Turun yliopisto
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PRODRUG STRATEGIES OF ANTIVIRAL NUCLEOTIDES:

STUDIES ON ENZYMATICALLY AND THERMALLY REMOVABLE PHOSPHATE PROTECTING GROUPS

Anna Leisvuori

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ABSTRACT

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LEISVUORI, ANNA: Prodrug Strategies of Antiviral Nucleotides: Studies on Enzymatically and Thermally Removable Phosphate Protecting Groups

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Antiviral nucleosides are compounds that are used against viruses, such as human immunodeficiency virus (HIV) and hepatitis C virus (HCV). To act as therapeutic agent, the antiviral nucleoside needs to be phosphorylated to nucleotide in the body in three consecutive phosphorylation steps by cellular or viral enzymes. The first phosphorylation to the nucleoside monophosphate is often inefficient and leads to poor antiviral activity. The antiviral efficacy can be improved by applying a prodrug strategy and delivering the antiviral nucleoside directly as its monophosphate. In prodrug strategies of antiviral nucleotides, the negative charges on the phosphate moiety are temporarily masked with protecting groups. Once inside the cell, the protecting groups are removed by enzymatic or chemical processes. Many prodrug strategies apply biodegradable protecting groups, the removal of which is triggered by esterase enzymes. Several studies have, however, demonstrated that the removal rate of the second and subsequent esterase labile protecting groups significantly slows down after the first protecting group is removed due to the negative charge on the phosphodiester intermediate, which disturbs the catalytic site of the enzyme. In this thesis, esterase labile protecting group strategies where the issue of retardation could be avoided were studied.

Prodrug candidates of antiviral nucleotides were synthesized and kinetic studies on the chemical and enzymatic stability were carried out. In the synthesized compounds, the second protecting group is cleaved from the monophosphate by some other mechanism than esterase triggered activation or the structure of the prodrug requires only one protecting group. In addition, esterase labile protecting group which is additionally thermally removable was studied. This protecting group was cleaved from oligomeric phosphodiesters both enzymatically and thermally and seems most attractive of the studied phosphate protecting groups. However, the rate of the thermal removal still is too slow to allow efficient protection of longer oligonucleotides and needs optimization.

Key words: antiviral, nucleotide, prodrug, protecting group, biodegradable

TIIVISTELMÄ

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Kemian laitos, Matemaattis-luonnontieteellinen tiedekunta

LEISVUORI, ANNA: Antiviraalisten nukleotidien aihiolääkestrategiat: entsyymin ja lämpötilan vaikutuksesta irtoavat fosfaatin suojaryhmät

Väitöskirja, 139 s.
Orgaanisen kemian ja kemiallisen biologian laboratorio
Syyskuu 2015

Antiviraaliset nukleosidit ovat yhdisteitä, joita voidaan käyttää esimerkiksi HI-virusta (human immunodeficiency virus) ja hepatiitti C virusta vastaan. Jotta antiviraalinen nukleosidi voi toimia lääkeaineena, se on fosforyloitava elimistössä nukleotidiksi viruksen tai solun entsyymien avulla kolmessa peräkkäisessä fosforylointireaktiossa. Ensimmäinen fosforylointireaktio nukleosidimonofosfaatiksi on kuitenkin usein tehoton ja johtaa heikkoon antiviraaliseen aktiivisuuteen. Antiviraalista vaikutusta voidaan tehostaa käyttämällä aihiolääkestrategiaa ja annostelemalla antiviraalinen nukleosidi suoraan monofosfaattina. Antiviraalisten nukleotidien aihiolääkestrategioissa fosfaatin negatiiviset varaukset naamioidaan suojaryhmillä, jotka irtoavat solun sisällä entsymaattisten tai kemiallisten prosessien avulla. Monissa suojaryhmästrategioissa käytetään biohajoavia suojaryhmiä, joiden irtoaminen tapahtuu esteraasientsyymien avulla. Useat tutkimukset ovat kuitenkin osoittaneet, että ensimmäisen suojaryhmän irrottua seuraavien suojaryhmien irtoaminen hidastuu merkittävästi johtuen fosfodiesterivälituotteen negatiivisesta varauksesta, joka häiritsee entsyymin katalyyttistä keskusta. Tässä väitöskirjassa tutkittiin esteraasien avulla irtoavia suojaryhmästrategioita, joissa suojaryhmien irtoamisen hidastuminen voitaisiin välttää.

Työssä valmistettiin antiviraalisten nukleotidien aihiolääke-ehdokkaita ja tutkittiin niiden suojaryhmien entsymaattisen ja kemiallisen irtoamisen kinetiikkaa. Valmistetuissa yhdisteissä toinen monofosfaatin suojaryhmistä irtoaa jollain muulla kuin esteraasin aktivoimalla mekanismilla tai yhdisteen rakenne vaatii ainoastaan yhden suojaryhmän. Lisäksi tutkittiin esteraasin avulla irtoavaa suojaryhmää, joka irtoaa myös lämpötilan vaikutuksesta. Tämä suojaryhmä irtosi oligomeerisista fosfodiestereistä niin entsymaattisesti kuin lämpötilan vaikutuksestakin ja oli tutkituista fosfaatin suojaryhmistä lupaavin. Lämpötilan aiheuttama suojaryhmän irtoaminen on kuitenkin liian hidas ja vaatii optimointia, jotta sitä voitaisiin käyttää pidempien oligonukleotidien suojaamiseen.

Asiasanat: antiviraalinen, nukleotidi, aihiolääke, suojaryhmä, biohajoava

PREFACE

This thesis is based on experimental work carried out in the Laboratory of Organic Chemistry and Chemical Biology at the Department of Chemistry, University of Turku during the years 2008-2014. The financial support of Alios BioPharma, Graduate School of Organic Chemistry and Chemical Biology, Magnus Ehrnrooth foundation and Turku University foundation are gratefully acknowledged.

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Turku, August 2015

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I Leisvuori, A., Aiba, Y., Lönnberg, T., Poijärvi-Virta, P., Blatt, L., Beigelman, L., Lönnberg, H.: Chemical and enzymatic stability of amino acid derived phosphoramidates of antiviral nucleoside 5'-monophosphates bearing a biodegradable protecting group. *Org. Biomol. Chem.*, **2010**, *8*, 2131-2141.

II Leisvuori, A., Ahmed, Z., Ora, M., Blatt, L., Beigelman, L., Lönnberg, H.: 5´,5´-Phosphodiesters and esterase labile triesters of 2´-*C*-methylribonucleosides. *Arkivoc*, **2012**, *5*, 226-243.

III Leisvuori, A., Ahmed, Z., Ora, M., Blatt, L., Beigelman, L., Lönnberg, H.: Synthesis of 3´,5´-cyclic phosphate and thiophosphate esters of 2´-*C*-methylribonucleosides. *Helv. Chim. Acta*, **2012**, *95*, 1512-1520.

IV Leisvuori, A., Ora, M., Lönnberg, H.: 4-Acetylthio-2,2-dimethyl-3-oxo butyl group as an esterase and thermolabile protecting group for oligomeric phosphodiesters. *Eur. J. Org. Chem.*, **2014**, 5816-5826.

ABBREVIATIONS

ABC	ATP binding cassette transporters
Ac	acetyl
ACV	acyclovir
ADAL1	adenosine deaminase-like protein 1
ADP	adenosine 5´-diphosphate
AIDS	acquired immune deficiency syndrome
AOB	acyloxybenzyl
Ar	aryl
araC	cytarabine, 1-(β-D-arabinofuranosyl)cytosine
ATP	adenosine 5´-triphosphate
AZT	azidothymidine, 3´-azido-3´-deoxythymidine
B	nucleobase
Bn	benzyl
Bz	benzoyl
CEM	a cell line derived from human T cells
CEM/TK$^-$	thymidine kinase deficient CEM cells
CD4	a glycoprotein present on the surface of immune cells
CdAMP	cladribine monophosphate, 2-chloro-2´-deoxyadenosine 5´-monophosphate
CycloSal	cyclosaligenyl
CYP	cytochrome P450 enzymes
d4T	stavudine, 2′,3′-didehydro-3′-deoxythymidine
DAA	direct-acting antivirals
DCM	dichloromethane
DCC	*N*,*N*′-dicyclohexylcarbodiimide
ddT	3´-deoxythymidine
DiPPro	4-acyloxybenzyl ester protected nucleoside diphosphates
DMAP	4-dimethylaminopyridine
DMTr	4,4´-dimethoxytrityl
DNA	2´-deoxyribonucleic acid
DQF-COSY	double quantum filtered correlation spectroscopy
EC_{50}	50 % effective concentration
ESI	electrospray ionization
Et	ethyl
GSH	glutathione
Gua	guanosine
HAART	highly active antiviral therapy
HBV	hepatitis B virus
HCV	hepatitis C virus

HepDirect	cyclic 1-aryl-1,3-propanyl ester
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HIV	human immunodeficiency virus
HMBC	heteronuclear multiple-bond correlation spectroscopy
HPLC	high-performance liquid chromatography
HSQC	heteronuclear single quantum coherence
HSV	herpes simplex virus
iPr	isopropyl
Lev	levulinoyl
Me	methyl
MMTr	4-monomethoxytrityl
MS	mass spectrometry
NMP	*N*-methyl-2-pyrrolidone
NMR	nuclear magnetic resonance
NTP	nucleoside 5´-triphosphate
Nu	nucleoside
PBS	phosphate buffered saline
PC3	prostate carcinoma cell extract
PepT1	peptide transporter 1
Piv	pivaloyl
PLE	porcine liver esterase
PMEA	9-(2-phosphonylmethoxyethyl)adenine
POC	isopropyloxymethyl
POM	pivaloyloxymethyl
RNA	ribonucleic acid
RP-HPLC	reversed phase HPLC
SATE	*S*-acyl-2-thioethyl
SDTE	*S*-[(2-hydroxyethyl)sulfidyl]-2-thioethyl
TBDMS	*tert*-butyldimethylsilyl
t-Bu	*tert*-butyl
TEA	trimethylamine
Thd	thymidine
THF	tetrahydrofurane
TK1	thymidine kinase 1
TK2	thymidine kinase 2
TMS	trimethylsilyl
TriPPPro	4-acyloxybenzyl ester protected nucleoside triphosphates
U	enzyme unit
Ura	uracil

1 INTRODUCTION

1.1 Discovery of antiviral nucleosides

For being just tiny simple particles, made up only of genetic material coated with protein, viruses cause a full spectrum of diseases from common cold to highly severe states, such as cancer. Vaccination is an effective method in preventing many viral infections. National vaccination programs give protection against childhood diseases, such as polio or measles, and systematic worldwide vaccination against smallpox, a contagious disease caused by variola virus, has even led to eradication of the virus in 1980.[1] Vaccines contain inactivated or weakened viruses or viral structural proteins, which do not cause infection, but stimulate the immune system to produce antigens and memory cells and thus, lead to an artificially acquired immunity against a virus. Vaccination is a preventive action and is not usually effective on already infected patients or patients with weakened immune response. In addition, it appears difficult to develop a vaccine for viruses, such as human immunodeficiency virus (HIV) and hepatitis C virus (HCV), with high genetic variability, because the composition of the immune system triggering glycoproteins present on the viral surface is so variable. Accordingly, antiviral therapies based on other mechanisms are needed. Viral infections can be treated with antiviral agents, compounds that inhibit the development of new virus particles in the host cell. Most of the antiviral agents on the market today are targeted against HIV, hepatitis B and C viruses, herpes viruses and influenza viruses. Nucleoside analogs constitute an important class of antiviral agents.

The utilization of nucleosides as antiviral agents dates back to early 1960's when the first two nucleoside analogs, idoxuridine[2,3] and trifluridine[4] (Figure 1), were launched for the treatment of herpetic keratitis. Due to poor selectivity for virus infected cells, the compounds were too toxic to be taken internally and were only licensed for external use. The actual mechanism of viral inhibition was at that time not known. A finding that greatly improved the understanding of inhibition mechanisms of antiviral nucleosides was the discovery of acyclovir[5] (ACV) as a selective inhibitor of herpes simplex virus (HSV). ACV is phosphorylated only by viral kinase, not by the kinases of the host cell, and it, hence, acts only in virus infected cells. The recognition of HIV as the cause of acquired immune deficiency syndrome[6] (AIDS) in 1983 truly started the extensive exploration of antiviral agents and in 1987 the first anti-HIV nucleoside, azidothymidine[7] (AZT), was approved. Over the past two decades, the treatment of HIV has evolved from taking a handful of pills a day to a one pill a day regimen. The

research to find new and more selective antiviral agents is ongoing. The increased mobility of people brings new challenges to the development of antiviral agents, as rare or new severe viral diseases are more easily spread around the world than ever before, the latest example being the Ebola virus outbreak in western Africa in 2014.

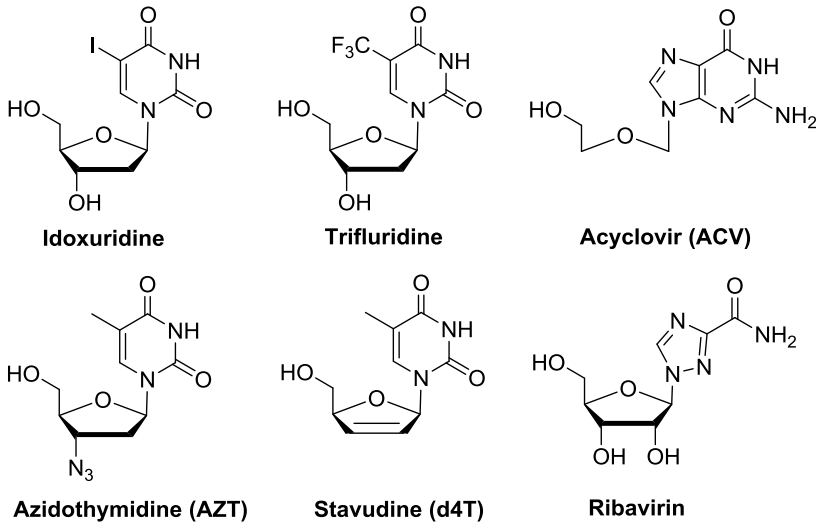


Figure 1 Structures of few key antiviral nucleosides.

There are currently 26 approved antiviral nucleosides and nucleotides for the treatment of viral infections, the majority of them being aimed at treatment of HIV and HSV.[8] The antiviral activity of nucleosides can be further improved by prodrug strategies, where the antiviral nucleoside is administered as its monophosphate, which is masked with appropriate phosphate protecting groups. Figure 2 represents three nucleoside derived prodrugs. Adefovir dipivoxil[9] (trade name Hepsera) has been used to the treat hepatitis B virus (HBV) infected patients since 2002. Tenofovir disoproxil fumarate[10] (trade name Viread) was accepted for the treatment of HIV in 2001 and in 2008 also for the treatment of HBV. The latest antiviral agent on the market is sofosbuvir[11] (trade name Sovaldi), which was accepted by the US Food and Drug Administration in December 2013. The standard treatment for HCV has, for more than a decade, been nucleoside analog ribavirin in combination with injections of pegylated interferon-α,[12] but sofosbuvir can be used to treat HCV without interferon-α injections.

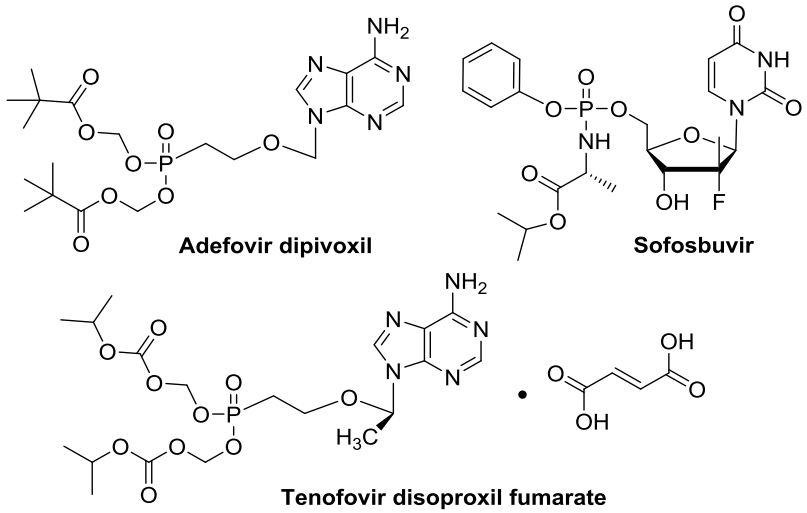


Figure 2 Approved prodrugs of antiviral nucleoside phosphates and phosphonates.

1.2 Antiviral nucleosides as drugs

1.2.1 Viral life cycle and targets for drug intervention

Viruses are small, non-cellular particles, consisting of genetic material coated by a protein capsid. The genetic material can consist of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) and it can be single or double stranded, depending on the virus. In some cases, the viral nucleocapsid also includes a few essential viral enzymes or is additionally surrounded by a membranous layer of lipids and glycoproteins. Viruses are obligate parasites: they don't have own cellular mechanisms and their reproduction is dependent on a host cell. Viruses are capable of infecting animal, plant or bacterial cells with their genetic material and exploiting the host cell metabolism to produce multiple copies of the virion.

The life cycle of viruses can be divided into separate steps which are all, in principle, potential targets for drug intervention.[13,14] The replicative cycle varies to some extent depending on the virus and the nature of its genetic material, but the following steps can be roughly separated for all viruses: adsorption of the virus to the host cell surface, penetration into the cell and uncoating of the genetic material, replication and transcription by the host-cell machinery, synthesis and assembly of nucleocapsids, and finally, departure of the mature virions from the host cell. The steps, which are carried out by the host cell enzymes, closely resemble the normal cellular processes. Antiviral agents

interfering with such processes most likely lead to cytotoxicity, since the compounds are not capable of distinguishing virus infected cells from the healthy ones. The best targets for antiviral agents are the most divergent steps requiring, in many cases, viral enzymes. This improves the selectivity of the antiviral agents for virus infected cells.

The viral life cycles of HIV and HCV are represented in Figures 3 and 4, respectively. These are both RNA viruses, the HIV genome constituting of two and the HCV genome of one positive-sense single-stranded RNA molecule. HIV is a retrovirus and the host cell nucleus is involved in the replicative cycle. CD4 glycoproteins on the surface of T-helper cells act as receptors for HIV. Once inside the cell, double stranded DNA is produced from the viral RNA by viral reverse transcriptase enzyme and the DNA is then integrated to the host cell genome by viral integrase, forming a so called provirus, which is then further transcribed. In contrast to HIV, the viral RNA of HCV is translated and replicated in the cytoplasm after uncoating. HCV targets the liver, infecting hepatocytes. [15,16]

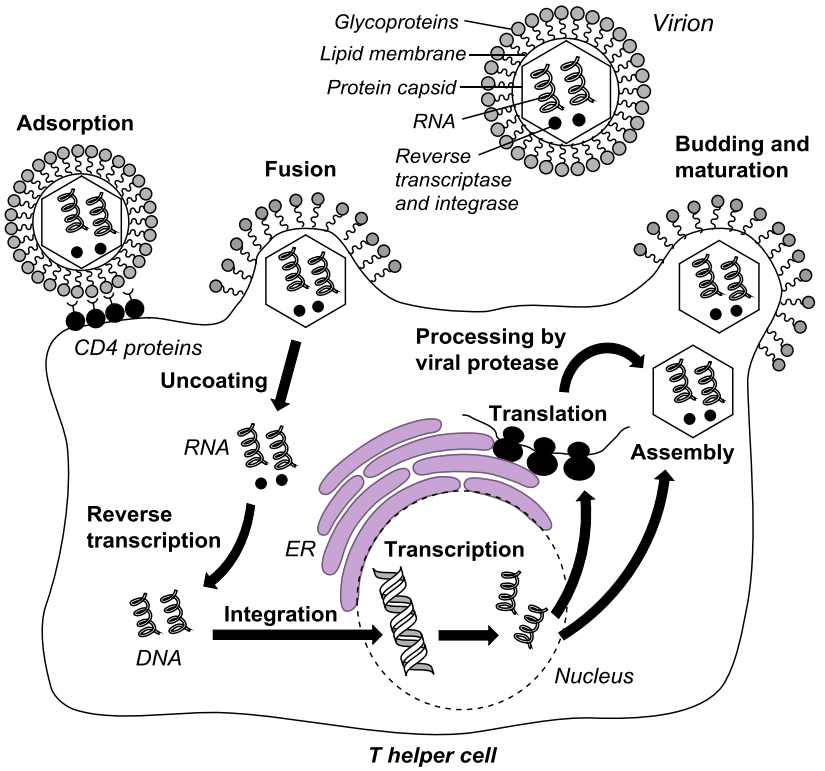


Figure 3 The HIV replicative cycle in T-helper cell. (ER = Endoplasmic reticulum)

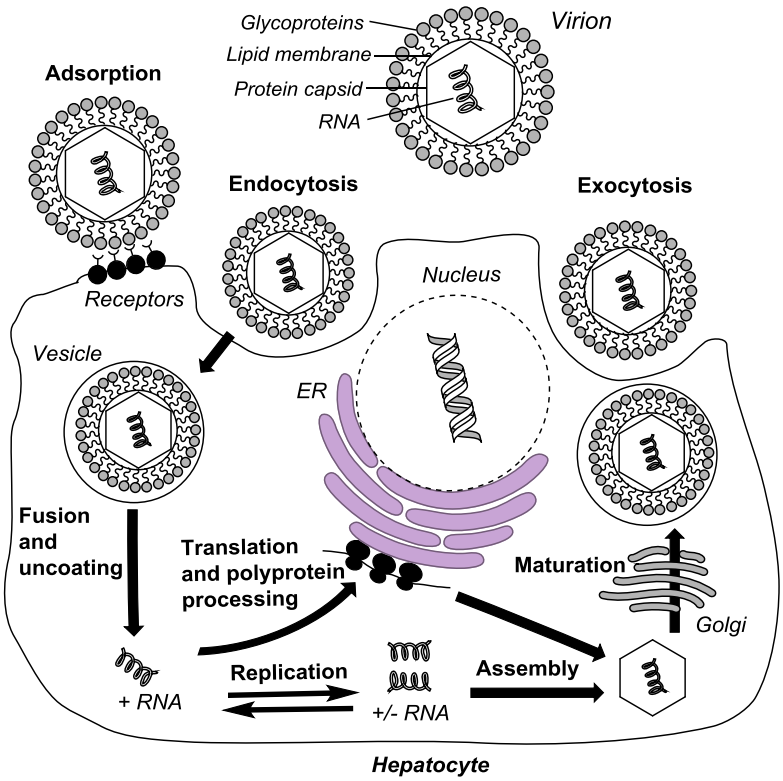


Figure 4 The HCV replicative cycle in hepatocyte. (ER = Endoplasmic reticulum)

The anti-HIV agents currently in use or in clinical trials include co-receptor antagonists (inhibition of adsorption), fusion inhibitors, reverse transcriptase inhibitors, integrase inhibitors and protease inhibitors. The antiviral nucleoside and nucleotide analogs act as reverse transcriptase (also called RNA-dependent DNA polymerase) inhibitors. The targets for anti-HCV agents include RNA replicase (RNA-dependent RNA polymerase) inhibitors and protease inhibitors, the nucleoside and nucleotide analogs belonging to the first mentioned class.[15,16]

Both HIV and HCV mutate readily because of short life-cycle and lack of proofreading enzymes. The viruses can develop resistance to antiviral drugs, but with combination therapy, by using more than one antiviral agent at a time, the chance for resistance development can be minimized. Since 1996 HIV infection has been treated with the so called highly active antiviral therapy (HAART) where at least three HIV inhibitors are combined into a single pill. For example, Atripla combines three antiviral agents: tenofovir disoproxil fumarate (a nucleotide reverse transcriptase inhibitor), emtricitabine (a nucleoside reverse transcriptase inhibitor) and efavirenz (a non-nucleoside reverse transcriptase inhibitor).[17]

The treatment of HCV aims to more precise regimens too. Combination of ribavirin and pegylated interferon-α, which was long the standard of care, has variable efficiency to HCV depending on the genotype of the virus. Interferons act usually as immunomodulatory agents, but in the combination therapy with ribavirin, their role seems to be different. Interferon acts as the antiviral agent, targeted at a phosphoprotein encoded by the NS5A gene of HCV, and ribavirin is the immunomodulatory agent by acting as inhibitor of inosine monophosphate dehydrogenase, and thus affecting the biosynthesis and levels of guanosine triphosphate in the cell.[18] Only 40-50% of patients with HCV genotype 1 achieve sustained virological response after 48 weeks treatment, while with genotypes 2 and 3 the rate can be as high as 75%.[19] Of the six genotypes of HCV, genotype 1 has been the most difficult one to treat and it also is the most common genotype in western countries. The treatment options for HCV are evolving with accelerated speed due to the new direct-acting antivirals (DAA), which are targeted to a specific step in the HCV life cycle, as has been the case with anti-HIV agents already for years.[20] Sofosbuvir has opened up the way towards all-oral treatment of HCV and several more direct-acting antivirals, which are in various states of the clinical trials today, are expected to be launched in the near future.[21]

The use of more than one antiviral nucleoside at the same time can cause drug-drug interactions and thus the different combinations have to be thoroughly evaluated. For instance, AZT (Figure 1) is able to inhibit the phosphorylation of stavudine (d4T, Figure 1) *in vitro*.[22] The development of drug resistance may be reduced by optimization of the drug delivery. If the drug reaches its target efficiently, the dosage may be lowered and the duration of treatment can be shortened. In addition to delivering the antiviral nucleosides as their monophosphates, prodrug strategies can also be used to achieve site-selective delivery and thus improve the efficiency of the drug.

1.2.2 Structure of antiviral nucleosides

Antiviral nucleosides are structural analogs of natural ribose and 2´-deoxyribose nucleosides *i.e.* the eight most common nucleosides constituting RNA and DNA (Figure 5). Antiviral properties result from chemical modifications in the structure of the nucleobase or sugar moiety or both. The modifications of some of the currently approved antiviral nucleosides include addition of functional groups or atoms, modification of the furanose ring by substitution of the heteroatom or removal of the ring structure, modification or suppression of the hydroxyl groups, modification of the nucleobase, and inversion of configuration.[23] In the cell, the nucleoside analogs are phosphorylated by

different kinases in multiple steps to become converted to nucleoside triphosphates, which are the active forms expressing the antiviral activity. This considerably restricts the number of feasible modifications of the nucleosides, as several kinases have to recognize the analogs as their substrates. The phosphorylation processes are discussed in more detail in the following chapter.

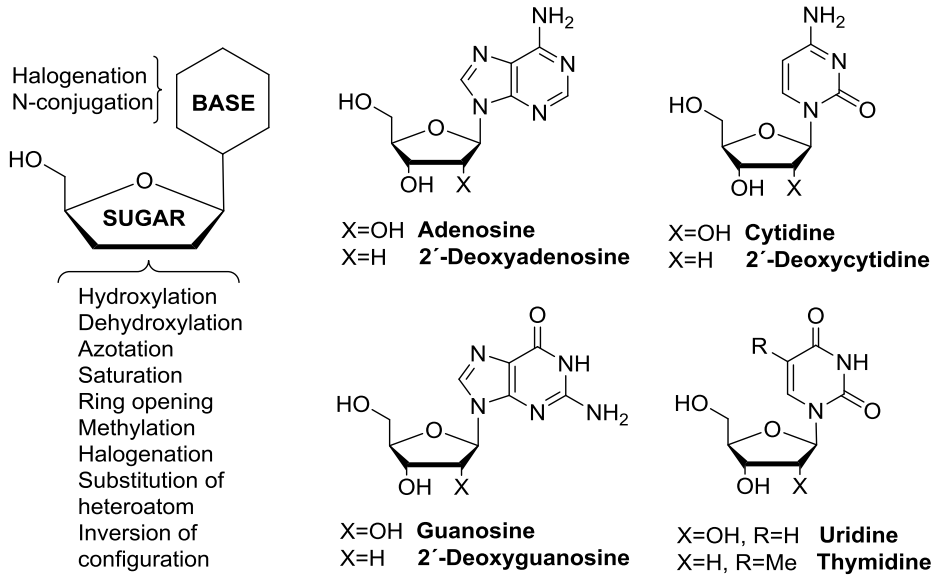


Figure 5 Some modifications of antiviral nucleosides and structures of natural ribose and 2´-deoxyribose nucleosides.

1.2.3 Cell delivery and phosphorylation

Cell membrane is a fluid structure constituting of phospholipid bilayer and proteins functioning as transporters. The nucleoside analogs enter the cell by passive diffusion or carrier-mediated transport. The transport is facilitated by members of the solute carrier superfamily, in particular by the equilibrative and concentrative families of nucleoside transporters, and organic anion transporters.[24,25] Due to the hydrophilicity of the nucleoside analogs, the carrier mediated transport has a major role in bringing the compounds inside the cell. Adenosine triphosphate (ATP) binding cassette transporters (ABC), in turn, efflux phosphorylated nucleoside analogs from the cell. Once inside the cell, the analogs can also be eliminated by various catabolic processes.

To achieve antiviral activity, the nucleoside analogs need to be phosphorylated to their triphosphate form. The initial phosphorylation to the nucleoside

monophosphate is carried out by a number of nucleoside kinases: 2´-deoxycytidine kinase, thymidine kinase 1 (TK1), thymidine kinase 2 (TK2) and deoxyguanosine kinase. The second phosphorylation step is catalyzed by nucleoside monophosphate kinases: thymidylate kinase, uridylate-cytidylate kinase, adenylate kinases 1 to 5 and guanylate kinases. The third phosphorylation to the nucleoside triphosphate is performed by nucleoside diphosphate kinase. In addition, some enzymes, such as phosphoglycerate kinase, pyruvate kinase and creatinine kinase, which are involved in reactions producing ATP, can also efficiently catalyze triphosphate formation of many nucleotide analogs. Nucleoside and nucleotide analogs can also be internalized into the mitochondria, where they can be further phosphorylated to the triphosphates. Nucleoside triphosphates have been found to inhibit the function of DNA-polymerase γ, which is responsible for mitochondrial DNA replication. This can be the cause of mitochondrial toxicity observed with some nucleoside analogs.[26] Figure 6 summarizes the discussed cellular processes of nucleoside analogs.

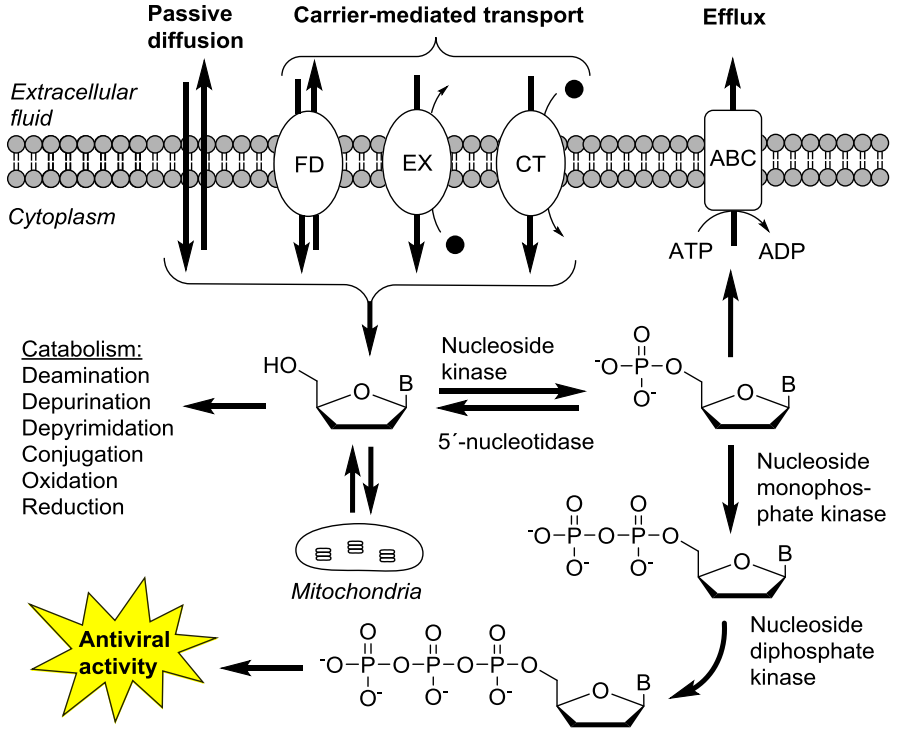


Figure 6 Cell related metabolism of nucleoside analogs. Mechanisms of carrier-mediated transport inside the cell: facilitated diffusion (FD), exchange (EX), cotransport (CO). (B = Nucleobase). Modified from ref. [27]

Some DNA viruses, like herpes viruses, encode their own viral kinases, which phosphorylate the nucleoside analogs to the monophosphate form in the infected cells. Viral thymidine kinase of herpes viruses phosphorylates ACV (Figure 1) to its monophosphate 100 times more efficiently than human thymidine kinase. This feature is extremely significant relative to antiviral therapy, because it enables selectivity of the antiviral nucleosides for virus infected cells.

1.2.4 Mechanism of action

The biologically active form of antiviral nucleoside analogs is the nucleoside 5´-triphosphate. The triphosphates interact with the viral or human polymerases. They can act as competitive inhibitors of polymerases or as alternative substrates that compete with the natural nucleoside triphosphates in DNA and RNA polymerization. Viral polymerases tend to be less substrate selective than the enzymes of the host cell and therefore nucleotide analogs become incorporated mainly in infected cells. The human replicative DNA polymerases have high fidelity which limits the incorporation of nucleotide analogs.

If incorporated into newly synthesized DNA or RNA, the antiviral nucleotide analogs act as chain terminators preventing further chain elongation. Many of the analogs lack 3´-hydroxyl function, which is required for the attachment of the incoming nucleotide. If the nucleotide analog possess the 3´-hydroxyl group (or a hydroxyl group at equivalent position), it can nevertheless act as chain terminator, if the hydroxyl group is conformationally constrained or sterically hindered. In addition, the incorporation of nucleotide analogs into viral DNA may lead to accumulation of mutations in the viral progeny, which weakens the virus and its ability to act as pathogen. [8,28]

1.3 Prodrug strategies of antiviral nucleotides

1.3.1 Applying the prodrug concept to antiviral nucleosides

A prodrug is a precursor of a drug.[29] It is an inactive agent that is metabolized in the body to the active drug by chemical or enzymatic reactions. Prodrug approaches are used to improve the physicochemical and, consequently, the pharmacological and toxicological properties of a drug. This is achieved by temporarily modifying the structure of the active drug with promoieties, functional groups that for example, make the molecule more metabolically stable, more lipophilic or hydrophilic, or help to target it to a specific cell type.

In an ideal case, the prodrug is converted to the parent drug as soon as it reaches its target and the remnants of the promoieties are eliminated into non-toxic compounds and rapidly excreted from the body.[30]

In many cases, the first phosphorylation of the antiviral nucleoside to the nucleoside 5´-monophosphate is the rate-limiting step in human cells. The first kinase in the phosphorylation cascade is the most substrate selective. AZT (Figure 1) makes an interesting exception to the rule: it is readily phosphorylated to the monophosphate and the bottleneck of the activation is the formation of the diphosphate leading to accumulation of AZT monophosphate in the cell.[31] If nucleoside analog is phosphorylated to the diphosphate, it will also most likely become phosphorylated to the triphosphate as well, because the enzymes that catalyze the final phosphorylation have wide substrate specificity.[32]

To avoid the problematic first phosphorylation step, the antiviral nucleoside should be delivered as its 5´-monophosphate. However, the nucleoside monophosphates are poor drug candidates as such. The negative charge on the molecule at physiological pH prevents crossing of biological membranes. In addition, the monophosphates are susceptible to degradation by phosphatases. In order to deliver an antiviral nucleoside monophosphate, a prodrug strategy is required (Figure 7). The phosphate group needs to be temporarily masked with protecting groups, which make the molecule more lipophilic and allow delivery into the cell. Several different types of protecting groups are available.[33] The removal of the protecting groups by chemical or enzymatic processes traps the anionic nucleoside monophosphate inside the cell. The released nucleoside monophosphate is still a prodrug, as it needs to be further converted to the triphosphate form to act as active drug.

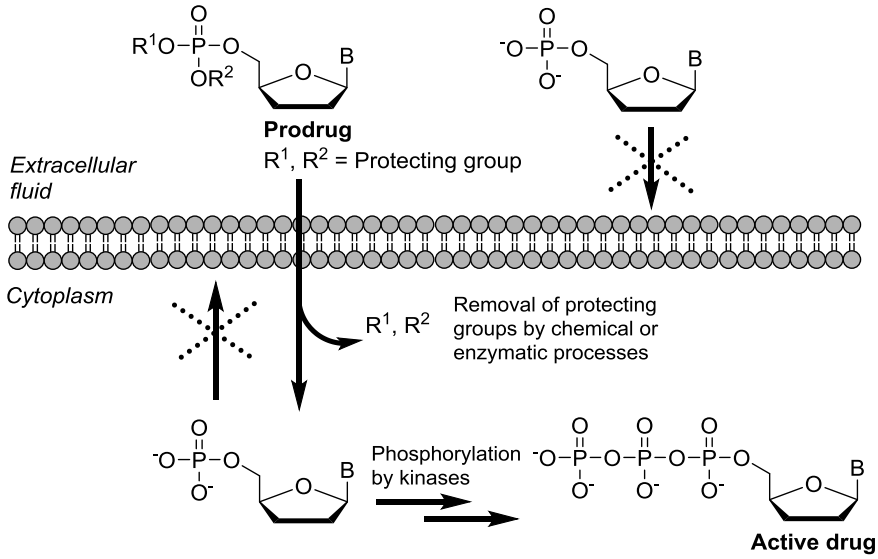


Figure 7 The prodrug approach of nucleoside monophosphates.

In addition, the prodrug strategies of nucleoside monophosphates enable the use of such nucleoside analogs as drugs, which have not shown antiviral activity due to lack of phosphorylation. The repertoire of antiviral nucleosides can, hence, be expanded. The process of activation of inactive nucleoside analogs has been termed as ''kinase by-pass''.[34]

To be successful as therapeutics, the prodrugs of antiviral nucleoside monophosphates should possess certain properties. The prodrug should be soluble in water, but also neutral and lipophilic enough to allow passive diffusion across the cell membrane. The prodrug should have sufficiently long half-life in serum to be able to reach the target cell. Once inside the cell, it should be converted to the active form with optimal kinetics. The prodrugs of nucleoside monophosphates as well as the by-products that are released upon conversion to active drug should be non-toxic. The prodrug should be stable enough to conditions in the gastrointestinal tract and allow formulation into an orally administered form to allow the most convenient way of treatment for the patient.[35,36,37]

1.3.2 Esterase labile protecting groups

The broad substrate specificity of esterases makes the esterase labile protecting groups an extensively studied prodrug class and a wide variety of these protecting groups have been reported in the literature. Figure 8 represents six

classes of esterase labile protecting groups: pivaloyloxymethyl[38,39,40] (POM), isopropyloxycarbonyloxymethyl[41] (POC), *S*-acyl-2-thioethyl[42] (SATE), 4-acyloxybenzyl[43,44,45,46] (AOB), 2,2-disubstituted 3-acyloxypropyl[47] and 2,2-disubstituted 4-acylthio-3-oxobutyl[48] groups. The last mentioned protecting group is additionally thermally removable and this feature is discussed in more detail in chapter 1.3.6.

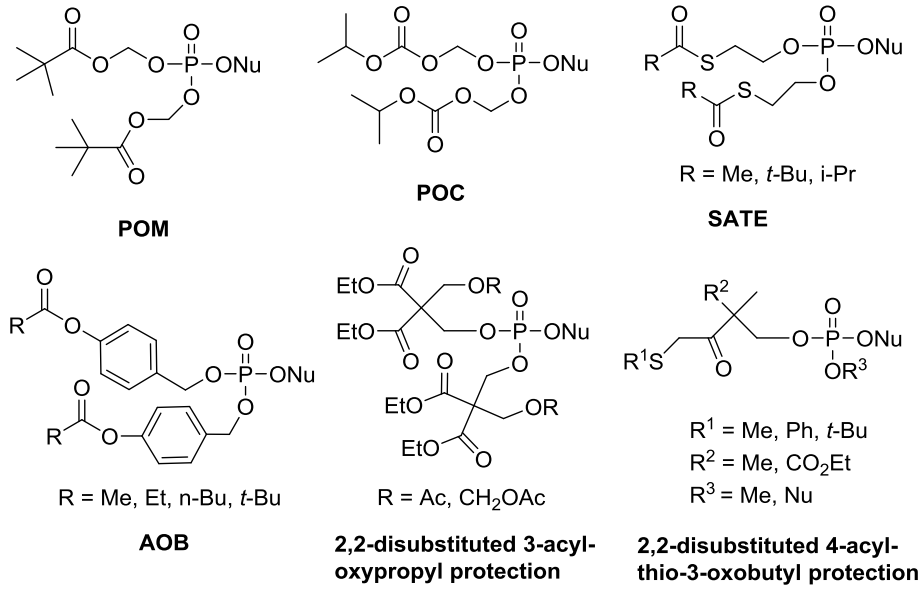
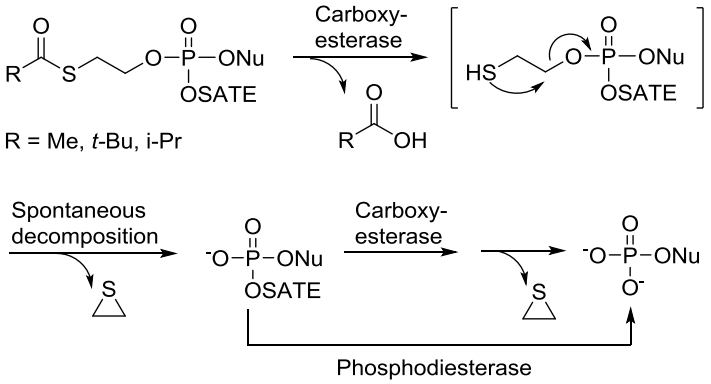


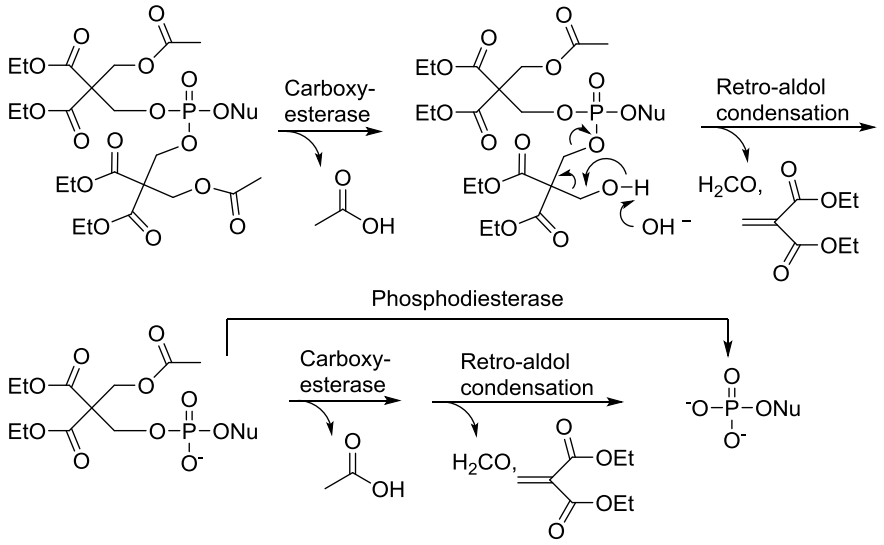
Figure 8 Structures of esterase labile protecting groups. (Nu = nucleoside).

The esterase labile protecting groups utilize carboxyesterase initiated hydrolysis for the release of nucleotides. Carboxyesterases are enzymes that are expressed at high levels in various tissues and they accept a wide variety of substrates.[49] First, the carboxyesterase catalyzes the cleavage of the acyl group. The stability of the esterase labile protecting groups can be adjusted by the nature of the acyl group: the more bulky the acyl group, the more stable the compound is against the degradation by carboxyesterase.[50] After deacylation, spontaneous elimination of the rest of the protecting group follows. The remnants of the esterase labile protecting groups are released as various side products depending on the group, but many of them are potentially toxic. The POM group releases formaldehyde and pivalate ion. The POC and 2,2-substituted 3-acyloxypropyl groups release formaldehyde and carbon dioxide together with isopropyl alcohol or enone, respectively. AOB groups release 4-hydroxybenzyl cation, which is further oxidized to 4-quinonemethidine or hydrolysed to 4-hydroxybenzyl alcohol. SATE and 2,2-disubstituted 4-acylthio-3-oxobutyl groups release a cyclic by-

product as a result of intramolecular cyclisation. As an example, the degradation pathways of bis(SATE) and bis(2,2-substituted 3-acyloxypropyl) nucleotides are represented in Schemes 1 and 2, respectively. In case of bis-protected nucleoside monophosphates, the second protecting group is expected to be removed stepwise in same manner or, alternatively, directly by a phosphodiesterase.



Scheme 1 Carboxyesterase triggered degradation of bis(SATE) nucleoside monophosphate.

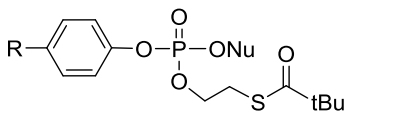


Scheme 2 Carboxyesterase triggered degradation of bis(2,2-substituted 3-acyloxypropyl) nucleoside monophosphate.

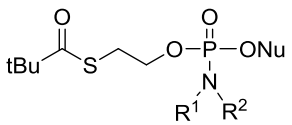
The POM and SATE groups[51,52,53] as well as the 2,2-substituted 3-acyloxypropyl groups[54,55] have also been applied to the protection of phosphorothioate bonds of

oligonucleotides. These studies demonstrate well that when the molecule contains more than one protecting group, the protections are removed at different rates by the enzyme. The first POM protection of a dodecathymidine bearing three POM protections was removed very rapidly by PLE (8 U mL^{-1}) with half-life of less than 2 minutes, while the remaining POM protections were removed much more slowly, the half-life for the fully deprotected oligomer being 2.4 hours.[53] In case of octathymidine bearing two 2,2-substituted 3-acyloxypropyl groups, the removal of the second protecting group was markedly retarded as well and additionally concurrent phosphorothioate backbone degradation took place.[55] Kinetic studies with bis-protected monophosphates bearing 2,2-substituted 3-acyloxypropyl groups also support the finding: enzymatic deacetylation of the negative phosphodiester is 10^3 times slower than deacetylation of the neutral phosphotriester.[47] Carboxyesterases prefer neutral substrates and the negative charge, which is formed after the removal of the first protecting group, disturbs the functioning of the catalytic site of the enzyme leading to retardation in removal of the subsequent esterase labile protecting groups.

The mixed SATE phosphoesters combine two different deprotection mechanisms. The nucleotide is protected with one SATE group and either an aryl, an amino or a glucosyl residue (Figure 9).[56] The SATE protection is first removed by carboxyesterase. The aryl derivative is then assumed to be removed by nucleotide phosphodiesterases, the amino residue by phosphodiesterase or phosphoramidase activation and the glucosyl moiety by glucosidases.

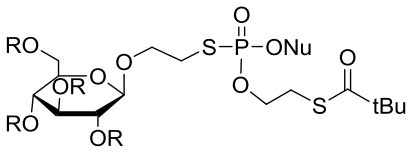


SATE aryl phosphotriesters



R^1, R^2 = methyl-esterified amino acid or non-amino acid derivatives

SATE phosphoramidate diesters



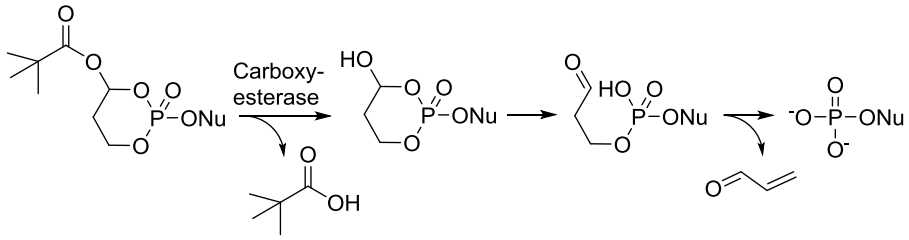
SATE glucosyl phosphorothiolates

Figure 9 Mixed SATE prodrugs.

The esterase labile SATE, POM and POC residues have also been used to protect 3´,5´-cyclic monophosphates of base-modified 2´-*C*-methylribonucleosides.[57] The 3´,5´-cyclic prodrugs showed enhanced potency in HCV inhibition studies

and improved antiviral activity against bovine viral diarrhea virus, a replicon model for HCV, compared to sheer nucleosides. The compounds were stable in simulated intestinal and gastric fluids as well as in human plasma, indicating that they are capable of reaching the hepatocytes intact. No significant cytotoxicity was observed. The esterase labile protecting groups suit well for the protection of 3´,5´-cyclic monophosphates, because only one protecting group is needed and thus the problem of slow removal of the subsequent esterase labile protections can be avoided. After removal of the esterase labile protecting group, the remaining 3´,5´-cyclic phosphate is hydrolyzed to 5´-monophosphate by phosphodiesterases.

Another cyclic approach, which requires only one esterase catalyzed activation step, utilizes 1-pivaloyloxypropan-1,3-diyl group as a cyclic phosphate protection of nucleoside monophosphate. [58] The removal of the pivaloyl group by carboxyesterase triggers the degradation of the protecting group (Scheme 3). The ring structure opens up to form an aldehyde. Spontaneous elimination of acrolein follows and the nucleoside monophosphate is released. The by-product acrolein is highly reactive. It can cause toxicity problems at high doses and has been associated with hemorrhagic cystitis. However, the toxicity can be controlled by concurrent administration of sodium 2-mercaptoethanesulfonate, which reacts with acrolein to produce non-toxic thioether. The 5´-[4-(pivaloyloxy)-1,3,2-dioxaphos-phorinan-2-yl]-2´-deoxy-5-fluorouridine was converted to the monophosphate with half-life of 5.9 hours, when exposed to hog liver carboxylate esterase (3 U mL^{-1}). When incubated with mouse plasma in 0.05 M phosphate buffer, the half-life for degradation was 30 minutes.



Scheme 3 Removal of 1-pivaloyloxypropan-1,3-diyl protecting group.

Some of the bis(AOB) esters of AZT monophosphates show increased cytotoxicity due to the side products released on deprotection. Attempts have been made to reduce the toxicity by using the AOB group to protect a 5´,5´-dimer of AZT, which releases only one potentially toxic 4-hydroxybenzyl cation per one nucleotide and one nucleoside.[59] In addition, the AOB esters have been used to deliver nucleoside diphosphates of d4T and AZT.[60,61] In the so called DiPPro-

approach the β-phosphate moiety is protected by two AOB groups (Figure 10). The α-phosphate is left unmasked, because the negative charge prevents the rapid cleavage of the anhydride bond by nucleophiles. However, acyloxybenzyl esters with short or branched acyl groups showed no antiviral activity in thymidine kinase deficient CEM cells (CEM/TK$^-$). The remaining polarity on the molecule possibly prevents effective cellular uptake. By increasing the lipophilicity of the molecule with longer chains of the acyl moieties, this obstacle was avoided and, for instance, 4-decanoyloxybenzyl ester of d4T diphosphate had 10-fold higher antiviral activity in HIV-1 infected wild-type CEM cells and 1570-fold higher antiviral activity in the CEM/TK$^-$ cell assay relative to d4T. However, when the DiPPro-strategy was applied to uridine nucleoside analogues 2´,3´-didehydro-2´,3´-dideoxyuridine and 2´,3´-didehydrouridine, the compounds showed low or no antiviral activity in HIV infected cells.[62] The uridine analogs were released as their nucleoside diphosphates, but studies with nucleoside diphosphate kinase showed that the released diphosphates are poor substrates for the enzyme and thus the triphosphates are not formed. This might explain the poor antiviral activity of the uridine analogs.

Recently, the 4-acyloxybenzyl ester strategy has also been applied to triphosphates in the TriPPPro-approach, where the γ-phosphate of the d4T triphosphate is protected with two identical or different 4-acyloxybenzyl groups (Figure 10).[63] The underlying idea is the same as in the DiPPro-approach, but more detailed information on the TriPPPro-approach is not yet available.

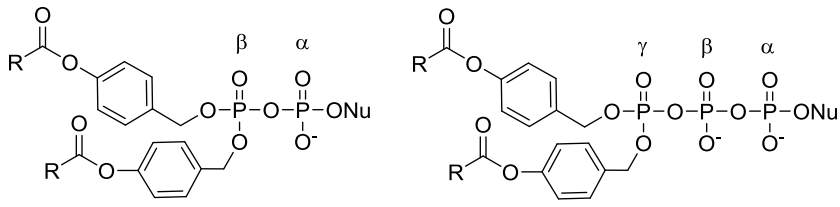
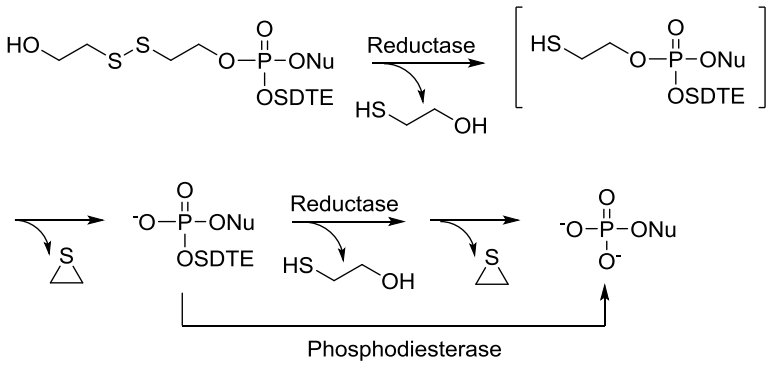


Figure 10 The 4-acyloxybenzyl esters studied in the DiPPro- and TriPPPro-approaches.

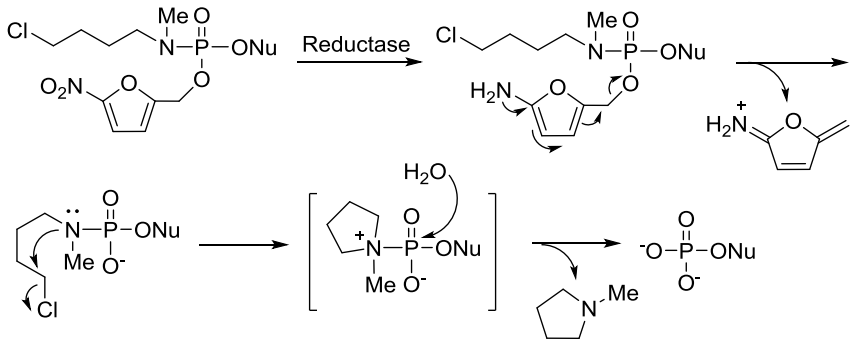
1.3.3 Reductase labile protecting groups

The removal of *S*-[(2-hydroxyethyl]sulfidyl]-2-thioethyl (SDTE) group is comparable to the removal of SATE groups, but the activating enzyme is different. The disulfide bond is first cleaved by reductase and then, an episulfide is spontaneously eliminated (Scheme 4). The limitations of this protecting group include the potential toxicity as a consequence of episulfide release and high susceptibility to serum mediated hydrolysis.[64,35]



Scheme 4 Reductase triggered degradation of bis(SDTE) nucleoside monophosphate.

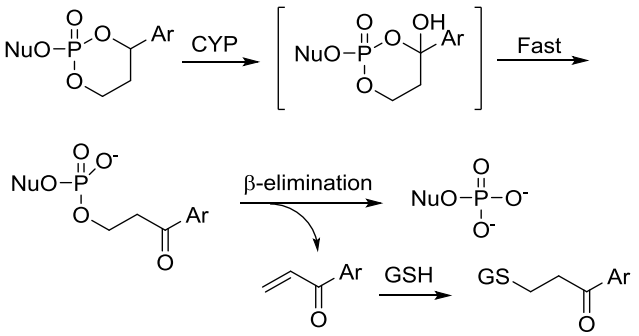
Nitrofuranylmethyl protection has been applied to protect phosphoramidates of anticancer nucleoside 5-fluoro-2´-deoxyuridine.[65] The removal of the protecting groups is triggered by enzymatic reduction of the nitrofuranylmethyl group, which leads to release of iminium and phosphoramidate ions (Scheme 5). The haloalkylamine group then undergoes spontaneous intramolecular cyclization and attack by water releases the nucleoside monophosphate.



Scheme 5 Proposed mechanism for the deprotection of nitrofuranylmethyl nucleoside phosphoramidates.

1.3.4 Oxidatively removable protecting groups

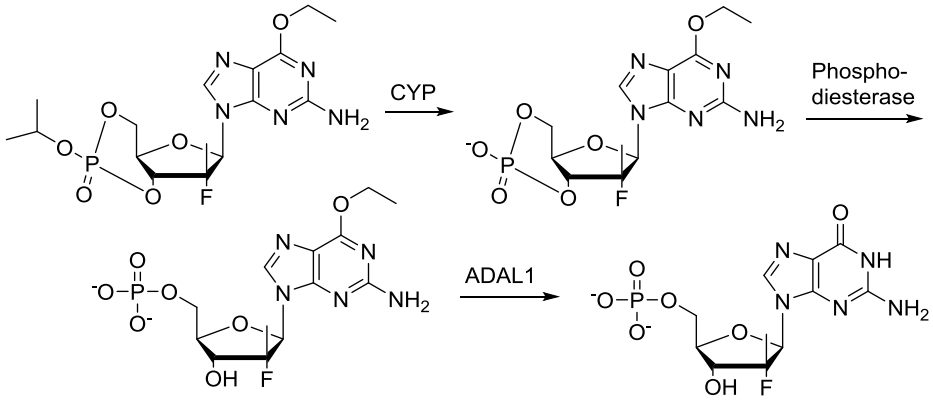
Cyclic 1-aryl-1,3-propanyl esters (HepDirect) of nucleoside monophosphates are targeted to treat liver diseases.[66] The removal of HepDirect protecting groups starts in the hepatocytes by oxidation catalyzed by cytochrome P450 (CYP) enzymes. The oxidation generates a cyclic hemiketal, which undergoes spontaneous ring opening, and an aryl vinyl ketone is cleaved by β-elimination (Scheme 6). The released aryl vinyl ketone is an alkylating agent and hence, potentially toxic, but cells expressing CYP enzymes also produce high levels of glutathione, which is expected to capture the toxic side product as glutathione conjugate.



Scheme 6 Degradation of HepDirect prodrugs.

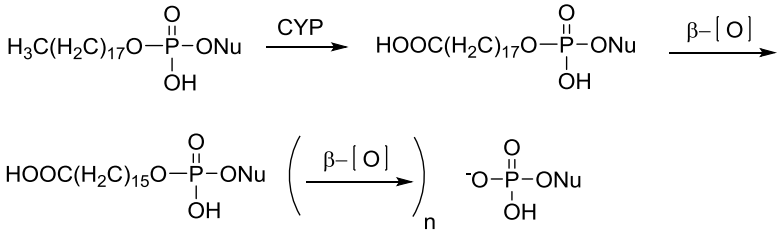
The HepDirect concept has been evaluated in human clinical trials for the delivery of adefovir to treat hepatitis B infection.[67] The prodrug was safe and well tolerated with only mild adverse effects. The HepDirect concept has also been applied to cytarabine [1-(β-D-arabinofuranosyl)cytosine, araC] monophosphate[68] to treat hepatocellular carcinoma. In mice, the prodrug was shown to generate high levels of araC triphosphate in the liver. In addition, the concept has been studied in the delivery of HCV antivirals 2´-methylcytidine[69] and 2´-methyladenosine[70].

3´,5´-Cyclic prodrugs with isopropyl as phosphate protection have been shown to be degraded by CYP enzymes and by subsequent ring opening by phosphodiesterases.[71] Adenosine deaminase-like protein 1 (ADAL1) catalyzes the removal of the O^6-ethyl group on the nucleobase and releases the nucleotide, a potential inhibitor of HCV replication (Scheme 7). In addition, other alkyl groups were studied as phosphate protections of 3´,5´-cyclic nucleoside monophosphates and cycloalkyl groups were shown to be significantly more potent as inhibitors of HCV replication than branched or straight chain alkyl groups.[72]



Scheme 7 Deprotection of isopropyl protected 3´,5´-cyclic monophosphate.

Nucleotide lipid esters are also oxidized in the hepatocytes.[73] The long chain alkyl group that is used to mask the monophosphate is first oxidized by cytochrome P450 species to the acid form, and then broken down in peroxisomes, two carbons at a time, by enzymatic β-oxidation (Scheme 8).[74] Lipid esters of nucleoside monophosphates are readily delivered inside the cell and they do not liberate any toxic side products.



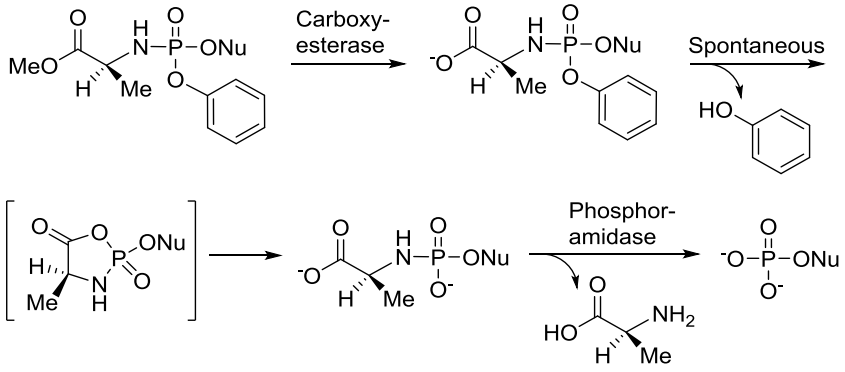
Scheme 8 Degradation of lipid ester nucleotide by enzymatic β-oxidation.

Lipids have been conjugated especially to anticancer nucleosides araC and gemcitabine (2´,2´-difluorodeoxycytidine),[8] but in addition, lipid esters of AZT monophosphate and tenofovir have been evaluated in clinical studies against HIV. A thioether lipid protecting group has been used to improve organ and cell selectivity of AZT monophosphate.[75] The prodrug is readily cleaved in mononuclear cells, but poorly in red blood cells and stem cells. This should reduce the hematologic toxicity, which is the main dose limiting adverse effect of AZT. Unlike tenofovir disoproxil fumarate, which is rapidly converted to tenofovir dianion by plasma esterases, hexadecyloxypropyl ester of tenofovir remains intact in plasma enabling more effective uptake by target cells.[76] The 50% lower effective concentration (EC_{50}) of the lipid ester of tenofovir may

reduce renal toxicity, which has been found to appear at elevated concentrations of tenofovir.

1.3.5 Phosphoramidase labile protecting groups

Phosphoramidates constitute an extensively studied class of protecting groups. In the first reported structure, an aryl group and an L-alanine methyl ester were used to mask the phosphate.[77] The removal of the protecting groups is initiated by esterase-mediated hydrolysis of the methyl ester (Scheme 9). This leads to intramolecular cyclization and release of a phenol. The five membered intermediate is rapidly hydrolyzed to phosphoramidic acid. The last step in the deprotection cascade of the nucleoside monophosphate may be catalyzed by a phosphoramidase or it may occur in the acidic environment of the lysosomes.[74,78,79]



Scheme 9 Degradation of aryl phosphoramidate prodrugs.

An amino acid is required for the antiviral activity. Studies have shown that simple amine substitution leads to a complete loss of antiviral activity.[80] Moreover, L-amino acids are preferred over D-amino acids, and especially L-alanine shows significant antiviral activity.[81] However, a natural amino acid is not necessarily required for the antiviral activity. An aryl phosphoramidate of d4T derived from the methyl ester of α,α-dimethylglycine is only 3 times less potent against HIV-infected cells than the corresponding derivative of L-alanine methyl ester.[82] In addition, it has been demonstrated that the amino acid derived phosphoramidates can act as alternative substrates for HIV-1 reverse transcriptase and may be incorporated into the DNA.[83] Hence, the antiviral effect of the amino acid phosphoramidate does not necessarily require the conversion of the 5´-phosphoramidic acid to the 5´-phosphate. The alaninyl d4T-monophosphate has been observed to accumulate and it has been suggested that

this intermediate could serve as intra- and/or extracellular depot for the nucleoside monophosphate.[84] The amino acid ester prodrugs of nucleoside analogues have also been shown to be substrates for the peptide intestinal transporter (PepT1).[85] PepT1 is stereoselective preferring L-amino acids.

With aryl phosphoramidates, the concerns that need to be taken into account are the toxicity of the phenol byproduct at modest concentrations, and the stereoisomerism that results from the attachment of two different groups to phosphorus atom. By attaching two identical amino acid groups to the phosphorus atom, both the toxicity and stereoisomer issues are avoided. Various diamide prodrugs of AZT monophosphate bearing two identical amino acids have been synthesized and tested against HIV-1 infected cells *in vitro* (Figure 11).[86] It was demonstrated that, in most cases, non-polar amino acid side-chains show greatest antiviral activity. In addition, the diamide strategy has been applied to a novel acyclic nucleoside phosphonate (GS-9219, Figure 11) and the compound has been evaluated in human clinical trials for the treatment of leukemia,[87] but the studies were terminated due to unacceptable safety profile.[88]

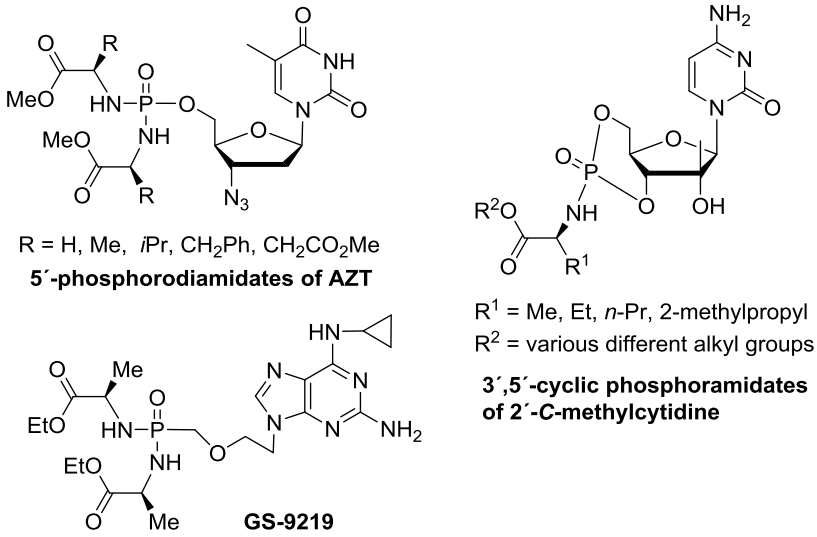


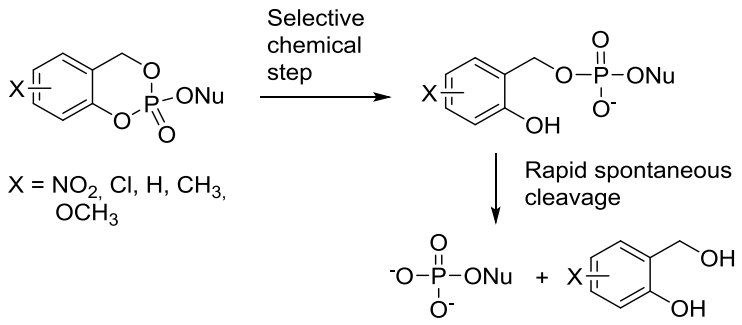
Figure 11 Structures of phosphorodiamidate and 3´,5´-cyclic phosphoramidate prodrugs.

Also with cyclic phosphoramidates the use of phenol protection is circumvented as only one protecting groups is needed to mask the 3´,5´-cyclic structure, but the formation of two diastereomers due to the 3´,5´-cyclic structure still has to be taken into account. A series of 3´,5´-cyclic phosphoramidates of 2´-*C*-methylcytidine, protected with 15 different amino acid esters, have been studied

as inhibitors of HCV (Figure 11).[89] The antiviral activity and conversion to nucleoside triphosphate (NTP) was examined in human hepatocytes. The 3´,5´-cyclic phosphoramidates were efficiently converted to the active triphosphate species. The levels of NTP formed depend on the structure of amino ester: the more lipophilic esters yield higher levels of NTP and also introduction of oxygen into the ester side chain raises NTP levels whereas branched esters did not have large effect on the NTP formation. There seem to be no preference in terms of diastereomers, when it comes to levels of NTP formation.

1.3.6 Chemically removable protecting groups

Cyclosaligenyl (*cyclo*Sal) phosphotriester approach is one of the few prodrug strategies that do not require enzymatic activity in any step of the release of the monophosphate. The concept has been widely studied and applied to several nucleoside analogs.[90] The removal of cyclosaligenyl protecting group proceeds stepwise by selective chemical hydrolysis (Scheme 10).[91,92,93] The selectivity is based on the different stability of the phenyl- and benzyl phosphate ester. The phenyl ester bond of the *cyclo*Sal monophosphate is the most labile of the three ester bonds, as the negative charge formed upon the bond breaking is delocalized into the benzene ring. After selective hydrolysis of the phenyl ester bond, the formed 2-hydroxybenzylphosphodiester then breaks down rapidly, releasing the nucleoside monophosphate and 2-hydroxybenzyl alcohol. The initial activation step can be controlled by the nature of the substituents in position 5 of the aromatic ring. Electron withdrawing substituents (NO_2, Cl) accelerate the cleavage of the phenyl ester bond, while electron donating substituents (Me) stabilize the same bond. In addition, the degradation of the *cyclo*Sal protecting group is pH dependent, the hydrolysis being slower under acidic conditions. In the *cyclo*Sal approach only one protecting group per nucleotide is needed.



Scheme 10 Removal of *cyclo*Sal protecting group.

The salicyl alcohols that are released as by-products have not shown cytotoxicity,[94] but *Cyclo*Sal phosphate triesters have been reported to inhibit butyrylcholinesterase in human and mouse serum.[95] The inhibitory effect is highly dependent on the nature of the nucleoside and the substituent on the *cyclo*Sal moiety. Bulky alkyl substituents in position 3 of the aromatic ring reduce the inhibition activity and the effect is even stronger with substituents at both *m*-positions, 3 and 5. The stereochemistry around the phosphorus atom also has a marked impact on the inhibitory effect: the S_P-diastereomers inhibit the enzyme while the R_P-diastereomers do not. The most promising cycloSal masking group thus far is the 3,5-di-*tert*-butyl-6-fluoro-*cyclo*Sal group.[96] In antiviral tests with HIV-1- and HIV-2-infected wild-type CEM cells the 3,5-di-*tert*-butyl-6-fluoro-*cyclo*Sal protected monophosphate of d4T showed enhanced antiviral activity compared to d4T.

There are several modifications of the *cyclo*Sal protecting group (Figure 12). The bis(*cyclo*Sal) approach delivers two nucleoside monophosphates per one protecting group.[97] The increased steric hindrance due to the size of the molecule reduces the butyrylcholinesterase inhibitor activity. *Cyclo*Sal protecting groups have also been modified by introducing enzymatically activated substituents to the aromatic ring. Due to the chemically triggered removal mechanism and lipophilic character of the initial *cyclo*Sal phosphotriesters, the compounds form concentration equilibrium through the cell membrane. The enzymatically activated *cyclo*Sal compounds, however, allow trapping of the molecule inside the cell, because the intracellular enzymatic removal of the substituent at the aromatic ring releases a more polar *cyclo*Sal derivative. After enzymatic activation, the rest of the *cyclo*Sal protection is hydrolyzed chemically. The substituents of these modified *cyclo*Sal derivatives include esterase labile groups[98,99,100,101,102] and amino acid esters[103] (Figure 12).

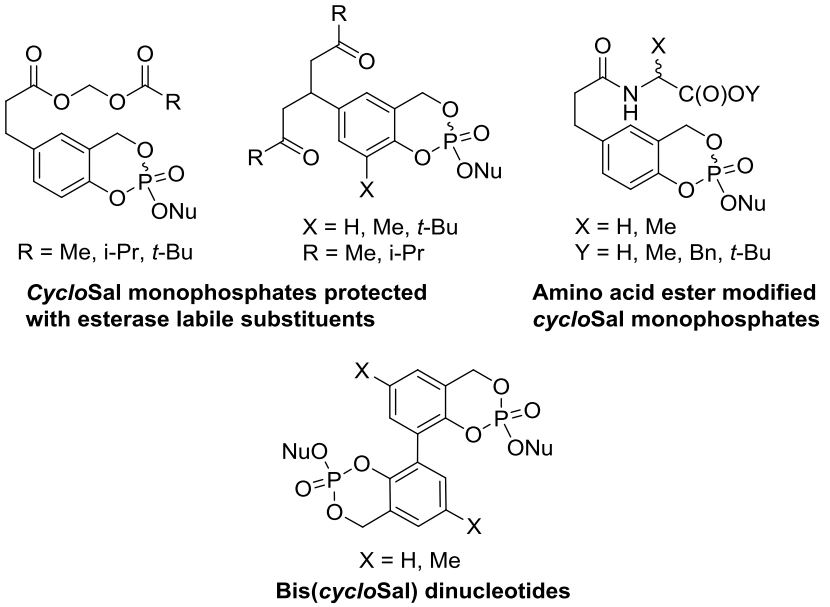
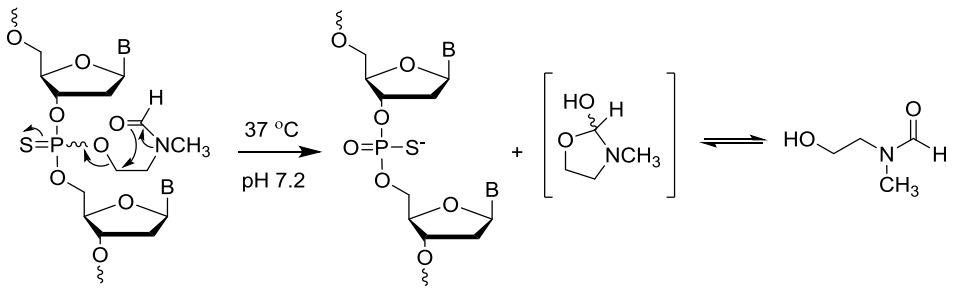


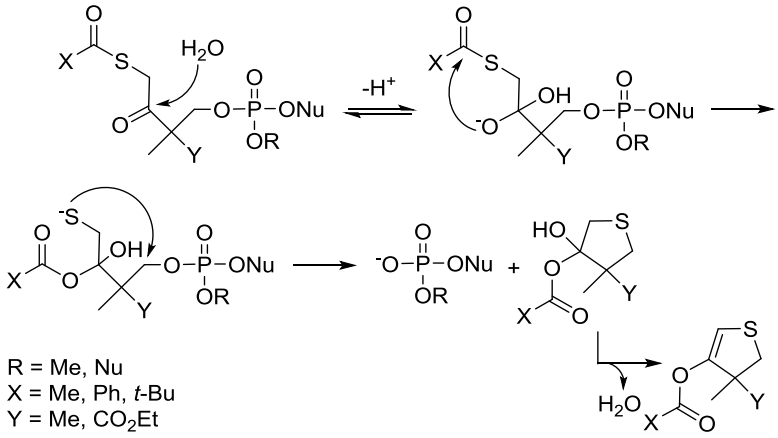
Figure 12 Structures of *cyclo*Sal protecting group modifications.

Another class of protecting groups that do not require enzymatic activation consists of the so called thermolabile protecting groups including *N*-formyl-*N*-methyl)-2-aminoethyl[104], 4-hydroxybutyl[105] and several ω-(alkylthio)alkyl[106] groups. These protecting groups are expected to be removed by intramolecular cyclization reaction at 37 °C in aqueous environment near neutral pH. The protecting groups have been studied as thermolabile thiophosphate protecting groups of oligonucleotides. The removal of the (*N*-formyl-*N*-methyl)-2-aminoethyl group is depicted in Scheme 11. The half-lives for the thermolytic deprotection have been studied with thymidine derived dinucleoside phosphorothioate triesters.[106] The half-lives for the conversions of Tps(OR)T to TpsT in phosphate buffered saline at 37 °C vary from 6.5 h to 38 h.



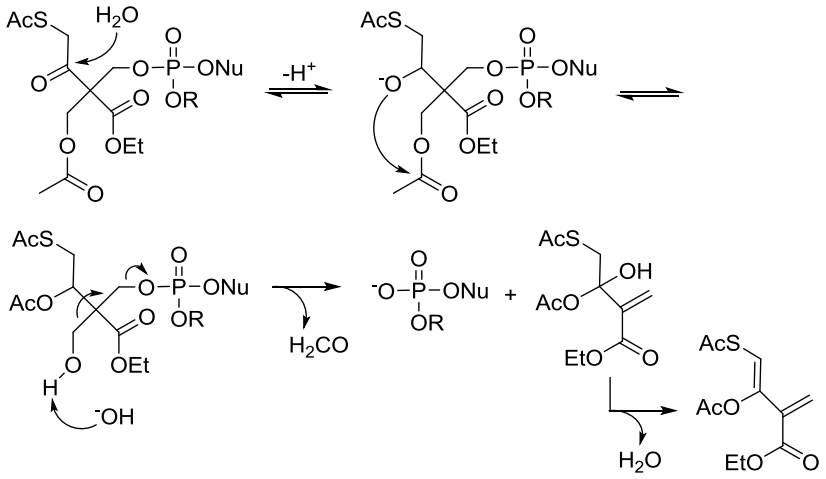
Scheme 11 Removal of (*N*-formyl-*N*-methyl)-2-aminoethyl protection by intramolecular cyclization.

2,2-disubstituted 4-acylthio-3-oxobutyl groups have been studied as phosphodiester bond protections.[48] These groups are esterase labile protecting groups that are additionally thermally removable. The underlying idea is that the protecting groups will be removed by thermolytic departure in case the enzymatic reaction markedly slows down, as can be the case when more than one esterase labile protection is applied to protect one molecule. The non-enzymatic removal of these groups is initiated by hydration of keto group, which enables the migration of acyl group from the sulfur atom (Scheme 12). The exposed mercapto group can then attack C1 leading to release of the phosphodiesters. The protecting group departs as 4,4-disubstituted 3-acyloxy-4,5-dihydrothiophene.



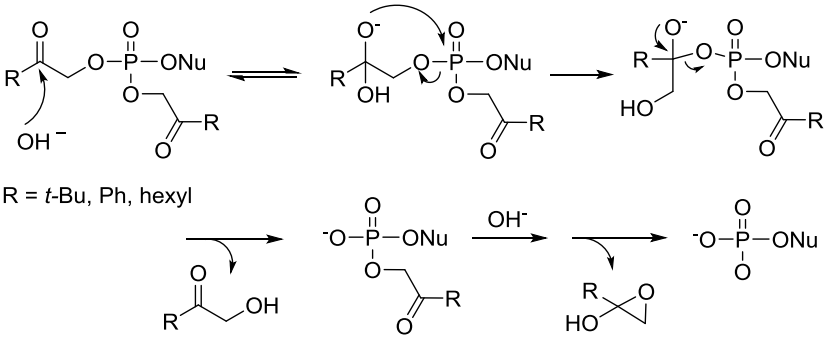
Scheme 12 Removal of 2,2-disubstituted 4-acylthio-3-oxobutyl groups by intramolecular cyclization.

2-[(Acetyloxy)methyl]-4-(acetylsulfanyl)-2-(ethoxycarbonyl)-3-oxobutyl group is also removable both enzymatically as well as thermally (Scheme 13).[107] Instead of intramolecular cyclization reaction, the non-enzymatic removal occurs by retro-aldol condensation releasing enone as by-product.



Scheme 13 Removal of 2-[(acetyloxy)methyl]-4-(acetylsulfanyl)-2-(ethoxycarbonyl)-3-oxobutyl group by retro-aldol condensation.

Three different bis-ketol protections have been introduced to 5´-monophosphates of 3´-deoxythymidine (ddT) and d4T as chemically removable phosphate protections.[108] The antiviral activity of the compounds against HIV has been studied. The bis-ketol protected phosphate triesters of ddT showed enhanced antiviral activity and the EC_{50} value was at least 10-fold lower when compared to the parent ddT. On the other hand, the bis-ketol protections did not improve the EC_{50} value of d4T significantly. The suggested pathway for the removal of bis-ketol protections is represented in Scheme 14.



Scheme 14 Proposed removal mechanism of bis-ketol protections.

1.3.7 Prodrug strategies of nucleotides in clinical trials

Enzymatically removable protecting groups constitute a versatile and well-established class of phosphate protections for the delivery of nucleotides into the cell. Two prodrugs based on esterase labile protecting groups have been approved for human use. Adefovir dipivoxil (Figure 2) is a bis(POM) protected monophosphate of adefovir, which is an acyclic adenosine phosphonate derivative and an anti-HBV nucleoside. Tenofovir disoproxil fumarate (Figure 2) is a bis(POC) protected prodrug of tenofovir, which is likewise an acyclic adenosine phosphonate derivative and has antiviral activity against HIV.

Another widely studied prodrug class is phosphoramidates. As Table 1 indicates, majority of the nucleotide prodrug candidates currently in clinical trials are phosphoramidate derivatives.[8] The potential of phosphoramidate prodrugs was recently proofed, when sofosbuvir was accepted for medical use against HCV infection in humans. Sofosbuvir is a prodrug of 2´-α-fluoro-2´-β-methyluridine monophosphate, where the phosphate moiety is protected with a phenyl group and an isopropyl ester of L-alanine (Figure 2). There have been drawbacks with phosphoramidate prodrugs as well. IDX-184 and IDX-19368 are 5´-phosphoramidates of 2´-*C*-methylguanosine that have been shown to be pan-genotypic against HCV.[109] In addition, the compounds show no inhibition of human polymerases and are readily converted into triphosphates in animal liver. Nevertheless, studies with practically all guanosine analogues have been discontinued after one volunteer died on cardiac failure and 40% of the volunteers had some kind of cardiac dysfunction in phase II studies of a guanosine derived phosphoramidate prodrug (BMS-986094).[110]

Other potential future prodrug classes that have proceeded to preclinical or clinical development are based on *cyclo*Sal, HepDirect, SATE and lipid ester protections. The advantageous feature of *cyclo*Sal and HepDirect prodrugs is that only one by-product molecule is released per one nucleoside monophosphate, due to the cyclic structure of the protecting groups. In most of the prodrug approaches two equivalents of potentially toxic by-products are released per one active molecule. Salicylic alcohols that are released as by-products of *cyclo*Sal protections have not shown cytotoxicity. Similarly, the lipid ester prodrugs do not liberate toxic by-products. The aryl vinyl ketone that is released from the HepDirect prodrugs, on the other hand, is toxic. Since the degradation of HepDirect compounds occurs in the liver, where high levels of glutathione is produced, this may not be a problem. Formaldehyde is a common by-product of many of the esterase labile protecting groups and has been named as a known human carcinogen.[111] However, formaldehyde is an essential metabolite of the body and we are also exposed to it from the environment and our food. Adefovir

dipivoxil and tenofovir disoproxil fumarate generate 1.2 mg and 28.1 mg of formaldehyde per dose of prodrug (10 mg and 300 mg) during bioconversion, respectively. It is estimated that the body uses 31 to 59 g of formaldehyde per day, so the additional formaldehyde from the prodrugs should be easily handled by the body.[112] The removal of SATE group releases an episulfide, which is an alkylating agent, but nevertheless at least two nucleotide prodrugs utilizing SATE protecting groups are studied in preclinical trials for cancer treatment.

Table 1 Approved nucleotide prodrugs and prodrug candidates in preclinical or clinical development, their target, year of approval or status of development and the applied prodrug strategy. Modified from ref.[8]

Name	Target	Status	Prodrug strategy	Ref., NB
Sofosbuvir	HCV	2013	Phosphoramidate	11,113
Adefovir dipivoxil	HBV	2002	POM	9,114,115
Tenofovir disopro. fum.	HIV, HBV	2001, 2008	POC	10,116
UA911	cancer	preclinical	SATE	117
Mixed SATE-araCMP	cancer	preclinical	SATE	118
*Cyclo*Sal-CdAMP	cancer	preclinical	*Cyclo*Sal	119
Phosphoramidate-araC	cancer	preclinical	Phosphoramidate	120
NUC-1031	cancer	Phase I	Phosphoramidate	121
GS-9212	cancer	Phase I/II	Diamide	87,122†
NB1011	cancer	Phase I/II	Phosphoramidate	123
GS-9131	HIV	Phase II	Phosphoramidate	124,125
GS-7340	HIV, HBV	Phase I/II	Phosphonoamidate	126,127
Stampidine	HIV	Phase I	Phosphoramidate	128
CMX157	HIV	Phase I	Lipid ester	76
Pradefovir	HBV	Phase II	HepDirect	129
PSI-353661	HCV	Phase I	Phosphoramidate	130
IDX-19368	HCV	Phase I	Phosphoramidate	109†
IDX-184	HCV	Phase II	SATE+Phosphora.	131†
INX-08189	HCV	Phase II	Phosphoramidate	132†

† Symbol refers to discontinuation of the studies.

In prodrug strategies, where diastereomers are formed, the different physical properties of single diastereomers need to be evaluated as well. Diastereomers are formed in synthesis of the mixed prodrug strategies, where two different protecting groups are used to mask the phosphate, and also in the cyclic

protecting group strategies. Sofosbuvir, for instance, is used as single diastereomer.

As for any drug, also in case of prodrugs of antiviral nucleotides, the issue of toxicity is not straightforward. How efficiently the compound reaches its target and acts as a drug is important in determining the risks in addition to the toxicity of the by-products. The efficiency determines on its behalf the size of the dose and hence, the amount of released toxic compound as well. In addition, how serious a disease is and the existence or lack of other treatment options counts in the assessment of the overall risks of a drug. If benefits are bigger than risks, the compound has potential for drug use. The toxicity issue becomes emphasized especially in the treatment of long term or chronic viral infections, such as HIV, where drugs need to be taken throughout lifetime.

2 AIMS OF THE THESIS

Antiviral nucleosides have proved their potential in the treatment of several viral infections. The bottle neck of the therapy utilizing antiviral nucleosides often is inefficient phosphorylation to the nucleoside monophosphate, which leads to low antiviral activity. The first phosphorylation step can be bypassed by delivering the antiviral nucleoside directly as its protected monophosphate derivative *i.e.* applying a prodrug strategy. Many prodrug strategies are based on enzymatically cleavable protections, esterase labile protecting groups in particular. However, several studies have demonstrated that, in case of carboxyesterase labile protecting groups, the rate of the removal of the second and subsequent protecting groups is significantly retarded. This is due to the negatively charged phosphodiester intermediate which is formed after the first protecting group is removed. The negative charge disturbs the active site of the enzyme.

This thesis is aimed at studying carboxyesterase labile protecting group strategies where the issue of retardation could be avoided. Potential prodrug candidates of antiviral nucleosides have been synthesized, including:

- Nucleoside phosphoramidates bearing a carboxyesterase labile protecting group and an L-alanine methyl ester.
- 5´,5´-dinucleoside monophosphates bearing a carboxyesterase labile protecting group.
- 3´,5´-cyclic nucleosides bearing a carboxyesterase labile protecting group.

In the synthesized compounds, the second protecting group is cleaved by some other mechanism than carboxyesterase triggered activation or the structure of the prodrug requires only one protecting group. Kinetic studies on the chemical and enzymatic stability of some of the synthesized compounds were carried out.

In addition, carboxyesterase labile protecting group which is additionally thermally removable was used as phosphate protecting group of short oligomeric phosphodiesters. The idea of this protecting group, which is removable by two mechanisms, is that in case the enzymatic removal becomes slow, the protecting group will undergo thermolytic departure instead. Kinetic studies with the synthesized thymidine derived model compounds were carried out to find out how the protecting group is removed from molecules with more than one such protection.

3 RESULTS

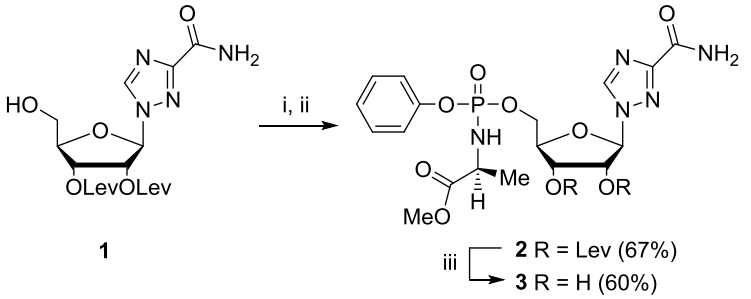
3.1 Synthesis

3.1.1 2,2-Bissubstituted 3-acyloxypropyl protected 5´-phosphoramidates (I)

Antiviral nucleosides ribavirin and 2´-*O*-methylcytidine were converted to L-alanine methyl ester derived 5´-phosphoramidates. 3-Acetyloxy-2,2-bis(ethoxycarbonyl)propyl and 3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)-propyl groups were used as esterase labile protecting groups.[47] To allow comparison, the extensively studied *O*-phenyl phosphoramidates were also synthesized.

The nucleosides were first appropriately protected. Levulinoyl (Lev) groups were used to protect the 2´- and 3´-hydroxyl functions of ribavirin and 3´-hydroxyl group of 2´-*C*-methylcytidine. To achieve this, 4-methoxytrityl (MMTr) and *tert*-butyldimethylsilyl (TBDMS) groups were first introduced to 5´-*O* of ribavirin and 2´-*O*-methylcytidine, respectively. The 4-amino function of 2´-*O*-methylcytidine was 4-methoxytritylated. The amino function of the ribavirin base moiety is not reactive and could be left unprotected. Levulinic anhydride was generated *in situ* from levulinic acid with *N,N'*-dicyclohexylcarbodiimide (DCC) using 4-dimethylaminopyridine (DMAP) as catalyst. Finally, the 5´-*O*-protections were removed. The synthetic details to obtain protected nucleosides **1** and **4** are given in Schemes 3 and 4 of paper I.

The 5´-*O*-phenylphosphoramidates of ribavirin (**3**) and 2´-*O*-methylcytidine (**6**) were obtained as a mixture of R_P- and S_P-diastereomers (Schemes 15 and 16) by following a previously published method.[133] The protected nucleosides **1** and **4** were first reacted with diphenylphosphite in pyridine under nitrogen atmosphere. The formed 5´-*H*-phosphonate phenyl esters were then oxidatively aminated with L-alanine methyl ester to obtain **2** and **5** in fairly good yields (67% and 70%, respectively). The levulinoyl protecting groups were removed with hydrazine hydrate in a mixture of acetic acid (AcOH) and pyridine. The MMTr-protection was removed in 80% aqueous AcOH.



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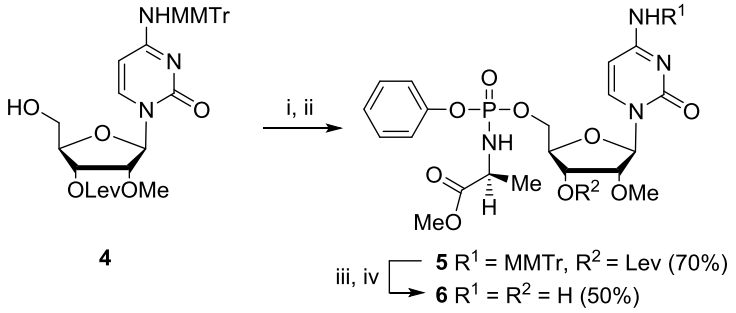
Scheme 15 Synthesis of compound **3**: i) diphenylphosphite, Py, 20 min, ii) L-alanine methyl ester in MeCN/Py (6:1), CCl_4, TEA, 70 min, iii) 0.5 M $N_2H_4 \cdot H_2O$ in AcOH/Py (4:1), 1 h.



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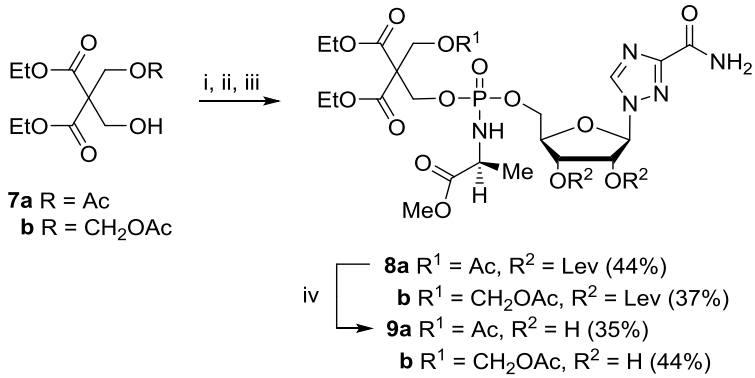
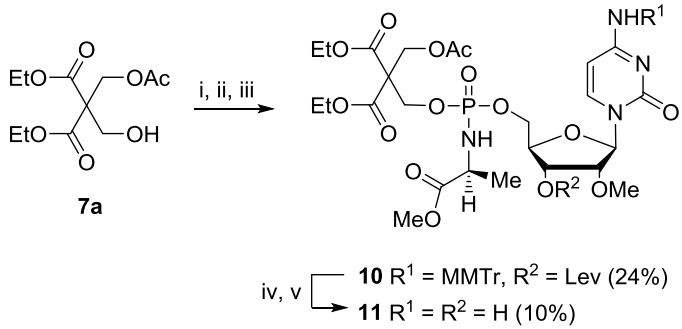
Scheme 16 Synthesis of compound **6**: i) diphenylphosphite, Py, 30 min, ii) L-alanine methyl ester in MeCN/Py (6:1), CCl_4, TEA, 70 min, iii) 1. 0.5 M $N_2H_4 \cdot H_2O$ in AcOH/Py (4:1), 5 h, iv) 80% aq. AcOH, 55 ºC for 2 h, 65 ºC for 4.5 h.

The 3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl and 3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)propyl protected phosphoramidates of ribavirin (**9a**, **9b**) and the 3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl protected phosphoramidate of 2´-*O*-methylcytidine (**11**) were obtained as a mixture of R_P- and S_P-diastereomers as depicted in Schemes 17 and 18, respectively. The syntheses of the starting materials, diethyl 2-acetyloxymethyl-2-hydroxymethylmalonate (**7a**) and diethyl 2-acetyloxymethoxymethyl-2-hydroxymethylmalonate (**7b**), have been previously described.[47] **7a** or **7b** was first reacted with diphenylphosphite under nitrogen. The phenol was then displaced from the mixed alkyl phenyl *H*-phosphonate diester with protected nucleoside **1** or **4**. Oxidative amination with L-alanine methyl ester gave **8a** and **8b** in moderate yields (44% and 37%, respectively). **10** was obtained in low yield (24%). The levulinoyl and MMTr-protections were removed as described above for **2** and **5**.



Scheme 17 Synthesis of compounds **9a** and **9b**: i) diphenylphosphite, Py, 30 min, ii) compound **1** in Py, 2 h iii) L-alanine methyl ester in Py, MeCN, CCl_4, TEA, 45 to 75 min, iv) 0.5 M $N_2H_4 \cdot H_2O$ in AcOH/Py (4:1), 0.5 to 4 hours.



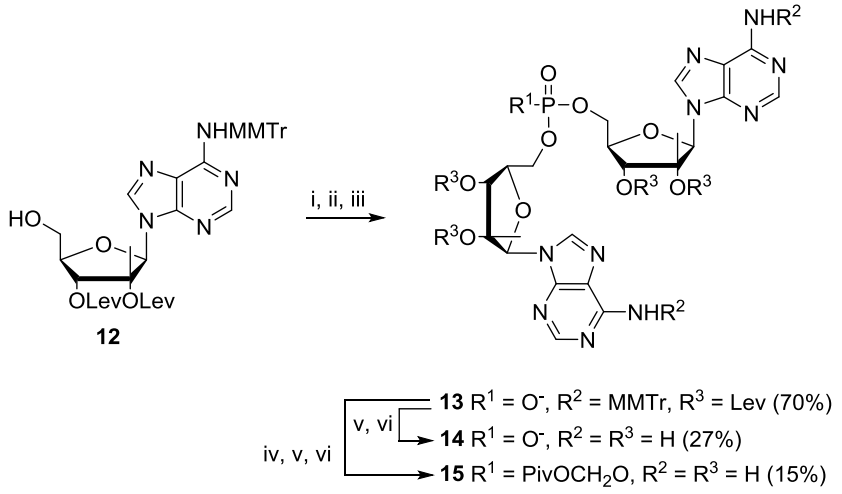
Scheme 18 Synthesis of compound **11**: i) diphenylphosphite, Py, 40 min, ii) compound **4** in Py, 2.5 h iii) L-alanine methyl ester in Py, MeCN, CCl_4, TEA, 60 min, iv) 0.5 M $N_2H_4 \cdot H_2O$ in AcOH/Py (4:1), 75 min, v) 80% aq. AcOH, overnight.

3.1.2 5´,5´-phosphodiesters and 3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)propyl and pivaloyloxymethyl protected 5´,5´-phosphodiesters (II)

A set of 5´5-phosphodiesters of 2´-*C*-methylnucleosides was synthesized. One of them, *viz.* bis(2´-*C*-methyladenosine) 5´5-phosphodiester was additionally protected with an esterase labile 3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)propyl or a pivaloyloxymethyl group. To allow 5´-phosphitylation, 2´-*C*-methyladenosine, 2´-*C*-methylguanosine and 2´-*C*-methyluridine were appropriately protected. With the purine nucleosides, the 5´-

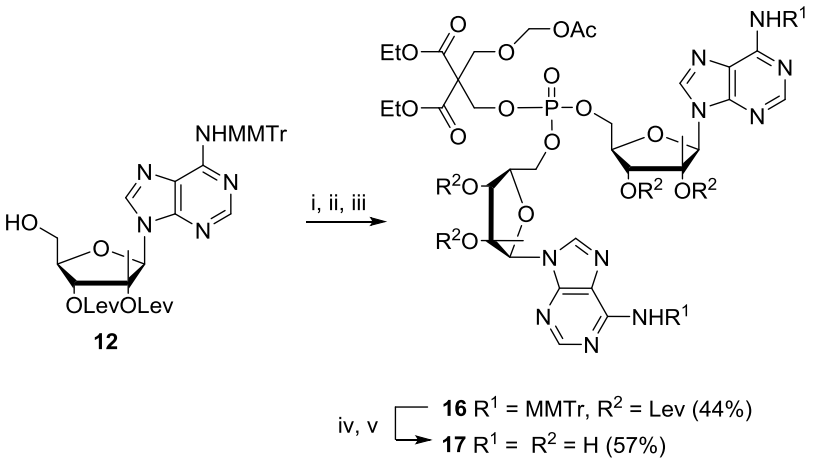
hydroxyl group was silylated and the base moiety amino groups monomethoxytritylated, as described above for **4**. With 2´-*C*-methyl uridine, the 5´-*O* was monomethoxytritylated. The 2´- and 3´-hydroxyl functions of 2´-*C*-methyladenosine and 2´-*C*-methyluridine were then protected with levulinoyl and benzoyl (Bz) groups, respectively. The 3´-hydroxyl function of 2´-*C*-methylguanosine was protected either with levulinoyl or benzoyl group, while the 2´-*O* was left unprotected. The 5´-*O*-protections were finally removed. The synthetic details are given in Schemes 1-3 of paper II.

The synthesis of 2´-*C*-methyladenosine derived di- and triesters are outlined in schemes 19 and 20. 2´,3´-Di-*O*-levulinoyl-N^6-(4-methoxytrityl)-2´-*C*-methyladenosine (**12**) was first phosphitylated with half equivalent of 1,1-dichloro-*N*,*N*-diisopropylphosphinamine in dry dichloromethane (DCM) and triethylamine (TEA) under nitrogen. To obtain bis[(2´,3´-di-*O*-levulinoyl-N^6-(4-methoxytrityl)-2´-*C*-methyladenosin-5´-yl] phosphate (**13**), the diisopropylamino group of the dinucleoside phosphoramidate was displaced with H_2O using 1*H*-tetrazole activation in MeCN and subsequent oxidation with iodine under aqueous conditions. The pivaloyloxymethyl group was introduced by reacting **13** with chloromethyl pivalate in *N*-methylpyrrolidone. The pivaloyloxymethyl bis(2´-*C*-methyladenosin-5´-yl) phosphate (**15**) was obtained in low yield (15%).



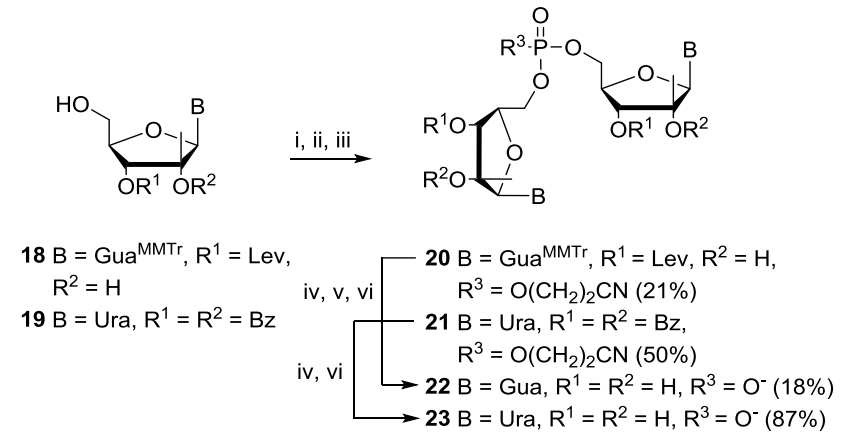
Scheme 19 Synthesis of compound **15**: i) iPr_2NPCl_2, TEA, DCM, 2 hours, ii) H_2O, 1*H*-tetrazole, MeCN, 75 min, iii) 0.15 M I_2 in a mixture of THF, H_2O and 2,6-lutidine (4:2:1), 2.5 hours, iv) $PivOCH_2Cl$, NMP, TEA, 60 °C, 4 days, v) H_2NNH_3OAc, DCM, MeOH, 18 hours, vi) 80% aq. AcOH, 24 hours.

To obtain 3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)propyl protected 5´,5´-phosphodiester **16**, the diisopropylamino group of the dinucleoside phosphoramidite was displaced with diethyl 2-acetyloxymethoxymethyl-2-hydroxymethylmalonate (**7b**) (Scheme 20). The product was then oxidized as above. The levulinoyl protections were removed with hydrazinium acetate in MeOH and the MMTr-protection in 80% aqueous AcOH. The 3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)propyl bis(2´-*C*-methyladenosiny-5´-yl) phosphate (**17**) was obtained in moderate yield (57%).

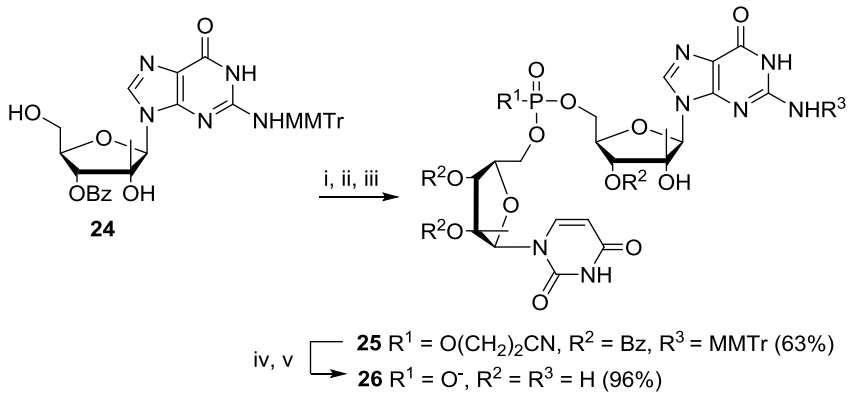


Scheme 20 Synthesis of compound **17**: i) iPr_2NPCl_2, TEA, DCM, 2 hours, ii) compound **7b**, 1*H*-tetrazole, MeCN, 60 min, iii) 0.15 M I_2 in a mixture of THF, H_2O and 2,6-lutidine (4:2:1), 2.5 hours, iv) H_2NNH_3OAc, DCM, MeOH, 23 hours, v) 80% aq. AcOH, 20 hours.

The 5´,5´-phosphodiesters of 2´-*C*-methylguanosine and 2´-*C*-methyluridine were synthesized as depicted in Schemes 21 and 22. A protected nucleoside was first phosphitylated with 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite, coupled with one equivalent of protected nucleoside and finally oxidized as previously. The levulinoyl and 2-cyanoethyl groups were removed with methanolic ammonia and the MMTr-protection with 80% aqueous AcOH. The ammonium ion was exchanged to sodium ion with strong cation exchange resin.



Scheme 21 Synthesis of compounds **22** and **23**: i) $NC(CH_2)_2O(iPr_2N)PCl$, TEA, DCM, 60 min, ii) compound **18** or **19**, 1*H*-tetrazole, MeCN, 1.5 to 2 hours, iii) 0.15 M I_2 in a mixture of THF, H_2O and 2,6-lutidine (4:2:1), 2.5 hours, iv) NH_3, MeOH, 20 hours, v) 80% aq. AcOH, 3 days vi) Dowex 50WX8 Na^+ form, H_2O.



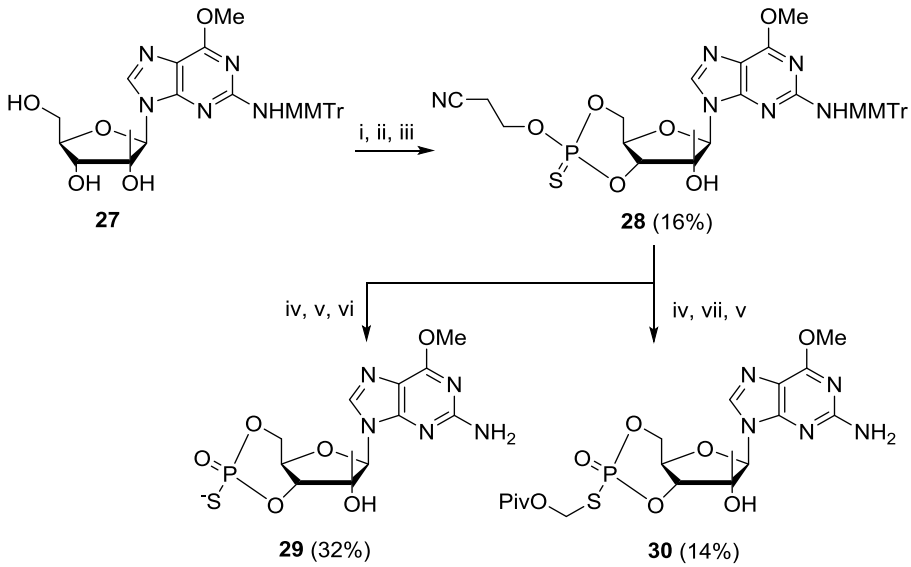
Scheme 22 Synthesis of compound **26**: i) $NC(CH_2)_2O(iPr_2N)PCl$, TEA, DCM, 60 minutes, ii) compound **19**, 1*H*-tetrazole, MeCN, 2 hours, iii) 0.15 M I_2 in a mixture of THF, H_2O and 2,6-lutidine (4:2:1), 2.5 hours, iv) NH_3, MeOH, 20 hours v) 80% aq. AcOH, overnight.

3.1.3 3´,5´-Cyclic phosphate and thiophosphate esters (III)

3´,5´-Cyclic phosphate and thiophosphate esters of antiviral nucleosides 2´-*C*-methylguanosine and 2´-*C*,O^6-dimethylguanosine were synthesized as potential prodrugs. 2´-*C*-Methylguanosine was prepared according to previously published

method by glycosylation of persilylated N^2-acetylguanine with 1,2,3,5-tetra-*O*-benzoyl-2-*C*-methylribofuranose.[134] 2´-*C*,O^6-Dimethylguanosine was prepared by using same glycosyl donor for glycosylation of 6-chloroguanine, followed by treatment with sodium methoxide in methanol.[135] The amino function was protected with 4-methoxytrityl group to allow selective 5´-*O*-phosphorylation. The 2´-hydroxyl function of the MMTr-protected 2´-*C*-methyl- and 2´-*C*,O^6-dimethylguanosine did not require a protecting group. The synthetic details are given in Scheme 1 of paper III.

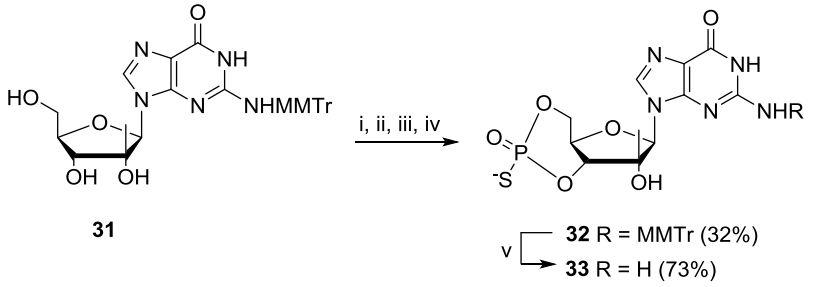
N^2-4-methoxytrityl-2´-*C*,O^6-dimethylguanosine (**27**) was phosphitylated with 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite and the 3´,5´-cyclic phosphite structure was formed using 1*H*-tetrazole activation in a large volume of MeCN (Scheme 23). The phosphite triester was sulfurized with elemental sulfur to form the thiophosphate ester **28**, which was obtained in low yield (16%). 2´-*C*,O^6-dimethylguanosine 3´,5´-cyclic phosphorothioate (**29**) was obtained by removal of the 2-cyanoethyl group in a mixture of TEA and DCM and subsequent detritylation with 80% aqueous AcOH. **29** was treated with iodomethyl pivalate in MeCN and MMTr-protection was removed with 80% aqueous AcOH to obtain 2´-*C*,O^6-dimethylguanosine cyclic 3´,5´-(*S*-[(pivaloyl)oxy-methyl] phosphorothioate) (**30**) in low yield (14%).



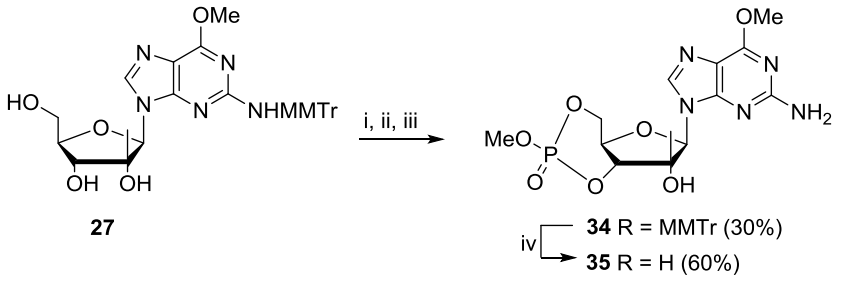
Scheme 23 Synthesis of compounds **29** and **30**: i) $NC(CH_2)_2O(iPr_2N)PCl$, TEA, DCM, 20-40 min, ii) 1*H*-tetrazole, MeCN, 2.5 hours, iii) S_8, Py, overnight, iv) TEA, DCM, 18 hours, v) 80% aq. AcOH, 5 hours, vi) Dowex 50WX8 Na^+ form, H_2O, vii) iodomethyl pivalate, MeCN, 2.5 hours.

2´-*C*-Methylguanosine 3´,5´-cyclic phosphorothioate (**33**) was prepared using a somewhat different method , *i. e.* by sulfurizing the 3´,5´-cyclic phosphite triester with 3*H*-1,2-benzodithiol-3-one 1,1-dioxide in MeCN with slightly better yield (32%) (Scheme 24). The 2-cyanoethyl group was cleaved during silica gel purification with DCM containing 1% of TEA. Detritylation with 80% aqueous AcOH gave **33** as triethylammonium salt.

To obtain 2´-*C*,O^6-dimethylguanosine 3´,5´-cyclic (methyl phosphate) (**35**), N^2-(4-methoxytrityl)-2´-*C*,O^6-dimethylguanosine (**27**) was phosphitylated with 1-chloro-*N*,*N*-diisopropyl-1-methoxyphosphinamine and the 3´-hydroxyl group was then displaced as described above (Scheme 25). Oxidation with iodine and detritylation in 80% aqueous AcOH gave triester **35** in moderate yield (60%).



Scheme 24 Synthesis of compound **33**: i) $NC(CH_2)_2(iPr_2N)PCl$, TEA, DCM, 40 min, ii) 1*H*-tetrazole, MeCN, 3 hours, iii) 3*H*-1,2-benzodithiol-3-one 1,1-dioxide, MeCN, overnight iv) SiO_2 chromatography with 15% MeOH and 0.5% TEA in DCM, v) 80% aq. AcOH.

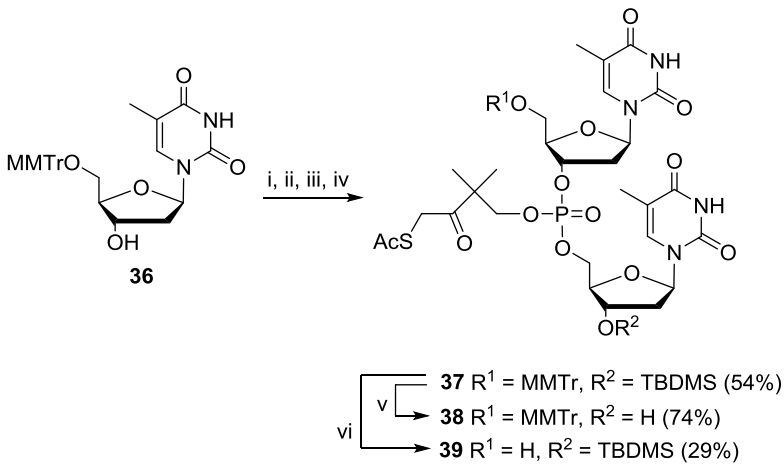


Scheme 25 Synthesis of compound **35**: i) $(iPr_2N)MeOPCl$, TEA, DCM, 30 min, ii) 1*H*-tetrazole, MeCN, 2 hours, iii) 0.15 M I_2 in a mixture of THF, H_2O and 2,6-lutidine (4:2:1), 16 hours, iv) 80% aq. AcOH, 24 hours.

3.1.4 4-Acetylthio-2,2-dimethyl-3-oxobutyl protected oligomeric phosphodiesters (IV)

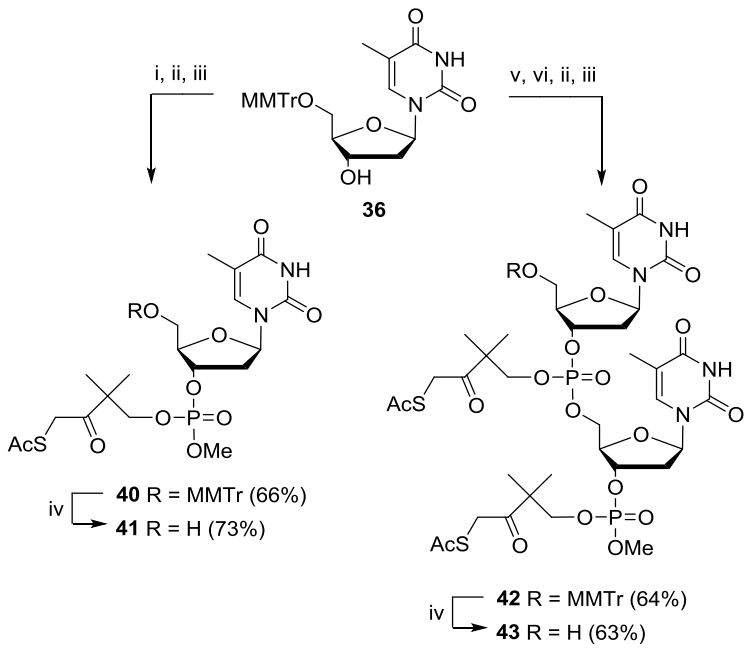
Thymidine trimer and tetramer, bearing three 4-acetylthio-2,2-dimethyl-3-oxobutyl moieties as esterase- and thermolabile phosphodiester protecting groups, were synthesized. The synthesis of *S*-(4-hydroxy-3,3-dimethyl-2-oxobutyl)ethanethioate has been previously published.[48]

Nucleotide building blocks **38** and **39** for the assembly of thymidine tetramer were synthesized as outlined in Scheme 26. 5´-*O*-(4-methoxytrityl)thymidine (**36**) was phosphitylated with 1-chloro-*N*,*N*,*N*´,*N*´-tetraisopropylphosphanediamine and the diisopropylamino ligands were displaced in a stepwise manner with 3´-(*tert*-butyldimethylsilyl)thymidine and *S*-(4-hydroxy-3,3-dimethyl-2-oxobutyl)ethanethioate in MeCN using 1*H*-tetrazole activation. Oxidation with iodine in aqueous conditions gave dimer **37** in moderate yield (54%). The *tert*-butyldimethylsilyl group was removed with tetrabutylammonium fluoride to obtain 5´-*O*-(4-methoxytrityl)thymidine-3´-yl thymidine-5´-yl 4-acetylthio-2,2-dimethyl-3-oxobutyl phosphate (**38**) and the 4-methoxytrityl group with 80% aqueous acetic acid to obtain thymidine-3´-yl 3´-*O*-(*tert*-butyldimethylsilyl)thymidine-5´-yl 4-acetylthio-2,2-dimethyl-3-oxobutyl phosphate (**39**).



Scheme 26 Synthesis of compounds **38** and **39**: i) $(iPr_2N)_2PCl$, TEA, DCM, 15 min, ii) 3´-*O*-TBDMS-thymidine, 1*H*-tetrazole, MeCN, 30 min, iii) *S*-(4-hydroxy-3,3-dimethyl-2-oxobutyl)ethanethioate, 1*H*-tetrazole, MeCN, 70 min, iv) 0.15 M I_2 in a mixture of THF, H_2O and 2,6-lutidine (4:2:1), overnight, v) TBAF, THF, AcOH, 4 days, vi) 80% aq. AcOH.

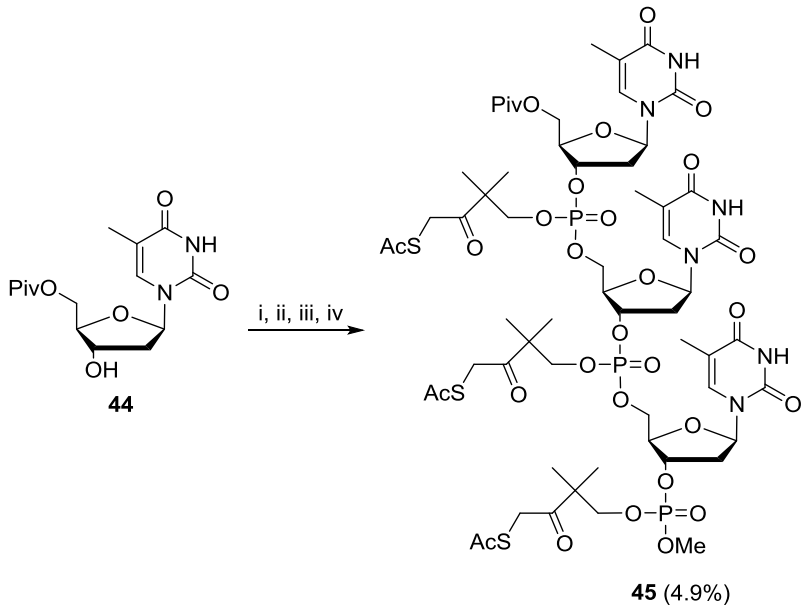
Nucleotide building blocks for the assembly of thymidine trimer were synthesized as illustrated in Scheme 27. 5´-*O*- (4-methoxytrityl)thymidine (**36**) was phosphitylated with 1-chloro-*N*,*N*-diisopropyl-1-methoxyphosphinamine and *S*-(4-hydroxy-3,3-dimethyl-2-oxobutyl)ethanethioate in MeCN was coupled using 1*H*-tetrazole activation. Oxidation with iodine in aqueous conditions gave nucleotide **40**, which was detritylated in 80% aqueous AcOH to give **41**. To form 4-acetylthio-2,2-dimethyl-3-oxobutyl protected dimer **43**, nucleoside **36** was first phosphitylated with 1-chloro-*N*,*N*,*N*´,*N*´-tetraisopropyl-phosphanediamine. The diisopropylamino ligands were then displaced with nucleotide **41** and *S*-(4-hydroxy-3,3-dimethyl-2-oxobutyl)ethanethioate using 1*H*-tetrazole activation and finally oxidized. MMTr-protection was removed with 80% aqueous AcOH.



Scheme 27 Synthesis of compounds **41** and **43**: i) (iPr_2N)MeOPCl, TEA, DCM, 30 min, ii) ethyl 4-(acetylthio)-2-(hydroxymethyl)-2-methyl-3-oxobutanoate, 1*H*-tetrazole, MeCN, 2 hours, iii) 0.15 M I_2 in a mixture of THF, H_2O and 2,6-lutidine (4:2:1), overnight, iv) 80% aq. AcOH, v) $(iPr_2)_2PCl$, TEA, DCM, 30 min, vi) compound **41,** 1*H*-tetrazole, MeCN, 1.5 hours.

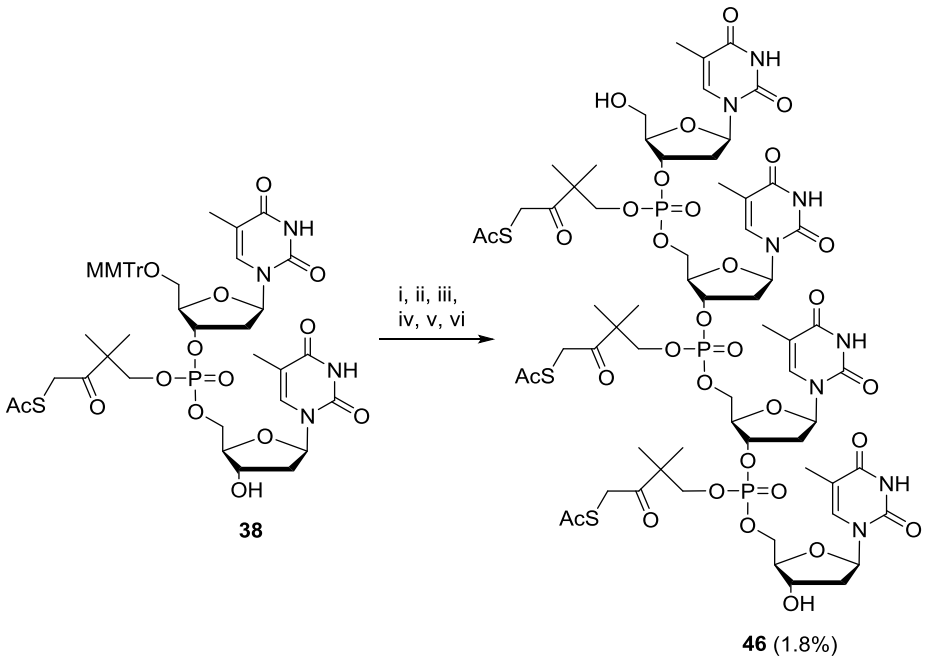
The synthesis of 4-acetylthio-2,2-dimethyl-3-oxobutyl protected phosphotriester **45** is presented in Scheme 28. 5´-*O*-Pivaloylthymidine (**44**) was first phosphitylated with 1-chloro-*N*,*N*,*N*´,*N*´-tetraisopropylphosphanediamine, then

coupled with dimer **43** and *S*-(4-hydroxy-3,3-dimethyl-2-oxobutyl)ethanethioate and finally oxidized with iodine in aqueous conditions.



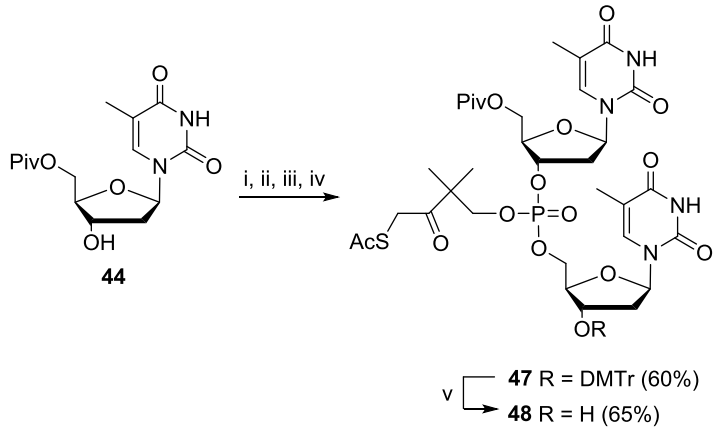
Scheme 28 Synthesis of compound **45**: i) $(iPr_2N)_2PCl$, TEA, DCM, 30 min, ii) compound **43**, 1*H*-tetrazole, MeCN, 1.5 hours, iii) *S*-(4-hydroxy-3,3-dimethyl-2-oxobutyl)ethanethioate, 1*H*-tetrazole, MeCN, 1.5 hours, iv) 0.15 M I_2 in a mixture of THF, H_2O and 2,6-lutidine (4:2:1), overnight.

To obtain 4-acetylthio-2,2-dimethyl-3-oxobutyl protected phosphotriester **46**, the nucleotide building block **38** was first phosphitylated with tris(diethylamino)phosphine using 1*H*-tetrazole as activator (Scheme 29). The diethylamino ligands were stepwise displaced with building block **39** and *S*-(4-hydroxy-3,3-dimethyl-2-oxobutyl)ethanethioate and oxidized. Desilylation with tetrabutylammonium fluoride and detritylation with 80% aqueous AcOH gave **46**.



Scheme 29 Synthesis of compound **46**: i) $(Et_2N)_3P$, 1*H*-tetrazole, MeCN, 3 hours, ii) compound **39**, 1*H*-tetrazole, MeCN, 1.5 hours, iii) *S*-(4-hydroxy-3,3-dimethyl-2-oxobutyl)ethanethioate, 1*H*-tetrazole, MeCN, 1.5 hours, iv) 0.15 M I_2 in a mixture of THF, H_2O and 2,6-lutidine (4:2:1), overnight, v) TBAF, THF, AcOH, 3 days, vi) 80% aq. AcOH, 22 h.

Synthesis of 4-acetylthio-2,2-dimethyl-3-oxobutyl 5´-O-pivaloylthymidin-3´-yl thymidine-5´-yl phosphate (**48**) is outlined in Scheme 30. 5´-*O*-Pivaloylthymidine (**44**) was phosphitylated with 1-chloro-*N*,*N*,*N*´,*N*´-tetraisopropylphosphanediamine and the diisopropylamine groups were displaced with 3´-*O*-(4,4´-dimehoxytrityl)thymidine and *S*-(4-hydroxy-3,3-dimethyl-2-oxobutyl)ethanethioate using 1*H*-tetrazole activation. The oxidation of the phosphite triester to phosphate triester was done as above with iodine.



Scheme 30 Synthesis of compound **48**: i) $(iPr_2N)_2PCl$, TEA, DCM, 40 min, ii) 3´-*O*-(4,4´-dimethoxytrityl)thymidine, 1*H*-tetrazole, MeCN, 30 min, iii) *S*-(4-hydroxy-3,3-dimethyl-2-oxobutyl)ethanethioate, 1*H*-tetrazole, MeCN, 60 min, iv) 0.15 M I_2 in a mixture of THF, H_2O and 2,6-lutidine (4:2:1), overnight, v) 80% aq. AcOH, 4 hours.

3.2 Kinetic studies

The synthesized 5´,5´-phosphodiesters and esterase labile 5´,5´-phosphotriesters as well as the 3´,5´-cyclic phosphate and thiophosphate esters have been further studied by Alios BioPharma. The data is owned by the company and is not discussed here. Kinetic measurements for the synthesized 2,2-bissubstituted 3-acyloxypropyl protected 5´-phosphoramidates and 4-acetylthio-2,2-dimethyl-3-oxobutyl protected oligomeric phosphodiesters are discussed below.

3.2.1 2,2-Bissubstituted 3-acyloxypropyl protected 5´-phosphoramidates (I)

3.2.1.1 Chemical stability

The non-enzymatic hydrolysis of 3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl substituted phosphoramidates was studied using the 2´-*O*-methylcytidine derivative **11** as a model compound. The hydrolysis reaction was followed at pH 7.5, 9.0 and 10.0 at 37 °C. The product composition of the samples taken from the reaction mixture at appropriate time intervals was determined by HPLC and mass spectrometric analysis.

The non-enzymatic hydrolysis begins with parallel formation of 2´-*O*-methylcytidine 5´-{*N*-[(*S*)-2-methoxy-1-methyl-2-oxoethyl]phosphoramidate} (**51**) and 2´-*O*-methylcytidine 5´-{*O*-[3-acetyloxy-2,2-bis(ethoxycarbonyl)-propyl-*N*-[(*S*)-2-hydroxy-1-methyl-2-oxoethyl]phosphoramidate} (**50**), as seen from the time-dependent product distribution obtained at pH 10.0 and 37 °C (Figure 13). Both intermediates are then converted to 2´-*O*-methylcytidine 5´-{*N*-[(*S*)-2-hydroxy-1-methyl-2-oxoethyl]phosphoramidate} (**53**).

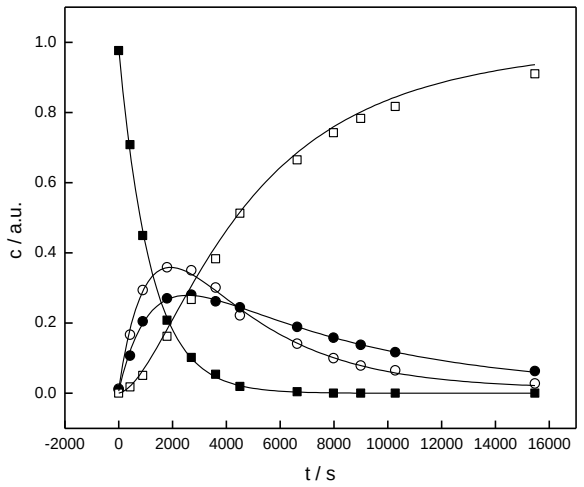
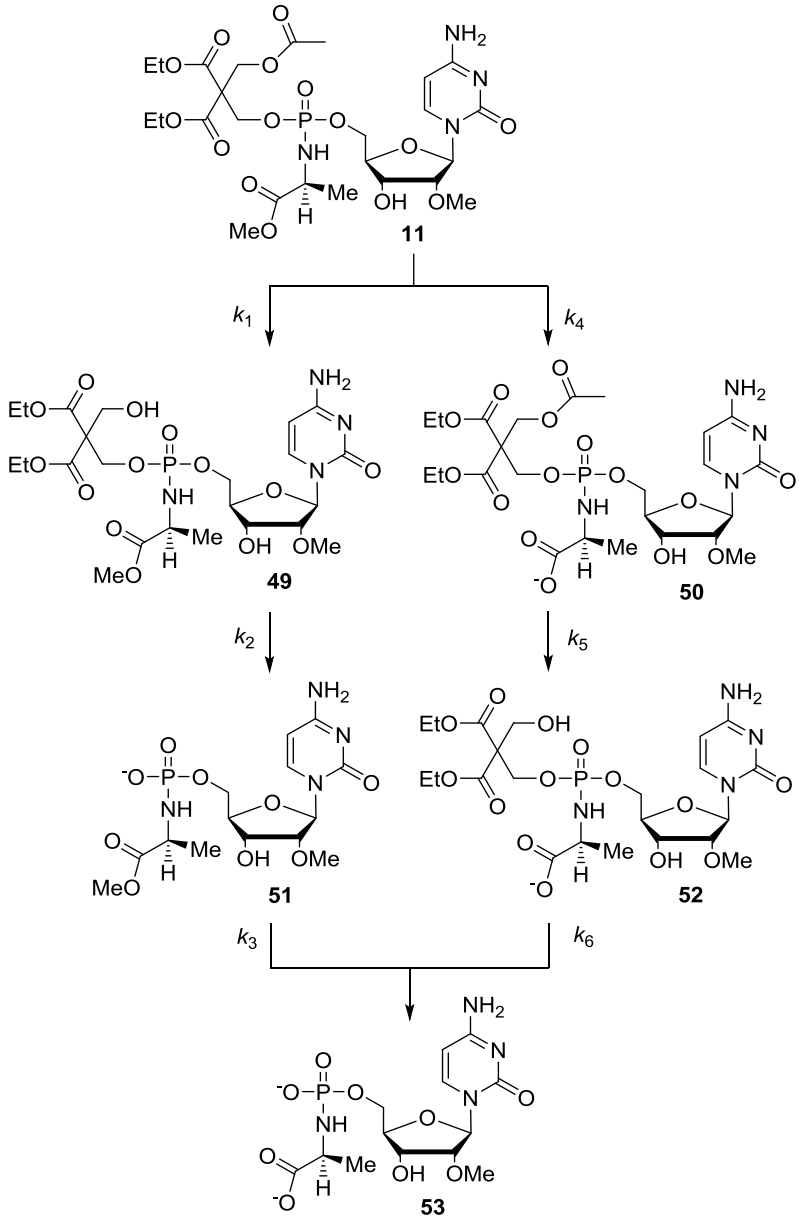


Figure 13 Time-dependent product distribution for the non-enzymatic hydrolysis of **11** at pH 10.0 (0.06 mol L^{-1} glycine buffer) and 37 °C. (■) **11**, (●) **51**, (○) **50**, (□) **53**. For the structures, see Scheme 31.

Scheme 31 represents the two most probable pathways for the non-enzymatic hydrolysis of **11**. The acetyl group of 3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl group is hydrolyzed to give **49**, which is not accumulated, but is rapidly converted to **51** via retro-aldol condensation and subsequent elimination (see also Scheme 2 on page 27). The final product **53** is then obtained from **51** by hydrolysis of the methyl ester of the alaninyl moiety. Alternatively, the methyl ester of the alaninyl moiety is hydrolyzed first to give **50** and deacetylation of the 3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl group to **52** follows. Again, intermediate **52** is not accumulated, but rapidly forms **53** by retro-aldol condensation and subsequent phosphate elimination.



Scheme 31 Non-enzymatic hydrolysis of 2´-*O*-methylcytidine 5´-{*O*-[3-acetyloxy-2,2-bis(ethoxycarbonyl)-propyl-*N*-[(*S*)-2-methoxy-1-methyl-2-oxoethyl]phosphoramidate} (**11**).

Table 2 compiles the obtained first-order rate constants and half-lives for the parallel consecutive reactions. The first-order rate constant k_d (= k_1 + k_4) for the disappearance of **11** was calculated by the integrated first-order rate law. Rate constants k_1, k_3, k_4, and k_5 were then determined from the time-dependent concentrations of **50** and **51** by applying the rate law of parallel consecutive first-order reactions (equations 1 and 2).

$$[50] = k_4 \frac{e^{-k_d t} - e^{-k_5 t}}{k_5 - k_d} [11]_0 \qquad (1)$$

$$[51] = k_1 \frac{e^{-k_d t} - e^{-k_3 t}}{k_3 - k_d} [11]_0 \qquad (2)$$

Rate constant k_2 was obtained by removing the acetyl group of **11** with porcine liver esterase (PLE) prior to kinetic run and applying then the first-order rate law. Rate constant k_6 is assumed to be approximately the same as k_2, based on the assumption that the lack of methyl group in **52** most likely has only a minor effect on the rate of the elimination step.

Table 2 First-order rate constants (k) and half-lives ($\tau_{½}$) for the partial reactions in the non-enzymatic hydrolysis of **11** to **53** at pH 7.5, 9.0 and 10.0 at 37 °C.

	k (pH 7.5)	$\tau_{½}$	k (pH 9.0)	$\tau_{½}$	k (pH 10.0)	$\tau_{½}$
k_d / (10^{-5} s^{-1})*	0.32 ± 0.02	60.0 h	8.9 ± 0.3	2.0 h	84 ± 2	14 min
k_1 / (10^{-5} s^{-1})	-	-	3.5 ± 0.1	5.5 h	33.0 ± 0.6	35 min
k_2 / (10^{-5} s^{-1})	67 ± 1	17 min	-	-	-	-
k_3 / (10^{-5} s^{-1})	-	-	2.0 ± 0.1	9.6 h	13.9 ± 0.4	1.4 h
k_4 / (10^{-5} s^{-1})	-	-	5.4 ± 0.2	3.6 h	51 ± 1	23 min
k_5 / (10^{-5} s^{-1})	-	-	3.4 ± 0.4	8.2 h	29 ± 1	40 min

* $k_d = k_1 + k_4$

For comparison, the non-enzymatic hydrolysis of the phenyl analog **6** was studied at pH 7.5 and 37 °C. The time-dependent product distribution of the reaction mixture is presented in Figure 14. The first-order rate constant for the hydrolysis of **6** is (3.1 ± 0.1) × 10-6 s^{-1}.

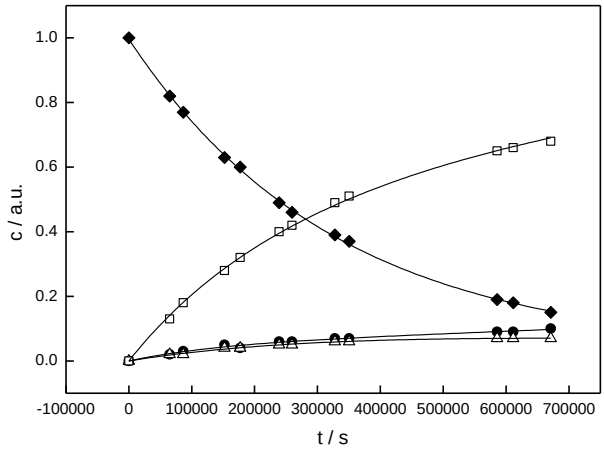
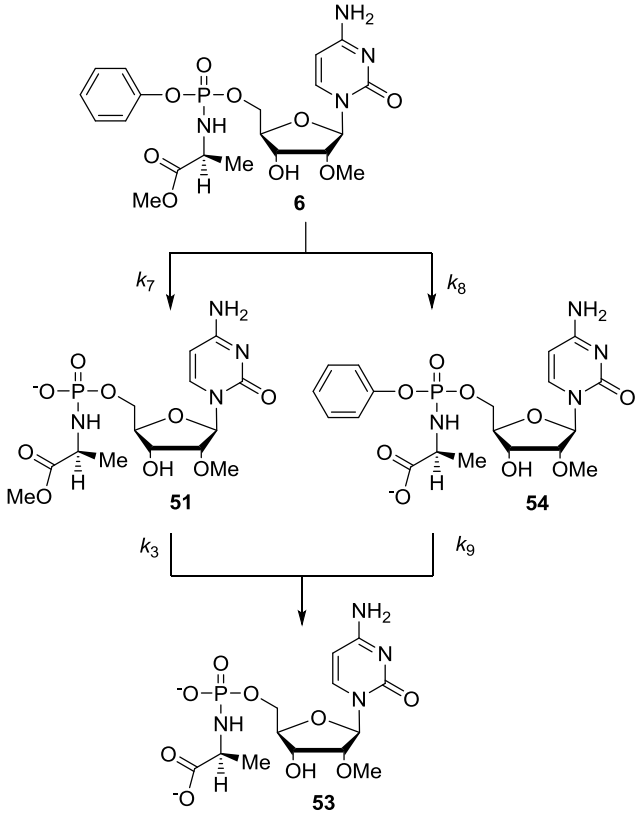


Figure 14 Time-dependent product distribution for the non-enzymatic hydrolysis of **6** at pH 7.5 (0.06 M HEPES buffer) and 37 °C: (◆) **6**, (●) **51**, (△) **54**, (□) **53**. For the structures see Scheme 32.

The possible degradation pathways of **6** are presented in Scheme 32. Two parallel reactions may occur: either the methyl ester is hydrolyzed to give **54** followed by displacement of the phenoxy group by assistance of the alanine carboxylate group or the hydroxide ion first displaces the phenoxy group to give **51** and the methyl ester hydrolysis then follows to give the final product **53**. The intermediates **51** and **54** do not accumulate significantly. Previous studies with the corresponding thymidine analog indicate that hydrolysis via **54** is one order of magnitude faster than via **51**.[136] This most likely applies to **6** too. Since the rate constant, k_3, for the conversion of **51** to **53** is of the order of 10^{-5} s^{-1} at pH 9.0 (Table 2), the rate constant k_3 at 30-fold lower hydroxide ion concentration at pH 7.5 may be estimated to be smaller than 10^{-6} s^{-1} and hence, the hydrolysis through intermediate **51** is too slow to be the main reaction pathway.



Scheme 32 Non-enzymatic hydrolysis of 2´-*O*-methylcytidine 5´-{*O*-phenyl-*N*-[(*S*)-2-methoxy-1-methyl-2-oxoethyl]phosphoramidate} (**6**).

3.2.1.2 Enzymatic stability

The enzymatic stability of compounds **6** and **11** was studied with PLE (26 U mL^{-1}) in HEPES buffer at pH 7.5 and 35 ºC. The enzymatic removal of the protecting groups from **11** can again take place through intermediates **49** and **50** (Figure 15 A). The predominant reaction is formation of *N*-[2-methoxy-1-methyl-2-oxoethyl)phosphoramidate **51** through **49**. The hydrolysis of the alanine ester bond to produce **50** represents about 20% of the overall disappearance of **11**. In addition, the formation of **53** is observed. The half-life for the disappearance of **11** is 5.7 h [$k = (3.4 \pm 0.2) \times 10^{-5}\ s^{-1}$]. Of the two diastereomers, R_P-**11** and S_P-**11**, one seems to react slightly faster than the other, but which one, cannot be decided on the basis of the data available. The only reaction detected with **6** is the conversion to **53** with the half-life of 43 hours [$k = (4.5 \pm 0.4) \times 10^{-6}$ s^{-1}) (Figure 15 B). Thus, the conversion of **6** to **53** is an order of magnitude

slower than the conversion of **11** to **51**. As in case of **11**, the diastereomers of **6** react at somewhat different rate.

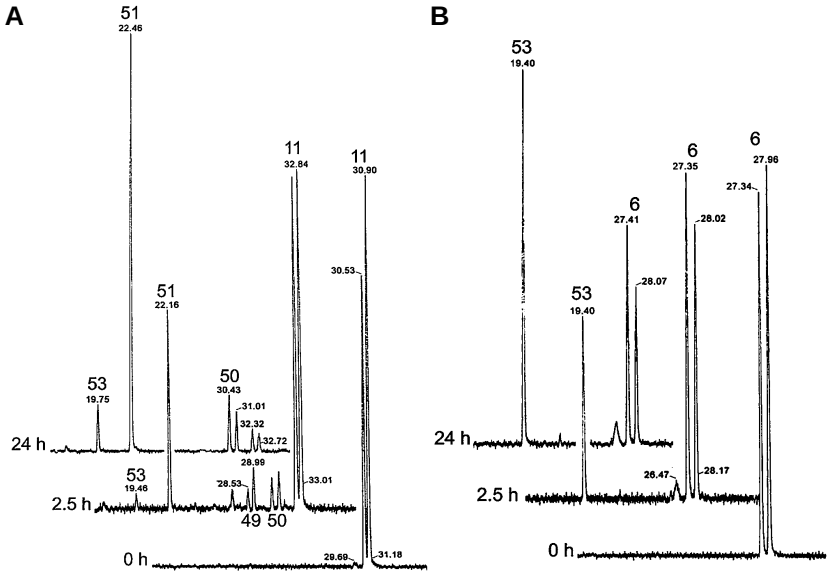


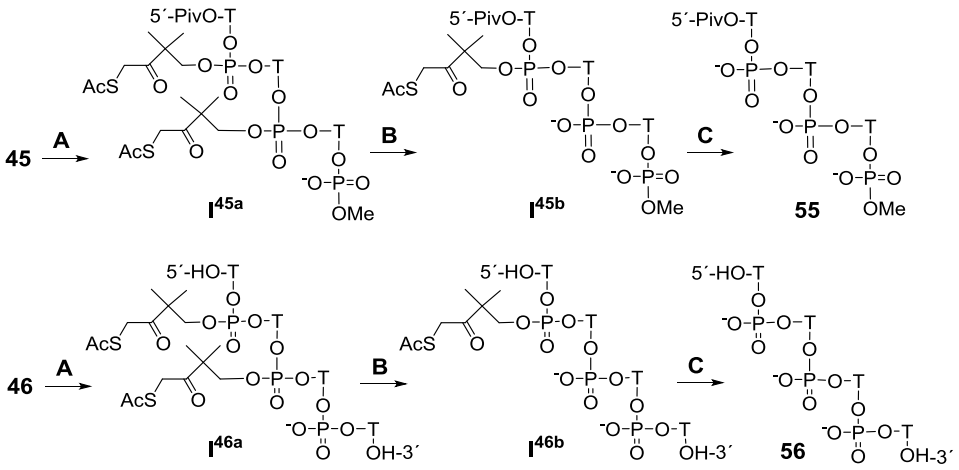
Figure 15 RP-HPLC traces for the PLE-catalyzed (26 U mL^{-1}) hydrolysis of **11** to **53** (A) and **6** to **53** (B) in HEPES buffer (20 mmol, pH 7.5, I = 0.1 mol L^{-1} with NaCl) at 35 °C. For the structures see Schemes 31 and 32.

The removal of the protecting groups was also studied at a lower PLE concentration (1 U mL^{-1}) and in prostate carcinoma cell extract (PC3) at pH 7.5 and 35 ºC. 36% of **9a** was degraded after incubation for one day at this low PLE concentration. With **9b**, 78% of the starting material was degraded in six hours under similar conditions. The enzymatic removal of the 3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)propyl protecting group from **9b** is thus at least one order of magnitude faster than the removal of the 3-acetyloxy-2,2-bis(ethoxycarbonyl)-propyl protecting group from **9a**. In PC3 cell extract, compounds **9a** and **11** degraded at similar rate: 48% and 55% of the compounds were degraded after one day, respectively.

3.2.2 4-Acetylthio-2,2-dimethyl-3-oxobutyl protected oligomeric phosphodiesters (IV)

3.2.2.1 Chemical stability

The non-enzymatic hydrolysis of 4-acetylthio-2,2-dimethyl-3-oxobutyl protected oligomeric phosphotriesters **45** and **46** was followed in HEPES buffer at pH 7.5 and 37 °C (for the mechanism see Scheme 12 on page 37). The mixtures of eight possible R_P/S_P-diastereomers were fractionated by HPLC to simplify the product distribution. For both compounds, a fraction showing a single chromatographic signal was selected for the measurements. The products were identified by spiking with authentic samples, by HPLC/ESI-MS analysis or by collecting a signal and identifying it by mass analysis. No attempt was made to find out which of the three possible diester intermediates were accumulated in each of the consecutive steps (Scheme 33).



Scheme 33 Deprotection of phophotiesters **45** and **46** to phosphodiesters **55** and **56** through intermediate mixtures **I**.

The departure of the first 4-acetylthio-2,2-dimethyl-3-oxobutyl group from **45** gave a mixture of diprotected intermediates $\mathbf{I}^{45a}$ (Reaction A in Scheme 33). The half-life for the disappearance of **45** was 7.8 hours [$k = (2.5 \pm 0.1) \times 10^{-5}\ s^{-1}$]. The half-life for the next conversion to monoprotected intermediates $\mathbf{I}^{45b}$ (Reaction B) was 10.7 hours [$k = (1.8 \pm 0.2) \times 10^{-5}\ s^{-1}$]. Removal of the third protecting group gave the desired final product **55** (Reaction C). The diastereomers react with somewhat different rates as seen from Figure 16. The removal of the last

protecting group from the slowest eluting isomer in intermediate mixture $\mathbf{I}^{\mathbf{45b}}$ is slower than the removal from the two faster eluting isomers. Accordingly, the microenvironment seems to have some effect on the rate of the removal of the protecting group by cyclization.

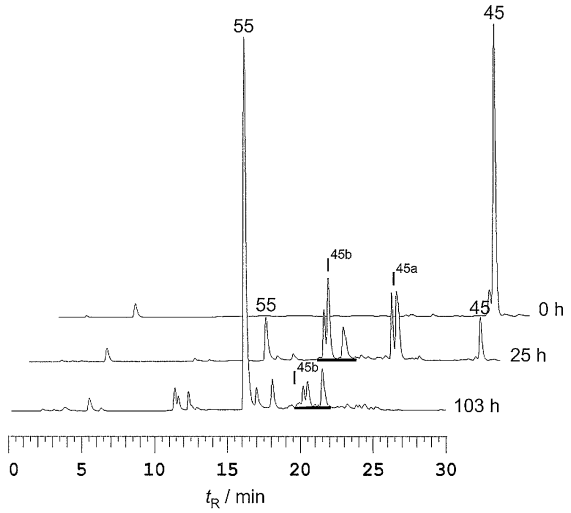


Figure 16 RP-HPLC traces for the hydrolysis of **45** to **55** in HEPES buffer (20 mmol, pH 7.5, I = 0.1 mol L^{-1} with NaCl)) at 37 °C.

The reaction of oligomer **46** proceeds similarly through mono- and dideprotected intermediate mixtures $\mathbf{I}^{\mathbf{46a}}$ and $\mathbf{I}^{\mathbf{46b}}$ to produce the fully deprotected final product **56** (Scheme 33 and Figure 17). The half-lives for the disappearance of **46** and $\mathbf{I}^{\mathbf{46a}}$ were 6.2 hours [$k = (3.1 \pm 0.2) \times 10^{-5} s^{-1}$] and 7.2 hours [$k = (2.7 \pm 1.0) \times 10^{-5} s^{-1}$], respectively. More than 70% of **45** and **46** were converted to the unprotected phosphodiesters **55** and **56**, respectively, in 4 days. In the reaction of **45**, only small amount of side products were detected by MS analysis The identified side products included TpT, PivO-TpTpTp, PivO-TpTpT, PivO-TpT, PivO-Tp-$CH_2C(Me_2)C(O)CH_2SAc$. The amount of side products was somewhat more marked with **46**, including TpT and Tp-$CH_2C(Me_2)C(O)CH_2SAc$, but all of them were not characterized.

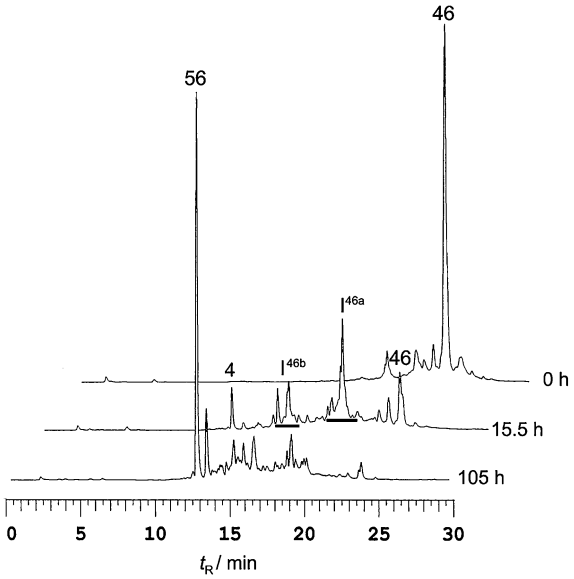


Figure 17 RP-HPLC traces for the hydrolysis of **46** to **56** in HEPES buffer (20 mmol, pH 7.5, I = 0.1 mol L^{-1} with NaCl)) at 37 °C.

The possibility of chain cleavage by P-O bond fission at a 4-acetylthio-2,2-dimethyl-3-oxobutyl protected phosphodiester linkage was also studied. The occurrence of such cleavage had been earlier detected with 4-benzoylthio-2,2-dimethyl-3-oxobutyl or 2-ethoxycarbonyl-2-methyl-3-oxo-4-pivaloylthiobutyl protected phosphodiester bonds.[48] 4-Acetylthio-2,2-dimethyl-3-oxobutyl 5´-*O*-pivaloylthymidin-3´-yl thymidine-5´-yl phosphate (**48**) was used as a model compound. Figure 18 shows that although the formation 5´-*O*-pivaloylthymidylyl-3´,5´-thymidine (Piv-TpT) was the predominant reaction, also thymidine and 5´-*O*-pivaloylthymidine (**44**) were formed. These two compounds constituted about 9% of the final products. The signal at 20.2 min most likely refer to 5´-(4-acetylthio-2,2-dimethyl-3-oxobutyl)phosphate and 5´-pivaloylthymidine 3´-(4-acetylthio-2,2-dimethyl-3-oxobutyl)phosphate, but this could not be verified by MS. The half-lives for the disappearance of the diastereomers of **48** were 9.9 h [k = (1.94 ± 0.02) × 10^{-5} s^{-1}] and 10.2 h [k = (1.88 ± 0.03) × 10^{-5} s^{-1}].

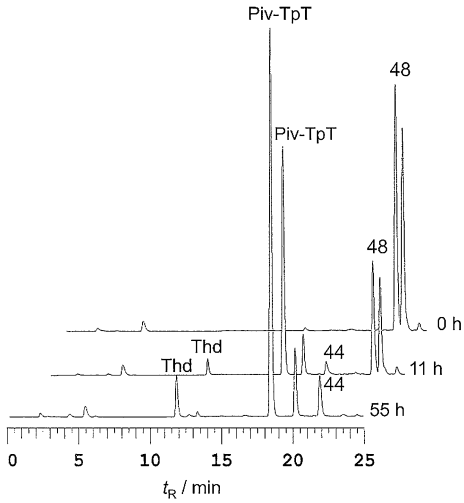


Figure 18 RP-HPLC traces for the hydrolysis of **48** in HEPES buffer (20 mmol, pH 7.5, I = 0.1 mol L^{-1} with NaCl)) at 37 °C.

3.2.2.2 Enzymatic stability

To study the enzymatic stability of phosphotriesters **45** and **46**, the removal of 4-acetylthio-2,2-dimethyl-3-oxobutyl group was followed in the presence of porcine liver esterase (2.6 U mL^{-1}) in HEPES buffer at pH 7.5 and 37 °C (Figures 19 and 20). Phosphotriester **45** was converted to phosphodiester **55** in two days. The half-life for the disappearance of **45** was 2.7 minutes [$k = (4.3 \pm 0.3) \times 10^{-3}$ s^{-1}]. The identified side products included TpTpMe, pTpTpMe and PivO-TpT. The enzymatically activated deprotection most likely proceeds through deacetylated intermediates, but these did not accumulate. An interchain disulfide bond formation competed as side reaction with the chemical removal of the remnants of the protecting group, lending support for the formation of deacetylated intermediates. The S-S bond mediated dimerization was observed with both phosphotriesters. The conversion of **46** to **56** proceeds as with **45**, but it is somewhat slower. The half-life for the disappearance of **46** was 36 minutes [$k = (3.2 \pm 0.1) \times 10^{-4}$ s^{-1}] and the deprotection to **56** was not complete even after 5 days. A considerable amount of an unknown byproduct was formed at t_R = 12.8 (Figure 20). The deprotection of dimer **48** produced 5´-*O*-pivaloylthymidylyl-3´,5´-thymidine quantitatively. The half-lives for the enzymatic deacetylation of the two diastereomers of dimer **48** were 1.21 min [$k = (9.5 \pm 0.5) \times 10^{-3}$ s^{-1}] and 1.7 min [$k = (6.7 \pm 0.6) \times 10^{-3}$ s^{-1}].

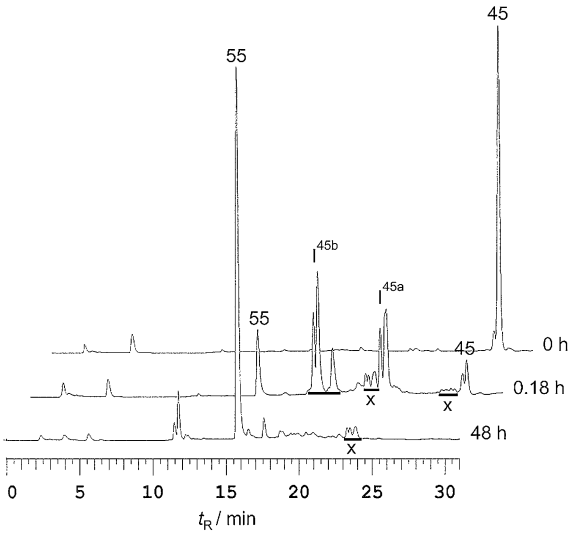


Figure 19 RP-HPLC traces for the enzymatic hydrolysis of **45** to **55** in PLE (2.6 U mL^{-1}) containing HEPES buffer (20 mmol, pH 7.5, I = 0.1 mol L^{-1} with NaCl)) at 37 °C. Signals marked with **X** refer to disulfide products.

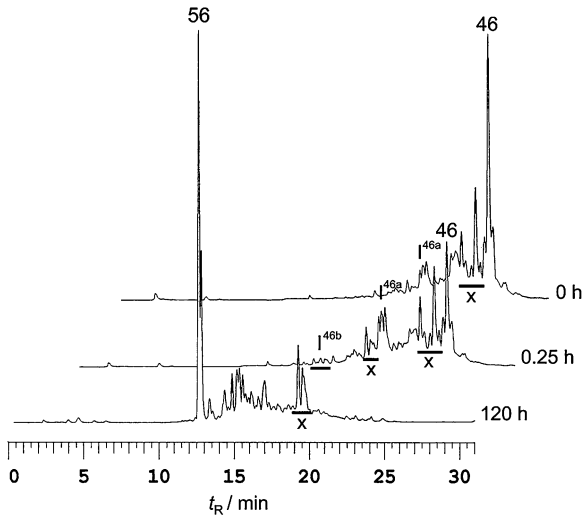


Figure 20 RP-HPLC traces for the enzymatic hydrolysis of **46** to **56** in PLE (2.6 U mL^{-1}) containing HEPES buffer (20 mmol, pH 7.5, I = 0.1 mol L^{-1} with NaCl) at 37 °C. Signals marked with **X** refer to disulfide products.

4 DISCUSSION

4.1 Background

The phosphate protecting groups studied in this thesis include 3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl,[47] 3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)-propyl[47] and 4-acetylthio-2,2-dimethyl-3-oxobutyl[48] groups. The removal of the protecting groups is triggered by carboxyesterase activity. With the 3-acetyloxy-2,2-bis(ethoxycarbonyl)group, the remnant of the enzymatically deacetylated group is removed by retro-aldol condensation and subsequent elimination (see Scheme 2). With its 3-acetyloxymethoxy analog, hydrolytic removal of the hydroxymethyl group precedes the retro-aldol condensation. The removal rate of these protecting groups can be altered in two ways.[137] Firstly, the nature of the acyl group determines the rate of the enzymatic deprotection step: bulky acyl groups are removed slower than simple ones. The stability of the deacylated product may, in turn, be tuned by the polarity of the substituents at C2: substituents that allow charge delocalization facilitate the departure of the protecting group. In case the chemical removal is considerably slower than the enzymatic deacylation, doubly protected monophosphate group may become fully deacylated before the departure of the remnants of either group. This helps to avoid the inhibition of enzymatic deacylation by accumulation of negative charge on the phosphate.

Another useful feature of the 3-acetyloxy- and 3-acetyloxymethoxy-bis(ethoxycarbonyl)propyl groups is that conjugate groups aimed at targeting the prodrug to a certain cell type or enhancing cellular uptake may be attached to C2 by ester or amide linkages without interfering with the retro-aldol condensation. The drawback of these groups is that they, upon deprotection, produce enones that are somewhat alkylating; formation of glutathione adducts has been reported.[138]

The enzymatic deacetylation of 4-acetylthio-2,2-dimethyl-3-oxobutyl group is, in turn, followed by intramolecular attack of the exposed mercapto group on C1 of the group, resulting in departure by cyclization. The group is not only esterase labile but additionally thermally removable at 37 °C.[48] In case the enzymatic deprotection slows down, the protecting group is released by intramolecular cyclization to a substituted tetrahydrothiophenone. As mentioned above, the rate of this process may be tuned by the electronegativity of the 2-substituents. This enzyme-independent removal plays a role with oligophosphate compounds, since it is known that the removal of esterase labile protecting groups significantly slows down, if there is negative charge present close to the enzyme cleavage

site.[53] These groups should not suffer from toxicity problems, since they are removed by cyclization to five-membered structures that do not form glutathione adducts.[48]

4.2 Synthesis

Potential nucleoside monophosphate prodrugs of antiviral nucleosides bearing either 3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl or 3-acetyloxymethoxy -2,2-bis(ethoxycarbonyl)propyl group, as well as oligomeric thymidine phosphodiesters with multiple 4-acetylthio-2,2-dimethyl-3-oxobutyl protecting groups, were synthesized. To obtain the appropriately protected nucleosides for the synthesis of the desired final products, conventional nucleic acid protecting group chemistry was applied. Amino functions of nucleobases were protected with MMTr-group by using TBDMS-group as transient 5´-*O*-protection or by persilylating the hydroxyl functions with trimethylsilyl group (TMS). MMTr-group was additionally used as transient 5´-*O*-protection. 2´- and 3´-OH groups were protected with Lev-, Bz-, DMTr- or TBDMS-groups, when needed. The synthesis carried out in this thesis proceeded mainly well with good to moderate yields, but few considerations are worth mentioning.

4.2.1 2,2-Bissubstituted 3-acyloxypropyl protected 5´-phosphoramidates (I)

Two antiviral nucleosides, ribavirin and 2´-*O*-methylcytidine, were converted to their 3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl and 3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)propyl protected 5´-phosphoramidates derived from L-alanine methyl ester. Usually potentially toxic phenyl and naphthyl groups are used to this purpose.[77, 139] The removal is initiated by enzymatic hydrolysis of the alaninyl ester linkage, followed by intramolecular displacement of the aryloxy group (see Scheme 9). In our approach, the *O*-protecting group is removed first, leaving the alaninyl group esterified. In both cases, the final conversion to 5´-monophosphate depends on phosphoramidase activity. The synthesis of the desired final products proceeded well with good to moderate yields. The phenyl compounds were obtained by reacting the protected nucleoside with diphenylphosphite followed by oxidative amination with L-alanine methyl ester. The phosphoramidates bearing the 2,2-bissubstituted 3-acetyloxypropyl and 3-acetyloxymethoxypropyl protecting groups were obtained by reacting the protecting group alcohol with diphenylphosphite. The phenol was displaced from the obtained *H*-phosphonate with the appropriately protected nucleoside and oxidative amination with L-alanine methyl ester followed.

4.2.2 5´,5´-phosphodiesters and 3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)propyl and pivaloyloxymethyl protected 5´,5´-phosphodiesters (II)

The 3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)propyl group was additionally studied as the phosphate protecting group of 5´,5´-phosphodiesters. After removal of the protecting group by carboxyesterases, the released 5´,5´-phosphodiester is thought to be cleaved to a nucleoside and a nucleoside monophosphate by phosphodiesterases. The 2´-*C*-methyl derivatives of adenosine, guanosine and uridine have shown antiviral activity against HCV and were chosen for the synthesis. These nucleosides were converted to 5´,5´-diesters containing two similar or dissimilar nucleosides, and the 2´-*C*-methyladenosine 5´,5´-diester was protected with pivaloyloxy-carbonyl and 3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)propyl groups.

The protection of the tertiary 2´-OH of purine nucleosides that were used as building blocks in the synthesis of 5´,5´-phosphodi- and triesters was not straightforward. To obtain 2´,3´-di-*O*-levulinoyl-N^6-(4-methoxytrityl)-2´-*C*-methyladenosine (**12**), the levulinoylation with an excess of levulinic anhydride had to be done twice. The first levulinoylation in a mixture dioxane and pyridine yielded the mono levulinoylated product, but also a trace amount of **12** was obtained. The second levulinoylation of this mixture in DCM using DMAP as catalyst yielded the desired product. In case of benzoyl protection, 2.5 equivalents of benzoyl chloride in the reaction did not yield the di-benzoylated 2´-*C*-methylguanosine. However, both the 2´- and 3´-OH groups were protected with same amount of benzoylchloride in the reaction with 2´-*C*-methyluridine. The lower reactivity of the tertiary 2´-OH of purine nucleosides may, at least in the case of 2´-*C*-methylguanosine, result from rise in steric hindrance caused by the MMTr-protection of the base moiety. When removing the transient TBDMS-protection, acetic acid was added to the reaction mixture in order to reduce basicity of TBAF and prevent premature removal of the levulinoyl groups.[140] This reaction proceeds fairly slowly.

Marked tailing severely complicated the purifications of 5´,5´-diesters **14** and **22** and 5´,5´-triester **20**, the 2´-*C*-guanosine derivatives in particular, and largely for this reason the yields of these compounds remained low. In addition, the yield of compound **15** was low due to the difficulties in introducing the pivaloyloxymethyl protection. The reaction of compound **13** with chloromethyl pivalate was pursued in different solvents including MeCN, dioxane and *N*-methyl-2-pyrrolidone (NMP) at room temperature or by heating or refluxing the reaction mixture. The best results were obtained in a mixture of NMP and TEA and heating the reaction mixture at 60 °C for four days,[141] but still with a poor yield (15%).

4.2.3 3´,5´-Cyclic phosphate and thiophosphate esters (III)

3´,5´-Cyclic nucleoside monophosphates have more recently been used for the delivery of nucleoside monophosphates. Removal of the protecting group releases a 3´,5´-cyclic phosphodiester which is expectedly cleaved to the nucleoside 5´-monophosphate by 3',5'-cyclic nucleotide phosphodiesterases. 2´-*C*-methylguanosine and 2´-*C*,O^2-dimethylguanosine, which show antiviral activity against HCV, were chosen for the synthesis of their 3´,5´-phosphorothioates. The latter was additionally transformed to *S*-pivaloyloxymethyl triester.

The appropriately protected nucleoside was first phosphitylated to phosphoramidate, which was then coupled to 3´,5´-cyclic structure with 1*H*-tetrazole as activator in a large volume of MeCN and oxidation or sulphurization followed. The formation of the 3´,5´-cyclic structures that still bore the MMTr-group as amino protecting group were obtained at low yields. The sulfurization of the 3´,5´-cyclic phosphite structure of N^2-(4-methoxytrityl)-2´-*C*,O^6-dimethylguanosine with elemental sulfur gave the desired product in 16% yield. Somewhat better yield (32%) was obtained for the sulfurization of the N^2-(4-methoxytrityl)-2´-*C*-methylguanosine derivative with 3*H*-1,2-benzodithiol-3-one 1,1-dioxide (Beaucage reagent). The 3´,5´-cyclic methyl phosphate triester **35** was obtained in similar yield (30%) by oxidation with aqueous iodine. The introduction of the pivaloyloxymethyl group to the 3´,5´-cyclic phosphorothioate as iodomethyl pivalate turned out to be inefficient with only 14% total yield.

Introduction of the enzymatically and thermally removable 4-acetylthio-2,2-dimethyl-3-oxobutyl protection to 3´,5´-cyclic nucleoside monophosphates was attempted by phosphoramidate method. Bis(diisopropyl-amino)chlorophosphine was used as phosphitylating agent, but the synthesis failed. No cyclization took place. In addition, the synthesis was attempted with enzymatically and thermally removable 2-ethoxycarbonyl-2-methyl-3-oxo-4-pivaloylthiobutyl group by using bis(diisopropylamino)chlorophosphine or bis(diethylamino)chlorophosphine as phosphitylating agent, but again unsuccessfully. The 3´,5´-cyclic monophosphate should give two ^{31}P NMR signals at about 6 to 7 ppm difference, referring to the *cis*- and *trans*-conformers of the 3´,5´-cyclic phosphite.[142] The successful 3´,5´-cyclization reactions that resulted compounds **28**, **31**, and **34** showed peaks at about 123 and 129 ppm. In contrast, only one peak at about 115 ppm was observed. The 4-acetylthio-2,2-dimethyl-3-oxobutyl protecting group has, however, been later introduced to the 3´,5´-cyclic monophosphate by applying the recently published method, where the 3´,5´-cyclic structure was generated from a nucleoside 3´-*H*-phosphonate by intramolecular cyclization using pivaloylchloride as an activator in a mixture of DCM and pyridine (95:5) at

0 ºC.[143] The resulting 3´,5´-cyclic *H*-phosphonate diester was then further oxidized to iodophosphate and the iodine was replaced with *S*-(4-hydroxy-3,3-dimethyl-2-oxobutyl)ethanethioate to obtain 4-acetylthio-2,2-dimethyl-3-oxobutyl protected 3´,5´-cyclic nucleoside triester.[144]

4.2.4 4-Acetylthio-2,2-dimethyl-3-oxobutyl protected oligomeric phosphodiesters (IV)

The 4-acetylthio-2,2-dimethyl-3-oxobutyl group was studied as protecting group of oligomeric phosphodiesters to find out the enzymatical and thermal degradation properties in molecules containing more than one such protecting group. The synthesis of the building blocks for the assembly of the thymidine derived trimer **45** and tetramer **46** proceeded with good to moderate yields. The low overall yields of the final compounds **45** and **46** (4.9% and 1.8%, respectively) result mainly from the difficulties in purification, which arise from the low polar nature of the compounds. The small amounts of **45** and **46** did not enable the separation of R_P- and S_P-diastereomers. The R_P/S_P-mixtures were only fractionated and a fraction showing a single chromatographic signal was used in the kinetic measurements. If the trimer and tetramer were synthesized on solid support, the yields might be better and especially the purification might be simpler. However, the conventional solid phase methods are not suitable with phosphate protecting groups which are base labile and leave readily by intramolecular cyclization. The development of solid phase procedures was not the object of this work.

4.3 Kinetic studies

4.3.1 2,2-Bissubstituted 3-acyloxypropyl protected 5´-phosphoramidates (I)

Treatment of 3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl and 3-acetyloxy-methoxy-2,2-bis(ethoxycarbonyl)propyl protected nucleoside phosphoramidates with PLE or PC3 cell extract resulted in the removal of the protecting groups and release of the phosphoramidate monoanion. These protecting groups have been previously studied as bis-protected derivatives of nucleoside 5´-monophosphates.[47] With the 3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl group, the deacetylation of the diester obtained by removal of the first protecting group was significantly slower ($\tau_{½}$ = 150 h) than the initial deacetylation ($\tau_{½}$ = 194 s). The additional CH_2O-group of 3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)-

propyl protection accelerated the initial deacetylation of the phosphotriester 20-fold ($\tau_{½}$ = 10 s) and, more importantly, the deacetylation of the monoanionic phosphodiester more than 50-fold ($\tau_{½}$ = 2.7 h). The negative charge on the substrate somehow prevented proper action of carboxyesterases, but the additional CH_2O-group brought the enzyme cleavage site a bit further from the negative charge of the phosphodiester intermediate.

The same observation was done with phosphoramidates **9a** and **9b**. When the compounds were treated with PLE at low enzyme concentration (1 U mL^{-1}), **9b** bearing a 3-acetyloxymethoxy-2,2-bis-(ethoxycarbonyl)propyl group was deacetylated one order of magnitude faster than **9a**. The enzymatic hydrolysis of 3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl group from **11** is relatively slow and the hydrolysis of alanine methyl ester linkage competes representing 20% of the overall disappearance of **11**. With 3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)-propyl protection this is not observed, since the enzymatic reaction is faster. The enzymatic hydrolysis of the phenyl analog **6** is very slow, one order of magnitude slower than the hydrolysis of **11**. Table 3 summarizes the obtained reaction rate constants and half-lives for the disappearance of compounds **6** and **11**.

Table 3 Obtained reaction rate constants (*k*) and half-lives ($\tau_{½}$) for the disappearance of compounds **6** and **11** in the presence (26 U mL^{-1} of PLE) and absence of enzyme.

Compound	*k* (PLE) / s^{-1}	$\tau_{½}$ (PLE)	*k* / s^{-1}	$\tau_{½}$
6	$(4.5 \pm 0.4) \times 10^{-6}$	43 h	$(3.1 \pm 0.1) \times 10^{-6}$	62 h
11	$(3.4 \pm 0.2) \times 10^{-5}$	5.7 h	$(0.32 \pm 0.02) \times 10^{-5}$	60 h

4.3.2 4-Acetylthio-2,2-dimethyl-3-oxobutyl protected oligomeric phosphodiesters (IV)

The 4-acetylthio-2,2-dimethyl-3-oxobutyl groups were released from the oligomeric thymidine model compounds, **45** and **46**, both enzymatically and thermally. The reaction rate constants and half-lives for the non-enzymatic removal of 4-acetylthio-2,2-dimethyl-3-oxobutyl group from **45**, **46** and **48** (Table 4) are of the same order of magnitude as previously reported for 2´,3´-*O*-isopropylideneuridine 5´-(methyl 4-acetylthio-2,2-dimethyl-3-oxobutyl) phosphate and 4-acetylthio-2,2-dimethyl-3oxobutyl bis(2´-*C*-methyluridin-5´-yl) phosphate under the same conditions ($\tau_{½}$ = 11.2 h and $\tau_{½}$ = 6.9 h, respectively).[48] The thermal removal of the protecting groups from intermediate mixtures $\mathbf{I}^{\mathbf{45a}}$ and $\mathbf{I}^{\mathbf{46a}}$ occurred at comparable rate too (Table 4). The rate of the enzymatic

deacylation of 2´,3´-*O*-isopropylideneuridine 5´-(methyl 4-acetylthio-2,2-dimethyl-3-oxobutyl) phosphate ($\tau_{½}$ = 4.7 min)[48] is also consistent with the rates obtained for compounds **45**, **46** and **48**. The hydrolysis of 4-acetylthio-2,2-dimethyl-3-oxobutyl group has been reported to occur at comparable rate also as protecting group of thymidine 3´,5´-cyclic monophosphate under similar conditions (enzymatic removal $\tau_{½}$ = 6 min, non-enzymatic removal $\tau_{½}$ = 9.3 h min). [144]

Table 4 Obtained reaction rate constants (*k*) and half-lives ($\tau_{½}$) for the disappearance of compounds **45**, **46** and **48** and intermediate mixtures $\mathbf{I^{45a}}$ and $\mathbf{I^{46a}}$ in the presence (2.6 U mL^{-1} of PLE) and absence of enzyme.

Compound	*k* (PLE) / s^{-1}	$\tau_{½}$ (PLE)	*k* / s^{-1}	$\tau_{½}$
45	$(4.3 \pm 0.3) \times 10^{-3}$	2.7 min	$(2.5 \pm 0.1) \times 10^{-5}$	7.8 h
$\mathbf{I^{45a}}$	-	-	$(1.8 \pm 0.2) \times 10^{-5}$	10.7 h
46	$(3.2 \pm 0.1) \times 10^{-4}$	36 min	$(3.1 \pm 0.2) \times 10^{-5}$	6.2 h
$\mathbf{I^{46a}}$	-	-	$(2.7 \pm 1.0) \times 10^{-5}$	7.2 h
48[a]	$(9.5 \pm 0.5) \times 10^{-3}$	1.2 min	$(1.94 \pm 0.02) \times 10^{-5}$	9.9 h
48[b]	$(6.7 \pm 0.6) \times 10^{-3}$	1.7 min	$(1.88 \pm 0.03) \times 10^{-5}$	10.2 h

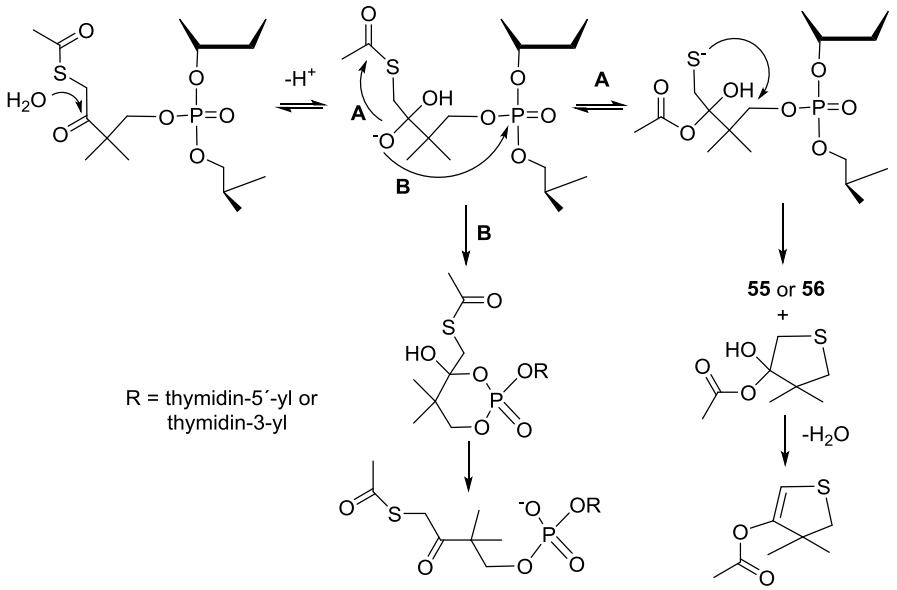
[a] slower eluting diastereomer
[b] faster eluting diastereomer

The enzymatic hydrolysis of 4-acetylthio-2,2-dimethyl-3oxobutyl bis(2´-*C*-methyluridin-5´-yl) phosphate, however, is somewhat slower ($\tau_{½}$ = 83 min) than the deacylation of compounds **45**, **46** and **48**. For some reason, the protecting group seems to be more available for the deacylation by the enzyme in the oligomeric structures than in the 5´,5´-phosphodiester. Interestingly, also in case of 3´,5´-cyclic monophosphates, the enzymatic removal of 3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl group from 2´-*C*-methyluridine derivative was markedly slower ($\tau_{½}$ = 35 h) than from thymidine derivative ($\tau_{½}$ = 93 min).[144] The additional steric hindrance of 2´-methyl group in the already more crowded 2´-position of the ribose sugar most likely has a retarding effect on the reaction rate.

A side reaction of internucleosidic P-O bond cleavage was observed, when the non-enzymatic removal of 4-acetylthio-2,2-dimethyl-3-oxobutyl group from 5´-*O*-pivaloylthymidin-3´-yl thymidine-5´-yl phosphate was studied. The side reaction represents about 9% of the total disappearance of the starting material **48** and is about the same magnitude as previously described for the thermally removable 4-benzoylthio-2,2-dimethyl-3-oxobutyl and 2-ethoxycarbonyl-2-methyl-3-oxo-4-pivaloylthiobutyl groups (15% and 5%, respectively).[48]

Although the side reaction is rather marked with **48**, it is not much increased with oligomer **45** which is protected with three 4-acetylthio-2,2-dimethyl-3-oxobutyl groups. The competing chain cleavage starts to play a role only when the enzymatic removal becomes so slow that the protecting groups are removed non-enzymatically.

The non-enzymatic departure of 4-acetylthio-2,2-dimethyl-3-oxobutyl groups from the oligomeric phosphotriesters **45** and **46** most likely occur as described earlier.[48] Hydration of the 3-oxo group forms a geminal diol, which allows the acetyl migration from the sulfur atom and the exposed mercapto group then attacks C1 releasing the protecting group as 4,4-dimethyl-3-acetyloxy-4,5-dihydrothiophene (Reaction A, Scheme 34). After hydration of the 3-oxo group, the competing side reaction can occur by intramolecular attack of an oxyanion at the phosphorus atom (Reaction B, Scheme 34).



Scheme 34 Mechanism of non-enzymatic removal of 4-acetylthio-2,2-dimethyl-3-oxobutyl protecting group (reaction A) and the observed side reaction of P-O3´/P-O5´ bond cleavage (reaction B).

4.4 Conclusions

This thesis includes studies on the feasibility of 3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl, 3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)propyl and 4-acetylthio-2,2-dimethyl-3-oxobutyl groups as phosphate protecting groups.

All of the protecting groups were shown to undergo conversion to fully deprotected products in the presence of carboxyesterase. The enzymatical removal of 3-acetyloxymethoxy-2,2-bis(ethoxycarbonyl)propyl group from nucleoside 5´-phosphoramidate was at least one order of magnitude faster than the removal of 3-acetyloxy-2,2-bis(ethoxycarbonyl)propyl group, thus making it more potential biodegradable phosphate protecting group, but the removal rate is nevertheless rather slow.

Of the three studied phosphate protecting groups, the 4-acetylthio-2,2-dimethylbutyl group seems most attractive. The weakness of prodrug strategies based on enzymatically removable protecting groups is that many unpredictable issues may have an effect on the enzymatic activity. In addition to the problem of negative charge disturbing the catalytic site of the enzyme, differences between individuals affect the enzymatic activity, including genetic polymorphism, age-related differences in physiological conditions and drug interactions.[145] The behavior and release of chemically removable protecting groups is more predictable. The problem with the chemically removable groups, on the other hand, is how to control the release of the protecting group and make difference between the target site and the rest of the body. With enzymatically removable protecting groups that are additionally thermally removable, the feature of two release mechanisms allows controlled removal of the protecting groups and, in case the enzymatic reaction becomes too slow, the thermolability ensures the removal of the protecting group. In addition, the five-membered byproducts that are released from the enzymatically and thermally removable protecting groups do not appear toxic, unlike the enones released from the esterase labile protecting groups.

The 4-acetylthio-2,2-dimethyl-3-oxobutyl group show encouraging results as phosphate protecting group of oligomeric phosphodiesters. The protecting groups were cleaved from the studied oligomeric phosphodiesters enzymatically and were additionally cleavable non-enzymatically. The carboxyesterase catalyzed removal of the protecting group proceeded readily. Accumulation of the negative charge on the intermediates did not markedly retard the non-enzymatic removal of the protecting group. However, the rate of the thermal removal still is too slow to allow efficient protection of longer oligonucleotides and needs optimization. By increasing the electronegativity of substituents at C2, the thermal stability may be decreased. For instance, the replacement of one of the methyl substituents with an ethoxycarbonyl group increases the non-enzymatic removal 20-fold.[48]

The side reaction of chain cleavage, which occurred with oligomeric phosphodiesters protected with 4-acetylthio-2,2-dimethyl-3-oxobutyl group,

should be avoided. This too could possibly be achieved by increasing the electronegativity of substituents at C2 and, hence, making the protecting group a better leaving group. Furthermore, the substituents at C2 should not cause steric hindrance in order to allow efficient intramolecular attack of the mercapto group to C1 and formation of the cyclic by-product. In addition, the acyl migration should occur so fast that the intramolecular attack of the oxyanion cannot take place. The rate of the acyl migration can be influenced by steric effects: bulky acyl groups migrate slower than simple ones. However, the 4-acetylthio-2,2-dimethyl-3-oxobutyl group already employs a simple acetyl group.

Indeed, the side reaction did not occur with 4-acetylthio-2-ethoxycarbonyl-2-methyl-3-oxobutyl group protected 3´-*O*-levulinoylthymidine 5´-methylphosphate.[48] The protecting group was readily cleaved both enzymatically and non-enzymatically with half-lives of 2.9 min and 34 min, respectively. Hence, by employing the 4-acetylthio-2-ethoxycarbonyl-2-methyl-3-oxobutyl group as phosphate protecting group of oligomeric phosphodiesters (Figure 21) instead of 4-acetylthio-2,2-dimethyl-3-oxobutyl group, both the side reaction and the problem of too slow non-enzymatic removal could possibly be avoided.

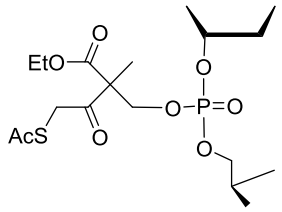


Figure 21 4-Acetylthio-2-ethoxycarbonyl-2-methyl-3-oxobutyl group as protecting group of phosphodiester bond.

5 EXPERIMENTAL

More detailed information about the synthesis, kinetic measurements and characterizations of the discussed compounds is found in chapter *Results* and in the original publications (papers I-IV).

5.1 Characterization of the synthesized compounds

The synthesized compounds were characterized with ^{1}H NMR, ^{13}C NMR and high resolution mass spectrometric methods and with ^{31}P NMR when applicable. 2D NMR spectra (DQF-COSY, HSQC, HMBC) were used for peak assignment. The NMR spectra were recorded on a Bruker Avance 400, 500 or 600 NMR spectrometers equipped with a BBI or BBO-5mm-Zgrad probes. The operating frequencies were: 400 MHz, 500 MHz or 600 MHz for ^{1}H; 101 MHz, 126 MHz or 151 MHz for ^{13}C; 162 MHz, 202 MHz or 243 MHz for ^{31}P, respectively. The chemical shifts (δ) are given in parts per million (ppm) and the coupling constants (J) in hertz (Hz). The exact masses (m/z) of the synthesized compounds were determined on Bruker Daltonics microTOF-Q mass spectrometer equipped with an ESI source. ^{31}P NMR was also used to monitor the progress of the separate reaction steps in the phosphorylation reactions by acquiring a ^{31}P NMR spectrum of a sample from the reaction mixture. The ^{31}P NMR signals of the products in each step occur at characteristic frequencies.

5.2 Kinetic measurements

The kinetic measurements were carried out in sealed tubes on a thermostated water bath in HEPES buffer at pH 7.5 [0.02 mol L^{-1} at (35.0 ± 0.1) °C and 0.06 mol L^{-1} at (37.0 ± 0.1) °C] or in glycine buffer at pH 9 and 10 [0.06 mol L^{-1}, (37.0± 0.1) °C]. The ionic strength was adjusted to 0.1 mol L^{-1} with NaCl. The initial substrate concentration was 0.10 mmol L^{-1} (paper I) or 0.15 mmol L^{-1} (paper IV). The enzymatic hydrolysis was carried out in PLE concentrations 1 U mL^{-1} and 26 U mL^{-1} (paper I) and 2.6 U mL^{-1} (paper IV). In addition, the enzymatic hydrolysis was studied in a 1:2 mixture of human prostate carcinoma (PC3) cell extract and HEPES buffer (paper I). Samples were taken from the reaction mixture at different time intervals and made acidic (pH 2) with aqueous hydrogen chloride and cooled on an ice-bath to stop the reaction. Samples containing PLE or PC3 cell extract were filtered with 0.2 µm SPARTAN 13A filters (paper I) or 0.2 µm RC4 syringe (paper IV). The product composition of the samples was analyzed by HPLC using Thermo Scientific ODS Hypersil C18

column [4 × 250 mm, 5 µm (paper I) and 4 × 260 mm, 5 µm (paper IV)] and a mixture of acetic acid/sodium acetate buffer (0.045/0.015 mol L^{-1}) and acetonitrile, containing ammonium chloride (0.1 mol L^{-1}). Signals were recorded on a UV-detector at a wavelength of 220 (ribavirin derivatives) or 267 nm. The products were identified by HPLC-ESI-MS methods on Perkin-Elmer Sciex-API-365 triple-quadrupole spectrometer and Phenomenex Gemini C18 column (2.5 × 150 mm, 5 µm) eluting with a mixture of water and MeCN containing 0.1% formic acid, spiking with authentic samples or by isolating and analyzing a peak by MS.

First-order rate constants were calculated by applying the integrated first-order rate equation to the time-dependent diminution of the concentration of the starting material. The first-order rate constants for the disappearance of intermediates were obtained by least-squares fitting of the time-dependent concentration of the compounds with the rate law of two consecutive first-order reactions.

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