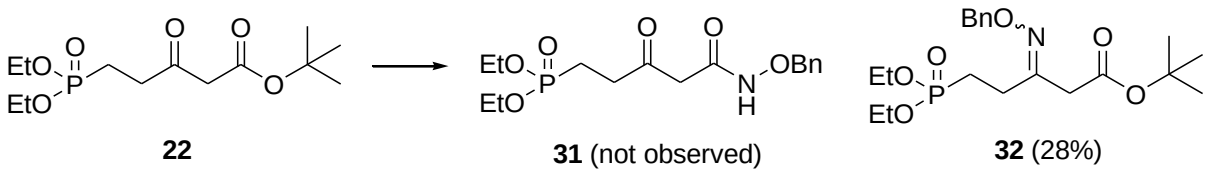
Two attempts were made to reduce the number of steps in this synthetic route. First, we tried to make the synthesis a little more convergent by trying to use the enolate of protected oxyamide **29** in the Claisen condensation with (diethylphosphono)propionate **21**, alas without success. To the best of our knowledge, the enolization and subsequent use as a nucleophile of protected acetohydroxamates has only very rarely been reported in literature.



Reagents and Conditions: (a) Boc_2O, DMAP, Et_3N, CH_2Cl_2, rt, on.;
(b) LICA, THF, -70° C to rt, on.

Scheme IV.6: Attempted oxyamide enolate formation and addition to methyl ester **21**

Secondly, we attempted the direct displacement of the *t*-butyl ester in **22** with *O*-benzylhydroxylamine in toluene[15], but only the oxime formation (**32**) from reaction with the ketone was noticed here, and no trace of the desired oxyamide **31** could be detected.



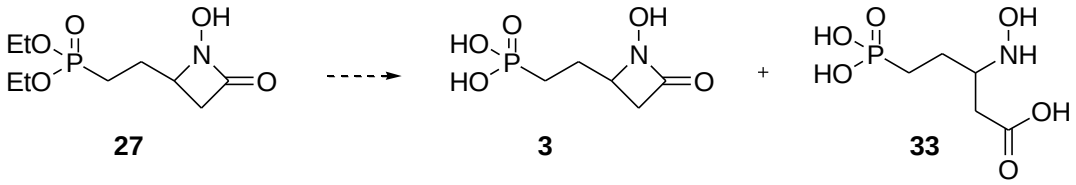
22 **31** (not observed) **32** (28%)

Reagents and Conditions: $BnONH_2$, toluene, 100°C, 1h

Scheme IV.7: Attempted direct ester – oxyamide conversion

In the end, a sufficient amount of penultimate intermediate **26** was synthesized using the 'original' protocol and final deprotection was attempted. The first step of the final deprotection of **26**, i.e. reductive debenzylation of the NHA, proceeded smoothly under standard conditions (Pd/C, ethanol, hydrogen at atmospheric pressure for 1 hour) resulting in a very pure and clean product (**27**).

Unfortunately, attempts to deprotect the phosphonate moiety of **27** with TMSBr led to extensive hydrolytic opening of the NHA ring with formation of β-oxyamino acid **33** as demonstrated by MS analysis. Also when we tried acidic hydrolysis of the phosphonate esters (aqueous HCl), the same ring-opening occurred, even at a faster rate than the ester removal itself. These facts once more illustrate the susceptibility of the NHA structure toward hydrolysis and ring-opening.



27 **3** **33**

Reagents and Conditions: HCl, H_2O; or: (i) TMSBr, CH_2Cl_2, (ii) THF, H_2O

SchemeIV.8: Hydrolytic NHA-ring opening upon phosphonate deprotection

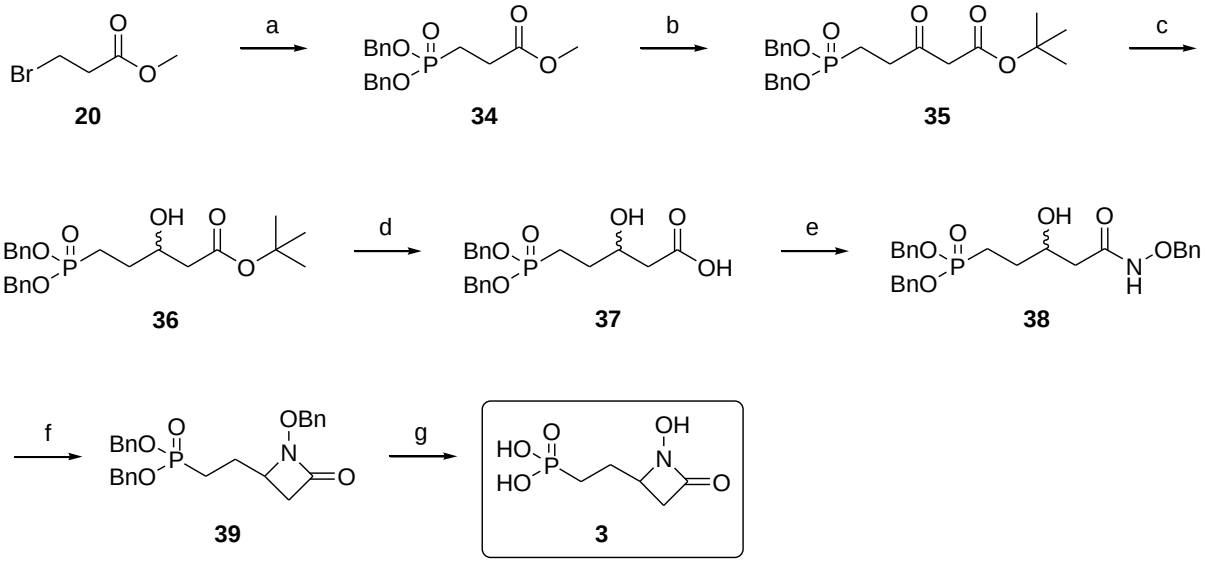
On a positive note, however, the ring-structure perfectly survived the hydrogenative debenzylation in the penultimate step. This called for an "all-benzyl" protection strategy for this molecule with only a final debenzylation as the last synthesis step. We did not use this strategy in the

first place because, on the one hand, most phosphonate building blocks are commercially available as ethyl esters. On the other hand, as was also described in Chapter II, benzyl phosphonate esters are more susceptible toward basic/nucleophilic conditions such as the Claisen reaction employed here. Moreover, the more (types of) benzyl protecting groups present in a molecule, the longer one potentially needs to hydrogenate in order to remove them all. In the case of oxyamines there is always the risk of overreduction resulting in oxyamine *N*-deoxygenation. With regard to phosphonate protecting groups (particularly in fosmidomycin), in the synthesis of analogues it is often a dilemma to choose for (potentially too) reductive conditions using Pd/C, or to use (potentially too) nucleophilic/acidic conditions using TMSBr. Anyhow, we decided to try and resynthesize the 4-phosphonoethyl NHA using benzyl protecting groups for both the phosphonate and the hydroxamide moieties.

IV.D.4. Fourth Approach: Benzyl protected phosphonate

Methyl (dibenzylphosphono)propionate **34** was synthesized by a Michaelis-Becker reaction from methyl 3-bromopropionate **20** and dibenzyl phosphite in excellent yield. Because of the low volatility of benzyl halides, Arbuzov reactions involving tribenzyl phophite are seldom successful and for the synthesis of dibenzyl phosphonates the Michaelis-Becker reaction is preferred.

Much to our delight, the phosphonate benzyl esters survived the subsequent Claisen-reduction-hydrolysis-amidation cascade without any problems, affording **38** in an acceptable overall yield.



Reagents and Conditions: (a) $(BnO)_2POH$, NaH, DMF, 0°C, 99%; (b) LICA, tBuOAc, THF, -75°C, 78%; (c) $NaBH_4$, MeOH, 0°C, 71%; (d) TFA, Et_3SiH, CH_2Cl_2, 0°C, quant; (e) EDC.HCl, $BnONH_2$, THF, H_2O, rt°, 66%; (f)DIAD, Ph_3P, THF, 79%; (g)H_2, Pd/C, MeOH, rt°, 3h, quant.

Scheme IV.9: Synthesis of NHA analogue **3** via benzylphosphonate intermediates

Also the intramolecular Mitsunobu reaction went flawless, apart from the tedious separation of triphenylphosphine oxide from the product afterwards. Finally, both the hydroxamate benzyl ether and phosphonate benzyl esters in **39** were removed by hydrogenation over Pd/C in methanol. Use of a non-protic/non-nucleophilic solvent would be preferable here, but to our knowledge no such solvent that is also capable of dissolving both the perbenzylated starting material as well as all reaction intermediates and the deprotected phosphonate **3** exists. Fortunately, upon careful monitoring of the reaction by MS we were able to restrict the reaction time to a minimum and no breakdown of the NHA was noticed, resulting in clean deprotected **3**. This product could be stored dry at -38 °C for several months without breakdown. However, upon dissolving the product in water or aqueous buffer it hydrolizes quite fast to the ring-opened product. Because of this inherent low hydrolytic stability and a low DXR inhibitory potential in preliminary enzyme inhibition tests (18.83 % EcDXR inhibition at 100nM), we decided to cancel all efforts to synthesize the hydroxamate-based isomer **4**.

IV.E Experimental Details

General Methods and Materials. See III.F.

Methyl 5-hydroxy-3-oxopentanoate (19). Methyl 5-(benzyloxy)-3-oxopentanoate (398 mg, 1.7 mmol) was dissolved in methanol (16 mL) and formic acid (0.85 mL). Pd (10 % wt. on activated carbon) was added and the mixture was stirred overnight at room temperature. The reaction mixture was filtered over a Celite pad, the pad was rinsed with ethyl acetate, and all volatiles were evaporated. The resulting crude material was purified by flash column chromatography (hexanes/ethyl acetate: 50/50), yielding 142 mg (57 %) of **19** as a colorless liquid. Analytical data were in accordance with previously reported data[16].

Methyl 3-(diethoxyphosphoryl)propanoate (21). Triethylphosphite (5.2 mL, 30 mmol) and methyl 3-bromopropionate (3.27 mL, 30 mmol) were mixed in a three necked flask and a distillation setup was installed. The flask was heated at 80 °C for 24 hours under a N_2 atmosphere, and then a slow stream of N_2 was blown into the reaction flask, over the reaction mixture and outward via the distillation cooler and receiver flask in order to enhance ethyl bromide boiling out of the reaction mixture. While maintaining this N_2 stream, the temperature was raised to 100 °C and stirring was continued for 12 hours, after which ^{31}P-NMR confirmed the total consumption of triethyl phosphite. Remaining traces of starting materials were then distilled out of the reaction mixture under vacuum, leaving pure **21** (6.70 g, quant.) as a colorless liquid. Analytical data were in accordance with previously reported data[17].

***tert*-Butyl 5-(diethoxyphosphoryl)-3-oxopentanoate (22).** *N*-isopropylcyclohexylamine (1.74 mL, 10.5 mmol) was dissolved in THF (5 mL) and cooled to -60 °C. nBuLi (4 mL of a 2.5 M solution in hexanes) was added, the solution was allowed to warm to room temperature and cooled again to -60 °C. *tert*-Butyl acetate (1.49 mL, 11 mmol) was added and the mixture was stirred for 20 minutes before it was cannulated into a flask containing a solution of methyl 3-(diethoxyphosphoryl)propanoate **21** (1.12 g, 5 mmol) in THF (10 mL) cooled to -60 °C. After 25

minutes of stirring the reaction was quenched by the addition of acetic acid (1 mL) and saturated aqueous NH_4Cl, and extracted three times with ethyl acetate. The combined organic fractions were washed with saturated aqueous $NaHCO_3$, brine and dried over anhydrous Na_2SO_4. Purification of the crude product by flash column chromatography (hexanes/ethyl acetate: 50/50 to 0/100) yielded 998 mg (65 %) of pure **22** as a colorless oil. ^{1}H NMR (300 MHz, CHLOROFORM-*d*) δ ppm 1.30 (t, *J*=7.03 Hz, 6 H) 1.39 - 1.54 (m, 9 H) 1.87 - 2.14 (m, 3 H) 2.70 - 2.94 (m, 2 H) 3.36 (s, 2 H) 3.95 - 4.19 (m, 4 H); HRMS (ESI) m/z 309.1477 [$(M+H)^+$, calcd for $C13H26O6P^+$ 309,1462]

***tert*-Butyl 5-(diethoxyphosphoryl)-3-hydroxypentanoate (23).** To a stirred solution of **22** (100 mg, 0.32 mmol) in methanol was added sodium borohydride (13 mg, 0.36 mmol) at 0 °C. After 30 minutes, the reaction was quenched by the addition of saturated aqueous NH_4Cl, and extracted three times with ethyl acetate. The combined organic fractions were washed with brine and dried over anhydrous Na_2SO_4. Purification of the crude product by flash column chromatography (hexanes/ethyl acetate: 75/25 to 50/50) yielded 94 mg (95 %) of **23** as a colorless oil. ^{1}H NMR (300 MHz, acetone) δ ppm 1.27 (t, *J*=7.03 Hz, 6 H) 1.38 - 1.49 (m, 9 H) 1.59 - 1.99 (m, 4 H) 2.36 (dd, *J*=6.44, 1.76 Hz, 2 H) 3.89 - 4.17 (m, 5 H); HRMS (ESI) m/z 311.1605 [$(M+H)^+$, calcd for $C13H28O6P^+$ 311,1618]

5-(Diethoxyphosphoryl)-3-hydroxypentanoic acid (24). *tert*-Butyl 5-(diethoxyphosphoryl)-3-hydroxypentanoate **23** (479 mg, 1.54 mmol) was dissolved in dichloromethane, followed by the addition of triethylsilane (0.62 mL, 3.86 mmol) and TFA (1.77 mL, 23 mmol) at 0 °C. The reaction mixture was stirred at room temperature for 1.5 hours, and all volatiles were removed *in vacuo*. Coevaporation with toluene afforded 568 mg (quantitative) of an off-white oil that was used as such in the next reaction.

Diethyl (5-((benzyloxy)amino)-3-hydroxy-5-oxopentyl)phosphonate (25). 5-(Diethoxyphosphoryl)-3-hydroxypentanoic acid **24** (391 mg, 1.54 mmol) and O-benzylhydroxylamine (free base, 284 mg, 2.31 mmol) were dissolved in THF (8 mL) and water (3 mL). EDC (442 mg, 2.31 mmol) was added and the reaction mixture was stirred overnight at room

temperature. Aqueous HCl (0.1 M) was added and the mixture was extracted three times with ethyl acetate. The combined organic fractions were washed with saturated aqueous K_2CO_3, brine and dried over anhydrous Na_2SO_4. Purification of the crude product by flash column chromatography (dichloromethane/methanol: 95/5) yielded 406 mg (73 %) of **25** as a colorless oil. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 1.22 (t, *J*=7.18 Hz, 6 H) 1.33 - 1.53 (m, 1 H) 1.53 - 1.92 (m, 3 H) 2.07 (d, *J*=6.44 Hz, 2 H) 3.69 - 3.89 (m, 1 H) 3.89 - 4.13 (m, 4 H) 4.78 (s, 2 H) 4.86 (d, *J*=5.56 Hz, 1 H) 7.19 - 7.53 (m, 5 H) 10.96 (s, 1 H); HRMS (ESI) m/z 360.1575 [$(M+H)^+$, calcd for $C16H27NO6P^+$ 360,1571]

Diethyl (2-(1-(benzyloxy)-4-oxoazetidin-2-yl)ethyl)phosphonate (26). Diethyl (5-((benzyloxy)amino)-3-hydroxy-5-oxopentyl)phosphonate **25** (377 mg, 1.05 mmol) and triphenylphosphine (304 mg, 1.16 mmol) were dissolved in THF (20 mL) and DIAD (0.23 mL, 1.16 mmol) was added while stirring at room temperature. After 7 hours, all volatiles were removed *in vacuo* at 30 °C. The resulting crude was purified by two consecutive flash columns (I: dichloromethane/methanol: 95/5; II: hexanes/acetone: 50/50 to 0/100), giving 326 mg (91 %) of **26** as a colourless oil. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 1.22 (t, *J*=7.18 Hz, 6 H) 1.55 - 1.93 (m, 4 H) 2.42 (dd, *J*=13.77, 2.34 Hz, 1 H) 2.71 (dd, *J*=13.77, 5.27 Hz, 1 H) 3.84 (dd, *J*=5.27, 2.34 Hz, 1 H) 3.89 - 4.10 (m, 4 H) 4.77 - 5.00 (m, 2 H) 7.27 - 7.51 (m, 5 H); HRMS (ESI) m/z 342.1460 [$(M+H)^+$, calcd for $C16H25NO5P^+$ 342,1465]

Diethyl (2-(1-hydroxy-4-oxoazetidin-2-yl)ethyl)phosphonate (27). Diethyl (2-(1-(benzyloxy)-4-oxoazetidin-2-yl)ethyl)phosphonate **26** (287 mg, 0.84 mmol) was dissolved in methanol (9 mL), Pd (10% wt. on activated carbon) was added and the mixture was stirred under a hydrogen atmosphere at room temperature. After 1 hour, the reaction mixture was filtered over a celite pad and the filter was rinsed with ethyl acetate. The combined organic fractions were evaporated and the resulting oil was purified by flash column chromatography (dichloromethane/methanol: 95/5 to 90/10) to give 195 mg (92 %) of **27** as a colourless oil. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 1.09 - 1.32 (m, 6 H) 1.58 -

1.94 (m, 4 H) 2.36 (dd, *J*=13.47, 2.34 Hz, 1 H) 2.70 (dd, *J*=13.47, 4.98 Hz, 1 H) 3.64 - 3.83 (m, 1 H) 3.86 - 4.14 (m, 4 H) 9.97 (s, 1 H); HRMS (ESI) m/z 252.1003 [$(M+H)^+$, calcd for $C_9H_{19}NO_5P^+$ 252,0995]

Methyl 3-(dibenzoxyphosphoryl)propanoate (34). To a cooled (0 °C) suspension of sodium hydride (1.53 g of a 60 % suspension in mineral oil, 38.3 mmol) in DMF (100 mL) was added dibenzyl phosphite (10.58 mL, 38.3 mmol) under vigorous stirring. The icebath was removed and the solution was allowed to warm to room temperature. Methyl 3-bromopropionate (2.87 mL, 25.5 mmol) was then added and the reaction mixture was stirred overnight at room temperature. The reaction was quenched by the addition of saturated aqueous NH_4Cl, diluted with aqueous 0.1 M HCl and extracted three times with ethyl acetate. The combined organic fractions were washed with saturated aqueous $NaHCO_3$, brine and dried over anhydrous $MgSO_4$. Purification of the crude product by flash column chromatography (toluene/hexanes/ethyl acetate: 35/35/30 to 10/10/80) yielded 10.77 g (99 %) of **34** as a colorless oil. ^{1}H NMR (300 MHz, acetone) δ ppm 1.99 - 2.21 (m, 2 H) 2.54 (dt, *J*=13.11, 7.80 Hz, 2 H) 3.59 (s, 3 H) 4.90 - 5.19 (m, 4 H) 7.08 - 7.48 (m, 10 H); HRMS (ESI) m/z 349.1203 [$(M+H)^+$, calcd for $C_{18}H_{22}O_5P^+$ 349,1199]

***tert*-Butyl 5-(dibenzoxyphosphoryl)-3-oxopentanoate (35).** The title compound was synthesized from methyl 3-(dibenzoxyphosphoryl)propanoate **34** (10.08g, 28.9 mmol) using the same method as described for **22**, giving 9.69 g (78%) of **35** as a colourless oil that was used in the next reaction without further purification. HRMS (ESI) m/z 433.1794 [$(M+H)^+$, calcd for $C_{23}H_{30}O_6P^+$ 433,1775]

***tert*-Butyl 5-(dibenzoxyphosphoryl)-3-hydroxypentanoate (36).** The title compound was synthesized from *tert*-butyl 5-(dibenzoxyphosphoryl)-3-oxopentanoate **35** (8.65 g, 20.0 mmol) using the same method as described for **23**, giving 6.13 g (71%) of **36** as a colourless oil. ^{1}H NMR (300 MHz, acetone) δ ppm 1.30 - 1.50 (m, 9 H) 1.62 - 2.02 (m, 4 H) 2.23 - 2.40 (m, 2 H) 3.85 - 4.04 (m, 1 H) 4.07 (br. s., 1 H) 5.04 (qd, *J*=12.16, 8.05 Hz, 4 H) 7.19 - 7.51 (m, 10 H); HRMS (ESI) m/z 435.1922 [$(M+H)^+$, calcd for $C_{23}H_{32}O_6P^+$ 435,1931]

5-(Dibenzoxyphosphoryl)-3-hydroxypentanoic acid (37). The title compound was synthesized from *tert*-butyl 5-(dibenzoxyphosphoryl)-3-hydroxypentanoate **36** (1.21 g, 2.58 mmol) using the same method as described for **24**, giving 1.18 g of crude **37** that was used as such in the next reaction. HRMS (ESI) m/z 377.1171 [(M-H^+), calcd for C19H22O6P^- 377,1159]

Dibenzyl (5-((benzyloxy)amino)-3-hydroxy-5-oxopentyl)phosphonate (38). The title compound was synthesized from crude 5-(dibenzoxyphosphoryl)-3-hydroxypentanoic acid **37** (1.04 g, 2.41 mmol) using the same method as described for **25**, giving 780 mg of **38** (66 %) as a colorless oil. ^{1}H NMR (300 MHz, acetone) δ ppm 1.67 - 2.09 (m, 4 H) 2.14 - 2.29 (m, 2 H) 3.84 - 4.09 (m, 1 H) 4.87 (m, 3 H) 4.91 - 5.29 (m, 4 H) 7.20 - 7.49 (m, 15 H) 10.27 (br. s., 1 H); HRMS (ESI) m/z 484.1892 [(M-H^+), calcd for C26H31NO6P^+ 484,1884]

Dibenzyl (2-(1-(benzyloxy)-4-oxoazetidin-2-yl)ethyl)phosphonate (39). The title compound was synthesized from dibenzyl (5-((benzyloxy)amino)-3-hydroxy-5-oxopentyl)phosphonate **38** (776 mg, 1.60 mmol) using the same method as described for **39**, giving 588 mg of **39** (79 %) as a colorless oil. ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 1.55 - 1.95 (m, 4 H) 2.36 (dd, *J*=13.77, 2.34 Hz, 1 H) 2.66 (dd, *J*=13.77, 5.27 Hz, 1 H) 3.70 - 3.91 (m, 1 H) 4.76 - 4.92 (m, 2 H) 4.92 - 5.11 (m, 4 H) 7.17 - 7.54 (m, 15 H); HRMS (ESI) m/z 466.1782 [(M-H^+), calcd for C26H29NO5P^+ 466,1778]

(2-(1-Hydroxy-4-oxoazetidin-2-yl)ethyl)phosphonic acid (3). Dibenzyl (2-(1-(benzyloxy)-4-oxoazetidin-2-yl)ethyl)phosphonate **39** (48 mg, 0.103 mmol) was dissolved in methanol (1.5 mL), Pd (10% wt. on activated carbon) was added and the mixture was stirred under a hydrogen atmosphere at room temperature. After 3 hour, the reaction mixture was filtered over a 20 µm cellulose acetate syringe filter and the filter was rinsed with methanol. The combined organic fractions were evaporated *in vacuo* at 26 °C, giving 19 mg (99 %) of **3** as a clear colorless sticky gum. ^{1}H NMR (300 MHz, METHANOL-d_4) δ ppm 1.67 - 1.99 (m, 3 H) 1.99 - 2.18 (m, 1 H) 2.32 - 2.44 (m, 1 H) 2.80 (dd, *J*=13.47, 4.98 Hz, 1 H) 3.81 - 3.96 (m, 1 H); ^{13}C NMR (75 MHz, METHANOL-d_4) δ ppm 23.93 (d,

J=140.15 Hz), 26.96 (d, J=4.15 Hz), 37.80, 59.93 (d, J=18.52 Hz) 166.17; ^{31}P NMR (121 MHz, METHANOL-d_4) δ ppm 29.07; HRMS (ESI) m/z 194.0177 [(M-H$^+$), calcd for C5H9NO5P$^-$ 194,0224]

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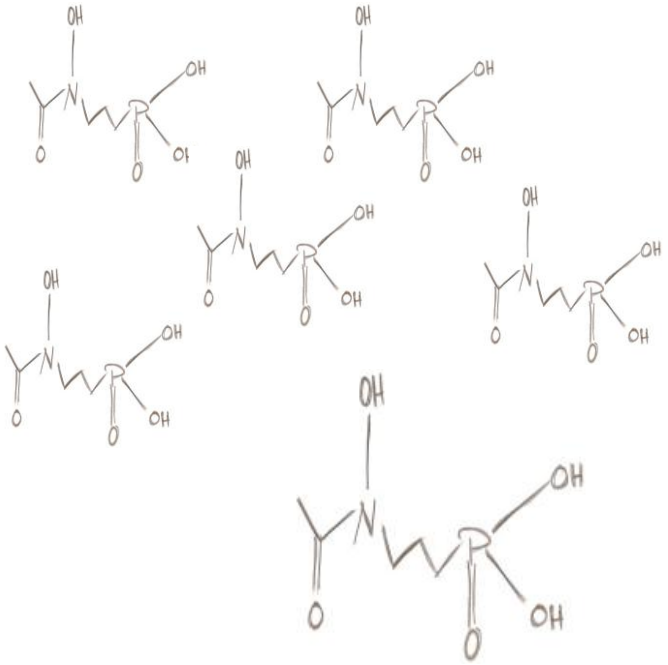


Chapter V

Alpha-heteroatom derivatized analogues of FR900098

V.Alpha-heteroatom Derivatized Analogues of FR900098

V.A Introduction

As described in the general introduction (Chapter I), derivatisation of the three-carbon spacer of fosmidomycin has afforded very promising antimalarials. Alkyl and aryl groups have been appended to the α- and the γ-position of the spacer, but substitution of the latter led to a detrimental loss in activity. Concerning the α-position, only α-aryl substituents gave an increase in antimalarial activity whereas α-(hydroxyl)alkyl and α-arylalkyl substituents were less favorable. All these modifications share a carbon-carbon bond between the substituent and the α-position of the spacer. Except for the α-halogenated analogues described in Chapter III, no fosmidomycin derivatives bearing a heteroatom in α-position have been described.

Therefore, we decided to devise a series of FR900098 analogues starting from α-OH and α-NH_2 FR900098, in which a variety of substituents are linked via different *O*- or *N*-based functional groups to the α-carbon of the spacer. This strategy has several advantages such as:

- **Relative ease of synthesis of starting materials.** The synthesis of (α-hydroxy)alkylphosphonates is well documented. The most common methods rely on the addition of dialkyl or trialkyl phosphites to aldehydes or ketones (Pudovik resp. Abramov reaction). Also the synthesis of (α-amino)alkylphosphonates is a well described field, due to the importance of these molecules as amino acid analogues. The one-pot three component reaction between a phosphite, an amine and an aldehyde or a ketone leading to α-aminoalkylphosphonates is known as the Kabachnik-Fields reaction.

- **Relative ease of further derivatisation.** The hydroxyl- and aminogroups in α-position serve as excellent handles for further derivatisation, allowing divergent synthesis from common precursors.

- **Possibility of broad SAR 'scanning'.** A large variety of substituents can be linked to the α-carbon via different functional groups based on OH (ether, ester) and NH (amide, urea, amine, sulfonamide, triazole,…)

V.B Retrosynthesis

As described above, both envisaged α-hydroxy and α-amino alkylphosphonates can be synthesized from a common aldehyde precursor (**8**, Figure V.1). Reaction of this aldehyde with a phosphite alone (Pudovik reaction) leads to α-OH FR900098 (**6**), whereas the same reaction in the presence of an ammonia source (Kabachnik-Fields reaction) may give α-NH_2 FR900098 (**7**). The α-OH analogue can then be converted to different ether analogues, or serve as a starting material for the synthesis of α-azido FR900098 (**2**). The latter may be tested as such, but also serves as a starting material for the synthesis of triazole derivatives (**1**) by means of a copper catalyzed azide-alkyne cycloaddition reaction. α-NH_2 FR900098 (**7**) on the other hand, can straightforwardly be transformed into α-amido, α-urea and α-sulfonamide derivatives.

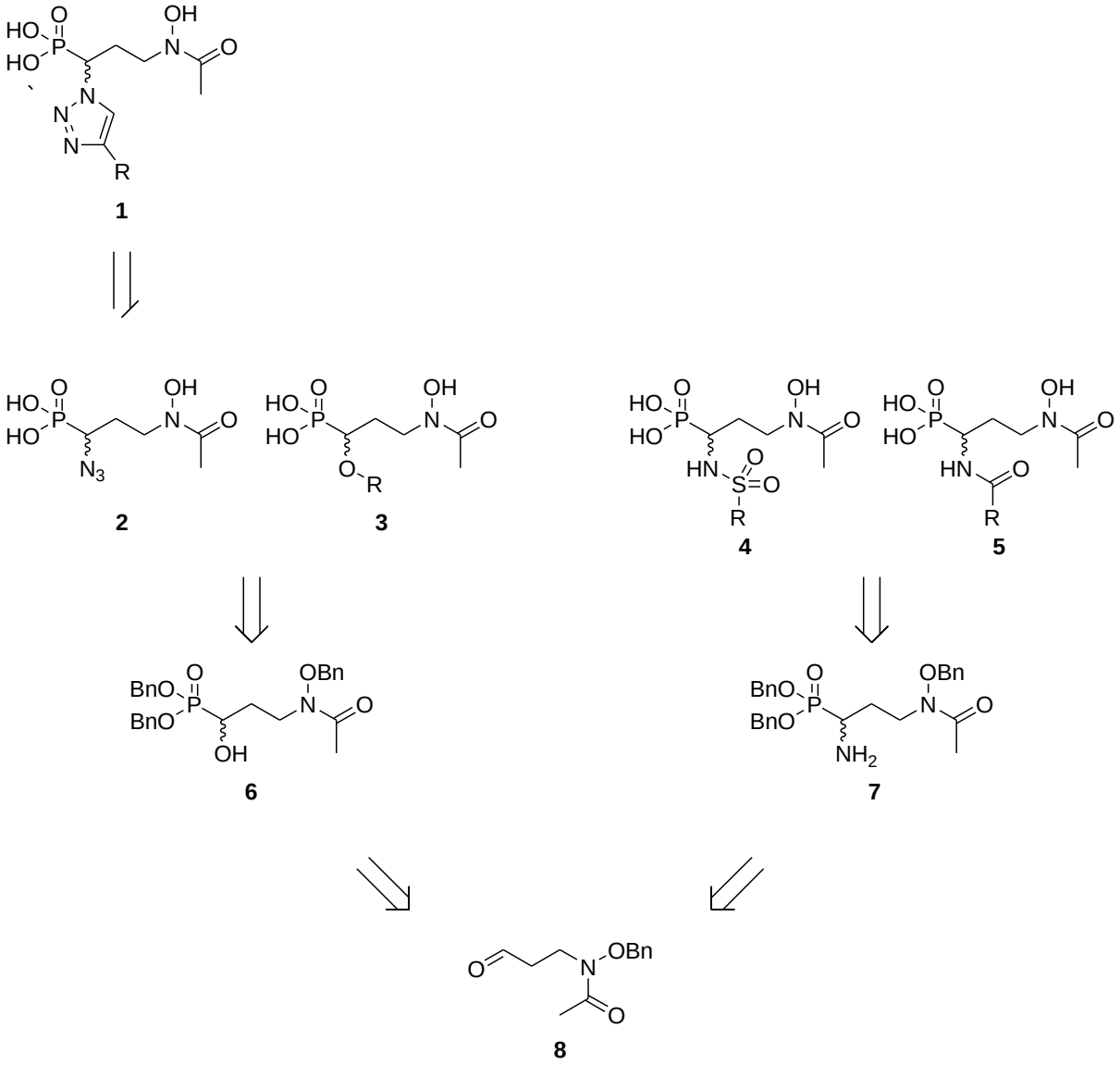
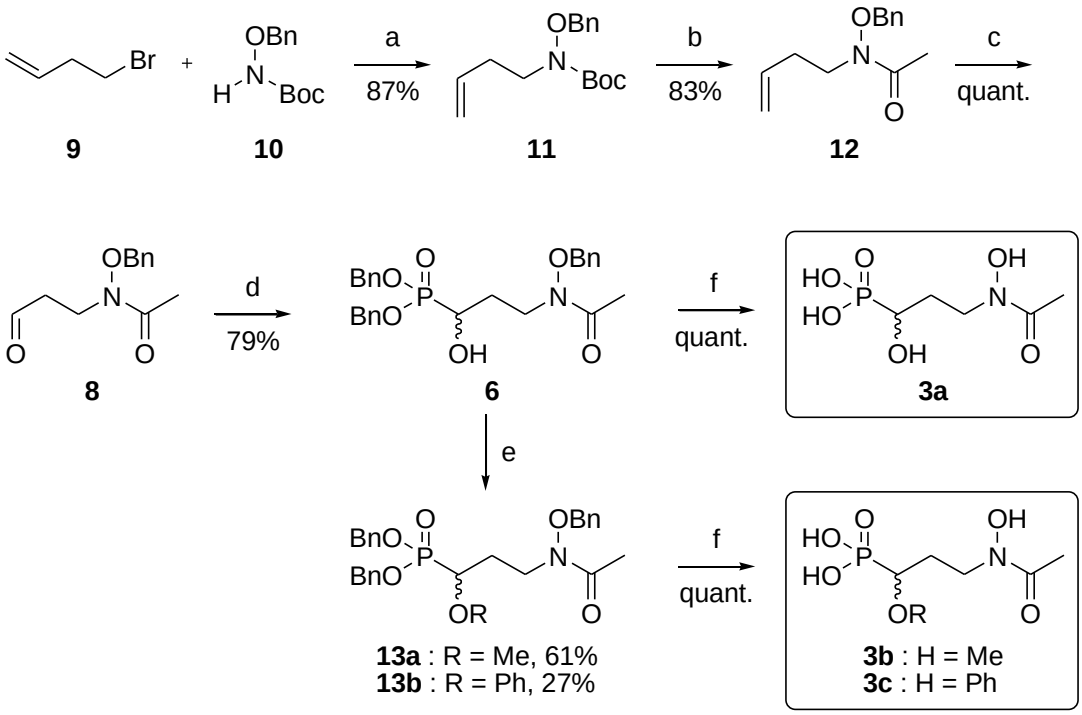


Figure V.1: Retrosynthetic analysis toward α-heteroatom substituted analogues of FR900098

V.C Chemistry

Aldehyde **8**, already equipped with a protected retrohydroxamate moiety, served as a starting point for the synthesis of α-O-based analogues via a Pudovik reaction. Its synthesis started with the alkylation of *N*-Boc-*O*-benzylhydroxylamine (**10**) with bromobutene (**9**) in DMF[1], (Scheme V.1) followed by a one-pot deprotection-acetylation[2] to afford terminal alkene **12** that can be stored indefinitely. Oxidative cleavage of the double bond with periodate and osmate gave the desired aldehyde **8** in good yields.

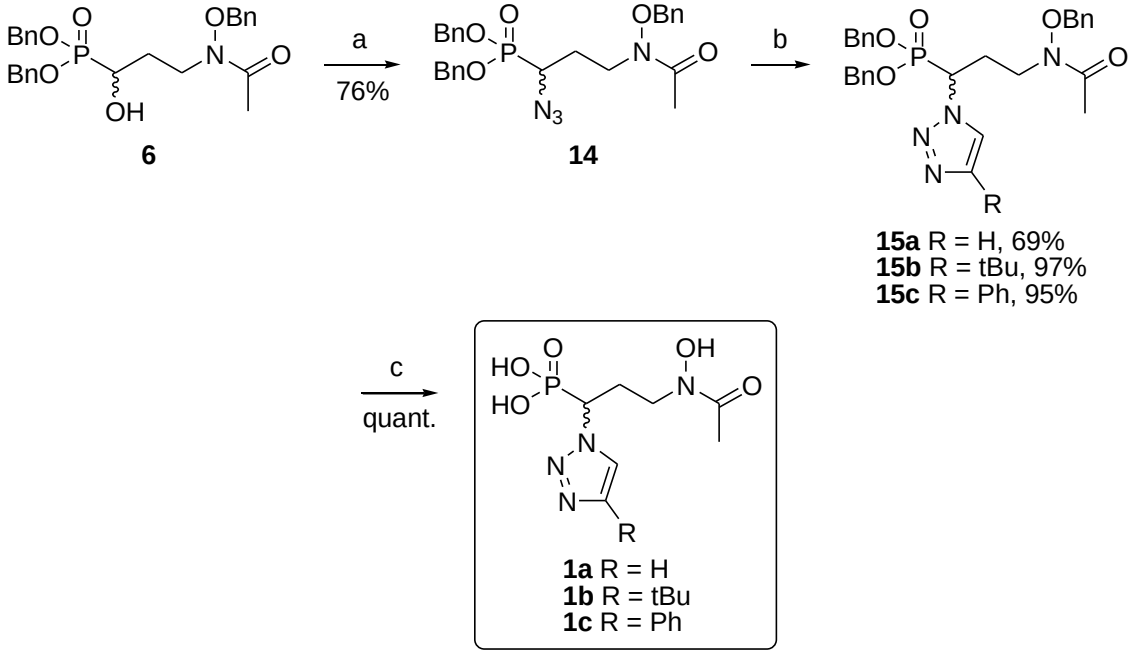


Reagents and conditions: (a) NaH, DMF, rt; (b) (i) acetyl chloride, MeOH, NaI, MeCN, 1h, rt (ii) Et_3N, DMAP; (c) $NaIO_4$, $K_2OsO_4.2H_2O$, H_2O, THF; (d) $(BnO)_2POH$, LiHMDS, THF, -78 °C; (e) **13a**: MeI, Ag_2O, DMF, rt OR **19b**: PhOH, PPh_3, DIAD, THF, ultrasound; (f) NH_4OOCH, MeOH, reflux, 20min

Scheme V.1: Synthesis of the common aldehyde precursor **8** and α-OH based analogues

For the synthesis of benzyl protected α-hydroxy-FR900098 **6** we chose a base-assisted Pudovik reaction with dibenzyl phosphite at low temperature for it resulted in a cleaner reaction mixture and higher yields than when using 'classical' Pudovik conditions. Using benzyl protection for both the phosphonate and the retrohydroxamate allowed us for a convenient one-step final deprotection

under very gentle conditions. α-Hydroxy-FR900098 **6** was converted into its methyl ether **13a** by a Williamson reaction with iodomethane and silver oxide. Attempted synthesis of α-phenyl ether **13b** under standard Mitsunobu conditions in THF proved unsuccessful. This was ascribed to a combination of the steric hindrance of the α-carbon by the phosphonate group and the sterical hindrance and/or too low acidity of phenol. Therefore, we switched to more forcing conditions as described by Lepore et al.[3]: using a high concentration of reactants in very little THF, resulting in a highly viscous reaction mixture that was mixed by sonication. This allowed us to synthesize **13b** albeit the complex reaction mixture called for fractionating by flash chromatography followed by purification by preparative HPLC.



6 **14**

Reagents and conditions: (a) PPh_3, DIAD, HN_3, toluene, rt; (b) **21a**: vinyl acetate, μW OR **21b**: 3,3-dimethylbut-1-yne, $CuSO_4$, Na-ascorbate, DMF, μW, OR **21c**: phenylacetylene, $CuSO_4$, Na ascorbate, DMF, μW; (c)**11b**: Pd/C, H_2, MeOH OR **11a,c**: Pd/C, NH_4OOCH, MeOH, reflux

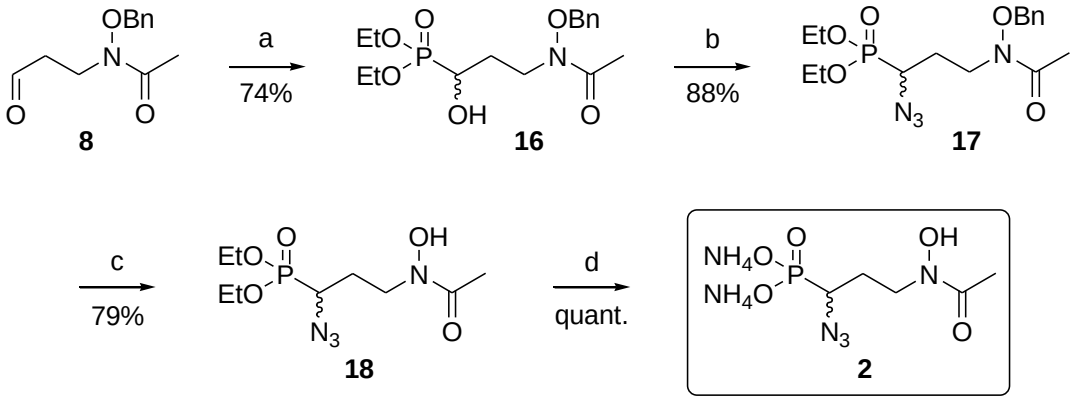
Scheme V.2: Synthesis of α-(1,2,3-triazolyl) analogues

Starting from the tri-benzylated α-hydroxy-FR900098 **6**, α-azidophosphonate **14** was synthesized by means of a Mitsunobu reaction with hydrazoic acid as the pronucleophile[4] (Scheme V.2) (***Caution*: HN_3 is volatile, highly toxic and explosive!**). Using 'classical' Mitsunobu conditions (2 equiv. of both triphenyl phosphine and diethylazodicarboxylate (DEAD), excess hydrazoic acid, submolar

concentrations in toluene) we were able to synthesize the desired α-azide in what appeared to be pure form according to TLC or HPLC. However, persistent ethyl signals were seen in the ^{1}H-NMR spectrum of the product. Originally assigned to ethyl acetate trapped in the oily product, it later turned out that these signals arose from the α-ethylcarbonate formed by attack of α-hydroxy-FR900098 **6** on one of the carbonyl groups of DEAD. As this unwanted product co-elutes with α-azidophosphonate **14** both on TLC and RP-HPLC it took us some time to identify it. This problem was circumvented by switching to the more sterically hindered diisopropylazodicarboxylate (DIAD) and mixing the reactants in a different order (precomplexing triphenylphosphine and DIAD, followed by adding the hydrazoic acid solution and finally the α-hydroxyphosphonate). The obtained benzyl-protected α-azidophosphonate **14** subsequently served in the synthesis of α-1,4-triazole-substituted analogues by copper-catalyzed azide-alkyne cycloaddition (CuAAC) with two acetylene derivatives. To the best of our knowledge, no literature data on the CuAAC of α-azidophosphonates exists and only thermal, hence non-regiospecific, phosphonomethylazide-alkyne cycloadditions have been performed before.[5] Attempted CuAAC using copper(II)sulfate and ascorbic acid with phenyl- or *t*-butylacetylene at room temperature or with conventional heating failed, but upon switching to microwave heating clean products were obtained in good yields. The unsubstituted triazole **15a** was synthesized using the protocol of Hansen et al.[6]: heating azide **14** in vinyl acetate under microwave irradiation for several hours.

We considered α-azido-FR900098 also of interest as a potential DXR inhibitor, but for obvious reasons total deprotection by reductive debenzylation could not be performed on precursor **14**. As we also feared that debenzylation of **14** with boron trichloride would lead to an unresolvable complex mixture of very polar compounds, we chose to synthesize a different α-azido precursor with orthogonal protective groups, *in casu* a benzyl ether for the retrohydroxamate and ethyl esters for the phosphonate (Scheme V.3). Therefore, a Pudovik reaction analogous to the one described above was performed with diethyl phosphite, leading to diethyl α-hydroxyphosphonate **16**, which was converted to the α-azide **17** as described above. The retrohydroxamate moiety of compound **17** was

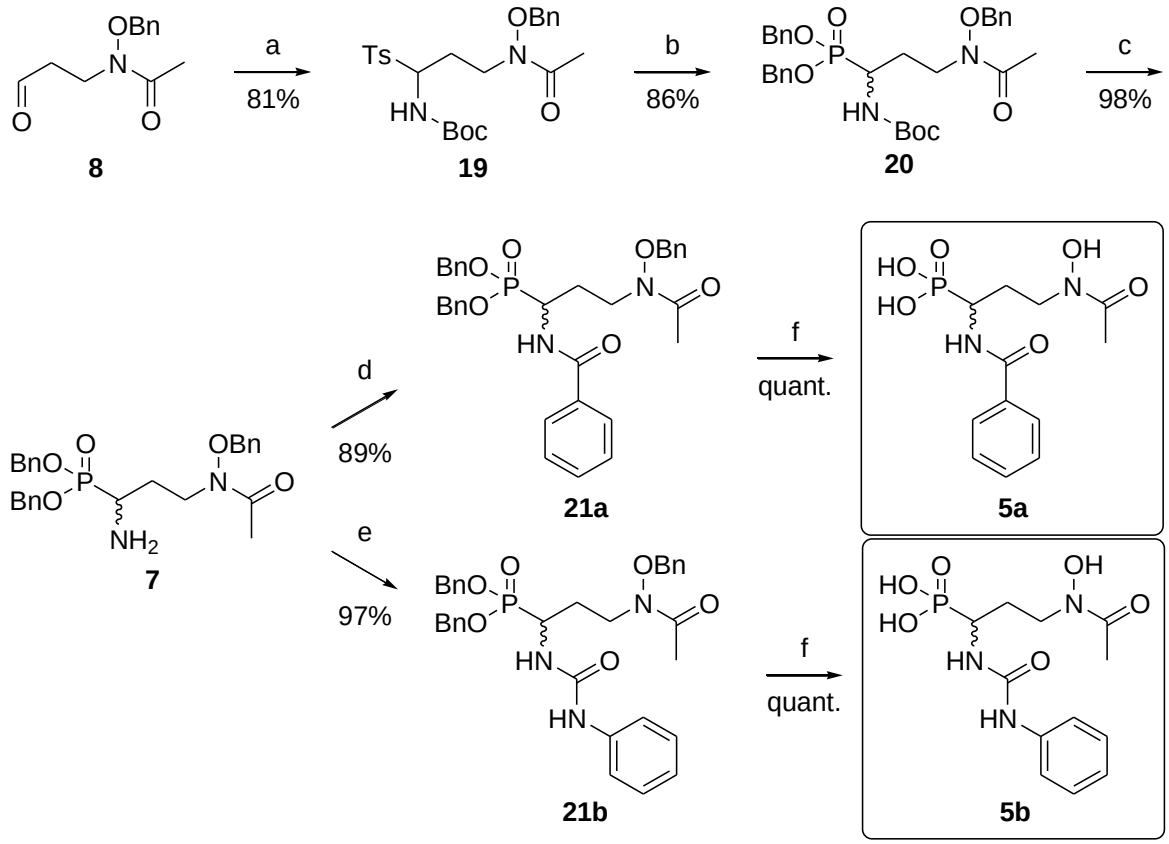
first debenzylated with boron trichloride, followed by purification of **18** on deactivated silica gel, prior to removal of the phosphonate esters by trimethylsilyl bromide in acetonitrile and basic workup yielding α-azido-FR900098 **2** as a bisammonium salt.



Reagents and conditions: (a) $(EtO)_2POH$, LiHMDS, THF, 78 °C; (b) PPh_3, DIAD, HN_3, toluene, rt; (c) BCl_3, CH_2Cl_2, 75 °C; (d) (i) TMSBr, BSTFA, MeCN, (ii) aq. NH_4OH, MeCN

Scheme V.3: Synthesis of α-azido-FR900098

Synthesis of the α-N based series of analogues (Scheme V.4) started with the synthesis of benzyl protected α-Boc-amino-FR900098 **20** as described by Klepacz et al.[7] in two steps: first aldehyde **8** was converted to sulfone **19** in a three-component reaction with *t*-butylcarbamate and sodium *p*-toluenesulfinate, followed by displacement of the sulfone with dibenzyl phosphite. The Boc-protecting group on the amine facilitated purification of **20** and was removed without subsequent purification before further reactions. Although numerous alternatives for the synthesis of α-aminophosphonates are known, such as a Kabachnik-Fields reaction with HMDS or ammonium carbonate (which only gave marginal results in our hands), or the evident Staudinger reduction of azide **16** (which would be a much less economic way of synthesizing amine **7**), this method benefits from high yields and easy purification. Conversion of amine **7** into benzamide **21a** and phenylurea **21b** was carried out under standard conditions as depicted in Scheme V.4.



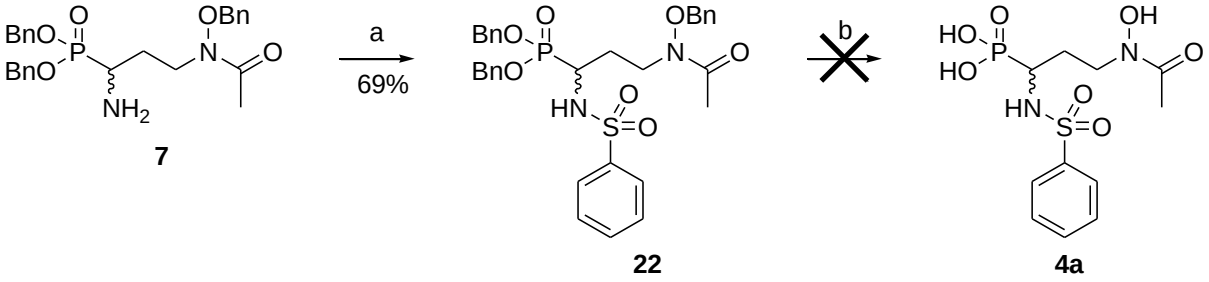
Reagents and conditions: (a) Na-*p*-toluenesulfinate, *t*-butylcarbamate, HCOOH, THF, MeCN, H_2O, rt, o.n.; (b) $(BnO)_2POH$, NaH, THF, rt; (c) TFA, CH_2Cl_2, 0 °C; (d) BzCl, DMAP, Et_3N, CH_2Cl_2; (e) phenylisocyanate, DMAP, Et_3N, CH_2Cl_2; (f) Pd/C, H_2, MeOH/H_2O/tBuOH

Scheme V.4: Synthesis of α-N based analogues

As stated before, the use of only benzyl protecting groups allowed mild final deprotection in one step upon hydrogenation on palladium on carbon. First attempts were made by bubbling hydrogen gas through methanolic solutions of the starting materials, and we noticed the importance of working 'metal free' here: apparently upon the use of standard metal hydrogenation needles the reaction was poisoned by metal ions coming off these needles. Therefore, hydrogen gas was bubbled into the reaction mixture through a glass capillary, resulting in clean deprotection for analogues **5a**, **5b** and **1b**. Other analogues showed acid-mediated breakdown of the retrohydroxamic acid moiety during deprotection, calling for the incorporation of a base in the reaction in order to neutralize the formed phosphonic acid. We found a solution to this problem in using catalytic transfer

hydrogenation with ammonium formate as the hydrogen donor, the ammonium acting as a base to neutralize the formed phosphonates. Excess ammonium formate was removed by lyophilization with mixed success, resulting in the end products as their monoammonium salts.

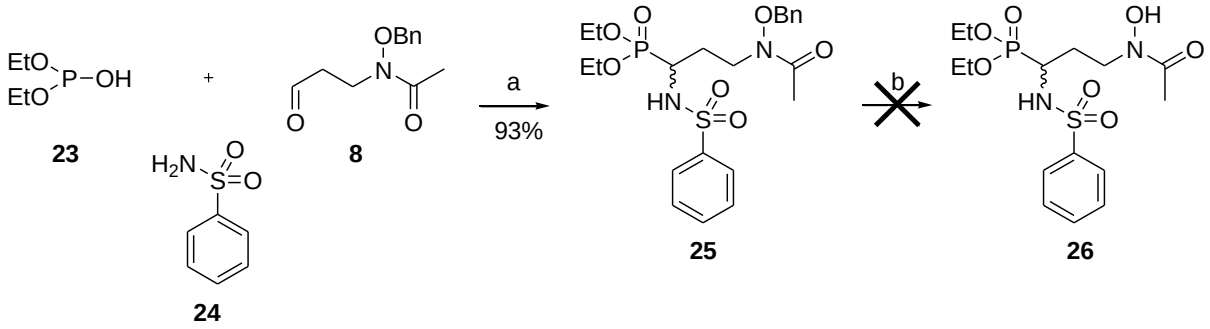
Attempts were also made to synthesize α-phenylsulfonamide FR900098 (**4a**, Scheme V.5) by reacting α-amine **7** with phenylsulfonyl chloride. Although this yielded penultimate α-phenylsulfonamide **22** in an affordable yield, the subsequent debenzylation got stuck after the two phosphonate benzyl esters were removed. Applying more forcing conditions (Pd black, hydrogen pressure up to 50 bar) only resulted in breakdown of the molecule.



Reagents and conditions: (a) phenylsulfonyl chloride, Et_3N, CH_2Cl_2, 0 °C to rt, 24h; (b) Pd/C, H_2, MeOH/H_2O/tBuOH

Scheme V.5: Attempted synthesis of α-phenylsulfonamide analogue

We then decided to try a boron trichloride mediated debenzylation for this compound, analogous to the procedure followed for the α-azido analogue above. This implied synthesis of the diethyl phosphonate precursor **25** (Scheme V.6), for which we used a three-component reaction between diethyl phosphite, phenylsulfonamide and aldehyde **8** in acetyl chloride[8]. The use of acetyl chloride as a solvent seems a bit strange but probably it serves as a dehydrating agent. α-Phenylsulfonyl diethylphosphonate **25** was formed in excellent yield by this procedure.



Reagents and conditions: (a) acetyl chloride, 0 °C to rt, o.n.; (b) Pd/C, H_2, MeOH/H_2O/tBuOH

Scheme V.6: Attempted synthesis of α-phenylsulfonamide analogue via the diethylphosphonate

Before attempting the planned deprotection of the retrohydroxamate with boron trichloride, we decided to give reductive debenzylation another try on this analogue. To our delight, simple hydrogenation over palladium on carbon in ethyl acetate at room temperature quickly and cleanly debenzylated **25**. Unfortunately, the resulting free retrohydroxamate spontaneously broke down upon flash column purification, probably under influence of the acidic sulfonamide. These stability issues made us stop the development of α-phenylsulfonamide FR900098 (**4a**).

V.D Biological Evaluation

The title compounds were tested their capacity to inhibit EcDXR using a spectrophotometric assay, i.e. by monitoring the decrease in NADPH absorption at 340 nm. Inhibition rates for all analogues as well as references fosmidomycin, FR900098 and α-(3,4-dichlorophenyl)fosmidomycin, incubated at 100nM are shown in Table V.1.

Table V.1: *In vitro* EcDXR inhibition and *P. falciparum* growth inhibition by α-heteroatom substituted FR900098 analogues.

		EcDXR Enzyme Inhibition			*In vitro* growth inhibition (IC_{50} in µM)
Compound	α-substituent	% inhibition at 100 nM	K_i (nM)	IC_{50} (nM)	*P. falciparum* K1
Fosmidomycin		75.57	-	-	1.73
FR900098		74.71	15.11	30.23	0.42
α-(3,4-dichlorophenyl) fosmidomycin		-	-	-	0.16
3a	-OH	48.86	-	-	1.82
3b	-OMe	4.14	-	-	4.00
3c	-OPh	No Inhibition	-	-	13.02
1a	-triazol-1-yl	3.32	-	-	8.96
1b	-4-tBu- triazol-1-yl	12.66	1918	3837	2.75
1c	-4-Ph- triazol-1-yl	1.15	207.3	414.7	7.76
2	$-N_3$	77.48	74.6	149	1.98
5a	-benzamide	1.42	-	-	49.02
5b	-phenylurea	1.30	-	-	>64.00

Remarkably, promising DXR inhibition is confined to analogues with the smallest substituents (azido and hydroxy) at the α-position. In light of the appreciable DXR affinity obtained with α-phenylpyridyl substituents[9], it is surprising that none of the α-triazoles showed significant inhibitory activity. Possibly the triazole ring is too electron rich to form strong π-stacking with Trp 211 described in this study. The activity seen with the α-azido derivative is encouraging, as this is the first

example of a DXR inhibitor equaling FR900098's DXR inhibitory potency. Crystallography studies should give us more insight in the binding mode of this molecule and the interaction of the rigid linear azide moiety with DXR.

All α-heteroatom analogues were subsequently tested for their inhibitory effect against intraerythrocytic forms of *P. falciparum* (K1 strain) using a microdilution assay (Table V.1). The same trends are noticeable here: both α-N based analogues **5a** and **5b** hardly showed antiplasmodial activity, and the same is true for both α-ether derivatives **3b** and **3c**. The three α-triazolyl analogues **1a**, **1b** and **1c** show encouraging activity and follow the same trend as observed in the enzymatic assay: the *t*-butyl substituted triazole shows the highest activity, the phenyl substituted and the unsubstituted show a comparable lower activity. The best antiplasmodial activity in the cellular assay is seen with α-OH and α-azido FR900098 **3a** and **2**. The latter surpassed the *in vitro* antiplasmodial potency of parent FR900098 and is a promising analogue for further *in vivo* testing and crystallographic investigation.

All α-heteroatom analogues were also tested for their cytotoxicity against MRC-5 SV2 cells, human fetal lung fibroblast, where they showed IC_{50} values of more than 64 µM. Furthermore, the title compounds were screened for their activity against *T. cruzei, L. infantum, T. brucei, T. rhodesiense, E. coli, S. aureus, C. albicans, T. rubrum* and *A. fumigatus*. No activity (IC_{50} >64 µM) was seen for all analogues against any of these organisms, confirming their selectivity for *Plasmodium* species.

V.E Experimental Details

General Methods and Materials. See section III.F.

Spectrophotometric DXR inhibition assay. See section III.F.

***In vitro P. falciparum* growth inhibition assay and microbial screening.** See section III.F.

Cytotoxicity. See section III.F.

***tert*-Butyl *N*-(benzyloxy)-*N*-(but-3-enyl)carbamate (11).** To a solution of *t*-butyl *N*-benzyloxycarbamate (6300 mg, 28.22 mmol) in dry DMF (60mL) was added sodium hydride (1242 mg of a 60% dispersion in mineral oil, 31.04 mmol) in small portions under vigorous stirring at room temperature. After 30 minutes, neat 4-bromobut-1-ene (3.15 mL, 4190 mg, 31.04 mg) was added dropwise via syringe to the clear solution. The reaction mixture was stirred for 90 minutes, quenched by the addition of saturated aqueous NH_4Cl and partitioned between aqueous 0.1N HCl and diethyl ether. The extraction with diethyl ether was repeated two times, the combined organic phases were washed with brine, dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. The resulting crude mixture was purified by dry column vacuum chromatography (DCVC)[10] with a gradient of ethyl acetate in hexanes to yield 6.81g (87 %) of **11** as a colourless oil. ^{1}H NMR (300 MHz, CHLOROFORM-d) δ ppm 1.50 (s, 9 H) 2.20 - 2.50 (m, 2 H) 3.32 - 3.61 (m, 2 H) 4.83 (s, 2 H) 5.05 (ddt, J=3.84, 2.60, 1.32, 1.32 Hz, 1H) 5.11 (q, J=1.46 Hz, 1 H) 5.77 (ddt, J=17.06, 10.25, 6.85, 6.85 Hz, 1 H) 7.27 - 7.51 (m, 5 H) ^{13}C NMR (75 MHz, CHLOROFORM-d) δ ppm 28.30 ($(CH_3)_3$) 31.44 (CH_2) 49.23 (CH_2N) 76.91 (OCH_2) 81.19 ($\underline{C}(CH_3)_3$) 116.68 (=CH_2) 128.39 (=CH-) 128.44 (=CH-) 129.31 (=CH-) 135.27 (=CH-) 135.69 (C_q) 156.54 (C=O); HRMS (ESI) m/z 278.1745 [$(M+H)^+$, calcd for $C_{16}H_{24}NO_3^+$ 278.1751].

***N*-(benzyloxy)-*N*-(but-3-enyl)acetamide (12).** *tert*-Butyl *N*-(benzyloxy)-*N*-(but-3-enyl)carbamate **11** (2774 mg, 10 mmol), NaI (2998 mg, 20 mmol) and methanol (dry, 0.81 mL, 20 mmol) were

dissolved in 40 mL of dry acetonitrile, resulting in a clear yellow solution. Acetyl chloride (2.85 mL, 40 mmol) was added dropwise while stirring the reaction mixture at room temperature. After 60 minutes the reaction mixture was cooled to 0° C in an icebath and Et_3N (5.6 mL, 40 mmol) was added as well as DMAP (122 mg, 1 mmol). The icebath was removed after 5 minutes and the reaction mixture stirred at room temperature for another 60 minutes. The reaction mixture was then poured into a separating funnel containing 1M aqueous HCl and extracted 3 times with diethyl ether. The combined organic fractions were washed with aqueous $NaHCO_3$, aqueous $Na_2S_2O_3$ and brine and dried over anhydrous Na_2SO_4. Purification of the crude material by DCVC (hexanes/ethyl acetate) yielded 2.07g (94%) of **12** as a slightly yellow oil. ^{1}H NMR (300 MHz, CHLOROFORM-d) δ ppm 2.07-2.08 (2s (rotamers), 3 H) 2.28 - 2.53 (m, 2 H) 3.69 (t, J=7.03 Hz, 2 H) 4.81 (s, 2 H) 4.93 - 5.22 (m, 2 H) 5.59 - 5.94 (m, 1 H) 7.28 - 7.51 (m, 5 H) ^{13}C NMR (75 MHz, CHLOROFORM-d) δ ppm 20.57 (CH_3) 31.26 (CH_2) 45.04 (NCH_2) 76.28 ($PhCH_2$) 116.97 (=CH_2) 128.68 (=CH-) 128.90 (=CH-) 129.11 (=CH-) 134.57 (=CH-) 134.93 (C_q) 172.48 (C=O); HRMS (ESI) m/z 220.1320 [$(M+H)^+$, calcd for $C_{13}H_{18}NO_2^+$ 220.1332].

***N*-(benzyloxy)-*N*-(2-formylethyl)acetamide (8).** To a solution of **12** (2200 mg, 10 mmol) in THF (50 mL) was added 40 mg $K_2OsO_4.2H_2O$ and a hot (50° C) solution of $NaIO_4$ (10700 mg, 50 mmol) in 50 mL water. The resulting off-white thick suspension was shielded from light and stirred for 4 hours, after which TLC (90:10 dichloromethane/methanol) confirmed the complete conversion of the starting material. The reaction mixture was then filtered over a glassfibre pad, the filter was rinsed with diethyl ether, the resulting biphasic filtrate was transferred to a separating funnel and aqueous Na_2SO_4 was added followed by threefold extraction with diethyl ether. The combined ether phases were washed with brine, dried over anhydrous Na_2SO_4 and evaporated *in vacuo*, yielding 1.85g (84%) of a brown oil that was used without further purification in the next reaction. ^{1}H NMR (300 MHz, CHLOROFORM-d) δ ppm 2.05 (s, 3 H) 2.68 (td, J=6.59, 1.46 Hz, 2 H) 3.95 (t, J=6.59 Hz, 2 H) 4.80 (s, 2 H) 7.22 - 7.50 (m, 5 H) 9.73 (t, J=1.46 Hz, 1 H) ^{13}C NMR (75 MHz, CHLOROFORM-d) δ ppm 20.50 (CH_3) 39.54 (NCH_2) 41.32 (CH_2) 76.37 ($PhCH_2$) 128.70 (=CH-) 129.01 (=CH-) 129.36 (=CH-) 134.20 (C_q) 172.80 (C=O) 200.19 (HC=O); HRMS (ESI) m/z 222.1136 [$(M+H)^+$, calcd for $C_{12}H_{16}NO_3^+$ 222.1125].

Dibenzyl 3-(*N*-(benzyloxy)acetamido)-1-hydroxypropylphosphonate (6). Dibenzyl phosphite (4330 mg, 16.5 mmol) was dissolved in THF (20 mL), the solution was cooled to -78°C and LiHMDS (15 mL of a 1M solution in THF) was slowly added. After 15 minutes a solution of aldehyde **8** (3320 mg, 15 mmol) in 30 mL dry THF was added via syringe and the icebath was removed. After another 15 minutes of stirring the reaction mixture had warmed up to room temperature at which point it was quenched by the addition of saturated aqueous NH_4Cl and extracted three times with ethyl acetate. The combined organic phases were washed with brine, dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. The resulting crude mixture was purified by dry column vacuum chromatography (DCVC) with a gradient of ethyl acetate in toluene containing 0.1% formic acid to yield 5.22g (72 %) of **6** as an off-white solid. ^{1}H NMR (300 MHz, CHLOROFORM-d) δ ppm 1.85 - 2.03 (m, 1 H) 2.05 (s, 3 H) 2.07 - 2.25 (m, 1 H) 3.56 - 3.72 (m, 1 H) 3.83 - 3.96 (m, 1 H) 4.05 (br. s., 1 H) 4.57 (br. s., 1 H) 4.71 - 4.85 (m, 2 H) 4.98 - 5.14 (m, 4 H) 7.11 - 7.42 (m, 15 H) ^{13}C NMR (75 MHz, CHLOROFORM-d) δ ppm 20.32 (CH_3) 29.21 (CH_2) 42.09 (NCH_2) 65.02 (d, $^{1}J_{C-P}$= 166.96Hz, P-CHOH) 68.01 (d, $^{2}J_{C-P}$= 5.80Hz, $POCH_2$) 68.10 (d, $^{2}J_{C-P}$= 5.80Hz, $POCH_2$) 76.43 ($PhCH_2$) 127.92 (=CH-) 128.31 (=CH-) 128.50 (=CH-) 128.70 (=CH-) 128.99 (=CH-) 129.19 (=CH-) 134.12 (C_q)136.22 (d, $^{3}J_{C-P}$= 2.21Hz, C_q) 136.3 (d, $^{3}J_{C-P}$= 2.21Hz, C_q) 173.12 (C=O) ; HRMS (ESI) m/z 484.1852 [$(M+H)^+$, calcd for $C_{26}H_{31}NO_6P^+$ 484.1884].

Dibenzyl 3-(*N*-(benzyloxy)acetamido)-1-methoxypropylphosphonate (13a). Dibenzyl 3-(*N*-(benzyloxy)acetamido)-1-hydroxypropylphosphonate (**6**) (488 mg, 1 mmol) was dissolved in DMF (4 mL) and Ag_2O (348 mg, 1.5 mmol) was added under vigorous stirring. Neat iodomethane (0.62 mL, 1419 mg, 10 mmol) was added via syringe, the reaction mixture was shielded from light and stirred overnight at room temperature. The reaction mixture was then filtered over a glassfibre pad, the filtrate was concentrated and filtered again, resulting in a clear solution that was evaporated *in vacuo*. The resulting crude material was purified by DCVC with a gradient of ethyl acetate in hexanes to yield 304 mg (61%) of **13a** as a colourless oil. 1H NMR (300 MHz, CHLOROFORM-d) δ ppm 1.85 - 2.02 (m, 1 H) 2.02 - 2.09 (m, 3 H) 2.09 - 2.29 (m, 1 H) 3.41 - 3.57 (m, 4 H) 3.59 - 3.76 (m, 1 H) 3.80 -

4.07 (m, 1 H) 4.63 - 4.86 (m, 2 H) 4.91 - 5.21 (m, 4 H) 7.14 - 7.45 (m, 15 H); HRMS (ESI) m/z 498.2035 [$(M+H)^+$, calcd for $C27H33NO6P^+$ 498,204].

Dibenzyl 3-(*N*-(benzyloxy)acetamido)-1-phenoxypropylphosphonate (13b). Dibenzyl 3-(*N*-(benzyloxy)acetamido)-1-hydroxypropylphosphonate (**6**) (725 mg, 1.5 mmol), triphenylphosphine (393 mg, 1.5 mmol) and phenol (141 mg, 1.5 mmol) were dissolved in 0.5 mL THF under sonication at 30° C to give a clear viscous jelly. To this mixture was added diisopropyl azodicarboxylate (0.30 mL, 1.5 mmol) and sonication at 30°C was continued for one hour. Then another half equivalent of phenol (47 mg, 0.5 mmol), triphenylphosphine (131 mg, 0.5 mmol) and diisopropyl azodicarboxylate (0.10 mL, 0.5 mmol) dissolved in 0.250 mL THF were added and the mixture was sonicated at room temperature overnight. Subsequently, all volatiles were removed *in vacuo* and the crude material was fractionated by DCVC (hexanes/ethyl acetate). All fractions containing product were pooled and evaporated and the resulting material was purified by preparative HPLC (50/50 to 100/0 acetonitrile – water containing 0.2% formic acid) to give 224 mg (27%) of **13a** as a colourless oil. 1H NMR (300 MHz, CHLOROFORM-d) δ ppm 1.99 (s, 3 H) 2.10 - 2.45 (m, 2 H) 3.62 - 4.01 (m, 2 H) 4.46 - 4.81 (m, 3 H) 4.82 - 5.15 (m, 4 H) 6.82 - 7.10 (m, 3 H) 7.12 - 7.50 (m, 17 H); HRMS (ESI) m/z 560.2203 [$(M+H)^+$, calcd for $C32H35NO6P^+$ 560,2197].

Dibenzyl 3-(*N*-(benzyloxy)acetamido)-1-azidopropylphosphonate (14). Hydrazoic acid solution (***Caution*: HN_3 is volatile, highly toxic and explosive!**): Sodium azide (4000 mg, 61.5 mmol) was dissolved in water (10 mL). Toluene (50 mL) was added and the resulting biphasic system was cooled on ice to 0°C. Under vigorous stirring, concentrated sulfuric acid (8 mL) was added dropwise. After 30 min of stiring, the organic layer was separated and stored on anhydrous Na_2SO_4.
Triphenylphosphine (5250 mg, 20 mmol) was dissolved in toluene (60 mL), cooled to 0°C and diisopropylazodicarboxylate (3.96 mL, 20 mmol) was added, followed by 10 mL of the freshly prepared hydrazoic acid stock solution. Then a solution of compound **6** (4880 mg, 10 mmol) in toluene (25 mL) was added and the icebath was removed. Stirring was continued for 2 hours during

which the appearance of the reaction mixture shifted from turbid and yellow to clear and almost colourless. The reaction mixture was concentrated *in vacuo*, the residue was taken up in diethyl ether and triphenylphosphine oxide was crystallized out by addition of heptane and seeding with triphenylphosphine oxide. The resulting crude product was then purified by DCVC (0 to 100% ethyl acetate in hexanes) to yield 3865 mg (76 %) of **14** as an oil. 1H NMR (300 MHz, CHLOROFORM-d) δ ppm 1.69 - 1.93 (m, 1 H) 2.00 - 2.09 (m, 3 H) 2.15 (ddt, J=14.50, 7.18, 3.81, 3.81 Hz, 1 H) 3.54 (td, J=11.42, 3.22 Hz, 1 H) 3.63 - 3.91 (m, 2 H) 4.77 (s, 2 H) 4.92 - 5.19 (m, 4 H) 7.17 - 7.54 (m, 15 H); HRMS (ESI) m/z 509.1952 [$(M+H)^+$, calcd for $C_{26}H_{30}N_4O_5P^+$ 509,1948].

Dibenzyl (3-(*N*-(benzyloxy)acetamido)-1-(1*H*-1,2,3-triazol-1-yl)propyl)phosphonate (15a). Azide **14** (508 mg, 1.01 mmol) was dissolved in vinyl acetate (10 mL, 108 mmol) and the solution was heated in a microwave at 120° C for 6.5 hours, followed by removal of all volatiles *in vacuo*. The resulting crude was purified by DCVC (0 to 100% ethyl acetate in hexanes followed by 2% ethanol in ethyl acetate) to yield 370 mg (69 %) of **15a** as an oil. 1H NMR (300 MHz, CHLOROFORM-d) δ ppm 2.01 (s, 3 H) 2.36 - 2.68 (m, 2 H) 3.27 - 3.67 (m, 2 H) 4.55 - 4.72 (m, 2 H) 4.72 - 5.14 (m, 5 H) 7.09 - 7.46 (m, 15 H) 7.58 - 7.80 (m, 2 H); HRMS (ESI) m/z 535.2098 [$(M+H)^+$, calcd for $C_{28}H_{32}N_4O_5P^+$ 535,2105].

Dibenzyl (3-(*N*-(benzyloxy)acetamido)-1-(4-(*tert*-butyl)-1*H*-1,2,3-triazol-1-yl)propyl)phosphonate (15b). To a solution of **14** (530 mg, 1.04 mmol) in DMF (10 mL) was added $CuSO_4$ (0.1 mL of a 0.1M aqueous stock solution, 0.01 mmol), sodium ascorbate (0.5 mL of a freshly prepared 0.1M aqueous stock solution, 0.05 mmol) and 3,3-dimethylbut-1-yne (2 mL, 1340 mg, 16.3 mmol). The reaction mixture was heated in a microwave oven for 30 minutes at 60° C. Assessment of the reaction mixture by TLC (25:75 toluene – ethyl acetate) showed the presence of unreacted azide, so another 0.5 mL of sodium ascorbate stock solution and 0.1 mL of $CuSO_4$ stock solution were added and the reaction mixture was irradiated for another 45 minutes at 70° C. At that point all azide was converted and the reaction mixture was concentrated, followed by purification of the resulting crude

by DCVC (0 to 100% ethyl acetate in hexanes) to yield 594 mg (97 %) of **15b** as a colourless oil. 1H NMR (300 MHz, CHLOROFORM-d) δ ppm 1.16 - 1.45 (m, 9 H) 2.01 (s, 3 H) 2.37 - 2.68 (m, 2 H) 3.35 - 3.67 (m, 2 H) 4.57 - 4.74 (m, 2 H) 4.74 - 4.91 (m, 2 H) 4.91 - 5.11 (m, 3 H) 7.07 - 7.40 (m, 15 H) 7.44 (d, J=0.88 Hz, 1 H); HRMS (ESI) m/z 591.2731 $[(M+H)^+$, calcd for $C32H40N4O5P^+$ 591,2731].

Dibenzyl (3-(*N*-(benzyloxy)acetamido)-1-(4-phenyl-1*H*-1,2,3-triazol-1-yl)propyl)phosphonate (15c). To a solution of **14** (536 mg, 1.1 mmol) in DMF (10 mL) was added $CuSO_4$ (0.5 mL of a 0.1M aqueous stock solution, 0.05 mmol), sodium ascorbate (1 mL of a freshly prepared 0.1M aqueous stock solution, 0.1 mmol) and phenylacetylene (3 mL, 2790 mg, 27.3 mmol). The reaction mixture was heated in a microwave oven for 1 hour at 80° C, followed by removal of all volatiles *in vacuo*. The resulting crude was purified by DCVC (0 to 95% ethyl acetate in hexanes) to yield 610 mg (95 %) of **15c** as an oil. 1H NMR (300 MHz, CHLOROFORM-d) δ ppm 1.89 - 2.14 (m, 3 H) 2.41 - 2.73 (m, 2 H) 3.34 - 3.74 (m, 2 H) 4.51 - 4.74 (m, 2 H) 4.76 - 4.93 (m, 2 H) 4.93 - 5.16 (m, 3 H) 7.08 - 7.45 (m, 18 H) 7.69 - 7.83 (m, 2 H) 7.83 - 7.91 (m, 1 H); HRMS (ESI) m/z 611.2417 $[(M+H)^+$, calcd for $C34H36N4O5P^+$ 611,2418].

Diethyl 3-(*N*-(benzyloxy)acetamido)-1-hydroxypropylphosphonate (16). Diethyl phosphite (684 mg, 4.95 mmol) was dissolved in THF (5 mL), the solution was cooled to -78°C and LiHMDS (4.5 mL of a 1M solution in THF) was slowly added. After 30 minutes a solution of aldehyde **8** (996 mg, 4.5 mmol) in 10 mL dry THF was added via syringe. After 20 minutes of stirring at -78°C the reaction was quenched by the addition of saturated aqueous NH_4Cl and extracted three times with ethyl acetate. The combined organic phases were washed with brine, dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. The resulting crude mixture was purified by DCVC (0 to 100 % acetone in toluene continued by 0 to 20 % methanol in acetone) to yield 1200 mg (74 %) of **16** as an oil. 1H NMR (300 MHz, CHLOROFORM-d) d ppm 1.26 - 1.41 (m, 6 H) 1.81 - 2.04 (m, 1 H) 2.09 (s, 3 H) 2.10 - 2.25 (m, 1 H) 3.70 (dt, J=14.64, 4.83 Hz, 1 H) 3.86 (dddd, J=11.35, 8.57, 5.86, 2.93 Hz, 1 H) 3.94 - 4.28 (m, 5

H) 4.48 - 4.73 (m, 1 H) 4.74 - 4.96 (m, 2 H) 7.38 (s, 5 H); HRMS (ESI) m/z 360.1578 [$(M+H)^+$, calcd for $C16H27NO6P^+$ 360,1571].

Diethyl 3-(*N*-(benzyloxy)acetamido)-1-azidopropylphosphonate (17). Hydrazoic acid solution (***Caution*: HN_3 is volatile, highly toxic and explosive!**): Sodium azide (4000 mg, 61.5 mmol) was dissolved in water (10 mL). Toluene (50 mL) was added and the resulting biphasic system was cooled on ice to 0°C. Under vigorous stirring, concentrated sulfuric acid (8 mL) was added dropwise. After 30 min of stiring, the organic layer was separated and stored on anhydrous Na_2SO_4. Triphenylphosphine (525 mg, 2.0 mmol) was dissolved in toluene (5 mL), cooled to 0°C and diisopropylazodicarboxylate (0.40 mL, 2.0 mmol) was added, followed by 5 mL of the freshly prepared hydrazoic acid stock solution, resulting in the formation of copious yellow precipitate. Then a solution of compound **16** (360 mg, 1.0 mmol) in toluene (5 mL) was added and the icebath was removed. Stirring was continued for 4 hours during which the appearance of the reaction mixture shifted to clear and almost colourless. The reaction mixture was concentrated *in vacuo*, the residue was taken up in diethyl ether and triphenylphosphine oxide was crystallized out by addition of heptane and seeding with triphenylphosphine oxide. The resulting crude product was then purified by DCVC (25 to 100 % ethyl acetate in toluene continued by 0 to 10 % methanol in ethyl acetate) to yield 336 mg (88 %) of **17** as an oil. 1H NMR (300 MHz, CHLOROFORM-d) δ ppm 1.34 (td, J=7.10, 0.73 Hz, 6 H) 1.71 - 1.95 (m, 1 H) 2.10 (s, 3 H) 2.12 - 2.32 (m, 1 H) 3.56 (td, J=11.50, 3.37 Hz, 1 H) 3.68 - 3.95 (m, 2 H) 4.08 - 4.29 (m, 4 H) 4.84 (s, 2 H) 7.38 (s, 5 H); HRMS (ESI) m/z 385.1628 [$(M+H)^+$, calcd for $C16H26N4O5P^+$ 385,1635].

Diethyl 3-(*N*-hydroxyacetamido)-1-azidopropylphosphonate (18). A solution of **17** (334 mg, 0.87 mmol) in dichloromethane (8 mL) was cooled to -75°C and BCl_3 (2.6 mL of a 1M solution in dichloromethane, 2.6 mmol) was added dropwise. After 45 minutes of stirring at -75°C the reaction mixture was poured into aqueous $NaHCO_3$ and extracted 4 times with dichloromethane. The combined organic fractions were washed with brine, dried over anhydrous Na_2SO_4 and evaporated *in*

vacuo. The resulting crude mixture was purified by DCVC on a silica column that was previously 'deactivated' by rinsing with 5% triethylamine in hexanes (0 to 20 % methanol in ethyl acetate containing 1% triethylamine) to yield 201 mg (79 %) of **18** as an oil. 1H NMR (300 MHz, METHANOL-d4) δ ppm 1.22 - 1.49 (m, 6 H) 1.62 - 1.88 (m, 1 H) 1.98 - 2.25 (m, 4 H) 3.60 - 3.99 (m, 3 H) 4.08 - 4.32 (m, 5 H); HRMS (ESI) m/z 295.1169 [$(M+H)^+$, calcd for $C9H20N4O5P^+$ 295,1166].

***tert*-Butyl 3-(*N*-(benzyloxy)acetamido)-1-tosylpropylcarbamate (19).** A mixture of aldehyde **8** (1106 mg, 5 mmol), *tert*-butyl carbamate (650 mg, 5.5 mmol), anhydrous sodium *p*-toluenesulfinate (980 mg, 5.5 mmol), water (5 ml), THF (2 ml), acetonitrile (5 mL) and formic acid (540 µL, 14 mmol) was stirred overnight at room temperature, forming a clear solution. As the reaction was not finished at this point according to TLC (25:75 toluene – ethyl acetate), the reaction mixture was stirred for another 6 hours at 50°C. After cooling to room temperature, aqueous $NaHCO_3$ was added and the mixture was extracted three times with diethyl ether, the combined organic phases were washed with brine, dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. The resulting crude mixture was purified by DCVC (0 to 50 % ethyl acetate in hexanes) to yield 1920 mg (81 %) of **19**. 1H NMR (300 MHz, CHLOROFORM-d) δ ppm 1.10 - 1.32 (m, 9 H) 1.88 - 2.22 (m, 4 H) 2.29 - 2.46 (m, 3 H) 2.46 - 2.69 (m, 1 H) 3.66 - 3.96 (m, 2 H) 4.83 (s, 2 H) 4.90 (td, J=10.54, 4.10 Hz, 1 H) 5.41 (d, J=10.54 Hz, 1 H) 7.18 - 7.35 (m, 2 H) 7.39 (s, 5 H) 7.61 - 7.88 (m, 2 H); HRMS (ESI) m/z 477.2058 [$(M+H)^+$, calcd for $C24H33N2O6S^+$ 477,2054].

***tert*-Butyl 1-((benzyloxy)phosphono)-3-(*N*-(benzyloxy)acetamido)propylcarbamate (20).** To a solution of **19** (1920 mg, 4.03 mmol) in THF (15 mL) was added NaH (360 mg of a 60% dispersion in mineral oil, 8.06 mmol) in one batch while stirring at room temperature, resulting in a grey suspension. After 15 minutes, a solution of dibenzyl phosphite (1160 mg, 4.42 mmol) in THF (5 mL) was added dropwise and the reaction mixture was stirred for 1 hour at room temperature. The reaction mixture was then cooled to 0°C, quenched with saturated aqueous NH_4Cl and extracted three times with ethyl acetate. The combined organic phases were washed with brine, dried over

anhydrous Na_2SO_4 and evaporated *in vacuo*. The resulting crude mixture was purified by DCVC (50 to 100 % ethyl acetate in hexanes) to yield 2020 mg (86 %) of **20** as an oil. 1H NMR (300 MHz, CHLOROFORM-d) δ ppm 1.24 - 1.52 (m, 9 H) 1.84 (dt, J=9.15, 4.65 Hz, 1 H) 2.04 (s, 3 H) 2.24 (d, J=8.20 Hz, 1 H) 3.54 - 3.89 (m, 2 H) 4.05 - 4.35 (m, 1 H) 4.68 - 4.82 (m, 2 H) 4.87 (d, J=10.25 Hz, 1 H) 4.92 - 5.12 (m, 4 H) 7.11 - 7.51 (m, 15 H); HRMS (ESI) m/z 583.2568 [$(M+H)^+$, calcd for $C31H40N2O7P^+$ 583,2568].

Dibenzyl 3-(*N*-(benzyloxy)acetamido)-1-aminopropylphosphonate (7). A solution of **20** (1660 mg, 2.85 mmol) in dichloromethane (30 mL) was cooled to 0°C and TFA (11 mL, 142 mmol) was added dropwise. After 20 minutes of stirring at 0°C the reaction mixture was poured into aqueous K_2CO_3 and extracted 4 times with chloroform. The combined organic fractions were washed with brine, dried over anhydrous Na_2SO_4 and evaporated *in vacuo* which resulted in 1350 mg (98 %) of crude **7** that was used as such in the following reactions. HRMS (ESI) m/z 483.2050 [$(M+H)^+$, calcd for $C26H32N2O5P^+$ 483,2043]

Dibenzyl 3-(*N*-(benzyloxy)acetamido)-1-(benzamido)propylphosphonate (21a). To a solution of **7** (241 mg, 0.5 mmol) in dichloromethane (2.5 mL) was added triethylamine (140 µL, 1 mmol), DMAP (6 mg, 0.05 mmol) and benzoylchloride (87µL, 0.75 mmol). The reaction mixture was stirred at room temperature for 3.5 hours, then poured into aqueous 0.2N HCl and extracted three times with ethyl acetate. The combined organic phases were washed with brine, dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. The resulting crude mixture was purified by DCVC (50 to 100 % ethyl acetate in hexanes continued by 0 to 10 % ethanol in ethyl acetate) to yield 261 mg (89 %) of **21a** as an oil. 1H NMR (300 MHz, CHLOROFORM-d) δ ppm 1.87 (s, 3 H) 1.96 - 2.19 (m, 1 H) 2.21 - 2.44 (m, 1 H) 3.83 (t, J=6.88 Hz, 2 H) 4.43 - 4.62 (m, 2 H) 4.62 - 4.80 (m, 1 H) 4.86 - 5.10 (m, 4 H) 6.98 (d, J=6.74 Hz, 2 H) 7.07 - 7.47 (m, 16 H) 7.50 - 7.65 (m, 2 H); HRMS (ESI) m/z 587.2311 [$(M+H)^+$, calcd for $C33H36N2O6P^+$ 587,2305]

Dibenzyl 3-(*N*-(benzyloxy)acetamido)-1-(3-phenylureido)propylphosphonate (21b). To a solution of **7** (241 mg, 0.5 mmol) in dichloromethane (2.5 mL) was added triethylamine (70 µL, 0.5 mmol), DMAP (6 mg, 0.05 mmol) and phenylisocyanate (65µL, 0.6 mmol). The reaction mixture was stirred at room temperature for 4 hours, then poured into aqueous 0.2N HCl and extracted three times with ethyl acetate. The combined organic phases were washed with brine, dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. The resulting crude mixture was purified by DCVC (50 to 100 % ethyl acetate in hexanes continued by 0 to 10 % ethanol in ethyl acetate) to yield 291 mg (97 %) of **21b** as an oil. 1H NMR (300 MHz, CHLOROFORM-d) δ ppm 1.80 - 2.01 (m, 4 H) 2.27 (br. s., 1 H) 3.46 - 3.85 (m, 2 H) 4.51 - 4.72 (m, 1 H) 4.72 - 4.87 (m, 2 H) 4.89 - 5.12 (m, 4 H) 6.73 (d, J=9.67 Hz, 1 H) 6.90 - 7.09 (m, 1 H) 7.13 - 7.43 (m, 19 H) 7.49 (s, 1 H); HRMS (ESI) m/z 602.2410 $[(M+H)^+$, calcd for $C33H37N3O6P^+$ 602,2414]

Phenyl 1-((benzyloxy)phosphono)-3-(*N*-(benzyloxy)acetamido)propylsulfonamide (22). A solution of **7** (436 mg, 0.90 mmol) and triethylamine (190 µL, 1.35 mmol) in dichloromethane (5 mL) was cooled to 0°C and phenylsulfonylchloride (120 µL, 0.9 mmol) was added dropwise. The icebath was removed and the reaction mixture was stirred overnight, gradually warming to room temperature. Saturated aqueous NH_4Cl was then added to quench the reaction, the reaction mixture was partitioned between water and ethyl acetate and the aqueous phase was extracted three times with ethyl acetate. The combined organic phases were washed with brine, dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. The resulting crude mixture was purified by DCVC (50 to 100 % ethyl acetate in hexanes continued by 0 to 10 % methanol in ethyl acetate) to yield 384 mg (69 %) of **22** as a white solid. 1H NMR (300 MHz, CHLOROFORM-d) d ppm 1.72 - 1.97 (m, 4 H) 2.05 - 2.19 (m, 1 H) 2.99 (br. s., 2 H) 4.41 - 4.70 (m, 1 H) 4.84 - 5.16 (m, 6 H) 6.46 - 6.76 (m, 1 H) 7.14 - 7.37 (m, 15 H) 7.39 - 7.54 (m, 2 H) 7.54 - 7.64 (m, 1 H) 7.72 - 7.88 (m, 2 H); HRMS (ESI) m/z 623.1968 $[(M+H)^+$, calcd for $C32H36N2O7PS^+$ 623,1975]

Diethyl (3-(*N*-(benzyloxy)acetamido)-1-(phenylsulfonamido)propyl)phosphonate (25). Benzenesulfonamide (473 mg, 3 mmol) was suspended in acetyl chloride (20 mL), cooled to 0° C and diethyl phosphite (0.384 mL, 3 mmol) and aldehyde **8** (733 mg, 3.31 mmol) were added dropwise. After 20 minutes of stirring at 0° C the icebath was removed and stirring was continued overnight at room temperature. All volatiles were removed *in vacuo* and the resulting crude was purified by DCVC (50 to 100 % ethyl acetate in hexanes continued by 0 to 10 % methanol in ethyl acetate) to yield 1737 mg (93 %) of **25** as a white solid. 1H NMR (300 MHz, CHLOROFORM-d) δ ppm 1.08 - 1.43 (m, 6 H) 1.85 - 2.24 (m, 5 H) 3.55 - 3.71 (m, 2 H) 3.71 - 3.87 (m, 1 H) 3.87 - 4.23 (m, 4 H) 4.73 (s, 2 H) 6.94 (d, J=8.20 Hz, 1 H) 7.21 - 7.56 (m, 8 H) 7.79 - 8.02 (m, 2 H); HRMS (ESI) m/z 499.1668 [$(M+H)^+$, calcd for $C22H32N2O7PS^+$ 499,1662]

3-(*N*-hydroxyacetamido)-1-azidopropylphosphonic acid, bisammonium salt (2). Compound **18** (165 mg, 0.561 mmol) was coevaporated with toluene (3 × 10 mL), taken up in acetonitrile (5 mL) and BSTFA (600 µL, 2.24 mmol) was added. After 15 minutes of stirring at room temperature, an icebath was installed and TMSBr (2.5 mL, 19 mmol) was added. The icebath was removed after 10 minutes and the reaction was stirred further at room temperature until, after 2.5 hours, ^{31}P-NMR confirmed that the starting phosphonate was completely deprotected (shift from δ = 23 ppm to 3 ppm). All volatiles were removed *in vacuo*, followed by coevaporation with toluene (3 × 10 mL). The resulting oil was taken up in acetonitrile, concentrated ammonia was added, the mixture was stirred at room temperature for 30 minutes and evaporated to give the crude material as a brown oil. This was dissolved in methanol, decolorized over activated carbon and lyophilized from water to give the product as a hygroscopic resin in quantitative yield. 1H NMR (300 MHz, METHANOL-d4) δ ppm 1.63 - 1.87 (m, 1 H) 2.03 - 2.35 (m, 4 H) 3.18 - 3.37 (m, 1 H) 3.45 - 3.69 (m, 1 H) 3.88 - 4.11 (m, 1 H); 13C NMR (75 MHz, METHANOL-d4) δ ppm 20.57, 28.60, 46.73 (d, J=12.99 Hz), 59.09 (d, J=143.47 Hz), 173.94; 31P NMR (121 MHz, METHANOL-d4) δ ppm 16.45; HRMS (ESI) m/z 237.0423 [$(M-H^+)$, calcd for $C5H10N4O5P^-$ 237,0394].

3-(*N*-hydroxyacetamido)-1-(benzamido)propylphosphonic acid (5a). To a solution of **21a** (309 mg, 0.53 mmol) in a mixture of methanol – water – *tert*-butanol (10 mL) was added 10% Pd/C. Hydrogen gas was bubbled through via a glass capillary at atmospheric pressure for 3.5 hours after which the reaction mixture was filtered and concentrated *in vacuo*. The residue was taken up in *tert*-butanol, frozen and lyophilized to give the product as a white foam in quantitative yield. 1H NMR (300 MHz, METHANOL-d4) δ ppm 1.72 - 2.11 (m, 3 H) 2.42 (ddd, J=14.21, 6.59, 3.22 Hz, 1 H) 3.52 - 3.79 (m, 1 H) 3.90 (d, J=4.69 Hz, 1 H) 4.34 (t, J=12.59 Hz, 1 H) 7.23 - 7.51 (m, 5 H) 7.61 (d, J=7.03 Hz, 1 H)); 13C NMR (75 MHz, METHANOL-d4) δ ppm 21.53, 26.55, 30.04, 44.60 (d, J=155.91 Hz), 129.90, 128.12, 130.33, 134.59, 172.20; 31P NMR (121 MHz, METHANOL-d4) δ ppm 22.53; HRMS (ESI) m/z 315.0767 [(M-H^+), calcd for C12H16N2O6P^- 315,0751].

3-(*N*-hydroxyacetamido)-1-(3-phenylureido)propylphosphonic acid (5b). To a solution of **21b** (321 mg, 0.53 mmol) in a mixture of methanol – water – *tert*-butanol (10 mL) was added 10% Pd/C. Hydrogen gas was bubbled through via a glass capillary at atmospheric pressure for 3 hours after which the reaction mixture was filtered and concentrated *in vacuo*. The residue was taken up in a mixture of water and *tert*-butanol, frozen and lyophilized to give the product as a white foam in quantitative yield. 1H NMR (300 MHz, DMSO-d6) d ppm 1.58 - 1.82 (m, 1 H) 1.82 - 1.91 (m, 3 H) 1.96 - 2.17 (m, 1 H) 3.29 - 3.58 (m, 2 H) 4.04 (ddd, J=16.69, 10.84, 3.81 Hz, 1 H) 6.82 - 7.06 (m, 1 H) 7.14 - 7.34 (m, 2 H) 7.50 - 7.72 (m, 2 H); 13C NMR (75 MHz, DMSO-d6) d ppm 22.51, 22.56, 27.10, 44.25 (d, J=153.70 Hz), 47.52 (d, J=14.93 Hz), 119.19, (s, 1 C) 119.30, (s, 1 C) 122.16, 128.39, 139.37, 157.39, 169.03; 31P NMR (121 MHz, DMSO-d6) δ ppm 21.12; HRMS (ESI) m/z 330.0901 [(M-H^+), calcd for C12H17N3O6P^- 330,0860].

3-(*N*-hydroxyacetamido)-1-(4-tert-butyl-1H-1,2,3-triazol-1-yl)propylphosphonic acid (1b). To a solution of **15b** (84 mg, 0.14 mmol) in a mixture of methanol – water – *tert*-butanol (10 mL) was added 10% Pd/C. Hydrogen gas was bubbled through via a glass capillary at atmospheric pressure for 4 hours after which the reaction mixture was filtered and concentrated *in vacuo*. The residue was

taken up in *tert*-butanol, frozen and lyophilized to give the product as a white foam in quantitative yield. 1H NMR (300 MHz, METHANOL-d4) d ppm 1.37 (s, 9 H) 2.06 (s, 3 H) 2.41 - 2.79 (m, 2 H) 3.35 - 3.51 (m, 1 H) 3.61 (dd, J=14.35, 7.32 Hz, 1 H) 4.76 - 4.99 (m, 1 H) 7.91 (s, 1 H); 13C NMR (75 MHz, METHANOL-d4) δ ppm 20.20, 28.34, 30.49, 31.73, 44.49 (d, J=12.72 Hz), 57.82 (d, J=148.17 Hz), 122.30, 157.58, 174.002; 31P NMR (121 MHz, METHANOL-d4) δ ppm 14.83; HRMS (ESI) m/z 319.1200 [(M-H^+), calcd for $C_{11}H_{20}N_4O_5P^-$ 319,1177].

3-(*N*-hydroxyacetamido)-1-hydroxypropylphosphonic acid, ammonium salt (3a). A mixture of compound **6** (261 mg, 0,54 mmol), ammonium formate (520 mg, 8.10 mmol) and 10% Pd/C in methanol (10 mL) was heated at reflux for 20 minutes, followed by filtration over a glass microfiber pad. The filter was rinsed with methanol and water and the filtrate was concentrated *in vacuo*. The resulting residue was lyophilized from a mixture of water and *tert*-butanol to give the product as an extremely hygroscopic resinous solid in quantitative yield. 1H NMR (300 MHz, METHANOL-d4) δ ppm 1.67 - 1.98 (m, 1 H) 2.13 (s, 4 H) 3.46 - 3.80 (m, 2 H) 3.80 - 4.12 (m, 1 H); 13C NMR (75 MHz, METHANOL-d4) d ppm 20.64, 30.98, 46.70 (d, J=14.93 Hz), 67.99 (d, J=157.84 Hz); 31P NMR (121 MHz, METHANOL-d4) δ ppm 19.86; HRMS (ESI) m/z 212.0351 [(M-H^+), calcd for $C_5H_{11}NO_6P^-$ 212,0329].

3-(*N*-hydroxyacetamido)-1-methoxypropylphosphonic acid (3b). Was prepared according to the same procedure as **3a** yielding an off-white hygroscopic solid (quant.). ^{1}H NMR (300 MHz, METHANOL-d_4) δ ppm 1.83 (dquin, *J*=14.33, 7.47, 7.47, 7.47, 7.47 Hz, 1 H) 2.05 - 2.29 (m, 4 H) 3.16 - 3.28 (m, 1 H) 3.43 - 3.64 (m, 4 H) 3.88 - 4.08 (m, 1 H); ^{13}C NMR (75 MHz, METHANOL-d_4) δ ppm 20.56, 29.50, 29.52 (d, J=3.59 Hz), 46.02 (d, J=12.99 Hz) 60.24, 78.15 (d, J=158.40 Hz) 173.70; 31P NMR (121 MHz, METHANOL-d4) δ ppm 18.83; HRMS (ESI) m/z 226.0520 [(M-H^+), calcd for $C_6H_{13}NO_6P^-$ 226,0486].

3-(*N*-hydroxyacetamido)-1-phenoxypropylphosphonic acid (3c). Was prepared according to the same procedure as **3a** yielding a white hygroscopic solid (quant.). ^{1}H NMR (300 MHz, METHANOL-d_4)

δ ppm 1.92 - 2.22 (m, 4 H) 2.22 - 2.54 (m, 1 H) 3.58 - 3.97 (m, 2 H) 4.29 - 4.59 (m, 1 H) 6.89 (t, *J*=7.18 Hz, 1 H) 7.05 (d, *J*=7.91 Hz, 2 H) 7.23 (t, *J*=7.91 Hz, 2 H) 8.52 (s, 1 H); ^{13}C NMR (75 MHz, METHANOL-d_4) δ ppm 20.36, 29.64, 46.27 (d, J=8.85 Hz), 75.05 (d, J= 158.67Hz), 117.31, 121.93, 130.331, 160.70 (d, J=6.63 Hz) 173.46; 31P NMR (121 MHz, METHANOL-d4) δ ppm 16.52; HRMS (ESI) m/z 288.0633 [(M-H$^+$), calcd for C11H15NO6P$^-$ 288,0642].

3-(*N*-hydroxyacetamido)-1-(1*H*-1,2,3-triazol-1-yl)propylphosphonic acid (1a). Was prepared according to the same procedure as **3a** yielding a white solid (quant.). ^{1}H NMR (300 MHz, METHANOL-d_4) δ ppm 2.07 (s, 3 H) 2.22 - 2.49 (m, 1 H) 2.51 - 2.76 (m, 1 H) 3.18 - 3.43 (m, 2 H) 3.64 (dt, *J*=14.28, 7.36 Hz, 1 H) 4.55 - 4.91 (m, 1 H) 7.72 (s, 1 H) 8.08 (s, 1 H); ^{13}C NMR (75 MHz, METHANOL-d_4) δ ppm 20.57, 29.98, 46.28 (d, J= 12.72 Hz), 59.92 (d, J= 136.56 Hz), 125.93, 134.15, 173.89; 31P NMR (121 MHz, METHANOL-d4) δ ppm 11.88; HRMS (ESI) m/z 263.0589 [(M-H$^+$), calcd for C7H12N4O5P$^-$ 263,0551].

3-(*N*-(benzyloxy)acetamido)-1-(4-phenyl-1*H*-1,2,3-triazol-1-yl)propylphosphonic acid (1c). Was prepared according to the same procedure as **3a** yielding a white solid (quant.). ^{1}H NMR (300 MHz, METHANOL-d_4) δ ppm 2.07 (s, 3 H) 2.32 - 2.56 (m, 1 H) 2.56 - 2.81 (m, 1 H) 3.33 - 3.52 (m, 1 H) 3.72 (dt, *J*=14.06, 7.32 Hz, 1 H) 4.59 - 4.86 (m, 1 H) 7.23 - 7.51 (m, 3 H) 7.73 - 7.95 (m, 2 H) 8.39 (s, 1 H); 31P NMR (121 MHz, METHANOL-d4) δ ppm 12.27; HRMS (ESI) m/z 339.0839 [(M-H$^+$), calcd for C13H16N4O5P$^-$ 339,0864].

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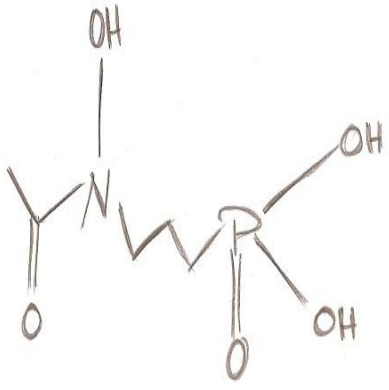
Chapter VI

Summary



VI.Summary

The current worldwide malaria burden, combined with the issue of resistance and the difficulties associated with the development of a vaccine form the major motivation for the development of new antimalarial drugs. The non-mevalonate pathway for isoprenoid biosynthesis, which is vital for the malaria causing *Plasmodium* parasite and absent in humans forms an interesting and validated target for the development of new antimalarials. Fosmidomycin and its acetyl congener FR900098 inhibit DXR, the second enzyme of the non-mevalonate pathway, and the former was found to be a safe and effective antimalarial when combined with clindamycin or artesunate in Phase III clinical trials. The major drawbacks of fosmidomycin are its high polarity and moderate potency, thus creating the necessity for high daily doses to cure malaria. In an effort to unravel the mode of action of fosmidomycin-based DXR inhibitors and to design more potent antimalarials, many analogues of fosmidomycin have been synthesized and published over the past decade. An overview is given in Chapter I, together with an attempt to derive structure-activity relationships. This thesis describes our efforts toward the synthesis of novel fosmidomycin analogues expected to show potent DXR inhibition based on this SAR. Toward this end, three major modifications of fosmidomycin/FR900098 were proposed:

First, we explored α-halogenation of FR900098 so as to increase the acidity of the phosphonate and to obtain a better phosphate mimic, thereby leading to enhanced DXR inhibition (Chapter III). Based on a literature procedure for electrophilic α-halogenation of alkylphosphonates, diethyl butenylphosphonate **1** was converted to versatile α-chloro and α-fluoro butenylphosphonate building blocks **2a** and **2b** (Figure VI.1). These were further transformed into the α-monochloro and the α-monofluoro FR900098 analogues **3a** and **3b**, as well as the α-monofluoro reversed hydroxamate **4**.

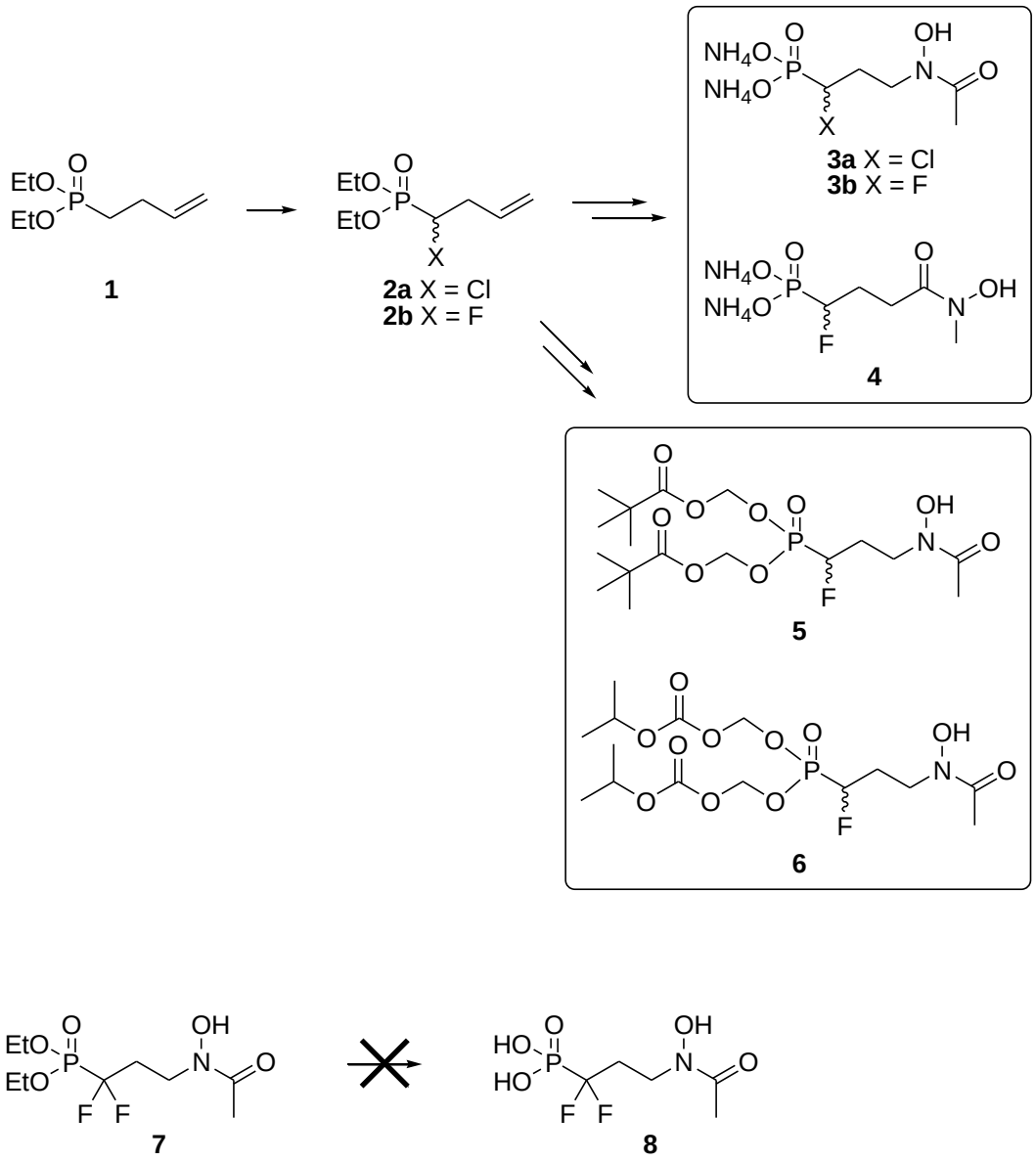


Figure VI.1: Development of α-halogenated analogues of FR900098

Many efforts were undertaken to synthesize α,α-difluoro FR900098 (**8**), but final deprotection of **7** always caused rapid hydrolysis of the retrohydroxamate functionality, probably mediated by the (increased) acidity of the α,α-difluorophosphonate moiety.

The expected pKa_2 decrease in the monohalogenated analogues was assessed by a ^{31}P-NMR monitored acid-base titration. Biological testing of the three halogenated free phosphonates revealed enhanced DXR inhibition and a stronger antiplasmodial effect compared to parent fosmidomycin. Following these good results, a synthetic strategy towards two prodrugs of α-fluoro

FR900098 (**5**, **6**) was optimized, resulting in the bis-POM (**5**) and the bis-carbonate (**6**) prodrugs. These two compounds showed the highest in vitro antiplasmodial activity so far for fosmidomycin based DXR inhibitors. Upscaling of these prodrugs to allow *in vivo* evaluation would be desirable.

The retrohydroxamate moiety of fosmidomycin/FR900098 makes an important contribution to the overall binding affinity for DXR and modifications of this functionality generally led to detrimental losses in DXR inhibition. Nevertheless, we inferred that incorporation of the (retro)hydroxamate in a four-membered ring structure (an *N*-hydroxyazetidinone, NHA) might lock it in its active (metal chelating) conformation and thus increase the potency of such an analogue, while the steric implications of such a modification should be limited. In Chapter IV we describe the development of a straightforward synthetic route toward the 4-(ω-phosphonoethyl)-NHA (or 'retrohydroxamate *N*-hydroxybetalactam') analogue, featuring one final deprotection under mild conditions (Figure VI.2).

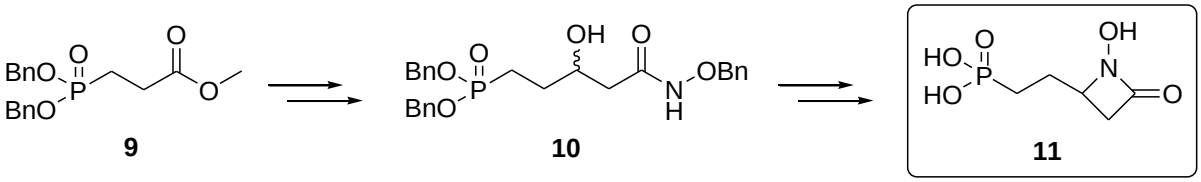


Figure VI.2: Development of a conformationally restricted FR900098 analogue

Unfortunately, **11** proved very sensitive to hydrolysis. The low potency observed *in vitro* is probably due to fast hydrolytic breakdown under testing conditions. On the other hand, good interaction with DXR may be impeded by sterical hindrance, as γ-methyl fosmidomycin was previously shown to be inactive. Because of the inherent sensitivity of the NHA structure toward hydrolysis, the 3-(ω-phosphonoethyl)-NHA (or 'hydroxamate *N*-hydroxybetalactam') was not pursued.

In the third major part of this work, described in Chapter V, we tried to extend the successful series of α-substituted fosmidomycin analogues. By introducing a hydroxyl or an amine group on the

α-carbon of FR900098, divergent α-substitution became possible. All envisaged analogues were synthesized in few steps and usually good yields from common aldehyde building block **12** (Figure VI.3). A high yield straightforward synthesis towards this aldehyde was also developed.

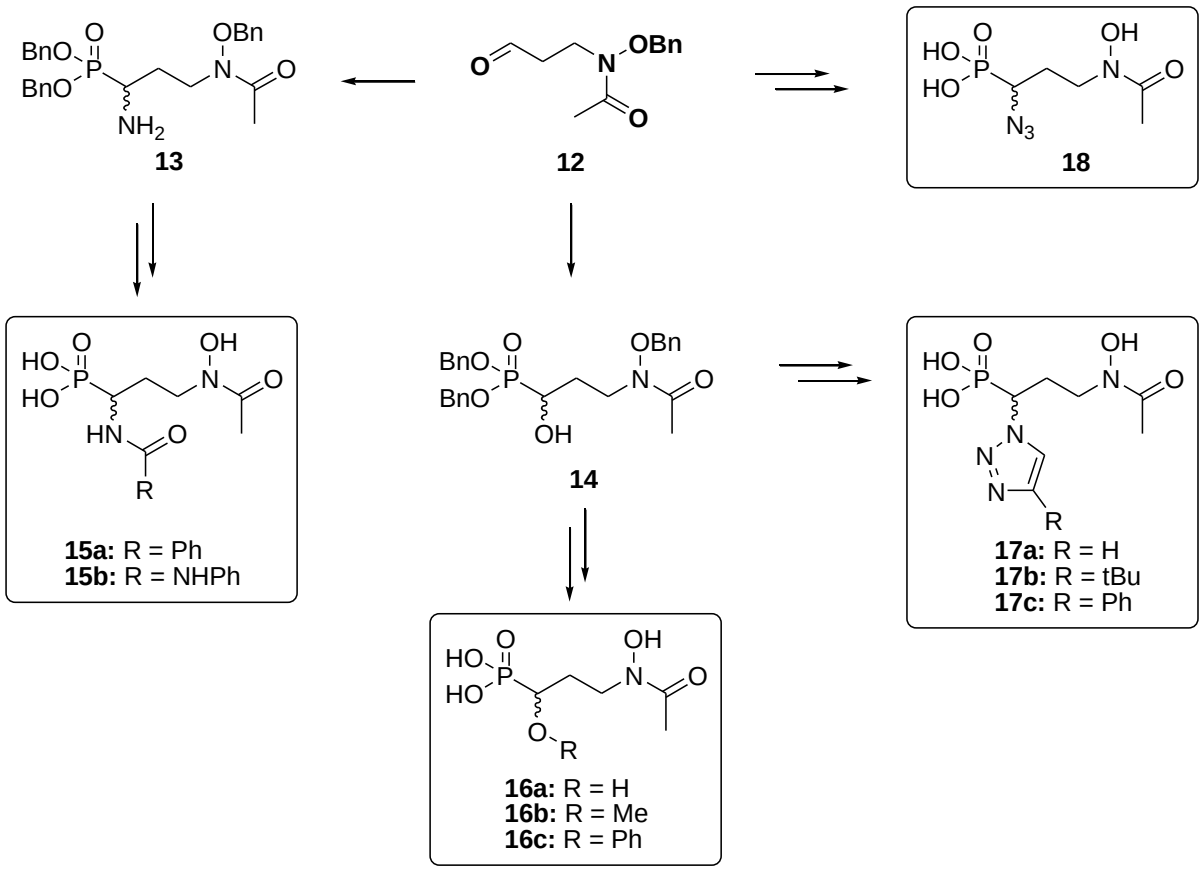


Figure VI.3: Development of α-heteroatom substituted analogues of FR900098

Four major analogues/groups of analogues were synthesized and biologically tested:

1. The α-benzamide **15a** and α-phenylurea **15b** failed to inhibit EcDXR or *P. falciparum* growth.

2. The α-hydroxy analogue of FR900098 (**16a**) showed considerable EcDXR inhibition as well as *P. falciparum* growth inhibition, while its methyl and phenyl ether (**16b**, **16c**)were virtually inactive.

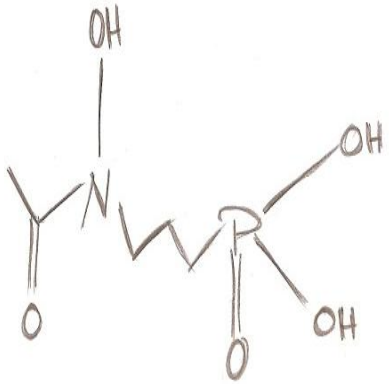
3. Surprisingly, none of α-triazolyl analogues **1a**, **1b** and **1c** suppressed *P. falciparum* growth to a significant extent and only *t*-butylated triazole **1b** showed EcDXR inhibition (albeit rather weak). Probably the triazole moiety in these molecules is too electron rich in order to obtain the π-π-stacking interaction with Trp211 of the enzyme that consolidates the binding of the aforementioned α-aryl fosmidomycins.

4. The α-Azido FR900098 (**18**) showed the highest activity in this series, surpassing both parent compounds (fosmidomycin and FR900098) in EcDXR inhibition and almost equaling fosmidomycin in inhibiting *P. falciparum* growth. It is also the first fosmidomycin analogue bearing a rigid, linear, heteroatom-based α-substituent. Perhaps its enhanced potency is due to the halogen-mimicking effect of the azide, increasing the acidity of the phosphonate moiety but other, new interactions with the enzyme are also possible. X-ray studies could provide more insight in this matter. The unique reactivity of the azide group in this potent DXR inhibitor also opens the way for a target-guided, in situ click chemistry approach as already successfully employed for discovering acetylcholinesterase (AChE) inhibitors by incubating the DXR/azide **3** combination with a variety of acetylene reagents in an effort to identify new binding partners for the flexible loop of DXR, e.g., for interaction with other residues, neighboring Trp211.

In summary, new and potent inhibitors of DXR were developed during this doctoral research, which may lead to novel antimalarials. The introduction of a halogen or an azide group in α-position of the phosphonate of FR900098 led to the most interesting DXR inhibitors and the formulation of prodrugs robustly enhanced the antimalarial potency of α-fluoro FR900098. Further research into such phosphonate prodrugs, as well as the binding mode of the α-azido analogue are warranted.

Chapter VII

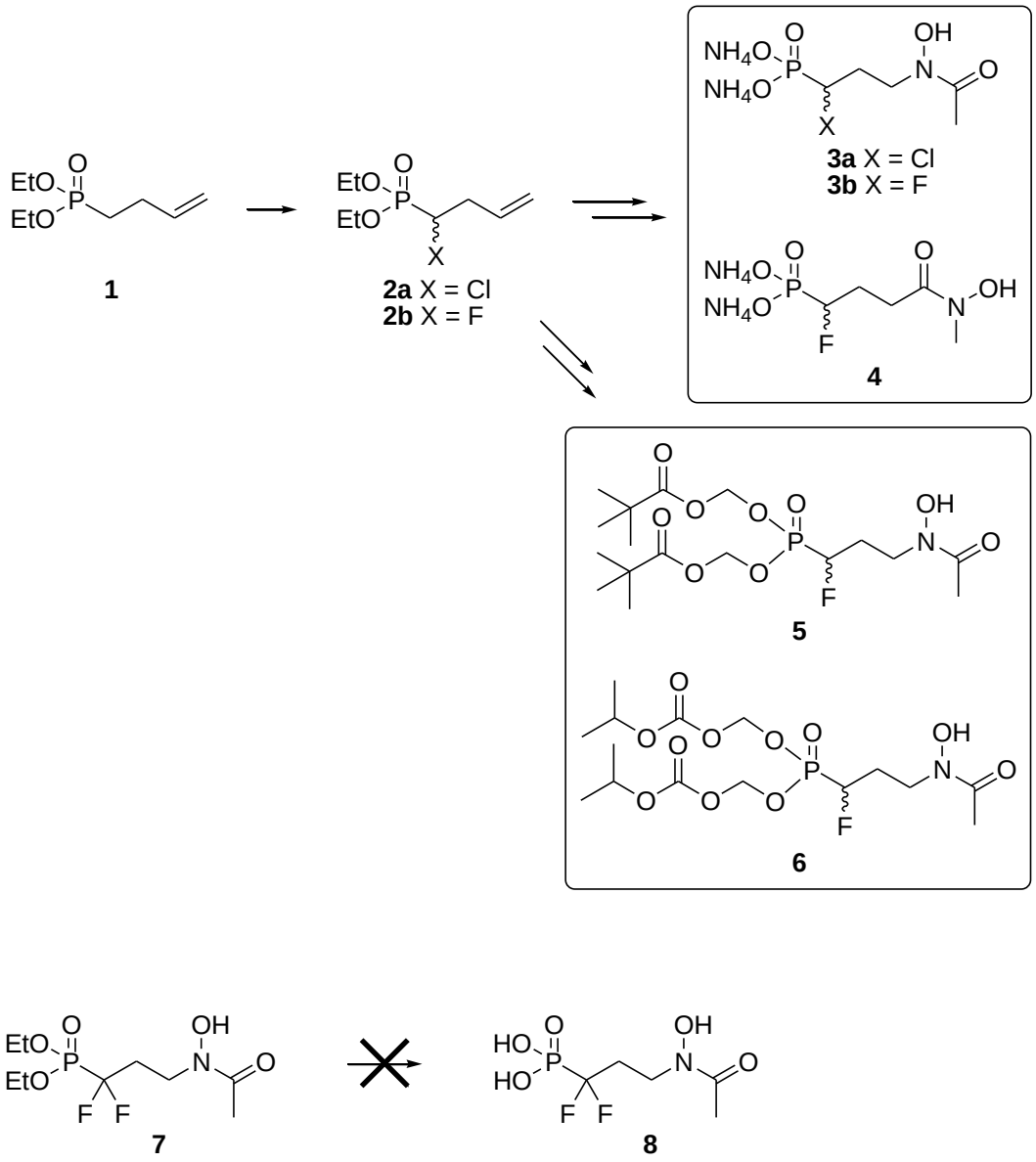
Samenvatting



VII.Samenvatting

De huidige wereldwijde dreiging van malaria, gecombineerd met de resistentieproblematiek en de moeizame vordering in de ontwikkeling van een vaccin vormen een sterke motivatie voor de ontwikkeling van nieuwe antimalariamiddelen. De non-mevalonaat *pathway* voor de biosynthese van isoprenoiden, welke levensbelangrijk is voor de pathogene *Plasmodium* parasiet maar afwezig in de mens, is een interessant en gevalideerd doel voor de ontwikkeling van nieuwe antimalariamiddelen. Fosmidomycine en zijn acetyl-analoog FR900098 inhiberen DXR, het tweede enzym in de non-mevalonaat *pathway* en fosmidomycine bleek reeds een veilig en effectief antimalariamiddel in combinatie met clindamycine of artesunaat in Fase III klinische studies. De voornaamste nadelen van fosmidomycine zijn zijn hoge polariteit en matige sterkte, die hoge dagelijkse dosissen nodig maken in de behandeling van malaria. Gedurende de voorbije tien jaar is een veelvoud aan fosmidomycine-gebaseerd DXR-inhibitoren gesynthetiseerd en gepubliceerd, met het oog op de studie van het mechanisme van DXR en het ontwikkelen van krachtiger antimalariamiddelen. Een overzicht hiervan wordt gegeven in Hoofdstuk I, samen met een poging structuur-activiteitsrelaties bloot te leggen. In dit doctoraatswerk wordt de synthese beschreven van fosmidomycine-analogen waarvan, gebaseerd op deze gegevens, een sterke DXR inhibitie wordt verwacht. Hiertoe werden drie modificatiestrategieën van fosmidomycine/FR900098 uitgetest:

Eerst werd de α-halogenering van FR900098 verkend als strategie om de zuursterkte van de fosfonaatgroep te verhogen en een beter fosfaat-analoog te bekomen, wat moet leiden tot een sterkere inhibitie van DXR (Hoofdstuk III). Diëthyl butenylfosfonaat **1** werd daartoe omgezet in de veelzijdige bouwstenen **2a** en **2b** (Figuur VII.1) door middel van een literatuurgebaseerde procedure voor de elektrofiele α-halogenering van alkylfosfonaten. Deze bouwstenen dienden voor de synthese van α-monochloro en α-monofluoro FR900098 analogen **3a** en **3b**, en ook voor synthese van α-monofluoro omgekeerd hydroxamaat **4**.



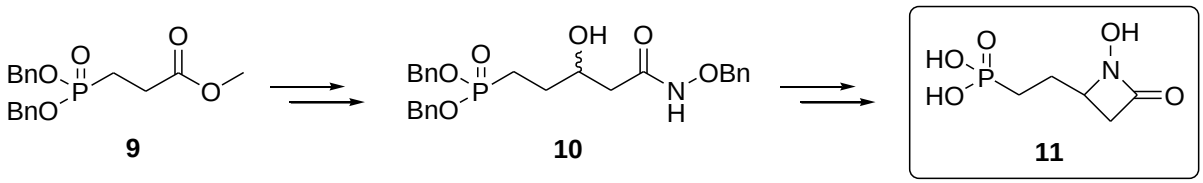
Figuur VI.1: Ontwikkeling van α-gehalogeneerde analogen van FR900098

Veel aandacht werd besteed aan de synthese van α,α-difluoro FR900098 (**8**), maar finale ontscherming van precursor **7** leidde steevast tot snelle hydrolyse van de retrohydroxamaatfunctionaliteit, mogelijks onder invloed van (of versneld door) de zure α,α-difluorofosfonaatgroep.

De veronderstelde daling in pKa_2 van de fosfonaatgroep in de α-monogehalogeneerde analogen werd aangetoond met behulp van een zuur-base titratie opgevolgd mbv ^{31}P-NMR . In biologische

testen vertoonden alledrie de gehalogeneerde vrije fosfonzuren een verhoogde DXR inhibitie en een sterker antimalaria effect dan moedermolecule fosmidomycine. Gevolg gevend aan deze hoopvolle resultaten werd een synthesestrategie op punt gesteld voor twee prodrugs van α-fluoro FR900098 (**3b**): de bis-POM ester (**5**) en bis-carbonaat **6**. Deze moleculen vertoonden de sterkste *in vitro* antimalaria activiteit totnogtoe voor fosmidomycine-gebaseerde DXR inhibitoren. Opschaling van deze analogen voor *in vivo* tests is nu zeer wenselijk.

De retrohydroxamaatgroep van fosmidomycine/FR900098 draagt in belangrijke mate bij tot de algemene bindingsaffiniteit voor DXR, en modificatie van deze functionaliteit leidde totnogtoe in quasi alle gevallen tot grote verliezen in DXR inhibitie. Nochtans zou het incorporeren van de (retro)hydroxamaatgroep in een vierledige ringstructuur (een *N*-hydroxyazetidinon, NHA) ervoor zorgen dat het (retro)hydroxamaat wordt vastgezet in zijn actieve (metaalbindende) conformatie. Dit zou moeten leiden tot een hogere activiteit voor zulk analoog terwijl de sterische hinder veroorzaakt door de modificatie eerder beperkt is. In Hoofdstuk IV wordt de ontwikkeling beschreven van een syntheseroute voor het 4-(ω-fosfonoethyl)-NHA (of 'retrohydroxamaat *N*-hydroxybetalactam') analoog, gekenmerkt door één finale ontschermingsstap onder milde condities (Figuur VII.2).

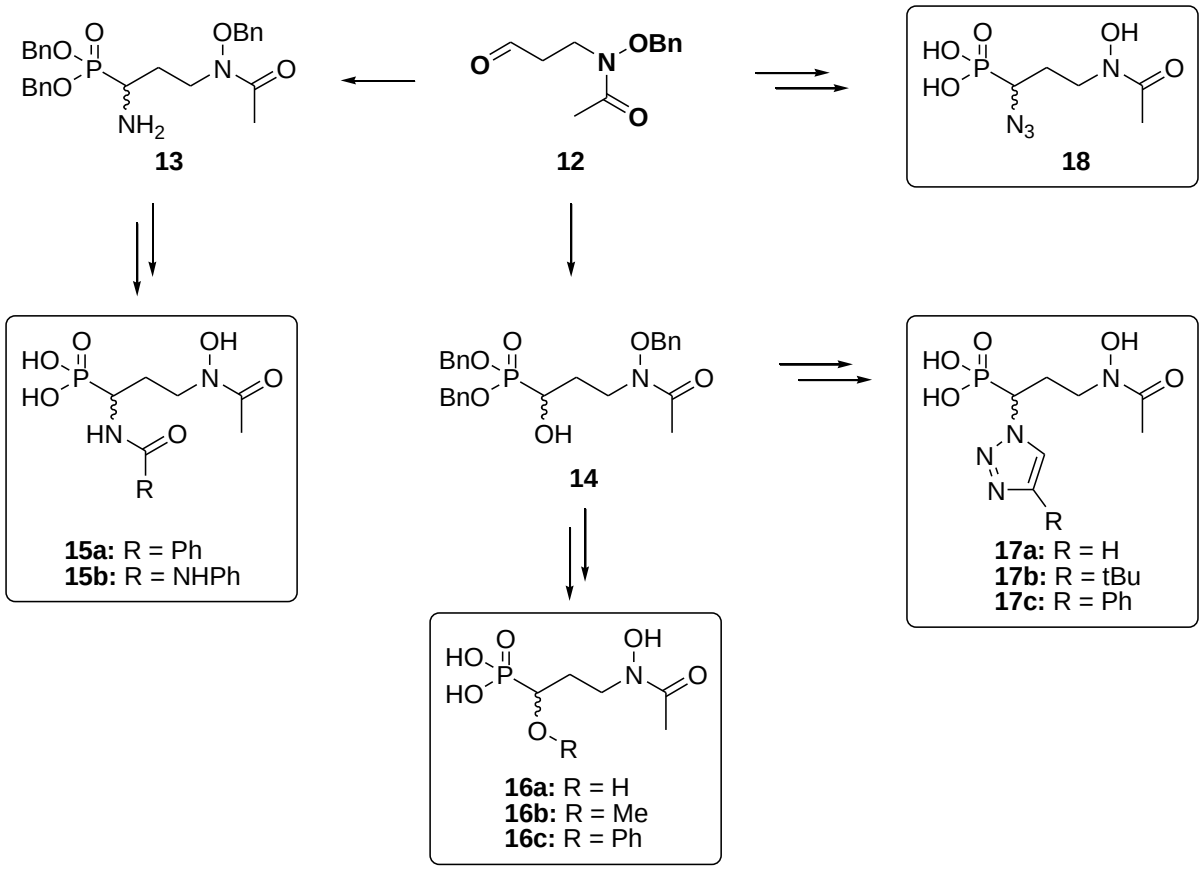


Figuur VII.2: Ontwikkeling van een conformationeel verstard FR900098 analoog

Helaas bleek **11** bijzonder hydrolysegevoelig te zijn. De lage activiteit die *in vitro* werd waargenomen is mogelijk het gevolg van een snelle hydrolytische afbraak tijdens de test. Anderzijds is het ook mogelijk dat een goede interactie van **11** met DXR wordt belet door sterische hinder, aangezien eerder werd aangetoond dat γ-methylfosmidomycine geen DXR inhiberende activiteit

bezit. Gezien de inherente, hoge hydrolysegevoeligheid van de NHA-structuur werd besloten de synthese van het 3-(ω-fosfonoethyl)-NHA (of 'hydroxamaat *N*-hydroxybetalactam') te staken.

Het derde grote deel van dit werk, beschreven in Hoofdstuk V, omvat een poging tot uitbreiding van de succesvolle reeks van α-arylfosmidomycines. Divergente α-substitutie werd hier mogelijk gemaakt door invoeren van een hydroxyl- of aminegroep op de α-koolstof van FR900098. Alle voorziene analogen werden gesynthetiseerd in slechts enkele stappen en in goede rendementen uitgaande van gemeenschappelijk aldehyde bouwsteen **12** (Figuur VII.3). Een snelle methode voor de synthese van dit aldehyde in hoog rendement werd eveneens ontwikkeld.



Figuur VII.3: Ontwikkeling van α-heteroatoom gebaseerde analogen van FR900098

De gesynthetiseerde en geteste analogen kunnen worden ingedeeld in vier grote klassen:

1. Het α-benzamide **15a** en α-phenylureumderivaat **15b** bleken noch EcDXR te inhiberen, noch de groei van *P. falciparum* te remmen.

2. Het α-hydroxy analoog van FR900098 (**16a**) vertoonde behoorlijke EcDXR inhibitie en *P. falciparum* groei-inhibitie, terwijl de corresponderende methyl en fenyl ethers (**16b**, **16c**) nagenoeg inactief bleken.

3. Tot onze verbazing onderdrukte geen enkele van de α-triazolyl analogen **17a**, **17b** en **17c** de groei van *P. falciparum* en vertoonde enkel *t*-butyl triazol **17b** inhibitie van EcDXR (zij het eerder zwak). Waarschijnlijk is de triazolfunctionaliteit in deze moleculen te elektronenrijk om de π-π-stapelingsinteractie met Trp211 van het enzym aan te gaan die de binding van voornoemde α-arylanaloga versterkt .

4. Het α-azido FR900098 (**18**) vertoonde de hoogste activiteit in deze reeks analogen: het verslaat zowel fosmidomycine als FR900098 in EcDXR inhibitie en is bijna even sterk als fosmidomycine in het afremmen van de groei van *P. falciparum*. Daarenboven is dit het eerste fosmidomycine-analoog dat een rigiede, lineaire, heteroatoom-gebaseerde α-substituent draagt. Mogelijks dankt het zijn verhoogde activiteit aan het halogeen-nabootsend effect van het azide, nl. de verhoging van de zuursterkte van de fosfonaatgroep. Nieuwe interacties met het enzym zijn evenwel niet uit te sluiten, en X-straal onderzoek zouden hier meer inzicht kunnen verschaffen. De unieke reactiviteit van de azidogroep in deze sterke DXR-inhibitor opent verder ook de deur naar *target-guided, in situ click*-chemie voor het identificeren van nieuwe bindingspartners voor de flexibele lus van DXR, bv voor interactie met zijketens van de aminozuren naast Trp211. Deze aanpak bestaat erin het DXR/**3** complex te incuberen met een reeks acetyleenreagentia en werd reeds met succes toegepast in de ontdekking van acetylcholinesterase (AChE) inhbitoren.

Als besluit kan worden gesteld dat tijdens dit doctoraatswerk nieuwe en potente inhibitoren van DXR werden ontwikkeld die kunnen leiden tot nieuwe antimalariamiddelen. Het invoeren van een halogeen of een azidegroep in α-positie van het fosfonaat van FR900098 leidde tot de meest interessante DXR inhibitoren, en het formuleren van prodrugs versterkte het antimalaria effect van α-fluor FR900098 in belangrijke mate. Verder onderzoek naar zulke fosfonaatprodrugs en de binding van het α-azide analoog aan DXR is wenselijk.

Appendix

Curriculum Vitae

Curriculum Vitae

Personal Data

Name:	Thomas Verbrugghen
Date of birth:	May 4, 1982
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Address:	Gaversesteenweg 786, 9820 Melsen
Phone:	+32 479 93 00 08
E-mail:	thomas.verbrugghen@pandora.be

Education

2006-present	**PhD** Pharmaceutical Sciences title: "Synthesis of Inhibitors of DXR as Antimalarials" Laboratory for Medicinal Chemistry, UGent promoter: prof. Serge Van Calenbergh
2001-2006	**Master** Pharmaceutical Sciences – Pharmacist, UGent diploma: June 30, 2006, graduated with distinction Thesis: "Optimalisatie en Validatie van een Methode voor de In-line Controle van de Kritische Parameters bij de Productie van een Farmaceutische Suspensie" (Apr. Thomas De Beer, prof. W.R.G. Baeyens)
2000-2001	1st year Bio-Engineer, UGent
1994-2000	**Sciences-Maths**, EDUGO Oostakker graduated with distinction

Additional Education

2009-2010	**Swedish**, level I & II University Language Centre, UGent

Research Fellowships

2007-2011	Research Fellowship of the Agency for innovation by Science and Technology (IWT Vlaanderen)

Teaching Experience

2008-2011	Tutor "Stereochemistry", Medicinal Chemistry for 3rd bachelor Pharmaceutical Sciences Prof. Serge Van Calenbergh
2008-2010	Tutor "Problem-based Pharmaceutical Education", 3rd bachelor Pharmaceutical Sciences Prof. Niek Sanders

Master Students Supervised

2006-2007	Pieter Glibert ("Nieuwe Ontwikkelingen in het Onderzoek naar Antimalariamiddelen")
2007-2008	Maaike Van Craen ("Synthese van α-Gehalogeneerde FR900098 Analogen als Inhibitoren van DXR")
2009-2010	Marjolijn Lowie ("Synthese van α-Gehalogeneerde Prodrugs van FR900098 als Inhibitoren van DXR")

Scientific Publications

- Alpha-heteroatom derivatized analogues of FR900098 as antimalarials.
  Verbrugghen T., Vandurm P., Pouyez J., Maes L., Wouters J., Van Calenbergh S.
  Manuscript in preparation

- Resistance of the Burkholderia cepacia complex to fosmidomycin and fosmidomycin derivatives.
  Messiaen A.S., **Verbrugghen T.**, Declerck C., Ortmann R., Schlitzer M., Nelis H., Van Calenbergh S., Coenye T.
  International Journal of Antimicrobial Agents 38(3):261-264 (2011)

- Synthesis and evaluation of alpha-halogenated analogues of 3-(acetylhydroxyamino)propylphosphonic acid (FR900098) as antimalarials.
  Verbrugghen T., Cos P., Maes L., Van Calenbergh S.
  Journal of Medicinal Chemistry. 53(14): 5342-5346 (2010)

- Synthesis of beta- and gamma-oxa isosteres of fosmidomycin and FR900098 as antimalarial candidates.
  Haemers T., Wiesner J., Giessmann D., **Verbrugghen T.**, Hillaert U., Ortmann R., Jomaa H., Link A., Schlitzer M., Van Calenbergh S.
  Bioorganic and Medicinal Chemistry. 16: 3661-3371 (2008)

Symposia

- Posterpresentation on: 11th Chemistry Conference for Young Chemists (ChemCYS 2012) (Blankenberge, March 1-2, 2012)
  Alpha-heteroatom derivatized Analogues of FR900098 as Antimalarials
  Verbrugghen T., Vandurm P., Pouyez J., Wouters J., Van Calenbergh S.

- Posterpresentation on: Antimal meeting: "Antimalarial Drugs: Chemistry, Development and Future Challenges" (London, March 15-16, 2011)
  Synthesis and Evaluation of alpha-Substituted Analogues of FR900098 as Antimalarials
  Verbrugghen T., Cos P., Maes L., Van Calenbergh S.

- Posterpresentation on: XXIst International Symposium on Medicinal Chemistry (EFMC-ISMC) (Brussels, September 5-9, 2010)
  Synthesis and Evaluation of alpha-Halogenated Analogues of FR900098 and Their Prodrugs as Antimalarials
  Verbrugghen T., Cos P., Maes L., Van Calenbergh S.

- Oral presentation on: 10e Vlaams Jongerencongres van de Chemie (Jong KVCV) (Blankenberge, March 1-2, 2010)
  Synthesis and Evaluation of alpha-Halogenated Analogues of FR900098 as Antimalarials
  Verbrugghen T., Cos P., Maes L., Van Calenbergh S.
  PRIZE FOR BEST ORAL PRESENTATION MEDICINAL CHEMISTRY

- Posterpresentation on: 13th Sigma-Aldrich Organic Synthesis Meeting
  (Spa, December 3-4, 2009)
  Synthesis and Evaluation of alpha-Halogenated Fosmidomycin Analogues
  Verbrugghen T., Matheeussen A., Maes L., Van Calenbergh S.

- Keystone Symposium Drug Design for Protozoan Parasites
  (Breckenridge, CO, USA, March 22-26, 2009)

- 12th Sigma Aldrich Organic Synthesis Meeting
  (Spa, December 4-5, 2008)

- Small Molecules, Antibodies and Natural Products: Multiple Faces of Medicinal Chemistry
  (Leuven, November 7, 2008)

- Belgian Organic Synthesis Symposium (BOSS)
  (Gent, July 13-18, 2008)

- 10th Sigma Aldrich Organic Synthesis Meeting
  (Spa, December 3-4, 2006)

- Personalized Medicine: New Opportunities for Drug Discovery
  (Antwerpen, November 10, 2006)

Dankwoord

Dankwoord

Eindelijk, eindelijk is het zover: de thesis staat op papier, verbeteringen zijn aangebracht, besloten verdediging afgerond en openbare verdediging volgt. Bijna zes jaar, een hele periode die bijzonder leerzaam is geweest op vele vlakken, bij momenten echt zwaar maar ook vaak enorm amusant. Tijd om een aantal mensen te bedanken voor hun rol tijdens die voorbije periode!

Eerst en vooral: mijn promotor, prof. Serge Van Calenbergh, want hij was het die mij de mogelijkheid heeft gegeven het doctoraat aan te vatten. Ik heb even moeten wennen aan je altijd-openstaande-deur, maar eenmaal ik doorhad dat ik gewoon vaak genoeg door die deur moest binnenlopen kon ik altijd terecht voor advies, een babbel, een goeie roddel… Je begeleiding, die zich bijvoorbeeld uitte in razendsnel (en grondig) verbeterde manuscripten, en tegelijk de vrijheid die ik kreeg tijdens dit doctoraat, heb ik enorm geapprecieerd. Ook je diplomatie en het onderhouden van vele binnen- en buitenlandse contacten waren zeer leerzaam voor mij. Dank ook voor de financiële steun tijdens het eerste jaar, waardoor ik nadien prima voorbereid naar het IWT kon trekken.

Het IWT wil ik bedanken voor vier jaar financiële steun (doctoraatsbeurs strategisch basisonderzoek).

Professoren Louis Maes en Paul Cos en hun team, in het bijzonder Ing. An Matheeussen, van de Universiteit Antwerpen wil ik bedanken voor het uitvoeren van de *in vitro* en *in vivo* tests en screenings en het aanleveren van de vele bruikbare biologische data.

A big thanks to professor Johan Wouters, Jenny Pouyez and dr. Pierre Vandurm for the recent but invaluable collaboration. Your work on the enzyme kinetics and modeling is enormously appreciated and I am very thankful for all these nice data.

Ook aan onze eigen faculteit werden de fosmidomycine-analogen nuttig aangewend in antimicrobiële tests, waarvoor dank aan Anne-Sophie en prof. Tom Coenye van het Labo voor Farmaceutische Microbiologie.

Uiteraard mogen hier de collega's niet ontbreken, want zij waren het vooral die het dagelijks leven op het labo en aan de faculteit kleur gaven. Zo eentonig als de term "de collega's" is, zo divers is de groep waarover ik het heb. Om te beginnen, aan het eigen labo:

First of all, a special thanks for Jeff (dr. Carl Jeffrey Lacey), for you were the one who guided me on my first steps in synthetic chemistry. Whenever I redid one of your syntheses during the past years, following an elaborate, extensively documented protocol in curly handwriting, I heard Dean Martin singing in the back of my head. Thanks for your patient company and guidance!

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Kiran, your smile and your natural calm but also your knowledge of all reactions involving toxic metals or unstable reagents made a chat with you always amusing. René, your presence in the lab was never unnoticed. I already miss the theological discussions with you and Martijn, the Pidgin lessons, the Dutch rehearsals, your adoration for owls and snakes… All the best for the future, and good luck with those phosphonates!

Natuurlijk mag ik hier Izet, vaste waarde in het Labo Medicinale Chemie, niet vergeten. Bedankt Izet, voor de hulp allerhande: met de NMR, de LCMS, de HPLC, de Parr, voor de eerste hulp bij labo-ongevallen, voor de zeer geslaagde traktaties van eigen makelij… Je optimisme en relativeringsvermogen zijn soms alles wat een vermoeide doctorandus nodig heeft.

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Melanie, enorm bedankt voor het cover-ontwerp. Met het simpele schetsje dat ik doorgaf maakte je nog iets veel beter dan wat ik voor ogen had: knap werk!

Hoewel daar wellicht af en toe aan werd getwijfeld, had ik de voorbije jaren ook nog een leven buiten het labo. Ook daar ben ik heel wat mensen dankbaar voor hun steun.

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nodig had! Ann en Hendrik, bij deze zie je dat jullie belastingsgeld alweer nuttig is gespendeerd, waarvoor dank. Alinetje, bedankt voor de uitbundige lachjes: niks beter dan dat om alles te leren relativeren.

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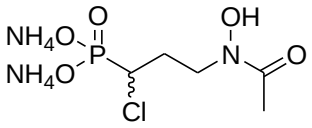
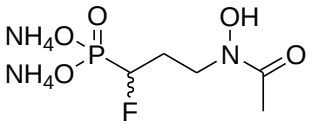
Jo en Henri, jullie hulp maakte 'de schrijfperiode' een stuk lichter. Een heel welgemeend Bedankt voor de morele, logistieke, culinaire en andere steun de voorbije maanden, en ook daarvóór. Jullie bezorgdheid om ons is hartverwarmend.

En tot slot, last but not least, mijn allergrootste steun: Katleen. Ik ben enorm blij dat ik je, enkele jaren geleden, kon overtuigen om te blijven doctoreren. En nog blijer om wat daarna allemaal kwam… Ik wil je bedanken voor alle steun de voorbije jaren, jij kon als geen ander mijn frustraties over mislukkende experimenten of uitblijvende resultaten begrijpen. Jij wist mij aan te moedigen, hielp mij gemotiveerd blijven en doorzetten. Het schrijven van deze thesis was niet bepaald mijn favoriete bezigheid, en de voorbije maanden heb ik je geduld af en toe nogal op de proef gesteld. Dank je voor alle steun, hulp, aanmoedigingen… Dank je dat je er bent!

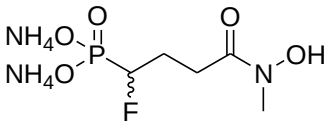
Overview of synthesized and evaluated compounds



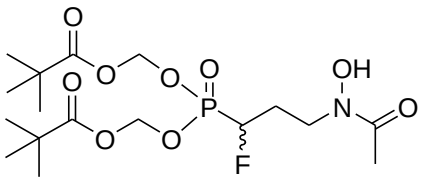
III. 4a



III. 4b



III. 5



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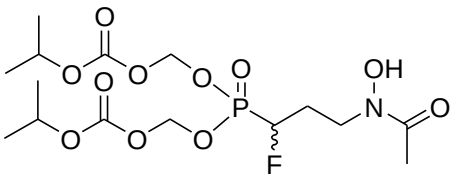

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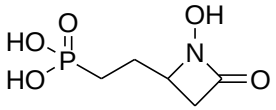
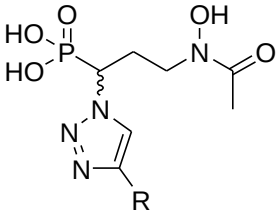
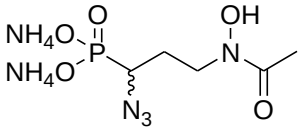


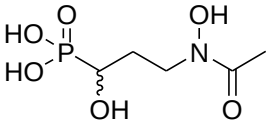
IV. 3



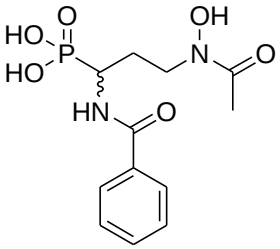
V. 1a R = H
V. 1b R = tBu
V. 1c R = Ph



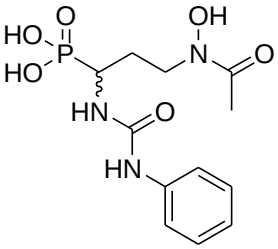
V. 2



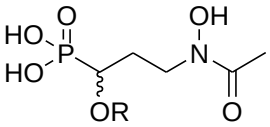
V. 3a



V. 5a



V. 5b



V. 3b : R = Me
V. 3c : R = Ph