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SEMISYNTHETIC PREPARATION OF C-NUCLEOSIDE 5′-TRIPHOSPHATES

As Bacterial RNA Polymerase Substrates

Petja Rosenqvist

TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
SARJA – SER. AI OSA – TOM. 660 | ASTRONOMICA – CHEMICA – PHYSICA – MATHEMATICA | TURKU 2021

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ABSTRACT

Interest in natural products as pharmaceutical leads has recently resurfaced due to increasing understanding towards the biosynthesis of natural compounds as well as advancements in methodologies that facilitate the semisynthetic preparation of novel compounds. Natural C-nucleosides are one class of bioactive compounds that have seen their biosynthetic background being elucidated in the last few years. Furthermore, the higher stability of the C-nucleosides against enzymatic and chemical hydrolysis compared to the N-nucleosides makes these compounds interesting for their potential medicinal properties.

The presented thesis focuses on semisynthesis of C-nucleoside 5´-triphosphates for studying their activity against RNA polymerases (RNAP) that are common drug targets for nucleoside-based pharmaceuticals. The natural C-nucleosides showdomycin, oxazinomycin and pseudouridimycin were produced as starting materials by cultivating strains of *Streptomyces* soil bacteria. Additionally, a small compound library of showdomycin analogues was prepared, by modifying the maleimide-type base moiety. The chemical 5´-phosphorylation was carried out by using the commonly used Yoshikawa-Ludwig protocol. However, the unmodified showdomycin unexpectedly isomerized during the phosphorylation affording isoshowdomycin-5´-triphosphate. In the RNAP assays, the efficiency of oxazinomycin-5´-triphosphate as a substrate was comparable to that of uridine-5´-triphosphate. In addition, in certain sequence contexts the incorporation of oxazinomycin to the RNA arrested the transcription process. On the other hand, the most promising showdomycin analogue, 4-(ethylthio)showdomycin-5´-triphosphate showed modest inhibition activity and slight selectivity towards the bacterial RNAP.

KEYWORDS: C-nucleosides, natural products, semisynthesis, RNAP inhibitors, drug design

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Mielenkiinto luonnonyhdisteiden käyttöä kohtaan lääkekehityksen johtoyhdisteinä on viime aikoina kohonnut uudelleen. Tähän ovat osaltaan vaikuttaneet lisääntynyt ymmärrys luonnonyhdisteiden biosynteesiä kohtaan sekä kehittyneet menetelmät, jotka helpottavat uusien yhdisteiden puolisynteettistä valmistusta. Luonnolliset C-nukleosidit ovat yksi bioaktiivinen yhdisteryhmä, jonka biosynteettinen alkuperä on selventynyt viimeisimpien vuosien aikana. Lisäksi C-nukleosidien suurempi pysyvyys entsymaattista ja kemiallista hydrolyysiä kohtaan verrattuna N-nukleosideihin tekee näistä yhdisteistä mielenkiintoisia mahdollisten lääkinnällisten ominaisuuksiensa vuoksi.

Tämä väistöskirjatyö keskittyy C-nukleosidien 5´-trifosfaattien puolisynteesiin niiden aktiivisuuden tutkimiseksi RNA polymeraasien (RNAP) kanssa, jotka ovat yleinen nukleosidipohjaisten lääkeaineiden vaikutuskohde. Lähtöaineiksi tuotettiin luonnollisiin C-nukleosideihin kuuluvat showdomysiini, oksatsinomysiini ja pseudouridimysiini viljelemällä *Streptomyces*-suvun maaperäbakteereja. Lisäksi valmistettiin pieni, showdomysiini-johdannaisista koostuva yhdistekirjasto modifioimalla sen maleimidi-tyyppistä emäsosaa. Valmistetut C-nukleosidit fosforyloitiin kemiallisesti käyttäen yleisestä Yoshikawa-Ludwig -menetelmää. Muokkaamaton showdomysiini kuitenkin isomeroitui odottamattomasti fosforylaation aikana antaen tuotteeksi *iso*-showdomysiini-5´-trifosfaatin. RNAP–kokeissa oksatsinomysiini-5´-trifosfaatin kyky toimia substraattina oli verrattavissa uridiini-5´-trifosfaattiin. Lisäksi oksatsinomysiinin liittyminen RNA:han tietyissä emäsjärjestyskonteksteissa keskeytti transkription. Sen sijaan showdomysiinijohdannaisista lupaavin, 4-(etyylitio)showdomysiini osoitti kohtalaista inhibitioaktiivisuutta ja lievää selektiivisyyttä bakteeriperäistä RNA polymeraasia kohtaan.

ASIASANAT: C-nukleosidit, luonnonyhdisteet, puolisynteesi, RNAP-inhibiittori, lääkekehitys

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Abbreviations

A	adenosine
AcOH	acetic acid
APDA	4-amino-1*H*-pyrazole-3,5-dicarboxylic acid
BAD	benzamide adenine dinucleotide
Bu_3P	tributylphosphine
C	cytidine
dA	deoxyadenosine
DADMe	4′-deaza-1′-aza-2′-deoxy-1′-methylene
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
dC	deoxycytidine
DCM	dichloromethane
dG	deoxyguanosine
DMSO	dimethyl sulfoxide
DMF	dimethylformamide
DNA	deoxyribonucleic acid
DP	diphosphate
DTNB	(5,5-dithio-bis-(2-nitrobenzoic acid)
EBOV	Ebola virus
FDA	(The United States) Food and Drug Administration
FOR	formycin
G	guanosine
GSH	glutathione
HBV	hepatitis B virus
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HPDA	4-hydroxy-1*H*-pyrazole-3,5-dicarboxylic acid
hTP	human thymidine phosphorylase
Imm	immucillin
IMPDH	inosine-5′-monophosphate dehydrogenase
LC-MS	liquid chromatography-mass spectrometry
MAL	malayamycin

MeCN	acetonitrile
MP	monophosphate
MTAN	5′-methylthioadenosine nucleosidase
$NaBH_4$	sodium borohydride
NAD	nicotinamide adenine dinucleotide
NMR	nuclear magnetic resonance
NsA	nucleoside analogue
NTP	nucleoside-5′-triphosphate
OZM	oxazinomycin
Ph_3P	triphenylphosphine
PNP	purine nucleoside phosphorylase
$POCl_3$	phosphoryl chloride
PPN	tris{bis(triphenylphosphoranylidene) ammonium}
PRPP	5-phosphoribosyl-1-pyrophosphate
PSU	pseudouridine
Py	pyridine
PYR	pyrazofurin
PUM	pseudouridimycin
RNA	ribonucleic acid
RNAP	RNA polymerase
RP	reverse phase
SARS-CoV2	severe acute respiratory syndrome coronavirus 2
$SnCl_2$	tin(II) chloride
SDM	showdomycin
T	thymidine
TEA	triethylamine
TEAA	triethylammonium acetate
TEC	transcription elongation complex
TEP	triethyl phosphate
THF	tetrahydrofuran
TP	triphosphate
U	uridine

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Palmu, K., Rosenqvist, P., Thapa, K., Ilina, Y., Siitonen, V., Baral, B., Mäkinen, J., Belogurov, G., Virta, P., Niemi, J. and Metsä-Ketelä, M. Discovery of the Showdomycin Gene Cluster from *Streptomyces showdoensis* ATCC 15227 Yields Insight into the Biosynthetic Logic of C-Nucleoside Antibiotics. *ACS Chem. Biol.*, 2017; 12: 1472 – 1477.

II Rosenqvist, P., Palmu, K., Prajapati, R. K., Yamada, K., Niemi, J., Belogurov, G. A., Metsä-Ketelä, M. and Virta, P. Characterization of C-nucleoside Antimicrobials from *Streptomyces albus* DSM 40763: Strepturidin is Pseudouridimycin. *Sci. Rep.* 2019; 9: 8935.

III Prajapati, R. K., Rosenqvist, P., Palmu, K., Mäkinen, J. J., Malinen, A. M., Virta, P., Metsä-Ketelä, M. and Belogurov, G. A. Oxazinomycin arrests RNA polymerase at the polythymidine sequences. *Nucleic Acids Res.*, 2019; 47: 10296 – 10312.

IV Rosenqvist, P., Mäkinen, J.J., Palmu, K., Prajapati, R. K, Korhonen, H. J., Virta, P., Belogurov, G. A., Metsä-Ketelä, M. Modulation of the Antimicrobial and Trancriptional Effects of Showdomycin by Modification of the Maleimide Ring System. Unpublished Manuscript.

List of Contemporaneous Publications

- Siddiqui, F. A., Alam, C., Rosenqvist, P., Ora, M., Sabt, A., babu Manoharan, G., Bindu, L., Okutachi, S., Catillon, M., Taylor, T., Abdelhafez, O. M., Lönnberg, H., Stephen, A. G., Papageorgiou, A. C., Virta, P. and Abankwa D. PDEδ inhibitors with a new design principle selectively block K-Ras activity in cancer cells, *ACS Omega*, 2020; 5: 832–842.
- Rosenqvist P, Sabt A, Dyunyasheva V, Abankwa D, Virta P, Ora M. Stability of the Phosphotriester PDE6D Inhibitors. *ChemistrySelect*, 2021; 6: 488–493.

1 Introduction

C-nucleosides are nucleoside analogues (NsA) characterized by a carbon-carbon glycosidic bond. This class of compounds was discovered in 1957 by the identification of pseudouridine[1], a modified nucleoside unit found in ribonucleic acid (RNA) structures. The following decades witnessed further the discovery of multiple microbe produced C-nucleoside antimicrobials.[2–9] These biologically active natural compounds have inspired synthesis and clinical evaluations of both natural and artificial C-nucleosides, although none of the trials to date has resulted in full approval by the The United States Food and Drug Administration (FDA).[10,11] However, a few cases of limited use of C-nucleosides in clinic have emerged; Tiazofurin has received an orphan drug status for treatment of chronic myelogenous leukemia[12], and Japan has permitted the use of Forodesine (aka immucillin H) for treatment of relapsed peripheral T-cell lymphoma since 2017.[13] In 2020, the experimental C-nucleoside antiviral Remdesivir was administered on compassionate-use basis to patients showing severe symptoms of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) infection[14], although the medicinal effect of Remdesivir was eventually assessed as only modest.[15] Despite the failures with C-nucleosides in the clinical trials thus far, the overall development in the synthetic C-glycosylations and the recent appearance of novel medicinally promising C-nucleosides have refreshed the interest towards further exploration of this class of compounds for their medicinal value.[10]

In contrast to C-nucleosides, analogues of the traditional N-nucleosides have a strong established position as pharmaceuticals, especially as anticancer and antiviral agents.[16–18] The primary targets of NsA drugs include the various viral and human DNA polymerases (DNAP) and RNA polymerases (RNAP), while bacterial RNAPs on the other hand are not targeted by any currently approved NsA drug.[19] Furthermore, the discovery of the first nucleoside analogue that selectively inhibits bacterial RNAPs, was reported only recently, in 2017.[9,20] The compound in question, pseudouridimycin, belongs to the C-nucleoside class and its novel mechanism of action implies a possibility of a new type of designed antibacterials urgently required against emerging multiple drug resistant bacteria.[21]

In the recent years, the genes and enzymes related to the biosynthesis of natural C-nucleosides have been clarified to a large extent, which has also contributed to the raised interest for C-nucleosides.[22–27, I] Genome mining efforts enabled by these findings may facilitate the discovery of other related natural products. In addition, engineering of these biosynthetic pathways could provide additional means for production of novel C-nucleosides, alone or in combination with synthetic methods.

1.1 Properties of C-nucleosides

The traditional N-nucleosides including the canonical ones listed in Figure 1a, are composed of a pentofuranose sugar connected to a nucleobase by a *N*-glycosidic bond (C1′–N), whereas C-nucleosides are defined by a *C*-glycosidic bond (C1′-C). The majority of bioactive nucleosides are defined as *β*-nucleosides, whereas their *α*-anomers are usually inactive and only a few *α*-nucleosides have been identified from natural sources.[3,28] The most distinctive property of C-nucleosides is the increased stability of the glycosidic bond towards enzymatic and chemical cleavage compared to the *N*-glycosidic bond that owes its lability to the chemical nature of the hemiaminal ether structure [O4′-C1′-N].[29] However in some cases, C-nucleosides may be prone to isomerization under acidic or alkaline conditions, while epimerization of C-nucleoside pyrazofurin is observed even in neutral aqueous solution.[3,30–33] Exchanging the nitrogen atom of a glycosidic C1′-N bond to a carbon often alters the biological activity of the nucleoside more than may be expected from a single atom replacement.[11] For example, the C-nucleoside analog of adenosine, 9-deazaadenosine (Figure 1b) is cytotoxic and has been studied for its antitumour properties.[34] In turn, a C-nucleoside analogue of 2′-*C*-methyladenosine (an anti-hepatitis C virus agent) was found mostly inactive.[35]

The differences in bioactivities of C-nucleosides (and all base-modified nucleosides in general) in respect to the N-nucleosides can arise from different tautomeric equilibria and acid-base properties of the pseudonucleobase, as well as alterations to the furanose conformation caused by the C1′-substituent, all of these factors affecting the interactions with enzymes and other biomolecules.[36–39]

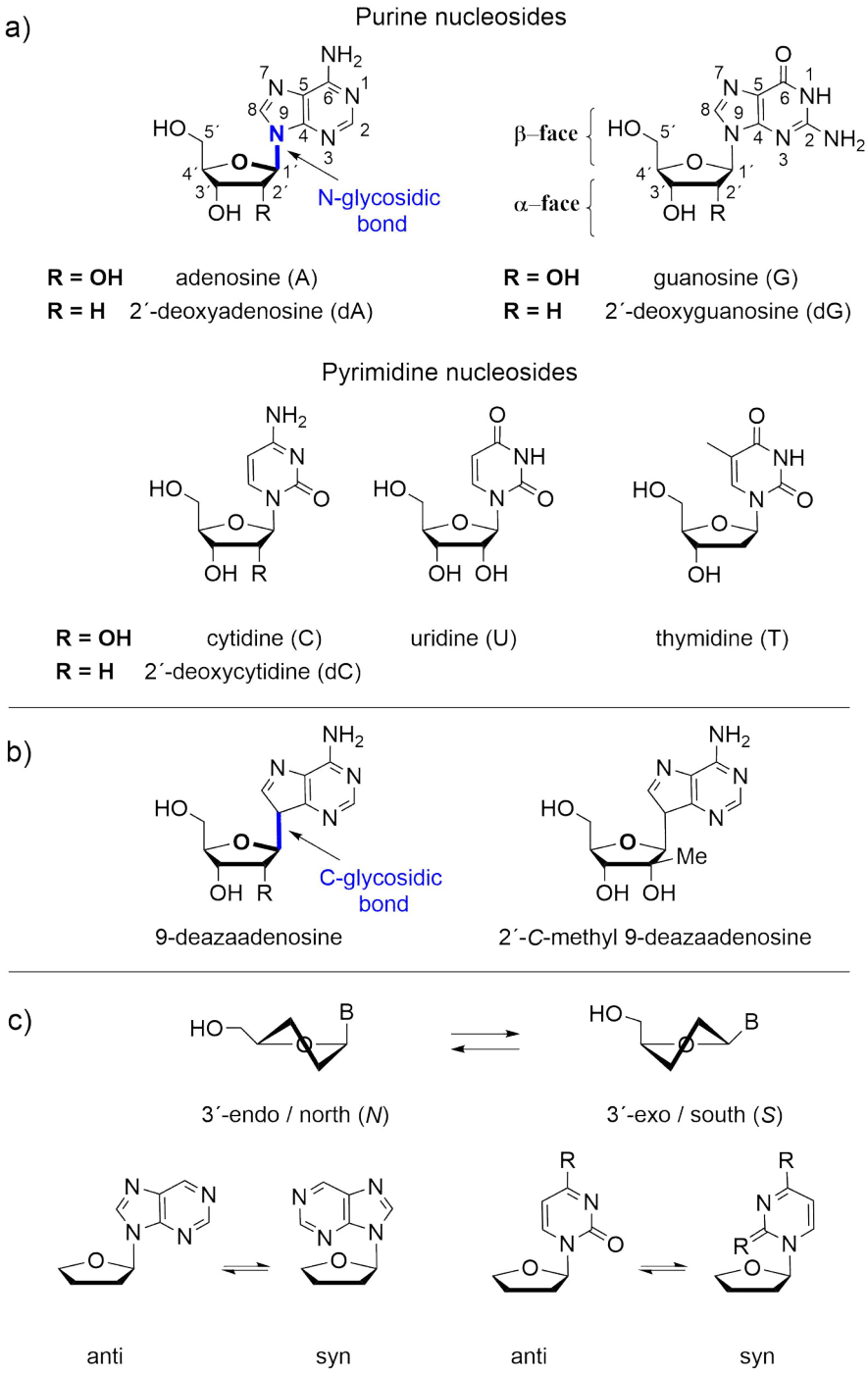


Figure 1. **a)** Structures of canonical N-nucleosides, **b)** examples of C-nucleosides, and **c)** the major conformations of nucleosides.

The C1′-substituent of C-nucleosides (i.e. the nucleobase) contributes to the pseudorotational[40] equilibria of the furanose sugar between 3′-exo/south (*S*) and 3′-endo/north (*N*) conformations (Figure 1c) by steric and stereoelectronic interactions including the anomeric effect.[41,42] In the case of *β*-nucleosides the steric effect drives the C1′-substituent towards pseudoequatorial orientation in *S*-type conformation. The anomeric effect in contrast has a stabilizing effect on the pseudoaxial orientation of the nucleobase in *N*-type conformation. In C-nucleosides the anomeric effect is diminished (and negligible in carbocyclic C-nucleosides where the ribose ring oxygen is also replaced with carbon) compared to that of the most similar N-nucleoside counterpart, and therefore the pseudorotation is more biased towards *S*-type conformation.[42,43] However, it is worth noting that in solution the *S* conformation predominates for most nucleosides whereas 2′,3′-dideoxy nucleosides are more biased towards the *N* conformation.[44]

The weaker anomeric effect also reduces the difference between the C1′-O4′ and C4′-O4′ bond lengths ($\Delta \approx 0.01$ Å) in most C-nucleosides. In N-nucleosides the length of the C1′-O4′ bond is typically shorter compared to the C4′-O4′ bond ($\Delta \approx 0.03$ Å).[42,45,46] Additionally, the modified nucleobases and the furanose pseudorotamer populations may affect the equilibrium between the *syn* and *anti* rotamers of the nucleobase.[47] Altogether, the conformational changes contribute to the sum of the binding interactions between the NsA substrate and enzymes, either increasing or decreasing the affinity. For example, most nucleotide polymerases bind nucleoside-5′-triphosphates (NTP) in the *N* conformation whereas nucleotide analogues locked in the *S* conformation have been found inactive.[48–50]

1.2 Cellular metabolism and drug targets of nucleoside analogues

The principal role of nucleosides in biological context is to act as intermediates for the formation of nucleotides and the nucleotide polymers, RNA and DNA. The RNA structures consist of the ribonucleosides adenosine (A), guanosine (G), cytidine (C), and uridine (U) connected by phosphodiester linkages, whereas for DNA the units are the deoxyribonucleosides deoxyadenosine (dA), deoxyguanosine (dG), deoxycytidine (dC) and thymidine (T) presented in Figure 1. Additionally, nucleosides may act as ligands for cell signalling receptors such as the ubiquitous adenosine receptors[51,52], whereas phosphorylated nucleosides have further roles in cell signalling and in storing and transferring cellular energy.[53,54]

A simplified representation of the cellular metabolism of NsAs is summarized in Figure 2. Nucleosides are produced and maintained at optimal levels inside cells by the *de novo* and salvage pathways. The *de novo* biosynthesis produces the nucleobases from simpler molecules over multiple catalytic steps and connects them

to a ribose unit along the pathway. In contrast, the salvage pathway recycles nucleosides, nucleotides and nucleobases recovered from degraded RNA and DNA, and taken up from extracellular medium by nucleoside transporter proteins.[55,56] The activities of these pathways vary depending on the type of cell and organism. For example, protozoan parasites such as *Plasmodium falciparum* (malaria) rely on purine nucleoside salvage and pyrimidine *de novo* synthesis[57], whereas for rapidly replicating cells such as cancer cells, the *de novo* synthesis is often vital.[58] Thus, inhibition of the more vital pathway is a potential therapeutic strategy.

A major fraction of bioactive C-nucleosides inhibits the *de novo* and salvage pathways.[10] The stability of the *C*-glycosidic bond has been utilized for design of inhibitors of nucleoside phosphorylase enzymes of the salvage pathway (section 1.4.1).[59] The natural C-nucleoside Pyrazofurin inhibits pyrimidine *de novo* biosynthetic enzyme orotidine-5′-monophosphate decarboxylase (section 1.3.5)[60], whereas some synthetic C-nucleosides are metabolized to analogues of nicotinamide adenine dinucleotide (NAD) that inhibit the inosine-5′-monophosphate dehydrogenase, vital for the *de novo* biosynthesis of guanine nucleotides (section 1.4.2).[61]

C-nucleosides, as most NsAs in general, rely on enzymatic recognition by nucleoside transporter enzymes for active uptake into cells.[56,62] Once inside cells, NsAs are often transformed to the active form by metabolic enzymes.[63,64] The usual transformation is the stepwise phosphorylation to either nucleoside-5′-monophosphate (NMP), -diphosphate (NDP) or -triphosphate (NTP) by nucleoside and nucleotide kinases where the initial phosphorylation is rate limiting.[65,66] In contrast, enzymes such as 5′-nucleotidases and nucleoside deaminases may deactivate some active forms of NsA drugs by dephosphorylation of the 5′-phosphates or deamination of possible amine groups in the nucleobases (of cytidine/adenosine analogues), respectively. Upregulation of deactivating enzymes or downregulation of the activating enzymes or nucleoside transporters often contribute to emergence of drug resistance.[56,67–69] Additionally, increased production of the natural substrate competing for the drug target binding, as well as mutations in the target or metabolic enzymes can increase the drug resistance.[70]

The phosphorylated NsAs may target many types of enzymes but often the main targets include the RNA and DNA polymerases, and viral reverse transcriptases.[71–73] As triphosphates the NsAs may be incorporated by the polymerase into the nascent RNA or DNA and either terminate the elongation or cause the formation of non-functioning RNA or DNA.[74] The chain terminators are divided into obligate and non-obligate chain terminators.[75] By definition, obligate chain terminators lack the 3′-hydroxyl necessary for the formation of the phosphodiester bond[76] whereas non-obligate chain terminators possess the 3′-hydroxyl but cause the termination by other mechanism. The non-obligate termination can occur immediately after the

incorporation of the NsA (pseudo-obligate)[77], or alternatively a delayed chain termination can occur after incorporation of a few additional nucleotides.[78,79]

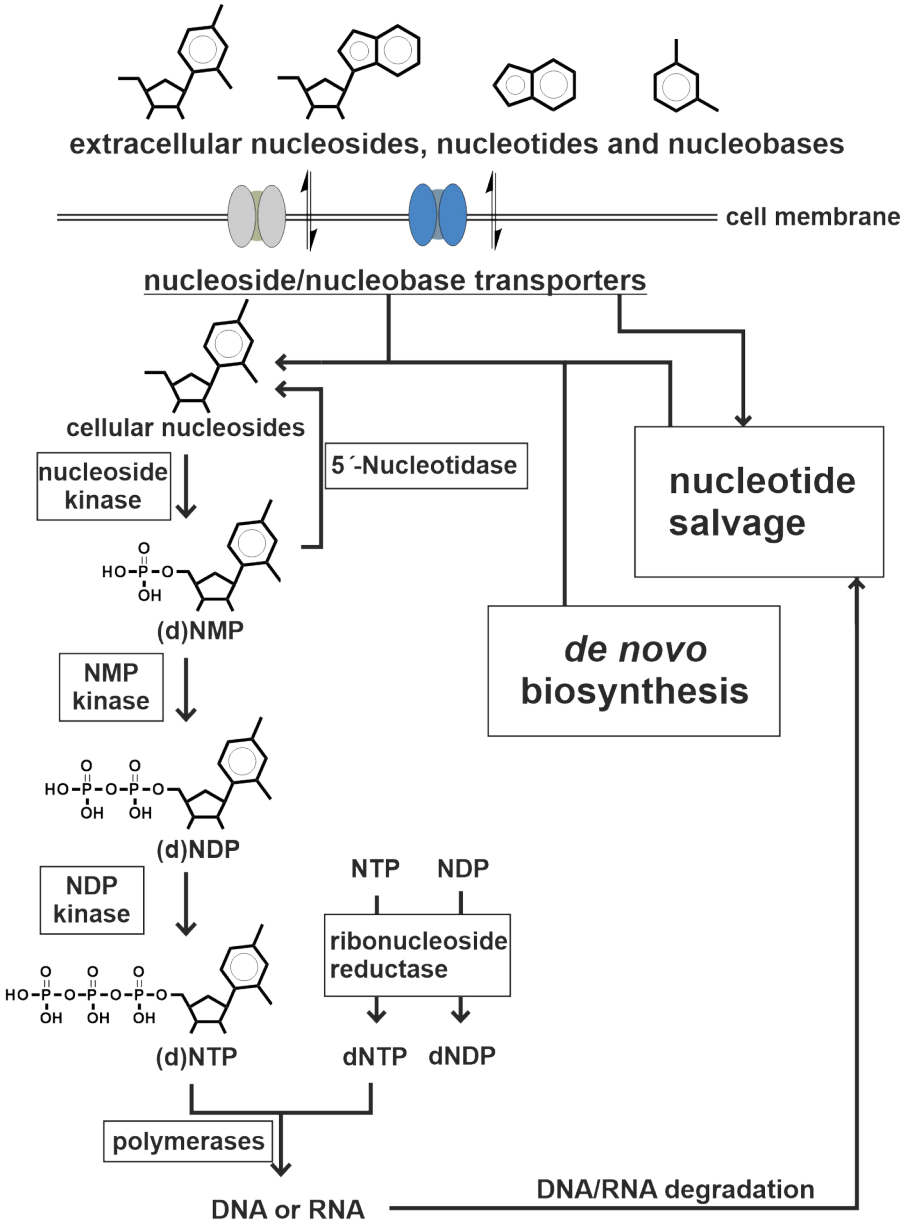


Figure 2. Simplified scheme of cellular nucleoside/nucleotide metabolic cycle.

In addition to the main mechanism of action, alternative targets for NsAs are common which might expand the utility of the drug for repurposing against other diseases.[80–82] In addition, 'self-potentiating' effects may result from secondary target activity.[56] For example, inhibition of ribonucleotide reductase decreases the natural 2′-deoxynucleotide levels and therefore the competition is decreased for the primary target such as DNA polymerases.[83] The off-target activities may also cause toxic side effects that often limit the use and dosage of a NsA drug.[84,85]

1.2.1 Structural motifs of nucleoside analgoues targeting polymerases

The DNA and RNA polymerizing enzymes bind the nucleotides based on the base complementary with the template strand, pairing G/dG with C/dC, and A/dA with U or T. Additionally, the DNA and RNA polymerases are able to discriminate against nucleotides with or without the 2′ hydroxyls, respectively. Numerous modifications in the natural nucleoside structures have been explored and many of such structures have found use as antivirals and anticancer agents targeting the various polymerases.[16–18]

1.2.1.1 Modifications of the ribose moiety

In general, the sugar moieties of NsAs seem to tolerate modifications more than the nucleobases in order to retain enzymatic recognition and binding affinity by the principal target as well as the metabolic enzymes required for the activation of the drug. As the 3′-hydroxyl of a nucleotide is required for the formation of the phosphodiester bond, removing or substituting this group leads to obligate chain termination after incorporation of such a nucleotide. The group of obligate chain terminators can be exemplified by the 2′,3′-dideoxy nucleoside Didanosine[86] (Figure 3) and by the 3′-N_3 substituted thymidine (AZT, Zidovudine)[87,88], both of which target the human immunodificiency virus (HIV) reverse transcriptase. Additionally, 3′-Deoxyadenosine (Cordycepin), a bioactive metabolite produced by *Cordyceps militaris* fungus,[89] has been shown to target RNA polymerases among its other mechanisms of action.[90] The immediate terminators are, however, susceptible to cleavage from the 3′-end by proofreading enzymes. L-nucleoside[91] anticancer compound Troxacitabine, is also phosphorylated by kinases and incoporated by DNA polymerase despite its unusual chirality.[92] Unlike its D-form, however, it is not cleaved from the DNA 3′-end by 3′-5′ exonucleases during elongation, although it still may be removed by the APE-1 excision repair mechanims.[93] Acyclic sugar containing nucleotides are also recognized by

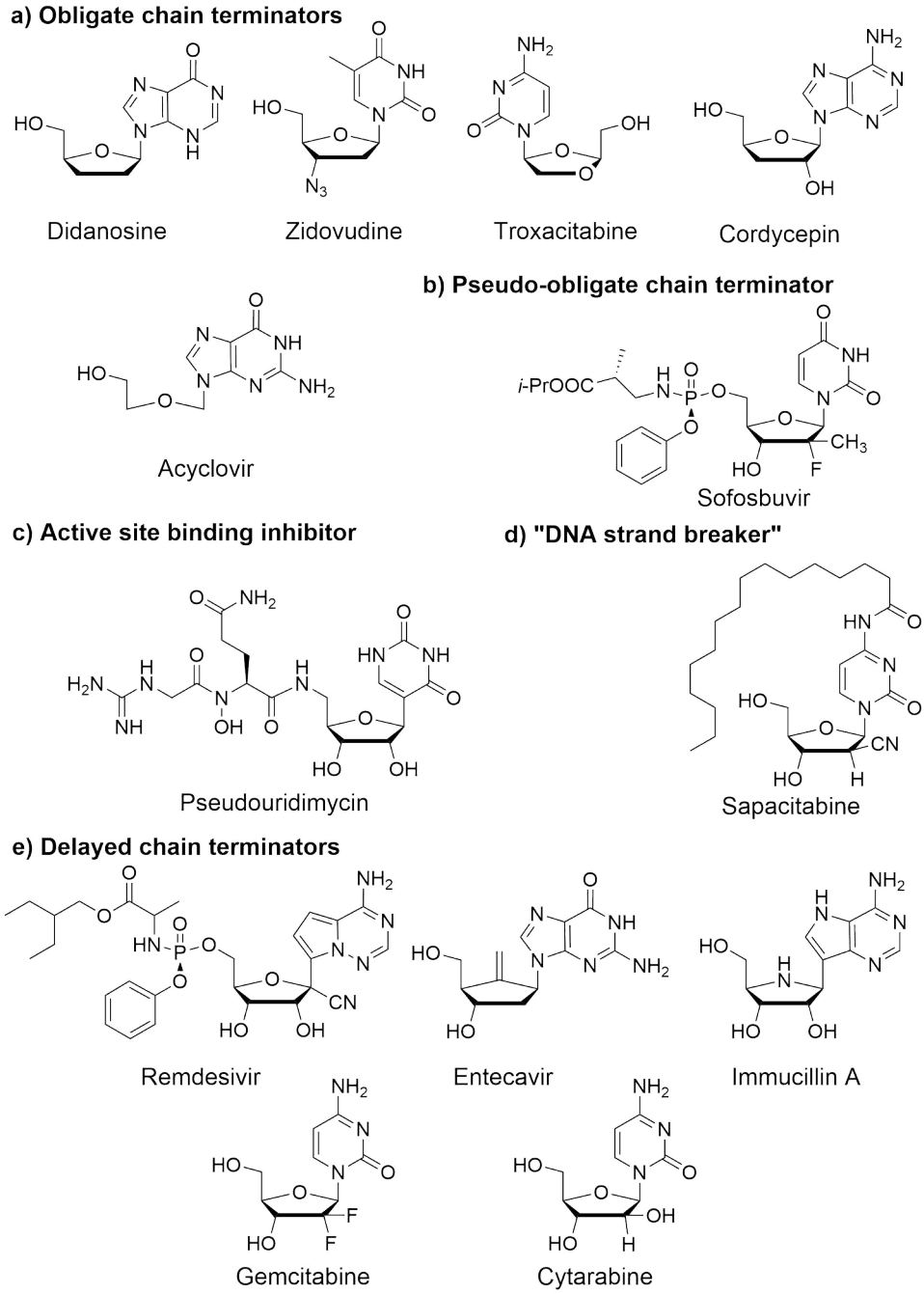


Figure 3. Examples of sugar modified nucleoside analogues affecting polymerase/oligonucleotide functions by **a)** obligate or **b)** pseudo-obligate chain termination, **c)** occupying the nucleotide addition site, **d)** causing DNA strand breaks, or **e)** by delayed chain termination.

polymerases. For example, the acyclic dG analogue, Acyclovir, is selectively activated by the viral thymidine kinase of human herpes virus in infected cells.[73,94] Moreover, application of a phosphoramidate prodrug strategy on Acyclovir bypasses the first phosphorylation expanding the drugs effectiveness against other types of viral infections.[65]

Other common sugar modifications causing polymerase dysfuntion include 2′-substituents on the *β*-side of the sugar, such as 2′-*C*-methyl group.[95] Incorporation of 2′-*C*-methyl modified nucleotides to the 3′-end of the oligonucleotide by viral RNAP prevents the incorporation of the next incoming substrate, and therefore terminates the elongation in a pseudo-obligate manner.[77] The *in vivo* activity of such compounds is benefitted significantly by the 5′-phosphate prodrug strategies implying that 2′-*β* substituents may hinder the first enzymatic phosphorylation.[72,96–98] For example, the parent compound of a clinically approved antiviral prodrug Sofosbuvir[99,100] (2′-deoxy-2′-fluoro-2′-methyluridine) was found inactive in Hepatitis C virus (HCV) replicon assays.[95]

In contrast to chain terminators, 2′-*β*-*C*-cyano group of an prosepective leukemia drug, Sapacitabine, functions by a different mechanism.[74] The cyano group increases the acidity of the 2′-proton making it susceptible to *β*-elimination that cleaves the phosphodiester bond and leads to DNA strand breaks. Another rare inhibition mechanism is exhibitied by the selective inhibitor of bacterial RNAP, pseudouridimycin (PUM).[9,20,101] PUM is modified at the 5′ position with a dipeptide moiety that mimics the triphosphate interactions. As such, the compound cannot be incorporated to the nascent RNA but instead it blocks the nucleotide addition site when the template DNA strand codes for UTP addition. Unlike most NsAs, PUM does not require metabolic activation.

Some sugar moity substituents may interact with additional binding sites in the target enzyme increasing affinity and selectivity. 1′-*α*-Cyano group[97,102], that is present in the antiviral Remdesivir[103,104], has been discovered to increase selectivity towards viral RNA dependent RNA polymerases. In a similar manner, an exocyclic double bond of Entecavir that replaces the ribose ring oxygen, is thought to occupy an additional hydrophobic binding site in hepatitis B virus (HBV) polymerase.[105] Both Remdesivir and Entecavir cause a delayed chain termination after a number of further nucleotide additions. Delayed chain termination has also been observed to be caused by the imino-sugar and 2′-difluoro-2′-deoxyribose structures of Immucillin A[106] and Gemcitabine[107], respectively. The delayed chain terminators are also beneficially masked by the further nucleotide additions, which in some cases protects the misincorporated NsA against cleavage from the 3′-end.[79]

1.2.1.2 Modifications of the base moiety

Base modifications of approved anticancer and antiviral drugs include purine position C2 halogenations and 6-thioketone modification, and various substituents at the pyrimidine position C5 (Figure 4). The thiopurines, after being metabolized to nucleotides, are incorporated into DNA and thereafter, are susceptible to nonenzymatic methylation which eventually causes replication errors by incorrect base pairing.[108] The C2-chloro modified deoxyadenosine (Cladribine), among its other mechanisms of actions, disrupts methyltransferase function leading to hypomethylation of DNA.[109] However, it has also been observed to induce a termination of DNA elongation after three consecutive Cladribine incorporations.[110]

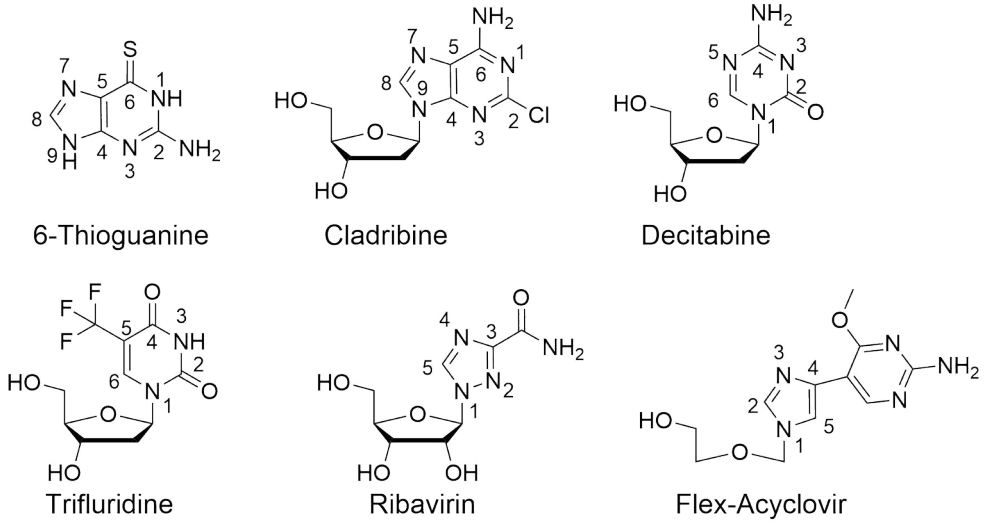


Figure 4. Examples of nucleobase modified nucleoside analogues affecting polymerase functions.

5-Azacytosine nucleosides such as Decitabine which inhibits DNA methyltransferases leading to hypomethylation.[111] Mechanistically it is likely that Decitabine forms a permanent covalent complex with the methyltransferase enzyme due to a missing proton at N5 which is required for the enzymatic β-elimination.[112] A cancer drug Trifluridine containing a 5-trifluoromethyl substituent, is instead effectively misincorporated into DNA leading to erroneous base pairing and a degree of DNA strand breaks.[113] On the other hand, 1,2,4-triazole-3-carboxamide nucleoside, Ribavirin, when incorporated by viral RNA dependent RNAP, can cause a lethal mutagenesis during RNA replication.[114,115]

More pronounced base modifications exemplified by the fleximer nucleosides where the bicyclic nucleobases have been disconnected by one bond length, have also shown potential biological activities.[116] A compound with a flex-guanosine base combined to the Acyclovir-type sugar moiety (Flex-Acyclovir), has shown activities against RNA viruses.[117,118] 2′-deoxyribose fleximer nucleotides were shown to be substrates for DNA polymerases, and therefore polymerase inhibition could be a possible mechanism of action of the Flex-Acyclovir.[116]

1.3 Natural C-nucleosides

1.3.1 Pseudouridine

C-nucleosides are quite rare in nature and only a small number have been discovered so far. The most common natural C-nucleoside is pseudouridine (PSU), a 5-ribosyl isomer of uridine (Figure 5), that occurs in all lifeforms mainly as a modification in ribosomal RNA, transfer RNA and small nuclear and nucleolar RNA.[119–121] In addition, methylated forms of PSU have been detected as RNA modifications,[122,123] formed by the action of methyltransferases on pseudouridine moieties at specific locations.[124]

PSU contains the uridine-type imide face for Watson-Crick base pairing with adenine. PSU has been proposed to enhance base stacking in RNA structures and it offers an additional hydrogen bond donor (NH-1), which are thought to rigidify and stabilize the three dimensional structures especially in ribosomal RNA and to regulate the intermolecular interactions in transfer RNA.[119,125] PSU is introduced into RNA post transcriptionally and the free nucleoside is mostly observed as a byproduct of RNA breakdown.[120] In plants and bacteria, the catabolism of PSU from degraded RNA has been elucidated to involve enzymes that first transform PSU to PSU-5′-monophosphate (PSU-MP) and then cleave the PSU-MP to uracil and 5-phosphoribosyl-1-pyrophosphate (PRPP).[126,127]

1.3.2 Ezomycin, malayamycin and pseudouridimycin

The pseudouracil base is also found in the structures of Ezomycin B, C and D,[128,129] malayamycin[8], as well as pseudouridimycin (PUM)[9]. Malayamycins and ezomycins additionally comprise bicyclic perhydrofuropyran motifs, whereas PUM contains a 5′-aminoribose bonded to a guanidylated glycine-glutamine moiety.

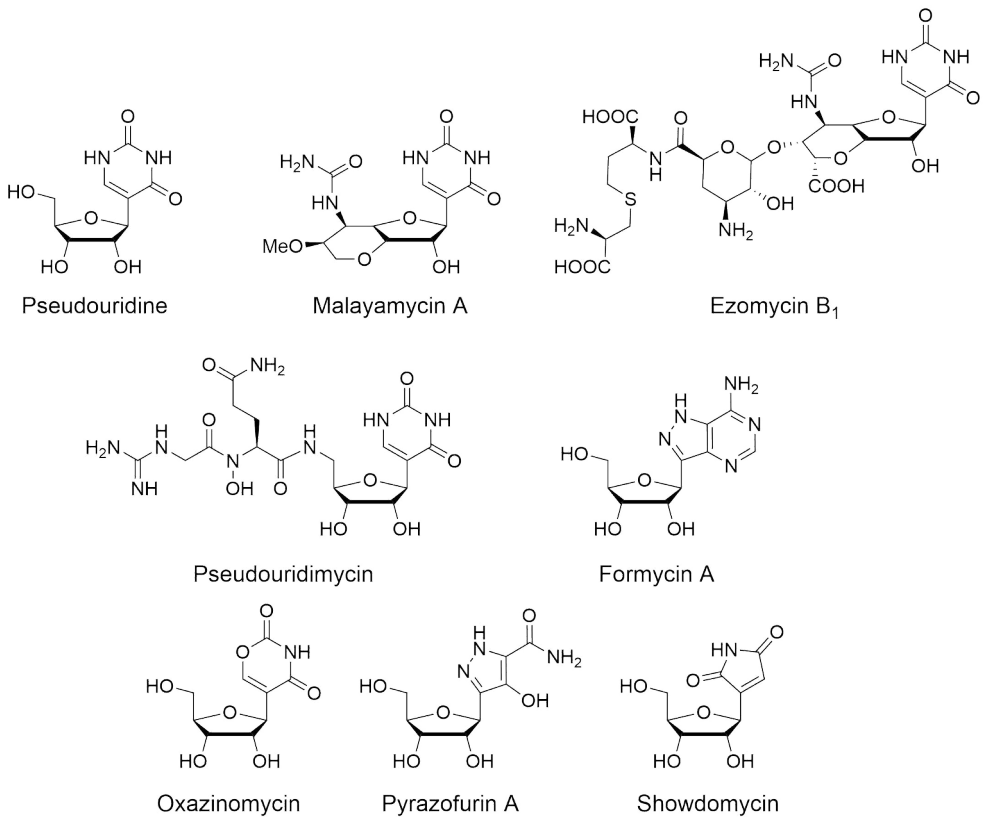


Figure 5. Examples of naturally occurring C-nucleosides.

The ezomycin series of compounds, including also N-nucleosides, exhibit broad-spectrum antifungal activities.[6] Similarly, Malayamycin A (MAL-A), isolated from *Streptomyces malaysiensis*, is an antifungal compound which has been shown to inhibit sporulation in plant pathogenic fungi.[130] The exact stereochemistry of the substituted perhydrofuropyran motif of MAL-A is vital for the activity, whereas the *C*-glycosidic bond is not essential because the synthesized N-nucleoside analogue retained equal activity.[131] The exact mechanisms of action of malayamycins and ezomycins are, however, still unclear.

The peptidyl nucleoside PUM was recently identified in hit extracts of two *Streptomyces* strains screened for bacterial RNAP inhibition.[9,132] Earlier in 2014, a compound designated as strepturidin was isolated from *Streptomyces albus*,[133] but it has since been reassigned as PUM[24] (see also section 3.3 and original publication II). In the hit extracts, PUM was identified as the active component that acts by binding competitively to the nucleotide addition site of the RNAP during transcription and inhibiting the addition of UTP. Moreover, PUM represents the first

NsA inhibitor of the bacterial RNAP whereas the single-subunit viral RNAP of a bacteriophage is not inhibited. PUM was found to be active *in vitro* against multi-drug-resistant strains of *Streptococcus*, *Staphylococcus* and *Enterococcus* species.[9] The Watson-Crick base pairing between PUM and the template DNA adenine is apparent since additions of G, A or C are not inhibited. The Gln moiety of PUM mimics the interactions of the triphosphate moieties of NTPs, and the *N*-hydroxy and guanidyl groups provide additional interactions with the 3′-end of the nascent RNA.[9,134]

1.3.3 Formycins

The purine nucleoside analogues with pyrazolopyrimidine bases, formycin A (FOR-A), formycin B (FOR-B), and oxoformycin B are structural isomers of adenosine, inosine and xanthosine, respectively.[80] FOR-A and FOR-B have been isolated from *Nocardia interforma*, *Streptomyces lavendulae* and *Streptomyces kaniharaensis* whereas oxoformycin B is a metabolite of FOR-A and FOR-B produced by *N. interforma*.[2,135,136] FOR-B is also generated enzymatically from FOR-A by adenosine deaminase, which can be prevented by the deaminase inhibitor, coformycin, that is often coproduced with FOR-A.[137,138]

The FOR-A and -B possess activities against some viruses and cancer types. As structurally closely related to the N-nucleosides adenosine and inosine, they act as substrates for a variety of enzymes.[80,139] They inhibit bacterial (*Escherichia coli*) and parasitic (*Plasmodium lophurae*) purine nucleoside phosphorylases (PNP) of the nucleoside salvage pathway, while the mammalian enzyme is not inhibited by FOR-A and is only moderately inhibited by FOR-B.[140–142] However, the antiviral and anticancer activity of FOR-A requires intracellular phosphorylation, and therefore it is suggested that the activity of FOR-A is principally based on FOR-A-5′-monophosphate inhibiting PRPP biosynthesis while some evidence exists that FOR-A-5′-triphosphate is eventually incorporated into nucleic acids.[143] Synthetically produced FOR-A-5′-triphosphate was utilized by *E. coli* RNAP with 47 – 68 % efficacy compared to ATP, and with a poly d(T-C) template the efficacy of incorporation was comparable with ATP.[144] FOR-B on the other hand is more efficiently converted to the 5′-monophosphate in some parasitic protozoa than in mammals, and thereon converted to cytotoxic analogues of FOR-A by succino-AMP synthetase/lyase system.[145] Formycins have exhibited toxic effects on mammalian cells too, preventing their use in clinical applications.[146,147]

The stability of the *C*-glycosidic bond of formycins, the similarity to natural N-nucleosides, and their special UV absorption and fluorescent properties[148] make them useful as molecular probes, substrates and ligands for studying biochemical nucleoside related events.[80] Thus, formycins have aided the elucidation of the

structures and catalytic mechanisms of PNPs as well as other nucleoside related enzymes. The fluorescent properties of formycins have been utilized to follow enzymatic events such as nucleoside transportation over cell membrane[149,150], deamination of nucleosides by adenosine deaminase[151] and production of 3′,5′-cyclic nucleoside monophosphates by membrane-bound nucleoside cyclases.[152,153] In addition, formycins have been used for affinity chromatography in purification of PNP enzymes.[80]

1.3.4 Oxazinomycin

Oxazinomycin (OZM, also known as Minimycin) produced by some *Streptomyces and Pseudomonas* species, has shown activity against gram negative and positive bacteria and transplantable tumors.[5,154,155] However, analyses on the target enzymes of OZM have not been reported. OZM differs from PSU by an oxygen atom in place of PSU's NH-1. Considering the role of the NH-1 of PSU in stabilizing RNA secondary structures, the OZM O-1 atom could be speculated to have a distorting effect on RNA structures in the case that OZM is metabolized into the 5′-triphosphate and incorporated into RNA. For the effects of synthetic OZM-5′-triphosphate on RNAPs, see the results and discussion section 3.3.2.

In the presence of thioglycerol, OZM underwent Michael addition with a simultaneous decarboxylation, which has been speculated as a possible origin of the antibiotic activity.[155] The hydrolytic instability of the oxazinedione ring, resulting in unstable nucleic acids, has also been suspected as a possible reason for the anticancer activity of OZM.[156] However, increased concentration of uridine has been shown to provide protection against OZM.[23] The same study demonstrated that the biosynthetic gene cluster responsible for OZM production, also encodes enzymes that produce UMP and dephosphorylate OZM-5′-monophosphate. Thus, it was deduced that these enzymes may act as mechanism of self-resistance for OZM producing microbes, and that the OZM is active as a phosphorylated species.

1.3.5 Pyrazofurin

Pyrazofurin A (PYR-A) and its α-anomer Pyrazofurin B are produced by *Streptomyces candidus* (which also produces OZM).[33] Pyrazofurin B is however, biologically mostly inactive, and is likely formed due to the susceptibility of pyrazofurins to anomerization in aqueous solution.[3,33]

Pyrazofurin A has been regarded as the most promising of the natrual C-nucleosides for clinical use[10], and it has been evaluated in clinical phase I and II studies as a chemotherapeutic, although not reaching phase III due to toxicity.[84] The main mechanism of action of PYR-A is the inhibition of *de novo* synthesis of

pyrimidine nucleotides. Therefore PYR-A has been also assayed against the malaria parasite *Plasmodium falciparum*, which is dependent on the *de novo* pathway for pyrimidine nucleotides.[157,158] Additionally, PYR-A has been shown to have potent activity against several classes of viruses[10,159,160] although in *in vivo* context, the inhibitors of pyrimidine *de novo* synthesis generally exhibit a limited antiviral activity due to the possibility of nucleotide salvage.[161]

The active metabolite, PYR-A 5′-monophosphate, formed by adenosine kinase[63], inhibits an enzyme included in the *de novo* pyrimidine biosynthesis, namely the orotidine-5′-phosphate decarboxylase that catalyzes the formation of uridine-5'-monophosphate as an intermediate for (d)UTP, (d)CTP and TTP. Accordingly, supplied uridine has a protective effect against PYR-A.[162] Additionally, resistance towards PYR-A has been attributed to a decreased adenosine kinase activity[69] or increased affinity of mutated viral RNAP for CTP and UTP.[68]

Chemical synthesis of a PYR-A 5′-triphosphate was recently achieved by a simultaneous phosphorylation of the 5′-hydroxyl and the enolic hydroxyl on the base moiety with *N*,*N*-diisopropylphophoramidite, and subsequent removal of the base ring phosphite ester with H_2O_2 oxidation.[163] In addition, enzymatic formation of diphosphate and triphosphate metabolites of PYR-A has been observed.[63] Still, at this date, any direct effects of pyrazofurins on RNA polymerases have not been reported.

1.3.6 Showdomycin

Showdomycin (SDM), isolated from *Streptomyces showdoensis*, is a broad-spectrum antibiotic with a maleimide group as the pseudonucleobase.[4] The maleimide moiety combines the uracil-type imide face with a strong alkylating capability as a Michael acceptor. SDM readily reacts with nucleophilic thiols and amino groups forming covalent bonds, which is most likely the main mechanism behind its biological activity.[164,165] On the other hand, the alpha-anomer of SDM does not possess significant activity which suggests that the activity of SDM is not based only on the alkylating nature of the maleimide moiety.

Even though SDM exhibits activities against multiple cancer types as well as gram negative and positive bacteria, its toxicity towards mammalian cells has prevented its medicinal use.[166,167] *In vitro*, SDM inhibits various enzymes of the pyrimidine nucleoside metabolic pathway[168–170] but also some enzymes with purine nucleoside substrates such as (Na^++ K^+)-ATPases.[171] SDM is taken up inside cells by membrane nucleoside transporters, but also inhibits those on cancer cells which has been speculated as a mechanism for its anticancer activity.[62,172,173] In addition, some SDM-resistant bacteria have been observed to possess decreased nucleoside transport activity.[174]

By tagging SDM with a 5-hexynoic acid ester at the 5′-hydroxyl, target enzymes have been identified in *Staphylococcus aureus* bacteria.[165] The SDM probes were covalently bound by enzymes via Michael addition and were subsequently labeled *in situ* with fluorescent tags, revealing a variety of enzymes targeted by SDM. The essential enzyme targets included muramyl ligase A that is involved in bacterial cell wall biosynthesis with an uridine-based natural substrate (UDP-GlcNAc). Moreover, the 5′-modified SDM probe exhibited equal antibiotic activity to the non-modified SDM which implies that bioactivation by phosphorylation at 5′-OH is not required for its antibacterial activity.

1.4 Artificial C-nucleosides

The diverse biological activities of natural C-nucleoside antibiotics have been an inspiration for design and synthesis of numerous artificial C-nucleosides.[10] Due to the natural C-nucleosides being potent at inhibiting nucleoside metabolic pathways, many artificial C-nucleosides have also been designed to target these systems. Most promising applications include inhibitors of nucleoside phosphorylases[59] and NAD dependent enzymes.[175] Moreover, potent C-nucleoside inhibitors of polymerases have been designed recently[97,103], most notably exemplified by the GS-5734 (Remdesivir), that was rapidly trialled as a treatment against SARS-CoV2 infections.[15]

1.4.1 Nucleoside phosphorylase inhibitors

Glycosidic bond cleaving reactions catalyzed by nucleoside phosphorylases and hydrolases are proposed to proceed through an oxocarbenium-type transition state (Figure 6a).[176,177] Rationally designed imino-C-nucleosides such as the cyanophenyl imino-C-nucleoside[178] (Figure 6b) mimic the positively charged transition state with the protonated imino sugar, thus achieving highly efficient binding in accordance to transition state theory.[179,180] The most notable compounds in this category are the immucillins (developed by BioCryst Pharmaceuticals), inhibiting PNPs with picomolar affinities and therefore interfering with purine salvage.[59] In treatment of malignant cells, the inhibition of PNP results in excessive buildup of nucleotide pools that leads to apoptosis.[181]

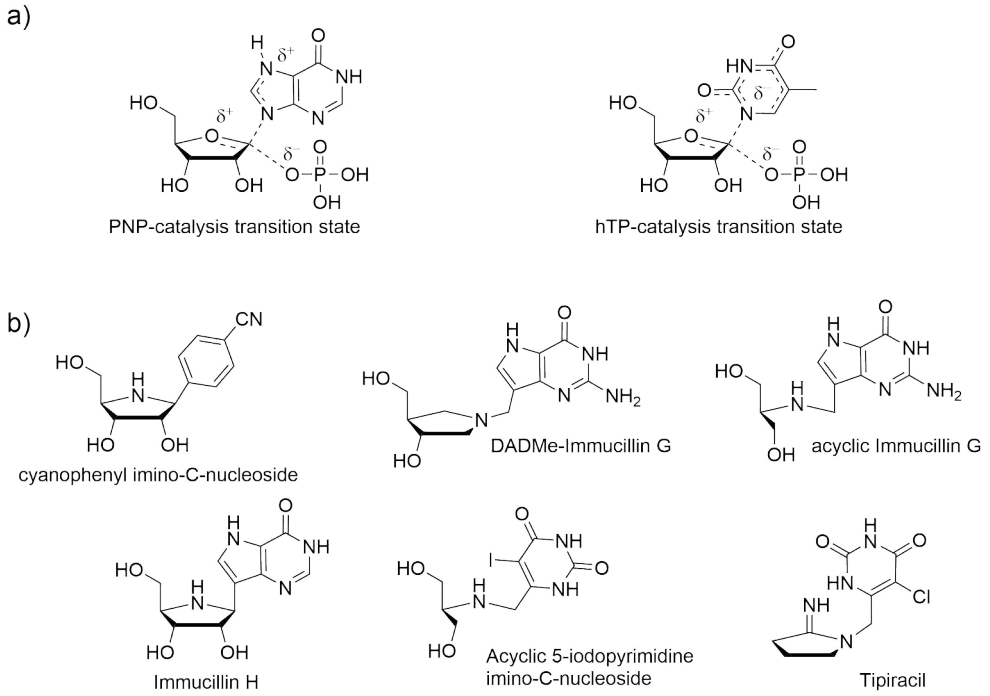


Figure 6. **a)** Proposed transition states of purine nucleoside phosphorylase (PNP) and human thymidine phosphorylase-catalysed reactions. **b)** Structures of nucleoside phosphorylase C-nucleoside inhibitors.

Immucillins as a whole exhibit a broad spectrum of activities against multiple types of diseases.[59] Immucillin H (ImmH, aka Forodesine) is orally active and efficient against T-cell lymphoma and was approved for clinical use against relapsed peripheral T-cell lymphoma in Japan in 2017.[182] Immucillin A (ImmA), and ImmH were identified as effective treatments for visceral Leishmaniasis parasite.[183] ImmA, although first designed mainly as a PNP inhibitor, was revealed as a potent antiviral prodrug by acting as a chain terminator in viral RNAPs.[184] Additionally, the ImmA-5′-triphosphate was shown to disrupt bacterial phosphonate metabolism by inhibiting an ATP nucleosidase-like enzyme of a carbon-phosphorus lyase complex.[185] These types of inhibitors could work as antibiotics against bacteria that are able to metabolize phosphonates in phosphate-limited environments.

The second generation of immucillins, known as the 4′-deaza-1′-aza-2′-deoxy-1′-methylene-immucillins (DADMe-Imm), are also potent inhibitors of PNPs and have shown efficiency against hyperuricemia (DADMe-ImmH aka Ulodesine)[186], malaria (DADMe-ImmG)[187] and solid tumours (MT-DADMe-ImmA).[188] Acyclic and achiral analogues of immucillins were designed as 3rd generation immucillins

obtainable with more simple syntheses.[189] The acyclic Immucillin G analogue inhibits PNP with picomolar affinity.

Some bacteria such as *Helicobacter pylori* (infecting mucous lining of the stomach) utilize the specific enzyme, 5′-methylthioadenosine nucleosidase (MTAN) to hydrolyze adenine in a pathway that produces menaquinone (electron transfer agent). Since the MTAN enzyme is absent from other gut bacteria, it has been a target for design of specific MTAN immucillin inhibitors.[190,191] Several DADMe derivatives of ImmA have reached picomolar affinities for MTAN.

Similar principles has been employed in the design of human thymidine phosphorylase (hTP) inhibitors.[192] The hTP is involved in purine and pyrimidine metabolism and catalyzes a similar reaction as PNPs with a transition state possessing oxocarbenium character.[193] An inhibitor of thymidine phosphorylase, Tipirasil (Figure 6b), is distantly structurally similar to the DADMe immucillins. Tipiracil is included in an orally administered anticancer combination drug Lonsurf™ (also known as TAS-102), where it protects trifluridine, the main drug component, from rapid metabolism.[194]

1.4.2 Inhibitors of NAD dependent enzyme

NAD acts as a cofactor during redox reactions catalyzed by NAD-dependent enzymes such as inosine-5′-monophosphate dehydrogenases (IMPDH). A C-nucleoside analogue of the nicotinamide riboside, benzamide riboside (Figure 7), was found to be metabolized first to a monophosphate form, and further to a NAD analogue, benzamide adenine dinucleotide (BAD).[64] BAD binds to the IMPDH as a NAD mimic but is unable to partake normally in the hydride transfer, thus inhibiting the enzyme and halting the *de novo* biosynthesis of GTP/dGTP. As a secondary target, the benzamide riboside or its metabolites inhibit NAD kinase, preventing the formation of nicotinamide adenine dinucleotide phosphate ($NADP^+$) that is required to maintain sufficient dihydrofolate reductase levels for cell viability.[195]

Tiazofurin and the analogous compounds such as Selenazofurin, Furanfurin and Imidazofurin also act after being metabolized to NAD analogues.[61,196,197] Tiazofurin has undergone phase I, II and III trials, and while exhibiting notable toxic side effects, it has been granted an orphan drug status (as Tiazole) for treatment of chronic myelogenous leukemia.[198] The approved antiviral N-nucleoside Ribavirin is structurally related to tiazofurin, and also inhibits the IMPDH, however by mimicking the substrate inosine-5′-monophosphate. However, the principal mechanism of Ribavirin is likely related to mutagenic interference with nucleotide polymerases.[199] On comparison, Tiazofurin and Ribavirin both can decrease GTP

levels in Hantaan virus infected cells but only Ribavirin increased mutation frequency of the viral RNA.[114]

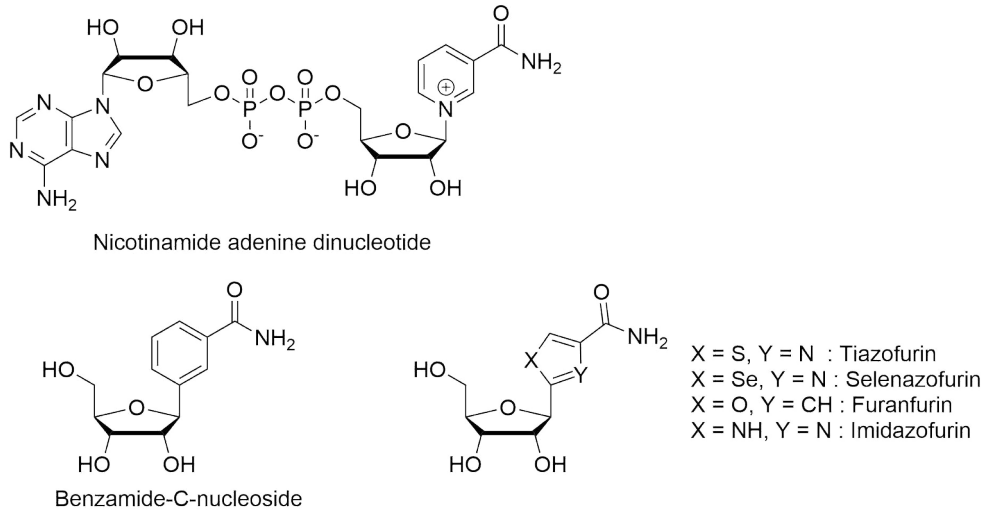


Figure 7. Structure of nicotinamide adenine dinucleotide and C-nuceoside inhibitors of NAD dependent enzymes.

Other C-nucleoside isomers of Ribavirin such as the 3-β-D-ribofuranosyl-1,2,4-triazole-5-carboxamide[200] and SRO-91[201] (Figure 8), also show potential anticancer activities. However, the former isomer presumably acts as an OMP decarboxylase inhibitor similarly as mentioned in the case of natural C-nucleoside PYR-A, whereas the exact mechanism of action of SRO-91 is not yet discovered. In comparison to Ribavirin, SRO-91 shows similar activity on cancer cells, but promisingly exhibits lower cytotoxicity to non-cancerous cells.[202]

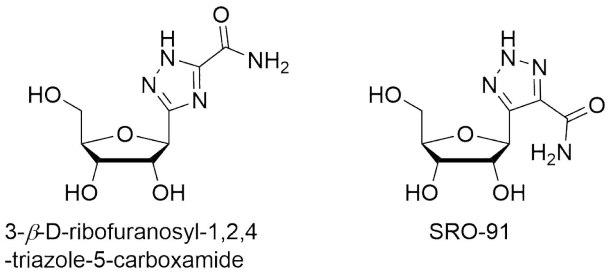


Figure 8. Triazole C-nucleoside isomers of Ribavirin.

1.4.3 Polymerase inhibitors

Originally designed as a PNP inhibitor, Immucillin A (Figure 9), as already described above, has more recently been found effective as an antiviral, especially against flaviviruses such as Ebola and Marburg viruses.[59,184] The nucleoside is converted by kinases to a triphosphate that acts as a delayed non-obligate chain-terminator following the incorporation to the nascent RNA in place of adenosine nucleotide by viral RNA dependent RNAP. In addition, inhibition of human DNA or RNA polymerases was not detected. Immucillin A is currently studied in phase 1 clinical trials (as Galidesivir; ClinicalTrials.gov Identifier: NCT03891420) with subjects suffering from yellow fewer or SARS-CoV2 infections.

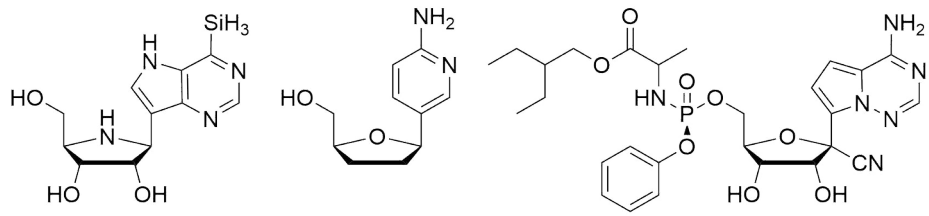


Figure 9. C-nucleoside inhibitors of polymerase enzymes.

A 2′,3′-dideoxy C-nucleoside analogue of cytidine (dd2APy), exemplifying an obligate chain terminator, was designed on the basis that mammalian DNA polymerases in contrast to viral polymerases, interact more with the functionalities on the minor groove side of the nucleobases.[76] Therefore, dd2APy lacking the position C2 carbonyl group, was selective towards HIV reverse transcriptase but still not as good as a substrate as dCTP.

A series of C-nucleoside analogues of adenosine, containing a 2′-*C*-methyl, was synthesized as inhibitors of hepatitis C virus polymerase (NS5B). The most promising one containing a 4-aza-7,9-dideazaadenine base group, MK-608, was further modified to reduce its toxicity on mitochondrial RNAP.[203] An analogue with a 1′-cyano group proved to be the most active while providing a good selectivity towards the viral RNAP. A phosphate prodrug strategy was further employed to bypass the initial phosphorylation which was liming the efficacy of the nucleoside on whole cell replicon assays.[97] The prodrug (GS-6620) was a potent inhibitor but its clinical utility has been limited by highly varying pharmacokinetic and pharmacodynamic properties in preliminary trials.

A similar compound was conceived by screening a library of nucleosides for activity against Ebola virus (EBOV) infected cells, and a subsequent implementation of a ProTide[66,204] prodrug protection. The resulting compound GS-5734

(Remdesivir, developed by Gilead Sciences), competes against ATP for incorporation into EBOV RNAP.[103] Again, the presence of 1′-CN group causes a near 500-fold selectivity over human mitochondrial RNA polymerase. Moreover, Remdesivir has been found effective against other RNA-viruses[205] and was regarded as one of the most promising treatments for the currently ongoing epidemic caused by SARS-CoV-2.[14,206] The drug was rapidly evaluated in clinical trials but the efficacy was only modest and it did not significantly reduce mortality.[15]

Mechanistically, Remdesivir and GS-6620 act as a non-obligate chain terminators after replacing ATP in transcription. The 2′-C-methyl of GS-6620 in the 3′-end of nascent RNA may prevent the next nucleotide from binding to nucleotide addition site in accordance to other 2′-C-methyl containing polymerase inhibitors.[97] In contrast, Remdesivir acts as a delayed chain terminator because the transcription by EBOV RNAP halted principally after 5 additional nucleotide incorporations following Remdesivir.[104] Similar observations were made with SARS-CoV-2 RdRP.[207]

Although the prodrug protected phosphorylated compound would in theory be superior to the nucleoside precursor, a pharmacokinetic analysis revealed that Remdesivir is prematurely hydrolyzed and dephosphorylated in serum.[208] Consequently, it was argued that the nucleoside precursor of Remdesivir (designated as GS-441524), would be more efficient as a drug while also being easier to synthesize.

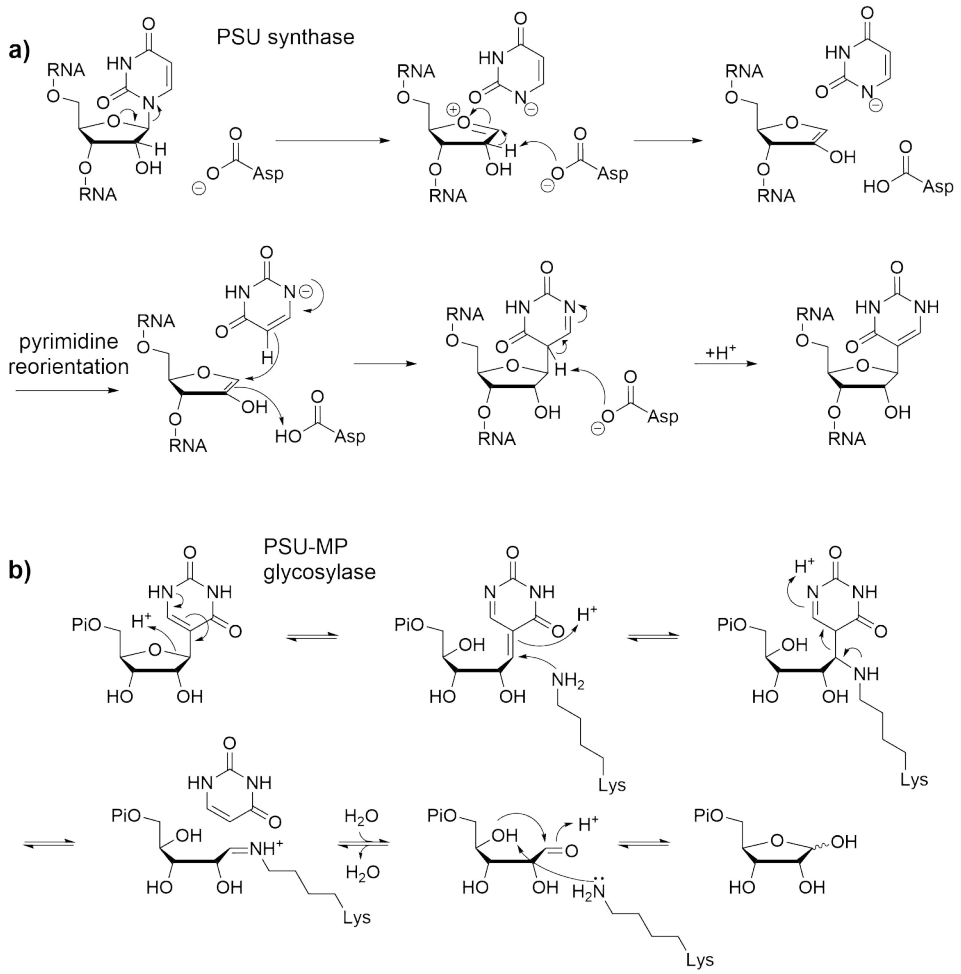
1.5 Formation of *C*-glycosidic bonds in C-nucleosides

1.5.1 Biosynthesis and biocatalytic prospects

In living organisms, PSU is formed by post-transcriptional isomerization of RNA bound uridine moieties by six families of pseudouridine synthases (PSU-synthase).[120,209,210] RNA-independent PSU-synthases are single protein enzymes while RNA-dependent PSU-synthases contain four core proteins and an RNA-guide to direct the pseudouridylation at a specific location. All PSU-synthases contain some conserved active site amino acid residues, including an aspartic acid residue that may act as a base or a nucleophile.[211]

The basic steps in the formation of PSU modification include the cleavage of the C-N anomeric bond, the alignment of the uracil C5 to the vicinity of the anomeric carbon, and the formation of a glycosidic C-C bond. Three mechanisms have been proposed for this enzymatic isomerization. The first, the so-called Michael addition mechanism suggests an initial attack of the catalytic aspartic acid on the base ring C-6 followed by dissociation of an oxocarbenium and the enzyme bound

pyrimidine.[212] Secondly, in the acylal mechanism, the aspartic acid would attack the anomeric carbon with simultaneous detachment of the base ring by a S_N1 or S_N2 type reaction.[213] A third mechanism suggests a glycal intermediate formed through deprotonation at C-2′ by the Asp residue as shown in Scheme 1a.[214] Quantum chemical calculations that are in favor of the glycal mechanism, suggest that the glycosidic C-N bond cleavage precedes the deprotonation of the 2′-proton.[211] A kinetic isotope effect study with 2′-deuterated uridine also supported the glycal mechanism by indicating that deprotonation or reprotonation at C-2′ was partly rate-limiting.[214]



Scheme 1. **a)** The steps of the proposed glycal mechanism catalyzed by PSU synthases, and **b)** the proposed mechanism of PSU-MP glycosylase.

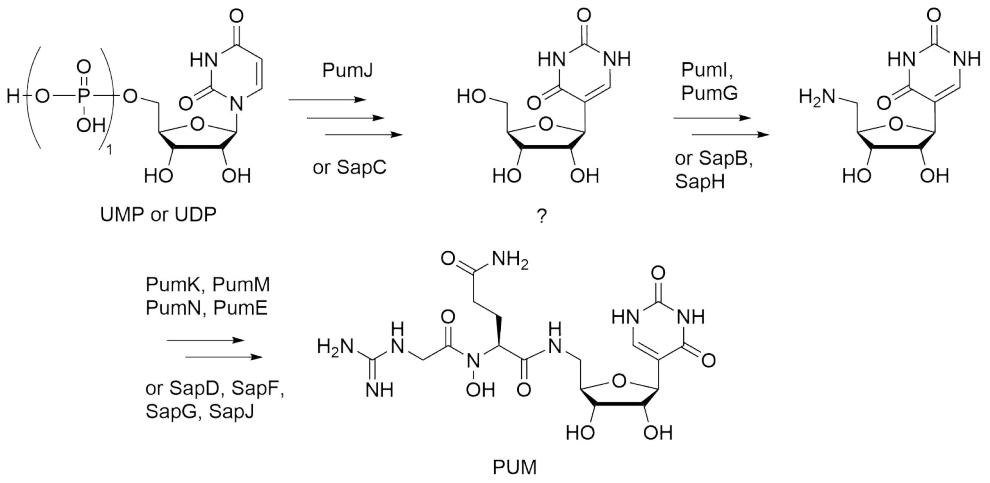
In mammals, free PSU resulting from degraded RNA is not catabolized but excreted instead.[215] In other lifeforms, pathways have been discovered where free PSU is transformed into PSU-MP and the *C*-glycosidic bond is subsequently cleaved by PSU-MP glycosylase enzyme.[126,216] By X-ray crystallographic studies performed on *E. coli* PSU-MP glycosylase, a mechanism was suggested, where an imine/Schiff base is formed between a lysine N-6 and the anomeric carbon of PSU (Scheme 1b).[127] Reverse action of PSU-MP glycosylases forming *C*-glycosidic linkages is also observed *in vitro*.[217] Furthermore, the *E. coli* PSU-MP glycosylase dubbed YeiN, was shown to be catalytically competent for preparation of PSU-MP from ribose-5-monophosphate (5 mM) and uracil (1 mM) in buffered aqueous solution in 99 % yield.[218] Additionally, the scope of the reverse C-glycosylase reaction was probed with different substrates. Varying activates were observed with the 5-monophosphates of 2-deoxyribose, xylose, arabinose and 2-deoxy-2-fluororibose, and with 3-methyluracil, 6-aminouracil, 4-thiouracil and 2-thiouracil as nucleobase analogues.[218]

Homologs of the PSU synthases were located in the *pum* gene cluster responsible for the enzymatic machinery producing PUM.[27] The first enzymes of the PUM biosynthesis pathway are proposed to construct free PSU from a uridine nucleotide, including the enzyme PumJ that likely catalyzes the isomerization step (Scheme 2). It was also suggested that the 3′-hydroxyl could be phosphorylated prior to the pseudouridylation, as to mimic the RNA phosphodiester backbone. Subsequently, PSU is activated by transformation into the 5′-amino form by the action of an oxidoreductase and an aminotransferase. Enzymes PumK, PumM, and PumN would be responsible for production of the 5′-peptidyl moiety and finally, PumE may conduct the *N*-hydroxylation. An analogous gene cluster designated as *sap* (*Streptomyces albus* pseudouridimycin) was discovered from *S. albus*, producing PUM with a similar biosynthetic scheme as depicted with the *pum* cluster (see original publication II).

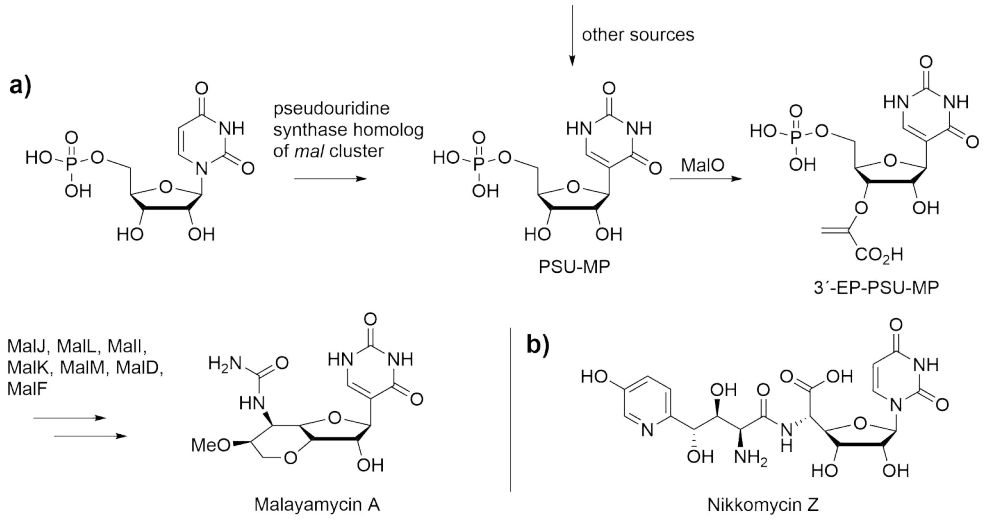
A PSU synthase homolog was located also in the Malayamycin A biosynthetic gene cluster (*mal)* producing PSU-MP presumably in the beginning of the MAL-A biosynthesis by *S. malaysiensis* (Scheme 3a).[24] However, a mutant strain without the enzyme could still produce MAL-A, albeit in lower amounts. It was suggested that the next enzyme of the pathway, MalO, could utilize PSU-MP generated in alternative ways such as RNA catabolism.

The MalO enzyme that converts PSU-MP to the 3′-enolpyruvoyl-PSU-MP (3′-EP-PSU-MP), is specific towards the C-nucleoside substrate. An analogous enzyme (NikO) in the pathway of the *N*-glycosidic nikkomycin Z (Scheme 3b), accepted both 3′-EP-UMP and 3′-EP-PSU-MP. Engineering of the biosyntheses of MalA and nikkomycin Z by interchanging the MalO and NikO enzymes between the pathways enabled the biosynthetic production of the *N*-glycosidic N-malayamycin and *C*-

glycosidic PSU-nikkomycin Z. As an alternative to the complex multi-step synthesis of malayamycins,[8] this kind of biosynthetic pathway engineering is intriguing for exploration of analogous compounds.

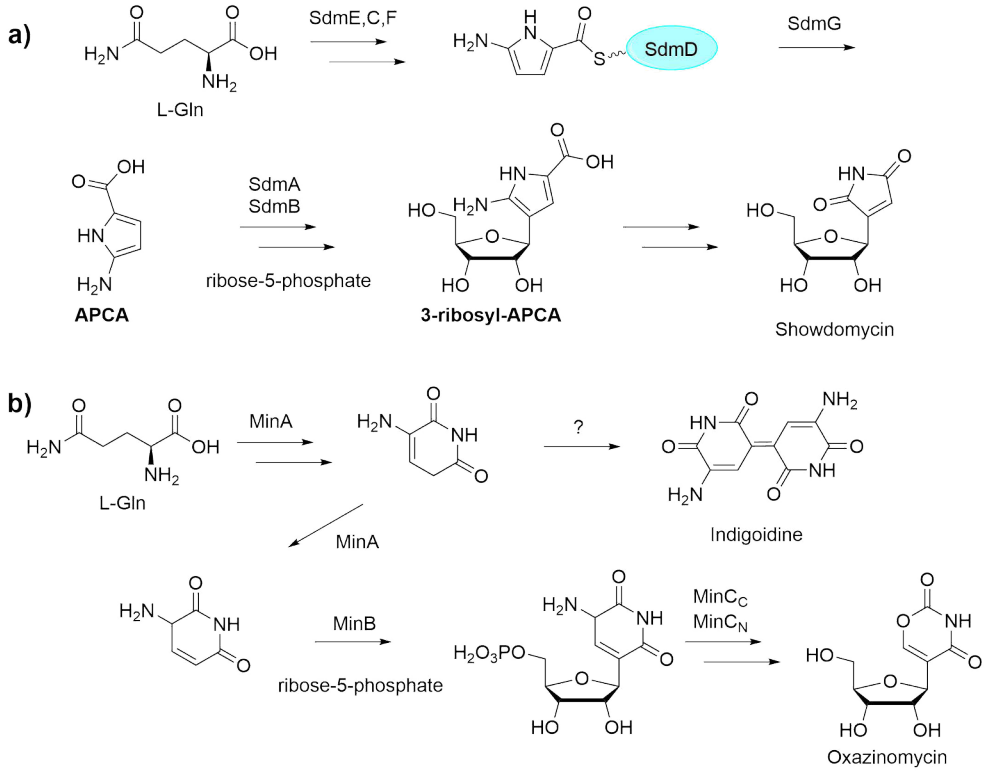


Scheme 2. Proposed biosynthetic pathways of PUM by the enzymes of the *pum* and *sap* gene clusters.



Scheme 3. **a)** Proposed biosynthetic pathway of MAL-A production and **b)** structure ofnikkomycin Z.

Earlier tracer studies have shown that the other natural C-nucleoside bases of FOR, OZM, PYR and SDM are constructed form L-glutamine.[219–221] The showdomycin biosynthetic gene cluster *sdm* was identified from *S. showdoensis* by searching for enzymes homologous to another known enzyme, AlnA (see original publication I). The AlnA is a PSU-MP-glycosylase homolog, discovered in the alnumycin A biosynthetic pathway where it catalyzes a C-ribosylation as an intermediate step.[222] In the biosynthesis of SDM, the C-glycosylation is proposed to be carried out by an AlnA-homolog SdmA that connects ribose-5-phosphate with the maleimide base precursor. It was recently shown that this precursor is 2-amino-1*H*-pyrrole-5-carboxylic acid (APCA) (Scheme 4a), which is susceptible to autoxidation and decarboxylation under aerobic conditions yielding 2-iminopyrrolone that further hydrolyzes into maleimide.[32] Likewise, the product of C-ribosylation and dephosphorylation (3-ribosyl-APCA) was transformed into showdomycin when it was exposed to atmospheric oxygen and subsequently treated at pH 3.[32] However, it is still unclear whether showdomycin is the actual biosynthesis end product, and therefore generated also enzymatically from 3-ribosyl-APCA.



Scheme 4. Proposed pathways of **a)** showdomycin and **b)** oxazinomycin biosynthesis.

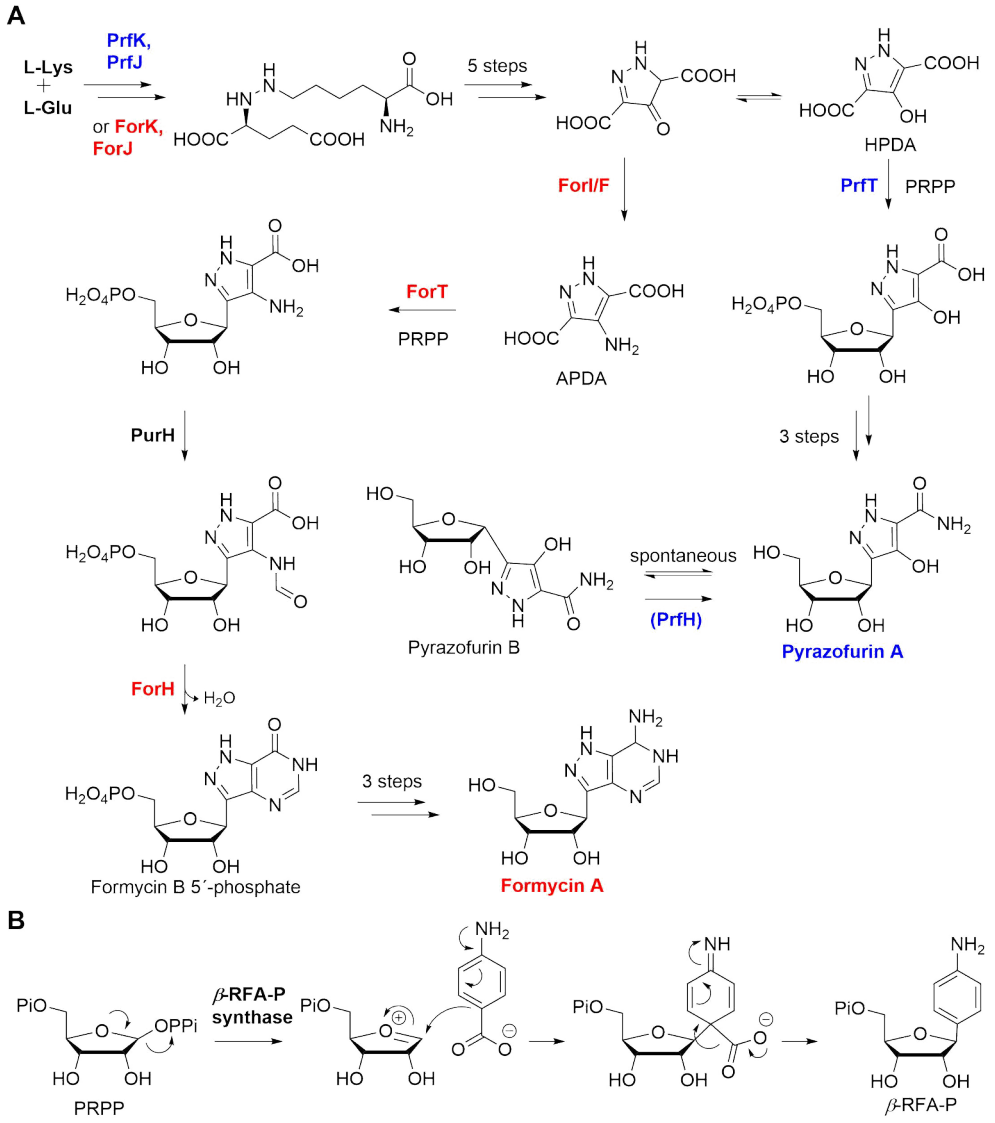
Similarly, the gene cluster responsible for OZM biosynthesis designated as *min*, contains an enzyme MinB, with 49 % identity with SdmA (Scheme 4b).[23] Therefore, MinB most likely catalyzes the C-glycosylation following the construction of the nucleobase by the multiple catalytic domains of enzyme MinA that, in addition, co-produces a blue pigment compound indigoidine as a side product.

The pyrazole-base rings of FOR-A and PYR-A are formed by partially analogous pathways until the formation of the intermediate 4-hydroxy-1*H*-pyrazole-3,5-dicarboxylic (HPDA) (Scheme 5a).[26,136,223] The two adjacent nitrogen atoms of the pyrazole ring originate from l-lysine and l-glutamic acid.

The C-glycosylations are suggested to be catalyzed by PrfT and ForT that are homologous to a hypothetic *β*-RFA-P synthase (with 81 % and 64 % identities, respectively), that forms the C-glycosyl linkage between phosphoribosyl pyrophosphate (PRPP) and *p*-aminobenzoate in methanopterin biosynthesis (Scheme 5b).[26] It is suggested that the *C*-glycosidic bond forms in an electrophilic aromatic substitution type reaction with an oxocarbenium ion formed from the PRPP. The mechanism is further supported by a recently determined crystal structure of ForT/PRPP complex, which implied that the negative charge of the dissociating 1′-pyrophosphate is stabilized by the enzyme leading to an S_N1-like transition state.[25]

The ForT is more substrate-specific, and only catalyzes the C-glycosylation after amination of HPDA to 4-amino-1*H*-pyrazole-3,5-dicarboxylic (APDA). The PrfT in contrast can employ both HPDA and APDA as substrates but only HPDA yields the correct product.[224] The PYR-A biosynthesis is completed by amidation of the carboxylic acid and 5′-dephosphorylation. As already mentioned, PYR-A is prone to spontaneous anomerization in aqueous solutions affording PYR-B. However, the *prf* gene cluster encodes a possible isomerase enzyme (PrfH) that was speculated to restore the *β*-anomer from PYR-B.[26]

The bicyclic base of formycins is completed by amidation of the carboxylic acid, formylation of the amine group and cyclization by imine formation. Interestingly, an enzyme of purine primary metabolism, PurH, catalyzes the formylation step.[136] The formed FOR-B-5′-monophosphate is finally transformed into FOR-A by amination and dephosphorylation.



Scheme 5. **a)** Proposed pathways of pyrazofurin A and formycin A biosynthesis, and **b)** a proposed mechanism of reactions catalyzed by β-RFA-P synthase-type enzymes.

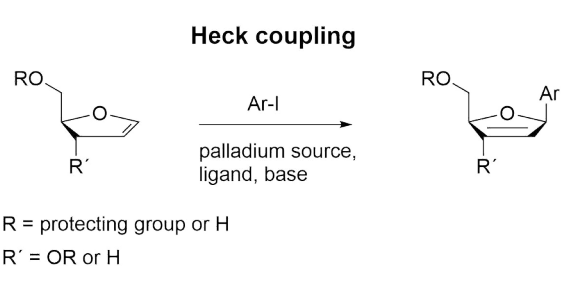
1.5.2 Synthetic methods

The first chemical synthesis of PSU was achieved nearly 60 years ago by a nucleophilic substitution with a lithiated dimethoxy pyrimidine on protected ribosyl chloride, which afforded the correct anomer in 2 % yield.[225] Since then, synthetic methods of C-glycoside formation have been developed with increasing yields and control of the stereochemistry.[226] Currently, the two most used approaches are the

Heck-type coupling[227] and the nucleophilic addition on furanolactones.[228] These methods are examples of direct couplings between preformed carbohydrate and nucleobase moieties in a convergent manner.[11,229] Optionally, the nucleobase moiety can be constructed stepwise upon a suitable functional group located at the carbohydrate C-1.[11,230] In a similar stepwise manner, the carbohydrate can be constructed upon a functionalized nucleobase moiety. In this section, an introductory selection of chemical C-glycosylation methods is presented.

1.5.2.1 Direct couplings of carbohydrates and nucleobases

The Heck coupling of aryl halides with typically silyl protected glycals can be used to prepare 2′-deoxy or 2′,3′-dideoxy-C-nucleosides[76] in a regio- and stereoselective manner.[11,227,229] The reaction includes, in addition to the glycal and the aryl halide, a palladium(0) source, a palladium complexing ligand and a base in aprotic solvents (DMF, MeCN, THF, dioxane).[227] Some typical reagents are presented in Scheme 6. The stereochemical outcome of the coupling can be controlled with a bulky substituent introduced to the glycal 3-hydroxyl directing the attack of the organopalladium reagent to the β-face.[231] Conversely, the use of a 3′-unprotected glycals in most cases leads to predominant formation of α-anomers.[232] The coupling and removal of the 3′-hydroxyl protection affords a 3′-ketonucleosides. The reduction of the 3′-keto group can be carried out stereoselectively from the more hindered side with e.g. sodium triacetoxyborohydride[233] or from the less hindered side with e.g. K-selectride[232], affording 2′-deoxyribose or 2′-deoxyarabinose nucleosides, respectively. The yields of the Heck couplings are generally case dependent, ranging from modest to excellent.[227]

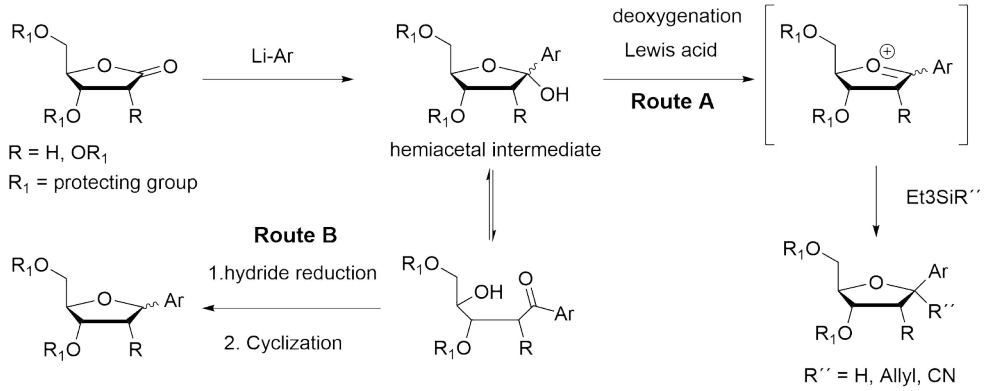


Scheme 6. General scheme of C-glycosylation by Heck coupling.

Contrary to Heck couplings, the addition of organometallic nucleophiles to furanolactones can be used to prepare ribose-C-nucleosides, in addition to 2′-deoxyribose-C-nucleosides.[228] Typically, the coupling step is carried out at low

temperatures with lithiated aromatics or a Grignard reagent. The coupling generates a hemiacetal intermediate that can be reduced by Lewis acids such as BF_3•OEt_2 producing an oxocarbenium intermediate (Scheme 7, Route A). When silanes are used to reduce the oxocarbenium, the stereochemistry of the anomeric center is determined by the relative reactivities of the oxocarbenium E_3/^3E conformers, but typically a good β-selectivity can be achieved.[228,234] Furthermore, depending on the type of the silyl reducing agent, additional 1´-substituents can be introduced during this step. Thus, the use of triethylsilane, allyltrimethylsilane or trimethylsilyl cyanide adds 1´-H, 1´-allyl or 1´-nitrile substituents, respectively.[235] Alternatively, vinyl magnesium bromide and ethynyl magnesium chloride have been used to treat the hemiacetal intermediate, followed by a ring closure with methanesulfonic acid, to afford the respective 1´-vinyl and 1´-ethynyl substituted C-nucleosides.[236]

The method has been used in the synthesis of Remdesivir, which includes the 1´-cyanation during the reduction of the oxocarbenium intermediate.[103] The envisioned potential of Remdesivir in the treatment of SARS-CoV2 infections has prompted optimizations of the synthesis for large scale production. Initially, the C-glycosylation step was carried out via lithium halogen exchange reaction at – 78 °C in 25% yield. An improved reaction benefitted from the presence of diisopropylamine which was thought to promote the silylation of the primary amine of the base group and to stabilize the lithiated base intermediate.[237] Another optimized method suitable to be carried out at more accessible temperatures (– 20 °C), proceeded through a Grignard reaction where neodymium(III) chloride and tetra-*n*-butylammonium chloride facilitated the formation of the *C*-glycosidic bond.[238]



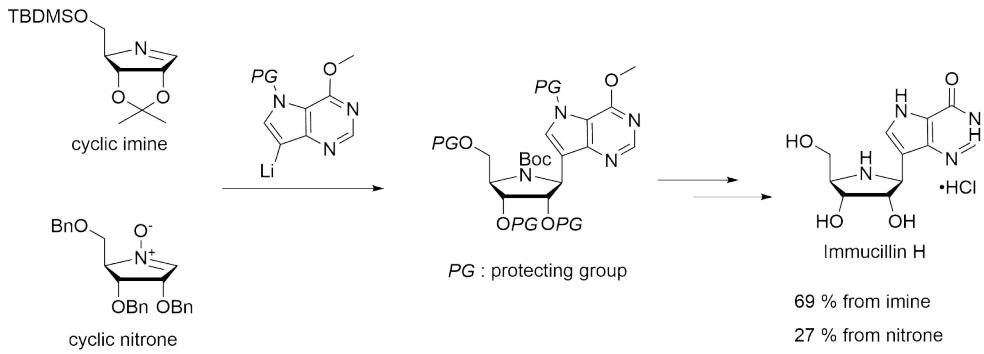
Scheme 7. C-glycosylation by a nucleophilic addition to furanolactones.

The hemiacetal intermediate generated from the furanolactone is in equilibrium with acyclic hydroxyketone form, which may be reduced and subsequently cyclized for example under Mitsunobu conditions (Scheme 7, Route B). In a stereoselective synthesis of PSU anomers, the reduction step performed with L-selectride in the presence or absence of zinc chlorde yielded β or α products, respectively.[239] The method was then applied to a total synthesis of the natural C-nucleoside Malayamycin A containing a pseudouracil base.[8]

Mixtures of acyclic 1′-*S* and 1′-*R* intermediates are also produced during a nucleophilic addition of organometallic reagents to the aldehyde functionality of aldoses.[240] Acid catalyzed cyclization of the intermediate has been shown to afford the α and β products in the ratio of the 1′-*S* and 1′-*R* epimers, respectively.[241] Cyclization under standard Mitsunobu conditions (with diethyl azodicarboxylate and Ph_3P) provides similar product mixtures.[240] However, a modified Mitsunobu cyclization (with *N*,*N*,*N*′,*N*′-tetramethylazodicarboxamide and Bu_3P) is stereochemically directed by the 2′-substituent. Accordingly, a synthesis with 2′-benzylated ribose as starting material provided stereoselectively the β-anomer[242–244], while 2′-benzylated arabinofuranose selectively afforded the α-anomer.[245]

Lewis acid catalyzed Fridel-Crafts type conditions can be used to couple electron rich aryls with, for example, acetoxy sugars or chloro sugars.[246,247] In a study with peracetylated ribose and 4-bromoanisole, $SnCl_4$ was found to be the most suitable Lewis acid affording the product in 70 % yield.[248] In addition, an unreactive electron-deficient thiazole was successfully coupled after trimethylsilylation of the thiazole in a reaction reminiscent of the Vorbrüggen protocol commonly used to prepare N-nucleosides. However, low regioselectivity and generality of the method limits the use of the Friedel-Crafts type C-glycosylation.

Direct Heck-type couplings to cyclic imines[249] with a lithiated deazapurine derivatives were developed to prepare the imino-C-nucleoside immucillin H on practical scale for clinical studies (Scheme 8).[250] In this reaction an ether/anisole mixture was necessary as the solvent in contrast to THF in which no reaction was observed. However, the preparation of immucillin A and G by this method was impractical due to instability of the lithiated 9-deazaadenine and 9-deazaguanine derivatives under the reaction conditions. An alternative route to immucillin H was developed utilizing a cyclic nitrone in place of a cyclic imine.[251] From the nitrone, the ImmH was obtained in 27% yield in contrast to the 69% from the cyclic imine. However, the nitrone precursor is stable compared to the imine and can be obtained from D-ribose in 7 steps (31% overall yield), whereas the cyclic imine can be produced from D-gulonic acid-γ-lactone in 11 steps.[251] The acyclic 3rd generation immucillins, on the other hand, can be synthesized by a simple reductive amination with 2-amino-1,3-propanediol and aldehyde derivatives of purine nucleobases.[189]

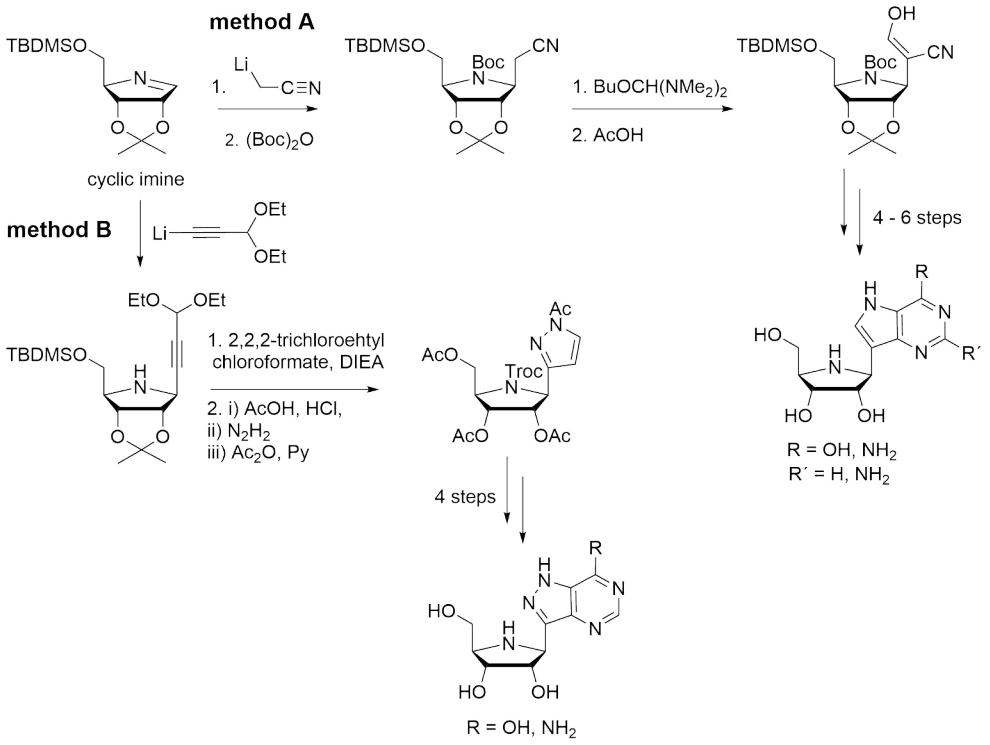


Scheme 8. Immucillin H synthesis by direct coupling of a cyclic imine.

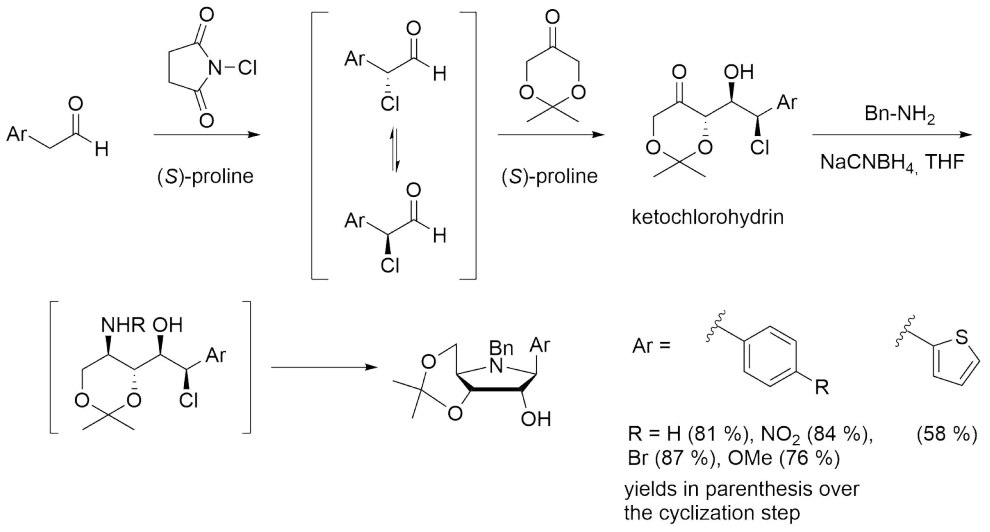
1.5.2.2 Stepwise construction of glycone or aglycone upon functional groups

The first generation synthesis of immucillins employed a linear construction of the heterocycle aglycones, commencing with coupling of the cyclic imine precursor to a lithiated acetonitrile (Scheme 9, Method A).[252] Another method for preparation of 8-aza-immucillins[253] was adapted from the syntheses of the pyrazole C-nucleosides formycin and pyrazofurin.[254–257] Accordingly, coupling of an alkynyl group at the C-1′ followed by a cyclization with hydrazine afforded the pyrazole ring (Scheme 9, Method B) that could be further expanded to 8-aza-9-deazapurines in 4 steps.

Cyclization of ketochlorohydrins has been explored for synthesis of various imino-C-nucleosides with simple aryl 1′-substituents (Scheme 10).[258] The ketochlorohydrin intermediates were prepared stereoselectively in one step from acetaldehyde derivatives that were reacted with a mixture of *N*-chlorosuccinimide and 2,2-dimethyl-1,3-dioxan-5-one in the presence of (*S*)-proline. The reaction proceeds through initial α-chlorination, while the (*S*)-proline catalyzes the racemization of the intermediate as well as controlling the enantioselectivity of the subsequent aldol reaction with the dioxanone. Reductive amination of the ketochlrohydrins with benzylamine simultaneously closed the pyrrolidine ring, yielding the *N*-substituted imino-C-nucleoside analogues in a stereospecific manner.

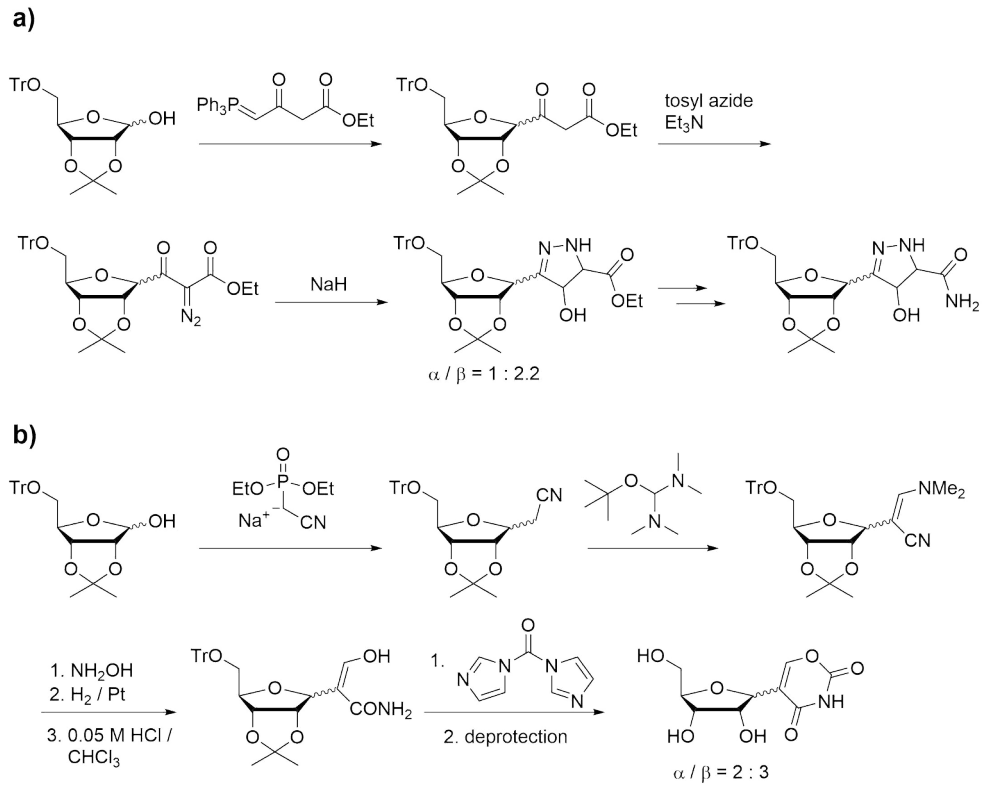


Scheme 9. Synthesis of imino-C-nucleosides by stepwise base construction.



Scheme 10. Synthesis of imino-C-nucleosides by ketochlorohydrin cyclization.

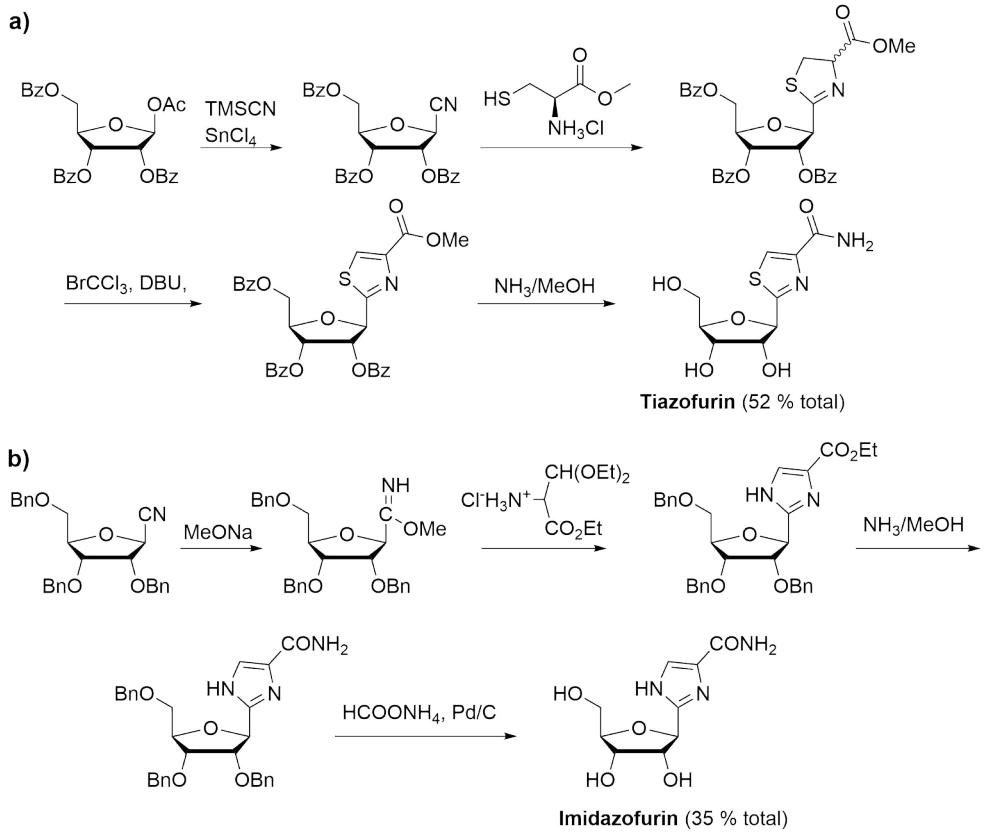
The Wittig reaction is a suitable method for formation of *C*-glycosidic bonds with 1-unsubstituted sugars.[11] Pyrazofurins were prepared by a reaction of protected riboside with the [3-(Ethoxycarbonyl)-2-oxopropylidene]-triphenylphosphorane (Scheme 11a), affording a diketone product which was then diazotized followed by cyclization in the presence of sodium hydride.[259,260] An anomeric mixture of the products was then obtained after aminolysis and deprotection of the cyclization product. In the synthesis of oxazinomycin, a cyanomethyl group was introduced to the anomeric carbon of a protected ribose by the Horner modification of the Wittig reaction (Scheme 11b).[261] The 1-cyanomethyl group was subsequently aminomethylenated with *tert*-butoxy bis(dimethylamino)methane. The obtained enamine intermediate was transformed in three steps to a formylacetamide intermediate. The enol tautomer of the formylacetamide could be cyclized with *N*,*N*-carbonyldiimidazole affording the oxazine ring. Furthermore, the 1-cyanomethyl group can be also transformed to 9-deazapurine bases[34] analogously to that presented in Scheme 9.



Scheme 11. Utilization of Wittig reaction in the synthesis of **a)** pyrazofurins and **b)** oxazinomycin.

The nitrile group in 1-cyano-furanoses has been used as a scaffold for heterocycle construction. In the synthesis of tiazofurin the 1-cyano furanose was obtained from protected 1-acetyl ribose by Lewis acid catalyzed substitution with trimethylsilyl cyanide (Scheme 12a).[262,263] Cyclization with L-cysteine methyl ester hydrochloride and subsequent oxidation in the presence of bromotrichloromethane and DBU provided the thiazole ring. The benzoyl protections were simultaneously removed during the ammonolysis of the ester group.

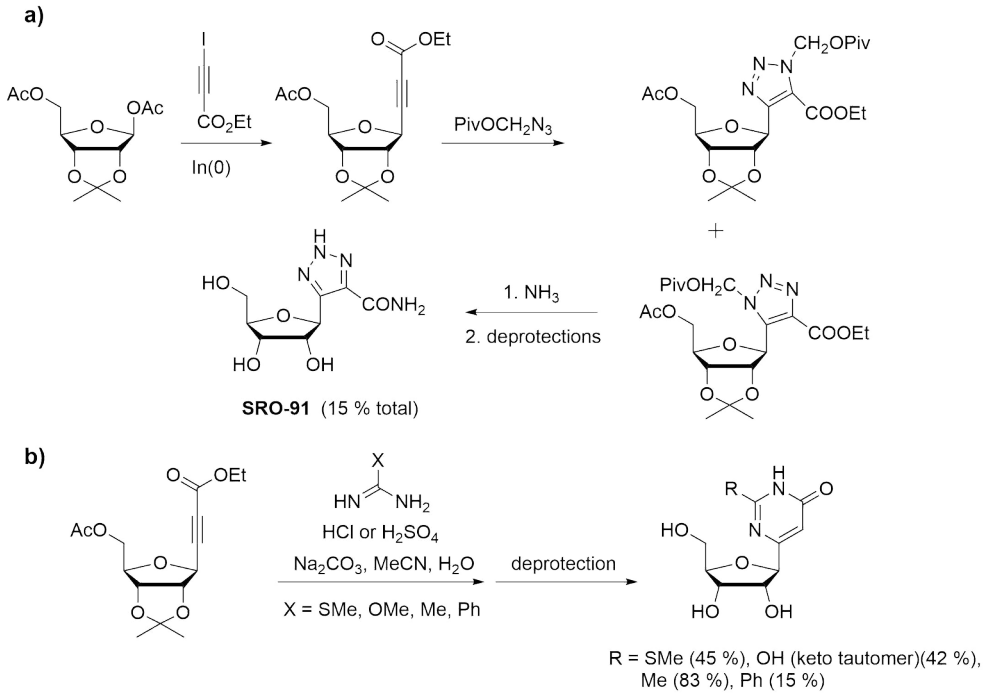
Synthesis of imidazofurin starting from benzyl protected 1-cyano-ribofuranose was realized in 4 steps (Scheme 12b).[197] Treatment of the starting material with sodium methoxide afforded an imidate product which was cyclized with 2-amino-3,3-diethoxypropionate hydrochloride. Final ammonolysis and deprotection with ammonium formate and Pd/C catalyst afforded the imidazofurin in 35 % yield.



Scheme 12. Constuction of **a)** tiazofurin and **b)** imidazofurin nucleobases on 1´-cyanogroup.

The azide-alkyne cycloadditions are common reactions related to alkynes and provide a route towards triazole-C-nucleosides.[264] Synthesis of the Ribavirin analogue SRO-91 was accomplished with indium catalyzed alkynylation of protected 1-acetyl-riboside with ethyl iodopropiolate (Scheme 13a).[201] Subsequent cycloaddition with azidomethyl pivalate provided the two protected C-nucleoside isomers. Ammonolysis and deprotection of the isomers afforded the final product in overall 15 % yield starting from D-ribose.

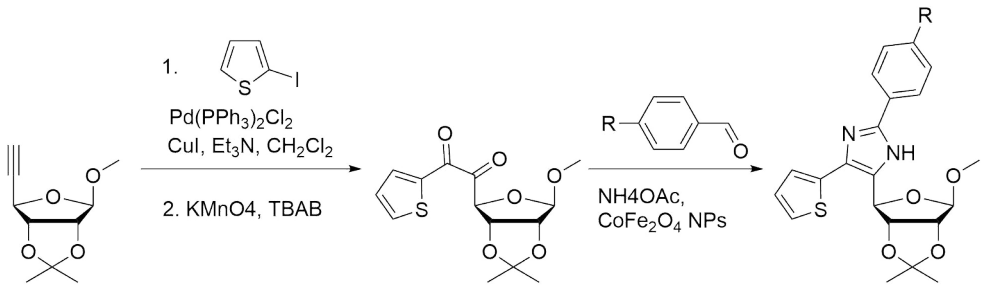
The 1´-alkyne could also be condensed with amidine reagents in the presence of a catalytic amount of water to produce pyrimidine-6-ribosides (Scheme 13b).[265] The yields varied depending on the amidine reagent used. Treatment with *S*-methylpseudourea, acetamidine, benzamidine or *O*-methylisourea gave the pyrimidine C-nucleosides with thiomethyl, keto, methyl or phenyl groups in the base ring position 3, respectively.



Scheme 13. Constuction of **a)** SRO-91 and **b)** 6-pyrimidine ribosides on 1´-alkynyl.

Alkynyl sugars were also used to prepare three-component imidazole C-nucleosides in one pot (Scheme 14).[266] Sonogashira coupling of aryl iodides with the alkyne and immediate oxidation of the product with $KMnO_4$ and tetra-n-butylammonium bromide (TBAB) afforded a diketo-intermediate. The diketo functionality was then

cyclized with an aryl aldehyde in a reaction catalyzed by cobalt ferrite nanoparticles ($CoFe_2O_4$ NPs). In all reported cases, the products were obtained in good yields (70 – 93 %) although the yields were generally higher in cases of pyranose sugars.



Scheme 14. Synthesis of three component imidazole C-nucleoside analogues.

2 Aims of the Study

The natural C-nucleoside secondary metabolites have been known to be highly bioactive for more than half a century.[2,3,5,164,215] Still, their activities with RNA polymerases remain mainly unsolved in detail. On the basis of their structure, most of the natural C-nucleosides could have much potential for interesting polymerase activity. Showdomycin and oxazinomycin both have the uracil type imide face complementary to adenine and therefore could be misincorporated into RNA structures as uridine mimics, whereas the C-nucleoside formycin A could imitate adenosine units. On the other hand, pyrazofurin A structurally resembles the N-nucleoside antiviral Ribavirin. Ribavirin is known to induce error catastrophes by incorporation to viral RNA and therefore, mechanistic similarities of pyrazofurin would be worth investigating. For these enzyme assays, 5′-triphosphates of the nucleosides are needed. Numerous methods have been developed to phosphorylate nucleosides.[267] Still, the triphosphates of showdomycin and oxazinomycin have not yet been reported. Additionally, the synthesis of pyrazofurin 5′-triphosphate was first reported only in 2018, but its RNAP activity has not been elucidated to this date.[163]

The discovery of antibiotic strepturidin was reported in 2017. Structurally the compound has a degree of resemblance toward pseudouridimycin which is the first known selective nucleoside analogue inhibitor of bacterial RNAPs. Therefore, strepturidin was one compound of interest for studying its RNAP activity.

Semisynthesis is a lucrative alternative to total synthesis of natural products as the production of compounds by synthetic biology bypasses often laborious and stereochemically demanding chemical procedures.[268] Thus, the C-nucleoside starting materials for this study are obtained by bacterial cultivations and the chemical transformations are then applied to prepare the corresponding 5′-triphosphates for RNAP activity assays. Additionally, other modifications to the C-nucleoside precursors are introduced when applicable. The results of the RNAP assays are hoped to yield insight into the mechanisms of action of the C-nucleoside analogues as well as into the mechanisms of the RNAP enzymes themselves. The most promising compounds could be considered as lead compounds for further development of RNAP targeting drugs.

In summary, the aims of this thesis are:

- To semisynthetically prepare phosphorylated C-nucleoside analogues of showdomycin, oxazinomycin, formycin and pyrazofurin, and to isolate strepturidine.
- To assess the effects of the prepared compounds on transcription by different RNA polymerases.
- To explore chemical modifications on showdomycin and to evaluate their effects in RNAP-assay

3 Results and Discussion

3.1 Production and isolation of natural C-nucleosides

Showdomycin (**1**), Oxazinomycin (**2**) and pseudouridimycin (**3**) (Figure 10) were produced by cultivating soil bacteria strains *Streptomyces showdoensis* ATCC 15227, *Streptomyces hygroscopicus* JCM 4712 and *Streptomyces albus* DSM 40763, respectively. The bacterial cultures were maintained in conditions adapted from previously reported procedures.[133,154,269] For production of **1**, a suitable culture medium contained potato juice as an essential ingredient. Additionally, the type of peptone ingredient in the medium was crucial in the case of **1**. For each fermentation of compounds **1**, **2** and **3**, a preculture was grown for 1 – 3 days and was subsequently used to inoculate the production culture. Large-scale cultivations were set up either in a 3 L bioreactor or in multiple Erlenmeyer flasks incubated on a reciprocal shaker.

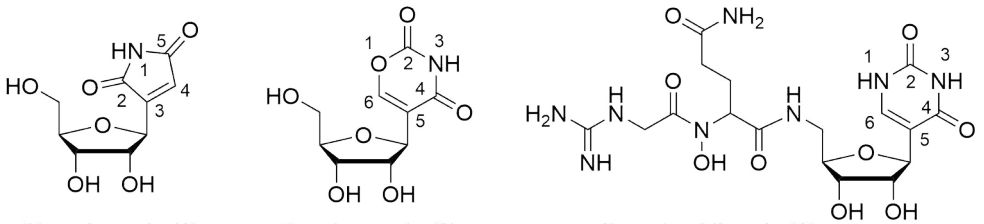


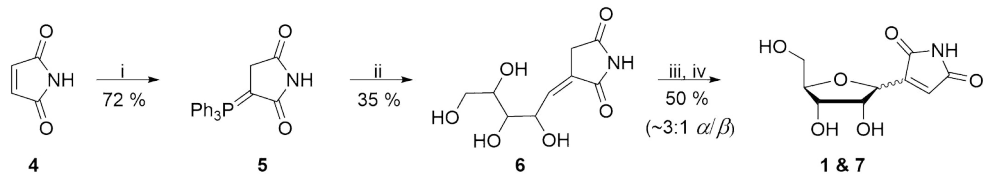
Figure 10. Structures of the isolated C-nucleoside secondary metabolites.

The formation of **1** in the cultures was monitored by a colorimetric assay based on the reactivity of the maleimide moiety with sulfhydryls. In this assay, a known excess of glutathione (GSH) was first used to consume **1**, and the remaining GSH could then be detected by treating it with Elman's reagent (5,5′-dithiobis(2-nitrobenzoic acid)) (DTNB). The amount of DTNB-GSH derivative was measured spectrophotometrically at 412 nm. The amount of **1** that had been consumed by GSH in the equimolar reaction could then be calculated using a molar extinction coefficient 14 150 M^{-1} cm^{-1}.[270] In the case of cultures incubated in Erlenmeyer flasks

on a reciprocal shaker the peak concentration was observed approximately at 20 hours from inoculation, after which the concentration began to steadily decrease. In a bioreactor, the level of **1** in the broth did not follow this rule as strictly and the maximum concentration was usually obtained in one to three days.

The completed fermentation mixtures were centrifuged to remove the bacterial mycelia and the compounds in the supernatants were adsorbed to activated charcoal. The crude materials of compounds **1** and **2** were easily extracted from the respective charcoal batches with a mixture of acetone and water. Compound **3** was more tightly bound to the charcoal and required extensive extraction with the water-acetone mixture. Charcoal extraction has been reported previously in cases of **1** and **2**.[154,269] The isolation of compound **3** has previously been carried out by direct fractioning on a column of ion exchange resin[9] or by binding as a heptanesulfonate ion pair on an Amberlite XAD-16 resin.[133] The crude materials of compounds **1**, **2** and **3** were purified by column chromatography. For **1**, normal phase silica column chromatography was sufficient and **2** was more easily purified by a short reverse phase silica column. The crude material of **3** was first fractioned on a reverse phase silica column and further purified by semipreparative HPLC.

1 was also prepared synthetically from maleimide and D-ribose by a method where the *C*-glycosidic bond is formed in a Wittig reaction.[165,271] (Scheme 15). Maleimide (**4**) and triphenylphosphine were refluxed in glacial acetic acid affording the triphenylphosphoranylidene succinimide (**5**). Wittig reaction between the phosphoranylidene and D-ribose yielded the (*E*)-3-(2,3,4,5-tetrahydroxypentylidene)pyrrolidine-2,5-dione (**6**) which was then subjected to selenoxide elimination affording the desired anomer **1** and 1′-*epi*-showdomycin (**7**) in respective 1:3 ratio.



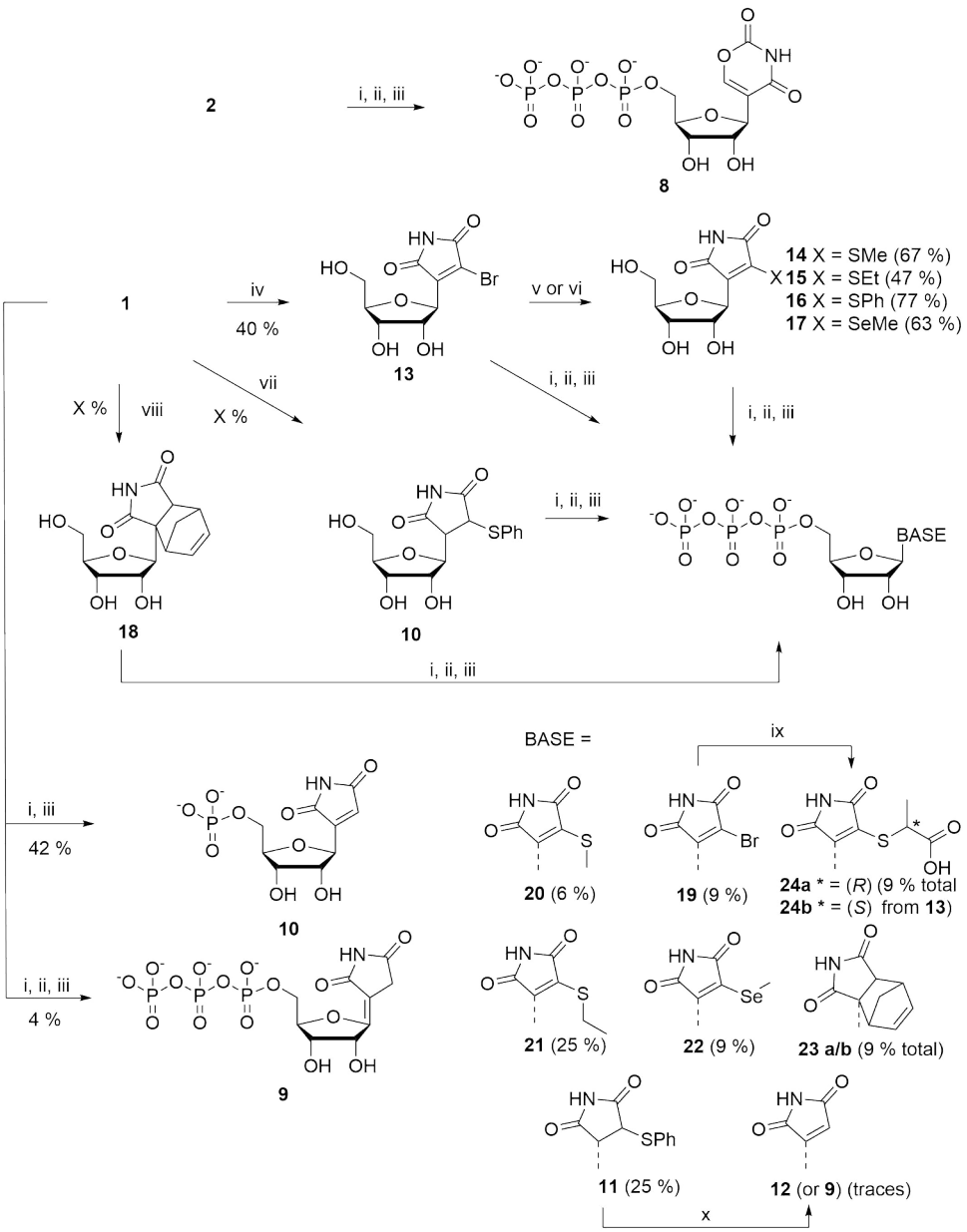
Scheme 15. Reagents and conditions: (i) PPh_3, AcOH, 100 °C, 30min; (ii) D-ribose, THF, 80 °C, 210 h; (iii) PhSeCl, MeCN, 65 °C, 29 h; (iv) 10 % H_2O_2, RT, 1 h.

Comparing the methods of showdomycin production described above, namely the bacterial cultivation and the chemical synthesis, both have some advantages to them. In general, the biosynthetic production is advantageous for complex products that would otherwise require often laborious multi-step syntheses with low yields. Here, the chemical synthesis of **1** comprises only four steps (in 3 bottles) and none of the steps are especially sensitive and can be reproduced reliably. Therefore, both

methods (synthesis and bacterial cultivation) were found to require similar amounts of effort in a small laboratory-scale production. However, the fermentation method may be more attractive for industrial applications, especially in terms of green chemistry. Considering the downstream processing, the synthetically obtained epimeric α/β-mixture with a modest total yield of the desired β-isomer (5 %) may also require additional HPLC purification to obtain the pure product. The cultivation produces only the desired β-anomer, which facilitates chromatographic purification. Furthermore, **1** can be obtained by the fermentation method slightly faster, provided that the method is already established and optimized. In addition, a lower amount of overall toxic waste is produced in contrast to the synthetic method. However, the bacterial cultures are sensitive towards contaminations as well as towards slightly altered conditions such as different brands of ingredient in the culture medium. Here, the costs of showdomycin synthesis on small (one gram) scale were approximately 3.5 times higher compared to isolation from bacterial cultures in regards to the required reagents and solvents.

3.2 Chemical transformations of the natural C-nucleosides

The C-nucleosides were converted to the 5′-triphosphates by the Yoshikawa-Ludwig phosphorylation method.[272,273] Initial treatment of the nucleosides with phosphoryl chloride produces the phosphorodichloridate intermediate which is subsequently treated with a nucleophilic pyrophosphate salt to afford the triphosphate. Hereby, the oxazinomycin-5′-triphosphate (**8**) was obtained after HPLC purification in 14 % yield (Scheme 16). However, the triphosphate of **1** was not obtained as expected. Instead the structure had isomerized around the *C*-glycosidic bond yielding the isoshowdomycin-5′-triphosphate (**9**). Similar transformation has been reported to occur in the presence methanolic triethylamine or enzymatically by *Streptomyces* sp. No 383.[274] However, showdomycin-5′-monophosphate (**10)** that was synthetized also by Yoshikawa phosphorylation, retained its anomeric integrity. Therefore, under the assumption that the isomerization into **9** occurs during the pyrophosphate treatment, different reagents were tried. The base, tributylamine, was exchanged either with diisopropylethyl amine or 2,4,6-trimethylpyridine. Additionally, in place of the initially used tris(tetrabutylammmonium hydrogen pyrophosphate salt, tris{bis(triphenylphosphoranylidene) ammonium} (PPN) pyrophosphate was tried.[275] Unfortunately, these changes in conditions did not prevent the isomerization.



Scheme 16. Chemical transformations of showdomycin (**1**) and oxazinomycin (**2**). Reagents and conditions: (i) $POCl_3$, 2,4,6-trimethylpyridine, triethylphosphate, - 10 ° C – 4 ° C, 1.5 – 20 h; (ii) tris(tetrabutylammonium) hydrogen pyrophosphate, tributylamine, 0 ° C – RT, 20 h; (iii) 0.05 M triethylammonium acetate, $CHCl_3$, RT, 30 min; (iv) Br_2, Fe (s), H_2O, RT, 72 h; (v) MeSNa or EtSH or PhSH, DIEA, DMF, RT, 4 – 20 h; (vi) MeSeSeMe, $NaBH_4$, EtOH/DMF, 0 °C – RT, 40 min; (vii) PhSH, DMF, 10 min, RT; (viii) cyclopentadiene, THF, 16 h, RT; (ix) thiolactic acid, DIEA, DMSO, RT, 4 h; (x) *N*-methylmaleimide, DMSO, 40 °C, 72 h.

1 reacts rapidly with sulfhydryls affording succinimide derivatives such as 1′-(4-phenylthiosuccinyl)riboside (**10**) that, in theory, could regenerate **1** through a retro Michael addition. This possibility was utilized in an attempt to produce unaltered showdomycin-5′-triphosphate from the triphosphate of **10** (**11)** in a retro Michael addition in the presence of excess *N*-methylmaleimide that would trap the eliminated thiophenol. Unfortunately, only traces of a possible product **12** were observed on LC-MS while degradation of the nucleotides predominated.

Other derivatives of **1** were synthesized *via* initial bromination of the maleimide base, carried out in saturated aqueous solution of bromine (Br_2) in the presence of a catalytic amount of *in situ* generated iron(III) bromide ($FeBr_3$) (Scheme 16). The iron catalyst efficiently reduced reaction time and the formation of side products. Treating the product, 4-bromoshowdomycin (**13**) with sodium thiomethoxide, ethanethiol or thiophenol in presence of a base afforded the 4-(methylthio)showdomycin (**14**), the 4-(ethylthio)showdomycin (**15**) and the 4-(phenylthio)showdomycin (**16**), respectively. Similar treatment with methylselenol, generated by reduction of dimethyl diselenide with $NaBH_4$, gave the 4-(methylseleno)showdomycin (**17**).

Diels-Alder addition is another reaction that can be applied to maleimides. Protection of the maleimide functionality as a 2,5-dimethylfuran-maleimido Diels-Alder adduct has been utilized for oligonucleotide conjugation purposes.[276] This protection can be removed by heating at 90 °C for 3 – 4 hours. The suitability of such strategy was examined for protection of **1**.

Diels-Alder reactions of **1** with cyclopentadiene and furan were attempted. The cyclic diene can approach the maleimide ring of **1** from each side in two orientations (exo/endo), therefore generating four stereo isomers. Cyclopentadiene readily reacted at room temperature producing mainly two of the stereo isomers (**18**), and the other two isomers in small quantities. With furan, the reaction did not proceed as desired. The Diels-Alder adduct **18** proved surprisingly stable and, unfortunately, the maleimide moiety could not be regenerated as desired at appropriate temperatures.

The derivatives of **1** modified at the C4 position were mostly stable under the phosphorylation conditions affording the nucleotides **19** – **24**. However, the 4-bromoshowdomycin-5′-triphosphate (**19**) was not stable towards buffered solutions used in HPLC purification. Therefore, the crude material **19** obtained by precipitation as a sodium salt from acetone was treated with pyrophosphatase in order to remove the pyrophosphate impurity that would otherwise interfere with the RNAP assays. Also, the triphosphate of the 4-(phenylthio)showdomycin was unstable and degraded in aqueous solutions during and after the HPLC purification. The4-(methylthio)showdomycin-5′-triphosphate (**20**), the 4-(ethylthio)-showdomycin-5′-triphosphate (**21**) and the 4-(methylseleno)showdomycin-5′-triphosphate (**22**) were obtained as stable triethylammonium or sodium salts.

Likewise, the triphosphate of the cyclopentadiene modified showdomycin was obtained as two main stereo isomers **23a** and **23b**.

The bromine at C4 position could be substituted also after the phosphorylation. Different functionalities can be thus introduced that would otherwise interfere with the phosphorylation step. Accordingly, the thiolactic acid modified showdomycin 5´-triphosphate isomers **24a** and **24b** were prepared by treating crude triphosphate **19** with a racemic mixture of (2*S*)- and (2*R*)-thiolactic acid and subsequently isolating the isomers by RP-HPLC. The isomers were identified by HPLC using a reference sample of pure 4-((1*S*)-1-carboxyethyl)thio)showdomycin-5´-triphosphate (**24b**) prepared on small scale with enantiopure (2*S*)-thiolactic acid (Figure 11).

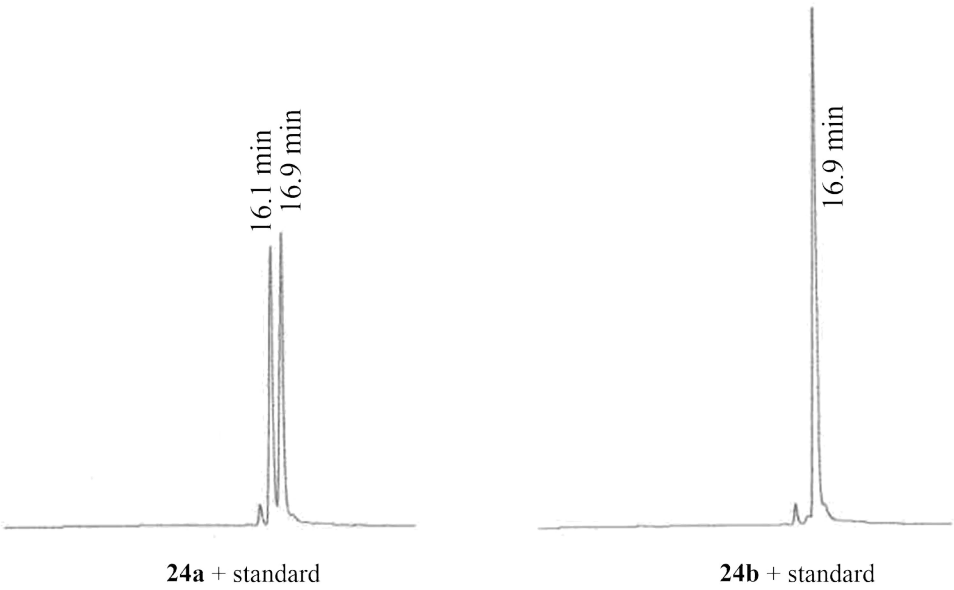
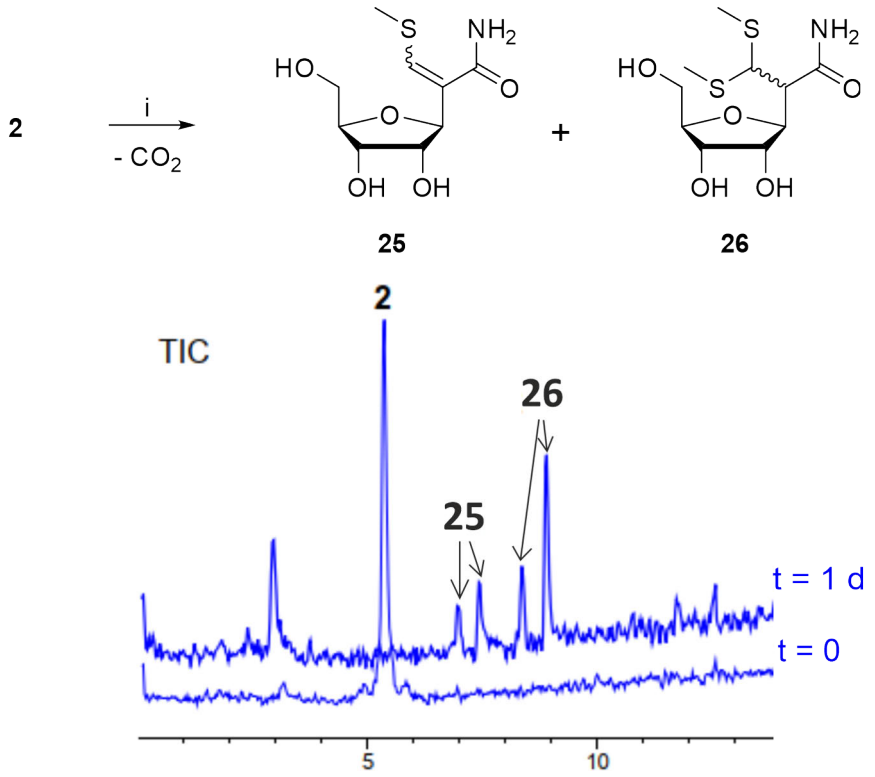


Figure 11. HPLC chromatograms with samples of isolated isomers **24a** and **24b** spiked with an enantiopure standard of 4-(((1*S*)-1-carboxyethyl)thio)showdomycin-5´-triphosphate.

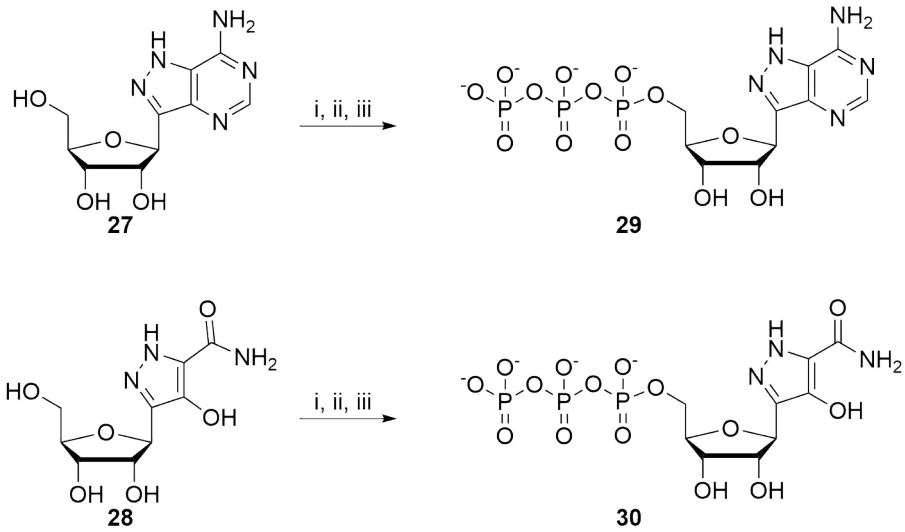
Reactivity with thiols was also observed with compound **2** which underwent Michael addition in the presence of a thiolate. Treatment with sodium thiomethoxide with simultaneous decarboxylation yielded isomeric mixtures of products with m/z values corresponding to the structures **25** and **26** as observed on LC-MS analysis (Scheme 17). With less nucleophilic thiols, analogous reactions were not observed. Similar reactivity has also been reported before between **2** and thioglycerol, and the covalent trapping of sulfhydryls has been speculated as a possible source of the antibiotic activity.[155]



Scheme 17. Reaction of oxazinomycin (**2**) with sodium thiomethoxide produced compounds with m/z values corresponding to the expected structures **25** and **26** observed on the LC-MS total ion chromatogram (TIC). Reagents and conditions: (i) sodium thiomethoxide (excess), DMF, RT, 1 d.

Because formycin A (**27**) and pyrazofurin A (**28**) were not detected in the bacterial cultures here, commercial samples were obtained instead. When subjected to phosphorylation by the Yoshikawa-Ludwig protocol[273], the formycin A 5′-triphosphate (**29**) was produced in 30 % yield, and the pyrazofurin A 5′-triphosphate (**30**) was afforded in 9 % yield (Scheme 18). Pyrazofurin 5′-triphosphate has been previously prepared by treating 2′,3′-*O*-isopropylidene protected pyrazofurin A with dibenzyl *N*,*N*-diisopropylphosphoramidite with concurrent phosphorylation of the 5′ and 4-hydroxyls.[163] Then, oxidation of the 5′-phosphorus by hydrogen peroxide simultaneously removed the phosphityl from the the 4-hydroxyl. Removal of the isopropylidene protecting group, activation of the 5′-phosphate with piperidine and introduction of pyrophosphate gave the 5′-triphosphate in overall 34 % yield.[163] Although the yield in the Yoshikawa-Ludwig protocol was lower in comparison, the overall simplicity of this one-pot protocol makes it an appealing alternative. As mentioned before, pyrazofurin is prone to anomerization in aqueous solutions (see

section 1.3.5).[3,163] The anomerization was also observed with **30** dissolved in D_2O during NMR measurements (β/α = 10:1 after one night).



27 **29** **28** **30**

Scheme 18. Synthesis of formycin A 5′-triphosphate (**29**) and pyrazofurin A 5′-triphosphate (**30**). Reagents and conditions: (i) $POCl_3$, 2,4,6-trimethylpyridine, triethylphosphate, -10 °C – 4 °C, 20 h; (ii) tris(tetrabutylammonium) hydrogen pyrophosphate, tributylamine, 0 °C – RT, 20 h; (iii) 0.05 M triethylammonium acetate, $CHCl_3$, RT, 30 min;

3.3 Reassigning strepturidine as pseudouridine

An isolated compound from fermentation broth of *Streptomyces albus* DSM 40763 culture, presumed to be strepturidin (**31**) at first, was characterized as pseudouridine (**3**) (Figure 12). Both structures **3** and **31** have the same empirical formula, and thus they could not be distinguished by m/z value. The pseudouridimycin structure consists of a guanidylated and N-hydroxylated glycine-glutamine moiety linked to 5′-aminopseudouridine. Compared to **3**, the structure of **31** is different by the acyclic carbohydrate and in addition, the atoms of the guanidylated glycine of **3** are rearranged as a cyclic moiety linked to the C2′ position in **31**. In theory, the long-range ^{1}H-^{13}C NMR correlations could provide information about the adjacent moieties. In the case of the isolated compound such a correlation was not revealed by the ^{1}H-^{13}C-HMBC experiment. However, the same bacterial culture contained the deoxy-analogue **32**, that could be also generated from **3** by a reduction with titanium(III) chloride.[9] Fortunately, the structure of **32** could be reliably assigned by NMR spectroscopy, which revealed the coupling between the glycine and

glutamine moieties as depicted in Figure 12. Furthermore, MS/MS-analysis on compound **3** showed similar fragmentation patterns reported for **31**[133], supporting the deduction that **3** and strepturidin are actually the same compound.

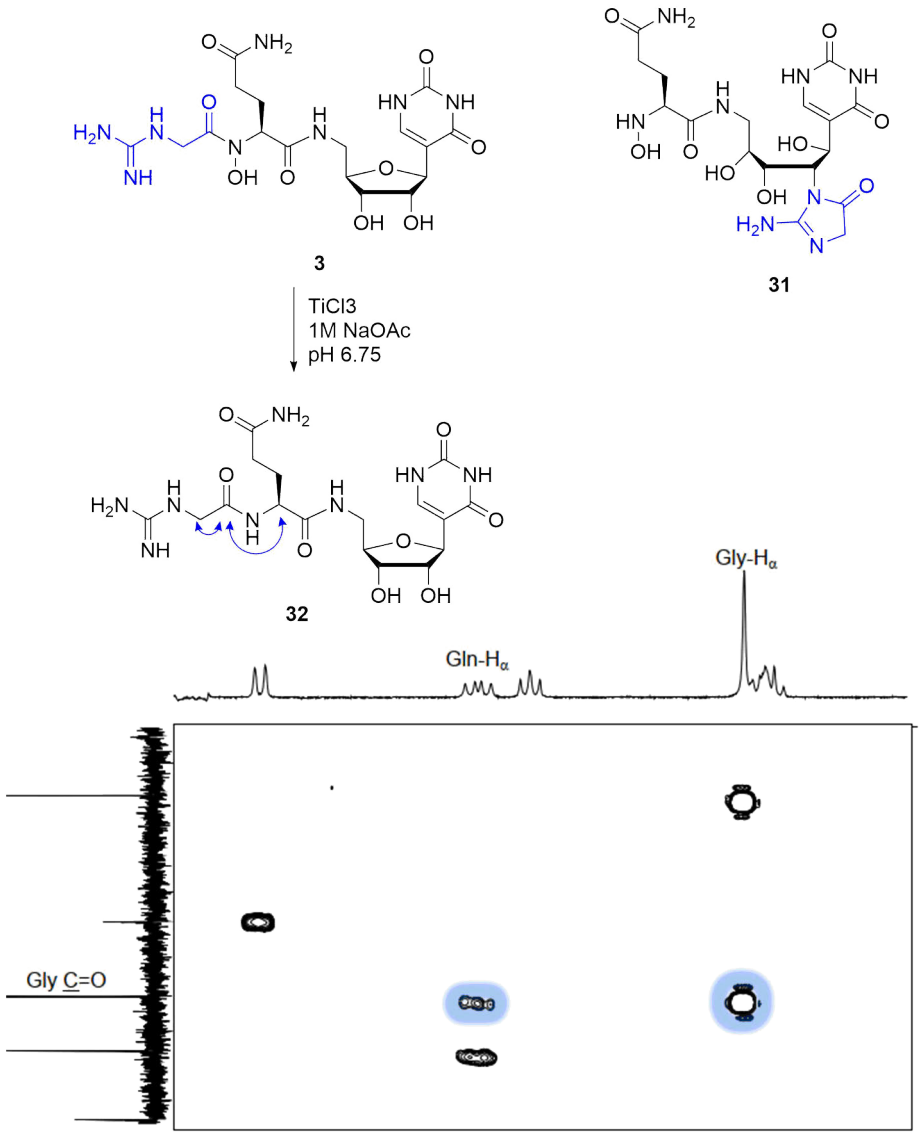


FIgure 12. The structural differences of pseudoridimycin (**3**) and strepturidine (**31**), and the reduction of **3** to desoxy-pseudouridimycin (**32**). Blue arrows indicate the key correlations highlighted on the 1H-13C-HMBC spectrum of **32**.

3.4 RNA polymerase assays

The prepared nucleosides triphosphates **9**, **19** – **24** as well as compound **3** were subjected to polymerase activity assays with the human mitochondrial RNAP (*Hsa* MT RNAP) and the bacterial and eukaryotic multisubunit polymerases from *Escherichia coli* (*Eco* RNAP) and *Saccharomyces cerevisiae* (*Sce* POL II), respectively. The RNAP activities of the triphosphates **29** and **30** are being studied at the time of writing and the results will be published in the near future.

The activity of **3** against RNAPs is well characterized and reported by Maffioli *et al*.[9] Here, the RNAP assays with **3** supported the conclusion that the compound obtained was indeed **3** instead of the previously proposed strepturidine (**31**). In agreement with previously reported results, **3** was found to have specificity towards the bacterial RNAP and to inhibit the incorporation of UTP. Equal level of inhibition of the eukaryotic multisubunit RNAP required 100-fold concentration of **3**. As expected, the processive transcription by *Eco* RNAP in the presence of **3** was strongly paused one base pair upstream from locations coding for uridine incorporation.

3.4.1 Bacterial RNA polymerase

To better understand the discussion and terminology related to the RNAP acitivity assays below, the structure and basic mechanisms related to RNAPs are briefly explained here. The focus is on the bacterial RNAP which is the simplest multi-subunit polymerase comprising the five core subunits (ααββ′ω).[19,277] Eukaryotic RNAPs Pol I, Pol II and Pol III contain a total of 14, 12 and 17 subunits respectively, including the five homologous core units. In contrast, the eukaryotic mitochondrial RNAP and the viral RNAPs are single subunit polymerases that are homologous to DNA polymerases.[278]

The core regions of the multi-subunit RNAPs feature the main channel, which accommodates the DNA template, and the secondary channel that acts as an entrance for the nucleotide substrates (Figure 13a).[19,277] The nascent RNA is ejected through the RNA exit channel. The active center is located around the middle of the main channel where the binding site ′i′ accommodates the 3′-end of the nascent RNA and the ′i+1′ site binds the incoming NTP substrates. The template DNA (tDNA) forms a 9-10 unit long RNA-DNA hybrid upstream from the active center and a duplex with the non-template DNA (ntDNA) downstream.

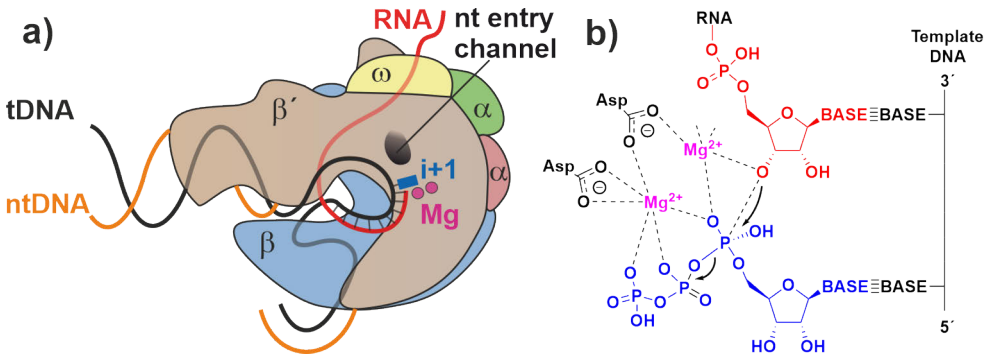


Figure 13. **a)** A simplified structure of the ααββ´ω multisubunit RNAP elongation complex, and **b)** the proposed catalytic mechanism of the phosphodiester formation.

The RNAPs conduct the transcription of genetic information into the various types of coding and non-coding RNAs. The transcription event starts by initiation, where the RNAP binds to the promoter region of a DNA template strand, partially opening the double helix.[279] The transcription elongation complex (TEC) that minimally comprises the RNAP, template DNA, and the nascent RNA strand, is formed after a conformational change that follows a successful synthesis of about 12 nucleotide long RNA. The RNAP binds ribonucleoside-5´-triphosphates to the active site based on the base complementarity with the template DNA. The elongation of the RNA happens by formation of a phosphodiester bond between the 3´-hydroxyl of the nascent RNA and 5´-hydroxyl of the substrate NTP with a concurrent release of a pyrophosphate. The reaction is catalyzed by one or two magnesium ions that stabilize the negative charges of the transition state (Figure 13b).[279,280] It has been proposed that one Mg^{2+} coordinates the 3´-hydroxyl attacking the α-phosphate while the other Mg^{2+} promotes the cleavage of the detaching pyrophosphate.[280] Alternatively, the 3´-hydroxyl may be coordinated and deprotonated by an arginine residue in the catalytic center.[279] The RNAP then translocates along the nucleic acids by ratchet-like one-step movements where the incorporated nucleotide moves away from the active site and the next unit of the tDNA is positioned to accommodate a new NTP into the ´i+1´ site (Figure 14). The TEC may also backtrack (reverse translocation) along the DNA, which is often caused by paused elongation process. During bactracking the nascent RNA extrudes into the secondary channel by one or more units.[277] Backtracking by a few nucleotides is reversible but the TEC becomes arrested by longer backtracking. In the latter case, RNA cleavage factors such as GreA and GreB are required to restore the catalytic ability of the TEC by cleaving the extruded RNA 3´-end in the secondary channel.[277] The transcription normally ends by a termination event where the elongation complex dissociates.

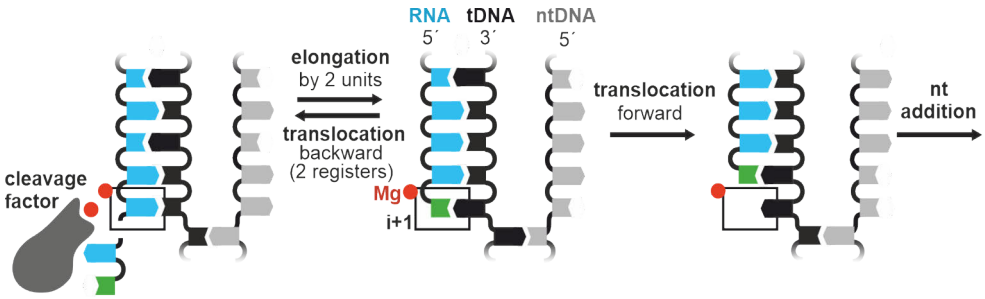


Figure 14. Forward and backward translocation and release of the backtracked RNA by a cleavage factor.

3.4.2 RNAP acitivity of oxazinomycin-5´-triphosphate

The nucleotide **8** was studied as a substrate for RNA polymerases in comparison to the natural substrates (ATP, UTP, CTP and GTP) and pseudouridine-5´-triphosphate (PSU-TP). The experiments are explained in detail in the original pubilication III. **8** was utilized mainly in place of UTP by all tested polymerases (Figure 15). The change in mobility of RNA on polyacrylamide gel electrophoresis (PAGE) upon incorporation of oxazinomycin-5´-monophosphate (**2**-MP) was low compared to that of RNA extended by UMP or PSU-MP. However, the subsequent GTP incorporation resulted in normal changes in electrophoretic mobility as can be observed on the PAGE gels (Figure 15b). **8** could also efficiently substitute UTP in processive transcript elongation by *Eco* RNAP (Figure 15c).

The affinity of **8** for *Eco* RNAP was determined to be equal to UTP based on a fluorescence translocation assay in a stopped flow instument. PSU-TP was also found to be incorporated and translocated in equal rates as UTP although binding to the active site 2-fold faster. In addition, the incorporations of next nucleotide (GMP), following the incorporation of **2**-MP, UMP or PSU-MP, occurred at similar rates.

The incorporation of **2**-MP by the *Eco* RNAP was found to cause an incomplete forward translocation. This translocation bias was deduced to be caused by increased susceptiblity of the elongation complex to enter into a backtracked state. The reasoning was supported by the fact that the GreA-facilitated RNA cleavage was 10-fold higher in case of a complex elongated by **2**-MP + GMP compared to elongation by UMP + GMP.

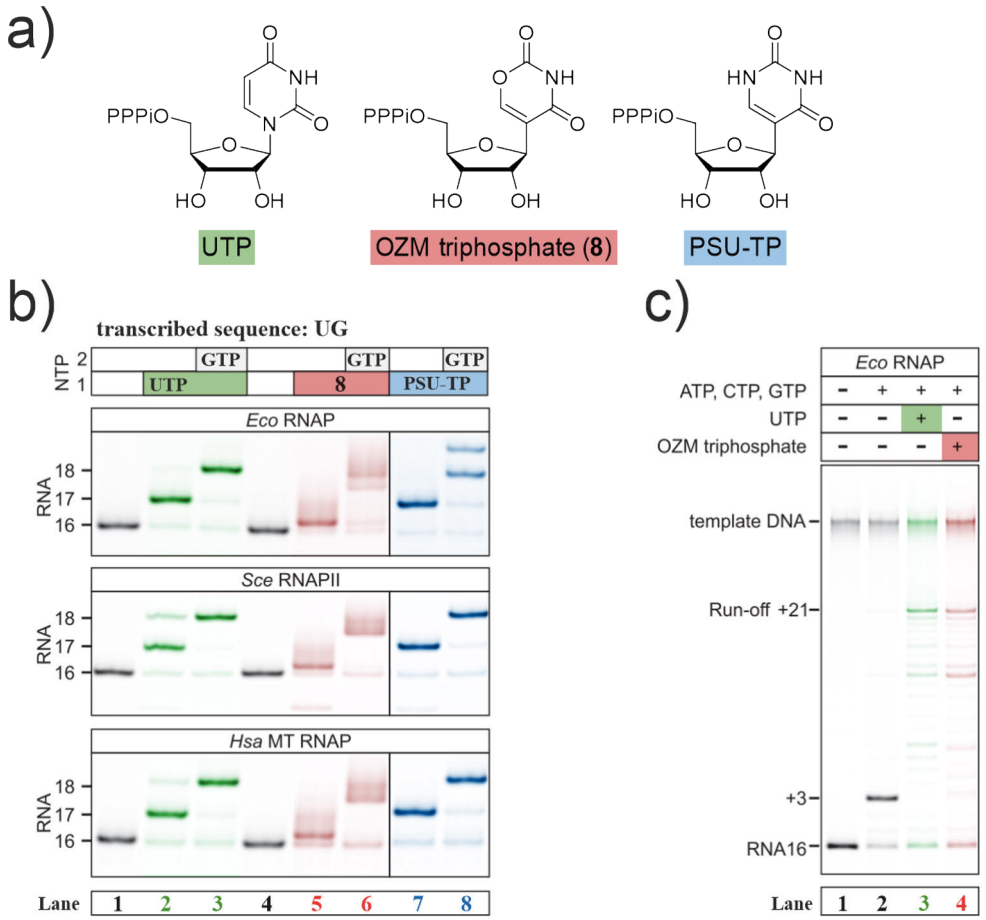


Figure 15. Incorporation of oxazinomycin-5´-monophosphate by RNAPs. **a)** The structures of UTP, **8** and PSU-TP for comparison, **b)** PAGE gels showing chain elongation by U+G (lanes 2 and 3, green highlight), OZM+G (lanes 5 and 6, red highlight) and PSU +G (lanes 7 and 8, blue highlight). **c)** Processive transcript elongation with RNA16 primer (lane 1) with all natural substrates (lane 3, green highlight) or ATP, CTP, GTP and **8** (lane 4, red highlight). Control experiment with only ATP, CTP and GTP (lane 2) halted the transcription at position coding for UTP addition (+3).

In a few specific cases, such as with a consensus-pause-like sequence that is more prone to backtrack, incorporation of **2**-MP fractionally arrested the transcript elongation (~20 % arrested at position of incorporation) in contrast to UMP (~6 % arrested). Transcriptional arrests caused by **8** were also observed with polythymidine sequences (2, 3, 4 or 7 thymidines) coding for multiple successive UMP additions (Figure 16). The fractional arrest at the polythymidine tracts were 2-fold higher with OZM-NTP mixture (**8**, ATP, CTP and GTP) compared to UTP or PSU-TP mixtures. In this case the GreA was unable to release the arrested fraction in the presence of **8.** However, when **8** was removed and substituted with UTP, the arrested complex was

released in the presence of GreA and the elongation process continued normally to full-length transcripts. The eukaryotic *Sce* RNAPII was also arrested by **8** in polythymidine sequences. The mithochondrial *Hsa* MT RNAP on the other hand showed less evident accumulation of RNA at the polythymidine tract although longer RNA transcripts were not visible on the gel either. However, longer RNAs containing oxazinomycin units were observed to poorly enter the PAGE gels which might explain why no long transcripts or arrested RNAs were detected.

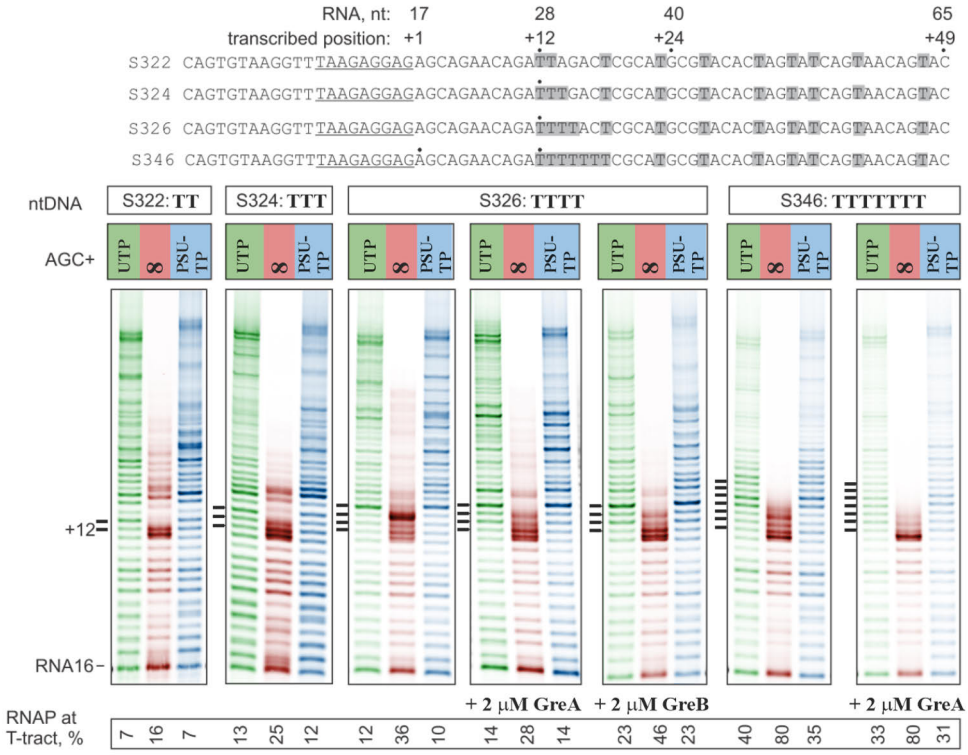


Figure 16. PAGE gel panels showing the effects of UTP (green), **8** (red), and PSU-TP (blue) on transcript elongation in the context of polythymidine seqeunces.

The RNAP activity of PSU-TP was nearly equal to the natural substrate in these experiments. Therefore, the *C*-glycosidic bond is not the sole factor contributing to the observed behaviour of **8**. Speculatively, the base ring oxygen, which distinguishes **8** from both the UTP and PSU-TP, could act as an additional hydrogen bond acceptor. Alternatively, the free electron pair of the base ring oxygen in **2** could participate in abnormal resonance structures. The incorporation of a **2**-MP would then distort the conformation at the RNA 3′-end, which could facilitate the backtrack motion of the transcription elongation complex and furthermore, cause the abnormal

behaviour of longer oxazinomycin containing RNAs with PAGE gels. The moderate reactivity of **2** with sulfhydryls (see Scheme 17) was not likely affecting the activity of **8** as substrate for RNAPs.

3.4.3 Biological acitivities of showdomycin analogues

In the RNAP assays, the showdomycin derivatives were incorporated to the nascent RNA as uridine mimics (Figure 17). However, the analogues derivatized with thiolactic acid (**24a/b**) or cyclopentadiene (**23a/b**) were likely not able to bind to the nucleotide addition site with notable affinity although some additonal PAGE gel bands were observed in the case of **24a**/**b** and *Eco* RNAP. Binding of the derivatives with smaller modification (MeS, EtS, MeSe or Br) (**19** – **22**) to ´i+1´ site and subsequent incorporation of the corresponding nucleoside-5´-monophosphates by the multi-subunit polymerases inhibited the elongation by the next incoming nucleotide. Additionally, the observed affinity of triphosphates **19** – **22** towards bacterial *Eco* RNAP was slightly higher than towards the eukaryotic *Sce* RNAPII. The most noticeable effect was induced by the 4-(ethylthio)showdomycin-5´-triphosphate (**21**) (see lanes highlighed with red in Figure 17). Although the derivatives were also incorporated by the single-subunit polymerase (*Hsa* MT RNAP), inhibition of further elongation was not apparent.

The prepared nucleotides (in 2 mM concentration) were also tested for inhibition activity in competition against all four natural ribonucleotides (100 µM each) in case of processive trancript elongation using a DNA template containing a polythymidine sequence (7T). Again, the derivatives with smaller modifications fractionally paused the elongation by multi-subunit polymerases at the 7T sequences whereas the single-subunit polymerase was not measurably inhibited. Within the experiment timeframe, the best inhibitor **21** increased the amount of paused (*Eco* RNAP) TEC fractions at the 7T tract nearly 3-fold compared to a control experiment. The inhbition was most likely caused by a stalled transcription after incorporation of the showdomycin derivatives, whereas chain termination with these compounds was not observed.

The antibacterial activity of 4-(ethylthio)showdomycin (**15)** against *Escherichia coli* was also studied and compared to that of showdomycin (**1**). The experiment was carried out by using bioluminescent *E. coli* cells expressing modified luciferace *luxABCDE* genes, which enables the monitoring of the metabolic activity and redox state of cells by measuring the bioluminence signal (counts per second, CPS).[281] In addition, the cell population growth could be followed with optical density (OD_{600}) measurements.[281] According to the OD_{600} curves, **15** acted as an antibiotic although with 4-fold lower efficacy compared to **1** (Figure 18, top row). The bioluminescence signal curves indicated that **1** caused a prompt decline in metabolic activity at 0.625 mM concentration whereas 2.5 mM of **15** was required for comparable effect.

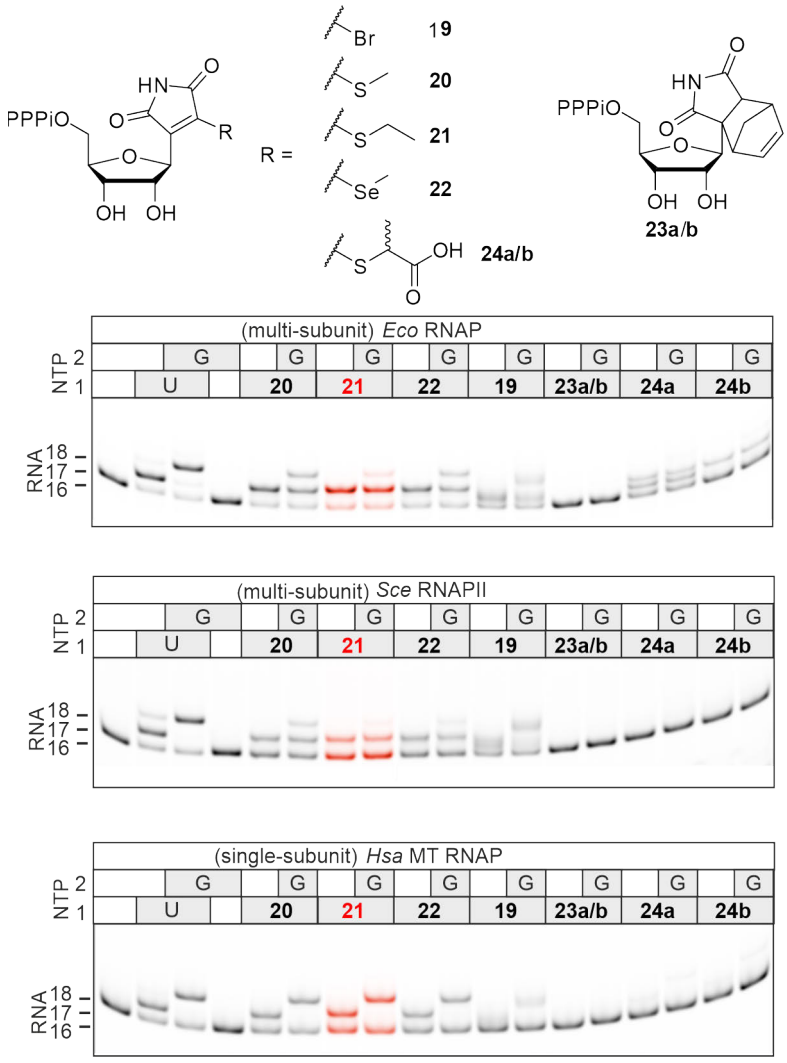


Figure 17. PAGE gel panels depicting the elongation of RNA by uridine (U) or shodomycin analogues followed by guanosine (G) by the bacterial (*Eco* RNAP), eukaryotic (*Sce* RNAPII) and human mitochondrial (*Hsa* MT RNAP) polymerases.

In contrast, apramycin inhibited the growth effectively at ≥ 40 μM concentrations while the efficacy of pseudouridimycin (**3**) was around the same order of magnitude as **1** and **15**. However, the different shapes of the bioluminescence signal curves caused by the tested compounds suggest different modes of action.

The activity of **1** is known to be reversed by the presence of other nucleosides,[282] that compete for entry into cells by the membrane nucleoside transporters. The experiment measuring the antibacterial activity was repeated with uridine added in the mixture. The presence of uridine diminished the antimicrobial activity of **1**

whereas **15** was mostly unaffected (Figure 18, bottom row). Therefore, the mechanism of entry of **15** into cells is not in competion with uridine. The reactivity of the maleimide moiety that is a significant contributor to the activity of **1**, is likely moderated in **15** by the ethylthio group located at the site of alkylation. However, it is unknown whether the reactivity of **15** is still biologically relevant or whether the ethylthio group affects the mechanism by other types of interactions. Regarding the RNAP activity of the triphosphate **21**, the phosphorylation of **15** to **21** by kinases was not studied and therefore, it cannot be confidently concluded whether the inhibiton of RNAP contributes to the observed antimicrobial activity.

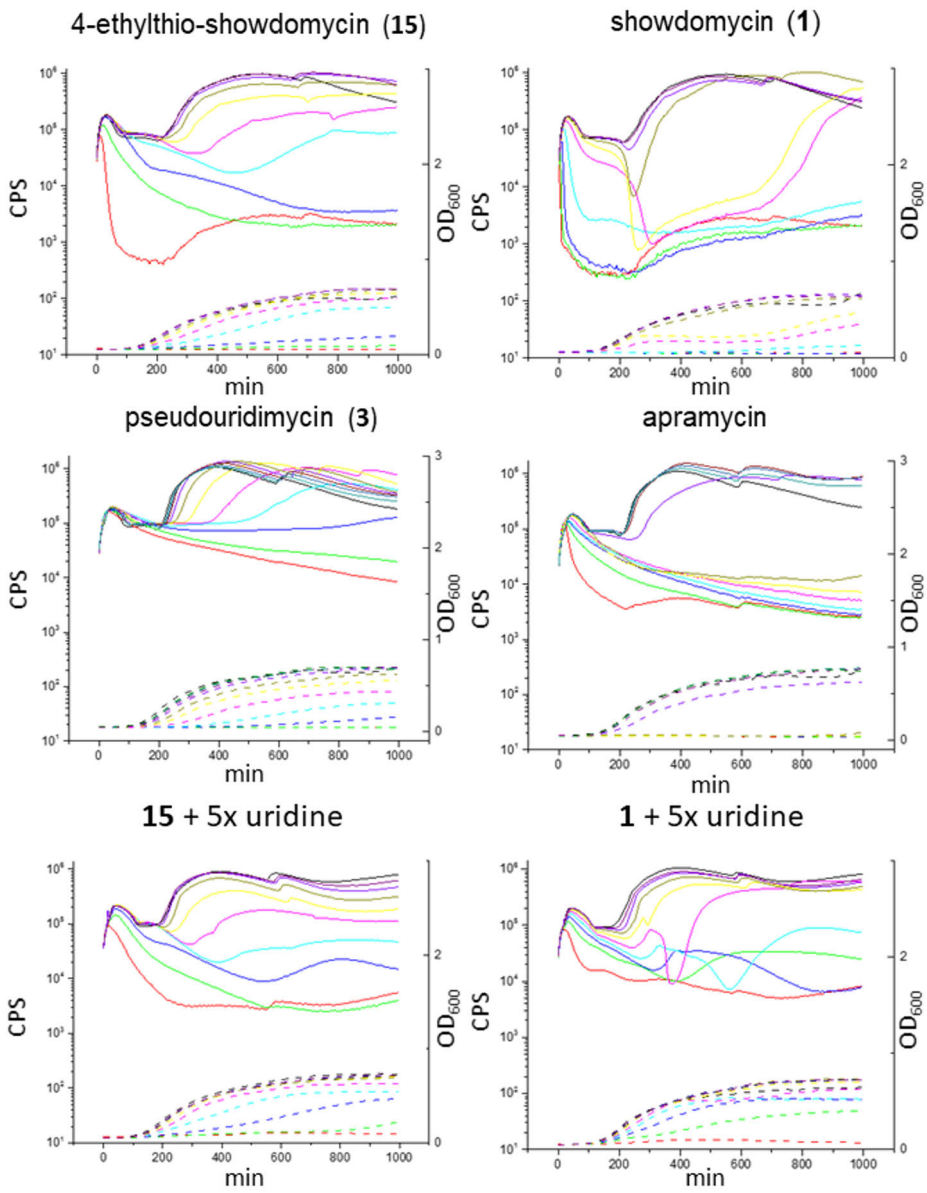


Figure 18. Antibiotic acitivities of **15**, **1**, **3**, apramycin, as well as of **15** and **1** in presence of 5 equivalents uridine. Solid line: luminescence in counts per second (CPS) and dotted line: optical density at 600 nm (OD_{600}). Black: control, no analytic added, red: 2.5 mM, green: 1.25 mM, blue: 0.625 mM, cyan: 0.3125 mM, pink: 0.156 mM, yellow: 0.078 mM, dark yellow: 0.039 mM, violet: 0.020 mM, Wine: 0.010 mM, dark cyan: 0.005 mM, olive: 0,002 mM compound analysed.

4 Summary

A combination of biosynthesis and chemical synthesis was utilized to produce a small library of C-nucleoside 5′-triphosphates for RNA polymerase assays. The natural C-nucleosides showdomycin (**1**) and oxazinomycin (**2**) were obtained from cultures of *Streptomyces showdoensis* and *Streptomyces hygroscopicus*, respectively. In addition, a C-nucleoside isolated from *Streptomyces albus* cultures was identified as pseudouridimycin. The structure of strepturidin, that was initially expected to be produced by *S. albus*, was consequently reassigned to match the structure of pseudouridimycin due to identical characteristic data. The C-nucleoside 5′-triphosphates were prepared by the Yoshikawa-Ludwig phosphorylation method. Under these conditions showdomycin concurrently isomerized affording the isoshowdomycin-5′-triphosphate. Most of the C4-modified showdomycin analogues were stable for phosphorylation and eventually a small library of eight showdomycin derivatives was obtained for RNA polymerase activity assays. The 5′-triphosphates of oxazinomycin, formycin A and pyrazofurin A were prepared similarly.

Transcriptional effects of the produced compounds were assessed against bacterial, eukaryotic and mitochondrial RNA polymerases. The 5′-triphosphates of formycin A (**29**) and pyrazofurin A (**30**) are being tested at the time of writing and the results will be published in due course. Oxazinomycin-5′-triphosphate (**8**) was shown to be efficiently utilized as a substrate by RNAPs in place of UTP. However, incorporation of oxazinomycin-5′-monophosphate promoted backtracking of the RNAP complexes which resulted in partial transcriptional arrests in the cases of consensus-pause-type sequences and polythymidine tracts. Considering the antimicrobial activity of **2**, the results obtained here may suggest that RNAPs are within its target scope, provided that **2** is first phosphorylated by kinases. Although the instances where the incorporation of oxazinomycin-5′-monophosphate into RNA leads to inhibition are infrequent, incorporation of unnatural nucleotides often renders he RNA unfit for proper post-transcriptional function.

The 5′-triphosphates of the showdomycin analogues with 4-Br (**19**), 4-MeS (**20**), 4-EtS (**21**) and 4-MeSe (**22**) substituents acted as substrates for RNAPs being utilized as uridine mimics, although with lower affinity than the natural substrate UTP. The analogues modified with cyclopentadiene (**23a/b**) and thiolactic acid

(**24a/b**) were not able to bind to the nucleotide addition site with significant affinity. Following incorporation by multi-subunit RNAPs, the showdomycin analogues moderatly inhibited further elongation by the next nucleotide. This inhibitory activity was slightly more pronounced with the bacterial RNAP compared to the eukaryotic RNAP. On the other hand, the nucleotides **19** – **22** did not induce measurable inhibitory effect on the single-subunit mitochondrial RNAP.

One of the prepared derivatives, 4-(ethylthio)showdomycin (**15)**, was chosen for antimicrobial activity assays to examine the effects of the position 4 modification in comparison to the unmodified **1**. The test proved that the 4-ethylthio modification did not abolish the antibacterial activity against *E. coli* although it was 4-fold lower compared to **1**. On the other hand, the activity of **15** was resistant towards the presence of uridine in contrast to **1**. The reactivity of the maleimide base of **1** with thiols is a major contributor to its biological activity. Therefore, it was also deduced that the modification of the maleimide moiety possibly attenuated its reactivity which could explain the lowered activity of **15**. In case the reactivity was decreased below the level of biological relevance, the observed activity of **15** could be explained by an altered mechansim of action that would be based mainly on the nucleosidic character. Additionally, these modifications may increase the analogues resistance against early deactivation by physiological thiol compounds.

5 Experimental

5.1 General

The production of C-nucleoside compounds **1** – **17** and **19 – 23** are described in the original publications. The syntheses of compounds **1**, **5** – **7**, **18**, **23a/b**, **29** and **30** are described below. The bacterial cultures used in C-nucleoside productions were grown in Biostat B or Fermentec FMT ST series bioreactors. The LC-MS analyses were performed on Agilent 1260 Infinity Binary LC and Agilent 6100 Series Quadropole mass spectrometer with a Phenomemex 150 × 4.6 Synergi^{TM} 4 μm Fusion-RP 80 Å column, 0.5 ml min^{-1} flow rate, eluting with 0.1% formic acid in water for 5 min and then with a gradient of 0.1% formic acid in water/MeCN from 100:0 to 80:20 in 20 min. Compounds were characterized by ^{1}H NMR, ^{13}C NMR, ^{31}P NMR and HRMS when applicable. Following abbreviations are used in reporting NMR data: s, singlet; d, doublet; aapd, apparent doublet; dd, doublet of doublets; dt, doublet of triplets; t, triplet; appt, apparent triplet; q, quartet; appq, apparent quartet; m multiplet. Formycin A and Pyrazofurin A were purchased from MilliporeSigma. The molar amounts of nucleoside triphosphates were measured by quantitative NMR, aquiring ^{1}H-NMR spectra with relaxation delay (d1) of 30 s, and using CH_3 singlet peak of known amount of acetonitrile as an internal standard.

5.2 RNA polymerase assays

Experiments with RNA polymerases were performed at the Department of Life Technologies at University of Turku. The TEC assemblies and the *in vitro* transcription reactions are described in detail in the original publications. The results of RNAP assays with compounds **3** and **8** are described in the publications II and III, respectively. The assays with the showdomycin analogues (**19** – **24**) are described in publication IV.

5.3 Antibiotic activity measurement

Antimicrobial activity of the compounds was analysed by cultivating *Escherichia coli* K-12 carrying plasmid pEGFPluxABCDEamp (*E. coli*/p*lux*).[281] The plasmid

expresses modified *luxABCDE* genes and the growth was followed by measuring bioluminescense (counts per second) and OD_{600} using a Hidex Sense Plate Reader 425-301 (Hidex,Turku, Finland). Bacteria were first inoculated from a glycerol stock (0.2 % inocula) in LB and mixed with the analysed compound to final concentration of 2.5 – 0.0098 mM on 96 well plates (PerkinElmer 6005020) and incubated at 37 °C for 16 h in the luminometer used for measurements. The concentration of uracil used in assays was 12.5 – 0.0098 mM. The results of the assay are described in publication IV.

5.4 Synthesis of Showdomycin (**1**)

5.4.1 3-(Triphenylphosphoranylidene)-2,5-pyrrolidinedione (**5**)

The synthesis was carried out analogously to a reported method.[165,271] To a solution of maleimide (**4**) (6.0 g, 61.8 mmol) in glacial acetic acid (120 ml) was added triphenylphosphine (15.9 g, 60.6 mmol) and the resulting solution was refluxed at 100 °C for 30 min. The solution was cooled to room temperature and diethyl ether was added until a red amorphous precipitation was formed. The supernatant was decanted and the precipitation was washed with diethyl ether. The precipitated product **5** was dried under a vacuum and ground to a pale red powder (15.7 g, 72 %). ^{1}H NMR δ_H (500 MHz, $CDCl_3$): = 7.65 – 7.58 (m, 9H, Ph), 7.55 – 7.51 (m, 6H, Ph), 3.01 (d, J = 1.4 Hz, 2H, CH_2). ^{13}C NMR δ_C (125 MHz, $CDCl_3$): 177.9 (d, $J_{C\text{-}P}$ = 17.2 Hz, C=O), 172.2 (d, $J_{C\text{-}P}$ = 13.6 Hz, C=O), 133.4 (d, $J_{C\text{-}P}$ = 10.0 Hz, Ph), 133.4 (d, $J_{C\text{-}P}$ = 10.0 Hz, Ph) 132.9 (d, $J_{C\text{-}P}$ = 2.7 Hz, Ph), 129.3 (d, $J_{C\text{-}P}$ = 12.7 Hz, Ph), 124.9 (d, $J_{C\text{-}P}$ = 92.6 Hz, Ph), 38.4 (d, $J_{C\text{-}P}$ = 136.1 Hz, CH_2), 38.1 (d, $J_{C\text{-}P}$ = 9.9 Hz, CH_2). ^{31}P NMR δ_P (202 MHz, D_2O): 12,34 (s).

5.4.2 (*E*)-3-(2,3,4,5-Tetrahydroxypentylidene)pyrrolidine-2,5-dione (**6**)

D-ribose (4.7 g, 31.3 mmol) was added to a suspension of **5** (13.2 g, 36.7 mmol) in dry THF (90 ml) under nitrogen. The mixture was refluxed at 80 °C for 56 h until completion of the reaction was indicated by TLC (DCM/MeOH 9:1). The solvent was evaporated under a vacuum and the crude material was partitioned between water (100 ml) and dichloromethane (100 ml). The aqueous phase was collected and evaporated to dryness under a vacuum. The crude product was purified by silica gel column chromatography eluting with a gradient of ethyl acetate and a mixture of MeCN/MeOH (4:1), starting with 100 % EtOAc and increasing the ratio of MeCN/MeOH (4:1) to 25 %. The product **6** was obtained as a solid in 64 % yield

(4.6 g). ^{1}H NMR δ_H (500 MHz, D_2O): = 6.69 (dt, J = 8.3, 2.4 Hz, 1H, C=C<u>H</u>), 4.55 (dd, J = 8.3, 4.3 Hz, C<u>H</u>-CH=C), 3.77 (dd, J = 8.0, 4.3 Hz, 1H, C<u>H</u>-$(CHOH)_2$), 3.75 – 3.58 (m, 2H, C<u>H_2</u>OH), 3.55 – 3.51 (m, 1H, C<u>H</u>-CH_2OH), 3.46 (dd, J = 7.2, 2.4 Hz, 2H, C<u>H_2</u>-C=O). ^{13}C NMR δ_C (125 MHz, D_2O): 179.0 (C=O), 173.1 (C=O), 134.4 (C=<u>C</u>H), 129.9 (<u>C</u>=CH), 73.6 (<u>C</u>H-$(CHOH)_2$), 71.8 (<u>C</u>H-CH_2OH), 69.7 (<u>C</u>H-CH=C), 62,8 (<u>C</u>H_2-OH), 33.3 (<u>C</u>H_2-C=O).

5.4.3 Showdomycin (**1**) and 1´-*epi*-showdomycin (**7**)

Phenylselenyl chloride (2.6 g, 13.6 mmol) was added to a suspension of **6** (3 g, 13.0 mmol) in dry acetonitrile (180 ml) being heated at 65 °C. After 29 hours the reaction mixture was allowed to cool to room temperature and 10 % aqueous H_2O_2 solution (65 ml) was added. The reaction was stirred at room temperature and monitored by TLC (EA/MeCN/MeOH 15:4:1). The selenium containing intermediate was visualized by staining the TLC plate with a solution of 1 % $PdCl_2$ in 0.1 M HCl. After completion, the reaction mixture was concentrated under a vacuum. The resulting crude material was purified on a silica gel column (EtOAc/EtOH 19:1) affording a mixture of epimers **1** and **7** (1.49 g, 50 %) approximately in 1:3 respective ratio as observed on LC-MS. The products were identified by comparing the retention time to a pure sample of **1** on LC-MS system. The epimers were separated by a second silica column purification using a 5 % MeOH in DCM as eluent.

Showdomycin (1). ^{1}H NMR δ_H (500 MHz, D_2O): 6.66 (d, J = 1.5 Hz, 1H, H-4), 4.75 (dd, J = 4.6, 1.5 Hz, 1H, H-1′), 4.22 (appt, J = 4.7 Hz, 1H, H-2′), 4.06 (appt, J = 5.2 Hz, 1H, H-3′), 4.99 – 3.93 (m, 1H, H-4′), 3.82−3.78 (dd, J = 3.1 and 12.6 Hz, 1H, H-5′), 3.68−3.64 (dd, J = 5.2 and 12.6 Hz, 1H, H-5″).

^{13}C NMR δ_C (125 MHz, D_2O): 173.1 (C-2), 172.4 (C-5), 146.9 (C-3), 129.2 (C-4), 83.3 (C-4′), 77.5 (C-1′), 74.5 (C-2′), 70.9 (C-3′), 61.4 (C-5′).

1´-*epi*-showdomycin (**7**). ^{1}H NMR δ_H (500 MHz, D_2O): 6.62 (d, J = 2.2 Hz, 1H, H-4), 5.01 (dd, J = 3.6, 2.2 Hz, 1H, H-1′), 4.44 (appt, J = 3.9 Hz, 1H, H-2′), 4.27 (dd, J = 8.6, 4.4 Hz, 1H, H-3′), 3.97 (m, 1H, H-4′), 3.85 (dd, J = 12.5, 2.6 Hz, 1H, H-5′), 3.66 (dd, J = 12.5, 5.0 Hz, 1H, H-5″).

^{13}C NMR δ_C (125 MHz, D_2O): 173.2 (C-2), 172.4 (C-5), 147.1 (C-3), 129.4 (C-4), 81.1 (C-4′), 76.4 (C-1′), 72.4 (C-2′), 72.0 (C-3′), 61.1 (C-5′).

5.5 Synthesis of the triphosphates of showdomycin Diels-Alder adducts

5.5.1 1´-(3a,4,7,7a-tetrahydro-1H-4,7-methanoisoindole-1,3(2H)-dion-3a-yl) riboside (**18**)

1 (60 mg, 0.26 mmol) dissolved in dry THF (3 ml) was treated with freshly distilled cyclopentadiene (44 µl, 0.52 mmol, 2 equiv) and the resulting solution was stirred for 16 h at room temperature. The solvent was removed under reduced pressure and the crude material was purified by silica column chromatography with 90:10 DCM/MeOH. The main fraction containing two product isomers in 43:57 ratio was evaporated to dryness under a vacuum giving **18** as a white crystalline solid (25.5 mg, 33 % yield). The obtained compound mixture was phosphorylated without further separation of the isomers. ^{1}H NMR δ_H (500 MHz, CD_3OD): 6.28 – 6.25 (m, 1H, H-7), 6.24 – 6.21 (m, 1H, H-8), 4.50 (d, J = 3.5 Hz, 0.43H, H-1´), 4.16 (appt, J = 4.2 Hz, 0.43H, H-2´), 4.12 (d, J = 7.5 Hz, 0.57H, H-1´), 4.10 (dd, J = 4.5 and 8.9 Hz, 0.43H, H-3´), 3.99 (dd, J = 5.6 and 7.5 Hz, 0.57H, H-2´), 3.97 (dd, J = 3.4 and 5.6 Hz, 0.57H, H-3´), 3.89 – 3.85 (m, 1H, H-4´), 3.79 (dd, J = 2.6 and 12.4 Hz, 0.43H, H-5´), 3.64 – 3.55 (m, 1.57H, H-5´ and H-5´´), 3.74 (d, J = 4.6 Hz, 0.43H, H-4), 3.33 – 3.31 (overlap with CD_3OD, 1.14H, H-4, and H-9), 3.28 – 3.25 (m, 0.43H, H-9), 3.23 – 3.22 (m, 0.57H, H-6), 3.14 – 3.13 (m, 0.43H, H-6), 2.08 (appd, J = 8.8 Hz, 0.43H, H-10), 1.86 (appd, J = 9.5 Hz, 0.57H, H-10), 1.70 (dt, J = 1.2 and 9.5 Hz, 0.57H, H-10´), 1.56 (dt, J = 1.4 Hz and 8.8 Hz, 0.43H, H-10´). ^{13}C NMR δ_C (125 MHz, CD_3OD): 183.4 (C-2), 182.8 (C-2), 182.6 (C-5), 181.6 (C-5), 136.7 (C-7), 136.2 (C-7), 136.1 (C-8), 135.7 (C-8), 84.0 (C-4´), 82.1 (C-1´), 81.6 (C-1´), 81.4 (C-4´), 72.3 (C-2´), 72.2 (C-2´), 71.7 (C-3´), 71.5 (C-3´), 61.8 (C-5´), 61.4 (C-5´), 60.5 (C-3), 59.6 (C-3), 50.9 (C-6), 50.8 (C-4), 50.2 (C-10), 49.5 (C-10), 49.3 (C-4), 46.5 (C-9), 45.4 (C-9), 45.3 (C-6). HRMS (ESI) *m/z*: $[M-H]^-$ calcd for $C_{14}H_{16}NO_6^-$ 294.0983; found 294.0986.

5.5.2 1´-(3a,4,7,7a-tetrahydro-1H-4,7-methanoisoindole-1,3(2H)-dion-3a-yl) riboside-5´-triphosphate (**23a/b**)

The isomeric mixture **18** (26 mg, 86 µmol) was dissolved in dry triethyl phosphate and cooled on an ice-salt bath to - 10 °C. Freshly distilled phosphoryl chloride (12.1 µl, 0.13 mmol) and 2,4,6-trimethylpyridine (11.4 µl, 0.086 mmol) were added and the solution was stirred overnight at -10 – +4 °C. To the reaction mixture was added a solution of tetrabutylammonium pyrophosphate (156 mg, 0.17 mmol) in MeCN (1.5 ml) (dried with 3 Å molecular sieves) and dry tributylamine (41 µl, 0.17 mmol) at 0 °C and the stirring was continued overnight at 0 °C – RT. The reaction was

quenched by addition of 50 mM triethylammonium acetate buffer (4 ml) and chloroform (4 ml) and stirring was continued for further 30 min. The aqueous phase was separated and washed with chloroform (2 ml). NaI (40 mg, 0.27 mmol) was added to the aqueous phase and the solution was diluted with acetone (30 ml). The mixture was vortexed for 5 min and cooled at – 20 °C for 30 min and the precipitated phosphorylated material was collected by centrifugation. The crude material was purified by HPLC (a Phenomemex Kinetex™ column, C18, 250 × 10 mm, 5 µm, flow rate 3 ml min-[1], eluted with a linear gradient from 0.025 M TEAA in acetonitrile/H_2O, 5:95, to 0.025 M TEAA in acetonitrile/H_2O, 70:30, *v/v*, over 20 min). The collected fractions of two stereo isomers were lyophilized and precipitated in acetone with NaI to obtain the the faster eluting isomer **23a** as sodium salt (11 µmol, 13 % yield) and slower eluting isomer **23b** as sodium salt (6.6 µmol, 7.7 % yield).

23a ^{1}H NMR δ_H (500 MHz, D_2O): = 6.26 (dd, 1H, J = 2.7 and 5.7 Hz, H-7), 6.21 (dd, 1H, J = 2.7 and 5.7 Hz, H-8), 4.23 (dd, J = 2.4 and 5.4 Hz, 1H, H-3′), 4.19 – 4.12 (overlapping m, 2H, H-1′ and H-2′), 4.04 – 4.00 (overlapping m, 2H, H-4′ and H-5′), 3.93 – 3.87 (m, 1H, H-5′′), 3.39 (d, 1H, J = 4.5 Hz, H-4), 3.33 – 3.30 (m, 1H, H-9), 3.17 – 3.15 (m, 1H, H-6), 1.90 (appd, J = 9.5 Hz, 1H, H10′), 1.69 (appd, J = 9.5 Hz, 1H, H10′). ^{13}C NMR δ_C (125 MHz, CD_3OD): 183.2 (C-2), 182.3 (C-5), 136.9 (C-7), 135.8 (C-8), 83.0 (d, J = 8.8 Hz, C-4′), 81.6 (C-1′), 71.8 (C-3′), 71.7 (C-2′), 65.7 (d, J = 5.6 Hz, C-5′), 60.8 (C-3), 49.8 (C-10), 48.9 (C-4), 46.3 (C-6), 45.2 (C-9). ^{31}P NMR δ_P (202 MHz, D_2O): -6.20 (d, J = 19.7 Hz, P-γ), -11.00 (d, J = 19.7 Hz, P-α), – 21.84 (appt, J = 19.7 Hz, P-β). HRMS (ESI) *m/z*: [M-H]$^-$ calcd for $C_{14}H_{19}NO_{15}P_3^-$ 533.9973; found 533.9985.

23b ^{1}H NMR δ_H (500 MHz, D_2O): = 6.25 (dd, 1H, J = 2.8 and 5.6 Hz, H-7), 6.22 (dd, 1H, J = 2.7 and 5.6 Hz, H-8), 4.57 (d, 1H, J = 3.6 Hz, H-1′), 4.35 (dd, J = 4.6 and 8.7 Hz, 1H, H-3′), 4.21 (appt, 1H, J = 4.1 Hz, H-2′), 4.17 – 4.13 (m, 1H, H-5′), 4.10 – 4.04 (m, 1H, H-5′′), 4.01 – 3.98 (m, 1H, H-4′), 3.49 (d, 1H, J = 4.5 Hz, H-4), 3.28 – 3.25 (m, 1H, H-9), 3.16 – 3.14 (m, 1H, H-6), 2.05 (appd, J = 8.9 Hz, 1H, H10), 1.57 (appd, J = 8.9 Hz, 1H, H10′). ^{13}C NMR δ_C (125 MHz, CD_3OD): 183.9 (C-2), 183.3 (C-5), 136.2 (C-7), 136.1 (C-8), 82.0 (C-1′), 80.2 (d, J = 9.0 Hz, C-4′), 72.3 (C-2′), 70.8 (C-3′), 64.8 (d, J = 5.7 Hz, C-5′), 59.7 (C-3), 51.1 (C-4), 50.7 (C-6), 50.3 (C-10), 45.4 (C-9). ^{31}P NMR δ_P (202 MHz, D_2O): -6.21 (d, J = 19.7 Hz, P-γ), -10.61 (d, J = 19.7 Hz, P-α), – 21.72 (appt., J = 19.7 Hz, P-β). HRMS (ESI) *m/z*: [M-H]$^-$ calcd for $C_{14}H_{19}NO_{15}P_3^-$ 533.9973; found 533.9959.

5.6 Synthesis of formycin A 5′-triphosphate (**29**)

Formycin A (**27**) (36 mg, 0.135 mmol) was dissolved in dry triethyl phosphate (0.8 ml) under nitrogen. The solution was cooled to -10 °C on an ice-salt bath followed

by addition of freshly distilled phosphoryl chloride (18.8 µl, 0.202 mmol) and 2,4,6-trimethylpyridine (17.8 µl, 0.135 mmol). The reaction was stirred overnight at -10 – +4 °C. A solution of tetrabutylammonium pyrophosphate (244 mg, 0.269 mmol) in MeCN (2 ml) dried with 3 Å molecular sieves and tributylamine (64 µl, 0.269 mmol) were added to the reaction solution under nitrogen at 0 °C and the stirring was continued overnight at 0 °C – RT. The reaction was quenched by addition of 50 mM triethylammonium acetate buffer (4 ml) and chloroform (4 ml) and stirring for further 30 min. The aqueous phase was separated and washed with chloroform (3 ml). NaI (60 mg, 0.40 mmol) was added and the solution was diluted with acetone (40 ml). The mixture was vortexed for 5 min and cooled at – 20 °C for 30 min and the precipitated phosphorylated material was collected by centrifugation. The product **29** was purified by HPLC (a Phenomemex Kinetex^{TM} column, C18, 250 × 10 mm, 5 µm, flow rate 3 ml min-^{1}) using a gradient 50 mM TEAA buffered water and acetonitrile (increasing the acetonitrile from 5 % to 70 % in 25 min). The collected fractions were lyophilized and the triethylammonium salt of the product was dissolved in water (0.5 ml) and precipitated from acetone as the sodium salt of **29** (41 µmol, 30 %) by addition of NaI (40 mg) and acetone (13 ml). ^{1}H NMR δ_H (500 MHz, D_2O): = 8.01 (s, H1, H-2), 5.20 (d, J = 6.9 Hz, 1H, H-1′), 4.57 (dd, J = 5.6 and 6.8 Hz, 1H, H-2′), 4.38 (dd, J = 4.2 and 5.6 Hz, 1H, H-3′), 4.24 (appq, J = 4.1 Hz, 1H, H4′), 4.19 – 4.11 (m, 2H, H5′ and H5′′). ^{13}C NMR δ_C (125 MHz, D_2O): 152.8 (C-6), 151.7 (C-2), 138.0 (C-9), 137.1 (C-4), 125.1 (C-5) 83.4 (d, $J_{C,P}$ = 8.5 Hz, C-4′), 75.8 (C-1′), 74.1 (C-2′), 71.0 (C-3′), 65.6 (d, $J_{C,P}$ = 5.5 Hz, C-5′). ^{31}P NMR δ_P (202 MHz, D_2O): -6.00 (d, J = 19.6 Hz, P-γ), -10.44 (d, J = 19.6 Hz, P-α), – 21.27 (appt, J = 19.6 Hz, P-β).

5.7 Synthesis of pyrazofurin A 5′-triphosphate (**30**)

Pyrazofurin A (**28**) (23mg, 89 µmol) was converted to the 5′-triphosphate (**30**) analogously to **29**. The product **30** was purified by HPLC (a Phenomemex Kinetex^{TM} column, C18, 250 × 10 mm, 5 µm, flow rate 3 ml min-^{1}) with 50 mM TEAA buffered eluents and a gradient increasing from 3 % MeCN to 70 % in 25 min. The product **30** as a sodium salt was then obtained in 9 % yield (8.1 µmol). ^{1}H NMR δ_H (500 MHz, D_2O): = 4.91 (d, J = 7.8 Hz, 1H, H-1′), 4.45 (dd, J = 5.4 and 7.7 Hz, 1H, H-2′), 4.36 (dd, J = 3.3 and 5.4 Hz, 1H, H-3′), 4.17 – 4.14 (m, 1H, H4′), 4.14 – 4.08 (m, 2H, H5′ and H5′′). ^{13}C NMR δ_C (125 MHz, D_2O): 165.2 (C-6) 144.2 (C-5) 143.6 (C-4), 131.6 (C-3), 83.6 (d, $J_{C,P}$ = 8.3 Hz, C-4′), 74.5 (C-1′), 73.7 (C-2′), 71.1 (C-3′), 65.6 (d, $J_{C,P}$ = 5.5 Hz, C-5′). ^{31}P NMR δ_P (202 MHz, D_2O): -6.45 (d, J = 19.6 Hz, P-γ), -10.71 (d, J = 20.0 Hz, P-α), – 21.95 (appt, J = 20.0 Hz, P-β). HRMS (ESI) *m/z*: $[M-H]^-$ calcd for $C_9H_{15}N_3O_{15}P_3^-$ 497.9721; found 497.9723.

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