CHARLES UNIVERSITY

FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ

DEPARTMENT OF ORGANIC AND BIOORGANIC CHEMISTRY

Diploma thesis

SYNTHESIS OF PHOSPHORAMIDATE PRODRUGS „PROTIDES“ AS NOVEL POTENTIAL THERAPEUTIC AGENTS FOR THE TREATMENT OF CONGENITAL DISORDERS OF GLYCOSYLATION AND MITOCHONDRIAL DNA DEPLETION SYNDROME

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HRADEC KRÁLOVÉ 2018

DECLARATION

I declare that this thesis has been composed by myself and the work presented is my own. All used literature and information sources are listed in the list of used literature and are properly cited. This work has not been used to gain equal or any other degree.

Hradec Králové 2018 Jana Sedláková

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Content

Abstract

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Title of diploma thesis: Synthesis of phosphoramidate prodrugs „ProTides“ as novel potential therapeutic agents for the treatment of congenital disorders of glycosylation and mitochondrial DNA depletion syndrome

At the present time, no effective treatment is available neither for the most of the congenital disorders of glycosylation (CDGs) nor the mitochondrial DNA depletion syndrome (MDS). Regarding the CDG therapy, D-mannose-1-phosphate (Man-1-P) offers considerable pharmacological potential to improve the pathological patterns in patients affected by phosphomannomutase 2 deficiency (PMM2-CDG), similarly as *N*-acetyl-D-mannosamine-6-phosphate (ManNAc-6-P) in case of GNE myopathy (GNEM). Administration of selected deoxyribonucleotides was proposed as a potential pharmacological strategy for the treatment of MDS. Unfortunately, the problematic membrane penetration of such polar molecules reduces their effect and limits their clinical application. Hydrophobic, membrane permeable derivatives of the sugar monophosphates and nucleotides, might represent more efficient potential therapeutics for CDGs and MDS, respectively.

In this work, various phosphoramidate prodrugs (ProTides) of Man-1-P, ManNAc-6-P and their peracetylated derivatives were synthesized. Two different synthetic approaches were used: a) coupling of the desired substrate with previously prepared phosphorochloridate in the presence of *t*BuMgCl, b) coupling of the appropriate substrate with the phosphorochloridate using *N*-methylimidazole (NMI). The ProTides were successfully prepared via the Grignard methodology, nevertheless, the NMI method did not provide the desired phosphoramidates even after several attempts to improve the reaction conditions.

In order to investigate the bioactivation of ProTides, an enzymatic experiment was carried out with one of the phosphoramidate derivatives synthesised.

The ProTide approach was also applied to 6-methoxyguanosine monophosphate with the aim to prepare potentially effective prodrugs for MDS treatment. Two derivatives were fruitfully synthetized via the nucleoside coupling with various (aryl) (*p*-nitrophenyl) phosphoramidates in the presence of *t*BuMgCl.

Abstrakt

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Název diplomové práce: Syntéza fosforamidátových proléčiv „ProTides“ jako nových potenciálních léčiv pro terapii vrozených poruch glykosylace a syndromu vyčerpání mitochondriální DNA

V současné době není dostupná efektivní léčba pro většinu vrozených poruch glykosylace (CDGs) ani pro léčbu syndromu vyčerpání mitochondriální DNA (MDS). Co se terapie týká, D-manosa-1-fosfát (Man-1-P) poskytuje značný farmakologický potenciál pro zlepšení patologických změn u pacientů zasažených deficitem fosfomanomutázy 2 (PMM2-CDG), podobně jako *N*-acetyl-D-mannosamin-6-fosfát (ManNAc-6-P) v případě GNE myopatie (GNEM). Podávání vybraných deoxyribonukleotidů bylo navrženo jako potenciální farmakologická strategie pro terapii MDS. Naneštěstí, problematický průnik přes membrány takto polárních molekul snižuje jejich efekt a omezuje jejich klinické využití. Hydrofobní, membránou prostupné deriváty sacharidových monofosfátů a nukleotidů, by mohly představovat efektivnější potenciální léčiva pro PMM2-CDG a GNEM, resp. MDS.

V rámci této práce byla syntetizována rozličná fosforamidátová proléčiva (ProTides) pro Man-1-P, ManNAc-6-P a jejich peracetylované deriváty. Pro syntézu byly použity dva různé přístupy: a) coupling požadovaného substrátu s dříve připraveným fosforochloridátem za přítomnosti *t*BuMgCl, b) coupling patřičného substrátu s fosforochloridátem pomocí *N*-methylimidazolu (NMI). ProTides proléčiva byla úspěšně připravena pomocí Grignardovy metody, nicméně NMI metoda nevedla k žádaným fosforamidátům, a to ani po několika pokusech vylepšit reakční podmínky.

Za účelem prozkoumání bioaktivace ProTide proléčiv byl proveden enzymatický experiment s jedním z připravených fosforamidátových derivátů.

ProTide přístup byl také aplikován na 6-methoxyguanosin monofosfát s cílem připravit potenciálně efektivní proléčiva pro terapii MDS. Dva deriváty byly úspěšně syntetizovány couplingem nukleosidu s různými (aryl) (*p*-nitrophenyl) fosforamidáty v přítomnosti *t*BuMgCl.

1 Introduction

1.1 Rare diseases

The rare diseases (also called orphan diseases) are characterized by the small number of people they affect, but there is no worldwide accepted definition. U.S. Food and Drug Administration (FDA) follows the Orphan Drug Act of 1983 and consider the rare diseases as disorders that occur in less than 200 000 individuals in the United States,[1] whereas the European Commission states the prevalence of the rare diseases lower than 5 in 10 000 persons in EU countries.[2] The worldwide prevalence is estimated to 350 million people.[3] According to the Global genes®, 1 in 10 Americans (approximately 30 million people) contends with the rare disease[3] and other estimates suggest that 6-8 % (27-36 million people) of the EU population suffer from 8 000 distinct rare diseases.[2]

Generally, the rare diseases are heterogeneous conditions, often chronic and progressive, which can impair any system.[4,5] Majority of the rare diseases originate from the altered genes or chromosomes, mostly acquired from previous generations, nevertheless, inherited genetic defects are not the only causes, but rare forms of auto-immune diseases and rare cancers are known as well.[2,4,6]

The rare diseases and patients' difficulties were firstly highlighted in the report of the National Commission on Orphan Disease of the U.S. Government in 1989. Until then, the public awareness of the issues related to the rare diseases, including overlooked development and limited availability of the treatments, was negligible.[5] Nowadays, most of the rare diseases are not curable, but the potential treatment and medical care may enhance the quality of life and increase the life expectancy of affected patients.[4] The development of the orphan drugs is not commercially attractive for the pharmaceutical companies which is one of the reasons why only few of the rare diseases have the effective treatment available. To support the research and development of the orphan drugs, the regulatory agencies, such as European Medicines Agency (EMA) or FDA offer granting of orphan designation.[7,8]

This project was focused on the development of potential treatment for the rare diseases, specifically for particular types of congenital disorders of glycosylation (CDGs) and mitochondrial depletion syndrome (MDS), which will be introduced in following chapters.

1.2 Congenital disorders of glycosylation (CDGs)

1.2.1 Introduction

Congenital disorders of glycosylation (CDGs) were firstly described in 1980 as "Carbohydrate Deficient Glycoprotein Syndrome (CDGS)".[9,10] They are rare genetic, metabolic disorders caused by the defects in the protein or lipid glycosylation, an essential process for the organisms' viability.[11] Mutations in genes encoding the enzymes directly involved in glycan processing, but also in genes coding the supporting systems, lead to the abnormal glycosylation which subsequently results in pathological modulation of physiological processes, clinical manifesting as a disease.[9,12]

Due to the tremendous diversity of the genes and glycans involved in the protein and lipid modification, the clinical presentation may be various, contributing to the difficult recognition of the patients with CDGs.[11] The impaired glycosylation pathways may hamper any tissue or organ, most notably nervous system.[13] There is no simple universal laboratory test which would reveal the defect in glycosylation process, making the diagnosis of CDGs challenging. Optimally, the CDGs should be considered in any case of multiorgan disease, especially with present neurologic symptoms.[9,11,13]

Approximately 250 genes encode proteins essential for the appropriate glycans synthesis and recognition, and approximately 50 % of proteins undergo the post-translation glycosylation. These facts contribute to the rapid expand of the list of the known glycosylation disorders. Until the present time, over 100 types of CDGs have been discovered.[11,14] The prevalence depends on the specific type of the disorder, but the frequencies of individual CDGs and CDGs collectively are mostly unknown.[9,11,14,15]

1.2.2 Glycosylation

Glycosylation is an ubiquitous process in which glycans are synthetized, modified and attached to the proteins and lipids to form the glycoproteins and glycolipids, respectively. A wide spectrum of biochemical pathways requires the glycosylation modification since the glycans are essential for the stabilization of glycoprotein folding, cellular communication, and trafficking, and modulation of protein, resp. lipid properties. The formed glycoconjugates then play important roles in all tissues and organs, including immune, coagulation or nervous system.[12,16]

Generally, the glycosylation process consists of three basic steps. First, the monosaccharides are synthetized and activated to nucleotide sugars in the cytoplasm. As the second step, the activated sugar donor is transported into endoplasmic reticulum (ER) or Golgi apparatus (GA), where the saccharide residue is ultimately processed and attached to the receiving molecule.[9] Nine distinct glycosylation pathways, defined by the first monosaccharide attached to the acceptor (protein or lipid), have been described in mammals.[14] The schematic representation of the glycosylation is captured in Figure 1.

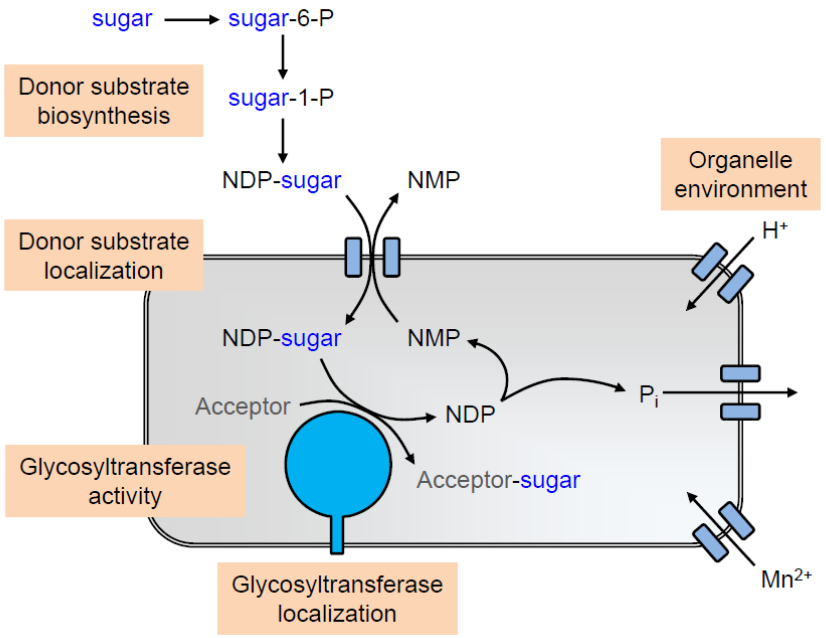


Figure 1. Adopted from Hennet, T. and J. Cabalzar (2015). The key players in the glycosylation reactions in GA. The nucleotide-activated sugars (NDP-sugar) are synthetized in the cytosol or nucleus and they are subsequently transported into the GA where the glycosyltransferases provides the attachment of the sugar to the acceptor. Transporters regulating pH, P_i export and Mn^{2+} import are necessary to maintain the optimal organelle homeostasis.[11]

Each glycosylation pathway requires activated sugar donors (NA-sugars, DolP-sugars), serving as the glycans building blocks, and a set of the glycosyltransferases, providing the sugar linkage. The sugar donors are synthetized and activated in the cytosol (CMP-Sia is activated in the nucleus), whereas the glycosyltransferases are located in the luminal side of ER/GA. The activated sugars, therefore, have to be trafficked into the organelles by specific transporter system so as to be utilized for the glycans assembly. The transferases, as well as the sugar donors, may be shared by several pathways, hence, any defect of the shared substrate or enzyme could affect more than one pathway.[13,17] The donor substrates are listed in Table 1.

a) NA sugars		
UDP-Glc	GDP-Man	CMP-Sia
UDP-GlcNAc	GDP-Fuc	
UDP-Gal		
UDP-GalNAc		
UDP-GlcA		
UDP-Xyl		
b) dolichol P linked sugars		
DolP-Man		
DolP-Glc		

Table 1: Adopted from Hennet, T. and J. Cabalzar (2015). Nine nucleotide-activated (NA) sugars and two dolichol-phosphate (P) linked sugars as donors for glycosylation pathways.[11]

1.2.3 Origin, biology of disorders of glycosylation

1.2.3.1 Genetic background

CDGs may be caused by mutations in extensive number of genes encoding different proteins important for the proper glycosylation.[16] Mostly, they are inherited autosomal recessive disorders, nevertheless, X-linked, dominant and *de novo* mutations in glycosylation-related genes have been identified as well.[14,16]

Any defect in sugar-donor activation or transport, dolichol and lipid-linked oligosaccharide (LLO) synthesis, biosynthesis of glycans in ER, compartment shifting, attachment to the protein/lipid, glycan trafficking and processing through the GA, final transport or secretion of matured glycan may result in clinically manifesting CDG.[9,18]

The lack of the glycosylation or incomplete glycosylation could be either toxic or insufficient for the CDG cell, depending on several factors. The type of the cell, glycosylation and metabolic load, and genes capable to compensate the deficiency determine the significance of the defect.[14]

1.2.3.2 Diagnosis

Patients with CDGs are usually diagnosed by the detection of the under-glycosylated transferrin (Tf) in human serum by isoelectric focusing (IEF). Tf is a sensitive marker indicating impaired glycosylation, however, the abnormal Tf is not characteristic for all CDGs and might normalise after infancy. Moreover, the Tf biochemical analysis identifies exclusively *N*-glycosylation disorders which explains why the *N*-glycosylation alterations belong to the majority of known CDGs. Identification of the specific mutation in gene related to glycosylation requires exome sequencing, which might be the only way to diagnose CDG, especially for mildly symptomatic or atypically presenting patients with normal transferrin pattern. Abnormal liver function, proteinuria, increased insulin level, hypoglycaemia or low plasma glycoproteins may also indicate altered glycosylation.[9,11,13,14,15,19]

1.2.3.3 Clinical manifestation

Due to the immense diversity of glycosylated proteins and lipids, resulting from the complex biosynthetic pathways, CDGs may affect any system of the human body resulting in broad spectrum of symptoms. The severity can vary from mild to severe, even lethal dysfunctions.[11,16] The symptoms are unspecific and highly differ from one type to another, nevertheless, several features are common for some CDG types, e.g., structural abnormalities, epileptic seizures, neuropathies, stroke-like episodes, cerebellar atrophy, developmental delay, coagulopathies, myopathies or immune system deficiency.[11,13] The diverse clinical manifestation implies that the CDGs are underdiagnosed.[11]

1.2.4 Classification

1.2.4.1 Type-I and Type-II

Originally, the CDGs were divided into two groups according the impaired section of the glycosylation pathway. CDG type-I involved defects in any step of the LLO assembly and its transfer to the acceptor proteins resulting in unoccupied glycosylation sites, whereas CDG type-II included defects in the following modifications, mostly *N*-glycan elongation defects, as well as any other alterations of glycosylation.[15,20,21]

In view of the fact that some defects impair several glycosylation pathways, the new consistent nomenclature has been developed and replaced the primal distinction between CDG type-I and II The congenital disorders of glycosylation are currently named according the impaired gene, of which name is enclosed with the suffix CDG.[22]

1.2.4.2 According to affected glycosylation pathway

Any of the described glycosylation pathways providing distinct types of glycoconjugates in the vertebrates may be impaired.[13,23] The main pathways and their roles are noted in Table 2. The basis of *N*- and *O*-glycosylation disorders are expound bellow.

Pathway	Typical clients	Functions
***N*-linked**	Nearly all cell-surface receptors, ECM, and secreted proteins	Folding assistance, stabilisation of target proteins, signalling
***O*-Xyl**	ECM proteins, heparan and chondroitin sulphate	Growth-factor binding, structure of ECM
***O*-Man**	α-Dystroglycan	Bridging neuromuscular 1α-receptor junction
***O*-Fuc**	Notch and notch ligands	Notch signalling, developmental patterning
***O*-GalNAc**	Mucins, leucocyte receptors	Pathogen decoys, lubrication, surface cell surface protection
***O*-Glc**	Notch and Notch ligands	Notch signalling, developmental patterning
GPI-anchor glycans	Proteins in lipid rafts, signalling molecules	Organisation of plasma-membrane domains
Glycosphingolipids	Proteins in lipid rafts, signalling molecules	Membrane organisation, signalling

Table 2: The main glycosylation pathways, their substrates and functions. Adopted from Freeze, H. H., et al. (2012).

1.2.4.2.1 *N*-Glycosylation disorders

Nascent proteins which possess Asn-X-Ser, Asn-X-Thr or Asn-X-Cys sequences may be modified in the ER during or not long afterwards their synthesis by the attachment of the previously synthetized LLO via *N*-glycosidic bond. The glycan part of the tribranched lipid (dolichol)-linked $Glc_3Man_9GlcNAc_2$ glycan (LLO) is transferred to the Asn residues of the acceptor protein by oligosaccharyltransferase complex (OST). The protein-bound chains are further processed and the glycoconjugate is then trafficked to the GA, where additional excision or addition of monosaccharides take place. Final glycoproteins are subsequently transferred to the various intracellular compartments, cell surface or extracellular matrix.[24]

The synthesis of LLO, attachment of the glycan to proteins and complete processing of *N*-glycans requires approximately 75 gene products. Any defects in any of these genes may alter the *N*-glycosylation process. The importance of possibly impaired *N*-glycoconjugate is unpredictable and may vary from insignificant to essential.[25]

1.2.4.2.2 *O*-glycosylation disorders

The presence of Ser or Thr in nascent proteins offers free hydroxyl groups available for the covalent modification by *O*-glycosidic linkage. The oligosaccharide chain may be attached via GalNAc, Xyl, Man, Glu or Fuc. Contrary to *N*-glycosylation, *O*-glycosylation only consist of monosaccharide assembly and the *O*-glycosylated proteins do not undergo further processing.[9,13,14]

Disorders of *O*-glycosylation were described to cause number of clinically manifesting diseases, affecting different organ systems with variable severity.[9,14]

1.2.4.3 According to the defect placement

Various altered glycan structures may originate in mutations of diverse genes, generally causing the defects of glycosyltransferases, donor substrates and their transporters or Golgi apparatus milieu.[11,14]

1.2.4.3.1 Impaired glycosyltransferases

Our genome encodes nearly 200 different glycosyltransferases, enzymes responsible for the formation of glycans through the creation of the glycosidic bonds.[11,26] The mutation in glycosyltransferase-related gene can lead to the impaired function of the enzyme or its abnormal localization. Generally, the severity of glycosyltransferases defects depends on the type of the enzyme (core or terminal), as well as on its functional redundancy.[11]

1.2.4.3.2 Deficient donor substrates

Contrary to the large number of glycosyltransferases, there is only 11 substrates of these enzymes, providing the building blocks of all human glycans. Considering that each substrate may serve to the considerable scope of the glycosylation pathways, the defects in the biosynthesis or transport of individual activated sugar affect wide range of glycoconjugates structures. Such impairments result in mild to severe multiorgan disorders, depending on the residual activity of the enzyme.[11]

1.2.4.3.3 Inconvenient organelle homeostasis

Since certain environment conditions are required for proper function of glycosyltransferases, mutations in genes providing proteins that regulate the acidity and ionic composition of the Golgi apparatus affect the glycosylation pathways.[11]

1.2.5 PMM2-CDG

Phosphomannomutase 2 (PMM2), encoded by *PMM2* gene, is an enzyme catalysing the conversion of Man-6-P to Man-1-P, which is subsequently involved in GDP-Man and DolP-Man biosynthesis (Figure 2), essential sugar donors for several glycosylation pathways.[14,15,27] Phosphomannomutase 2 deficiency (PMM2-CDG), previously called CDG-Ia, is the most prevalent type of *N*-glycosylation disorder (estimated frequency 1:20 000) caused by the mutation in *PMM2*.[28]

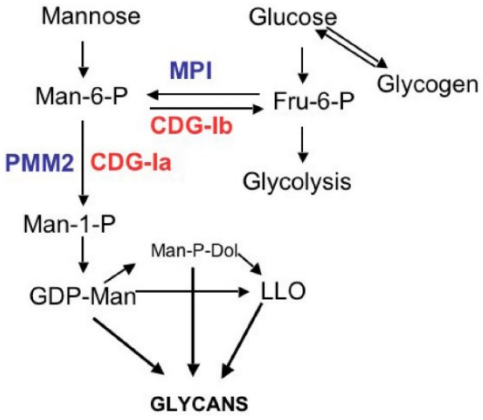


Figure 2: Adopted from Freeze, H. H. (2009). Mannose metabolic pathway. Man-6-P is formed in the cytosol by the phosphorylation of exogenous mannose catalysed by the hexokinase, or is interconverted from Fru-6-P by phosphomannose isomerase (MPI). PMM2 then transfer the phosphate to 1 position, providing Man-1-P as an intermediate for subsequent GDP-Man and ManP-Dol synthesis. The excessive amount of Man-6-P is processed by MPI, suppressing the formation of Man-1-P.[29]

More than 100 different mutations of *PMM2* causing decreased catalytic activity or/and PMM2 instability and thus leading to the reduced GDP-Man pool (10 fold lower) have been identified.[15,28] Complete loss of the enzyme is lethal in mice in early

embryogenesis and most likely also in humans.[29] The most frequent mutation in *PMM2* is fatal for homozygotes,[30] nevertheless, several mutations manifest with mild symptoms.[31]

The prime target is usually the nervous system, nevertheless, the clinical picture is highly variable and include broad spectrum of symptoms. The particular manifestation depends on several factors, e.g. specific mutation or involved organ system. PMM2-CDG can be sorted by the clinical manifestation at different life periods into three stages.[13,32]

Feeding difficulties, inverted nipples, fat pads, strabismus, developmental delay or cerebellar hypoplasia belong to typical features of the infantile multisystem stage. The infants may also suffer from generalized muscle hypotonia, hypertrophic cardiomyopathy, pericardial effusion, esotropia or facial abnormalities. Episodes of organ system failure and severe infections result in life-threatening conditions with 20% childhood mortality rate in the first 5 years.[9,13,32]

During the late-infantile and childhood ataxia-intellectual disability phase, the symptoms associated with underdeveloped brain dominate. Typically, affected children exhibit balance problems, difficult voluntary movement coordination, delayed language development, intellectual deficiency, retinopathy, demyelinating peripheral neuropathy or seizures. Coagulation system is affected as well, since the liver impairment leads to deficient synthesis of coagulation factors. Abnormal formation of blood clots causes the stroke-like episodes, occurring mostly during the intercurrent feverish infections.[9,13,32]

The adult stable disability stage can be characterised by stabilisation or remission of the clinical symptoms, mainly neurological. Skeletal abnormalities become more evident and hormonal abnormalities, such as hypergonadotrophic hypogonadism in adult females, may develop.[9,13,32]

1.2.6 GNE Myopathy

GNE gene encodes UDP-GlcNAc 2-epimerase/ManNAc kinase (GNE/MNK), the bifunctional enzyme, which catalyses the epimerization of UDP-GlcNAc to ManNAc and its subsequent phosphorylation to ManNAc-6-P, two early, rate-limiting steps in the biosynthesis of sialic acid (Sia) (Figure 3). The cell surface sialyation is an ubiquitous modification of the cells which is required for their proper functioning, including signal transduction or cell interactions.[33,34] In humans, *GNE* mutations lead to GNE myopathy (GNEM), a rare, autosomal recessive neuromuscular disorder. GNEM is also known as hereditary inclusion body myopathy (hIBM), allelic distal myopathy with rimmed vacuoles (DMRV) or Nonaka disease. Inclusion body myopathy type 2 (IBM2) or quadriceps-sparing myopathy are other commonly used names of GNEM.[34,35,36]

CMP-Sia serves as a substrate for sialyltransferases, which incorporate Sia into the terminal ends of proteins and lipids, producing negatively charged glycoconjugates.[34,37] Deficient GNE/MNK produces insufficient amount of ManNAc, resp. ManNAc-6-P, which subsequently leads to the decreased production of Sia, resp. CMP-Sia, ultimately causing impaired protein/lipid glycosylation.[38]

Over 140 *GNE* mutations are known to cause GNEM. Even though no patient with complete loss of GNE/MNK has been found, the embryonic lethality in GNE knockout mice shows the crucial functions of the GNE/MNK and sialoglycans in prenatal development.[36,39]

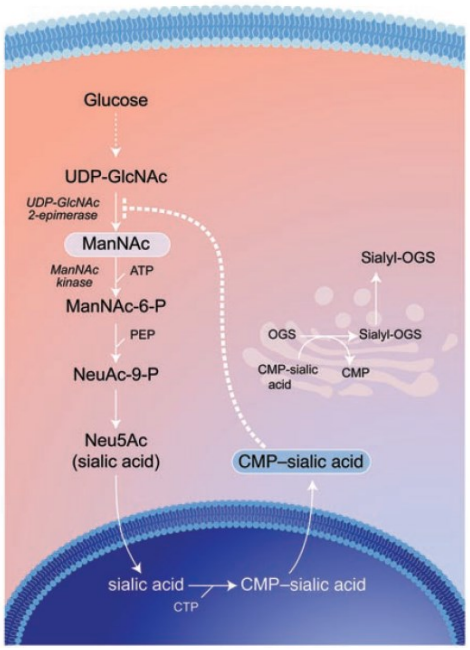


Figure 3: Adopted from Galeano, B., et al. (2007). Biosynthetic pathway and metabolism of Sia. The biosynthesis of Sia is started in the cytosol by the conversion of glucose to UDP-GlcNAc within several steps. Subsequent two steps, epimerization of UDP-GlcNAc to ManNAc and its phosphorylation, are catalysed by the bifunctional enzyme GNE/MNK. Produced ManNAc-6-P is afterwards converted to Sia, which is transferred into the nucleus, where the activation to CMP-Sia takes place. The biosynthesis is regulated by negative feedback effect of downstream CMP-Sia, which inhibits UDP-GlcNAc 2-epimerase activity by allosteric modulation.[38]

The clinical picture includes the distal muscle weakness with onset in early adulthood (typically around the age of 30), slowly progressing and affecting lower and proximal muscles of the upper limbs. Fibro-fatty tissue gradually replaces the skeletal muscle, resulting in loss of muscle strength.[36] The degree of impairment differs between specific muscles, which might suggest that variable Sia threshold levels are required by different muscles for their normal function. The relative quadriceps sparing in GNEM may be explained by naturally lower Sia levels in quadriceps muscles, contrary to e.g. gastrocnemius, which might protect the muscles from Sia deficiency.[34]

1.2.7 Current therapeutic options

Several approaches towards CDGs treatment have been attempted: supplementation of deficient monosaccharide, influencing of the metabolic flux, chaperone rescue, enzyme activation or enzyme replacement therapy. Unluckily, most of the CDGs are not causally treatable and the effective therapy is available for only three CDGs of all described to date.[9,12,29]

MPI-CDG (CDG-Ib), hepatic-intestinal disease caused by Man-P isomerase deficiency, was the first CDG effectively treated with dietary mannose supplementation.[11,14,40] Oral supplementation of fucose may moderate SLC35C1-CDG (CDG-2c), disorder of terminal fucosylation, also called leucocyte adhesion deficiency type II (LAD II).[9,11,41] Administration of butyrate, histone deacetylase inhibitor, to patients with mutations in a housekeeping *PIGM* gene, resulting in disruption of GPI-anchors synthesis, increases *PIGM* transcription, restores histone hypoacetylation and leads to clinical improvement of PIGM-CDG.[9,31]

1.2.7.1 Treatment of PMM2-CDG

Unfortunately, only supportive, symptomatic treatment can be offered to patients suffering from PMM2-CDG, the most prevalent CDG. The therapeutic goal is to increase the level of deficient GDP-Man and ManP-Dol precursors to secure the proper glycosylation.[29] Unfortunately, contrary to MPI-CDG, no signs of improvement were observed in PMM2-CDG patients treated with mannose,[42,43,44] but prophylactic prenatal mannose supplementation in women at risk for a PMM2-CDG child might overcome altered prenatal development and enable normal life thereafter.[9,27]

Supplying the deficient cells directly with Man-1-P might restore GDP-Man pool, but Man-1-P cannot enter the cell via specific membrane transporters and its high polarity limits its diffusion through the cell membranes.[45] Modification with biologically reversible protecting groups can provide a solution by increasing the lipophilicity and enabling the membrane penetration.[45] Synthesis of several derivatized compounds have already been reported.[21,45,46] Hydrophobic, cell permeable derivatives of Man-1-P prepared by Eklund, E. A., et al. (2005) were able to correct the altered glycosylation in cells from PMM2-CDG patients more efficiently than free mannose.[21] Treatment with hydrophobic Man-1-P derivatives thus may be a way to overcome PMM2-CDG, nevertheless, compounds with longer half-life and lower toxicity should be studied.[21]

Combining inhibition of Man-6-P catabolism with mannose supplementation might provide another therapeutic strategy for PMM2-CDG patients.[47,48] As seen from Figure 2, PMM2 and MPI compete for Man-6-P. Application of benzoisothiazolone, MPI inhibitor, successfully led to diversion of Man-6-P towards PMM2 with residual activity and improved the impaired *N*-glycosylation.[9,47]

Enzyme replacement therapy, gene therapy, stem-cell therapy, PMM2 activation by specific activators, or stabilisation of the enzyme by small molecules might present other approach to improve the altered glycosylation in PMM2-CDG patients, however, all strategies have certain drawbacks and further studies have to be carried out.[13,29]

1.2.7.2 Treatment of GNE Myopathy

So far, no effective therapy for GNEM patients is available, nevertheless, promising results of reported studies raise hope for the affected individuals. With the aim to circumvent the enzymatic blockage, one of the therapeutic approach is to compensate the insufficient Sia level by supplying free or bound form of Sia, or ManNAc, resp. ManNAc-6-P, as its precursor.[49] After entering the cell, ManNAc should be phosphorylated by MNK enzyme domain with residual activity or by other common sugar kinases (e.g. GlcNAc kinase) and provide ManNAc-6-P for subsequent biosynthetic steps.[35,39,49]

Prophylactic administration of naturally occurring sialic-acid related molecules, as well as ManNAc, to *GNE* deficient mice successfully increased Sia abundance in skeletal muscles and improved survival rate, muscle impairment, body weight and muscle strength, nonetheless, no dose-effect correlation was observed.[49,50] The therapeutic effect of administered exogenous Sia-related compounds is limited by the rapid excretion of Sia metabolites.[37] Potential slow-release drugs may be considered as a way to circumvent this issue and might offer suitable therapeutic strategy.[36]

Compared to natural compounds, oral administration of the synthetic tetra-*O*-acetylated *N*-acetylmannosamine (Ac_4ManNAc) to affected mice resulted in more significant improvement of sialylation with dramatic, dose-dependent effect, measurable by protein biomarkers.[37] Increased lipophilicity of Ac_4ManNAc facilitates its transmembrane diffusion into the cell, where the nonspecific esterases remove the acetyl groups and produce free ManNAc, which can be subsequently fluxed into the Sia biosynthetic pathway. Furthermore, *O*-acyl ManNAc analogues may be sequestered within the cell and provide a “reservoir” for this pathway.[51] Ac_4ManNAc was reported to be swiftly eliminated, but the positive results encourage further investigations of Sia sources as therapy for GNEM patients and clinical trials in humans.[36,37]

Intravenous application of IgG (containing Sia) or enzyme replacement therapy might be another treatment option, nonetheless, more studies should be carried out.[36,52]

1.3 Mitochondrial DNA depletion syndrome (MDS)

1.3.1 Introduction

Mitochondria are cell organelles involved in many essential biological processes, e.g. energy production in form of ATP. Mitochondrial DNA depletion syndrome (MDS) is group of rare, mostly recessively inherited disorders, characterized by mitochondrial dysfunction, due to the lack of mitochondrial DNA (mtDNA) in affected tissues and organs.[53,54,55] MDS are associated with reduced mtDNA copy number that is caused by mutations in nuclear genes (nDNA), which disrupt either mitochondrial nucleotide pool maintaining, or mtDNA replication. mtDNA depletion leads to inadequate synthesis of mitochondrial respiratory chain subunits, resulting in energy deficiency and subsequently organ dysfunction.[55,56,57]

The genetic heterogeneity results in broad spectrum of clinical manifestation, which vary between specific phenotypes.[55] Neonates, infants, or juveniles are the most frequently affected individuals. Unfortunately, the prognosis of MDS patients is poor and no cure is available.[55]

1.3.2 Origin, biology of mitochondrial depletion syndrome

1.3.2.1 Genetic background

Mitochondrial DNA is closed, circular double-stranded molecule, localized in the mitochondrial matrix in several copies. It encodes 37 genes, all involved, directly or indirectly, in ATP production.[57] Contrary to nDNA, mtDNA replication is independent of the cell division. Continuous mtDNA replication requires balanced mitochondrial deoxyribonucleotide triphosphate (dNTP) pools, permanently available for deoxyribonucleic acid synthesis and maintenance. Homeostasis of dNTP pools is crucial for genetic stability, since the lack, as well as the redundancy, of particular dNTP may lead to the disease. dNTPs are synthetized inside the mitochondria via salvage pathway by step-wise phosphorylation of pre-existing deoxyribonucleosides. Alternatively, they may be formed in the cytosol via *de novo* pathway during the S phase of the cell cycle by reduction of ribonucleotides and subsequently imported into the mitochondrial matrix via specific transporters (Figure 4).[55,57] In terminally differentiated, non-dividing cells, dNTPs levels depend mainly on the mitochondrial enzymes involved in the salvage pathway, since the cytosolic synthesis of dNTPs is down-regulated.[57] Impaired dNTP synthesis inside the mitochondria, caused by nDNA mutations, then cannot sufficiently meet the demands for dNTPs during mtDNA replication or reparation.[56,57,58]

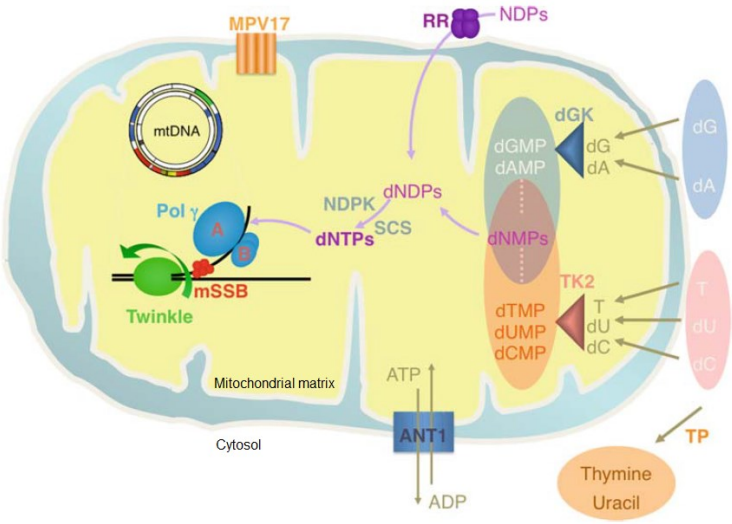


Figure 4: Adopted from Spinazzola A. et al. (2009). dNTPs metabolic pathways and mtDNA replication. Abbreviations: dNMP, dNDP, dNTP: mono-, di-, tri-phosphate deoxynucleotides, NDPK: nucleoside diphosphate kinase. SCS: succinate CoA synthase isoforms (encoded by SUCLA2 and SUCLG1 genes). ANT1: (muscle specific) isoform 1 of the adenine-nucleotide translocator. mtSSB: mitochondrial single-strand DNA binding protein. RR: ribonucleotide reductase (encoded by RRM2B gene). TP: thymidine phosphorylase (encoded by TYMP gene). T, dU, dC, dG, dA: deoxynucleosides. Poly γ: polymerase gamma (encoded by POLG gene). dGK: deoxyguanosine kinase (encoded by DGUOK gene). TK2: thymidine kinase 2 (encoded by TK2 gene). Twinkle: protein (encoded by C10orf2). MPV17: protein (encoded by MPV17 gene).[57]

DGUOK, TK2, SUCLG1, SUCLA2, TYMP and *RRM2B* are genes encoding proteins essential for maintaining dNTP pools, mainly through the salvage pathway. Mutation in any of these genes may lead to imbalances of any of dNTP pools and subsequent deficient mtDNA synthesis. *DGUOK* provides deoxyguanosine kinase (dGK) responsible for phosphorylation of purine nucleosides, whereas thymidine kinase 2 (TK2), the product of *TK2*, phosphorylates the pyrimidine nucleosides. dGK and TK2 catalyse the first and rate-limiting steps of the dNTPs synthesis via salvage pathway.[55,58]

The only DNA polymerase in humans, DNA polymerase γ, is responsible for mtDNA replication and reparation. For its proper functioning, catalytic subunit encoded by *POLG* is necessary, as well as the cooperation with DNA helicase, which requires the twinkle protein, encoded by *C10orf2 gene,* for its function. Mutations in *POLG* or *C10orf2* result in impaired mtDNA replication due to defective replication enzymes.[55]

MPV17 protein, encoded by *MPV17*, is mitochondrial inner membrane protein with unknown function, nevertheless, mutations in *MPV17* results in impaired mtDNA maintenance.[55,59]

1.3.2.2 Diagnosis

Besides the manifesting symptoms, the diagnosis is based on the laboratory tests (including mtDNA level detection) which reveal abnormal biochemical features. Lactic acidosis, hypoglycaemia or elevated serum enzymes may be present. Muscle biopsy or neuroimaging may be used to examine the muscular, resp. neurological pathology. Identification of the particular mutated gene by genetic testing ultimately confirms MDS diagnosis.[55,60]

1.3.2.3 Clinical manifestation

Considerable number of diverse genes, associated with dNTPs metabolism and mtDNA production, reflects in phenotypic heterogeneity of MDS manifestation. The diseases typically affect newborns or infants, however, *TYMP*-related MDS has been reported to occur usually during adolescence. The disorders may impair one specific organ or combination of organs, including brain, muscle, liver, or intestines.[55] Clinically, four phenotypes are distinguished according to the majorly involved organs: hepatocerebral, myopathic, encephalomyopatic and neurogastrointestinal.[55] Typical clinical features of the phenotypes are noted in Table 3.

Alpers-Huttenlocher syndrome (AHS), mitochondrial neuro-gastro-intestinal encephalo-myopathy (MNGIE), infantile onset spinocerebellar ataxia (IOSCA), and mitochondrial recessive ataxia syndrome (MIRAS) represent four syndromic mitochondrial disorders resulting from deficient mtDNA.[60]

Unfortunately, the prognosis is grave and no effective treatment is available. Most of the patients die during the infancy or childhood.[57]

Form of MDS	Mutated gene	Clinical features
Hepatocerebral	*DGUOK, MPV17, POLG, or C10orf2*	Hepatic dysfunction, psychomotor delay, hypotonia, seizures, ataxia, peripheral neuropathy, sense impairment
Myopathic	*TK2*	Muscle, facial, bulbar weakness, hypotonia, feeding difficulties, failure to thrive
Encephalomyopathic	*SUCLG1, SUCLA2, or RRM2B*	Muscle weakness, hypotonia, psychomotor delay, impaired hearing, seizures, movement disorders, failure to thrive
Neurogastrointestinal	*TYMP*	Peripheral neuropathy, ptosis, opthalmoplegia, gastrointestinal dysmotility, chronic diarrhoea, cachexia

Table 3: Adopted from Adopted from El-Hattab, A. W. and F. Scaglia (2013). Clinical phenotypes of MDS.

1.3.3 Current therapeutic options

Despite deepening of our knowledge about molecular mechanism of MDS, no effective causal treatment is available. So far, the clinically used therapeutic strategies are mainly supportive and symptomatic.[55] The liver transplantation is the only therapeutic option dealing with the hepatic dysfunction in patients suffering from hepatocerebral form of MDS.[55,56]

"Molecular bypass therapy" that modulates dNTP pools by deoxyribonucleotides supplementation, seems promising for particular MDS patients. Increasing the availability of deficient mitochondrial deoxyribonucleotides leads to the improvement of dNTP imbalances. Cultivation of human *DGUOK* deficient myotubes in medium containing dAMP/dGMP resulted in increased mtDNA copy number.[53,56,61] Oral administration of dCMP/dTMP to *TK-2* knock-in mice raised dTTP concentrations, improved mtDNA levels and prolonged mice lifespan.[62] Generally, dNMPs are charged, polar molecules, which cannot easily penetrate the plasma membranes, and, moreover, they are sensitive to dephosphorylation in the extracellular space. Due to the low bioavailability of such nucleotides, employment of protecting groups masking the charge and increasing the lipophilicity should be considered.[56] Nevertheless, increased dNTP levels may negatively regulate deoxyribonucleoside kinases and RRs. Further investigations are necessary to avoid the secondary imbalance.[53,56]

The key role in dNTP pool size maintenance plays the balance between mitochondrial and cytosolic synthetic pathways, incorporation into DNA, and enzymatic catabolism of dNTP, which is secured by deoxyribonucleotidases, triphosphohydrolases, and specific phosphorylases. Interfering with dNMPs degradation via inhibitors of catabolic enzymes might be an alternative way to improve mitochondrial dNTP pools.[53,56,57]

Exogenous stem cell therapy or gene therapy might offer other therapeutic options.[55,63,64] Gene therapy holds great potential for MDS, but problematic gene targeting and delivery, immune reactions, and short-lived effect make it challenging.[62,65]

1.4 Monophosphate prodrugs approach

Monophosphate-containing drugs has been developed for application as mainly antiviral and anticancer agents, but may be applicable in any felicitous therapeutic area.[66] To be incorporated into DNA, the nucleoside analogues, used in antiviral and anticancer treatment, must be phosphorylated to respective triphosphates. To circumvent the first slow phosphorylation step catalysed by nucleoside kinase, the nucleoside monophosphates already bear the phosphate moiety.[66,67] In cases of CDGs and MDS, appropriate monophosphate drugs should bypass the enzymatic blockage, enabling the downstream biosynthetic pathway of activated sugar, resp. improving dNTP pool. Unfortunately, monophosphates struggle with poor cell penetration, since they are strong acids, negatively charged at physiological pH. Such hydrophilic compounds are not able to simply penetrate the cell membrane, resulting in low intracellular bioavailability.[66,67] Moreover, they are rapidly converted to the parent molecules by blood and surface extracellular phosphohydrolases.[66]

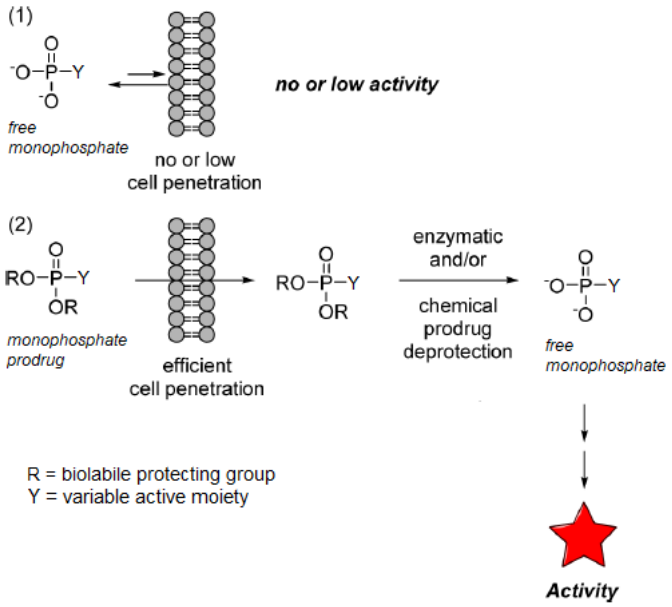


Figure 5: Adapted from Pradere, U., et al. (2014). Mechanism of activation of monophosphate prodrugs. "Protected" monophosphates are capable of cell membrane penetration by passive diffusion and can efficiently deliver the parent monophosphates into the cell. The prodrugs undergo intracellular enzymatic and/or chemical degradation of biolabile protecting groups, releasing the original monophosphate.[67]

To overcome the monophosphates limitations, several prodrug approaches have been devised. The prodrugs should promote passive diffusion and increase monophosphates bioavailability, thus enhancing their therapeutic potential.[66] The ionisable phosphate groups can be derivatized by various protecting groups, masking the negative charges, increasing the lipophilicity and facilitating the membrane penetration (Figure 5). The prodrug approach should afford extracellularly stable compound, which would rapidly undergo the hydrolysis once inside the cell, providing the original phosphate.[66]

This chapter will introduce several prodrug approaches applicable to monophosphate-containing molecules, focusing on ProTide methodology.

1.4.1.1 Bis(POM) + bis(POC)

Carbonyloxymethyl protecting groups were successfully applied to mask the negative charges of the phosphate (X = O), resp. phosphonate (X = CH_2). Introduction two pivaloyloxymethyl (POM) or two isopropoxycarbonyl groups (POC) effectively increased the oral bioavailability and systemic exposure to the parent phosphate, resp. phosphonate. The bioactivation of bis-(POM) and bis-(POC) prodrugs is provided by two-step esterase-mediated cleavage of the protecting groups. Adefovir dipivoxil and tenofovir disoproxil fumarate are examples of such prodrugs in clinical use, approved for HBV infections treatment and HIV-1 infections, respectively.[67,68]

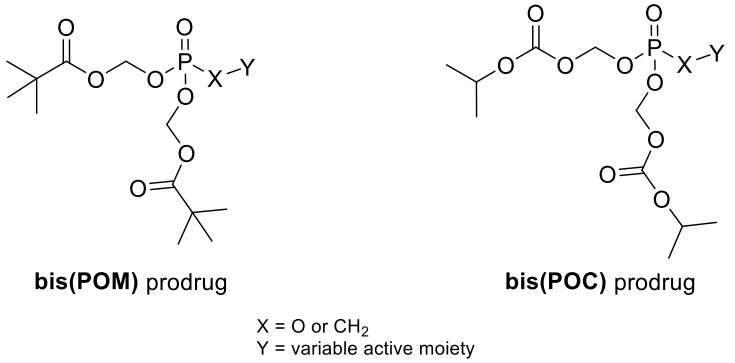


Figure 6: General structure of bis(POM) and bis(POC) prodrugs.

1.4.1.2 Bis(SATE) + bis(DTE)

Implementation of S-acyl-2-thioethyl (SATE) or S-[(2-hydroxyethyl)sulfidyl]-2-thioethyl (DTE) chains is another approach developed to increase the cellular permeability of monophosph(on)ates. After entering the cell, enzymatic activation generates original monophosph(on)ate, but also ethylene sulfide as potentially toxic byproduct, which limits the advancement of these prodrugs.[66,67,69]

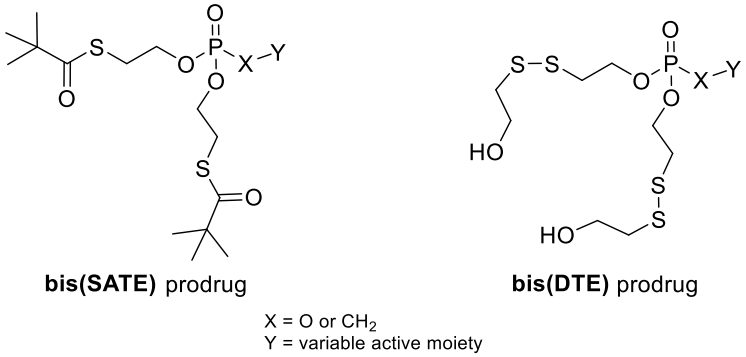
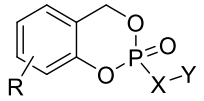


Figure 7: General structure of bis(SATE) and bis(DTE) prodrugs.

1.4.1.3 CycloSal

The cyclosalogenyl (cycloSal) prodrug approach has been explored as another method improving the phosphates or phosphonates intracellular delivery. The phosphate or phosphonate group bears molecule of salicyl alcohol, masking the negative charges. Tandem intracellular chemical hydrolysis cleave the protecting group, providing the free phosph(on)ate. Due to the low hydrolytic stability of cycloSal derivatives, the masking group may be also partially hydrolysed extracellularly, hampering prodrugs cellular import. Therefore, additional function may be attached to the aromatic ring, increasing the hydrolytic stability in the extracellular compartment.[66,68]



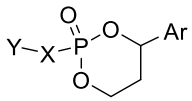
cycloSal prodrug

R = side chain
X = O or CH_2
Y = variable active moiety

Figure 8: General structure of cycloSal prodrug.

1.4.1.4 HepDirect

The cyclic 1-aryl-1,3-propanyl ester prodrugs (HepDirect) were developed with the aim to affect exclusively the liver and thus decrease the exposure to other tissues. The liver targeting is achieved by implementation of masking group, which is selectively cleaved by liver specific cytochrome P450 (CYP 3A4). Pradefovir mesylate, HepDirect prodrug of adefovir, is a small molecule undergoing clinical trial for the treatment of HBV.[68,69]



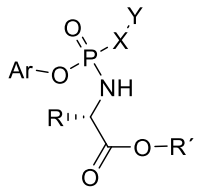
HepDirect prodrug

Ar = aryl
X = O or CH_2
Y = variable active moiety

Figure 9: General structure of HepDirect prodrug.

1.4.1.5 Aryloxy phosphoramidate / phosphonamidate triesters (ProTide approach)

The ProTide ("pronucleotide") approach was developed by McGuigan group in early 1990s as a prodrug technology, enabling the intracellular delivery of charged nucleotide analogues. General structure of ProTides (Figure 10) consists of the parent monophosphate (X = O), resp. monophosphonate (X = CH_2), of which negative charges are masked by aryl and amino acid ester moieties.[67,68] The ProTide approach was successfully applied to not only nucleoside derivatives, but also to *N*-acetyl-D-glucosamine analogues, improving their anti-osteoarthritic activity compared to the parent molecules.[70]



ProTide prodrug

Ar = aryl
R = amino acid side chain
R´ = alkyl / aryl
X = O or CH_2
Y = variable active moiety

Figure 10: General structure of ProTide prodrugs.

Owing to the increased lipophilicity achieved by the introduction of the protecting groups, the molecule of ProTide can penetrate the lipid-rich cell membrane by passive diffusion and thus deliver the parent phosph(on)ate inside the cell. To liberate the free active molecule, it has to undergo the intracellular bioactivation pathway (Figure 11). The putative activation mechanism is initiated by the carboxylic ester hydrolysis mediated by carboxylesterase or carboxypeptidase (Cathepsin A) enzyme, providing the intermediate A. After the ester cleavage, the internal nucleophilic attack of the carboxylate to the phosphorus centre, together with the aryloxy group displacement leads to formation of putative cyclic intermediate B. This unstable cyclic anhydride is rapidly hydrolysed to corresponding phosphoramidate, resp. phosphonamidate derivative C. The final P-N cleavage, releasing the desired monophosphate, resp. monophophonate, is provided by phosphoramidase-type, resp. phosphonamidase-type enzyme, or by spontaneous hydrolysis of the amino acid moiety inside the more acidic subcellular environment.[68,69]

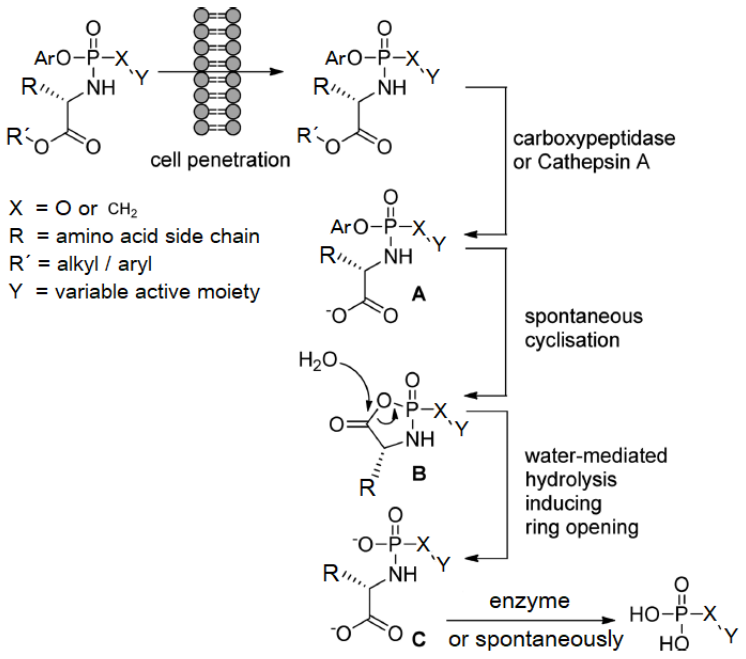


Figure 11: Adapted from Pradere, U., et al. (2014). Proposed mechanism of ProTides activation.

1.4.1.5.1 Aryloxy phosphoramidates triesters

The ProTide approach was firstly applied to antiretroviral agent zidovudine with positive impact on its intracellular releasement. This success led to the further application of the ProTide technology on several nucleoside analogues and structure-activity relationship (SAR) investigation. To identify the suitable substituents, affording the optimal prodrug activity, an extensive study was accomplished, evaluating the impact of different aryl, amino acid and ester moieties on the ProTide biological activity. The general outcomes are captured in Figure 12.[68,71]

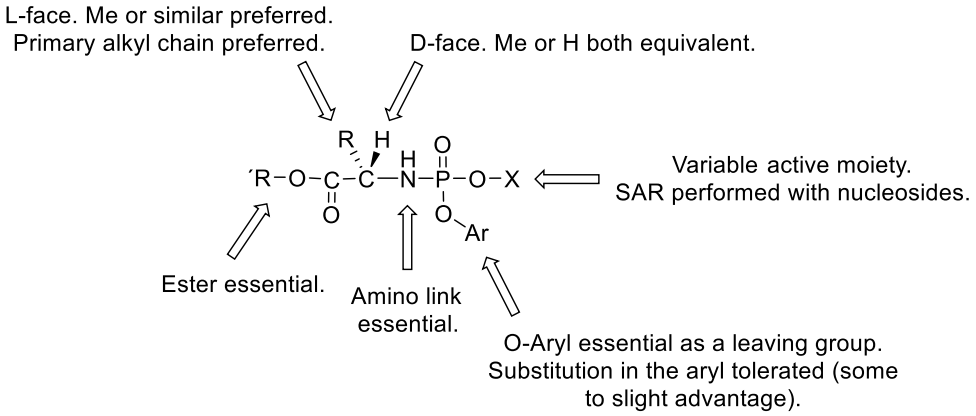


Figure 12: Adopted from Dominique, C., et al. (2004). SAR of aryloxy phosphoramidates triesters.

Structure-activity relationship (SAR)

The ester moiety is crucial for the bioactivation of the ProTide. The ester bond has to be sufficiently labile to be hydrolysed and achieve the relevant biological activity. A number of esters have been explored, from linear, branched, cyclic, up to aromatic. Generally, the more lipophilic the prodrug is, the more the compound is able to diffuse through the cell membrane. The lipophilic benzyl and naphthyl ester derivatives appeared to be the highest potent, while branched lipophilic *tert*-butyl ester derivatives exhibited poor activity. The steric hindrance of bulky *tert*-butyl moiety most likely interfere with carboxypeptidase-type enzyme susceptibility, which leads to the reduced ester bond cleavage and subsequent bioactivation pathway.[68,71]

The suitable aryl substituent (phenyl, 1-naphthyl) should act as a good leaving group, since its elimination during the bioactivation is not enzyme-mediated, but is based on the intramolecular cyclisation. Aryl substituents are preferred to alkyl and haloalkyl moieties because of their ability to delocalise the negative charge, that makes them better leaving groups. The leaving group ability may be increased by mild electron-withdrawing substituents, while implementation of strong electron-withdrawing group leads to reduced potency.[68,71]

The attachment of the α-amino acid moiety via P-N bond is essential for the ProTide bioactivation. The SAR studies showed that the substitution by the P-O bond or replacement with n-alkylamine chain resulted in considerable or complete activity loss. The distance between the nitrogen and the carboxyl is important as well. The extension of the chain length (β-amino acids) led to decreased activity, correlating with the fact that the potentially formed six-membered cyclic intermediate is not favoured. From stereochemical point of view, L-amino acids are preferred by the bioactivation enzymes, whereas unnatural D-amino acids poorly interact with these enzymes, which results in low activity of such derivatives. In general, the concrete α-amino acid structure may be variable. Stavudine ProTides with L-alanine provided the best antiviral activity, making the L-alanine the amino acid of choice for nucleoside prodrugs, whilst in case of *N*-acetyl-D-glucosamine phosphoramidates, L-proline derivatives performed the best results.[68,71]

The presence of chiral phosphorus, risen from the phosphorus asymmetrical substitution, in combination with other chiral centres (with fixed or variable configuration) of the ProTide molecule, results in the formation of a mixture of diastereoisomers. No correlation between absolute configuration at the phosphorus atom and the relative potency of the prodrug was found, nevertheless, the biological activity may be influenced by the configuration of phosphorus centre due to stereoselectivity of appropriate bioactivation enzymes.[68,71]

Synthesis

Generally, three distinct methods (summarised in Figure 13) are usually used to access aryloxy phosphoramidates triesters. Approach A involves coupling of the nucleophile (the proper active moiety, e.g. nucleoside, monosaccharide) with pre-prepared phosphorochloridate reagent in the presence of *t*BuMgCl or NMI. *t*BuMgCl acts as a strong base able to deprotonate the free hydroxyl group of the nucleophile, providing more nucleophilic alkoxide, whereas NMI serves as an activator of the phosphorochloridate (by formation of imidazolinium intermediate), thus increasing its reactivity towards nucleophiles. The phosphorochloridates are usually prepared by reacting the aryldichlorophosphate with desired amino acid ester in the presence of Et_3N. The alternative phosphite approach (B) consists of reaction of the nucleophile with previously prepared diaryl phosphite, followed by oxidative amination. To avoid potential formation of byproducts mixture, the synthesis may be accomplished via P(III) substitution and oxidation, and subsequent reaction with the amino acid ester. The third synthetic approach (C) is based on the preparation of aryloxy phosphoramidate intermediate (z) and final coupling with the desired amino acid ester.[67]

All approaches mentioned above generally yields diastereomeric mixture (1:1) of ProTides with different configuration at the phosphorus centre, which are usually not separable by flash chromatography. In view of the fact, that S_P and R_P isomers may possess different biological properties, diastereoselective approach (A´) was developed to obtain enantiomerically pure ProTides.[67]

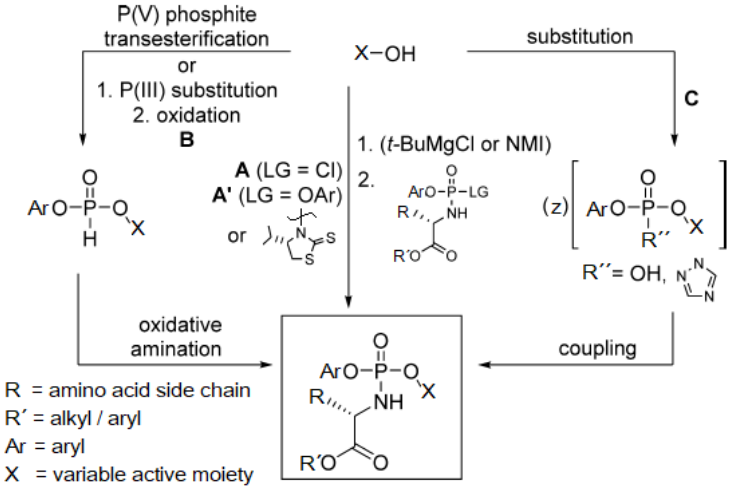


Figure 13: Adapted from Pradere, U., et al. (2014). Synthetic approaches towards aryloxy phosphoramidate prodrugs.

1.4.1.5.2 Aryloxy phosphonamidate triesters

After successful application of ProTide approach to nucleoside phosphates, the strategy was extended also to nucleoside phosphonates (X = CH_2). In case of phosphonates, the nucleoside is attached to the phosphorus via P-C instead of P-O bond, which increases the ProTide chemical and enzymatic stability. P-C bond is resistant to phosphodiesterases and phosphatases, preventing the hydrolysis of the phosphonate group, which makes the phosphonate analogues potentially more effective. Similarly to ProTides of the phosphates, negative charges are masked by aryloxy group and amino acid ester moiety linked via P-N bond, increasing the molecule lipophilicity and facilitating the passive diffusion.[69] Example of successful aryl phosphonamidate diester approach is application to adefovir by Ballatore *et al*., resulting in enhanced anti-HIV potency of the prodrug compared to original nucleotide derivative.[67,69]

1.4.1.6 Phosphoramidate / phosphonamidate diesters

Phosphoramidate diester (also known as amino acid amidate monoester) strategy has been explored by Wagner *et al*. as a modification of aryloxy phosphoramidate approach. Contrary to aryloxy phosphoramidates, which mask both negative charges, these prodrugs mask only one negative charge of the phosphate group by means of one amino acid ester, which improves the water solubility and moreover, the stability in human blood. The lack of aryloxy moiety attached to the phosphorus centre leads to the loss of phosphorus chirality and thus optical isomers. The direct cleavage of amino acid moiety is executed by intracellular phosphoramidase-type enzyme, releasing the parent monophosphate (Figure 14). Amino acid amidate monoester prodrugs of azidothymine, 1-β-arabinofuranosylcytosine, and 5-fluoro-2'-deoxyuridine showed increased antiviral activity *in vitro*, nevertheless, they are degraded in the gastrointestinal tract resulting in poor bioavailability *in vivo*.[67,68] Amino acid phosphonamidate monoester approach has been published in the literature less frequently. For example, application to cidofovir and adefovir molecules was reported.[67]

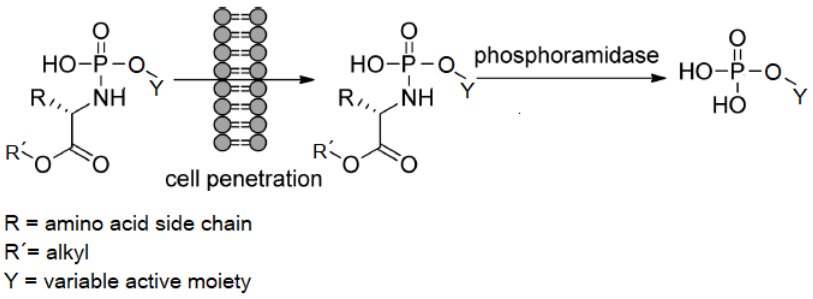


Figure 14: Adapted from Pradere, U., et al. (2014). Mechanism of phosphoramidate diesters activation.

1.4.1.7 Phosphorodiamidates / phosphonodiamidates

Phosphorodiamidate and phosphonodiamidate prodrugs incorporate two identical amino acid esters, serving as the masking groups of polar phosphate, which are linked via P-N bond to appropriate active moiety. This approach has been investigated after the ProTide application, offering two substantial benefits: phosphorus is achiral centre due to the symmetric structure of attached amino acid esters and no noxious byproducts are released since the displaced amino acids are nontoxic.[67,69]

The activation mechanism proposed by McGuigan, depicted in Figure 15, is supposed to be analogous to similar phosphoramidates, resp. phosphonamidates. After the cell membrane penetration, esterase-type enzyme cleaves one of the amino acid esters which leads to the spontaneous cyclization to five-membered cyclic intermediate while amino acid moiety is cleaved. Subsequent rapid spontaneous hydrolysis provides corresponding aminoacyl phosphoramidate ester, resp. phosphonamidate ester. Final P-N cleavage may be enzyme-mediated or may occur spontaneously generating parent monophosphate, resp. monophosphonate.[69]

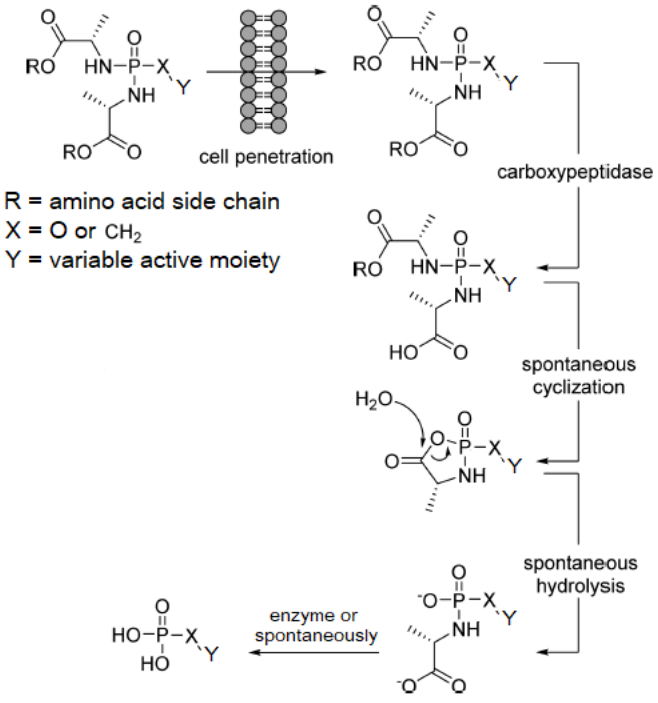


Figure 15: Adapted from Adapted from Pradere, U., et al. (2014).Mechanism of phosphorodiamidates, resp. phosphonodiamidates unmasking.

2 Aim of the work

Nowadays, there are no medications in clinical use available for the majority of CDGs. The research showed considerable pharmacological potency of Man-1-P to restore GDP-Man levels in patients with PMM2-CDG and the ability of ManNAc and its derivatives to improve deficient sialylation in patients suffering from GNEM.[45,49,51] Therapeutic supplementation with Man-1-P or ManNAc-6-P thus could be an effective way to bypass the appropriate enzymatic insufficiency. Despite the positive pharmacological effect, the physical and chemical properties of the monophosphates are unfavourable. As highly polar molecules, the monophosphates struggle with difficult transmembrane penetration resulting in decreased intracellular bioavailability. Moreover, they are substrates of the extracellular phosphorylases which are responsible for the elimination of the phosphate group from the parent monophosphate. Several prodrug strategies dealing with these issues have been developed.[67] As previously described, the ProTide technology represents an effective approach towards enhanced delivery of the monophosphates into the cells and improved *in vivo* stability, and may be used for Man-1-P or ManNAc-6-P prodrugs.

Unfortunately, either MDS patients cannot be offered with any causal therapy so far. The administration of deficient deoxyribonucleotides was reported to better the nucleotide imbalances. Similarly to sugar monophosphates, deoxyribonucleotides possess the negatively charged phosphate group, which hamper their intracellular penetration. In analogy with other prodrugs successfully used in other therapies, the ProTide technology can be also employed to deliver the nucleotides necessary to synthesize the mtDNA.[67,72]

This work was focused on chemical synthesis of ProTides. The aims of the project were:

- Application of the ProTide approach to Man-1-P, ManNAc-6-P and their peracetylated derivatives (Figure 16) and synthesis of various potential prodrugs for congenital PMM2-CDG (D-mannose derivatives) and GNEM (*N*-acetyl-D-mannosamine derivatives).

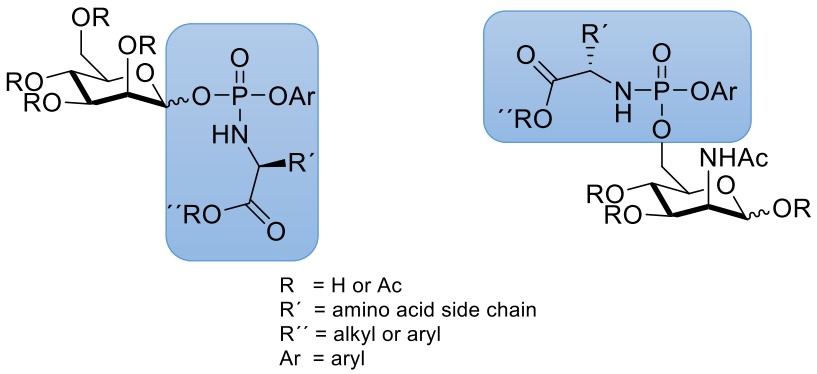


Figure 16: The first aim of this work. General structure of ProTides for CDGs.

- Preparation of ProTide prodrugs of 6-methoxyguanosine monophosphate (Figure 17), potentially useful for the treatment of mitochondrial DNA depletion syndrome.

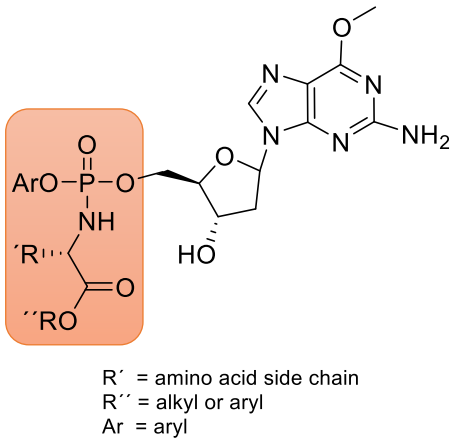


Figure 17: The second aim of this work. General structure of 6-methoxyguanosine ProTides for MDS.

Such prodrugs are expected to easily diffuse from the extracellular compartment to the cell cytosol owing to the increased lipophilicity and to release the corresponding monophosphates by intracellular transferases.

In the following chapters, the synthesis of novel ProTides will be described in detail. Furthermore, the enzymatic study carried out in order to confirm the ProTide affinity to the activation enzyme will be presented.

3 Results and Discussion

3.1 Synthesis of ProTides as potential medication for CDGs

3.1.1 Introduction

Supplementation of deficient Man-1-P in patients with CDG-Ia represents an option to compensate the inability of PMM2 to convert Man-6-P into Man-1-P leading to the insufficient level of GDP-Man-1-P which plays an important role in *N*-glycan biosynthesis. Similarly, administrated ManNAc-6-P could replace the missing product of impaired GNE in patients with GNEM. ManNAc-6-P is one of the sialic acid precursors, shortage of which leads to hyposialylation of glycolipids and glycoproteins, causing progressive muscle weakness.[45,49,51]

Because monosaccharide monophosphates are highly polar molecules, they cannot diffuse through the membrane into the cell. Therefore, this project was focused on the synthesis of phosphoramidates as more lipophilic prodrugs affording better intake of the molecules into the cells. In several steps, the intracellular bioactivation of the phosphoramidates provides the monophosphates of the parent molecules.[66]

This section will describe the synthesis of phosphoramidate prodrugs of Man-1-P and ManNAc-6-P as well as their peracetylated derivatives (Figure 18). Peracetylated sugars were previously reported to be able to penetrate the cells by the membrane diffusion more easily because of the increased lipophilicity.[73] Deacetylation of the core sugar is provided inside the cell by nonspecific esterases, releasing the free hydroxyl sugar available for the specific biosynthetic pathways.

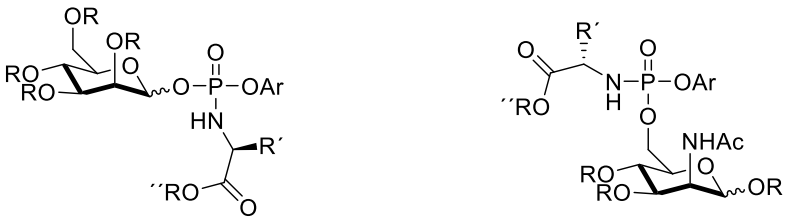


Figure 18: General structures of sugar phosphoramidate prodrugs synthetized in this work.

In general, the synthetic work of this section was divided into three parts. First, we had to prepare the necessary phosphoramidating agents. Secondly, the sugar substrates that would serve as the starting materials for the prodrugs synthesis had to be synthetized. As the third step, final compounds were prepared. Phosphoramidates are generally

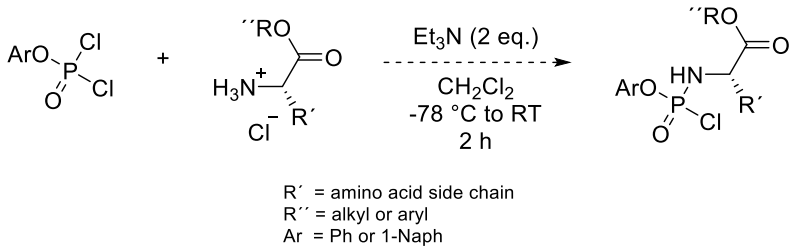
synthetized following two different approaches, both using phosphorochloridates as the phosphorus source.[67] They are coupled with the desired substrates via two different methodologies: a) nucleoside activation via a Grignard reagent, usually *t*BuMgCl and b) *N*-methylimidazole (NMI) activation of phosphorochloridate.

In the following chapter, the synthesis of phosphorylating agents (phosphorochloridates) will be described. Subsequently, we will explore the preparation of the starting materials and the proper synthesis of desired prodrugs of Man-1-P, ManNAc-6-P and their peracetylated derivatives.

3.1.2 Phosphorochloridates synthesis

3.1.2.1 Introduction

The preparation of phosphorochloridates (General procedure **A**) as the key intermediates in the ProTides synthesis is usually accomplished by reacting the appropriate amino acid ester salt with an aryldichlorophosphate in the presence of trimethylamine as shown in Scheme 1.[67]

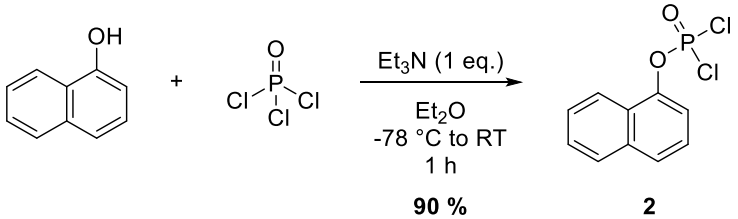


Scheme 1: General procedure for phosphorochloridates synthesis.

In this part, we will describe the synthesis of the aryldichlorophosphate followed by the description of the amino acid ester hydrochloride preparation and ultimately, the phosphorochloridates synthesis will be delineated.

3.1.2.2 Synthesis of aryldichlorophosphate

Phenyl dichlorophosphate (**1**) was commercially available whereas α-naphtyl dichlorophosphate (**2**) was previously prepared by our group as outlined in Scheme 2.

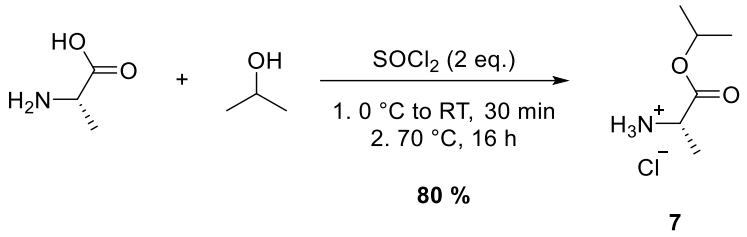


*Scheme 2: Previous synthesis of α-naphtyl dichlorophosphate (**2**).*

The substitution of phosphoryl chloride with α-naphtol was carried out in the presence of triethylamine which was added slowly to the cooled solution (-78 °C) of α-naphtol and $POCl_3$ in diethyl ether.[74] Triethylamine neutralizes the hydrogen chloride formed during the reaction whilst low temperature prevents the multiple coupling.

3.1.2.3 Synthesis of amino acid ester hydrochloride

All desired amino acid esters hydrochlorides (**3-6**) were available from commercial sources except L-alanine isopropyl ester hydrochloride (**7**) which was previously synthetized via esterification of L-alanine with isopropyl alcohol using thionyl chloride in excess (Scheme 3).[74]

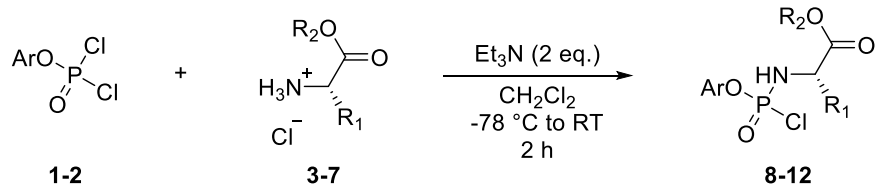


Scheme 3: Previous synthesis of L-alanine isopropyl ester hydrochloride (7).

3.1.2.4 Preparation of phosporochloridates

The synthesis of final phosphorochloridates **8-12** (Scheme 4) was accomplished by the reaction of 1 eq. of desired phosphorodichloridate (**1-2**) dissolved in anhydrous dichloromethane which was added to a suspension of the appropriate amino acid ester hydrochloride (**3-7**) (1 eq.) in anhydrous dichloromethane cooled to -78 °C. After 20 minutes, anhydrous triethylamine (2 eq.) was added dropwise over a period of 15 minutes.[74] Two equivalents were necessary. The first equivalent was necessary to produce the nucleophilic free amino group while the second equivalent was necessary to neutralise the HCl developed in the coupling reaction.

All compounds were synthetized according to the procedure **A**. The yields of the final, isolated products are listed in Table 4.



1 Ar = Ph
2 Ar = 1-Naph

3	$R_1 = CH_2Ph$	$R_2 = CH_2CH_3$	**8**	Ar = Ph	$R_1 = CH_2Ph$	$R_2 = CH_2CH_3$	**99 %**
4	$R_1 = CH(CH_3)_2$	$R_2 = CH_2Ph$	**9**	Ar = Ph	$R_1 = CH(CH_3)_2$	$R_2 = CH_2Ph$	**89 %**
5	$R_1 = H$	$R_2 = CH_3$	**10**	Ar = Naph	$R_1 = H$	$R_2 = CH_3$	**74 %**
6	$R_1 = CH_3$	$R_2 = CH_2Ph$	**11**	Ar = Naph	$R_1 = CH_3$	$R_2 = CH_2Ph$	**96 %**
7	$R_1 = CH_3$	$R_2 = CH(CH_3)_2$	**12**	Ar = Ph	$R_1 = CH_3$	$R_2 = CH(CH_3)_2$	**99 %**

*Scheme 4: Synthesis of phosphorochloridates **8-12**.*

Compound	Ar	R_1	R_2	Yield [%]
8	Ph	CH_2Ph	CH_2CH_3	99
9	Ph	$CH(CH_3)_2$	CH_2Ph	89
10	α-Naph	H	CH_3	74
11	α-Naph	CH_3	CH_2Ph	96
12	Ph	CH_3	$CH(CH_3)_2$	99

Table 4: Synthetized phosphorochloridates with reactions yields.

The slow addition of Et_3N was necessary to avoid the formation of bisamidate by-product, as evidenced by the additional peak in the ^{31}P NMR spectra (and previously observed and isolated in the group). During the synthesis of compounds **8**, **9** and **11**, a small amount of corresponding bisamidate was formed (Figure 19), nevertheless, the obtained compounds were not further purified since the molecules with both chlorine substituted with amino acid would not react in the following reaction.

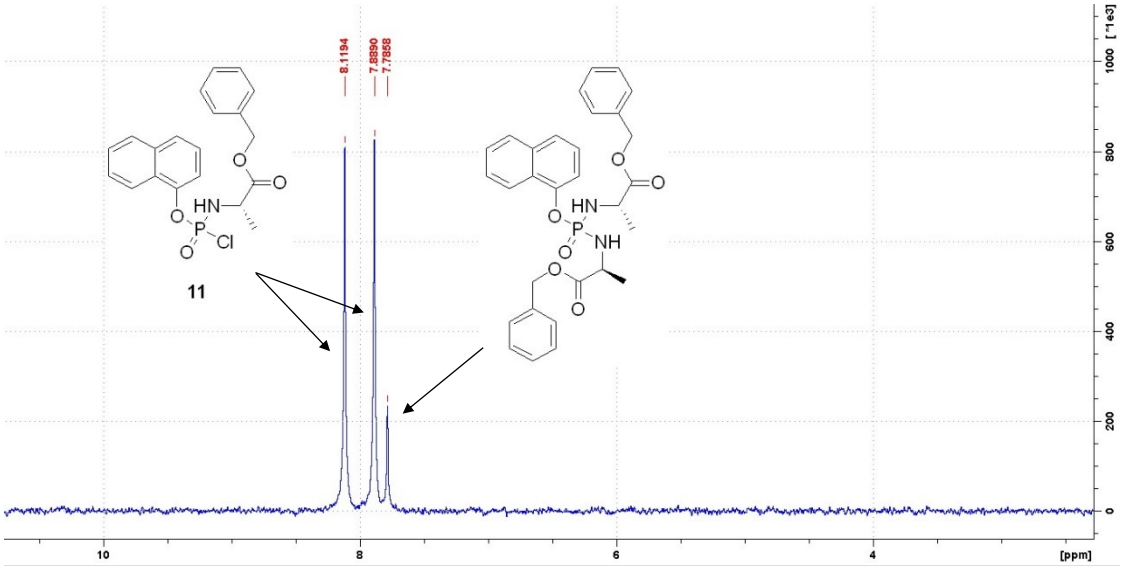


*Figure 19: ^{31}P NMR (202 MHz, $CDCl_3$) of **11**. Peak at 7.79 ppm represents the formed bisamidate by-product, other two peaks correspond to two diastereoisomers of phosphorochloridate **11**.*

All reactions were monitored by ^{31}P NMR (202 MHz, $CDCl_3$). Completed substitution was indicated by the disappearance of the singlet corresponding to the original phosphorodichloridate and detection of singlet (**10**) or doublet (**8**, **9**, **11**, **12**) of phosphorochloridate with ppm shifted towards lower field.

The removal of triethylamine hydrochloride was achieved by suspending the solid obtained after the evaporation of dichloromethane in diethyl ether, stirring for 15 minutes and filtering on sintered glass filter.

The coupling between aryldichlorophosphate and amino acid esters results in the formation of a new chiral centre at the phosphorus. In case of compound **10,** two enantiomers were formed with the only chiral centre on phosphorus since the amino acid ester used (glycine methyl ester, **5**) is achiral. Generally, enantiomers are not distinguishable by ^{31}P NMR spectra without special sample modification since they have identical properties in usually used achiral environment.[75] Therefore, only a single peak representing both enantiomers (R_P, S_P) of the phosphorochloridate **10** can be observed (Figure 20a). On the other hand, the rest of the phosphorochloridates (**8**, **9**, **11**, **12**) were synthetized from optically active L-amino acid esters. The presence of a chiral carbon of the amino acid (a stereocentre with fixed configuration) and phosphorus (the second stereocentre which configuration is variable) leads to the creation of two diastereoisomers (LR_P, LS_P). The chemical shift of the diastereoisomers differ from each other, hence two signals in the ^{31}P NMR spectra are detected (Figure 20b).

Given that the phosphorochloridates are sensitive to moisture, they were stored under inert atmosphere in the freezer.[74]

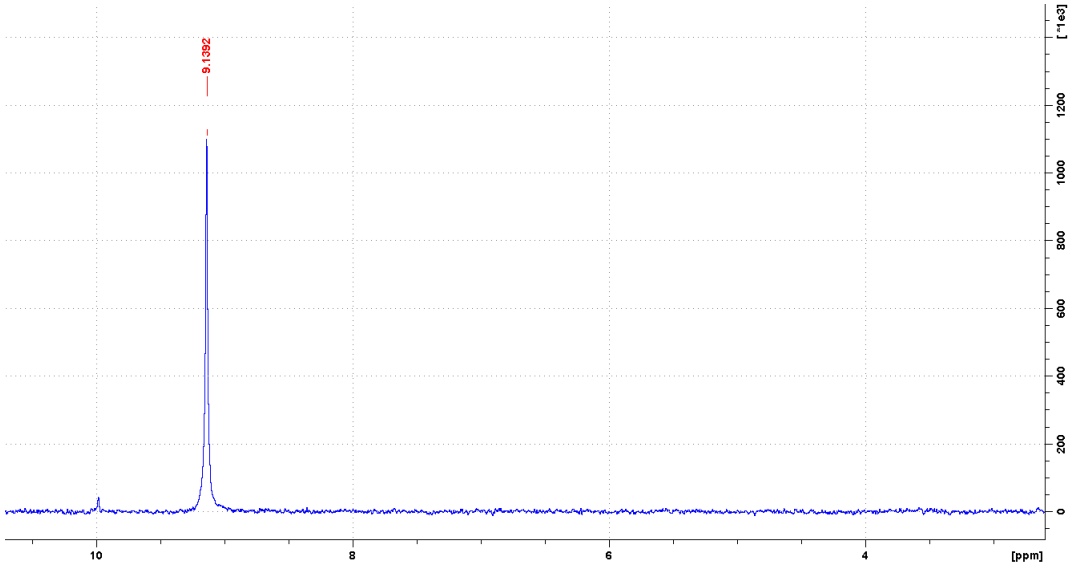


*Figure 20a: ^{31}P NMR (202 MHz, $CDCl_3$) of phosphorochloridate **10**. Single peak representing two enantiomers.*

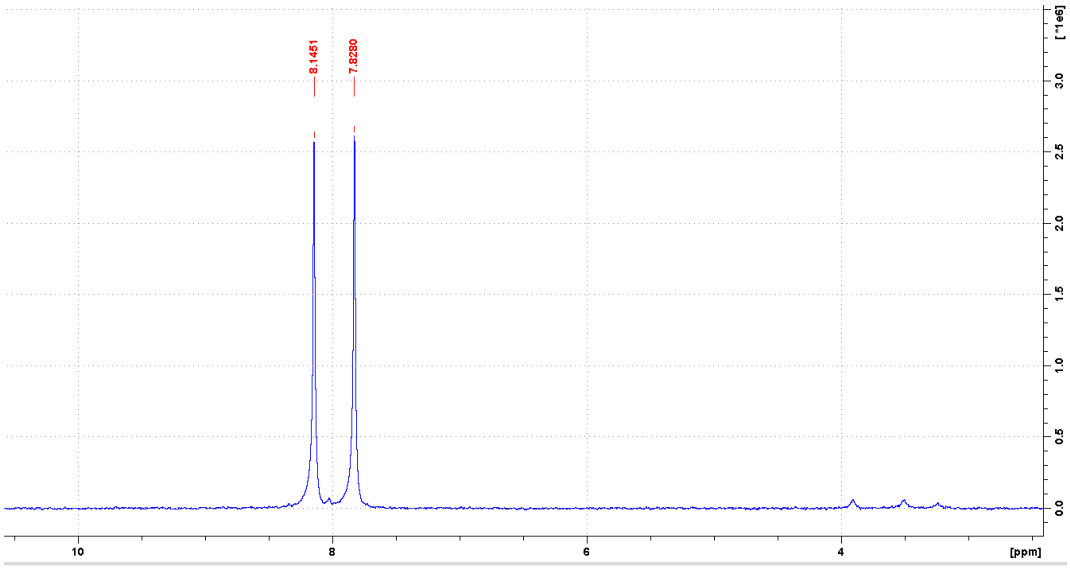


*Figure 20b: ^{31}P NMR (202 MHz, $CDCl_3$) of phosphorochloridate **12**. Two diastereoisomers distinguishable as two single peaks.*

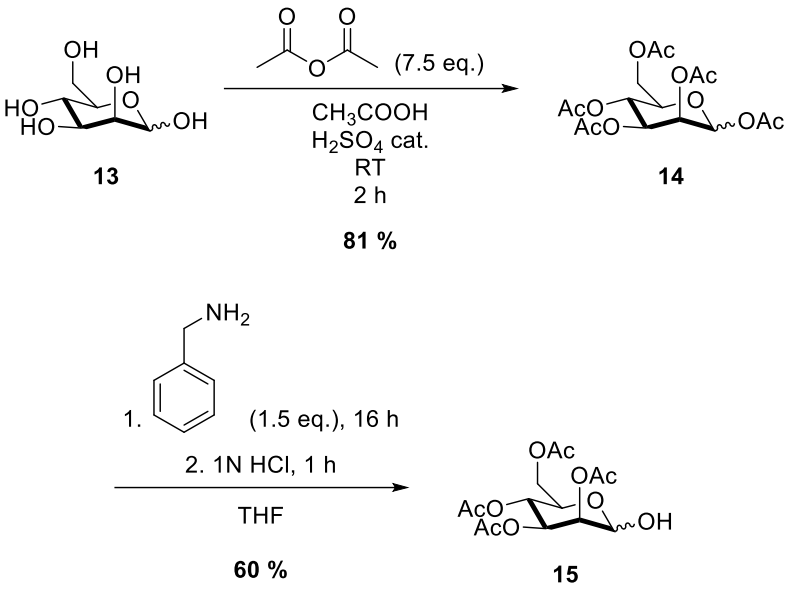
3.1.3 Tetraacetylated D-mannose ProTides synthesis

3.1.3.1 Introduction

Phosphomannomutase-2 deficiency results into a reduced availability of Mannose-1-P leading to impaired glycosylation causing multisystem disease affecting primarily the nervous system.[13] Therefore ProTides of tetraacetylated D-mannose with phosphoramidate moiety bonded to the anomeric position of the monosaccharide molecule were prepared in order to be used as potential source of Man-1-P and supply to its deficiency in patients affected with PMM2-CDG. The synthetic approach described below, included preparation of the phosphorochloridates (described in the previous chapter), appropriate D-mannose derivative and their coupling as the latest step.

3.1.3.2 Synthesis of 2,3,4,6-tetra-*O*-acetyl-D-mannopyranose (15) as the starting material

The proposed strategy for the tetraacetylated D-mannose ProTides synthesis included preparation of D-mannose derivatives which would provide the attachment of the phosphoramidate moiety exclusively to the hydroxyl group in the anomeric position. Adopting the published procedure,[76] 2,3,4,6-tetra-*O*-acetyl-D-mannopyranose (**15**) was prepared as a suitable starting material (Scheme 5). The synthesis was accomplished in two steps: 1. full acetylation of D-mannose, and 2. selective deprotection of the anomeric position (Scheme 5).

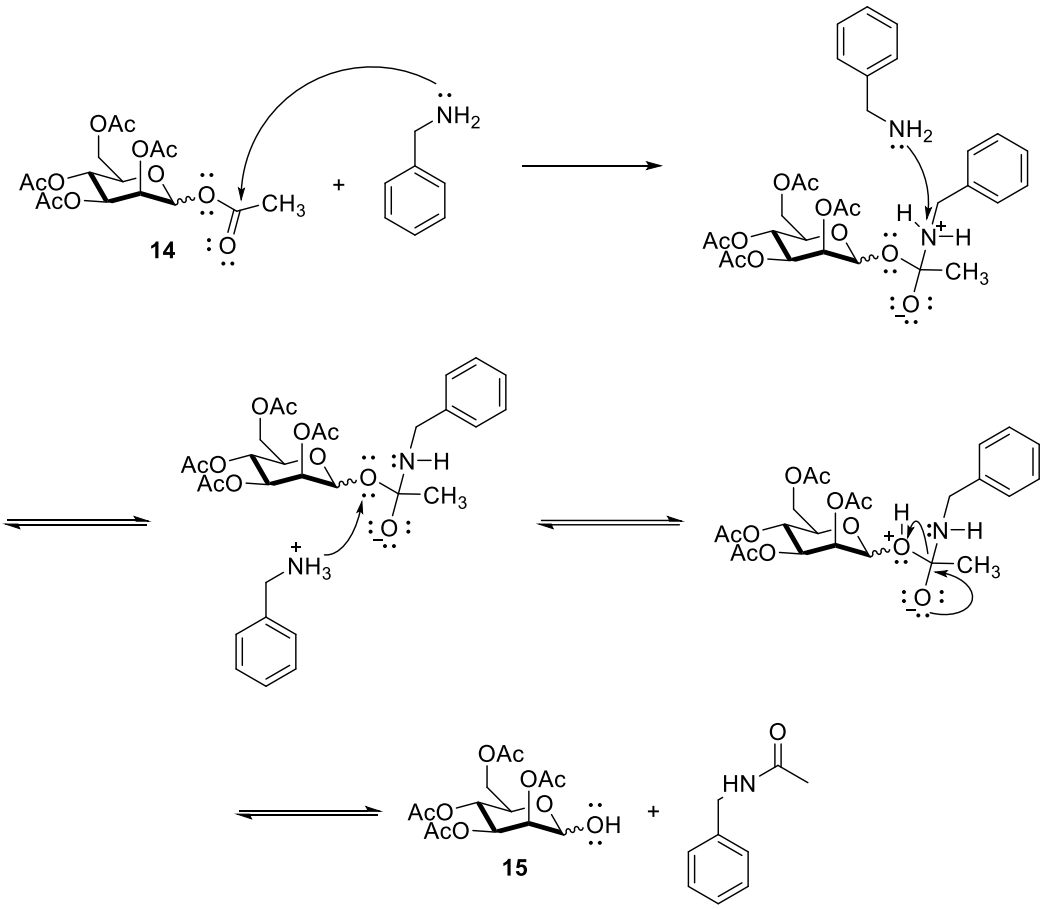


*Scheme 5: Approach to the 2,3,4,6-tetra-O-acetyl-D-mannopyranose (**15**) synthesis.*

Peracetylated D-mannose (**14**) was synthetized by the acetylation of commercially available D-mannose (**13**) using an excess of acetic anhydride (7.5 eq.) in acetic acid and catalytic amount of concentrated sulfuric acid. The complete conversion into the peracetylated compound was indicated when the suspension became a transparent solution. During the work-up, washing the clear solution with saturated sodium bicarbonate was necessary to remove the acidic impurities, therefore avoiding the purification by column chromatography. Pure compound **14** was obtained in 81% yield. According to ^{1}H NMR (500 MHz, $CDCl_3$), the reaction provided two anomers (α and β) judging from the detection of two different doublets corresponding to H-1. Because α and β anomers are diastereomeric, their NMR spectra can differ in achiral environment. Other signals were observed as multiplets because of the presence of two isomers, splitting and overlapping of the individual signals. Literature states typical $J_{1,2}$ value 1.6 Hz for α-D-mannose, and 0.8 Hz for β-D-mannose.[77]

Selective deacetylation of the anomeric position was achieved by the reaction of compound **14** (1 eq.) with benzylamine (1.5 eq.) at room temperature for 16 hours in tetrahydrofuran (THF) as a polar aprotic solvent.[76] The reaction mechanism (Scheme 6) involves nucleophilic attack of benzylamine on carbonyl attached to oxygen on C-1. The endocyclic oxygen is making the anomeric position a good leaving group thanks to the electron withdrawal effect, therefore benzylamine attacks primarily the acetyl group attached to this position.

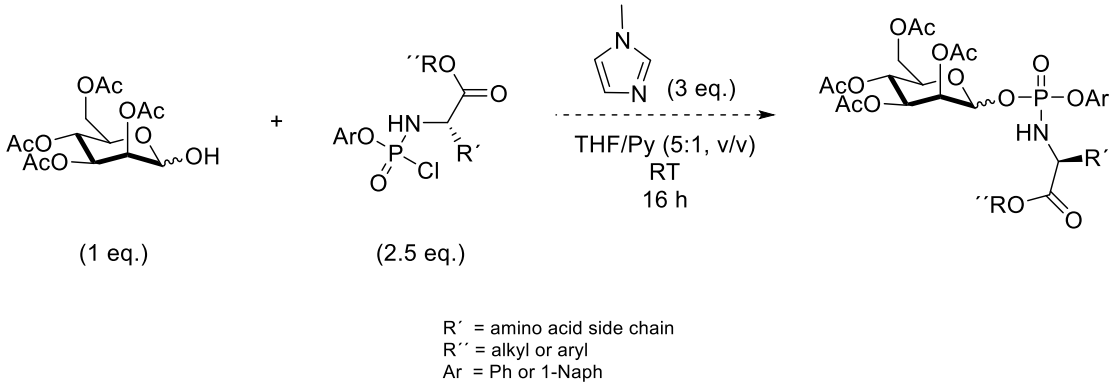
Benzylamine was removed by washing the solution with 1N HCl, and pure product **15** was obtained after the purification by column chromatography on silica as a mixture of α and β anomers with 60% yield.



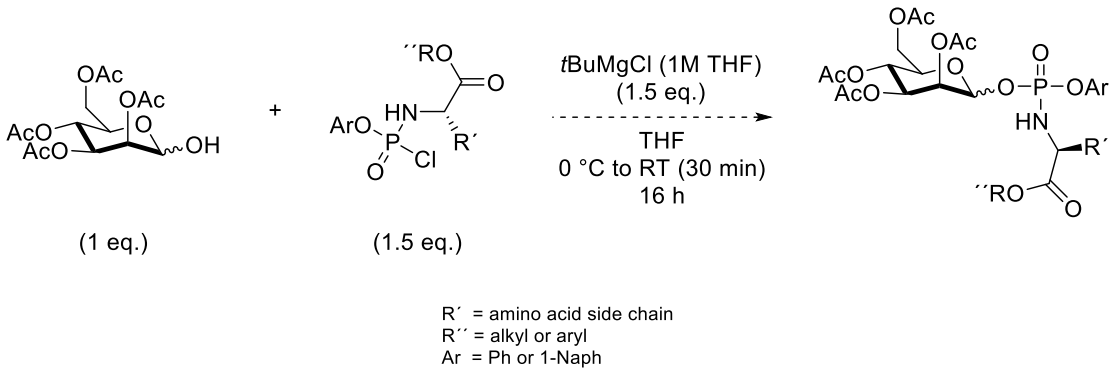
Scheme 6: Mechanism of the anomeric position deprotection.

3.1.3.3 2,3,4,6-tetra-*O*-acetyl-D-mannopyranose ProTides synthesis

With the synthetized phosphorochloridates (**8**-**11**) and 2,3,4,6-tetra-*O*-acetyl-D-mannopyranose (**15**) in hand, the couplings between these two compounds was carried out. Two different approaches were applied: NMI method (Scheme 7a) and Grignard method (Scheme 7b).



Scheme 7a: Synthesis of phosphoramidate prodrugs of 2,3,4 6-tetra-O-acetyl-D-mannopyranose via NMI methodology.

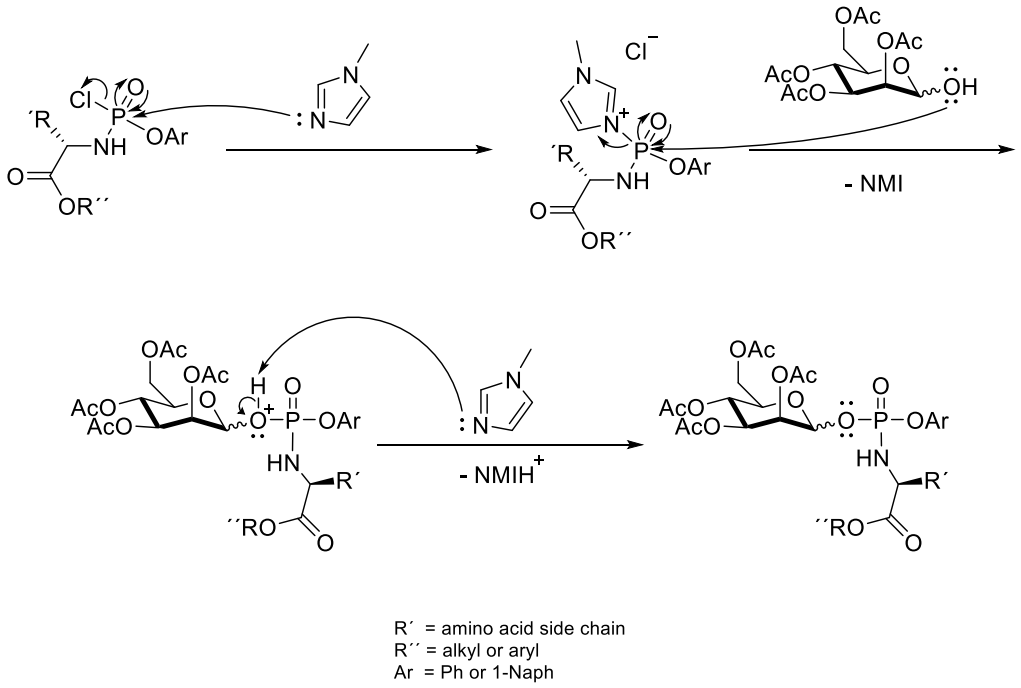


Scheme 7b: Synthesis of phosphoramidate prodrugs of 2,3,4,6-tetra-O-acetyl-D-mannopyranose via tert-butylmagnesium chloride methodology.

3.1.3.3.1 NMI method

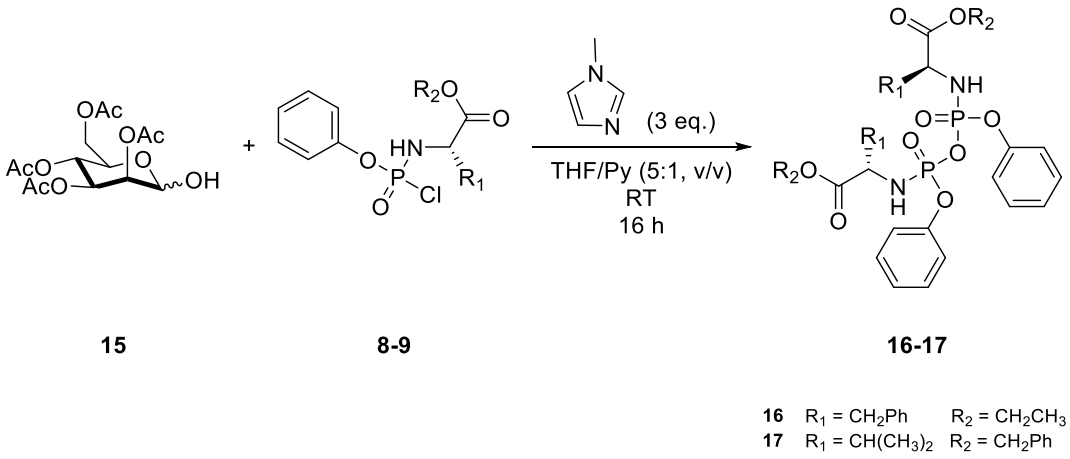
The synthesis of 2,3,4,6-tetra-*O*-acetyl-D-mannopyranose (**15**) ProTides was initially attempted using the *N*-methylimidazole as the coupling reagent.

The assumed mechanism of the reaction (Scheme 8) perceive NMI as the activator of the phosphorochloridate since it should displace the chlorine attached to the phosphorus providing the NMI cation as a better leaving group increasing the reactivity towards nucleophiles. The phosphorus should be attacked by the electron rich nitrogen of NMI causing the release of chloride anion. The hydroxyl group of the 2,3,4,6-tetra-*O*-acetyl-D-mannopyranose (**15**) then should attack the phosphorus releasing the molecule of NMI and the ProTide should be formed.[78,79]



Scheme 8: Theoretical mechanism of NMI activation of the phosphorochloridates.

Following the procedure for the coupling, previously successfully performed on nucleosides by McGuigan group,[74] Compound **15** (600 mg, 1 eq.) was dissolved in anhydrous THF (8 mL) and anhydrous pyridine (2.4 mL) under Argon atmosphere in a 50 mL round-bottom flask. NMI (3 eq.) was added dropwise and the resulting mixture was stirred for 30 minutes. After that time, a solution of phosphorochloridate **8** (Entry 1) or **9** (Entry 2) (2.5 eq.) in anhydrous THF (4 mL) was added dropwise and the reaction mixture was stirred overnight. Unfortunately, no traces of the desired ProTide was detected by mass spectrometry (MS). Instead, as shown in Table 5, in both cases, the mass spectra of the crude were characterised by major peak at m/z 681 and 709 $[M+H]^+$ and their respective sodium adducts (Entry 1 and Entry 2). By extensive NMR analysis (a combination of ^{31}P, ^{1}H, COSY, ^{13}C and HSQC) we were able to determine the structure of this product as the "dimerization" of two molecules of phosphorochloridate generating the symmetric phosphate derivatives (**16**-**17**) (Scheme 9).



Scheme 9: NMI methodology attempts.

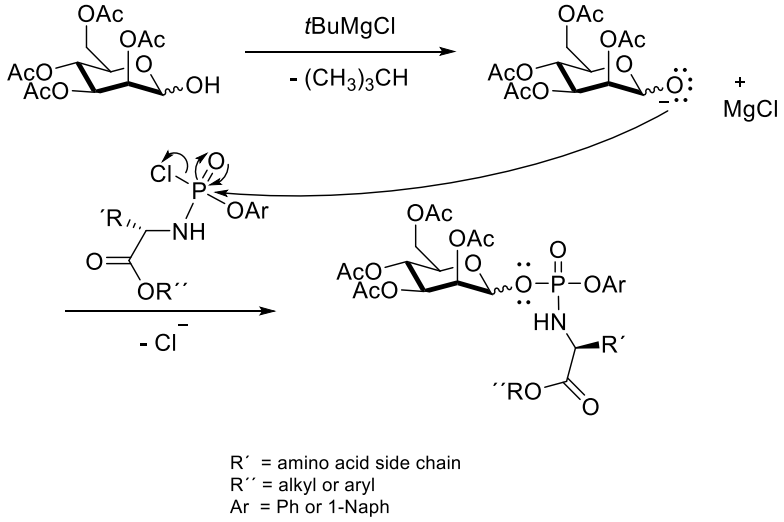
Entry	Phosphoro-chloridate	M.W. of expected ProTide [g/mol]	M.W. of phosphorochloridates adduct [g/mol]	m/z detected (ESI+)
1	**8**	679.61	680.63	681.20 $[M+H]^+$ 703.18$[M+Na]^+$
2	**9**	693.64	708.68	709.23$[M+H]^+$ 731.21$[M+Na]^+$

Table 5: NMI methodology attempts.

Further attempts to find the correct reaction conditions to obtain the ProTides with NMI methodology were unsuccessful. Firstly, we tried to reverse the addition of NMI and the phosphorochloridate with no success. Secondly, catalytic amount (2 mol%) of NMI was used, but no improvement was observed. Since we already had obtained the ProTides applying the Grignard method at that time, no further investigation of the reaction conditions was carried out.

3.1.3.3.2 Grignard method

The second approach to the ProTides synthesis is based on *tert*-butylmagnesium chloride (*t*BuMgCl) as a strong base which is able to deprotonate the free hydroxyl of 2,3,4,6-tetra-*O*-acetyl-D-mannopyranose. The formed alkoxide group is more nucleophilic than the original hydroxyl, allowing the attack on the phosphorus of the phosphorochloridate resulting in release of the chloride anion and formation of the desired phosphoramidate (Scheme 10). Considering that *t*BuMgCl chloride acts as a strong base (pK_a of the conjugate acid is 45), multiple phosphoramidate isomers would be provided if not protected D-mannose was used since no selectivity towards any of the hydroxyl groups would be evinced.[68,80]

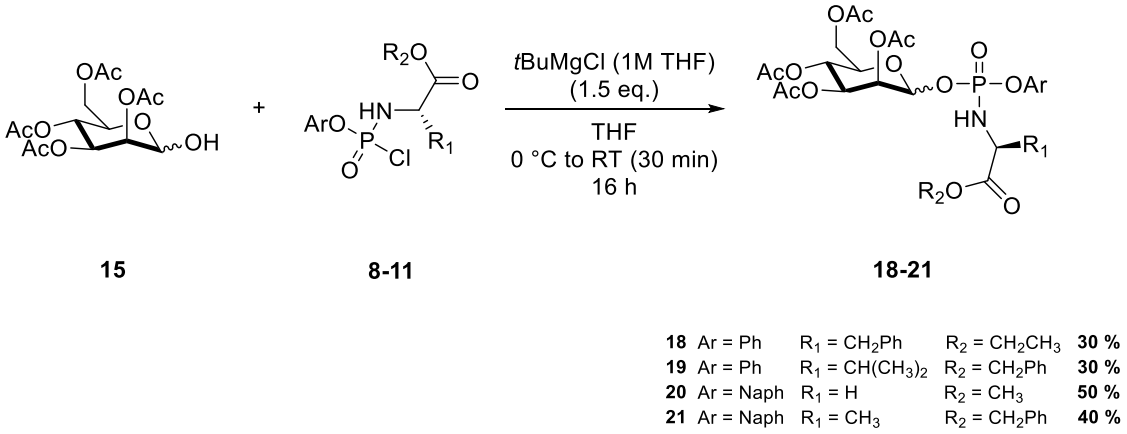


Scheme 10: The mechanism of Grignard method.

Standard procedure for the synthesis using the Grignard reagent (Scheme 11) was adopted from the previously described protocol (General procedure **B**).[74] Under Argon atmosphere, 1 eq. of compound **15** was dissolved in anhydrous THF and cooled to 0 °C in order to prevent the migration of the acetyl groups formerly observed by our group. After addition of 1M *t*BuMgCl in THF, a solution of 1.5 eq. of appropriate phosphorochloridate (**8-11**) (1.5 eq.) in anhydrous THF was added dropwise and the reaction mixture was stirred overnight, allowing the cooling bath to reach room temperature.

The formation of the phosphoramidate was confirmed by mass spectrometry. Compounds **18** and **19** were firstly purified by column chromatography on silica using dichloromethane (DCM)/MeOH (98:2) as the eluting system. Even though TLCs showed only one spot, according to the ^{31}P NMR the compounds were still impure. After investigating different chromatographic conditions, we eventually discovered that replacing DCM/MeOH with Hex/EtOAc (1:1) allowed the separation of some of the impurities and two spots corresponding to the ProTide isomers were isolated. Hex/EtOAc (1:1 or 2.3) was also used for the purification of compounds **20** and **21**. Products isolated after column chromatography on silica gel using the above conditions,

were analysed by reversed-phase HPLC (RP HPLC) to check their analytical purity. Unfortunately, some impurities were still present (except for compound **20**). Therefore, additional purification via preparative reversed-phase (RP) column chromatography (C-18, acetonitrile (ACN)/H_2O (90:10), 100 % ACN in 50 minutes) was necessary for the compounds **18**, **19** and **21**. After this step, all the phosphoramidates were obtained with a purity ≥ 95%.



Scheme 11: 3,4,6-tetra-O-acetyl-D-mannopyranose ProTides synthesis via Grignard method.

All synthetized compounds with eluting systems and final yields are listed in Table 6.

Compound	Ar	R_1	R_2	Purification	Yield [%]
18	Ph	CH_2Ph	CH_2CH_3	1. DCM/MeOH (98:2) 2. Hex/EtOAc (1:1) 3. RP	30
19	Ph	$CH(CH_3)_2$	CH_2Ph	1. DCM/MeOH (98:2) 2. Hex/EtOAc (1:1) 3. RP	30
20	α-Naph	H	CH_3	Hex/EtOAc (2:3)	50
21	α-Naph	CH_3	CH_2Ph	1. Hex/EtOAc (1:1) 2. RP	40

Table 6: ProTides of 2,3,4,6-tetra-O-acetyl-D-mannopyranose synthetized via Grignard method.

The obtained phosphoramidates possess four (**20**) or five (**18**, **19**, **21**) defined stereocentres (C2-C5 of the mannose and eventually C_α of the amino acid) and two variable stereocentres (anomeric carbon and phosphorus atom). Consequently, four diastereoisomers can be formed represented as four single peaks in ^{31}P NMR spectra, nonetheless, typically only two or three peaks were present as a result of the signals overlapping.

We did not explore different conditions for the RP HPLC analysis, thus we did not separate the signals of individual isomers. Usually, we observed only two signals in HPLC chromatogram corresponding to four diastereoisomers. Representative ^{31}P NMR (Figure 21a) and HPLC (Figure 21b) spectra of compound **19** are shown below.

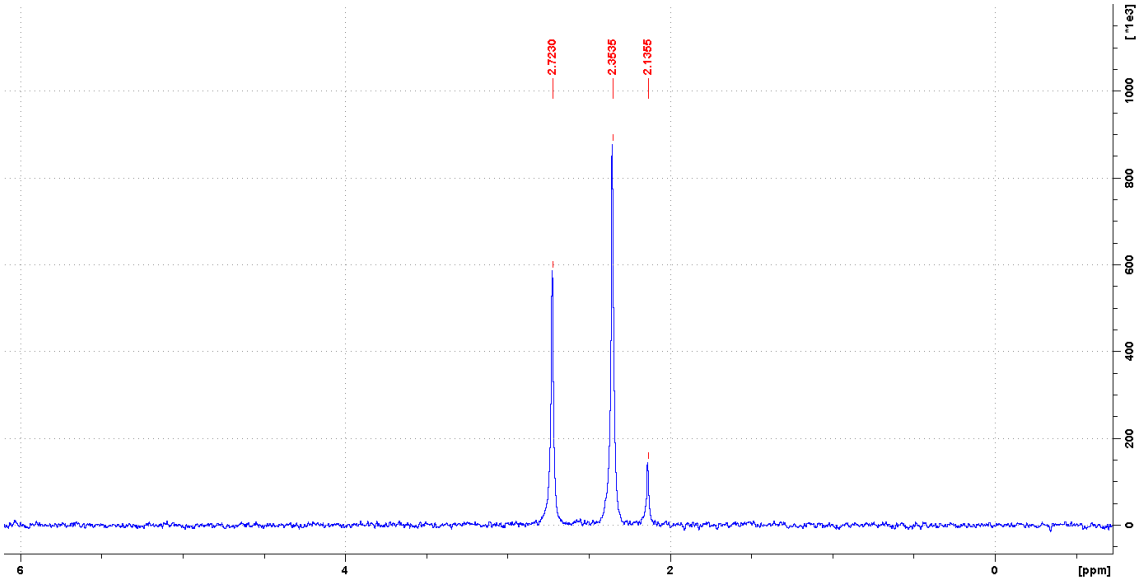


*Figure 21a: ^{31}P NMR spectrum (202 MHz, MeOD) of ProTide **19**.*

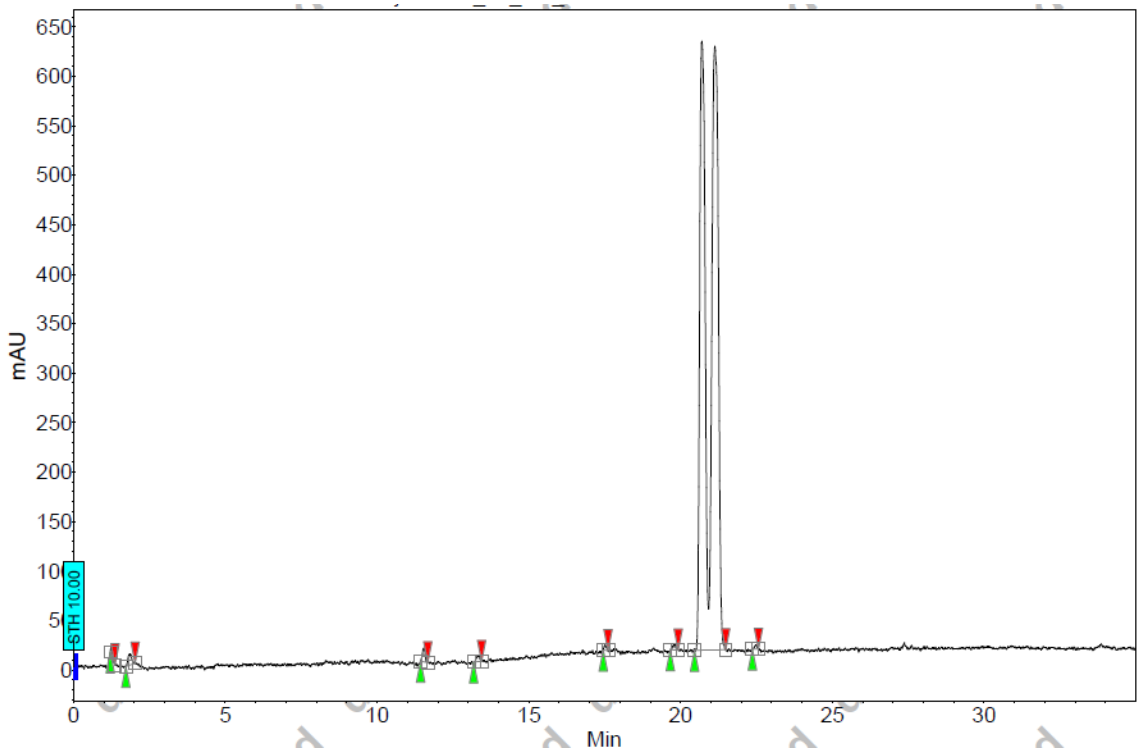


*Figure 21b: HPLC analysis of ProTide **19**. ACN/H_2O (90:10), 100 % ACN in 30 minutes, flow 1 mL/min.*

3.1.3.4 Carboxypeptidase assay

The proposed mechanism for the ProTides bioactivation (Figure 22) usually begin with the enzymatic hydrolysis of the ester moiety by carboxypeptidase-type enzyme. This is followed by the rapid attack of the amino acid carboxylate to the phosphorus centre forming the cyclic intermediate **B** with elimination of the aryloxy moiety. This cyclic anhydride is not stable and undergoes rapid ring opening caused by the attack of a nucleophile (usually water). The formed phosphoramidate monoester intermediate is then further hydrolysed by phosphoramidase-type enzyme providing the monophosphate of the parent drug.[68,69] To verify if the synthetized phosphoramidates can be substrates of the activating enzymes and potentially be metabolized in human cells according to the putative mechanism of their bioactivation, an enzymatic activation experiment with carboxypeptidase was performed.

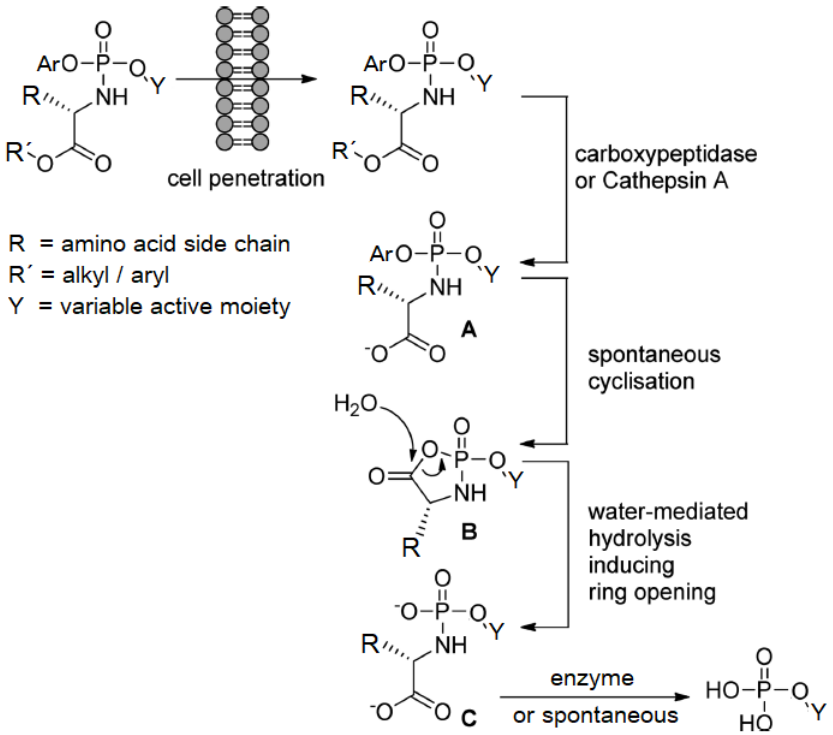
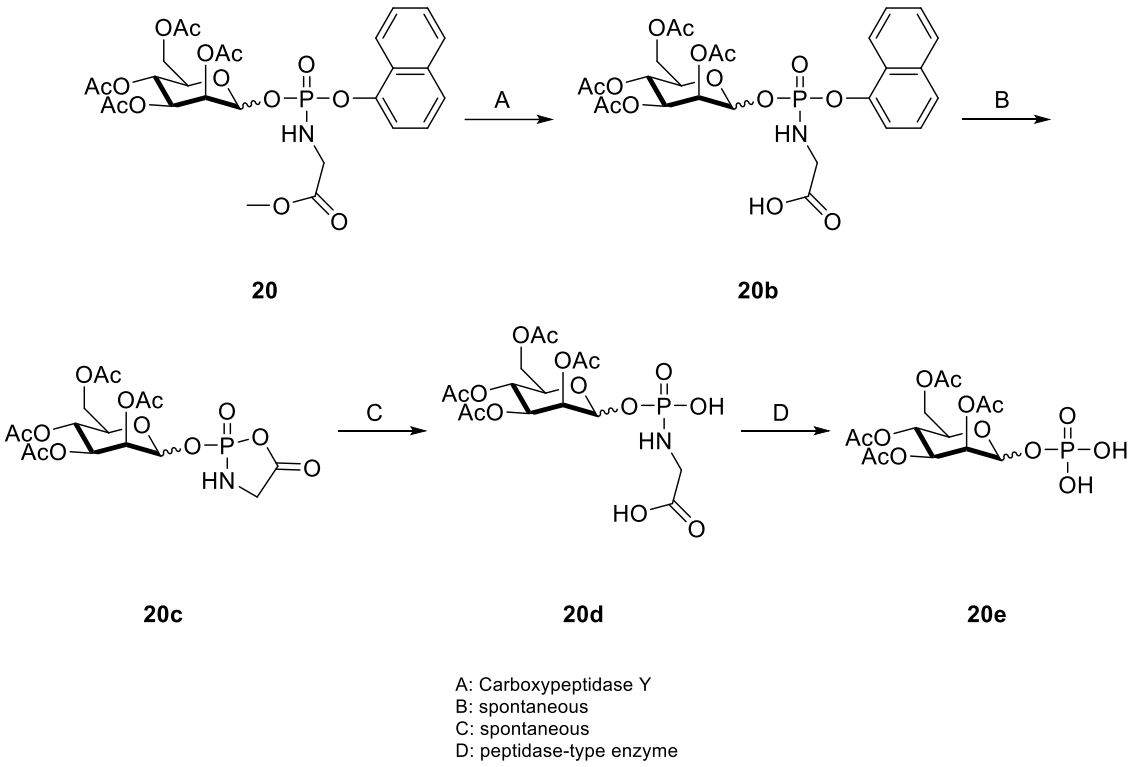


Figure 22: Adopted from Pradere, U., et al. (2014). Bioactivation of aryloxyphosphoramidate prodrugs..

The enzymatic study was carried out on product **20** according to the procedure previously designed by McGuigan group. The blank was prepared by dissolving of 1-2 mg of product **20** in 150 µL of acetone-d6 and 300 µL of Trizma buffer (pH 7.6). The solution was transferred into the NMR tube and ^{31}P NMR (202 MHz) was then recorded. After that, a small amount of Carboxypeptidase Y (CPY) from baker´s yeast (*Saccharomyces cerevisiae*) was dissolved in 150 µL of Trizma buffer and the resulting solution was added to the NMR tube. The incubation of compound **20** with CPY was monitored every 30 minutes by ^{31}P NMR.

According to the putative activation pathway of ProTide **20** (Scheme 12), the compound started to be metabolized within 30 minutes of incubation into intermediate **20b** which was converted into metabolite **20d** after further 90 minutes. The complete hydrolysis and aryl moiety cleavage of the starting material was accomplished after 18 hours (Figure 23). Two signals corresponding to two couples of diastereoisomers can be observed in the spectrum of the blank sample and of the metabolite **20d**.



*Scheme 12: Suggested activation of ProTide **20**.*

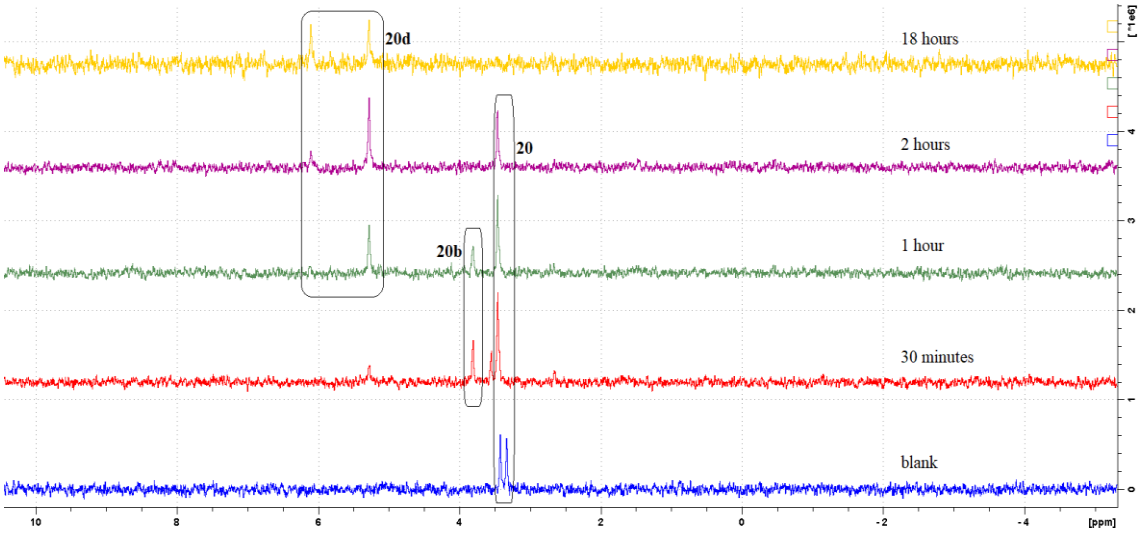


*Figure 23: ^{31}P NMR (202 MHz, acetone-d6) spectra of enzymatic assay performed on ProTide **20**.*

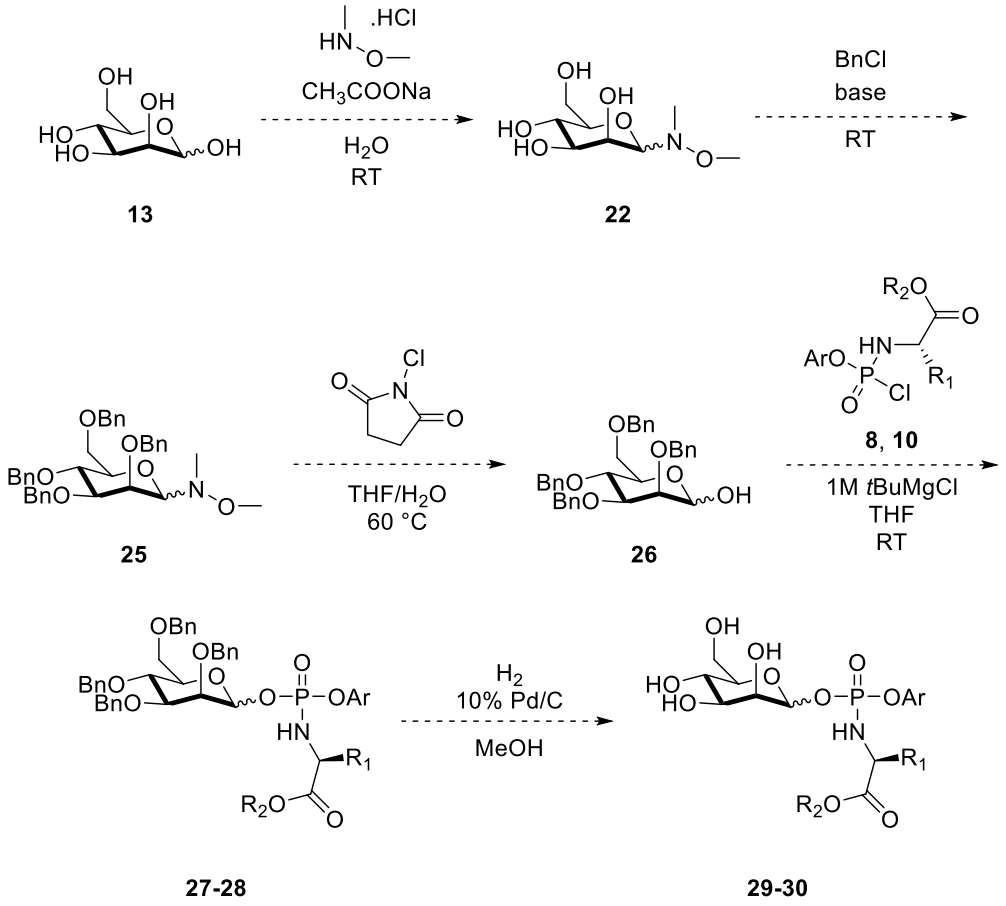
In order to confirm the formation of the monoester intermediate during the assay, MS of the solution was performed after 18 hours of incubation. The detected MS (ESI+) m/z = 508.08 $[M+Na]^+$ and MS (ESI-) m/z = 484.10 $[M-H]^-$ suggest the presence of metabolite **20d** (M.W. = 485.33). On the other hand, no traces of compounds **20**, **20b** or **20c** were observed. Judging from this result, the ProTide as a carboxypeptidase substrate could be metabolized inside the cells and provide the monoester that could be further converted into the desired monophosphate.

As seen from Figure 23, probably two of four diastereoisomers (represented as one singlet) were degraded more rapidly than the other two. After 10 hours, one of the peaks was still present in the sample whereas the second one was completely hydrolysed after 30 minutes. This fact probably refers to the different affinity of the compound according to the configuration on phosphorus.

3.1.4 D-mannose ProTides synthesis

3.1.4.1 Introduction

Since PMM2 produces Man-1-P, we planned to synthetize the prodrugs with phosphoramidate part attached to the anomeric position of the mannose. This chapter outlines the synthesis of Man-1-P ProTides using the Grignard methodology. Firstly, we prepared 2,3,4,6-tetra-*O*-benzyl-D-mannopyranose (**26**) as a suitable starting material which was then coupled with the appropriate phosphorochloridates (their synthesis is described above). Deprotection of the hydroxyl groups was accomplished by catalytic hydrogenation. The suggested synthetic strategy is captured in Scheme 13.



Scheme 13: Proposed synthetic strategy for the preparation of D-mannose ProTides.

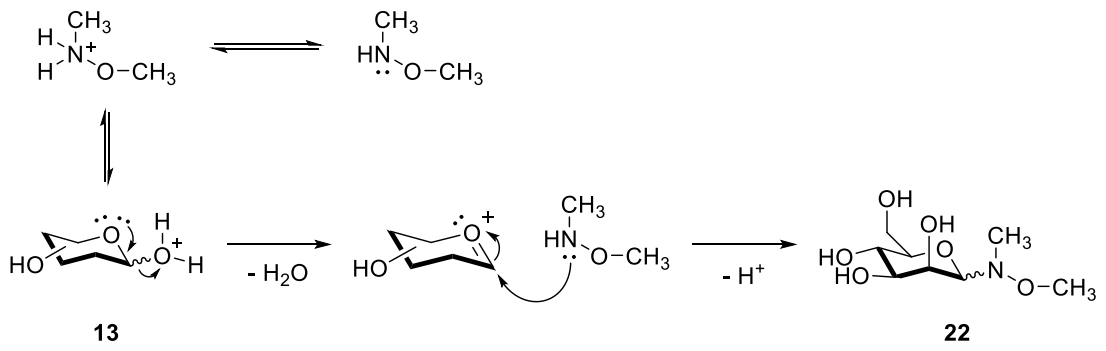
3.1.4.2 Synthesis of 2,3,4,6-tetra-*O*-benzyl-D-mannopyranose (26)

3.1.4.2.1 *N*,*O*-dimethyl-*N*-(D-mannopyranosyl) hydroxylamine (22)

The first step of the staring material preparation was carried out according to the published procedure, using *N,O*-dimethylhydroxylamine hydrochloride which provides selective protection of the hydroxyl on C-1 in mildly acidic (pH 4-5) aqueous conditions.[81] No activation of the anomeric position nor protection of the other hydroxyl groups was necessary. Another advantage of this reaction is the compatibility of the formed *N,O*-dimethyloxyamine-*N*-glycoside with numerous protecting groups, including benzyl group, and possibility to selectively hydrolyse C-N bond, e.g. with *N*-chlorosuccinimide (NCS), afterwards.[82]

The mechanism of the reaction is captured in Scheme 14. At pH 4-5, *N,O*-dimethylhydroxylamine hydrochloride is present with its corresponding deprotonated molecule in a propitious equilibrium. As an acidic catalyst, the hydrochloride protonates the anomeric hydroxyl of the sugar leading to elimination of a water molecule. The nucleophilic (deprotonated) hydroxylamine then attacks

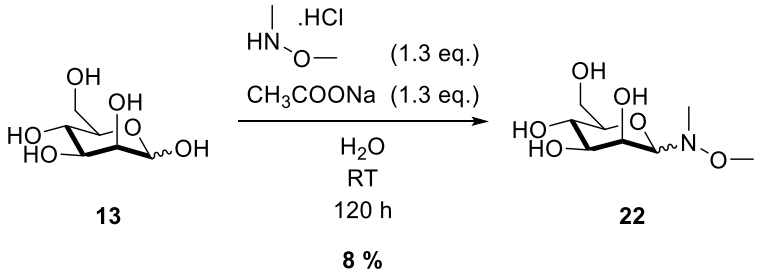
the anomeric position providing the desired *N*,*O*-dimethyl-*N*-(D-mannopyranosyl) hydroxylamine (**22**).



Scheme 14: The mechanism of anomeric protection with hydroxylamine group.

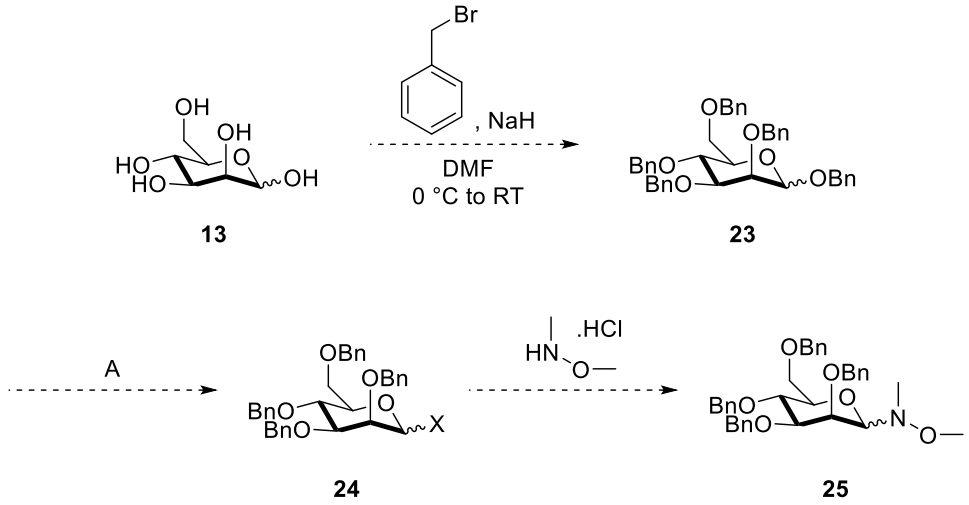
Following the reported procedure,[81] to a cooled solution (0 °C) of D-mannose (**13**) (1 eq.) and *N,O*-dimethylhydroxylamine hydrochloride (1.1 eq.) in water, a solution of sodium acetate (1.1 eq.) in water was added slowly to adjust the pH to 5. The reaction mixture was stirred for 20 hours at room temperature and monitored by TLC (DCM/MeOH, 4:1). Even though the reaction was not completed, the water was evaporated under reduced pressure yielding a white sticky crude. This was dissolved in a mixture of methanol and water and purified twice (because the similarity of the retention factors in several eluting systems) by column chromatography on silica using DCM/MeOH (4:1). *N*,*O*-dimethyl-*N*-(D-mannopyranosyl) hydroxylamine (**22**) was isolated in 8.02% final yield. Because the poor yield, we attempted to improve this methodology by increasing the number of equivalents (1.3 instead of 1.1) of *N,O*-dimethylhydroxylamine hydrochloride and sodium acetate (1.3 equivalents) (Scheme 15). The reaction mixture was then stirred for 5 days (instead of 20 hours) at room temperature. Even after five days, the reaction was not completed. After the solvent evaporation, the crude was suspended in methanol and filtered on sintered glass filter to eliminate the inorganic impurities and to ease the further purification. Nevertheless, we obtained only 8.38 % of pure product **22**.

We also tried to heat up (60 °C) the reaction mixture after sodium acetate addition at 0 °C, but with no improvement.



*Scheme 15: Synthesis of N,O-dimethyl-N-(D-mannopyranosyl) hydroxylamine (**22**).*

Due to the very low yield, we attempted to bypass the first two steps of the proposed synthetic strategy (Scheme 13) by the preparation of more reactive 2,3,4,6-tetra-*O*-benzyl-D-mannopyranosyl halide (**24**) which could react with *N,O*-dimethylhydroxylamine hydrochloride more easily (Scheme 16).



Scheme 16: Proposed modification. Conditions A are described in Table 7.

1,2,3,4,6-penta-*O*-benzyl-D-mannopyranose (**23)** was successfully obtained by the reaction of D-mannose (**13**) (1 eq.) with excessive amount of benzyl bromide (12 eq.) in the presence of NaH (10 eq.) as a base at room temperature.[83] After 16 hours, the reaction was quenched with methanol and purified by column chromatography on silica (Hex/EtOAc, 7:1) after work-up. Pure compound **23** was gained in 37% yield.

As the following step, 2,3,4,6-tetra-*O*-benzyl-D-mannopyranosyl halide (**24**) was attempted to be synthetized in several entries captured in Table 7.

Firstly, 33% hydrogen bromide solution in acetic acid (8 eq.) was added to a solution of compound **23** (1 eq.) in anhydrous dichloromethane cooled to 0 °C (Entry 1).[84] After the reaction mixture was stirred overnight at room temperature, mass spectrometry was carried out. No mass corresponding to 2,3,4,6-tetra-*O*-benzyl-D-mannopyranosyl bromide (M.W. = 603.55 g/mol) was detected. The staring material (**23**) (M.W. = 630.78 g/mol, found 653.29 $[M+Na]^+$) was observed, as well as the mass corresponding to the molecules of the starting material substituted with two molecules of bromine (M.W. = 576.33 g/mol; found 577.26 $[M+H]^+$) or lacking one benzyl protecting group (M.W. = 540.66 g/mol, found 536.24 $[M+Na]^+$).

Because the product with two benzyl groups substituted with two Br was detected, we envisaged, that 8 equivalents of HBr were not necessary. However, even with 2 equivalents of 33% HBr in acetic acid, identical products were obtained (Entry 2).

We then decided to replace the hydrobromic acid with 1.25 eq. of oxalyl chloride (Entry 3) which was added to benzylated mannose **23** dissolved in anhydrous dichloromethane at 0 °C.[83] After stirring for 20 hours at room temperature, no reaction occurred as judged by TLC and MS measurements.

The optimization of the reaction yields was not the main goal of this project, hence, no further experiments to improve the synthesis were attempted.

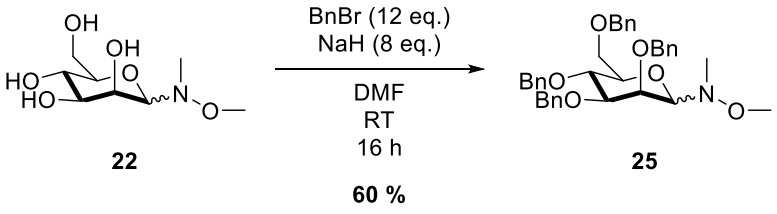
Entry	Conditions (A)	Observed mass
1	HBr in AcOH (33%), 8 eq. DCM 20 h	653.29 = SM $[M+Na]^+$ 577.26 = 2× Br $[M+H]^+$ 563.24 = -1× Bn $[M+Na]^+$
2	HBr in AcOH (33%), 2.eq. DCM 3 h	653.29 = SM $[M+Na]^+$ 577.26 = 2× Br $[M+H]^+$ 563.24 = -1× Bn $[M+Na]^+$
3	Oxalyl chloride, 1.25 eq. DCM 20 h	653.30 = SM $[M+Na]^+$

*Table 7: Attempts of synthesis of compound **24**.*

Since the attempts to get around the first step of the synthesis of compound **26** were unsuccessful, we decided to follow the initial strategy captured in Scheme 13.

3.1.4.2.2 *N*,*O*-dimethyl-*N*-(2,3,4,6-tetra-*O*-benzyl-D-mannopyranosyl) hydroxylamine (25)

After the protection of the anomeric position, we proceeded to the protection of the other hydroxyl groups of the mannose. This would than enable us to selectively deprotect the anomeric position that would be successively coupled with phosphorochloridates. The choice fell on the benzylation, since the protecting benzyl group was claimed to be compatible with conditions used for the deprotection of the anomeric position.[82] Moreover, this protecting group (PG) is generally easily removed by catalytic hydrogenation, a methodology highly compatible with the phosphoramidate moiety as well.



*Scheme 17: Entry 3 of N,O-dimethyl-N-(2,3,4,6-tetra-O-benzyl-D-mannopyranosyl) hydroxylamine (**25**) synthesis.*

N,*O*-dimethyl-*N*-(D-mannopyranosyl) hydroxylamine (**22)** (1 eq.) was then dissolved in anhydrous DMF and cooled to 0 °C. NaH was chosen as a strong base suitable for the deprotonation of the hydroxyl groups. Firstly, 4 equivalents of anhydrous NaH were added to the cooled solution of compound **22** and the mixture was stirred for 30 minutes. After that time, 6 equivalents of benzyl chloride were added dropwise (Entry 1). The cooling bath was removed and the reaction mixture was stirred overnight at room temperature.[82] The reaction was quenched with methanol, diluted with diethyl ether, washed with water and purified by column chromatography (Hex/EtOAc, 6:1) yielding 20 % of product **25**.

According to the MS analysis, only partially protected mannose species were present in the reaction mixture. We then replaced the benzyl chloride with the more reactive benzyl bromide (6 eq) in Entry 2 (Table 8). We also replaced anhydrous NaH with 60% dispersion of NaH in mineral oil (4 eq.). Nevertheless, we were able to obtain product **25** in only 20% isolated yield (Table 8).

Finally, the yield improvement was achieved by using 12 equivalents of benzyl bromide and 8 equivalents of NaH (60% dispersion in mineral oil) (Scheme 17). Using the double amount of the reagents provided 60 % of pure *N,O*-dimethyl-*N*-(2,3,4,6-tetra-*O*-benzyl-*D*-mannopyranosyl) hydroxylamine (**25**).

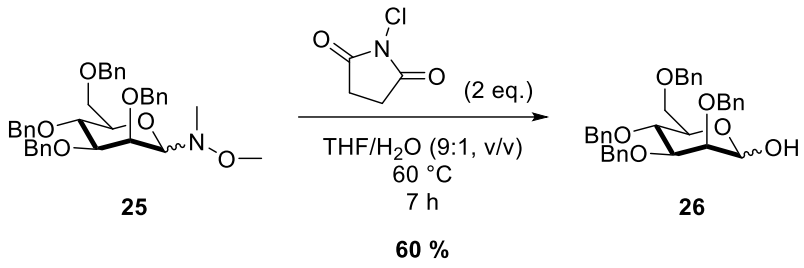
All attempts are noted in Table 8.

Entry	Benzyl halide	NaH	Conditions	Purification	Yield [%]
1	Benzyl chloride (6 eq.)	Dry NaH (4 eq.)	RT, 16 h	Hex:EtOAc 8:1	20
2	Benzyl bromide (6 eq.)	60% NaH in mineral oil (4 eq.)	RT, 16 h	Hex:EtOAc 6:1	20
3	Benzyl bromide (12 eq.)	60% NaH in mineral oil (8 eq.)	RT, 16 h	Hex:EtOAc 6:1	60

*Table 8: Synthesis of N,O-dimethyl-N-(2,3,4,6-tetra-O-benzyl-D-mannosyl) hydroxylamine (**25**).*

3.1.4.2.3 2,3,4,6-tetra-*O*-benzyl-D-mannopyranose (26)

The selective elimination of hydroxylamine group from the anomeric position was accomplished by hydrolysis with NCS as reported by Dasgupta, S. and Nitz, M. (2011). Use of *N*-iodosuccinimide and *N*-bromosuccinimide did not lead to the deprotection of the anomeric position, whereas NCS was able to hydrolyse the C-N bond in THF/H_2O at increased temperature[82] (Scheme 18).[82] After 7 hours, the reaction provided 60% yield of 2,3,4,6-tetra-*O*-benzyl-D-mannopyranose (**26)**.



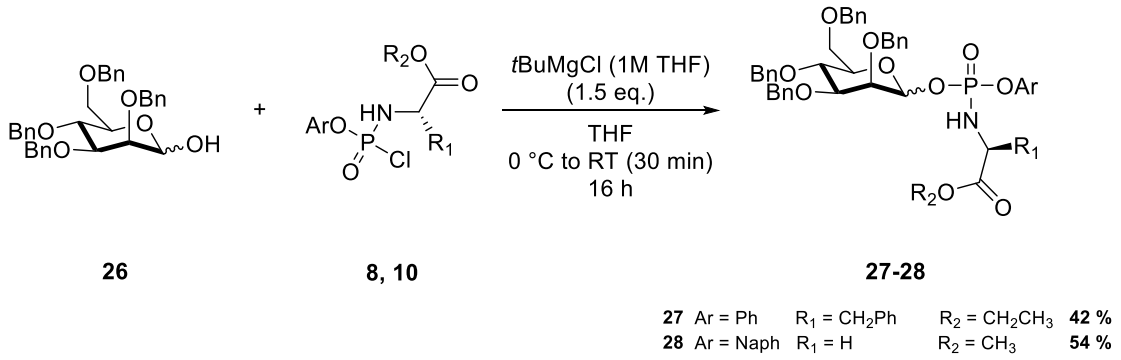
*Scheme 18: Synthesis of 2,3,4,6-tetra-O-benzyl-D-mannopyranose (**26**)*

3.1.4.3 Synthesis of D-mannose ProTides

The Grignard methodology was given the preference in D-mannose ProTides synthesis, since the NMI method was not successful in previous preparation of peracetylated D-mannose ProTides. Identical conditions were applied as for the synthesis of ProTides **18-21**. Compound **26** (1 eq.) was dissolved in anhydrous THF under Argon atmosphere and cooled to 0 °C. To this solution, 1M *t*BuMgCl in THF (1.5 eq.) was added followed by the phosphorochloridates (**8**, **10**) (1.5 eq.) dissolved in anhydrous THF. The cooling bath was removed and the suspension was allowed to reach room temperature and the reaction mixture was stirred overnight.[74]

Hex/EtOAc was used as the eluting system for the purification by column chromatography, providing sufficiently pure compounds for the final step. Because there are seven chiral centres in compound **27** (C1-C5 of the mannose, α carbon of the amino acid, phosphorus) and six chiral centres in compound **28** (lacking the chiral C_α of the amino acid), but only two of them can occur in variable configuration (anomeric carbon and phosphorus), we obtained compounds **27** and **28** as a mixture of four diastereoisomers.

Synthetic procedure (Scheme 19) and synthetized compounds with reaction yields (Table 9) are shown below.

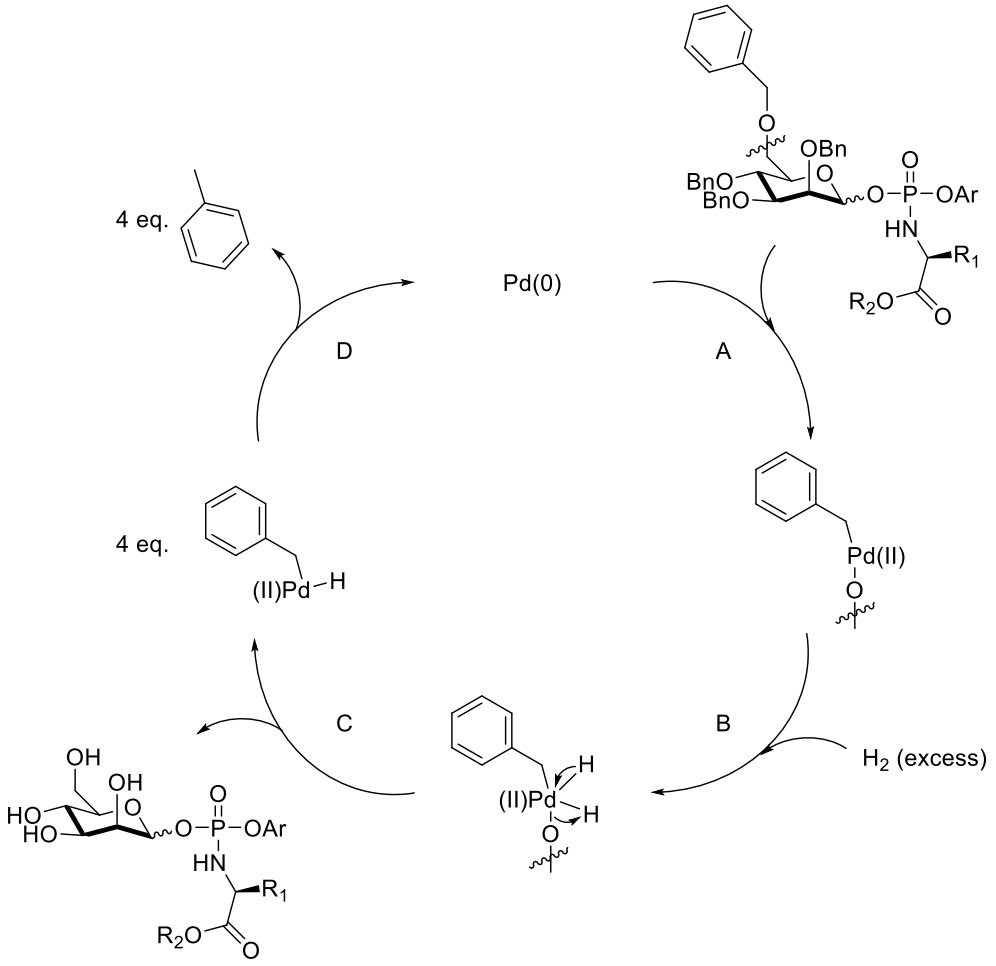


*Scheme 19: Synthesis of compounds **27-28** using the Grignard method.*

Compound	Ar	R_1	R_2	Purification	Yield [%]
27	Ph	CH_2Ph	CH_2CH_3	Hex/EtOAc (2:1)	42
28	α-Naph	H	CH_3	Hex/EtOAc (3:1)	54

*Table 9: Synthesis of compounds **27-28** with purification conditions and reaction yields.*

Prepared benzylated ProTides had to be deprotected to give phosphoramidate prodrugs of "free" mannose. Typically, the benzyl ether cleavage can be achieved by catalytic hydrogenation using palladium on carbon (Pd/C). According to the claimed mechanism (Scheme 20),[85] catalytic Pd (0) incorporates between oxygen and benzylic carbon by oxidative addition (A) converting to Pd (II). Applying of the excessive amount of hydrogen provides coordination of hydrogen atoms and Pd (II) (B) and subsequent deprotonation (C) releasing corresponding alcohol. Palladium is then recovered by reductive elimination (D) while toluene is formed as a side product.



Scheme 20: The accepted mechanism for the catalytic removal of benzyl protecting group.

In all experiments (Table 10), the starting material (**27** or **28**) was dissolved in anhydrous methanol. As the first attempt, 10 mol% of 10% Pd/C was added to the solution of compound **28** and hydrogen (1 atm via a balloon) was applied (Entry 1). To compare different catalysts, another attempts were performed using $Pd(OH)_2$ on carbon and 1 atm

of H_2 (Entry 5) and H_2 under pressure (Entry 6). The reactions were stopped after 7 and 5 days, respectively, but only partial hydrogenation occurred in both cases. Therefore, Pd/C appeared to be more suitable, because it provided the desired ProTides within the shortest time (3 days), nevertheless, the obtained compounds were degraded.

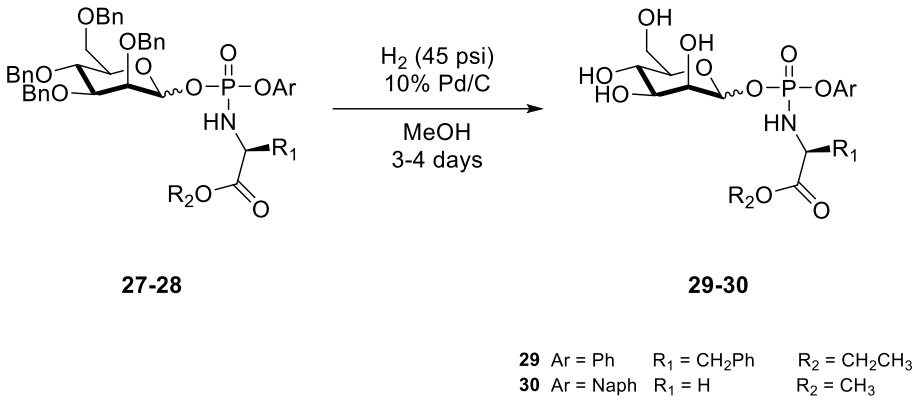
Entry	Starting material	Catalyst	H_2 pressure	Time	Yield
1	**28** (60 mg)	Pd/C (10 mol%)	1 atm	16 h	**28** detected
		Pd/C (+ 30 mol%)	1 atm	3 days	degradation
2	**28** (36 mg)	Pd/C (40 mol%)	1 atm	16 h	degradation
3	**28** (42 mg)	Pd/C (10 mol%)	45 psi	4 days	**30** detected
4	**27** (94 mg)	Pd/C (10 mol%)	45 psi	3 days	**29** isolated, degraded after evaporation
5	**27** (50 mg)	$Pd(OH)_2/C$	1 atm	7 days	partial hydrogenation
6	**27** (50 mg)	$Pd(OH)_2/C$ (10 mol%)	45 psi	5 days	partial hydrogenation

Table 10: Catalytic hydrogenation attempts.

The reaction mixture was stirred for 16 hours at room temperature. The mass spectrometry analysis of the reaction mixture was performed, detecting only the starting material (**28)** (M.W. = 817.87 g/mol, found 840.29 $[M+Na]^+$). To the same reaction mixture, further 30 mol% of 10% Pd/C was added to enhance the hydrogenation conditions. Unfortunately, after 3 days, MS and ^{31}P NMR spectrum (peak at -3.29 ppm) confirmed the degradation of most of the starting material into an unidentified compound which was not isolated.

Attempts to increase the amount of catalyst (40 mol% of 10% Pd/C) directly from the beginning of the reaction (Entry 2) was also unsuccessful, leading to the degradation of the starting material within 16 hours (^{31}P NMR: -3.30 ppm).

Because longer reaction time and high amount of catalyst were unproductive, we envisaged that performing the reaction with H_2 under pressure (Entry 3) with standard amount of catalyst (usually 10 mol%) would be a suitable procedure that would avoid degradation of the benzylated phosphoramidate. Pleasingly, when a methanolic solution of compound **28** was treated with 10 mol% of Pd/C and shaken under a 45 psi hydrogen atmosphere at room temperature (Scheme 21) for four days, no starting material (**28**) was detected by MS analysis. Under this set of conditions, we were able to detect the presence of desired ProTide (**30**) as well as the ProTide in which the naphthalene ring was partially hydrogenated to a 3,4,5,6-tetrahydronaphtyl residue. Due to the small amount of the reaction mixture, the ProTide was not isolated.



*Scheme 21: Accomplished hydrogenation of compounds **27-28**.*

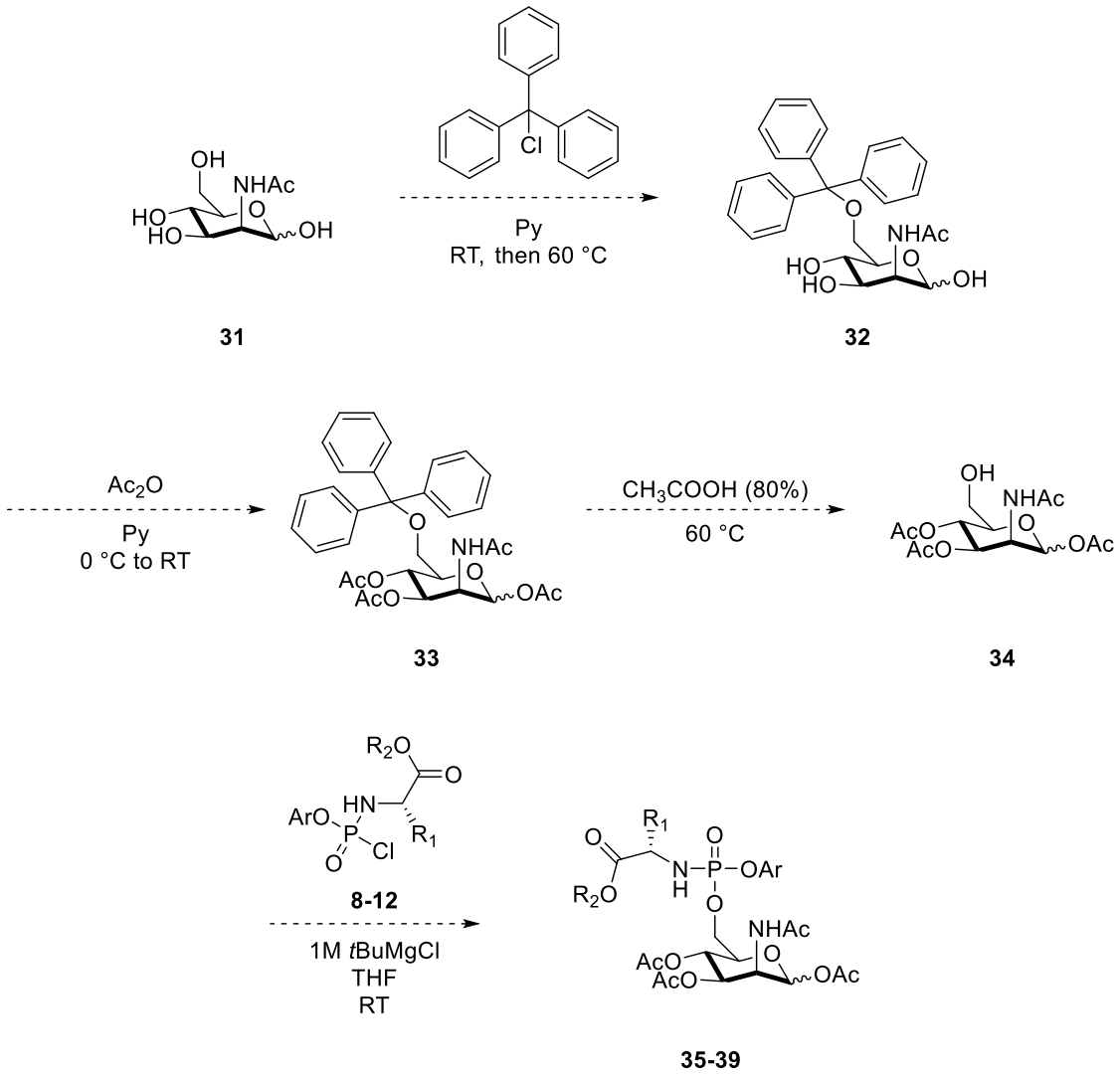
Similar conditions were used for the hydrogenation of 94 mg of compound **27** (Entry 4) (Scheme 21). After 3 days, removal of benzyl groups was completed (as judged by MS analysis (ESI+): m/z = 534.16 $[M+Na]^+$).

Unfortunately, these compounds proved to be highly unstable and any attempts to isolate them in analytically pure form were unsuccessful. Indeed, few minutes after the catalyst was filtered off and the solvent evaporated, the colourless oil isolated showed already a decomposition into a species with a ^{31}P NMR and HPLC analyses showing the degradation of the obtained ProTide into unidentified compounds. The instability could be caused by the free hydroxyl groups, since the protected derivatives were stable.

3.1.5 Triacetylated *N*-acetyl-D-mannosamine ProTides synthesis

3.1.5.1 Introduction

Impaired GNE/MNK causes the lack of ManNAc-6-P, a key intermediate in the biosynthesis of sialic acid.[33,34] Phosphoramidate prodrugs of peracetylated ManNAc-6-P were prepared according to the synthetic approach outlined in Scheme 22 as potential replacement for the ManNAc-6-P. In the following chapter, the synthesis of starting material for triacetylated *N*-acetyl-D-mannosamine ProTides and its coupling with phosphorochloridates will be described. Preparation of appropriate phosphorochloridates was accomplished as described above in paragraph 3.1.2.4.



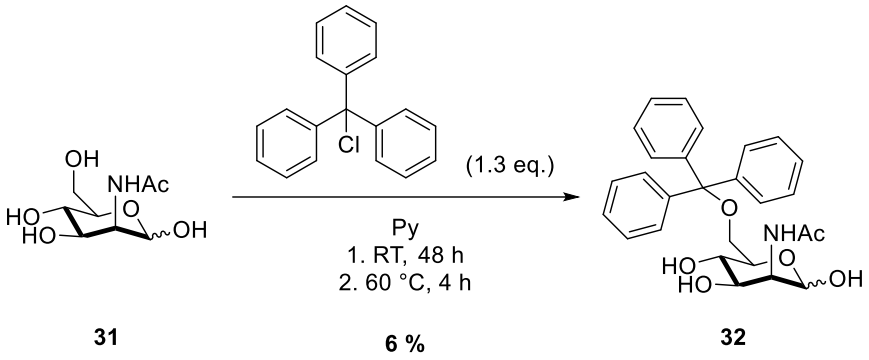
Scheme 22: Synthetic strategy for the preparation of peracetylated ManNAc-6-P phosphoramidate prodrugs.

3.1.5.2 Synthesis of 2-acetamido-1,3,4-tri-*O*-acetyl-2-deoxy-D-mannopyranose (34) as the starting material

The preparation of triacetylated *N*-acetyl-D-mannosamine-6-phosphate prodrugs was initiated with the synthesis of 2-acetamido-1,3,4-tri-*O*-acetyl-2-deoxy-D-mannopyranose (**34**) as the sugar derivative, which reaction with appropriate phosphorochloridate would provide coupling only with the oxygen bonded to C-6.

The direct preparation of triacetylated derivative is not feasible, because the primary hydroxyl group on C-6 would be acetylated faster than the secondary ones.[86] Therefore, 6-OH had to be firstly selectively protected with a different protecting group. As a suitable protecting group, bulky triphenylmethyl (trityl) group was chosen, because it shows high selectivity for primary OH groups in polyhydroxylated compounds. Moreover, it is stable under conditions intended to be used for the acetylation

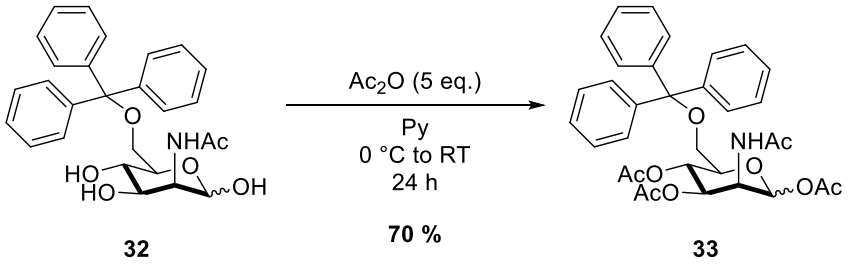
of secondary OH groups and it can be removed with acetic acid at increased temperature in relatively short reaction time in quantitate yield.[86,87] Reaction of *N*-acetyl-D-mannosamine (**31)** (1 eq.) with triphenylmethyl chloride (1.3 eq.) in pyridine (acting as a base) selectively yields 2-acetamido-2-deoxy-6-*O*-triphenylmethyl-D-mannopyranose (**32**) (Scheme 23).



*Scheme 23: Synthesis of 2-acetamido-2-deoxy-6-O-triphenylmethyl-D-mannopyranose (**32**).*

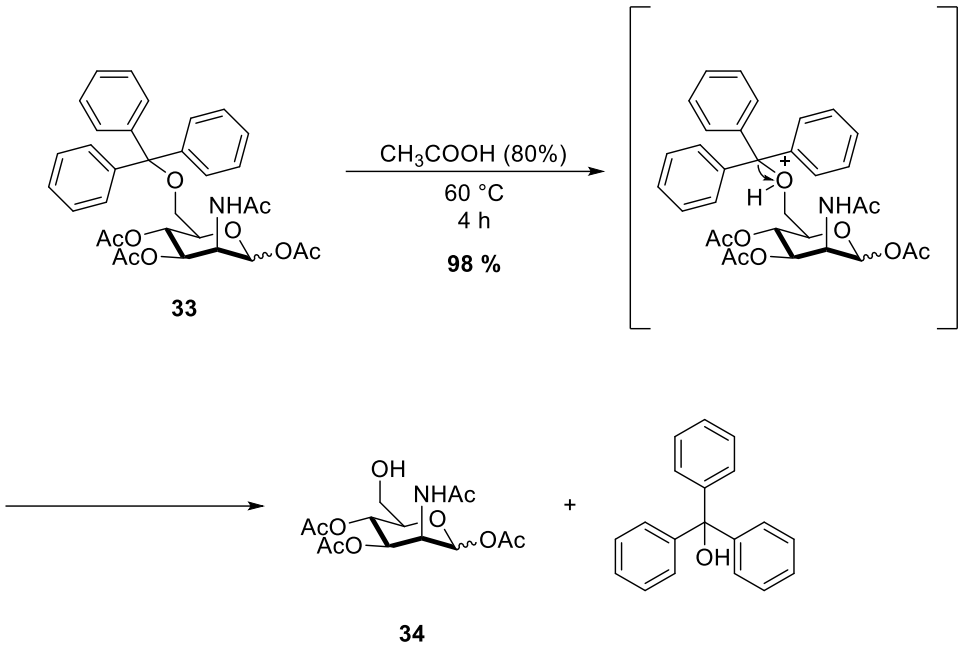
The reaction was stirred at room temperature for 48 hours and after that was heated to 60 °C.[88] The formation of desired protected compound (**32**) was confirmed by MS. Contrary to published procedure, we decided to purify the obtained crude after work-up yielding 6 % of pure compound **32**.

After the protection of primary hydroxyl, the acetylation could be performed (Scheme 24). Following a published procedure,[88] compound **32** (1 eq.) dissolved in pyridine was treated with acetic anhydride in excess (5 eq.). 70% yield of 2-acetamido-1,3,4-tri-*O*-acetyl-2-deoxy-6-*O*-triphenylmethyl-D-mannopyranose (**33**) was obtained after stirring at room temperature for 24 hours.



*Scheme 24: Synthesis of 2-acetamido-1,3,4-tri-O-acetyl-2-deoxy-6-O-triphenylmethyl-D-mannopyranose **(33).***

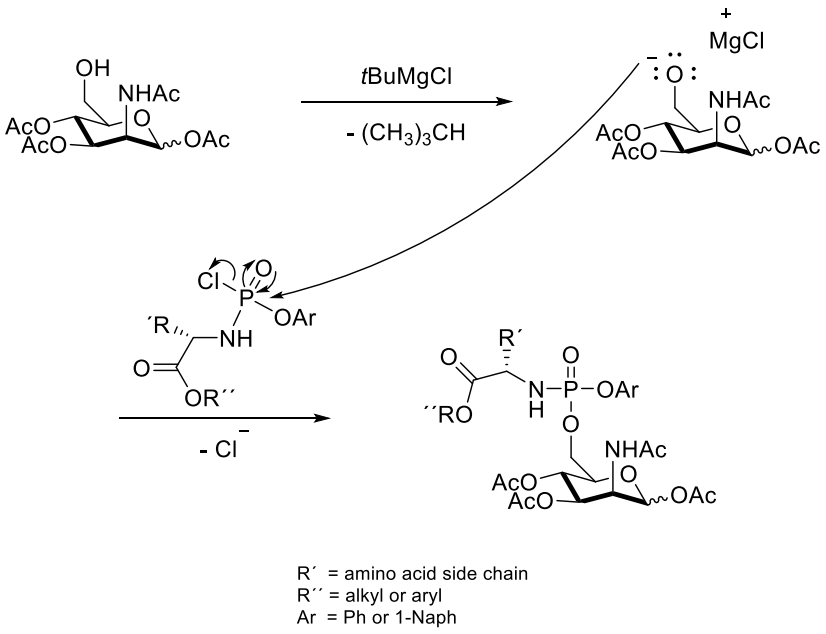
Since the properties of the present two types of *O*-protecting groups are different, selective cleavage of triphenylmethyl group can be achieved without affecting the acetyl groups. Usually, mild acidic conditions are applied to hydrolyse the O-Tr bond leaving untouched the acetylated alcohols (Scheme 25).[86] The increased stability of the formed triphenylmethyl carbocation can be explained by distribution of the positive charge and resonance stabilisation. After 4 hours at 60 °C,[88] 2-acetamido-1,3,4-tri-*O*-acetyl-2-deoxy-D-mannopyranose (**34**) was obtained in 98 % yield.



*Scheme 25: Selective detritylation of compound **33**.*

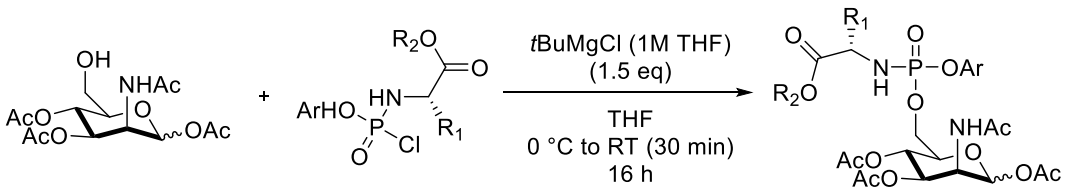
3.1.5.3 2-acetamido-1,3,4-tri-*O*-acetyl-2-deoxy-D-mannopyranose ProTides synthesis

Preferring the Grignard methodology, compound **34** was coupled with previously prepared phosphorochloridate (**8-12**) using *t*BuMgCl as a strong base able to deprotonate the free hydroxyl. Created nucleophilic alkoxide group attacks the phosphorus of appropriate phosphorochloridate and replaces the chlorine atom. The mechanism of the reaction is captured in Scheme 26.



Scheme 26: The mechanism of the Grignard methodology.

As typical procedure (General procedure **C**) (Scheme 27),[74] compound **34** (1 eq.) was dissolved in anhydrous THF under Argon atmosphere, cooled to 0 °C and 1M *t*BuMgCl (1.5 eq.) was added. Resulting solution was treated with desired phosphorochloridate (**8-12**) (1.5 eq.) in anhydrous THF at 0 °C and the reaction mixture was stirred at room temperature for 16 hours. Formation of the ProTide was confirmed by MS, the compound was isolated and purified by column chromatography on silica (DCM/MeOH, 98:2).



34 **8-12** **35-39**

35	Ar = Ph	$R_1 = CH_2Ph$	$R_2 = CH_2CH_3$	**45 %**
36	Ar = Ph	$R_1 = CH(CH_3)_2$	$R_2 = CH_2Ph$	**40 %**
37	Ar = Naph	$R_1 = H$	$R_2 = CH_3$	**49 %**
38	Ar = Naph	$R_1 = CH_3$	$R_2 = CH_2Ph$	**47 %**
39	Ar = Ph	$R_1 = CH_3$	$R_2 = CH(CH_3)_2$	**68 %**

Scheme 27: 2-acetamido-1,3,4-tri-O-acetyl-2-deoxy-D-mannopyranose ProTides synthesis via Grignard method.

Judging from the ^{31}P NMR spectra, we obtained a mixture of four diastereoisomers of the phosphoramidates (**35-39**). HPLC analysis performed after purification confirmed ≥ 95% purity of the final compounds. HPLC analysis of peracetylated D-mannose ProTides was performed with a different elution gradient. In cases of phosphoramidates **35-39**, elution began from 2 % of ACN and a slower gradient (100 % ACN in 40 minutes) was used. These conditions allowed us to separate the peaks corresponding to four isomers. In case of compounds **36, 37** and **39**, only three or two peaks on HPLC spectra were observed, since the signals are probably overlapping. Representative ^{31}P NMR and HPLC trace of compound **38** are shown in Figure 24a and Figure 24b, respectively.

Phosphoramidate prodrugs of peracetylated D-mannosamine synthetized via Grignard method are listed in Table 11.

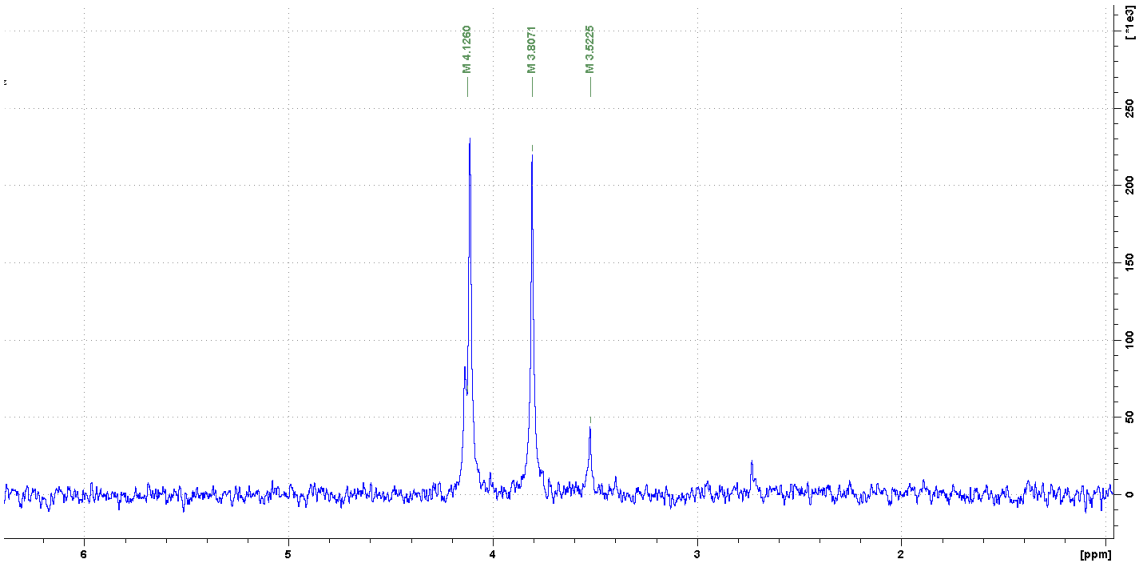


*Figure 24a: ^{31}P NMR (202 MHz, MeOD) of ProTide **38**: δ 4.12 (d, J = 4.5 Hz, 1P), 3.78 (s, 0.8P), 3.52 (s, 0.1 P) ppm corresponding to four diastereoisomers.*

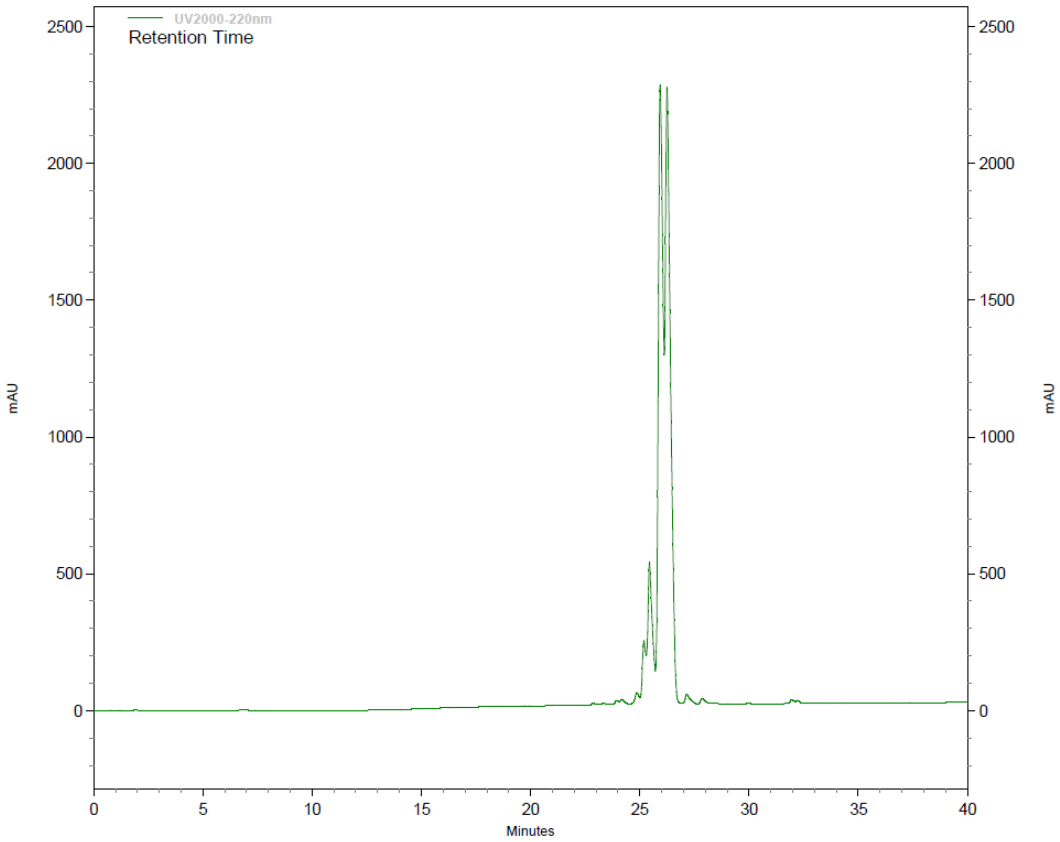


*Figure 24b: HPLC analysis of ProTide **38** (gradient ACN/H_2O 2:98, 100 % ACN in 40 min, flow: 1 mL/min).Rt: 25.20, 25.45, 25.93, 26.25 min corresponding to four diastereoisomers. Minor impurities are present.*

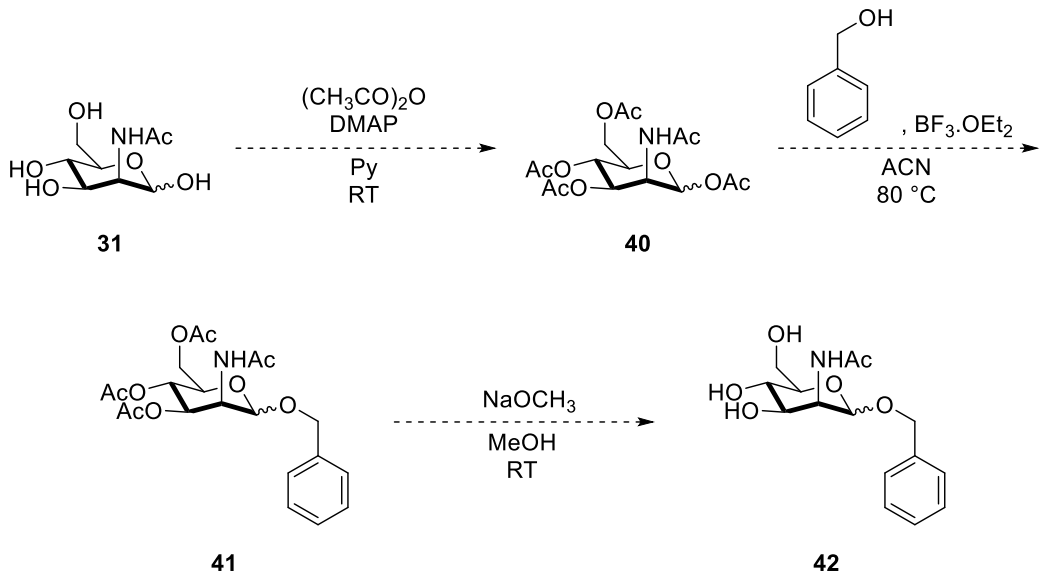
Compound	Ar	R_1	R_2	Yield [%]
35	Ph	CH_2Ph	CH_2CH_3	45
36	Ph	$CH(CH_3)_2$	CH_2Ph	40
37	α-Naph	H	CH_3	49
38	α-Naph	CH_3	CH_2Ph	47
39	Ph	CH_3	$CH(CH3)_2$	68

*Table 11: ProTides of 2-acetamido-1,3,4-tri-O-acetyl-2-deoxy-D-mannopyranose (**34**) synthetized via Grignard method*

3.1.6 Synthesis of the starting material for *N*-acetyl-D-mannosamine ProTides

3.1.6.1 Introduction

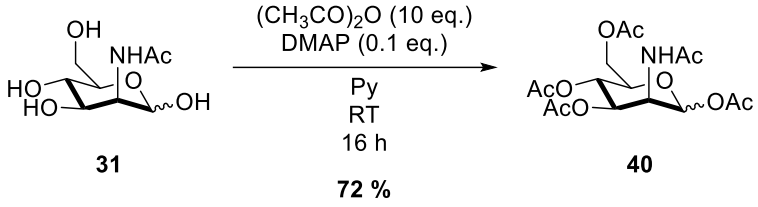
2-acetamido-1-*O*-benzyl-2-deoxy-D-mannopyranose (**42**) was opted to be prepared as a compound which would provide coupling with phosphorochloridate selectively in C-6 position. The synthetic strategy is outlined in Scheme 28. The protection of the anomeric position should prevent the formation of multiple phosphoramidates, since the anomeric hydroxyl is activated because the electron withdrawing effect of the endocyclic oxygen. In this chapter, we will describe the preparation of the sugar derivative as the starting material. Ultimate couplings with desired phosphorochloridates were not carried out due to the lack of time.



*Scheme 28. 2-acetamido-1-O-benzyl-2-deoxy-D-mannopyranose (**42**) synthetic strategy.*

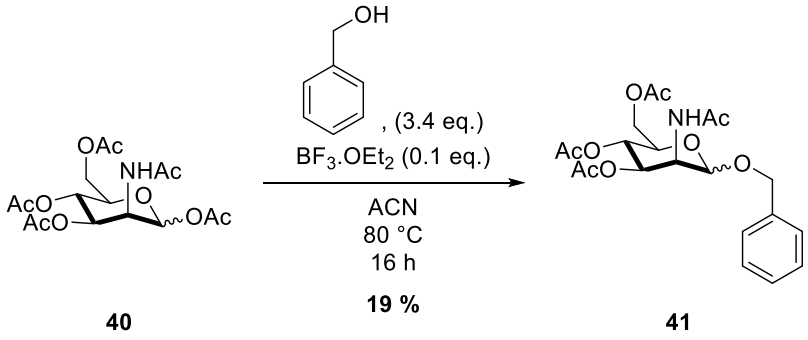
3.1.6.2 Synthesis of 2-acetamido-1-*O*-benzyl-2-deoxy-D-mannopyranose (42) as a starting material

Before selective benzylation of the anomeric position, protection of the rest of the hydroxyl groups was executed (Scheme 29). *N*-acetyl-D-mannosamine (**31**) (1 eq.) was acetylated via the reaction with acetic anhydride (10 eq.) in the presence of catalytic amount of 4-(dimethylamino)pyridine (DMAP).[89] According to the TLC (DCM/MeOH, 98:2), the conversion into peracetylated product (with higher Rf) was completed within 16 hours. After the work-up, 2-acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-D-mannopyranose (**40**) was obtained in 72% yield and no further purification was carried out.



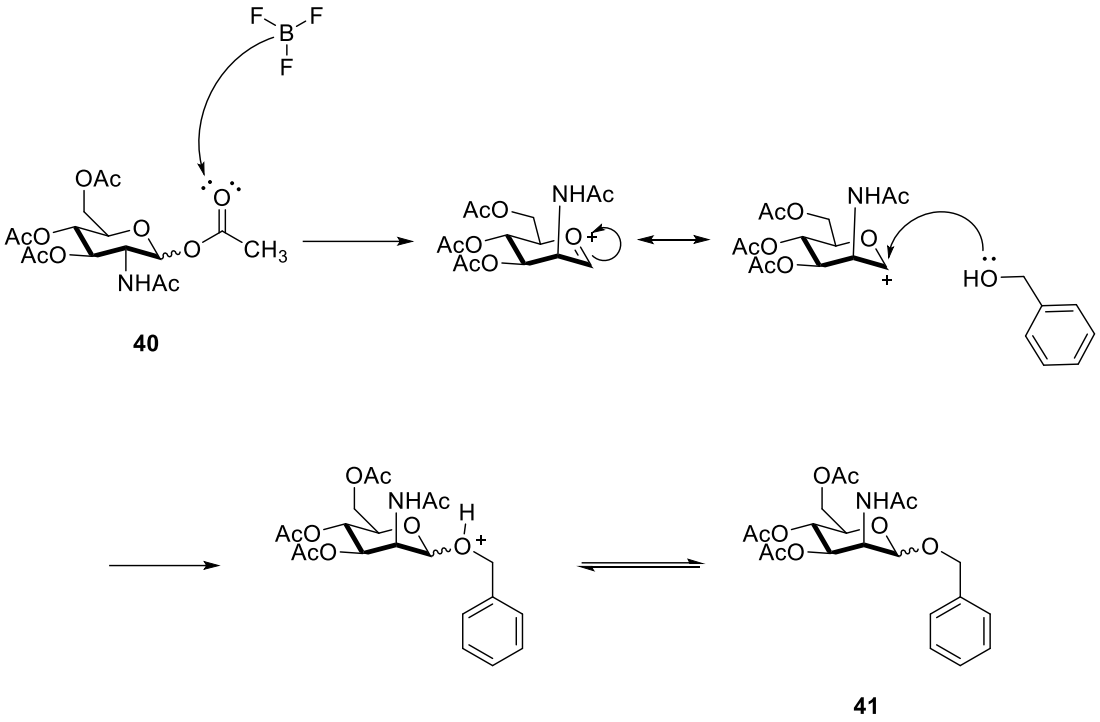
*Scheme 29: Acetylation of N-acetyl-D-mannosamine (**31**).*

Compound **40** (1 eq.) was then dissolved in anhydrous acetonitrile and treated with benzyl alcohol (3.4 eq.) followed by addition of boron trifluoride diethyl etherate (0.1 eq.).[90] The solution was heated overnight at 80 °C yielding 19 % of corresponding benzyl glycoside (**41**) (Scheme 30).



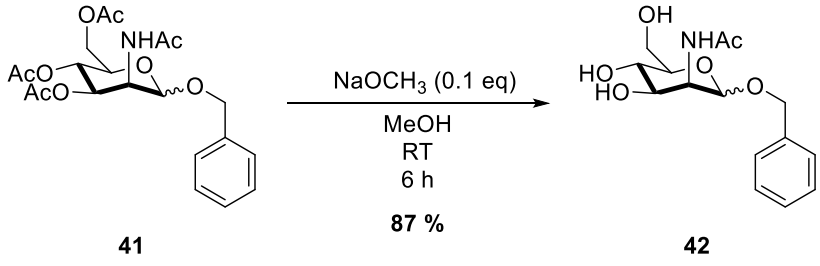
*Scheme 30: Synthesis of 2-acetamido-3,4,6-tri-O-acetyl-1-O-benzyl-2-deoxy-D-mannopyranose (**41**).*

The mechanism of the selective benzylation is shown in Scheme 31.



Scheme 31: Mechanism of the benzylation of the anomeric position.

Final deacetylation was accomplished via treating of compound **41** (1 eq.) dissolved in anhydrous methanol with a solution of sodium methoxide (0.1 eq.) in methanol (Scheme 32).[90] The methoxide anion attacks the carbon of the carbonyl group resulting in methyl acetate cleavage. After the acidification by the acidic cation exchange resin, pure 2-acetamido-1-O-benzyl-2-deoxy-D-mannopyranose (**42**) should be obtained by trituration of the crude mixture with diethyl ether, nevertheless, further purification by column chromatography (EtOAC/MeOH, 4:1) was required to obtain pure compound **42** in 87 % isolated yield.



*Scheme 32: Deacetylation of compound **41**.*

3.1.7 Conclusion and future work

In conclusion, we were able to prepare four phosphoramidate prodrugs (ProTides) of peracetylated D-mannose (**18-21**) and five prodrugs of peracetylated *N*-acetyl-D-mannosamine (**35-39**) using the Grignard methodology, as one of the reported synthetic strategies. We attempted to employ the NMI activation of the phosphorochloridate, nevertheless, desired compounds were not obtained.

The enzymatic experiment was performed with compound **20**, confirming the affinity of the ProTide to the active site of the carboxypeptidase enzyme. We can suggest, that the ProTide would be converted into the appropriate monoester inside the cell as well, and transformed into the desired monophosphate afterwards. The affinity probably depends on the phosphorus configuration, because we observed different rate of degradation of the present diastereoisomers.

Unfortunately, synthetized D-mannose ProTides (**29-30**) showed considerable instability, since we observed their degradation immediately after the solvent evaporation. Derivatization of the anomeric position (e.g. methoxy group) could represent an option to stabilize the monosaccharide prodrug.

We also began the preparation of anomeric benzylated *N*-acetyl-D-mannosamine (**42**) as precursor for the synthesis of its 6-phosphoramidate prodrugs. It can be anticipated that coupling of compound **42** with the appropriate phosphorochloridates, followed by cleavage of the anomeric benzyl group via catalytic hydrogenation will provide the desired ProTides.

In vitro biological evaluation of the synthetized compounds is ongoing. Based on the biological results, new molecules with different aryl and amino acid moieties can be explored. The obtained outcomes could help to determine the structure-activity relationships (SAR).

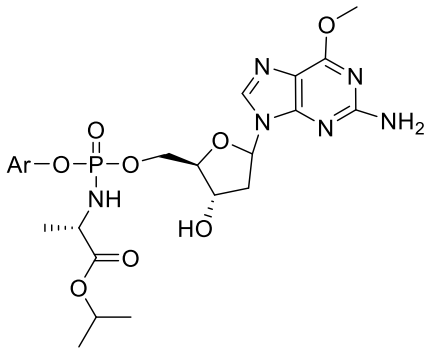
3.2 Synthesis of ProTides as potential medication for MDS

3.2.1 Introduction

Mitochondrial deoxyguanosine kinase (dGK), encoded by *DGUOK* gene, is an enzyme responsible for the phosphorylation of deoxyguanosine (dG) and deoxyadenosine (dA) to their monophosphates which are necessary for the mtDNA synthesis. Mutation in *DGUOK* leads to the deficiency of dGMP/dAMP and subsequently to the defects in mtDNA maintenance resulting in insufficient energy production in affected organs and tissues.[55,58] Administration of deficient nucleoside monophosphate in patients with impaired dGK could substitute the lacking dGMP/dAMP in the mitochondrial matrix.[61]

Nucleoside monophosphates are highly polar molecules, and as such they cannot pass through the cell membrane but require an active transporter. Phosphoramidate prodrugs (ProTides) are more lipophilic compounds enabling the molecule diffuse into the cell and release the original monophosphate after bioactivation within the cell.[67,69]

This part of the thesis will describe the preparation of 2′-deoxy-O^6-methylguanosine ProTides captured in Figure 25.



53 Ar = 1-Naph
54 Ar = Ph

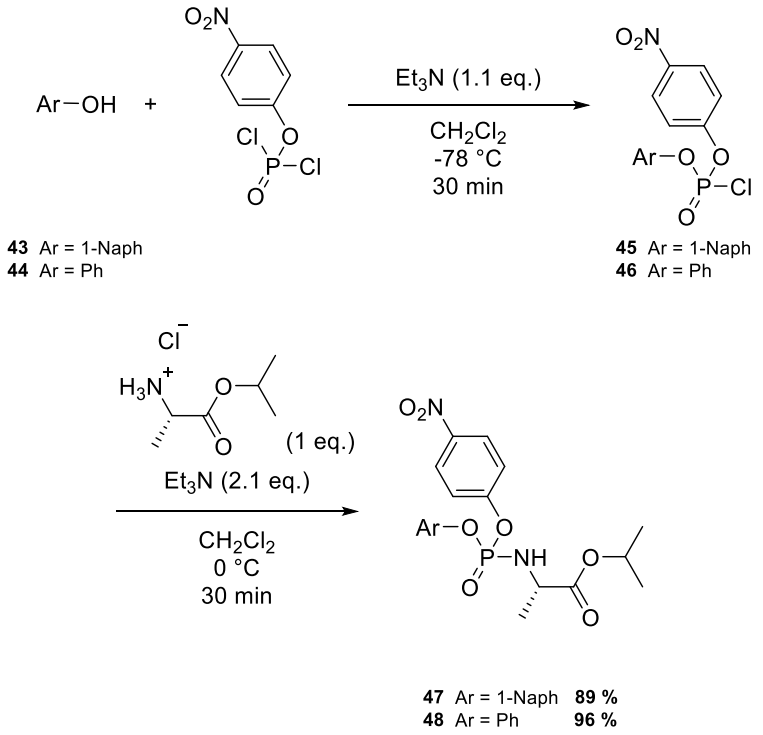
Figure 25: General structure of nucleoside phosphoramidate prodrugs synthetized in this work.

The synthetic work included the preparation of the phosphoramidate moiety donors, preparation of the nucleoside substrate and final synthesis of the desired prodrugs. We used the Grignard methodology for the ultimate coupling as one of the known approaches towards phosphoramidates synthesis.[67]

In the following chapters we will explore the synthesis of (aryl) (*p*-nitrophenyl) phosphoramidates serving as the phosphoramidating agents, then we will delineate the preparation of 2′-deoxyguanosine derivative as the starting material for the coupling and finally, the synthesis of the proper ProTides will be described.

3.2.2 Synthesis of (aryl) (*p*-nitrophenyl) phosphoramidates

The preparation of phosphoramidating agents (Scheme 33) began from the substitution of the commercial available *p*-nitrophenyl phosphorodichloridate (1 eq.) with appropriate aryl-alcohol (**43**-**44**) (1 eq.) in the presence of anhydrous trimethylamine (1.1 eq.). After 30 minutes at -78 °C, formation of the corresponding (aryl)(*p*-nitrophenyl) phosphorochloridate (**45**-**46**) was confirmed by ^{31}P NMR. Even though the formed phoshorochloridates possess one stereocentre (phosphorus), thus two enantiomers can be present in the reaction mixture, only one signal appears in ^{31}P NMR spectrum, since the enantiomers are generally not differentiated by NMR without using chiral environment. In our case, a singlet at -5.65 ppm (compound **45**) and -6.00 ppm (compound **46**) confirmed the complete conversion into the desired chloridate.



*Scheme 33: Synthesis of (aryl) (p-nitrophenyl) phosphoramidates (**47**-**48**) as the phosphoramidating agents.*

As the further step, the previous reaction mixture (compounds **45**-**46**) was transferred through syringe to a cooled solution (0 °C) of L-alanine isopropyl ester hydrochloride (**7**) dissolved in anhydrous dichloromethane. The resulting mixture was then treated with additional 2.1 equivalents of anhydrous Et_3N to create free amino acid with increased reactivity towards the phosphorus compared to its hydrochloride salt. After approximately 30 minutes at 0 °C, the substitution was completed which was observed in ^{31}P NMR spectrum. The singlet vanished whilst doublet shifted towards lower field appeared. Molecules of the phosphoramidates (**47**-**48**) exist as two diastereoisomers (LR_P, LS_P) with distinguishable ^{31}P NMR signals owing the chiral phosphorus with variable configuration and fixed L configuration of α carbon.

Therefore, two peaks are present in ^{31}P NMR spectra of phosphoramidates **47-48**. Representative ^{31}P NMR spectra of compound **45** and **47** are shown in Figure 26a and Figure 26b, respectively. Prepared compounds with NMR shifts and reaction yields are listed in Table 12.

Compound	**Ar**	**^{31}P NMR (202 MHz, $CDCl_3$)**	**Yield [%]**
47	α-Naph	-2.86 (d, J = 4.3 Hz)	89
48	Ph	-3.12 (d, J = 3.7 Hz)	96

Table 12: Synthetized (p-nitrophenyl) phosphoramidates with reaction yields.

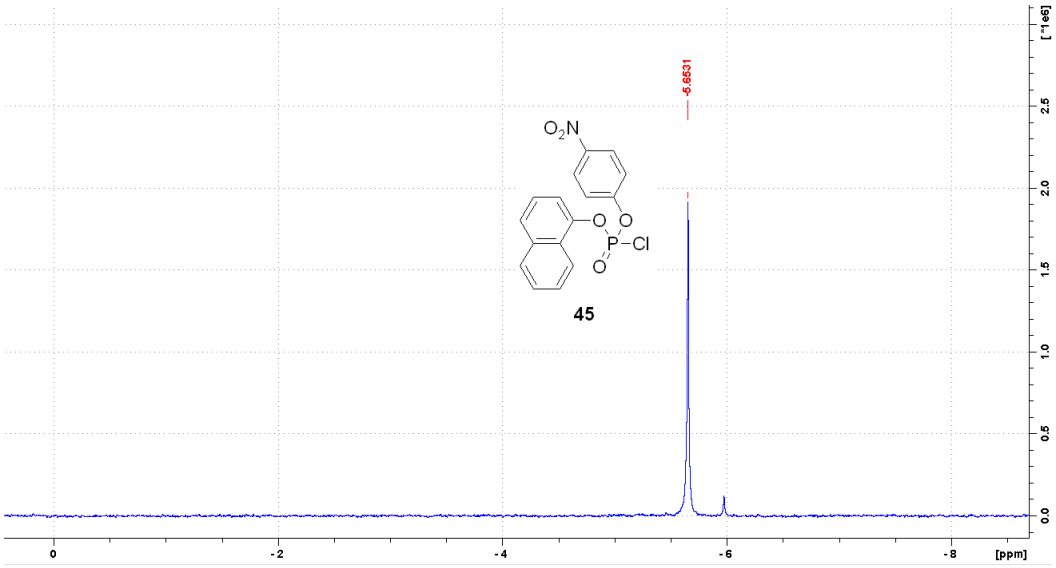


*Figure 26a: ^{31}P NMR (202 MHz, $CDCl_3$) of compound **45**. One peak representing two enantiomers.*

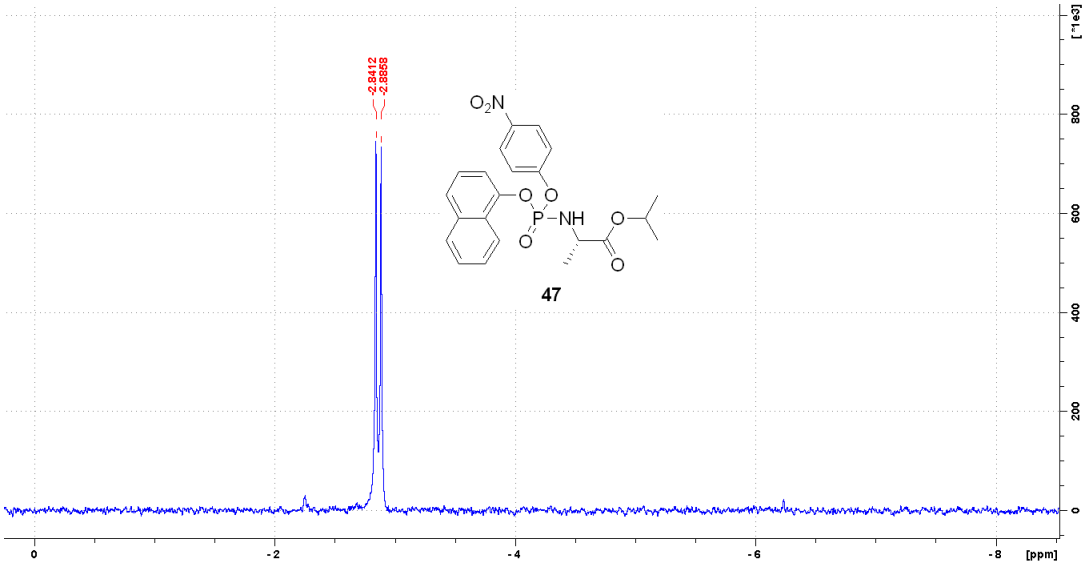
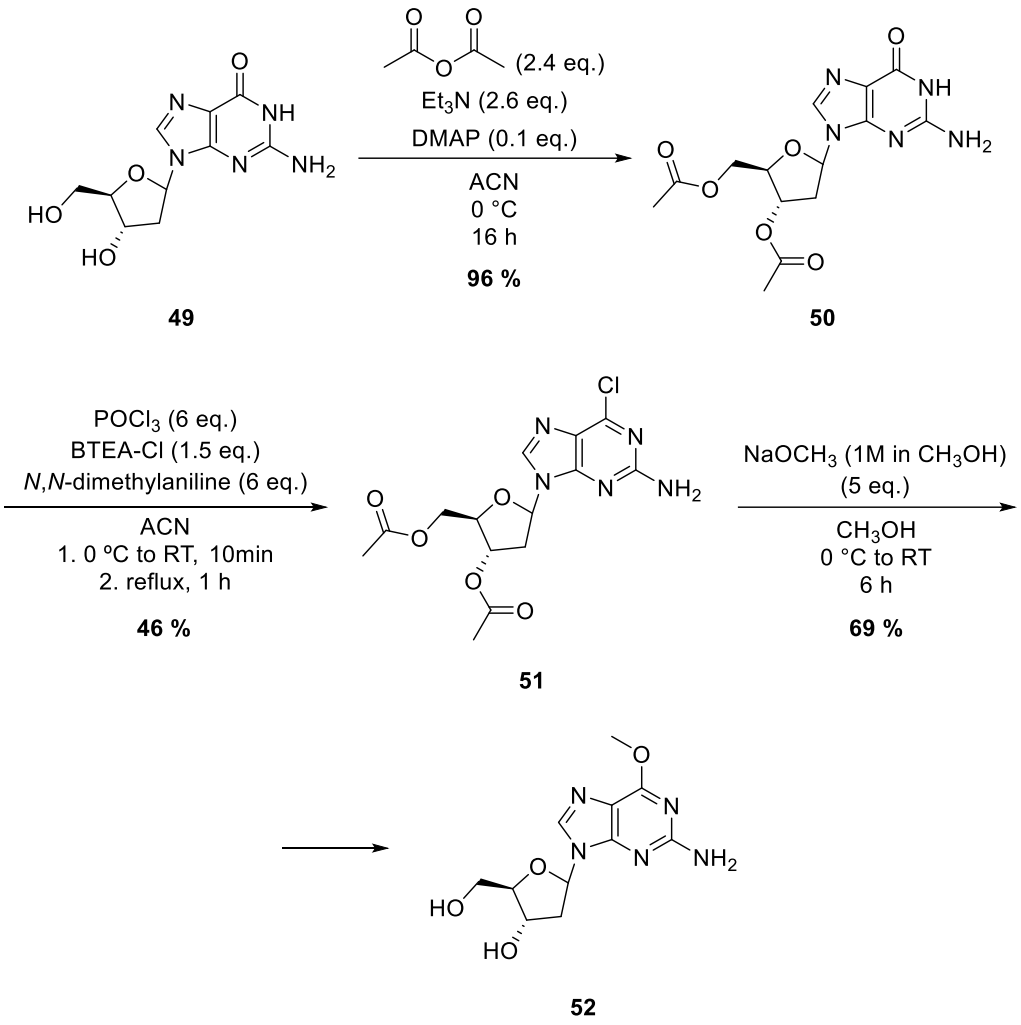


*Figure 269b: ^{31}P NMR (202 MHz, $CDCl_3$) of compound **47**. Two peaks corresponding to two diastereoisomers.*

3.2.3 Synthesis of 2′-deoxy-O^6-methylguanosine (52) as the starting material

Our goal was to prepare phosphoramidate prodrugs of the unnatural nucleotide, therefore, we had to prepare the appropriate nucleoside derivative before final prodrugs synthesis. On the contrary to naturally occurring 2′-deoxyguanosine, one of the DNA building blocks, 2′-deoxy-O^6-methylguanosine shows increased lipophilicity owing the methoxy group, nevertheless, it still belongs to the highly polar molecules.

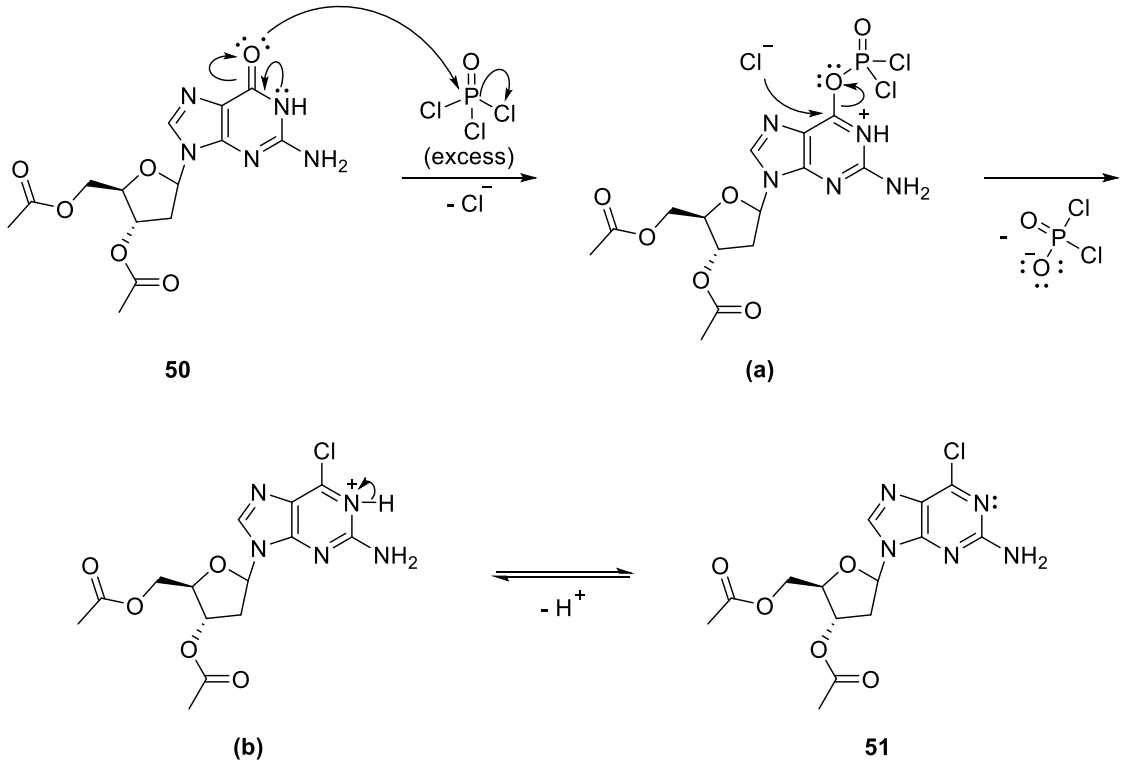
Adopting the published procedure,[91] the designed synthesis was based on the acetylation of the commercially available 2′-deoxyguanosine (**49**) yielding diacetylated analogue (**50**) which was chlorinated at the 6-position (compound **51**) using $POCl_3$ and ultimately derivatized to O^6-methyl analogue (**52**) by treating with $NaOCH_3$, with concomitant deacetylation of the hydroxyl groups. The all synthetic sequence is captured in Scheme 34.



*Scheme 34: Successful synthesis of 2′-deoxy-O^6-methylguanosine (**52**).*

Before the deoxychlorination step, protection of the free hydroxyl groups of 2′-deoxyguanosine (**49**) was necessary. The quantitative acetylation was achieved by the reaction of compound **49** (1 eq.) with acetic anhydride (2.4 eq.) using anhydrous Et_3N (2.6 eq.) and DMAP (0.1 eq.).[91] Addition of DMAP to the Ac_2O/Et_3N acetylating system was reported to improve the reaction yield, especially in more sterically hindered substrates. DMAP is supposed to act as a base, activating hydroxyl groups directly or by generation of the reactive acetyl group after being acetylated by acetic anhydride.[89] After 16 hours of stirring at 0 °C, the product was precipitated by addition of methanol, filtered and washed with methanol and hexane yielding 96% of pure 3′,5′-di-*O*-acetyl-2′-deoxyguanosine (**50**).

Chlorine, one of the most frequently used leaving groups at C-6 of purine nucleosides, was introduced to the molecule following the improved method for the acid-labile 2′-deoxynucleoside derivatives using $POCl_3$ (6 eq.), *N*,*N*-dimethylaniline (6 eq.) and benzyltriethylammonium chloride (BTEA-Cl) (1.5 eq.).[91] $POCl_3$ acts as a source of the chlorine atom into compound **50** as outlined in Scheme 35. Phosphoryl chloride reacts with the nucleophilic oxygen leading to the elimination of chloride and formation of an intermediate **(a)** possessing excellent leaving group.[92] Chloride than attacks the C-6 providing the desired 3′,5′-di-*O*-acetyl-6-deoxo-6-chloro-2′-deoxyguanosine (**53**).

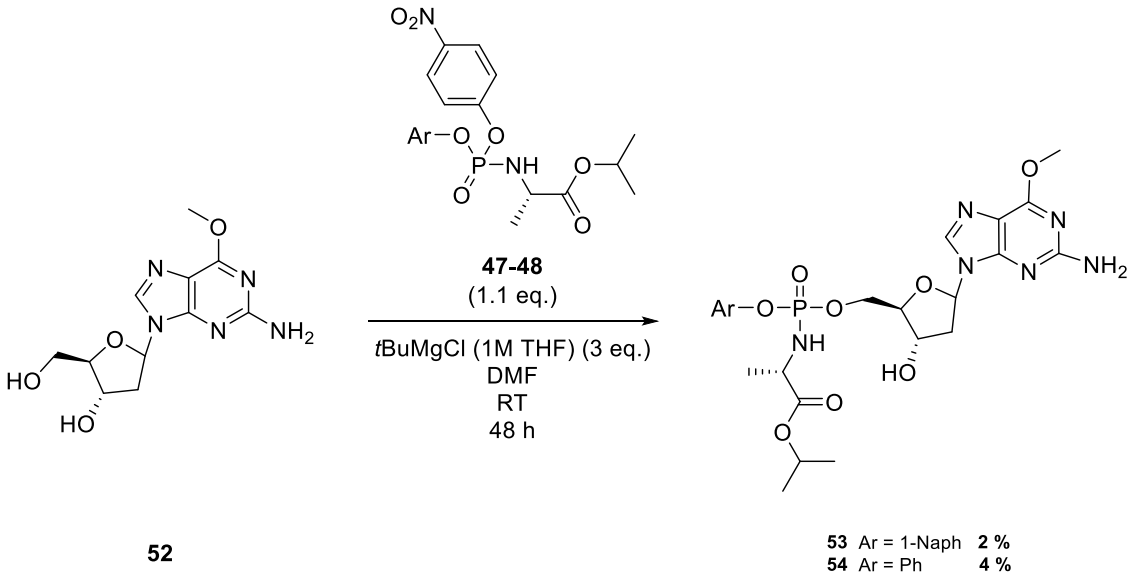


Scheme 35: Mechanism of deoxychlorination.

Final methoxylation was provided by treating of product **51** with freshly prepared 1M solution of sodium methoxide (5 eq.) in anhydrous methanol at 0 °C.[91] The addition-elimination mechanism of the substitution is explained by the nucleophilic attack of the methoxide anion to the carbon that bears the halogen (addition) and subsequent loss of the halide (elimination) and restoration of the ring aromaticity. Alongside with the SnAr, the methoxide anions attack the acetyl groups providing deprotection of the sugar hydroxyls. After 6 hours, the reaction was completed. Work up of the crude solution was performed by adjusting the pH of prepared water solution to pH 7 to remove the created salt. The formed solid was extracted with ethyl acetate and dichloromethane and the subsequent evaporation of organic extracts yielded 69 % of pure 2´-deoxy-*O*^{6}-methylguanosine (**52**).

3.2.4 2´-deoxy-*O*^{6}-methyl-guanosine ProTides synthesis

On the basis of the experience with 2´-deoxyguanosine ProTides synthesis performed by our group previously, we decided to prepare the desired prodrugs via the Grignard methodology (Scheme 36). The mechanism[68] involves deprotonation of the primary hydroxyl in the 5´-position by the *t*BuMgCl providing the 5´-alkoxide group which then attacks the phosphorus of the appropriate (aryl) (*p*-nitrophenyl) phosphoramidate. 4-nitrophenolate is cleaved and the desired nucleoside phosphoramidate is formed.



Scheme 36: Synthesis of 2´-deoxyguanosine ProTides using Grignard method.

No protection of 3´ position leads to the deprotonation of the secondary hydroxyl alongside the primary one, since *t*BuMgCl serves as strong a base with no preference towards primary or secondary hydroxyl groups.[68] Although both, the mono and bis- phosphosphoramidates were contained in the reaction mixture, we separated the desired compound and we further purified and characterized only the 5´-prodrug.

Generally, 1M *t*BuMgCl in THF (3 eq.) was added dropwise to a suspension of compound **52** (1 eq.) in anhydrous DMF under inert atmosphere. After 30 minutes of stirring, a solution of phosphoramidate **47** or **48** (1.1 eq.) in anhydrous DMF was dripped into the reaction mixture and the resulting mixture was stirred for 48 (ProTide **53**) or 96 (ProTide **54**) hours at room temperature. The formation of the monophosphoramidate was confirmed by MS. Purification by column chromatography on silica and following reversed-phase column chromatography provided ProTides **53** and **54** in ≥ 95% purity, as judged by HPLC analysis. In order to determine if the phosphoramidate prodrug was effectively at the 5′-position, a ^{13}C NMR experiment was conducted. Indeed, based on $^{2}J_{P\text{-}C}$ coupling observed in the ^{13}C NMR spectra, the phosphorus splits the signal of the C-5′ and not the C-3′ which proves the right isomer.

The obtained yield of compound **53** (2 %) was slightly improved in case of compound **54** (4 %) by prolongation of the reaction time. Nevertheless, both yields are very low (Table 13).

The molecules of prepared ProTides own four stereocentres, however, phosphorus is the only chiral atom which configuration is variable, and therefore, only two diastereoisomers can be formed. ^{31}P NMR, as well as the HPLC analysis, detected two peaks corresponding to two stereoisomers of the ProTide (**53**-**54**).

Compound	Ar	^{31}P NMR (202 MHz, MeOD)	Reaction time	Yield [%]
53	α-Naph	4.46 (s, 1P), 4.32 (s, 0.7P)	48 hours	2
54	Ph	4.08 (s, 0.4P), 3.88 (s, 1P)	96 hours	4

Table 13: Synthetized nucleoside ProTides.

3.2.5 Conclusion and future work

In summary, we successfully prepared two 5′-ProTides of 2′-deoxy-O^6-methyl-guanosine (**53**-**54**) using the Grignard methodology, without protecting the 3′-OH group. As a future work, improvement of the low yield of the final coupling providing the phosphoramidate prodrug should be attempted.

The *in vitro* biological studies of ProTides **53**-**54** are in progress. According to the obtained outcomes, further aryl and amino acid modifications can be investigated.

4 Experimental section

4.1 General experimental details

Anhydrous solvents were purchased from Sigma–Aldrich and NMR solvents were obtained from Cambridge Isotope Laboratories. All reagents commercially available were used without any purification. Reactions were monitored with ^{31}P NMR spectra and/or analytical thin layer chromatography (TLC) on Silica gel 60-F_{254} precoated aluminum plates (Merck) and visualized under UV (254 nm) or using Pancaldi reagent. Flash column chromatography was carried out using Biotage Isolera One purification system. High-performance liquid chromatography (HPLC) was performed by Spectra System SCM (with X-select C18 5µm, 4.8 × 150 mm column) or Varian Prostar HPLC System (LC Workstation-Varian ProStar 335 LC detector) to verify the purity of final compounds to be > 95%. Proton (^{1}H; 500 MHz), carbon (^{13}C, 125 MHz) and phosphorus (^{31}P; 202 MHz) NMR spectra were obtained by Bruker Avance 500 spectrometer at 25 °C. Spectra were autocalibrated to the deuterated solvent peak. All ^{13}C NMR and ^{31}P NMR were proton-decoupled. Chemical shifts (δ) are quoted in parts per million (ppm), coupling constants (*J*) are given in Hertz. The following abbreviations are used in the characterization of NMR signals: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), dd (doublet of doublets), ddd (doublet of doublet of doublets), dt (doublet of triplets). Low-resolution mass spectrometry was performed using electrospray spectrometry (micrOTOF, Bruker Daltonics) as a service by Cardiff University.

4.2 Synthesis of ProTides for CDGs

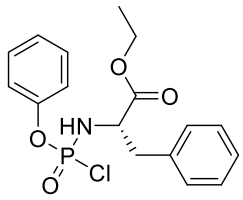
4.2.1 Preparation of phosphorochloridates

4.2.1.1 General procedure for the phosphorochloridates synthesis (A)

Under Argon atmosphere, the desired aminoacid ester (1 eq.) was placed in a round-bottom flask, suspended in anhydrous dichloromethane and then cooled to -78 °C by means of dry ice/acetone bath. A solution of desired phosphorodichloridate (1 eq.) in anhydrous dichloromethane was added and the resulting mixture was stirred for 20 minutes. Subsequently, anhydrous triethylamine (2 eq.) was added dropwise over a period of 15 minutes. The cooling bath was removed after 20 minutes of stirring and the mixture was allowed to reach room temperature. In approximately 60 minutes, when ^{31}P NMR of the reaction mixture confirmed completion of the reaction ($CDCl_3$, disappearance of the singlet corresponding to the phosphorodichloridate and detection of the doublet corresponding to the phosphorochloridate), the solvent was evaporated under reduced pressure without any contact with air. The solid residue was suspended in anhydrous diethyl ether and stirred vigorously under Argon atmosphere for 15 minutes.

The slurry was filtered on sintered glass filter and the solvent was finally evaporated under reduced pressure without any contact with air. The resulting crude containing a mixture of 2 diastereoisomers was used for the following reaction without any purification.

4.2.1.2 (2*S*)-ethyl 2-{[(chloro) (phenoxy) phosphoryl] amino} -3-phenylpropanoate (8)



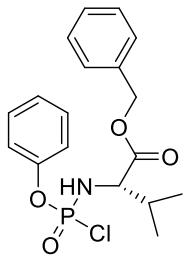
Compound **8** was synthetized following the procedure **A** using 4.00 g (17.41 mmol, 1 eq.) of L-phenylalanine ethylester hydrochloride (**3**), 2.6 mL (17.41 mmol, 1 eq.) of phenyl dichlorophosphate (**1**), 4.9 mL (34.83 mmol, 2 eq.) of anhydrous Et_3N and 100 mL of anhydrous CH_2Cl_2 in a 250 mL round-bottom flask.

$C_{17}H_{19}ClNO_4P$; M.W.: 367.77 g/mol; (6.33 g, 99 %)

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.49-7.02 (10H, m, CH_2Ph and OPh), 4.51-4.35 (1H, m, CH), 4.26-4.16 (2H, m, OCH_2CH_3), 3.24-3.10 (2H, m, CH_2Ph), 1.29-1.16 (3H, m, CH_3) ppm

^{31}P NMR (202 MHz, $CDCl_3$): δ 8.00 (d, J = 14.3 Hz) ppm

4.2.1.3 (2*S*)-benzyl 2-{[(chloro) (phenoxy) phosphoryl] amino}- 3-methylbutanoate (9)



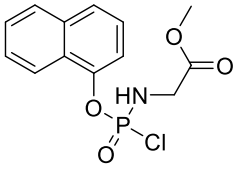
Compound **9** was synthetized following the procedure **A** using 3.00 g (12.31 mmol, 1 eq.) of L-valine benzylester hydrochloride (**4**), 1.8 mL (12.31 mmol, 1 eq.) of phenyl dichlorophosphate (**1**), 3.4 mL (24.62 mmol, 2 eq.) of anhydrous Et_3N and 100 mL of anhydrous CH_2Cl_2 in a 250 mL round-bottom flask.

$C_{18}H_{21}ClNO_4P$; M.W.: 381.79 g/mol; (4.43 g, 89 %)

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.48-7.09 (10H, m, CH_2Ph and OPh), 5.32-5.07 (2H, m, CH_2), 4.08-3.86 (1H, m, NHCH), 2.23-2.03 (1H, m, CH_3CHCH_3), 1.03 (3H, dd, J = 12.2, 6.8 Hz, CH_3CHCH_3), 0.96-0.90 (3H, m, CH_3CHCH_3) ppm

^{31}P NMR (202 MHz, $CDCl_3$): δ 9.48 (s, 1P), 8.95 (s, 0.9P) ppm

4.2.1.4 Methyl 2-{[(chloro) (naphtalen-1-yloxy) phosphoryl] amino} acetate (10)



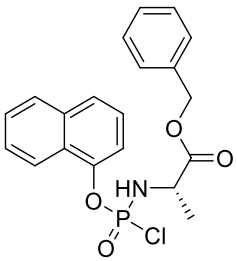
Compound **10** was synthetized following the procedure **A** using 1.25 g (9.96 mmol, 1 eq.) of glycine methylester hydrochloride (**5**), 2.60 g (9.96 mmol, 1 eq.) of α-naphtyl dichlorophosphate (**2**), 2.8 mL (19.92 mmol, 2 eq.) of anhydrous Et_3N and 50 mL of anhydrous CH_2Cl_2 in a 150 mL round-bottom flask.

$C_{13}H_{13}ClNO_4P$; M.W.: 313.67 g/mol; (2.30 g, 74 %)

^{1}H NMR (500 MHz, $CDCl_3$): δ 8.27-7.32 (7H, m, Naph), 4.05 (2H, d, J = 11.0 Hz, CH_2), 3.85 (3H, s, CH_3) ppm

^{31}P NMR (202 MHz, $CDCl_3$): δ 8.99 (s) ppm

4.2.1.5 (2*S*)-benzyl 2-{[(chloro) (naphtalen-1-yloxy) phosphoryl] amino} propanoate (11)



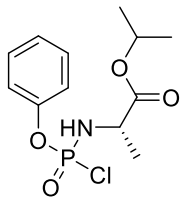
Compound **11** was synthetized following the procedure **A** using 4.00 g (18.55 mmol, 1 eq.) of L-alanine benzylester hydrochloride (**6**), 4.48 g (18.55 mmol, 1 eq.) of α-naphtyl dichlorophosphate (**2**), 5.2 mL (37.09 mmol, 2 eq.) of anhydrous Et_3N and 100 mL of anhydrous CH_2Cl_2 in a 250 mL round bottom-flask.

$C_{20}H_{19}ClNO_4P$; M.W.: 403.80 g/mol; (7.18 g, 96 %)

^{1}H NMR (500 MHz, $CDCl_3$): δ 8.16-7.05 (12H, m, Naph and Ph), 5.24-4.70 (2H, m, CH_2), 4.53-4.33 (1H, m, CH), 1.47 (3H, dd, J = 10.4, 7.1 Hz, CH_3) ppm

^{31}P NMR (202 MHz, $CDCl_3$): δ 8.16 (s, 1P), 7.91 (s, 1P) ppm

4.2.1.6 (2*S*)-isopropyl 2-{[(chloro) (phenoxy) phosphoryl] amino} propanoate (12)



Compound **12** was synthetized following the procedure **A** using 4.63 g (27.62 mmol, 1 eq.) of L-alanine isopropylester hydrochloride (**7**), 4.1 mL (27.62 mmol, 1 eq.) of phenyl dichlorophosphate (**1**), 7.7 mL (55.24 mmol, 2 eq.) of anhydrous Et_3N and 150 mL of anhydrous CH_2Cl_2 in a 250 mL round-bottom flask.

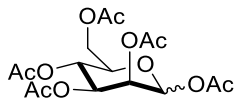
$C_{12}H_{17}ClNO_4P$; M.W.: 305.69 g/mol; (8.36 g, 99 %)

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.51-7.13 (5H, m, Ph), 5.19-4.96 (1H, m, CH_3CHCH_3), 4.51-4.29 (1H, m, NH), 4.24-3.95 (1H, m, NHCH), 1.54-1.50 (3H, dd, *J* = 7.11, 2.34 Hz, $NHCHCH_3$), 1.33-1.20 (6H, m, CH_3CHCH_3) ppm

^{31}P NMR (202 MHz, $CDCl_3$): δ 8.15 (s, 1P), 7.83 (s, 1P) ppm

4.2.2 Synthesis of tetraacetylated D-mannopyranose phosphoramidates

4.2.2.1 1,2,3,4,6-penta-*O*-acetyl-D-mannopyranose (14)

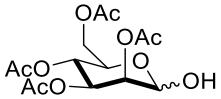


D-mannose (**13**) (2.00 g, 11.10 mmol, 1 eq.) was suspended in glacial acetic acid (20 mL) and acetic anhydride (7.8 mL, 83.26 mmol, 7.5 eq.) in a 100 mL round-bottom flask. Concentrated sulfuric acid (0.5 mL) was added dropwise and the resulting mixture was stirred at room temperature for approximately 2 hours. Once the reaction mixture became transparent, indicating completion of the reaction, the solution was dissolved in dichloromethane (50 mL), washed with cold water and then with saturated sodium bicarbonate to remove the acidic impurities. The organic layer was washed with brine, dried over sodium sulfate and the solvent was evaporated under reduced pressure to obtain pure 1,2,3,4,6-penta-*O*-acetyl-D-mannopyranose.

$C_{16}H_{22}O_{11}$; M.W.: 390.34 g/mol; (3.50 g, 81 %)

^{1}H NMR (500 MHz, $CDCl_3$): δ 6.11 (d, *J* = 1.8 Hz) – 5.88 (d, *J* = 1.2 Hz) (1H, H-1), 5.51 – 5.15 (3H, m, H-2 and H-3 and H-4), 4.35- 4.04 (3H, m, H-5 and 2H-6), 2.02-2.25 (15H, m, 5 × Ac) ppm

4.2.2.2 2,3,4,6-tetra-*O*-acetyl-D-mannopyranose (15)



To a solution of **14** (3.50 g, 8.97 mmol, 1 eq.) in THF (15 mL) in a 100 mL round-bottom flask, benzylamine (1.5 mL, 13.45 mmol, 1.5 eq.) was added and the reaction mixture was stirred at room temperature overnight. After addition of 1N HCl (7 mL), the mixture was stirred for further 60 minutes. The reaction mixture was diluted with 1N HCl (100 mL) and extracted with dichloromethane. The organic layers were combined, dried over sodium sulfate and concentrated under reduced pressure on rotary evaporator. The resulting crude was purified by column chromatography on silica using Hex/EtOAc (1:1) as the eluting system yielding pure 2,3,4,6-tetra-*O*-acetyl-D-mannopyranose as yellow oil.

$C_{14}H_{20}O_{10}$; M.W.: 348.30 g/mol; (1.87 g, 60 %)

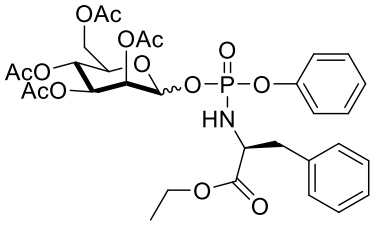
^{1}H NMR (500 MHz, $CDCl_3$): δ 5.37 (d, J = 3.5 Hz) – 5.35 (d, J = 3.5 Hz) (1H, H-1), 5.27-4.99 (3H, m, H-2 and H-3 and H-4), 4.23-4.02 (3H, m, H-5 and 2H-6), 2.12-1.91 (12H, m, 4 × Ac) ppm

$Rf_{(Hex/EtOAc,\ 1:1)}$ = 0.28

4.2.2.3 General procedure for the tetraacetylated D-mannopyranose phosphoramidates synthesis using Grignard method (B)

2,3,4,6-tetra-*O*-acetyl-D-mannopyranose (**15**) (1 eq.) was placed in a round-bottom flask under Argon atmosphere, dissolved in anhydrous THF and cooled to 0 °C. To the cold solution, 1M *t*BuMgCl in THF (1.5 eq.) was added. After 30 minutes, a solution of desired phosphorochloridate (1.5 eq.) in anhydrous THF was added dropwise while stirring. The cooling bath was allowed to reach room temperature and the reaction mixture was stirred overnight. After confirmation of the presence of the phosphoramidate in the mixture by MS, the solvent was evaporated under reduced pressure to obtain foamy residue. The crude was purified by column chromatography on silica using Hex/EtOAc as the eluting system. In order to obtain the compound in acceptable purity, reversed-phase chromatography (C-18) using ACN/H_2O (90:10), 100 % ACN in 50 minutes was carried out, yielding pure phosphoramidate as colourless oil.

4.2.2.4 (2*S*)-ethyl 2-{[((2,3,4,6-tetra-*O*-acetyl-D-mannopyranosyl) oxy) (phenoxy) phosphoryl] amino} 3-phenylpropanoate (18)



Compound **18** was prepared according to the procedure **B** using 0.60 g (1.72 mmol, 1 eq.) of **15**, 0.95 g (2.58 mmol, 1.5 eq.) of **8**, 2.6 mL (2.58 mmol, 1.5 eq.) of 1M *t*BuMgCl in THF and 8.6 mL of anhydrous THF in a 25 mL round-bottom flask.

$C_{31}H_{38}NO_{14}P$; M.W.: 679.61 g/mol; (0.35 g, 30 %)

^{1}H NMR (500 MHz, MeOD): δ 7.39-7.10 (10H, m, OPh and CH_2Ph), 5.69 (dd, *J* = 7.2, 1.4 Hz) – 5.42 (dd, *J* = 6.9, 1.5 Hz) (1H, H-1), 5.36-5.17 (3H, m, H-2 and H-3 and H-4), 4.28-3.88 (6H, m, OCH_2 and H-5 and 2H-6 and NHCH), 3.16-3.08 (1H, m, CH_2Ph- a), 2.96-2.88 (1H, m, CH_2Ph- b), 2.23-1.94 (12H, m, 4 × OCOCH_3), 1.20 (3H, t, *J* = 7.2 Hz, CH_2CH_3) ppm

^{13}C NMR (125 MHz, MeOD): δ 172.49 (d, *J* = 3.7 Hz) - 172.42 (d, *J* = 3.7 Hz) - 170.87 (d, *J* = 7.2 Hz) – 169.96 (d, *J* = 8.1 Hz) – 169.80 (s) – 150.51 (m) – 136.74 (s) – 136.63 (s) (7C, C$OOCH_2CH_3$ and 4 × O$COCH_3$ and 2 × Ph-C1), 129.50 (s) – 129.20 (s) – 128.19 (s) – 126.59 (s) – 125.03 (s) – 124.91 (s) – 120.23 (d, *J* = 5.3 Hz) – 119.92 (d, *J* = 4.5 Hz) (10C, OPh-C2-C6 and CH_2Ph-C2-C6), 94.81 (d, *J* = 5.5 Hz) – 94.48 (d, *J* = 5.5 Hz) (1C, C-1), 70.31 (s) – 70.11 (s) (1C, C-5), 68.84-68.47 (m) – 65.03 (d, *J* = 8.0 Hz) (3C, C-2 and C-3 and C-4), 61.57 (1C, d, *J* = 8.0 Hz, C-6), 61.11 (1C, d, *J* = 3.8 Hz, OCH_2), 56.41 (1C, d, *J* = 13.0 Hz, NHCH), 39.52 (1C, q, *J* = 3.6 Hz, CH_2Ph), 29.33 (s) – 19.28 (s) – 19.22 (s) – 19.17 (s) (4C, s, 4 × OCOCH_3), 13.03 (1C, s, CH_2CH_3) ppm

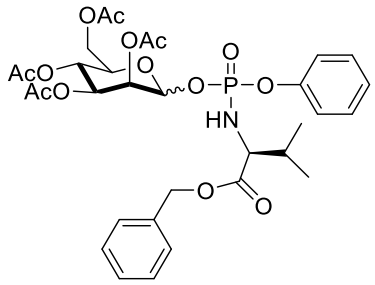
^{31}P NMR (202 MHz, MeOD): δ 1.53 (s, 1P), 0.87 (s, 0.25P) ppm

HPLC: Rt = 20.37 min (gradient ACN/H_2O 10:90, 100 % ACN in 30 min, flow: 1 mL/min)

MS (ESI+): m/z = 702.08 $[M+Na]^+$

$Rf_{(Hex/EtOAc,\ 1:1)}$ = 0.22, 0.31

4.2.2.5 (2*S*)-benzyl 2-{[((2,3,4,6-tetra-*O*-acetyl-D-mannopyranosyl) oxy) (phenoxy) phosphoryl] amino} 3-methylbutanoate (19)



Compound **19** was prepared according to the procedure **B** using 0.60 g (1.72 mmol, 1 eq.) of **15**, 0.99 g (2.58 mmol, 1.5 eq.) of **9**, 2.6 mL (2.58 mmol, 1.5 eq.) of 1M *t*BuMgCl in THF and 8.6 mL of anhydrous THF in a 25 mL round-bottom flask.

$C_{32}H_{40}NO_{14}P$; M.W.: 693.64 g/mol; (0.36 g, 30 %)

^{1}H NMR (500 MHz, MeOD): δ 7.44-7.18 (10, m, OPh and CH_2Ph), 5.78-5.50 (1H, m, H-1), 5.60-5.13 (5H, m, H-2 and H-3 and H-4 and CH_2Ph), 4.31-3.99 (2H, m, 2H-6), 3.99-3.73 (2H, m, H-5 and NHCH), 2.22-1.93 (13H, m, CH_3CHCH_3 and 4 × $OCOCH_3$), 0.97-0.83 (6H, m, CH_3CHCH_3) ppm

^{13}C NMR (125 MHz, MeOD): δ 172.56 (d, *J* = 3.7 Hz) – 172.50 (d, *J* = 2.9 Hz) – 172.33 (d, *J* = 3.7 Hz) – 172.19 (d, *J* = 4.4 Hz) – 170.87 (d, *J* = 6.4 Hz) – 170.45 (s) – 170.23 (s) – 170.08-169.81 (m) – 150.69-150.47 (m) – 135.90-135.76 (m) (7C, 4 × $OCOCH_3$ and $COOCH_2$Ph and 2 × Ph-C1), 129.64-129.32 (m) – 128.35-127.98 (m) – 125.03 (d, *J* = 12.9 Hz) – 120.36-120.06 (m) (10C, OPh-C2-C6 and CH_2Ph-C2-C6), 94.92 (d, *J* = 5.5 Hz) – 94.61 (d, *J* = 5.4 Hz) – 93.85-93.72 (t, *J* = 4.6 Hz) (1C, C-1), 72.54 (d, *J* = 11.8 Hz) – 70.61-70.15 (m) – 69.01-68.50 (m) – 65.29 (d, *J* = 10.9 Hz) – 65.02 (d, *J* = 7.2 Hz) (4C, C-2 and C-3 and C-4 and C-5), 66.72-68.48 (1C, m, CH_2Ph), 61.79 (d, *J* = 5.3 Hz) – 61.57 (d, *J* = 4.5 Hz) (1C, C-6), 60.63-60.22 (1C, m, NHCH), 31.88-31.62 (m) – 19.40-19.08 (m) (4C, CH_3CHCH_3 and 4 × $OCOCH_3$), 18.19 (t, *J* = 6.1 Hz) – 16.93 (d, *J* = 15.4 Hz) – 16.53 (d, *J* = 15.4 Hz) (2C, CH_3CHCH_3) ppm

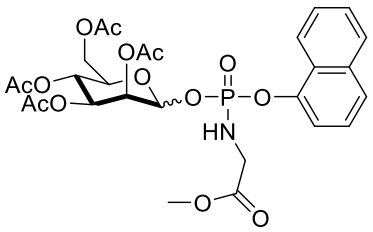
^{31}P NMR (202 MHz, MeOD): δ 2.72 (s, 0.66P), 2.35 (s, 1P), 2.13 (s, 0.15P) ppm

HPLC: Rt = 20.77, 21.19 min (gradient ACN/H_2O 10:90, 100 % ACN in 30 min, flow: 1 mL/min)

MS (ESI+): m/z = 716.20 $[M+Na]^+$

$Rf_{(Hex/EtOAc,\ 1:1)}$ = 0.32, 0.40

4.2.2.6 Methyl 2-{[((2,3,4,6-tetra-*O*-acetyl-D-mannopyranosyl)oxy) (naphtalen-1-yloxy) phosphoryl] amino} acetate (20)



Compound **20** was prepared according to the procedure **B** using 0.60 g (1.72 mmol, 1 eq.) of **15**, 0.81 g (2.58 mmol, 1.5 eq.) of **10**, 2.6 mL (2.58 mmol, 1.5 eq.) of 1M *t*BuMgCl in THF and 8.6 mL of anhydrous THF in a 25 mL round-bottom flask.

$C_{27}H_{32}NO_{14}P$; M.W.: 625.52 g/mol; (0.54 g, 50 %)

^{1}H NMR (500 MHz, MeOD): δ 8.28-7.44 (7H, m, Naph), 5.89-5.75 (1H, m, H-1), 5.59-5.04 (3H, m, H-2 and H-3 and H-4), 4.29-3.70 (m) - 3.31-3.27 (m) (8H, H-5 and 2H-6 and $NHCH_2$ and OCH_3), 2.20-1.91 (12H, m, 4 × $OCOCH_3$) ppm

^{13}C NMR (125 MHz, MeOD): δ 171.69 (s) – 171.48 (m) – 170.94 (s) – 170.74 (s) – 170.42 (s) – 170.02 (d, *J* = 1.8 Hz) – 169.82 (d, *J* = 3.6 Hz) – 146.46-146.20 (m) – 134.95 (d, *J* = 10.1 Hz) (8C, 4 × $OCOCH_3$ and CH_2COOCH_3 and Naph-C1 and Naph–C9 and Naph-C10), 127.51 (t, *J* = 9.1 Hz) – 126.65-126.10 (m) – 125.27-124.75 (m) – 121.35 (d, *J* = 4.1 Hz) – 115.94 (d, *J* = 3.6 Hz) – 115.07 (d, *J* = 3.6 Hz) (7C, Naph-C2-C8), 94.70 (d, *J* = 5.4 Hz) – 93.79 (d, *J* = 4.6 Hz) – 93.68 (d, *J* = 3.8 Hz) (1C, C-1), 72.62 (d, *J* = 12.1 Hz) – 70.47-69.63 (m) – 68.5-68.48 (m) – 65.33 (d, *J* = 6.3 Hz) – 64.65 (s) (4C, C-2 and C-3 and C-4 and C-5), 61.85-61.06 (1C, m, C-6), 51.38 (1C, d, *J* = 7.9 Hz, OCH_3), 42.34 (d, *J* = 9.0 Hz) – 42.23 (d, *J* = 9.7 Hz) (1C, $NHCH_2$), 19.27-18.93 (4C, m, 4 × $OCOCH_3$) ppm

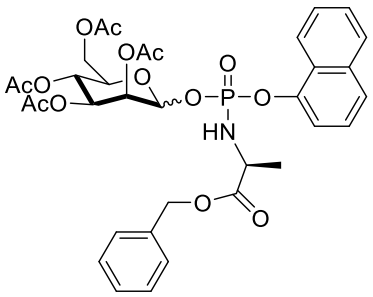
^{31}P NMR (202 MHz, MeOD): δ 3.57 (1P), 3.44 (s, 0.6P) ppm

HPLC: Rt = 18.16, 18.40 min (gradient ACN/H_2O 10:90, 100 % ACN in 30 min, flow: 1 mL/min)

MS (ESI+): m/z = 648.15 $[M+Na]^+$

$Rf_{(Hex/EtOAc,\ 2:3)}$ = 0.17, 0.24

4.2.2.7 (2*S*)-benzyl 2-{[((2,3,4,6-tetra-*O*-acetyl-D-mannopyranosyl) oxy) (naphtalen-1-yloxy) phosphoryl] amino} propanoate (21)



Compound **21** was prepared according to the procedure **B** using 0.60 g (1.72 mmol, 1 eq.) of **15**, 1.04 g (2.58 mmol, 1.5 eq.) of **11**, 2.6 mL (2.58 mmol, 1,5 eq.) of 1M *t*BuMgCl in THF and 8.6 mL of anhydrous THF in a 25 mL round-bottom flask.

$C_{34}H_{38}NO_{14}P$; M.W.: 715.64 g/mol; (0.49 g, 40 %)

^{1}H NMR (500 MHz, MeOD): δ 8.30–7.25 (12H, m, Naph and Ph), 5.87 (dd, *J* = 7.5, 1.8 Hz) – 5.74 (d, *J* = 7.8 Hz) – 5.67 (dd, *J* = 7.9, 1.25 Hz) (1H, H-1), 5.61-5.03 (5H, m, H-2 and H-3 and H-4 and CH_2Ph), 4.27-3.39 (4H, m, H-5 and 2H-6 and CH), 2.30-1.76 (12H, m, 4 × OCOCH_3), 1.53-1.23 (3H, m, CHCH_3) ppm

^{13}C NMR (125 MHz, MeOD): δ 173.38 (d, *J* = 4.5 Hz) - 173.20 (d, *J* = 4.5 Hz) - 170.90 (d, *J* = 2.7 Hz), 170.85 (s) – 170.73 (s) – 170.44 (s) – 170.11-169.71 (m) – 146.40 (d, *J* = 7.2 Hz) – 146.27 (d, *J* = 8.2 Hz) – 135.93-135.76 (m) – 134.94 (s) – 134.90 (s) – 134.86 (s) (9C, COOCH$_2$Ph and 4 × OCOCH$_3$ and Ph-C1 and Naph-C1 and Naph–C9 and Naph-C10), 128.30-128.14 (m) – 128.03-127.83 (m) – 127.64-127.38 (m) – 126.66-126.08 (m) – 125.29-124.69 (m) 121.53-121.26 (m) – 115.8 (d, *J* = 2.9 Hz) – 115.19-115.04 (m) (12C, Naph-C2-C8 and CH$_2$Ph-C2-C6), 94.94 (d, *J* =5.5 Hz) – 94.56 (d, *J* = 5.5 Hz) – 93.78 (d, *J* = 4.5 Hz) – 93.72 (d, *J* = 4.5 Hz) (1C, C-1), 72.56 (d, *J* = 14.7 Hz) – 70.44 (d, *J* = 10.7 Hz) – 70.04 (s) – 69.01-68.47 (m) – 65.37 (d, *J* = 8.2 Hz) – 64.99 (s) – 64.74 (s) (4C, C-2 and C-3 and C-4 and C-5), 66.85-66.48 (1C, m, CH_2Ph), 61.87-61.19 (1C, m, C-6), 50.39 (d, *J* = 9.5 Hz) – 50.27 (1C, CH), 19.33-18.80 (5C, m, CHCH_3 and 4 × OCOCH_3) ppm

^{31}P NMR (202 MHz, MeOD): δ 2.35 (s, 0.2P), 2.26 (s, 0.8P), 2.00 (s, 1P) ppm

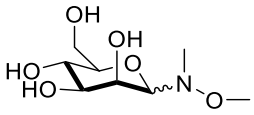
HPLC: Rt = 21.99, 22.36 min (gradient ACN/H_2O 10:90, 100 % ACN in 30 min, flow: 1 mL/min)

MS (ESI+): m/z = 738.17 $[M+Na]^+$

$Rf_{(Hex/EtOAc,\ 1:1)}$ = 0.25, 0.31

4.2.3 Synthesis of D-mannopyranose phosphoramidates

4.2.3.1 *N*,*O*-dimethyl-*N*-(D-mannopyranosyl) hydroxylamine (22)



D-mannose (**13**) (13.00 g, 72.16 mmol, 1 eq.) and *N,O*-dimethylhydroxylamine hydrochloride(9.15 g, 93.81 mmol, 1.3 eq.) were dissolved in water (90 mL) and cooled to 0 °C. To the stirring mixture, a solution of sodium acetate (7.69 g, 93.81 mmol, 1.3 eq.) in water (15 mL) was added slowly. The resulting reaction mixture was stirred for 5 days at room temperature. The water was evaporated under reduced pressure, the obtained white crude was suspended in methanol and the solid was filtered on sintered glass filter and the final crude was purified by column chromatography on silica using DCM/MeOH (4:1) as the eluting system to obtain pure *N*,*O*-dimethyl-*N*-(D-mannopyranosyl)hydroxylamine as white solid.

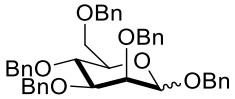
$C_8H_{17}NO_6$, M.W.: 223.23 g/mol; (1.35 g, 8 %)

^{1}H NMR (400 MHz, D_2O): δ 4.08 (1H, dd, *J* = 3.4, 2.1 Hz, H-2), 4.05 (1H, d, *J* = 2.1 Hz, H-1), 3.77 (1H, dd, *J* = 9.0, 3.4 Hz, H-3), 3.74 (1H, dd, *J* = 12.0, 6.0 Hz, H-6 a), 3.70 (1H, m, H-5), 3.62 (1H, dd, *J* = 12.0, 6.0 Hz, H-6 b), 3.50 (1H, dd, *J* = 9.0 Hz, H-4), 3.46 (3H, s, OCH_3), 2.54 (3H, s, NCH_3) ppm

MS (ESI+): m/z = 246.11 $[M+Na]^+$

$Rf_{(DCM/MeOH,\ 4:1)}$= 0.43

4.2.3.2 1,2,3,4,6-penta-*O*-benzyl-D-mannopyranose (23)



Under Argon atmosphere, D-mannose (**13**) (1.00 g, 5.55 mmol, 1 eq.) was dissolved in anhydrous DMF (30 mL) and the solution was cooled to 0 °C. After addition of NaH (60% suspension in mineral oil) (2.22 g, 55.50 mmol, 10 eq.) in anhydrous DMF (30 mL), the resulting mixture was stirred for 30 minutes at room temperature. Benzylbromide (7.9 mL, 66.61 mmol, 12 eq.) was added dropwise and the reaction mixture was stirred at room temperature overnight. The reaction was quenched with methanol (40 mL) and the mixture became transparent. The mixture was diluted with diethyl ether (100 ml) and washed with water (3 × 50 mL). The water layers was then once extracted with diethyl ether (50 mL) and the joined organic layers were dried over sodium sulfate and evaporated under reduced pressure. The obtained yellow oil crude was purified

by column chromatography on silica using Hex/EtOAc (7:1) as the eluting system to give pure 1,2,3,4,6-penta-*O*-benzyl-D-mannopyranose as colourless oil.

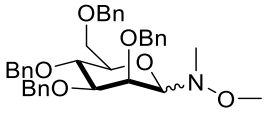
$C_{41}H_{42}O_6$, M.W.: 630.78 g/mol; (1.29 g, 37 %)

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.56-7.18 (25H, m, 5 × OCH_2Ph), 5.12-4.48 (11H, m, H-1 and 5 × OCH_2Ph), 4.11-3.78 (m) – 3.59-3.48 (m) (6H, H-2 and H-3 and H-4 and H-5 and 2H-6) ppm

MS (ESI+): m/z = 553.30 $[M+Na]^+$

$Rf_{(Hex/EtOAc,\ 7:1)}$= 0.29

4.2.3.3 *N,O*-dimethyl-*N*-(2,3,4,6-tetra-O-benzyl-D-mannosyl) hydroxylamine (25)



Under Argon atmosphere, **22** (1.26 g, 5.64 mmol, 1 eq.) was dissolved in anhydrous DMF (30 mL) and the solution was cooled to 0 °C. After addition of NaH (60% suspension in mineral oil) (1.81 g, 45.16 mmol, 8 eq.) in anhydrous DMF (30 mL), the resulting mixture was stirred for 30 minutes at room temperature. Benzylbromide (8.1 mL, 67.73 mmol, 12 eq.) was added dropwise and the reaction mixture was stirred at room temperature overnight. The reaction was quenched with methanol (20 mL) and the mixture became transparent. The mixture was diluted with diethyl ether (100 mL) and washed with water (3 × 50 mL). The water layers were then once extracted with diethyl ether (50 mL) and joined organic layers were dried over sodium sulfate and evaporated under reduced pressure. The obtained yellow oil crude was purified by column chromatography on silica using Hex/EtOAc (6:1) as the eluting system to give pure *N,O*-dimethyl-*N*-(2,3,4,6-tetra-*O*-benzyl-D-mannopyranosyl) hydroxylamine as colourless oil.

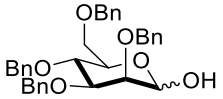
$C_{36}H_{41}NO_6$, M.W.: 583.73 g/mol; (1.97 g, 60 %)

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.55-7.22 (20H, m, 4 × OCH_2Ph), 5.01-5.49 (8H, m, 4 × OCH_2Ph), 4.32-3.93 (m) – 3.78-3.72 (m) (7H, H-1 and H-2 and H-3 and H-4 and H-5 and 2H-6), 3.48 (s) – 3.32 (s) (3H, OCH_3), 2.72 (s) – 2.57 (s) (3H, NHCH_3) ppm

MS (ESI+): m/z = 606.29 $[M+Na]^+$

$Rf_{(Hex/EtOAc,\ 6:1)}$= 0.25

4.2.3.4 2,3,4,6-tetra-*O*-benzyl-D-mannopyranose (26)



Under Argon atmosphere, to a solution of **25** (0.20 g, 0.34 mmol, 1 eq.) in THF : H_2O (10 mL, 9:1 v/v), *N*-chlorosuccinimide (0.92 g, 0.68 mmol, 2 eq.) was added and the resulting mixture was heated to 60 °C. The reaction was monitored by TLC (Hex/EtOAc, 4:1) and after 7h, when no further changes were observed on TLC plate, the solvent was evaporated under reduced pressure. The yellow crude oil was purified by column chromatography on silica using Hex/EtOAc (4:1) as the eluting system yielding pure 2,3,4,6-tetra-*O*-benzyl-D-mannopyranose as colourless oil.

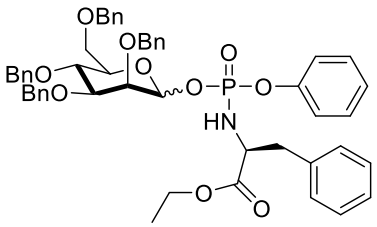
$C_{34}H_{36}O_6$, M.W.: 540.66 g/mol; (0.11 g, 60 %)

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.69-7.02 (20H, m, 4 × Ph), 5.55 (d, J = 4.0 Hz) – 5.29 (s) (1H, H-1), 5.15-4.39 (8H, m, 4× CH_2Ph), 4.39-3.79 (6H, m, H-2 and H-3 and H-4 and H-5 and 2H-6) ppm

MS (ESI+): m/z = 563.23 $[M+Na]^+$

$Rf_{(Hex/EtOAc,\ 4:1)}$= 0.12

4.2.3.5 (2*S*)-ethyl 2-{[((2,3,4,6-tetra-*O*-benzyl-D-mannopyranosyl) oxy) (phenoxy) phosphoryl] amino} 3-phenylpropanoate (27)



Compound **26** (0.35 g, 0.65 mmol, 1 eq.) was dissolved in anhydrous THF (3.5 mL) under Argon atmosphere, the solution was cooled to 0 °C and 1M *t*BuMgCl in THF (1.0 mL, 0.97 mmol, 1.5 eq.) was added dropwise. After 30 minutes of stirring, a solution of **8** (0.36 g, 0.97 mmol, 1.5 eq.) in anhydrous THF (1.0 mL) was added dropwise. The cooling bath was allowed to reach room temperature and the mixture was stirred overnight. The solvent was evaporated under reduced pressure and the crude oil was purified by column chromatography on silica using Hex/EtOAc (3:1) as the eluting system to obtain pure (2*S*)-ethyl 2-{[((2,3,4,6-tetra-*O*-benzyl-D-mannopyranosyl)oxy) (phenoxy) phosphoryl] amino} 3-phenylpropanoate as colourless oil.

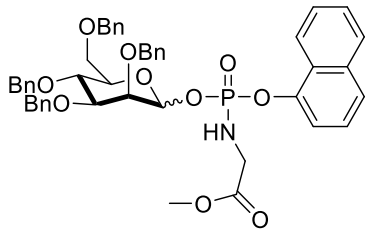
$C_{51}H_{54}NO_{10}P$, M.W.: 871.96 g/mol; (0.24 g, 42 %)

^{1}H NMR (500 MHz, MeOD): δ 7.31-6.89 (30H, m, OPh and $CHCH_2$Ph and 4 × OCH_2Ph), 5.68 (dd, *J* = 7.0, 1.9 Hz) – 5.60 (dd, *J* = 6.5,1.9 Hz) – 5.11 (d, *J* = 7.3 Hz) (1H, H-1), 4.70-4.28 (8H, m, 4 × OCH_2Ph), 4.24-3.35 (m) – 3.04-2.70 (m) (11H, H-2 and H-3 and H-4 and H-5 and 2H-6 and $CHCH_2$Ph and CH_2CH_3), 1.19-0.96 (3H, m, CH_2CH_3) ppm

^{31}P NMR (202 MHz, MeOD): δ 2.03 (s, 0.3P), 1.69 (s, 1P), 1.30 (s, 0.7P) ppm

$Rf_{(Hex/EtOAc,\ 3:1)}$ = 0.11, 0.18

4.2.3.6 Methyl 2-{[((2,3,4,6-tetra-*O*-benzyl-D-mannopyranosyl)oxy) (naphtalen-1-yloxy) phosphoryl] amino} acetate (28)



Compound **26** (74 mg, 0.14 mmol, 1 eq.) was dissolved in anhydrous THF (1 mL) under Argon atmosphere, the solution was cooled to 0 °C and 1M *t*BuMgCl in THF (0.2 mL, 0.21 mmol, 1.5 eq.) was added dropwise. After 30 minutes of stirring, a solution of **10** (64 mg, 0.21 mmol, 1.5 eq.) in anhydrous THF (0.5 mL) was added dropwise. The cooling bath was allowed to reach room temperature and the mixture was stirred overnight. The solvent was evaporated under reduced pressure and the crude oil was purified by column chromatography on silica using Hex/EtOAc (2:1) as the eluting system to obtain pure Methyl 2-{[((2,3,4,6-tetra-*O*-benzyl-D-mannopyranosyl)oxy) (naphtalen-1-yloxy) phosphoryl] amino} as colourless oil.

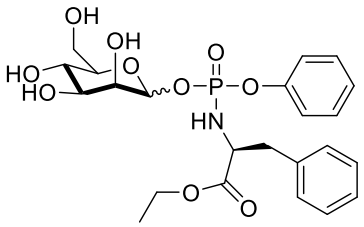
$C_{47}H_{48}NO_{10}P$, M.W.: 817.87 g/mol; (60 mg, 54 %)

^{1}H NMR (500 MHz, MeOD): δ 8.13-7.04 (27H, m, Naph and 4 × CH_2Ph), 5.79 (dd, *J* = 6.8, 2.0 Hz) – 5.42 (dd, *J* = 7.9, 0.9 Hz) (1H, H-1), 4.69-4.23 (8H, m, 4 × CH_2Ph), 4.02-3.24 (11H, m, H-2 and H-3 and H-4 and H-5 and 2H-6 and NHCH_2 and OCH_3) ppm

^{31}P NMR (202 MHz, MeOD): δ 3.46 (s, 0.8P), 3.35 (s, 1P) ppm

$Rf_{(Hex/EtOAc,\ 2:1)}$= 0.12, 0.21

4.2.3.7 (2*S*)-ethyl 2-{[((D-mannopyranosyl)oxy) (phenoxy) phosphoryl] amino} 3-phenylpropanoate (29)

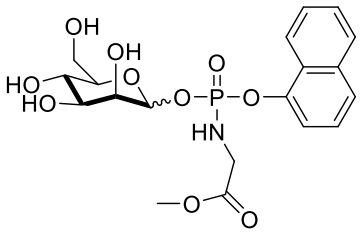


Compound **27** (94 mg, 0.11 mmol, 1 eq.) was dissolved in anhydrous methanol (15 mL), 10% Pd/C (11 mg, 10 mol%) was added and the mixture was shaken under an atmosphere of hydrogen (45 psi) at room temperature. After 3 days, when the MS confirmed completion of the reaction, the catalyst was filtered off through polypropylene filter and the solvent was evaporated under reduced pressure. Unfortunately, the compound could not be characterised by NMR or HPLC, because degradation was observed immediately after solvent evaporation.

$C_{23}H_{30}NO_{10}P$, M.W.: 511.46 g/mol; (47 mg, 85 %)

MS (ESI+): m/z = 534.16 $[M+Na]^+$

4.2.3.8 Methyl 2-{[((D-mannopyranosyl)oxy) (naphtalen-1-yloxy) phosphoryl] amino} acetate (30)



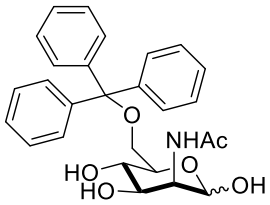
Compound **28** (42 mg, 0.05 mmol, 1 eq.) was dissolved in anhydrous methanol (10 mL), 10% Pd/C (5 mg, 10 mol%) was added and the mixture was shaken under an atmosphere of hydrogen (45 psi) at room temperature. After 4 days, the MS confirmed completion of the reaction. Due to the small remaining amount of the reaction mixture the product was not isolated.

$C_{19}H_{24}NO_{10}P$, M.W.: 457.37 g/mol

MS (ESI+): m/z = 480.34 $[M+Na]^+$

4.2.4 Synthesis of triacetylated *N*-acetyl-D-mannosamine phosphoramidates

4.2.4.1 2-acetamido-2-deoxy-6-*O*-triphenylmethyl-D-mannopyranose (32)

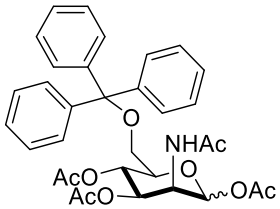


Triphenylmethyl chloride (16.38 g, 58.76 mmol, 1.3 eq.) was added to *N*-acetyl-D-mannosamine (**31**) (10.00 g, 45.21 mmol, 1 eq.) dissolved in anhydrous pyridine (150 mL) in a 500 mL round-bottom flask under Argon atmosphere while stirring. After 48 hours, the reaction mixture was heated to 60 °C and was left stirring for further 4 hours while monitoring by TLC (EtOAc). The reaction mixture was concentrated under reduced pressure and the rest of the pyridine was co-evaporated with dry toluene. The obtained solid was dissolved in ethyl acetate, washed with water and the organic layer was dried over sodium sulfate. Ethyl acetate was evaporated under reduced pressure to gain a crude product which was purified by column chromatography on silica using EtOAc/Hex (3:1) as the eluting system yielding pure 2-acetamido-2-deoxy -6-*O*-triphenylmethyl-D-mannopyranose.

$C_{27}H_{29}NO_6$; M.W.: 463.53 g/mol; (1.25 g, 6 %)

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.47-7.42 (m) – 7.35-7.24 (m) (15H, 3 × Ph), 6.13 (s) – 5.88 (s) (1H, H-1), 5.83 (d, *J* = 9.2 Hz) – 5.77 (d, *J* = 9.2 Hz) (1H, NH), 5.39-5.27 (m, H-3 and H-4), 5.01 (dd, *J* = 10.1, 3.9 Hz, H-3), 4.79-4.75 (m) – 4.68-4.53 (m) (1H, H-2), 3.96-3.90 (m) – 3.71-3.65 (m) (1H, H-5), 3.42-3.34 (m) – 3.13 (dd, *J* = 10.7, 4.1 Hz) – 3.08 (dd, *J* = 10.7, 3.7 Hz) (2H, 2H-6), 2.02 (3H, d, *J* = 5.2 Hz, Ac) ppm

4.2.4.2 2-acetamido-1,3,4-tri-*O*-acetyl-2-deoxy-6-*O*-triphenylmethyl-D-mannopyranose (33)



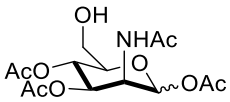
Compound **32** (19.93 g, 43.00 mmol, 1eq.) was dissolved in anhydrous pyridine (30 mL), the solution was cooled to 0 °C by an ice-water bath and acetic anhydride (20.5 mL, 214.06 mmol, 5 eq.) was added dropwise at that temperature while stirring.

The reaction mixture was allowed to reach room temperature and was monitored by TLC (EtOAc/Hex, 3:1). After 24 hours, the mixture was concentrated under reduced pressure and the residual pyridine was co-evaporated with dry toluene. The dry residue was suspended and extracted in a mixture of dichloromethane and water. The organic phase was dried over sodium sulfate and the solvent was evaporated under reduced pressure. The crude was purified by column chromatography on silica using EtOAc/Hex (3:1) as the eluting system to obtain pure 2-acetamido-1,3,4-tri-*O*-acetyl-2-deoxy-6-*O*-triphenylmethyl-D-mannopyranose as an oi.

$C_{33}H_{35}NO_9$; M.W.: 589.64 g/mol; (17.74 g, 70 %)

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.26-7.19 (15H, m, 3 × Ph), 6.00 (d, *J* = 9.6 Hz, NH), 5.97 (d, *J* = 2.0 Hz, H-1), 5.95 (d, *J* = 9.2 Hz, NH), 5.79 (d, *J* = 8.0 Hz, H-1), 5.33 (dd, *J* = 10.2, 4.4 Hz, H-3), 5.10 (t, *J* = 10.0 Hz, H-4), 5.04 (dd, *J* = 10.0, 4.4 Hz, H-3), 4.78 (m) – 4.58 (m) (1H, H-2), 3.75-3.65 (1H, m, H-5), 3.51 (2H, dd, *J* = 10.08, 2.4 Hz, 2H-6), 2.04-1.95 (12H, m, 4 × Ac) ppm

4.2.4.3 2-acetamido-1,3,4-tri-*O*-acetyl-2-deoxy-D-mannopyranose (34)



A stirred mixture of **33** (2.00 gr, 3.39 mmol, 1 eq.) in 80% aqueous acetic acid (45 mL) in a 150 mL round-bottom flask was heated at 60 °C and monitored by TLC (Hex/EtOAc, 3:1). After approximately 4 hours, the reaction mixture was concentrated under reduced pressure and the residue was co-evaporated with dry toluene. The obtained crude was purified twice by column chromatography on silica using 1. Hex/EtOAc (3:1) and 2. EtOAc as the eluting systems to obtain pure 2-acetamido-1,3,4-tri-*O*-acetyl-2-deoxy-D-mannopyranose.

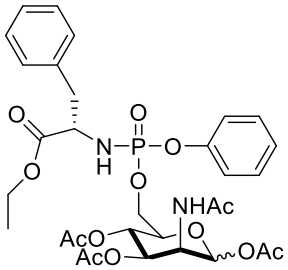
$C_{14}H_{21}NO_9$; M.W.: 347.32 g/mol; (1.15 g, 98 %)

^{1}H NMR (500 MHz, $CDCl_3$): δ 6.11 (d, *J* = 9.2 Hz) – 6.01 (d, *J* = 9.2 Hz) (1H, NH), 5.97 (d, *J* = 1.7 Hz) – 5.79 (d, *J* = 1.7 Hz) (1H, H-1), 5.32 (dd, *J* = 10.4, 4.5 Hz, H-3), 5.12 (t, *J* = 10.2, H-4), 5.05-5.03 (m, H-3 and H-4), 4.73-4.69 (m) – 4.59 (ddd, *J* = 9.3, 4.5, 1.7 Hz) (1H, H-2), 3.76-3.70 (1H, m, H-5), 3.66 (1H, dd, *J* = 12.9, 2.1 Hz, 2H-6 a), 3.52 (1H, dd, *J* = 12.9 Hz, 3.2 Hz, 2H-6 b), 2.10-1.93 (12H, m, 4 × Ac) ppm

4.2.4.4 General procedure for triacetylated *N*-acetyl-D-mannosamine phosphoramidates synthesis using Grignard method (C)

Compound **34** (1 eq.) was placed in a round-bottom flask, dissolved in anhydrous THF under Argon atmosphere and cooled to 0 °C. To the cold solution, 1M *t*BuMgCl in THF (1.5 eq.) was added. After 30 minutes, a solution of desired phosphorochloridate (1.5 eq.) in anhydrous THF was added dropwise while stirring. The cooling bath was allowed to reach room temperature and the reaction mixture was stirred overnight. After confirmation of the presence of the phosphoramidate in the mixture by MS, the solvent was evaporated under reduced pressure to obtain foamy residue. The crude was purified by column chromatography on silica using DCM/MeOH (98:2) as the eluting system yielding pure phosphoramidate as a mixture of 2 diastereoisomers and 2 anomers.

4.2.4.5 (2*S*)-ethyl 2-{[(3*S*,4*R*,5*S*,6*R*)-3-acetamido-2,4,5-triacetoxytetrahydro-2*H*-pyran-6-ylmethoxy) (phenoxy) phosphoryl] amino} 3-phenylpropanoate (35)



Compound **35** was prepared following the Grignard method **C** using 0.60 g (1.73 mmol, 1 eq.) of **34**, 0.95 g (2.59 mmol, 1.5 eq.) of **8**, 2.6 mL (2.59 mmol, 1.5 eq.) of 1M *t*BuMgCl in THF and 8.6 mL of anhydrous THF in a 25 mL round-bottom flask.

$C_{31}H_{39}N_2O_{13}P$; M.W.: 678.63 g/mol; (0.53 g, 45%)

^{1}H NMR (500 MHz, MeOD): δ 7.36-7.06 (10H, m, OPh and CH_2Ph), 5.99 (d, *J* = 1.6 Hz) – 5.96 (d, *J* = 1.8 Hz) – 5.94 (d, *J* = 1.6 Hz) – 5.92 (d, *J* = 1.8 Hz) (1H, H-1), 5.38-5.16 (2H, m, H-3 and H-4), 4.76 (dd, *J* = 4.1, 1.7 Hz) – 4.59 (dd, *J* = 4.1, 1.9 Hz) (1H, H-2), 4.19-4.03 (m) – 3.96-3.84 (m) (6H, H-5 and 2H-6 and OCH_2 and NHCH), 3.06 (ddd, *J* = 13.5, 6.9, 2.1 Hz) – 2.95 (dd, *J* = 13.5, 7.7 Hz) (2H, CH_2Ph), 2.20-1.97 (12H, m, 4 × Ac), 1.20 (3H, td, *J* = 7.2, 2.9 Hz, CH_2CH_3) ppm

^{13}C NMR (125 MHz, MeOD): δ 172.97 (s) -172.39 (m) – 170.32-169.92 (m) – 168.73-168.59 (m) – 150.67 (d, *J* = 6.2 Hz) – 136.61 (d, *J* = 7.3 Hz) (7C, OPh-C1 and CH_2Ph-C1 and CHCOOCH_2CH_3 and NHCHCOOCH_3 and 3 × OCOCH_3), 129.42-129.13 (m) – 128.13 (s) – 126.65-126.51 (m) – 124.82-124.56 (m) – 130.13-119.93 (m) (10C, OPh-C2-C6 and CH_2Ph-C2-C6), 91.76 (s) – 90.75 (d, *J* = 13.0 Hz) (1C, C-1), 73.70-73.57 (m) – 71.39-70.70.99 (m) – 69.30 (d, *J* = 10.1 Hz) – 65.37 (d, *J* = 10.1 Hz) – 65.28 (d, *J* = 5.5 Hz) – 65.08 (d, *J* = 5.5 Hz) – 61.00 (d, *J* = 10.0 Hz) – 56.42 (s) – 56.33 (s) – 56.21 (s) (6C, C-3 and C-4 and C-5 and C-6 and NHCH and OCH_2CH_3), 49.37 (s) – 49.30 (s) – 49.17 (d, *J* = 2.8 Hz) (1C, C-2), 39.71-39.55 (1C, m, CH_2Ph), 21.00 (d, *J* = 4.6 Hz) – 19.36-19.22 (m) (4C, NHCOCH_3 and 3 × OCOCH_3), 12.99 (1C, d, *J* = 3.7, OCH_2CH_3) ppm

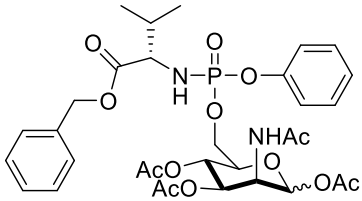
^{31}P NMR (202 MHz, MeOD): δ 3.39 (d, *J* = 3.4 Hz, 1P), 3.21 (s, 0.3P), 3.03 (s, 0.1P) ppm

HPLC: Rt = 23.11, 23.44, 23.79, 24.23 min (gradient ACN/H_2O 2:98, 100 % ACN in 40 min, flow: 1 mL/min)

MS (ESI+): m/z = 701.21 $[M+Na]^+$

$Rf_{(DCM/MeOH,\ 98:2)}$ = 0.35

4.2.4.6 (2*S*)-benzyl 2-{[((3*S*,4*R*,5*S*,6*R*)-3-acetamido-2,4,5-triacetoxytetrahydro-2*H*-pyran-6-ylmethoxy) (phenoxy) phosphoryl] amino} 3-methylbutanoate (36)



Compound **36** was prepared following the Grignard method **C** using 0.50 g (1.44 mmol, 1 eq.) of **34**, 1.10 g (2.88 mmol, 2 eq.) of **9**, 2.9 mL (2.88 mmol, 2 eq.) of 1M *t*BuMgCl in THF and 8.0 mL of anhydrous THF in a 25 mL round-bottom flask.

$C_{32}H_{41}N_2O_{13}P$; M.W.: 692.65 g/mol; (0.39 g, 40%)

^{1}H NMR (500 MHz, MeOD): δ 7.42-7.31 (m) – 7.25-7.17 (m) (10H, OPh and CH_2Ph), 6.00 (d, *J* = 1.6 Hz) – 5.97 (d, *J* = 1.7 Hz) – 5.94 (d, *J* = 1.7 Hz) – 5.92 (d, *J* = 1.9 Hz) (1H, H-1), 5.62-5.49 (m) – 5.39-5.12 (m) (4H, H-3 and H-4 and CH_2Ph), 4.81-4.76 (m) – 4.64-4.60 (m) (1H, H-2), 4.25-4.13 (m) – 4.02-3.90 (m) – 3.79-3.68 (m) (4H, H-5 and 2H-6 and NHCH), 2.19-1.96 (13H, m, CH_3CHCH_3 and 4 × Ac), 0.95-0.81 (6H, m, CH_3CHCH_3) ppm

^{13}C NMR (125 MHz, MeOD): δ 172.93 (s) – 172.79 (m) – 172.70 (d, *J* = 2.7 Hz) – 172.40 (d, *J* = 4.6 Hz) – 170.33 (s) – 169.99 (m) – 168.68 (d, *J* = 7.2 Hz) – 168.61 (s) – 150.87-150.72 (m) – 135.80 (d, *J* = 5.0 Hz) (7C, OPh-C1 and CH_2Ph-C1 and CHCOOCH$_2$Ph and NHCOCH$_3$ and 3 × OCOCH$_3$), 129.37 (t, *J* = 5.6 Hz), 128.30-128.01 (m) – 124.79-124.68 (m) – 120.27-119.97 (m) (10C, OPh-C2-C6 and CH_2Ph-C2-C6), 91.75 (s) – 90.82 (s) – 90.69 (s) (1C, C-1), 73.77 (d, *J* = 6.8 Hz) – 73.65 (d, *J* = 7.4 Hz) – 71.42-71.13 (m) – 69.33-69.23 (m) – 65.68-65.56 (m) – 60.66 (d, *J* = 7.6 Hz) – 60.49 (d, *J* = 5.3 Hz) (4C, C-3 and C-4 and C-5 and NHCH), 66.63 (d, *J* = 6.2 Hz) – 66.51 (s) (1C, CH_2Ph), 65.68-65.60 (1C, m, C-6), 49.45-49.10 (1C, m, C-2), 21.14 (d, *J* = 6.4 Hz) – 19.46-19.32 (m) (5C, CH_3CHCH$_3$ and NHCOCH_3 and 3 × OCOCH_3), 18.17 (s) – 17.10 (d, *J* = 10.5 Hz) (2C, CH_3CHCH_3) ppm

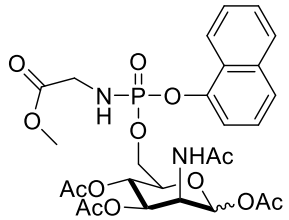
^{31}P NMR (202 MHz, MeOD): δ 4.30 (d, *J* = 5.9 Hz, 1P), 4.14 (s, 0.2P), 3.96 (s, 0.1P) ppm

HPLC: Rt = 25.23, 25.49, 25.90 min (gradient ACN/H_2O 2:98, 100 % ACN in 40 min, flow: 1 mL/min)

MS (ESI+): m/z = 715.24 $[M+Na]^+$

$Rf_{(DCM/MeOH,\ 98:2)}$ = 0.23

4.2.4.7 Methyl 2-{[((3*S*,4*R*,5*S*,6*R*)-3-acetamido-2,4,5-triacetoxytetrahydro-2*H*-pyran-6-ylmethoxy) (naphtalen-1-yloxy) phosphoryl] amino} acetate (37)



Compound **37** was prepared following the Grignard method **C** using 0.60 g (1.73 mmol, 1 eq.) of **34**, 0.81 g (2.59 mmol, 1.5 eq.) of **10**, 2.6 mL (2.59 mmol, 1.5 eq.) of 1M *t*BuMgCl in THF and 8.6 mL of anhydrous THF in a 25 mL round-bottom flask.

$C_{27}H_{33}N_2O_{13}P$; M.W.: 624.54 g/mol; (0.53 g, 49 %)

^{1}H NMR (500 MHz, MeOD): δ 8.23-8.18 (1H, m, Naph), 7.90 (1H, d, *J* = 8.0 Hz, Naph), 7.34 (1H, d, *J* = 8.0 Hz, Naph), 7.62-7.44 (4H, m, Naph), 6.02 (d, *J* = 1.8 Hz) – 6.00 (d, *J* = 1.8 Hz) – 5.98 (q, *J* = 1.9 Hz) (1H, H-1), 5.43-5.18 (2H, m, H-3 and H-4), 4.78 (dd, *J* = 9.5, 4.3, 1.8 Hz) – 4.61 (dd, *J* = 9.7, 4.5, 1.8 Hz) (1H, H-2), 4.48-4.26 (2H, m, 2H-6), 4.27-4.16 (m) - 4.09-4.04 (m) - 4.00-3.95 (m) (1H, H-5), 3.86-3.79 (2H, m, CH_2), 3.74-3.70 (3H, m, OCH_3), 2.21-1.95 (12H, m, 4 × Ac) ppm

^{13}C NMR (125 MHz, MeOD): δ 172.46 (d, *J* = 5.8 Hz) – 172.01-171.67 (m) – 170.24 (s) – 170.15-170.06 (m) – 168.78 (s) - 168.63 (d, *J* = 5.4 Hz) – 146.51 (s) - 134.90 (s) (8C, Naph-C1 and Naph-C9 and Naph-C10 and CH_2COOCH_3 and $NHCOCH_3$ and 3 × $OCOCH_3$), 127.48-127.39 (m) – 126.37 (s) – 126.09 (d, *J* = 4.2 Hz) – 125.23 (s) – 124.60 (s) – 121.51-121.27 (m) – 115.08 (d, *J* = 3.6 Hz) – 114.96 (d, *J* = 3.3 Hz) (7C, Naph-C2-C8), 91.76 (d, *J* = 8.1 Hz) - 90.71 (s) (1C, C-1), 71.35-71.07 (2C, m, C-3 and C-5), 69.21 (s), 65.28 (d, *J* = 3.5 Hz) (2C, C-3 and C-4), 66.67-65.43 (1C, m, C-6), 51.27 (1C, s, OCH_3), 49.38 (s) - 49.19 (d, *J* = 2.6 Hz) (1C, C-2), 42.42-42.21 (1C, m, CH_2), 21.05-20.96 (m) – 19.32-19.21 (m) (4C, $NHCOCH_3$ and 3 × $OCOCH_3$) ppm

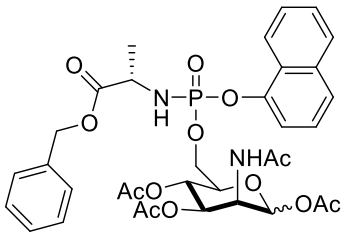
^{31}P NMR (202 MHz, MeOD): δ 5.57 (s, 0.8P), 5.32 (s, 0.4P), 4.75 (s, 1P), 4.60 (s, 0.2P) ppm

HPLC: Rt = 20.19, 20.40, 20.77 min (gradient ACN/H_2O 2:98, 100 % ACN in 40 min, flow: 1 mL/min)

MS (ESI+): m/z = 647.17 $[M+Na]^+$

$Rf_{(DCM/MeOH,\ 98:2)}$ = 0.23

4.2.4.8 (2*S*)-benzyl 2-{[(3*S*,4*R*,5*S*,6*R*)-3-acetamido-2,4,5-triacetoxytetrahydro-2*H*-pyran-6-ylmethoxy) (naphtalen-1-yloxy) phosphoryl] amino} propanoate (38)



Compound **38** was prepared following the Grignard method **C** using 0.60 g (1.73 mmol, 1 eq.) of **34**, 1.05 g (2.59 mmol, 1.5 eq.) of **11**, 2.6 mL (2.59 mmol, 1.5 eq.) of 1M *t*BuMgCl in THF and 8.6 mL of anhydrous THF in a 25 mL round-bottom flask.

$C_{34}H_{39}N_2O_{13}P$; M.W.: 714.66 g/mol; (0.58 g, 47%)

^{1}H NMR (500 MHz, MeOD): δ 8.24-7.24 (12H, m, Naph and Ph), 6.03 (d, *J* = 1.6 Hz) – 6.00 (d, *J* = 1.6 Hz) – 5.98 (d, *J* = 1.6 Hz) – 5.96 (d, *J* = 1.6 Hz) (1H, H-1), 5.50-5.17 (2H, m, H-3 and H-4), 5.15-5.04 (2H, m, CH_2Ph), 4.78 (td, *J* = 4.4, 1.7 Hz) – 4.73 (dd, *J* = 5.0, 1.7 Hz) – 4.61 (dd, *J* = 4.5, 1.5 Hz) (1H, H-2), 4.44-4.03 (m) – 3.96-3.90 (m) (4H, H-5 and 2H-6 and NHCH), 1.17-1.95 (12H, m, 4 × Ac), 1.37 (3H, dd, *J* = 18.7, 7.4 Hz, $CHCH_3$) ppm

^{13}C NMR (125 MHz, MeOD): δ 175.07 (s) – 173.64-173.45 (m) – 172.96 (s) – 172.47 (d, *J* = 7.5 Hz) – 170.25 (d, *J* = 1.7 Hz) – 170.13 (s) – 170.07 (s) – 170.00 (s) – 168.64 (d, *J* = 3.6 Hz) – 146.62-146.47 (m) – 135.81 (d, *J* = 1.8 Hz) – 134.89 (s) (9C, ONaph-C1 and ONaph-C9-C10 and CH_2Ph-C1 and CHCOOCH_2Ph and NHCOCH_3 and 3 × OCOCH_3), 128.21-128.14 (m) – 127.89 (d, *J* = 3.8 Hz) – 127.80 (d, *J* = 5.4 Hz) – 127.43 (d, *J* = 1.8 Hz) – 126.36 (d, *J* = 2.7 Hz) – 126.11 (d, *J* = 6.3 Hz) – 125.21 (s) – 124.55 (d, *J* = 4.9 Hz) – 121.54-121.36 (m) – 115.04 (d, *J* = 3.2 Hz) – 114.91 (d, *J* = 3.2 Hz) (12C, ONaph-C2-C8 and CH_2Ph-C2-C6), 91.76 (s) – 90.75 (d, *J* = 10.0 Hz) (1C, C-1), 71.33 (d, *J* = 6.3 Hz) – 71.24-71.11 (m) – 69.27 (s) – 65.42 (s) – 65.27 (s) – 65.17 (s) – 50.55-50.31 (m) (4C, C-3 and C-4 and C-5, NHCH), 66.65-66.38 (1C, m, CH_2Ph), 65.68 (1C, dd, *J* = 9.0, 5.4 Hz, C-6), 49.38 (s) – 49.25 (s) – 49.14 (s) (1C, C-2), 21.06-20.92 (m) – 19.35-19.18 (m) (4C, NHCOCH_3 and 3 × OCOCH_3), 18.90 (1C, dd, *J* = 7.2, 1.8 Hz CHCH_3) ppm

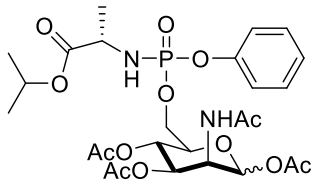
^{31}P NMR (202 MHz, MeOD): δ 4.12 (d, *J* = 4.5 Hz, 1P), 3.78 (s, 0.8P), 3.52 (s, 0.1 P) ppm

HPLC: Rt = 25.20, 25.45, 25.93, 26.25 min (gradient ACN/H_2O 2:98, 100 % ACN in 40 min, flow: 1 mL/min)

MS (ESI+): m/z = 737.23 $[M+Na]^+$

$Rf_{(DCM/MeOH,\ 98:2)}$ = 0.30

4.2.4.9 (2*S*)-isopropyl 2-{[((3*S*,4*R*,5*S*,6*R*)-3-acetamido-2,4,5-triacetoxytetrahydro-2*H*-pyran-6-ylmethoxy) (phenoxy) phosphoryl] amino} propanoate (39)



Compound **39** was prepared using the Grignard method **C** using 0.52 g (1.50 mmol, 1 eq.) of **34**, 0.92 g (2.99 mmol, 2 eq.) of **12**, 3.0 mL (2.99 mmol, 2 eq.) of 1M *t*BuMgCl chloride in THF and 8.0 mL of anhydrous THF in a 25 mL round-bottom flask.

$C_{26}H_{37}N_2O_{13}P$; M.W.: 616.56 g/mol; (0.63g, 68%)

^{1}H NMR (500 MHz, MeOD): δ 7.41-7.35 (m) – 7.29-7.19 (m) (5H, OPh), 6.00 (d, *J* = 1.7 Hz) – 5.99-5.97 (m) – 5.96 (d, *J* = 1.9 Hz) (1H, H-1), 5.40-5.25 (m) – 5.23-5.17 (m) (2H, H-3 and H-4), 5.05-4.94 (1H, m, CH_3CHCH_3), 4.78-4.75 (m) – 4.60 (dd, *J* = 4.4, 1.7 Hz) (1H, H-2), 4.39-4.29 (m) – 4.28-4.12 (m) – 4.12-4.03 (m) (3H, H-5 and 2H-6), 3.98-3.86 (1H, m, NHCH), 2.21-1.98 (12H, m, 4 × Ac), 1.40-1.33 (m) – 1.29-1.18 (m) (9H, NHCHCH_3 and CH_3CHCH_3) ppm

^{13}C NMR (125 MHz, MeOD): δ 173.23 (d, *J* = 4.7 Hz) – 172.94 (d, *J* = 4.2 Hz) – 172.44 (d, *J* = 4.6 Hz) – 170.25 (d, *J* = 4.5 Hz) – 170.14 (d, *J* = 2.7 Hz) – 170.04 (d, *J* = 5.4 Hz) – 168.62 (d, *J* = 2.7 Hz) – 150.82 (d, *J* = 7.2 Hz) (6C, OPh-C1 and NHCHCOO and $NHCOCH_3$ and 3 × $OCOCH_3$), 129.42-129.31 (m) – 124.74 (d, *J* = 4.2 Hz) – 120.23-120.00 (m) (5C, OPh-C2-C6), 91.75 (d, *J* = 1.8 Hz) – 90.70 (d, *J* = 4.4 Hz) (1C, C-1), 73.81 (m) – 71.42-71.07 (m) – 69.27 (d, *J* = 10.0 Hz), 68.79 (d, *J* = 2.7 Hz) – 68.74 (s) – 65.58-65.47 (m) – 65.39 (d, *J* = 1.4 Hz) – 65.31 (s) - 50.36 (d, *J* = 3.1 Hz) (6C, C-3 and C-4 and C-5 and C-6 and NHCH and CH_3CHCH_3), 49.37 (d, *J* = 8.1 Hz) – 4.17 (d, *J* = 7.7 Hz) (1C, C-2), 21.02 (d, *J* = 4.6 Hz) – 19.29 (d, *J* = 8.2 Hz) (4C, $NHCOCH_3$ and 3 × $OCOCH_3$), 20.64-20.49 (m) – 18.99 (dd, *J* = 7.2, 3.6 Hz) (3C, CH_3CHCH_3 and $NHCHCH_3$) ppm

^{31}P NMR (202 MHz, MeOD): δ 3.83 (s, 0.4P), 3.76 (s, 1P), 3.55 (s, 0.5P), 3.35 (s, 0.1P) ppm

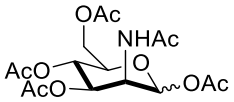
HPLC: Rt = 21.29, 21.70 min (gradient ACN/H_2O 2:98, 100 % ACN in 40 min, flow: 1 mL/min)

MS (ESI+): m/z = 639.20 $[M+Na]^+$

$Rf_{(DCM/MeOH,\ 98:2)}$ = 0.20

4.2.5 Preparation of starting material for *N*-acetyl-D-mannosamine phosphoramidates synthesis

4.2.5.1 2-acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-D-mannopyranose (40)



To a solution of *N*-acetyl-D-mannosamine (**31**) (10.00 g, 45.21 mmol, 1 eq.) and 4-(dimethylamino)pyridine (0.55 g, 4.52 mmol, 0.1 eq.) in anhydrous pyridine (200 mL) in a 500 mL round-bottom flask under Argon atmosphere, acetic anhydride (42.1 ml, 452.06 mmol, 10 eq.) was added slowly and the reaction mixture was stirred at room temperature overnight. After 16 hours, TLC (DCM/MeOH, 98:2) confirmed complete conversion of the starting material to a product with higher Rf. The reaction mixture was diluted with dichloromethane (100 mL), washed with 1M HCl (2 × 50 mL), saturated sodium bicarbonate (2 × 50 mL) and brine. The organic layer was dried over sodium sulfate and the solvent was evaporated under reduced pressure to obtain white gum of 2-acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-D-mannopyranose. The compound was used for the following reaction without further purification.

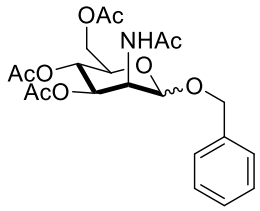
$C_{16}H_{23}NO_{10}$; M.W.: 389.36 g/mol; (12.65 g, 72 %)

^{1}H NMR (500 MHz, $CDCl_3$): δ 6.03 (d, *J* = 1.7 Hz) – 5.87 (d, *J* = 1.7 Hz) (1H, H-1), 5.34 (dd, *J* = 10.2, 4.5 Hz, H-3), 5.21-5.11 (1H, m, H-4), 5.07 (dd, *J* = 9.8, 4.0 Hz, H-3), 4.79 (ddd, *J* = 9.3, 4.1, 1.8 Hz) – 4.65 (ddd, *J* = 9.3, 4.5, 1.8 Hz) (1H, H-2), 4.32-4.27 (m) – 4.10 (dd, *J* = 12.6, 2.4 Hz) (2H, 2H-6), 4.07-4.03 (m) – 3.82 (ddd, *J* = 9.7, 5.5, 2.5 Hz) (1H, H-5), 2.19-2.00 (15H, m, 5 × Ac) ppm

MS (ESI+): m/z = 412.14 $[M+Na]^+$

$Rf_{(Hex/EtOAc,\ 1:4)}$ = 0.21

4.2.5.2 2-acetamido-3,4,6-tri-*O*-acetyl-1-*O*-benzyl-2-deoxy-D-mannopyranose (41)



Compound **40** (6.65 g, 17.07 mmol, 1 eq.) was dissolved in anhydrous acetonitrile (40 mL) in a 150 mL round-bottom flask under Argon atmosphere and anhydrous benzyl alcohol (6.0 mL, 57.98 mmol, 3.4 eq.) was added followed by boron trifluoride diethyl etherate (230 µL, 1.83 mmol, 0.1 eq.). The solution was heated to 80 °C and stirred overnight. The reaction mixture was cooled to room temperature, diluted with dichloromethane (400 mL) and washed with saturated sodium bicarbonate (3 × 100 mL). The organic layer was dried over sodium sulfate, concentrated under reduced pressure on rotary evaporator and the obtained crude was purified by column chromatography on silica using EtOAc/Hex (4:1) as the eluting system to gain pure 2-acetamido-3,4,6-tri-*O*-acetyl-1-*O*-benzyl-2-deoxy-D-mannopyranose as white gum.

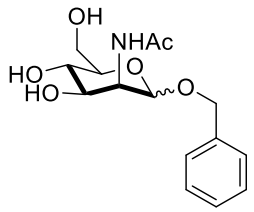
$C_{21}H_{27}NO_9$; M.W.: 437.45 g/mol; (1.12 g, 19 %)

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.40-730 (5H, m, Ph), 5.73 (1H, d, *J* = 8.5 Hz, NH), 5.37 (1H, dd, *J* = 10.0, 4.5 Hz, H-3), 5.11 (1H, t, *J* = 10.0 Hz, H-4), 4.83 (1H, d, *J* = 1.5 Hz, H-1), 4.69 (1H, d, *J* = 12.0 Hz, CH_2Ph a), 4.66 (1H, ddd, *J* = 9.0, 4.5 Hz, 1.5 Hz, H-2), 4.55 (1H, d, *J* = 12 Hz, CH_2Ph b), 4.27 (1H, dd, *J* = 12.5, 5.0 Hz, H-6 a), 4-04-3.98 (2H, m, H-5 and H-6 b), 2.13 (3H, s, Ac), 2.05 (3H, s, Ac), 2.04 (3H, s, Ac), 1.99 (3H, s, Ac) ppm

MS (ESI+): m/z = 460.16 $[M+Na]^+$

$Rf_{(Hex/EtOAc,\ 1:4)}$ = 0.49

4.2.5.3 2-acetamido-1-*O*-benzyl-2-deoxy-D-mannopyranose (42)



To a solution of **41** (1.00 g, 2.29 mmol, 1 eq.) in anhydrous methanol (10 mL) in a 50 mL round bottom-flask under Argon atmosphere, freshly prepared solution of sodium methoxide (12 mg, 0.23 mmol, 0.1 eq.) in anhydrous methanol (0.3 mL) was added and the reaction mixture was stirred at room temperature. After 6 hours, Amberlite© IR-120 hydrogen form was added and the solution was stirred for further 1 hour at room temperature. The reaction mixture was filtered through celite and the solvent was evaporated under reduced pressure. The obtained solid was triturated with diethyl ether and was further purified by column chromatography on silica using EtOAc/MeOH (4:1) as the eluting system to obtain 2-acetamido-1-*O*-benzyl-2-deoxy-D-mannopyranose as white solid.

$C_{16}H_{23}NO_{10}$; M.W.: 311.33 g/mol; (0.62 g, 87 %)

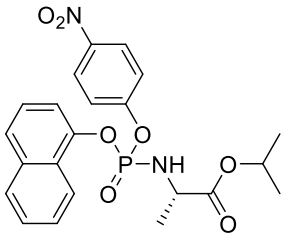
^{1}H NMR (500 MHz, $CDCl_3$): δ 7.40-7.25 (5H, m, Ph), 6.73 (1H, m) 4.81 (1H, s), 4.66 (1H, d, *J* = 12 Hz, CH_2Ph a), 4.47 (1H, d, *J* = 12 Hz, CH_2Ph b), 4.46-4.32 (1H, m), 4.30 (1H, s), 4.12-4.08 (1H, m), 4.07-3.99 (1H s), 3.95-3.90 (1H, m), 3.80-3.70 (2H, m), 3.66-3.61 (1H, m), 3.40-3.30 (1H, s), 2.01 (3H, s, Ac) ppm

MS (ESI+): m/z = 334.15 $[M+Na]^+$

$Rf_{(EtOAc/MeOH,\ 4:1)}$ = 0.41

4.3 Synthesis of ProTides for MDS

4.3.1 (2*S*)-isopropyl 2-{[(4-nitrophenyloxy) (naphtalen-1-yloxy) phosphoryl] amino} propanoate (47)

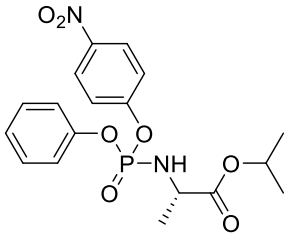


A solution of α-naphtol (**43**) (2.25 g, 15.62 mmol, 1 eq.) and anhydrous triethylamine (2.4 mL, 17.19 mmol, 1.1 eq.) in anhydrous dichloromethane (35 mL) was added dropwise to a solution of *p*-nitrophenyl phosphorodichloridate (4.00 g, 15.62 mmol, 1 eq.) in anhydrous dichloromethane (35 mL) under Argon atmosphere in a 250 mL round-bottom flask, cooled to -78 °C by means of dry ice/acetone bath. The resulting mixture was stirred at that temperature for 30 minutes and after that period, when ^{31}P NMR confirmed completion of the reaction ($CDCl_3$, a singlet at -5.65 ppm corresponding to desired phosphorochloridate was observed), the reaction mixture was transferred through syringe to a 250 mL round-bottom flask containing a cold solution (0 °C) of L-alanine isopropyl ester hydrochloride (**7**) (2.62 g, 15.62 mmol, 1 eq.) in anhydrous dichloromethane (35 mL). Subsequently, anhydrous triethylamine (4.6 mL, 32.82 mmol, 2.1 eq.) was added dropwise and the mixture was stirred at 0 °C for further 30 minutes. Once ^{31}P NMR confirmed completion of the reaction ($CDCl_3$, appearance of doublet at -2.86 ppm), dichloromethane was evaporated under reduced pressure without any contact with air. The residue was suspended in diethyl ether and stirred at 0 °C for 30 minutes. The white solid was filtered off and the filtrate was concentrated under reduced pressure on rotary evaporator without any contact with air to obtain yellow oil of (2*S*)-isopropyl 2-{[(4-nitrophenyloxy) (naphtalen-1-yloxy) phosphoryl] amino} propanoate**.**

$C_{22}H_{23}N_2O_7P$, M.W.: 458.41 g/mol; (6.42 g, 89 %)

^{31}P NMR (202 MHz, $CDCl_3$): δ -2.86 (d, J = 4.3 Hz) ppm

4.3.2 (2*S*)-isopropyl 2-{[(4-nitrophenyloxy) (phenoxy) phosphoryl] amino} propanoate (48)

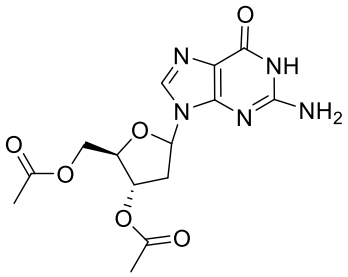


A solution of phenol (**44**) (1.47 g, 15.62 mmol, 1 eq.) and anhydrous triethylamine (2.4 mL, 17.19 mmol, 1.1 eq.) in anhydrous dichloromethane (35 mL) was added dropwise to a solution of *p*-nitrophenyl phosphorodichloridate (4.00 g, 15.62 mmol, 1 eq.) in anhydrous dichloromethane (35 mL) under Argon atmosphere in a 250 mL round-bottom flask, cooled to -78 °C by means of dry ice/acetone bath. The resulting mixture was stirred at that temperature for 30 minutes and after that period, when ^{31}P NMR confirmed completion of the reaction ($CDCl_3$, a singlet at -6.00 ppm corresponding to desired phosphorochloridate was observed), the reaction mixture was transferred through syringe to a 250 mL round-bottom flask containing a cold solution (0 °C) of L-alanine isopropyl ester hydrochloride (**7**) (2.62 g, 15.62 mmol, 1 eq.) in anhydrous dichloromethane (35 mL). Subsequently, anhydrous triethylamine (4.6 mL, 32.82 mmol, 2.1 eq.) was added dropwise and the mixture was stirred at 0 °C for further 30 minutes. Once ^{31}P NMR confirmed completion of the reaction ($CDCl_3$, appearance of doublet at -3.12 ppm), dichloromethane was evaporated under reduced pressure without any contact with air. The residue was suspended in diethyl ether and stirred at 0 °C for 30 minutes. The white solid was filtered off and the filtrate was concentrated under reduced pressure on rotary evaporator without any contact with air to obtain yellow oil of (2*S*)-isopropyl 2-{[(4-nitrophenyloxy) (phenyloxy) phosphoryl] amino} propanoate.

$C_{18}H_{21}N_2O_7P$, M.W.: 408.35 g/mol; (6.12 g, 96 %)

^{31}P NMR (202 MHz, $CDCl_3$): δ -3.12 (d, J = 3.7 Hz) ppm

4.3.3 3′,5′-di-*O*-acetyl-2′-deoxyguanosine (50)



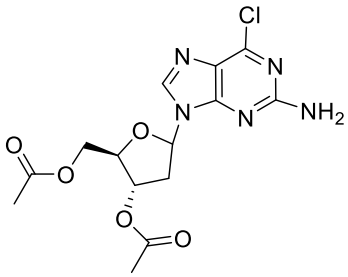
2′-deoxyguanosine (**49**) (10.00 g, 37.42 mmol, 1 eq.), 4-(dimethylamino)pyridine (0.46 g, 3.74 mmol, 0.1 eq.) and triethylamine (13.6 mL, 97.29 mmol, 2.6 eq.) were dissolved in anhydrous acetonitrile (500 mL) under Argon atmosphere in a 1000 mL round-bottom flask and the solution was cooled to 0 °C. Acetic anhydride (8.5 mL, 89.81 mmol, 2.4 eq.) was added dropwise and the resulting reaction mixture was stirred overnight at room temperature. After addition of methanol (app. 200 mL), the formed solid was filtered using a Büchner funnel and washed with methanol and hexane to obtain a white solid of 3′,5′-di-*O*-acetyl-2′-deoxyguanosine.

$C_{14}H_{17}N_5O_6$, M.W.: 351.32 g/mol; (12.60 g, 96 %)

^{1}H NMR (500 MHz, DMSO-d6): δ 10.67 (1H, s, NH), 7.92 (1H, s, H-8), 6.50 (2H, s, NH_2), 6.14 (1H, dd, *J* = 8.8, 5.9 Hz, H-1′), 5.30 (1H, dt, *J* = 6.2, 1.9 Hz, H-3′), 4.31-4.25 (1H, m, H-5′ a), 4.22-4.17 (2H, m, H-4′ and H-5′ b), 2.96-2.88 (1H, m, H-2′ a), 2.46 (1H, ddd, *J* = 14.2, 6.0, 2.1 Hz, H-2′ b), 2.09 (3H, s, CH_3), 2.05 (3H, s, CH_3) ppm

$Rf_{(DCM/MeOH,\ 9:1)}$ =0.47

4.3.4 3′,5′-di-*O*-acetyl-6-deoxo-6-chloro-2′-deoxyguanosine (51)



Compound **50** (6.00 g, 17.08 mmol, 1 eq.) was suspended in anhydrous acetonitrile (100 mL) under Argon atmosphere in a 250 mL round-bottom flask together with benzyltriethylammonium chloride (5.83 g, 25.62 mmol, 1.5 eq.) and *N,N*-dimethylaniline (13.0 mL, 102.47 mmol, 6 eq.). The resulting mixture was cooled to 0 °C and phosphoryl chloride (9.6 mL, 102.47 mmol, 6 eq.) was added dropwise. The mixture was stirred for 10 minutes at room temperature and then heated to reflux in preheated oil bath. The reaction was monitored every 10 minutes by TLC

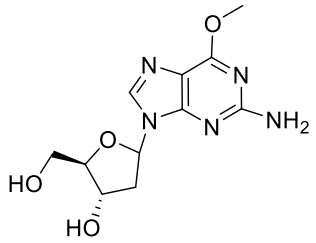
(DCM/MeOH, 9:1) and after 1 hour, when there were no further changes observed on TLC plate, the reaction mixture was cooled with an ice bath and concentrated to dryness under reduced pressure. Ice water (20 mL) was added under cooling in order to hydrolyse the remaining phosphoryl chloride, the mixture was stirred for 20 minutes and subsequently extracted with ethyl acetate. The organic layers were joined, dried over sodium sulfate and the solvent was evaporated under reduced pressure on rotary evaporator. The crude residue was purified by column chromatography on silica using DCM/MeOH (95:5) as the eluting system yielding a white foam of 3′,5′-di-*O*-acetyl-6-deoxo-6-chloro-2′-deoxyguanosine.

$C_{14}H_{16}ClN_5O_5$, M.W.: 369.76 g/mol; (2.90 g, 46 %)

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.94 (1H, s, H-8), 6.31 (1H, dd, *J* = 7.9, 6.2 Hz, H-1′), 5.45 (1H, dt, *J* = 6.3, 2.5 Hz, H-3′), 5.21 (2H, s, NH_2), 4.48 (1H, dd, *J* = 14.5, 6.01 Hz, H-5′ a), 4.41-4.36 (2H, m, H-4′ and H-5′ b), 3.00 (1H, ddd, *J* = 14.2, 7.9, 6.4 Hz, H-2′ a), 2.59 (1H, ddd, *J* = 14.2, 6.2, 2.6 Hz, H-2′ b), 2.16 (3H, s, CH_3), 2.11 (3H, s, CH_3) ppm

$Rf_{(DCM/MeOH,\ 95:5)}$ = 0.43

4.3.5 2′-deoxy-*O*^{6}-methylguanosine (52)



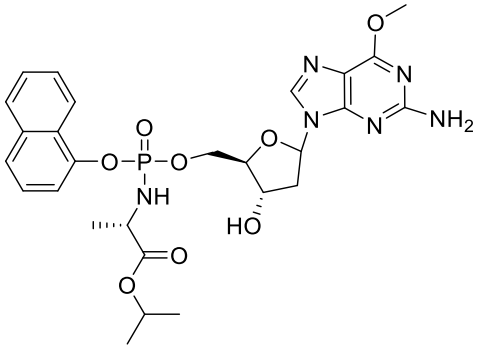
Freshly prepared 1M solution of $NaOCH_3$ (5.84 g, 108.18 mmnol, 5 eq.) in anhydrous MeOH (108.2 mL) was added dropwise to a solution of **51** (8.00 g, 21.64 mmol, 1 eq.) in anhydrous methanol (50 mL) under Argon atmosphere in a 500 mL round-bottom flask, cooled to 0 °C. The reaction mixture was stirred at room temperature for 6 hours, until no more starting material could be observed at TLC plate (DCM/MeOH, 9:1). The mixture was concentrated to dryness under reduced pressure, the residue was dissolved in small amount of water to obtain a transparent yellow solution. Acetic acid was used to adjust pH of the solution to pH 7, resulting in formation of a white solid of sodium acetate. Using decantation, the solid was extracted 5 times with ethyl acetate and 5 times with dichloromethane afterwards. The solid residue was then dissolved in as small amount of water as was necessary and extracted further two times with ethyl acetate and 2 times with dichloromethane. All organic layers were combined, dried over sodium sulfate and concentrated under reduced pressure on rotary evaporator yielding pure 2′-deoxy-*O*^{6}-methyl-guanosine.

$C_{14}H_{20}O_{10}$, M.W.: 281.27 g/mol; (4.21 g, 69 %)

^{1}H NMR (500 MHz, MeOD): δ 8.05 (1H, s, H-8), 6.34 (1H, dd, *J* = 8.3, 6.1 Hz, H-1′), 4.59-4.57 (1H, m, H-3′), 4.09-4.04 (4H, m, H-4′ and CH_3), 3.86 (1H, dd, *J* = 12.2, 3.1 Hz, H-5′ b), 3.76 (1H, dd, *J* = 12.2, 3.4 H-5′ b), 2.84-2.76 (1H, m, H-2′ a), 2.36 (1H, ddd, *J* = 13.4, 6.0, 2.6 Hz, H-2′ b) ppm

$Rf_{(DCM/MeOH,\ 9:1)}$ = 0.37

4.3.6 (2*S*)-isopropyl 2-{[((2′-deoxy-*O*^{6}-methyl-guanosine)-5′-yloxy) (naphtalen-1-yloxy) phosphoryl] amino} propanoate (53)



Compound **52** (3.00 g, 10.67 mmol, 1 eq.) was suspended in anhydrous DMF (80 mL) in a 250 mL round-bottom flask under Argon atmosphere, 1M *t*BuMgCl in THF (32.0 mL, 32.00 mmol, 3 eq.) was added dropwise and the resulting mixture was stirred for 30 minutes. A solution of **47** (5.38 g, 11.73 mmol, 1.1 eq.) in anhydrous DMF (20 mL) was added dropwise over a period of 20 minutes and the reaction mixture was stirred for 48 hours at room temperature. Afterwards, the solvent was evaporated under reduced pressure on rotary evaporator and the residue was diluted with water resulting in formation of a solid that was filtered off. The water phase was extracted two times with dichloromethane and two times with ethyl acetate. Organic layers were combined, dried over sodium sulfate and concentrated under reduced pressure. The crude was purified by column chromatography on silica using DCM/MeOH (9:1) as the eluting system. Fractions containing desired product were further purified by reversed-phase column chromatography (C-18) using ACN/H_2O (90:10), 100 % ACN in 50 minutes yielding pure (2*S*)-isopropyl 2-{[((2′-deoxy-*O*^{6}-methyl-guanosine)-5′-yloxy) (naphtalen-1-yloxy) phosphoryl] amino} propanoate as white solid.

$C_{27}H_{33}N_6O_8P$, M.W.: 600.57 g/mol; (101 mg, 2 %)

^{1}H NMR (500 MHz, MeOD): δ 8.18- 7.33 (8H, m, Naph and H-8), 6.33-6.28 (1H, m, H-1′), 4.95-4.89 (1H, m, OCH), 4.62-4.54 (1H, m, H-3′), 4.49-4.32 (2H, m, 2H-5′), 4.21-4.14 (1H, m, H-4′), 4.03 (3H, s, OCH_3), 4.00-3.91 (1H, m, NHCH) 2.75-2.52 (1H, m, H-2′ a), 2.38-2.26 (1H, m, H-2′ b), 1.31-1.27 (3H, m, NHCHCH_3), 1.17 (6H, dd, *J* = 8.9, 6.3 Hz, CH_3CHCH_3) ppm

^{13}C NMR (125 MHz, MeOD): δ 173.27 (d, *J* = 4.5 Hz) – 173.00 (d, *J* = 5.2)- 161.19 (d, *J* = 2.7 Hz) – 160.37 (s) – 153.13 (s) – 146.56 (t, *J* = 6.8 Hz)– 134.83 (d, *J* = 4.5 Hz) – 126.46 (s) – 114.29 (8C, Naph-C1 and Naph-C9 and Naph-C10 and COO and C-2 and C-4 and C-5 and C-6), 137.99 (s) – 137.75 (s) - 127.43 (d, *J* = 7.2 Hz) - 126.35 (s) – 126.08 (d, *J* = 5.3 Hz) - 125.08 (d, *J* = 7.5 Hz) - 124.56 (s) - 121.25 (s) - 114.84 (m) (8C, Naph-C2-C8 and C-8), 85.42 (d, *J* = 8.7 Hz) – 85.24 (d, *J* = 8.7 Hz) (1C, C-4′) 84.21 (1C, d, *J* = 4.1 Hz, C-1′), 71.09 (1C, d, *J* = 2.7 Hz, C-3′), 68.80 (1C, d, *J* = 4.1 Hz, OCH), 66.46 (1C, dd, *J* = 9.9, 5.5 Hz, C-5′), 52.75 (1C, s, OCH_3), 50.41 (1H, d, *J* = 8.4 Hz, NHCH), 38.82 (s) – 38.69 (s) (1C, C-2′) 20.53-20.42 (2C, m, $\underline{C}H_3CH\underline{C}H_3$), 19.10 (d, *J* = 6.8 Hz) – 18.94 (d, *J* = 7.2 Hz) (1C, NHCH$\underline{C}H_3$) ppm

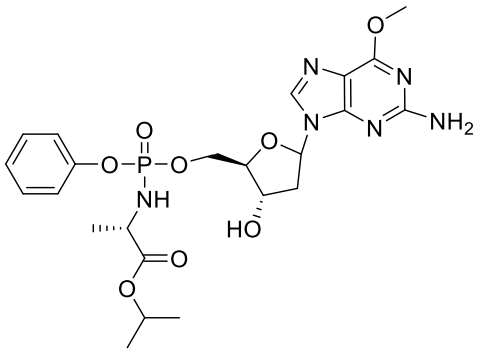
^{31}P NMR (202 MHz, MeOD): δ 4.46 (s, 1P), 4.32 (s, 0.7P)

HPLC: Rt = 15.15, 15.32 min (gradient ACN/H_2O, 10:90, 100 % ACN in 30 min, flow: 1 mL/min)

MS (ESI+): m/z = 601.19 $[M+H]^+$

$Rf_{(DCM/MeOH,\ 9:1)}$ = 0.33

4.3.7 (2*S*)-isopropyl 2-{[((2′-deoxy-*O*^{6}-methyl-guanosine)-5′-yloxy) (phenoxy) phosphoryl] amino} propanoate (54)



Compound **52** (1.30 g, 4.62 mmol, 1 eq.) was suspended in anhydrous DMF (35 mL) in a 150 mL round-bottom flask under Argon atmosphere, 1M *t*BuMgCl in THF (13.9 mL, 13.87 mmol, 3 eq.) was added dropwise and the resulting mixture was stirred for 30 minutes. A solution of **48** (2.07 g, 5.08 mmol, 1.1 eq.) in anhydrous DMF (10 mL) was added dropwise over a period of 15 minutes and the reaction mixture was stirred for 96 hours at room temperature. Afterwards, the solvent was evaporated under reduced pressure on rotary evaporator and the residue was diluted with water resulting in formation of a solid that was filtered off. The water phase was extracted two times with dichloromethane and two times with ethyl acetate. Organic layers were combined, dried over sodium sulfate and concentrated under reduced pressure. The crude was purified by column chromatography on silica using DCM/MeOH (9:1) as the eluting system. Fractions containing the desired product were further purified by reversed-phase

column chromatography (C-18) using ACN/H_2O (90:10), 100 % ACN in 50 minutes yielding pure (2*S*)-isopropyl 2-{[((2′-deoxy-O^6-methyl-guanosine)-5′-yloxy) (phenyloxy) phosphoryl] amino} propanoate as a white solid.

$C_{23}H_{31}N_6O_8P$, M.W.: 550.51 g/mol; (104 mg, 4 %)

^{1}H NMR (500 MHz, MeOD): δ 7.98 (1H, s, H-8), 7.38-7.14 (5H, m, Ph), 6.37-6.32 (1H, m, H-1′), 4.99-4.90 (1H, m, OCH), 4.64-4.60 (1H, m, H-3′), 4.44-4.25 (2H, m, 2H-5′), 4.20-4.12 (1H, m, H-4′), 4.06 (3H, d, *J* = 2.5 Hz, OCH_3), 3.93-3.83 (1H, m, NHCH), 2.86-2.71 (1H, m, H-2′ a), 2.44-2.37 (1H, m, H-2′ b), 1.32-1.26 (3H, m, NHCHCH_3), 1.23-1.18 (6H, m, CH_3CHCH_3) ppm

^{13}C NMR (125 MHz, MeOD): δ 173.24 (d, *J* = 4.6 Hz) – 172.99 (d, *J* = 5.4 Hz) – 161.23 (s) – 160.42 (d, *J* = 3.6 Hz) – 153.19 (d, *J* = 4.6 Hz) – 150.74 (d, *J* = 6.8 Hz) (6C, Ph-C1 and COO and C-2 and C-4 and C-5 and C-6), 138.04 (s) – 137.84 (s) (1C, C-8), 129.53 (s) – 129.36 (s) – 125.16 (s) – 124.74 (s) – 120.20-119.95 (m) (5C, Ph-C2-C6), 85.38 (d, *J* = 8.6 Hz) – 85.26 (d, *J* = 8.6 Hz) (1C, C-4′), 84.24 (1C, d, *J* = 8.9 Hz, C-1′), 71.13 (1C, s, C-3′), 68.76 (1C, s, OCH), 66.35 (d, *J* = 5.5 Hz) – 66.15 (d, *J* = 5.5 Hz) (1C, C-5′), 52.79 (1C, s, OCH_3), 50.37 (s) – 50.24 (s) (1C, NHCH), 38.98 (s) – 38.78 (s) (1C, C-2′), 20.57-20.45 (2C, m, CH_3CHCH_3), 19.10 (d, *J* = 6.4 Hz) – 18.94 (d, *J* = 7.2 Hz) (1C, NHCHCH_3) ppm

^{31}P NMR (202 MHz, MeOD): δ 4.08 (s, 0.4P), 3.88 (s, 1P)

HPLC: Rt = 13.84, 14.05 min (gradient ACN/H_2O, 10:90, 100 % ACN in 30 min, flow: 1 mL/min)

MS (ESI+): m/z = 551.20 $[M+H]^+$

$Rf_{(DCM/MeOH,\ 9:1)}$ = 0.54

List of abbreviations

Ac_4ManNAc – tetra-*O*-acetylated *N*-acetylmannosamine

ACN – acetonitrile

AHS – Alpers-Huttenlocher syndrome

ATP – adenosine triphosphate

BTEA-Cl – benzyltriethylammonium chloride

CDGs – Congenital disorders of glycosylation

CPY – Carboxypeptidase Y

cycloSal – cyclosalogenyl

dA – deoxyadenosine

dAMP – deoxyadenosine monophosphate

DCM – dichloromethane

dG – deoxyguanosine

dGK – deoxygunaosine kinase

dGMP – deoxyguanosine monophosphate

dCMP – deoxycytidine monophosphate

DMAP – 4-(dimethylamino)pyridine

DMF – *N*,*N*-dimethylformamide

DMRV – Allelic distal myopathy with rimmed vacuoles

dNMP – deoxyribonucleotide monophosphate

dNTP – deoxyribonucleotide triphosphate

DolP-Man – dolichol-phosphate mannose

DTE – S-[(2-hydroxyethyl)sulfidyl]-2-thioethyl

dTMP – deoxythymidine monophosphate

dTTP –deoxythymidine triphosphate

ECM – extracellular matrix

EMA – European Medicines Agency

ER – endoplasmic reticulum

FDA – U.S. Food and Drug Administration

GA – Golgi apparatus

GDP-Man – Guanosine diphosphate mannose

GNE/MNK – UDP-GlcNAc 2-epimerase/ManNAc kinase

GNEM – GNE myopathy

GPI – glycosylphosphatidylinositol

Hex – hexane

hIBM – Hereditary inclusion body myopathy

HPLC – high-performance liquid chromatography

IBM2 – Inclusion body myopathy type 2

IEF – isoelectric focusing

IgG – immunoglobulin G

IOSCA – Infantile onset spinocerebellar ataxia

LAD II (SLC35C1-CDG) – Leukocyte adhesion deficiency type II

LLO – lipid-linked oligosaccharide

Man-1-P – D-Mannose-1-phosphate

Man-6-P – D-Mannose-6-phosphate

ManNAc-6-P – *N*-acetyl-D-mannosamine-6-phosphate

MDS – Mitochondrial DNA depletion syndrome

MIRAS – Mitochondrial recessive ataxia syndrome

MNGIE – Mitochondrial neuro-gastro-intestinal encephalo-myopathy

MPI – Phosphomannose isomerase

MPI-CDG – Phosphomannose isomerase deficiency

MS – mass spectrometry

mtDNA – mitochondrial DNA

M.W. – molecular weight

NA-sugar – nucleotide activated sugar

NCS – *N*-chlorosuccinimide

nDNA – nuclear DNA

NMI – *N*-methylimidazole

OST – oligosaccharyltransferase complex

PG – protecting group

PIGM – Congenital disorder of glycosylation due to PIGM deficiency

PMM2 – Phosphomannomutase 2

PMM2-CDG – Phosphomannomutase 2 deficiency

POC – isopropoxycarbonyl

POM – pivaloyloxymethyl

Py – pyridine

Rf – retention factor

RP HPLC – reversed-phase high-performance liquid chromatography

RR – ribonucleotide reductase

RT – room temperature

SAR – structure-activity relationship

SATE – S-acyl-2-thioethyl

Sia – sialic acid

SM – starting material

SnAr – nucleophilic aromatic substitution

*t*BuMgCl – tert-butylmagnesium chloride

TLC – thin layer chromatography

Tf – transferrin

THF - tetrahydrofuran

TK2 – thymidine kinase 2

Tr-Cl (trityl chloride) – triphenylmethyl chloride

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