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Everyone is trying to accomplish something big,

not realizing that life is made up of little things.

Frank Clark (American politician)

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Abstract

Neisseria meningitidis is a gram-negative ß-proteobacterium and certain strains initiate life threatening diseases like meningococcemia and meningitis. The symptoms of these dangerous diseases are not very specific, so immunisation is the best strategy to protect people against meningococcal infections. A novel approach for vaccine development uses the inner core structure of lipooligosaccharide (LOS) of *N.meningitidis*, as the structure is similar in all serogroups. Within this thesis fragments of the inner core were successfully synthesized.

The monosaccharide Methyl 3-O-(2-aminoethylphosphate)- L-*glycero*-α-D-*manno*-heptopyranoside was prepared. Formation of a 2,3 orthoacetate and the selective opening allowed the introduction of the 2-aminoethylphosphate linker at position 3 *via* the phosphoramidite approach.

Furthermore, preliminary studies for the disaccharide Methyl-(*N*-acetyl-α-D-glucosamine)-(1→2)- L-*glycero*-α-D-*manno*-heptopyranoside with substitution at position 3 of the acceptor with 2-aminoethylphosphate should be considered. The donor 1, 3, 4, 6-tetra-O-acetyl-2-azido-2-deoxy-D-glucopyranose was created successfully by using a diazotransfer reagent to introduce the azide as temporary protecting group.

Zusammenfassung

Neisseria meningitidis ist ein Gram-negatives ß-Proteobakterium und bestimmte Stämme lösen lebensbedrohliche Krankheiten, wie beispielsweise Meningokokkeninfektion und Meningitis aus. Die Symptome dieser gefährlichen Krankheiten sind sehr unspezifisch, sodass eine Immunisierung gegen Meningokokkeninfektionen die beste Alternative ist die Bevölkerung zu schützen. Eine neue Methode in der Impfstoffentwicklung verwendet die Kernregion von Lipooligosacchariden (LOS) aus *N.meningitidis*, welche in allen Serogruppen ähnlich ist.

Das Monosaccharid Methyl 3-O-(2-aminoethylphosphate)- L-*glycero*-α-D-*manno*-heptopyranosid wurde synthetisiert. Die Ausbildung eines 2,3 Orthoesters und die selektive Öffnung ermöglichte die Einführung der 2-Aminoethylphosphat Kette an Position 3 mithilfe der Phosphoramidit Methode.

Außerdem konnten erste Überlegungen für das Disaccharid Methyl-(*N*-acetyl-α-D-glucosamine)-(1→2)- L-*glycero*-α-D-*manno*-heptopyranosid mit Einbeziehung des 2-Aminoethylphosphats an Position 3 des Akzeptors untersucht werden. Der Donor 1, 3, 4, 6-Tetra-O-acetyl-2-azido-2-desoxy-D-glucopyranose wurde erfolgreich hergestellt, indem ein Diazotransfer Reagenz für die Einführung des Azids als vorübergehende Schutzgruppe benutzt wurde.

Abbreviations

Å	Angstroem
ACN	Acetonitrile
AP	Alternative Pathway
BBB	Blood brain barrier
Bn	Benzyl group
BnBr	Benzyl bromide
BnOH	Benzyl alcohol
Bs	Broad signal
C1-inh	C1-inhibitor
C4BP	C4 binding protein
cat.	Catalytic
Cbz	Carboxybenzyl
COSY	Correlated spectroscopy
CP	Classical pathway
CPS	Capsular polysaccharides
CRM197	Non-toxic mutant of diphtheria toxin
CSA	Camphor sulfonic acid
D	Doublet
DCM	Dichloromethane
Dd	Doublet of Doublet
ddd	Doublet of Doublet of Doublet
dddd	Doublet of Doublet of Doublet of Doublet
DMF	Dimethylformamide
DSS	4,4-Dimethyl-4-silapentane-1-sulfonic acid
equiv.	Equivalent
ESI	Electrospray ionization
Et	Ethyl
Et_2O	Diethylether
EtOAc	Ethylacetate
EtOH	Ethanol
Exp.	Experiment
Fab	Fragment antigen binding
Fc domain	Fragment crystallisable region

fH	Factor H
fHbp	Factor H binding protein
fP	Properdin
Gly	Glycine
Hep	L,D- heptose
HMBC	Heteronuclear Multiple Bond Correlation
HPLC	High performance thin layer chromatography
HPTLC	High performance thin layer chromatography
HRMS	High- resolution mass spectrometry
HSQC	Heteronuclear Single Quantum Coherence
Hz	Hertz
IgG	Immunoglobulin G
IgM	Immunoglobulin M
J	Coupling constant
kDa	Kilodalton
Kdo	3-deoxy-D-*manno*-oct-2-ulusonic acid
LC-MS	Liquid chromatography- mass spectrometry
Leu	Leucine
LNT	Lacto-*N*-neotetraose
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
M	Multiplet
mAb	Monoclonal antibody
MAC	Membrane attack complex
MASP	Mannose binding lectin associated serine proteases
MBL	Mannose binding lectin
*m*CPBA	Meta-Chloroperoxybenzoic acid
Me	Methyl
MenA	Serogroup A
MenB	Serogroup B
MenC	Serogroup C
MeOH	Methanol
MS	Mass spectrometry
NadA	*Neisserial* adhesion A

NHBP	*Neisserial* Heparin Binding Antigen
NMR	Nuclear magnetic resonance
OAc	Acetate
OMV	Outer membrane vesicle
Opa	Opacity proteins
Pd/C	Palladium on Carbon
PEtn	2-aminoethylphosphate
Ph	Phenyl
PivCl	Pivaloyl chloride
PorA	Porin A
ppm	Parts per million
Prod.	Product
Q	Quartet
r.t.	Room temperature
Rf	Retention value
S	Singlet
SP-D	Surfactant protein D
STD	Saturation- Transfer Difference
T	Triplet
TBAI	*tert*-butylammonium iodide
TCA	trichloroacetimidates
TEA	Triethylamine
TEAB	Triethylammonium bicarbonate buffer
Temp.	Temperature
TFA	Trifluoroacetic acid
TfN_3	Trifluoromethanesulfonyl azide
Tfp	Type IV pili
THF	Tetrahydrofuran
TIPDS	1,1,3,3-tetraisopropyldisiloxane
TLC	Thin layer chromatography
TREAT	Triethylamine trihydrofluoride
Tyr	Tyrosine

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1 Introduction

1.1 Neisseria meningitidis

The genus *Neisseria*, part of the family Neisseriaceae, includes two important pathogens: *Neisseria meningitidis* and *Neisseria gonorrhoeae*. Both are gramnegative ß-proteobacteria and are 0.6- 1.0 µm in diameter. As both live in pairs, they are also called diplococci. *N. gonorrhoeae* is known to transmit gonorrhoea, one of the most widespread sexual diseases, whereas *N. meningitidis* (meningococcus) causes meningitis, so both are extremely dangerous pathogens.[1]

In 1887 Anton Weichselbaum, an Austrian pathologist, identified meningococcus from cerebrospinal fluid of a patient deceased on meningitis for the first time. Before, reports of meningococcal disease were published in 1805 by Vieusseux in Geneva and 1806 by Danielson in the United States (Massachusetts).[2,3]

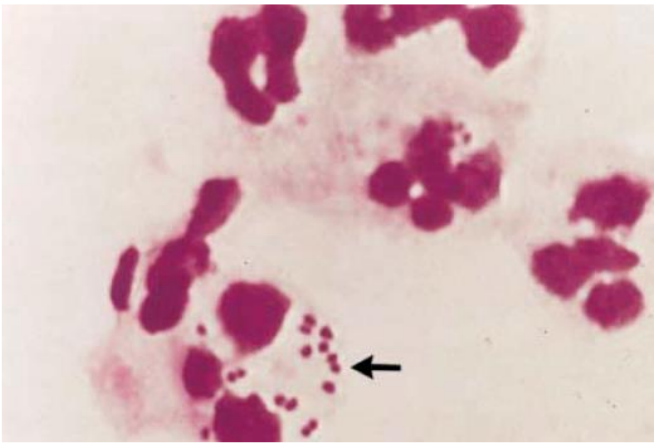


Figure 1 Cerebrospinal Fluid with the diplococcus *Neisseria meningitidis* (black arrow) [4]

N. meningitidis is a pathogen only in humans and is classified in 13 serogroups on the basis of their immunological reactivity and capsular polysaccharide structure: A, B, C, E-29, H, I, K, L, W-135, X, Y, Z, Z'. They can be further ordered into 20 serotypes, 10 subtypes and 12 immunotypes, based on the outer membrane protein antigens and the Lipopolysaccharide antigens. Six of the serogroups (A, B, C, W-135, X, Y) are known to cause life threatening disease.[1,2]

The natural reservoir of meningococcus is the human nasopharynx and up to 10 % of all adults are carrier of the pathogen. As the most common serotypes are not pathogenic and only a small number enters the bloodstream, the human innate immune system can mostly defend the attack.[4]

1.2 Meningococcal disease

When *N.meningitidis* (serogroup A, B, C, W-135, X, Y) enters the bloodstream, it can lead to septic shock and/or also can cross the blood brain barrier (BBB). Meningococcal sepsis (=meningococcemia) is a bloodstream infection and crossing of the BBB points to meningeal infection[5]. Characteristic for the meningococcal infection is the abrupt onset of fever and a petechial or purpuric rash on the skin. Furthermore the symptoms, e.g. headache and stiffness of the neck are not very specific, so the identification is complicated[6]. Meningococcemia is correlated with extensive thrombosis, vascular leakage and cardiovascular failure (e.g. purpura fulminans)[5]. In both infections of *N.meningitidis* a fast recognition and treatment with antibiotics is essential for a low mortality rate.

Meningococcus is only a human pathogen, so studies about its pathogenesis are difficult due to missing animal models. Most of the hypotheses were obtained by studying post-mortem patients. Bacteria are found on the endothelial cell and can form colonies on the apical surface of endothelial cell capillars[5]. For the interactions with endothelial cells several components on the surface of the bacteria are important e.g. type IV pili (Tfp) and Opa. With the help of Tfp *N. meningitidis* forms colonies on endothelial cells and succeeds to open BBB to infect the meninges which then lead to meningitis.[5]

Worldwide *N. meningitidis* causes more than 1.2 million cases per year and more than 135.000 are mortal. The mortality of meningococcal disease varies in geographic areas and age groups[2]. The highest rate appears in infants under 1 year and the secondary rate is during adolescence[7].

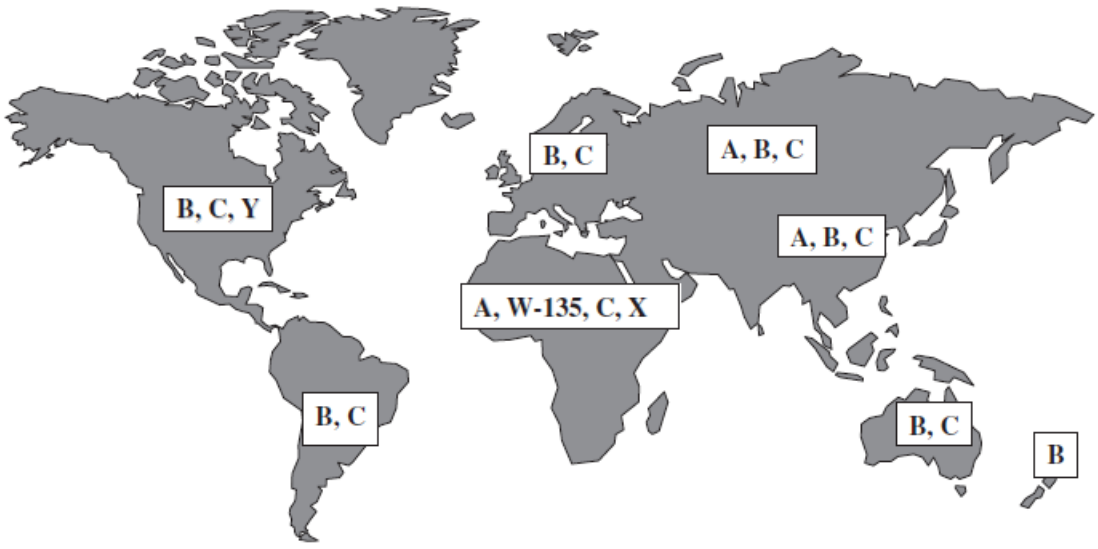


Figure 2 Global serogroup distribution of *Neisseria meningitidis*[8]

In the United States less than one case per 100.000 people is observed. In the first part of the twentieth century serogroup A was dominant in the U.S., but these days serogroup B, C, and Y are causing the most infections. Two thirds of all cases in the U.S. concern infants less than 6 months of age.[2]

In Europe the infection rate (more than two cases per 100.000 people) is higher than in the U.S. High infection rates in the U.K. of 5 cases per 100.000 people were noticed due to serogroup C and in 1999 a vaccine against serogroup C was launched in the U.K.. Thereupon meningococcal disease caused by serogroup C decreased as a result of immunisation. Now serogroup B causes the most frequent meningococcal disease.[2]

In Asia serogroup A, B and C are common and in Latin America serogroup B and C. Surprisingly in this two areas the outbreaks are very variable between the countries themselves. The cases in Japan are very low (0.1 cases per 100.000) whereas serogroup A has caused outbreaks in China in the last 25 years.[8]

In Africa serogroup A, C, W-135 and X are prevalent. In the Mid-late 1990s serogroup A led to 150.000 cases per year in the area of Ethiopia to Senegal, so this area is known as 'meningitis belt'. This belt includes 18 countries and more than 270 million people. Described cases vary from 20 to 1.000 per 100.000. Furthermore epidemics appear every 8-10 years, but it is difficult to predict outbreaks of meningococcal disease. Often the rash of meningococcal disease occurs in the dry season. Up to date *N. meningitidis* onsets in the meningitis belt remain still a challenge.[2]

1.3 Virulence factors of N. meningitidis and its interactions with the immune system

Neisseria meningitidis is more resistant in blood serum than many other gram negative bacteria like *Escherichia coli* or *Shigella flexneri*[9]. Meningococci have several factors to interact with the complement system and therefore resist killing.

The complement system is a component of innate immunity and plays an important role in host defence. Complement activation by foreign antigens leads to adaptive immunity and the system is also linked to the inflammatory responses[10]. Overall the system includes more than 30 soluble and surface-expressed proteins and a few are zymogens (=inactive precursors which are activated by cleavage)[11]. The complement system can be activated with the help of three activation pathways (**Figure 3**):

1) The classical pathway (CP)

2) The lectin pathway (MBL)

3) The alternative pathway (AP)

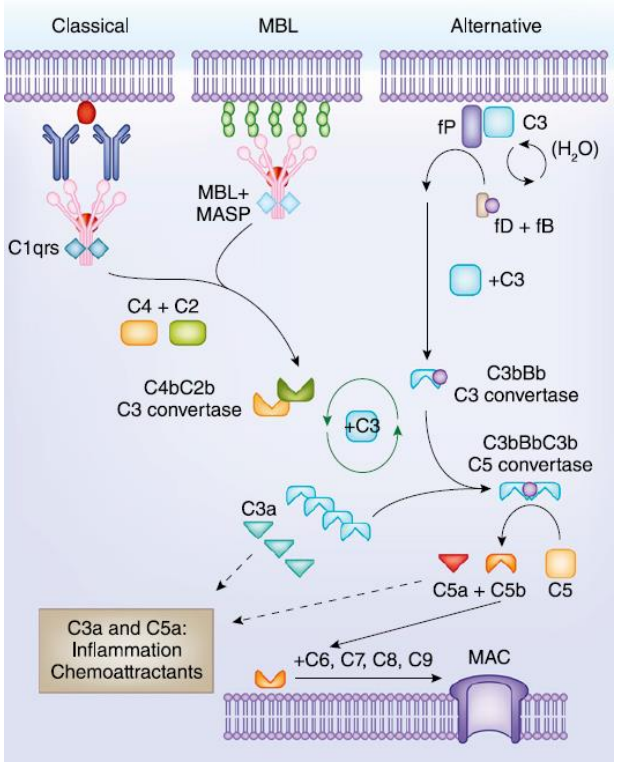


Figure 3 Overview of activation of the complement system *via* classical pathway (CP), Lectin pathway (MBL) and the Alternative pathway (AP)[11]

Classical pathway (CP)

The classical pathway is activated by binding of antibodies (IgG or IgM) to their antigens e.g. Lipopolysaccharide. The outcome of this is a conformational change in the hinge region of the Fc domain and as a result the C1-Complex (comprises C1q, C1r and C1s) binds to the Fc domain. Consequently this leads to conformational change in C1q and activates thereby C1r. Activated C1r cleaves C1s, which cuts out a 9 kDa fragment from the N-terminus of the α-chain of C4 and the outcome is C4a and C4b[11]. Activated C1s cleaves C2 in C2a and C2b and C4b can then bind to cell surface by a thioester and also to C2b to form the complex C4bC2b (C3 convertase), which can cleave C3 into C3a and C3b[7].

Lectin pathway (MBL)

The MBL pathway is very similar to the classical pathway and also ends by the formation of the C3 convertase. The lectin pathway can be activated by binding of lectin molecules to terminal monosaccharides on the surface of pathogens. The resulting MBL-associated serine protease (MASP) leads to formation of the C3 convertase (C4bC2b).[7]

Alternative Pathway (AP)

The activation of the alternative pathway is due to a "tickover" of C3 and the C3 convertase (C3bBb) can be built. In this pathway several factors are involved e.g. factor B, Properdin (fP) (**Figure 3**).[7]

All three pathways end in the formation of a C3 convertase and in the end of the cascade the membrane attack complex (MAC) occurs. MAC can insert in the cells and generates tissue injury, but does not lead mandatorily to cell lysis.[11]

Several proteins are responsible for the regulation of the complement system to avoid host cell damage. This factors can inhibit the formation of C3b or MAC. Additionally, C3 convertase is regulated by proteins e.g. C4 binding protein (C4BP), factor H (fH) or C1-inhibitor (C1-inh), which are present in the bloodstream. An efficient complement system is a requirement by host susceptibility of *Neisseria meningitidis.*[9]

N. meningitidis can interact *via* different factors on its cell surface with the complement system (**Figure 4**[12]. Different factors will be discussed later.

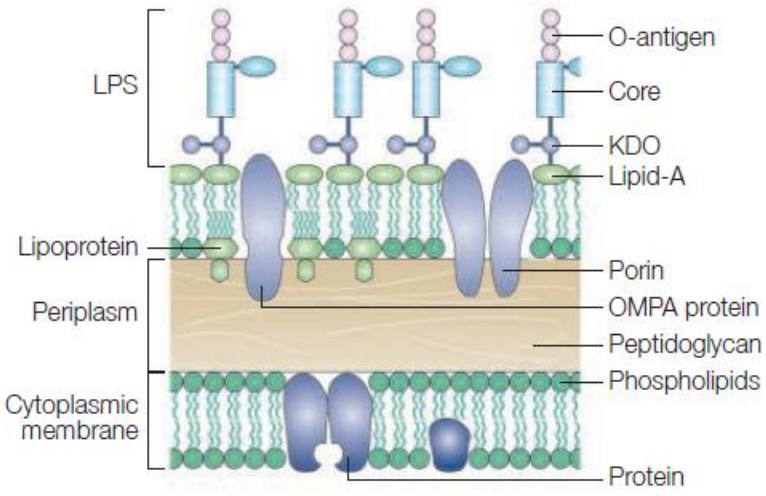


Figure 4 Surface Structure of gram-negative bacteria[12]

The Lipopolysaccharide (LPS)

LPS of *N. meningitidis* is found in the outer membrane and lacks a repeating O- side chain, and therefore it is also known as lipooligosaccharide (LOS). LOS contains three parts:[13]

1) lipid A anchored to the outer membrane
2) the inner core structure linked to lipid A *via* Kdo and to the outer core with a heptose
3) the outer core

The LPS structure is variable and until now 13 different immunotypes (L1-12) are known and most strains express more than one immunotype[2, 14]. Endotoxin (LPS or LOS) stimulates the release of inflammatory mediators in the immune system. Meningococcus in the bloodstream can release "blebs" which are fragments from outer membrane involving endotoxin. The amount of endotoxin which is circulating and the resulting proinflammatory cytokines and chemokines- including interleukin 1 and 6- reflect the severity of meningococcal disease.[7] Lipid A in *N.meningitidis* has six acyl chains and is highly biologically active. Besides, mutants which lack an acyl chain show less virulence than the wild type.[15]

The outer core in some immunotypes covers lacto-*N*-neotetraose (LNT), which is similar to a human blood antigen. This molecular mimicry is responsible for the non-existent activation of the complement system in some cases. Additionally, LNT can be modified with sialic acid by sialyltransferases. In *N. gonorrhoeae* sialylation leads to improved resistance to human serum blood. Due to disagreeing studies the function of sialylations in meningococcal infection is not certain. Serogroup B and C mutants, which lack the ability of sialylation are more sensitive to human serum blood than their corresponding wildtype.[7]

Capsule

Meningococcus can be either encapsulated or unencapsulated, but isolated species from the serum are almost always encapsulated. The capsule consists of repeating saccharides which are linked by glycosidic bonds. Due to the composition of these polysaccharides, *N.meningitidis* can be classified in 13 serogroups. The main issue of CPS is the additional protection, but also inhibits phagocytosis. Furthermore, with capsular polysaccharide the complement activation and also the insertion of MAC can be hindered. The upregulation of CPS also enhances serum resistance.[7]

In serogroup B, C, W and Y the capsule contains derivatives of sialic acid (Neu5Ac). Neu5Ac plays also in humans a key role in intercellular and/or intermolecular recognition. Due to Neu5Ac as building blocks in CPS the bacteria is less observable. This molecular mimicry makes the activation of the complement system more complicated.[2]

Another important virulence mechanism is the ability of capsule switching, which is due to the fact of genetic identity of parts of the capsule loci.[2]

Other virulence factors

There are several more factors how meningococci can interact with the complement system, but only a few will be discussed briefly. It is known that porin A (PorA) can bind C4BP, which can regulate the CP by inactivation the C3 convertase and by changing active C4b to its inactive form. Furthermore, factor H (fH) is also involved and can bind to fH binding protein (fHbp), which is a surface-exposed lipoprotein of the pathogen. Binding of fH to fHbp leads to down- regulation of AP. Furthermore different surface proteins are virulence factors e.g. *Neisserial* Heparin Binding Antigen (NHBP) and *Neisserial* adhesion A (NadA). NadA is important for adhesion and entry into nasopharyngeal epithelial cells.[7,16]

1.4 Vaccine progress against N. meningitidis

The six serogroups (A, B, C, W-135, X, Y), which cause meningococcal disease, are different in their capsular polysaccharides (CPS). Serogroup B, C, W-135, X and Y have structure similarity, because their CPS is covered with sialic acid (Neu5Ac, *N*-acetyl-neuraminic acid). In serogroup B (MenB) the sialic acid is α(2-8) linked. This linkage is identical to mammalian neural cell adhesion molecule, so MenB is not immunogenic. Serogroup C (MenC) is related to MenB, because it is a homopolymer of α(2-9) linked sialic acid. W-135 and Y contain heteropolymers combined of disaccharide items of sialic acid with either galactose or glucose and shows a similarity of more than 99 %. However in serogroup A (MenA) no sialic acid is present, but the capsule is expressed of (→6)- α-D-Man*p*NAc-(1→OPO_3→). Furthermore serogroup A, C, W-135 and Y can diversify by their rate of O-acetylation.[17]

In the late 1960s the first vaccine against MenC was discovered at the Walter Reed Army Institute of Research (Washington, D.C.) by Gottschliek, Artenstein, and Goldschneider and is based on the CPS of MenC[18]. Until now many polysaccharide meningococcal vaccines were licensed and they are highly successful drugs for military recruits or household contacts of concerned individuals[19]. In the early 1980 a quadrivalent A, C, W-135 and Y polysaccharide vaccine was licensed in the U.S. and showed over 85 % efficacy against MenA and MenC. In younger children (< 2 years) this vaccine showed poor immunogenicity except for the MenA component. Furthermore, a big hindrance of polysaccharide vaccines is their failure of generating memory cells for immunisation. So the protection only lasts a short term and every 3 - 5 years a new dose is necessary. The frequent donation can cause hyporesponsiveness (= reduced antibody response)[20]. In China and Egypt MenA polysaccharide vaccines are still in use for routine immunisation[19].

The short time protection can be increased by coupling the polysaccharides to a protein as a carrier molecule. The conjugation leads to T-cell dependent immunity and memory response. In 1999 the U.K. was the first country to license a polysaccharide- protein conjugate vaccine against MenC, where tetanus toxoid was used as carrier. The vaccine showed an effectiveness of 90 %, but only 66 % for infants at 4 months of age or younger. The amount of bactericidal antibodies resulting from the immunisation cannot be sustained in infants, so a booster injection for infants is necessary. Now tetravalent A, C, Y, W-135 vaccines coupled either on Diphtheria toxoid or Diphtheria toxin mutant are also licensed[21]. Recently the monovalent conjugate vaccine MenAfriVac against MenA was developed. Mass vaccination in the meningitis belt with MenAfriVac averted epidemics caused by serogroup A[20,22]. Now different polysaccharide vaccines based on CPS are licensed, which are different in their O-acetylation of the polysaccharide, protein- conjugate, conjugate chemistry or adjuvant (**Table 1**)[17].

Table 1 Overview of polysaccharide vaccines based on CPS with proteins as linker[19]

Vaccine	*Manufactor*	*Serogroups*	*Protein conjugate*
Menveo	Novartis Vaccines	A, C, Y, W- 135	Diphtheria cross
Menveo	Novartis Vaccines	A, C, Y, W-135	CRM197
Menactra	Sanofi Pasteur	A, C, Y, W-135	Diphtheria toxoid
Meningitec	Neuron Biotech	C	CRM197
Menjugate	Novartis Vaccines	C	CRM197
NeisVac-C	Baxter Bioscience	C	Tetanus toxoid
Menitorix	GlaxoSmithKline	C	Tetanus toxoid
MenAfriVac	Serum Institute of India	A	Tetanus toxoid
MenHibrix	GlaxoSmithKline	C & Y	Tetanus toxoid

Serogroup B causes the most frequent meningococcal disease. Due to the effect that the CPS of MenB is similar to human tissue, no polysaccharide vaccine based on CPS can be designed. This is why outer-membrane vesicles were used for vaccines, e.g. lipooligosaccharide, outer membrane proteins, periplasmic proteins and phospholipids. In Cuba an outer membrane vesicle (OMV) vaccine was the first licensed MenB vaccine and showed 83 % efficacy. The meningococcal rate of 14.4 per 100.000 people could be reduced to 0.8 cases due to a mass vaccination campaign. But the challenge was still the ineffectiveness in young children[23]. Porin protein A (PorA) also was used in OMV vaccines. In the 1990s a hexavalent OMV vaccine (Hexamen) was licensed. Hexamen comprised two OMVs, which express three PorA types[24]. Due to the effect that PorA is antigenically variable, the vaccines can protect against MenB disease, but the protection is limited to the vaccine strain. Furthermore, since meningococcal outer membrane proteins can subject an antigenic shift or gene deletion, OMV vaccine development is restricted[16]. Additionally, the ineffectiveness in infants remained still a challenge.

A new approach for vaccine development against MenB uses reverse vaccinology. The concept of reverse vaccinology describes the analysis of all surface proteins or secreted ones by its genome sequence to find novel antigens for the improvement of new vaccines. The proteins are screened whether they can bind onto an antibody and activate the immune system. MenB was the first pathogen using reverse vaccinology. Due to this approach 29 novel antigens, which can elicit an antibody, are found. 2013 the first MenB vaccine 4CMenB (Bexsero, Norvatis Vaccines and Diagnostics S.r.l.) was licensed by the European Medicines Agency based on reverse vaccinology. Bexsero contains three main antigens: *Neisserial* heparin-binding antigen (NHBA), factor H-binding protein (fHbp) and *N. meningitidis* adhesion A (NadA)[25]. Furthermore Bexsero is the first vaccine for infants, but still booster injections are necessary to guarantee immunsiation[26]. Now there is also rLP2086 vaccine (Trumenba, Pfitzer, Inc.) licensed in Europe since 2016.

1.5 Inner LOS core structure in N.meningitidis as potential vaccine component

The inner core of LPS or LOS is a well reported target in many pathogenic bacteria e.g. *Escherichia coli*, *Moraxella catarrhalis*. In *N. meningitidis* the inner core is a hexasaccharide, which is attached to the lipid A *via* 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) and to the outer core *via* glucose. Furthermore the α(1 → 2) substitution by *N*-acetyl-D-glucosamine (GlcNAc) to L-*glycero*-D-*manno*-heptopyranose (HepII) is exceptional. KdoI is further linked to another Kdo (KdoII) and to a Heptose (HepI). HepII can be substituted with 2-aminoethylphosphate (PEtn) linker. Substitutions are reported for 3-OH and 6-OH at HepII and monosubstituted 3-PEtn is present in 70 % of known serogroups (**Figure 5**).

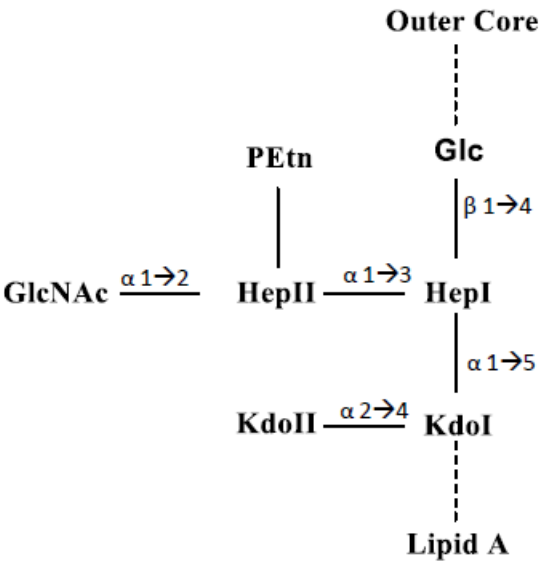


Figure 5 Inner core of *N.meningitidis*

Moreover, a polyclonal immune response to LOS inner core was reported and six murine monoclonal antibodies (mAb) based on the inner core were cloned and studied by Gidney et al.: L3B5, L4A4, L4-7, L5-10, L2-16 and LPT3-1 (**Table 2**)[27].

Table 2 Activitiy of the six murine cloned antibodies based on the inner core of N.meningitidis. Assumed from Parker et al.[28]

Antibody	*Subclass*	*HepII substitution*					
		None	3-PEtn	6-PEtn	3,6-di-PEtn	3-Glc	3-Glc, 6-PEtn
L3B5[c,d]	IgG3	-	++	-	-	-	-
L4A4[c]	IgG2a	++	-	++	-	++	++
L4-7[c]	IgG2a	++	-	++[a]	-	-	-
L5-10[c]	IgG1	-	-	+[a]	-	++	++
L2-16[c,d]	IgG2b	-	-	++	-	-	++
LPT3-1[c,d]	IgG2a	++	++	++[b]	-	-	-

[a] *acylated lipid A required for binding*

[b] *will only bind truncated* galE *mutants*

[c] *solid-phase indirect enzyme-linked immunosorbent assay (ELISA) with truncated* galE *mutants*

[d] *whole cell ELISA, immunofluorescence and IB assays with both wild-type and truncated* galE *mutants*

+ binding observed slightly over background

++ binding observed

-No binding observed

They differ in their cross reactivity whether they allow substitution with PEtn at various positions. All *N.meningitidis* strains from different collections can be recognised by using mAb L3B5, L2-16 and LPT3-1. So the panel of the six cloned mAB can be used for scanning antigens as potential vaccine candidates[27].To study the biological activity of mAb LPT3-1, Parker et al. performed X-ray crystallography of the antigen-binding fragment (Fab) with the dephosphorylate inner core structure (**Figure 6**). MAb LPT3-1 showed binding to 3-PEtn, 6-PEtn and to unsubstituted inner core. In total 9 hydrogen bonds between the antibody and non-substituted antigen could be observed. Except one bond all bind through GlcNAc and HepII. GlcNAc, HepII and Glc form the main epitope for mAb LPT3-1. Interestingly 3-OH of HepII forms two of the nine hydrogen bonds and substituted HepII with 3-PEtn can still bind. Furthermore, mAb LPT3-1 has some bactericidal activity, but a lower one than mAb L3B5. However mAb L3B5 only binds to 3-PEtn substituted inner core of *N. meningitidis*.[28]

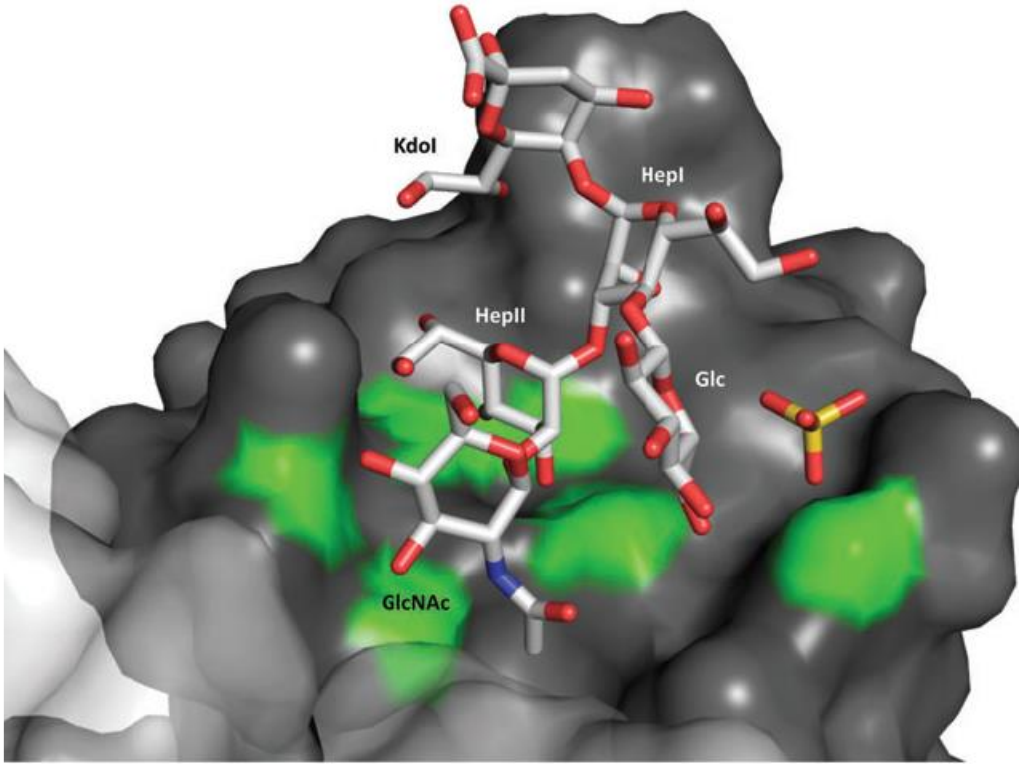


Figure 6 Surface of inner core antigen binding to Fab LPT3-1[28]

1.6 Biological relevance of phosphoethanolamine-substitution in N.meningitidis

Ram et al. identified LOS of *N.meningitidis* as acceptor for C4b that is part of the complement system (1.3). As the inner core is fluctuating by the presence of phosphoethanolamine linkage on HepII and of LNT, binding studies with mutants which lack different substitutions were considered and summarised in **Figure 7**. C4b can either bind via an amide bond to the phosphoethanolamine linker or *via* an ester bond to LNT. Binding of C4b to 3-PEtn was affected by the simultaneous substitution of LNT. When LNT is present, C4b binds preferred to LNT via an ester bond. Substitution of the phosphoethanolamine linker on HepII at position 6 shows a strong binding ability to C4b even in the presence of LNT. Furthermore, by bearing of 3-PEtn and 6-PEtn binding of 6-PEtn to C4b is favoured.[29]

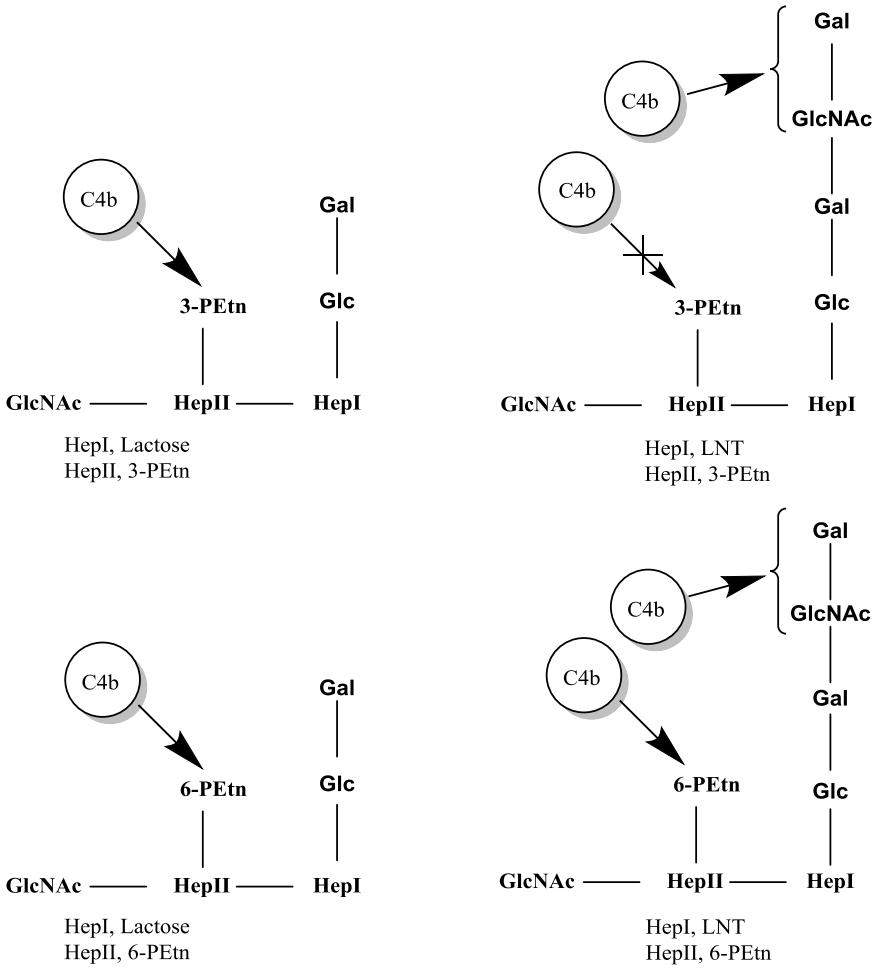


Figure 7 Schematic of the influence of different inner core structures in *N.meningitidis*, modified from Ram et al.[29]

6-PEtn substitution simplifies the activation of the complement system *via* C4b binding in comparison to 3-PEtn substitution. So these data could explain the preponderance of 3-PEtn (~70 % in all strains).[29]

2 Aim of the thesis

Due to isolation procedures from bacterial LPS to obtain the inner core structure of *N.meningitidis* the 2-aminoethylphosphate linker is not present in X-ray studies. Two phosphoethanolamine substitutions are possible and Olsson and Oscarson prepared fragments of the inner core with 6-PEtn substitution on HepII[30]. As shown in the biological studies in 1.6, the 3-PEtn substitution is of higher interest than the 6-PEtn substitution. The aim of the thesis was to synthesize components of the inner core structure of *N.meningitidis* with substitution on the HepII with a 2-aminoethylphosphate group.

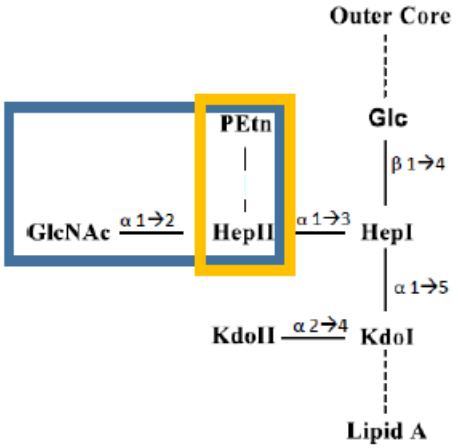


Figure 8 LOS inner core structure of N.meningitidis, with the planned synthesis of HepII substituted with 3-PEtn (yellow) and of disaccharide α-D-GlcNAc-(1→2)- α-Hep (blue)

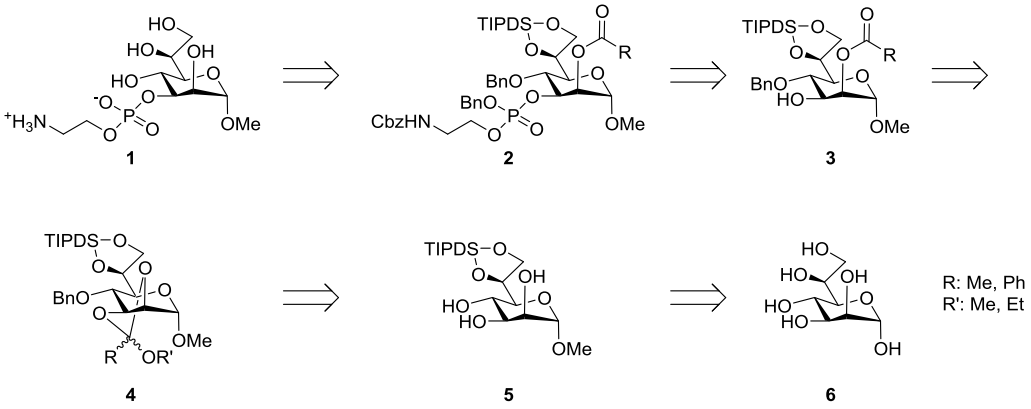
Two main goals were pursued within this thesis. On one hand the monosaccharide L-*glycero*-D-*manno*-heptose should be created by using different approaches to introduce a 2-aminoethylphosphate residue selective by the 3-hydroxyl group (**Figure 8**, yellow box).

On the other hand the approach towards the disaccharide α-D-GlcNAc-(1→2)- α-Hep (blue) which 2-aminoethylphosphate linker at 3-hydroxyl group of the heptose should also be investigated (**Figure 8**, blue box).

3 Results and discussion

3.1 Synthesis of Monosaccharide

3.1.1 Retrosynthesis of the Monosaccharide

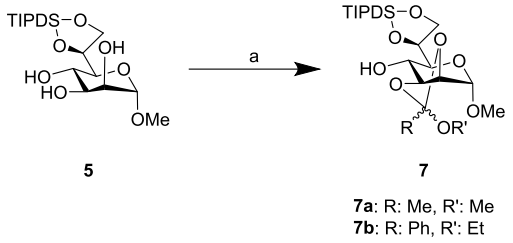


Scheme 1 Retrosyntetic analysis scheme of the monosaccharide (R=Me, Ph, R'=Me, Et)

Scheme 1 shows the retrosynthesis analysis of the final heptoside **1**, in which the 3- hydroxyl group is linked to 2-aminoethylphosphate linker. The protecting group pattern displays a fully protected heptoside **2**. The 6- and 7- hydroxyl groups are protected *via* a bifunctional silyl protecting group[31]. The formation of 2,3 orthoester in **4** allows the introduction of protecting groups e.g. a benzyl protecting group at the 4-hydroxyl group. The selective opening of the orthoester supports the phosphorylation at the 3-hydroxyl group to obtain **2**. The introduction of the 2-aminoethylphosphate can be either carried out by using the H-phosphonate method or by using the phosphoramidite approach[32,33]. In addition compound **2** could serve as glycosyl acceptor after selective cleavage of the 2-O-Ester. Finally the starting material **5** can be prepared from the fully unprotected heptose **6** in a two steps synthesis by using Fischer glycosylation to introduce the methyl group in α position and afterwards by using $TIPDSCl_2$ to introduce the bifunctional silyl protecting group[34,35].

3.1.2 Synthesis of the Monosaccharide in detail

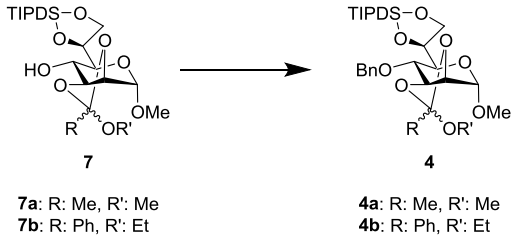
3.1.2.1 2,3 Orthoester formation



Scheme 2 Formation of the orthoester. a) trimethyl orthoacetate, CSA or triethyl orthobenzoate, CSA

The orthoester formation is a versatile diol protecting group strategy. For the TIPDS protected heptoside **5** the hydroxyl groups at 2-OH and 3-OH can be selectively protected by using trimethyl orthoacetate or triethyl orthobenzoate with catalytic amounts of CSA. The formation was fast (< 60 min, checked with TLC) and the products **7** were stable under neutral and basic conditions. **7** is a mixture of the exo and endo orthoester. As the acetate and benzoate orthoesters were very sensitive to hydrolysis, the following reactions were performed in a one pot reaction without further purification.

3.1.2.2 Benzylation approaches and outcome



Scheme 3 Introduction of the benzyl group at 4-OH

According to the retrosynthesis in 3.1.1 the next step was the introduction of a benzyl group at the 4-OH group. Benzyl ethers are important protecting groups for hydroxyl groups. They can selectively be introduced and removed easily by hydrogenation for example. As the silyl protecting group can be deprotected under acidic conditions, the introduction of the benzyl group was performed under neutral or basic conditions. Three different approaches were discussed[36,37].

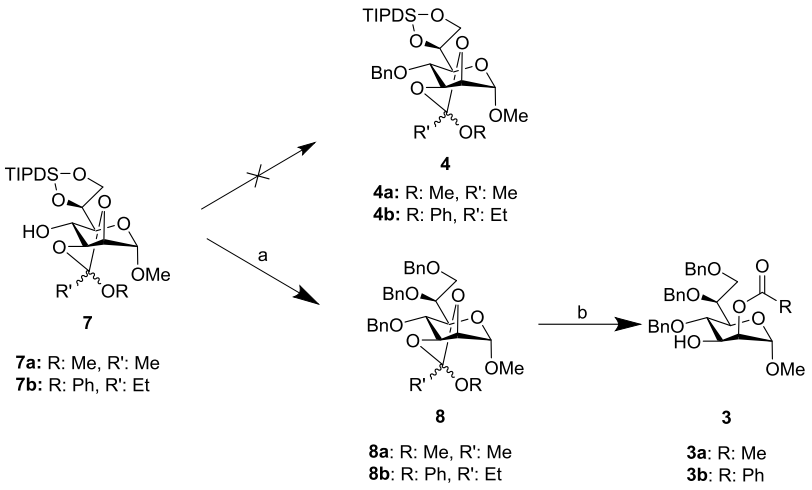
1) Benzyl halide and base
2) In situ activation of benzyl bromide
3) Benzyloxypyridinium triflate (Dudley's reagent) and MgO

1st approach:

Treatment of the orthoacetate **7a** and orthobenzoate **7b** with benzyl bromide and sodium hydride under varying conditions (**Table 3**) led to the same negative result.

Table 3 1st approach. Benzylation conditions with benzyl bromide and sodium hydride

Exp.	*R, R'*	*Benzylation reagents*	*Temp.*	*solvent*	*yield*	*Prod.*
1	R:Et, R':Ph	BnBr, NaH	r.t.	DMF	-	x
2	R: Me, R':Me	BnBr, NaH	r.t.	DMF	-	x
3	R: Me, R':Me	BnBr, NaH	0-4°C	DMF	-	x



Scheme 4 Benzylation method with desired product **4** and with the isolated product **8** and **3**.

a) benzyl bromide, NaH; b) CSA, H_2O

Treatment of 2,3 orthoacetate **7a** and 2,3 orthobenzoate **7b** with sodium hydride and freshly distilled benzyl bromide led to the same results and gave molecule **8**. The silyl protecting group was not stable during treatment with benzyl bromide and sodium hydride, a common base for the hydroxyl group deprotonation and the TIPDS group was cleaved off. Treatment of orthoesters **7a** and **7b** with sodium hydride and benzyl bromide led to deprotection of the silyl group and benzylation at the 4-, 6- and 7 hydroxyl group. Furthermore an experiment by adding just sodium hydride to the orthoester in DMF showed the instability under these conditions. Lower temperatures could also not prevent the deprotection of the TIPDS group. Treatment of **7a** with excess of benzyl bromide (> 6 equiv.) and sodium hydride (> 8 equiv.) to protect also 6-OH and 7-OH with benzyl groups was successful. Furthermore, the opening of orthoester **8** was carried out with CSA (cat.) and H_2O to obtain **3**.

The NMR spectrum in **Figure 9** however, showed the rearranged orthoester **3a**, whereas the shift of H2 indicates the 2-OAc protecting group pattern.

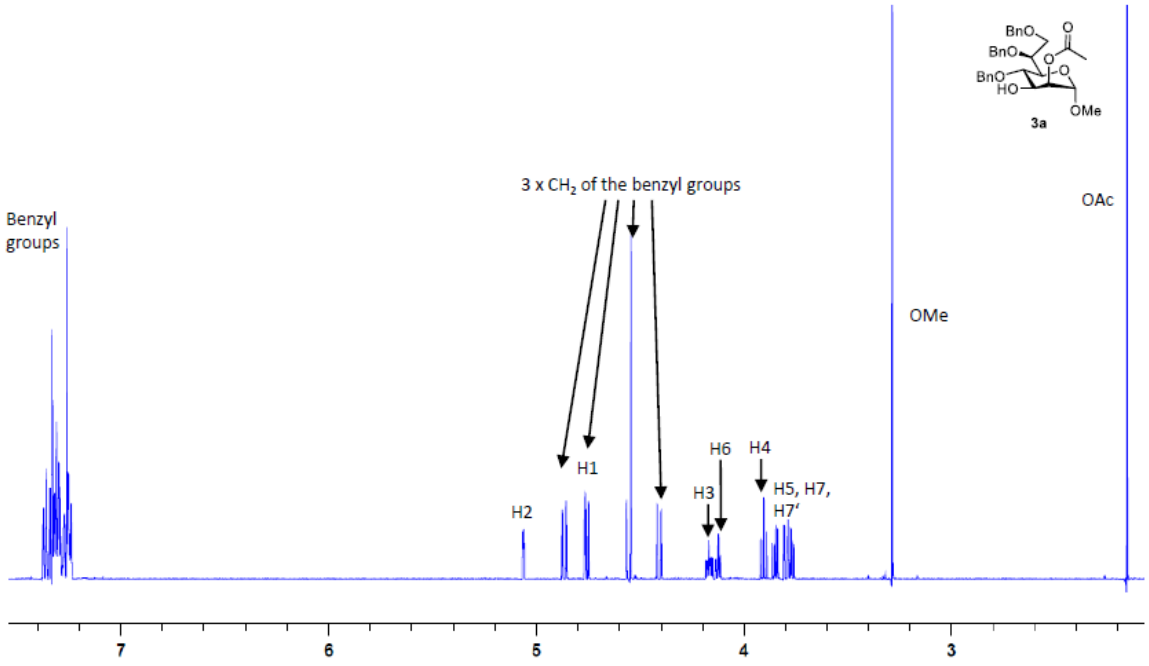


Figure 9 ^{1}H-NMR spectrum of **3a**

However, **3a** could not be isolated in a better yield than 12 %, thus alternative approaches were investigated.

2nd approach:

The 2nd approach was an in situ activation of the benzyl bromide. Either by activation of the carbon next to the bromide increasing the electrophilicity to facilitate the nucleophilic attack of the hydroxyl group, or by generating benzyl iodide in situ with TBAI (**Table 4**).

Table 4 2nd approach: Benzylation conditions by activation of benzyl bromide

Exp.	*R, R'*	*Benzylation reagents*	*Temp.*	*solvent*	*yield*	*Prod.*
1	R: Me, R':Me	Ag_2O, BnBr	r.t.	DCM	-	x
2	R: Me, R': Me	NaH, BnBr, TBAI	0-35 °C	THF	-	x

Scheme 5 Benzylation method by activation of benzyl bromide

The first benzylation method was mild and used using silver (I) oxide and benzyl bromide in DCM[38]. Silver (I) oxide can form complexes to the bromide to generate an electrophilic carbon so the oxygen can attack and form the benzylation product[39]. In our hands treatment of 2,3 orthoacetate with Ag_2O (2 equiv.) and benzyl bromide (3 equiv.) in a light protected flask was not successful and no conversion was observed. The other benzylation method was an in situ activation of benzyl bromide with *tert*-butylammonium iodide (TBAI), which led to the formation of benzyl iodide *via* a Finkelstein reaction[40]. Benzyl iodide is much more reactive than benzyl bromide, because the iodide is a better leaving group than the bromide. Also in this reaction sodium hydride reacts as base. Because no conversion at 0°C with TBAI (cat.), NaH and BnBr could be observed, the reaction was allowed to stir at r.t. up to 35 °C. But unfortunately the in situ activation was not successful, since also here sodium hydride deprotected the silyl group.

The 3rd benzylation method is known as Dudley and can be carried out under neutral conditions.

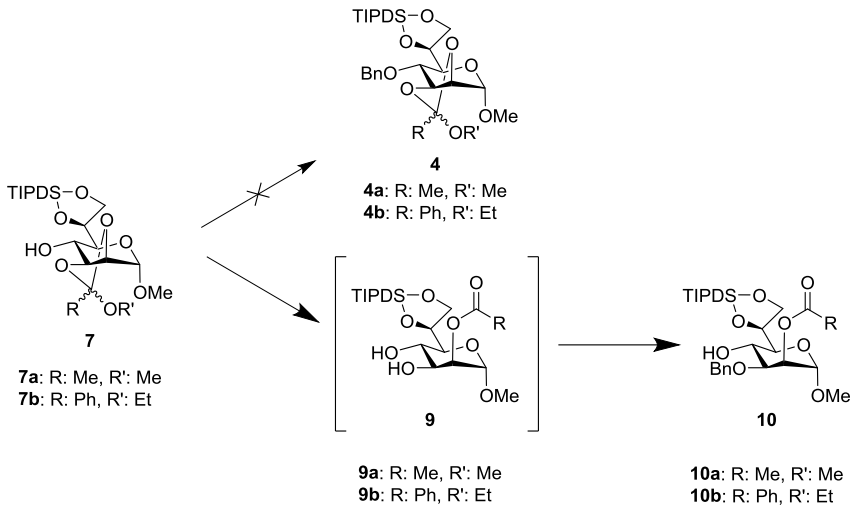
3rd approach:

Benzyloxypyridinium triflate (Dudley's reagent) can be prepared in a two steps synthesis starting from 2-chloropyridin[37]. This method allows neutral benzylation and the addition of MgO acts as acid scavenger.

In **Table 5** the conditions are summarised.

Table 5. 3rd approach. Benzylation conditions with benzyloxypyridinium triflate and MgO

Exp.	*R, R'*	*Benzylation reagents*	*temp*	*solvent*	*yield*	*Prod.*
1	R:Et, R':Ph	Dudley's, MgO	85°C	toluene	-	x
2	R: Me, R':Me	Dudley's, MgO	85°C	trifluorobenzene	-	x



Scheme 6 Benzylation with Dudley's reagent with the desired route to get **8** and the outcome to **10**.

Dudley's benzylation method was carried out with 2,3 orthoacetate **7a** as well as 2,3 orthobenzoate **7b**. Unfortunately the desired product **4** could not be isolated. It seems that 2,3 orthoacetate was not stable, so during the reaction it was opened to **9** as intermediate. **9** was benzylated, but interestingly only at 3-OH group. **10a** could be obtained in 43 % yield over two steps. Then 2,3 orthobenzoate **7b**, known to be a more stable orthoester, was treated with Dudley's reagent and MgO, but also this orthoester was not stable during the reaction and **10b** was isolated again in a lower yield (16 % over two steps). The lower yield for 2,3 orthobenzoate-reaction can be explained by the more stable 2,3 orthobenzoate than the comparable 2,3 orthoacetate. The orthoester was not stable during the reaction and hydrolysis opened it to **9**. Then only the 3-hydroxyl group was benzylated. Due to the failure of purification of the reaction only **10** can be isolated. The structure of **10** and the selective benzylation at 3- hydroxyl group was assigned by NMR data. **Figure 10** and **Figure 11** show the COSY and HSCQ spectra of **10.** The COSY spectrum showed a coupling of H4 to a hydroxyl group (marked in yellow), which has no carbon coupling in the HSQC spectrum. Furthermore, the orthoacetate signal was missing and an acetate signal ~2 ppm indicated the opened orthoester. The downfield shift of the H2 signal signified the 2-OAc protecting group pattern. **10a** however, could further be processed into a suitable glycosyl acceptor (e.g. by silylation at 4-OH group followed by deacetylation).

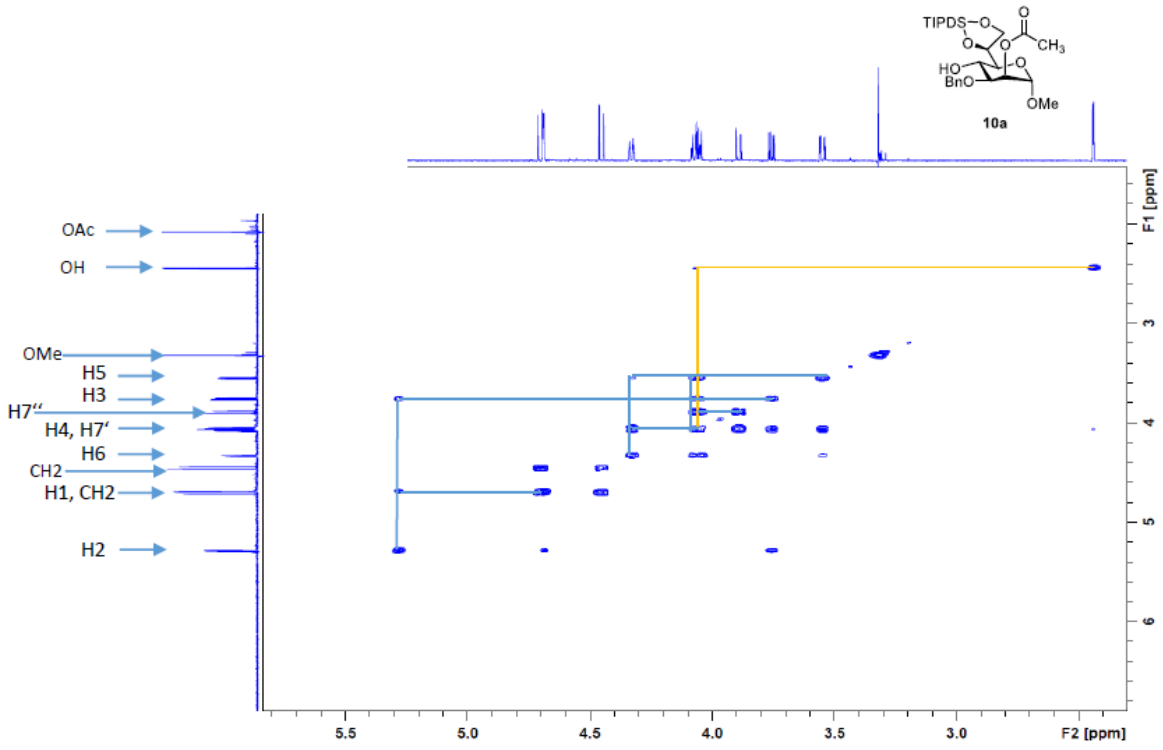


Figure 10 Detail of COSY spectrum of the undesired product **10a**

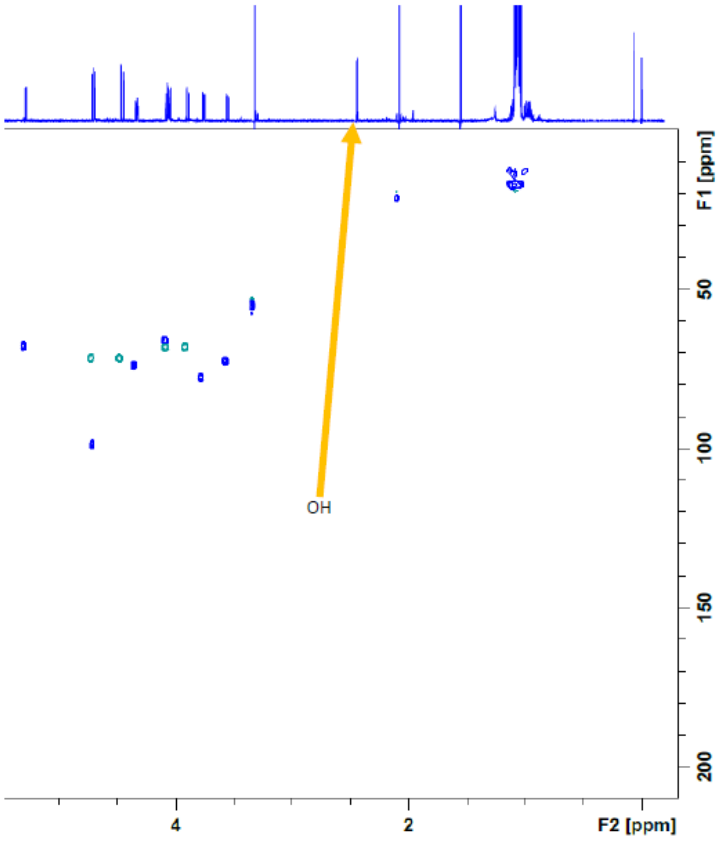
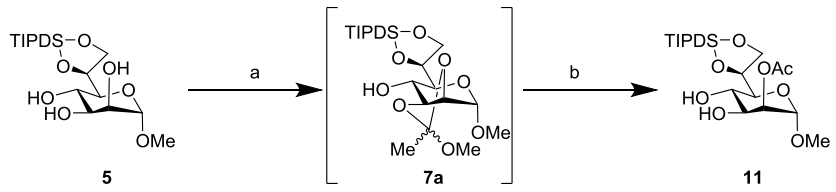


Figure 11 Detail of HSQC spectrum of **10a**

To summarise the 3rd approach. Unexpected hydrolysis during the reaction led to opening of both orthoesters. The opening of both orthoesters was much faster than the benzylation by Dudley, so the desired product could not be isolated. Interestingly **10** was detectable as main product, so that position 3 was more likely benzylated than the 4-hydroxyl group.

In all three approaches the desired product **4** was never observed nor isolated. The 1st described method showed stability of both orthoesters **7**, but sodium hydride could cleave the TIPDS protecting group. Also the activation of the benzyl bromide by using neither silver (I) oxide nor TBAI led to **4** as confirmed by the 2nd approach proofs. Benzylation with Dudley's reagent indicated that both orthoesters were not stable, however the benzylation occured selectively by 3-OH. This fact suggested that 3-OH was more reactive than 4-OH group. This result was surprising and raised the question whether one could observe the same effect by phosphorylation with the opened orthoester **11**. To test this hypothesis, the orthoester was initially installed and selectively opened to position 2 to generate the starting material **11** for the phosphorylation.



Scheme 7 Formation of 2,3 orthoacetate and selective opening to **11**. a) Trimethyl orthoacetate, CSA b) H_2O

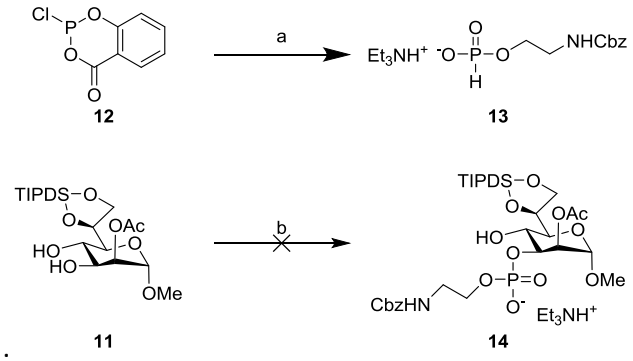
Since 2,3 orthoacetate is less stable than 2,3 orthobenzoate and the aim was to open the orthoester **7** immediately and selectively to 2-OAc, it is recommended to use the more labile **7a** to increase the yield. Treatment of **5** with trimethyl orthoacetate and CSA (cat.), led directly to the 2,3 orthoacetate **7a** (< 60 min). Hydrolysis of the intermediate **7a** induced the axial acetate in good yields (> 70 %). The opening to the more favoured axial position has steric and stereoelectronic effects[41]. Due to the effect of migration of acetyl groups, the acetyl group can also migrate to the equatorial hydroxyl group at the position 3 (< 10%), but the two isomers can be separated *via* HPLC[42].

3.1.2.3 Phosphorylation

As described in 3.1.2.2 the 3-OH group showed higher reactivity during the benzylation with Dudley's reagent than the 4-OH group. This effect should be exploited to introduce the 2-aminoethylphosphate linker selectively at 3-OH group. For the phosphorylation two approaches will be discussed.

3.1.2.3.1 H-Phosphonate methodology

Van der Boom and Seta described first a way to introduce phosphodiesters in carbohydrates by using an H-phosphonate[43,44]. H-phosphonates can be prepared using tri (1-imidazolyl)phosphine, 2-chloro-1,2-benzodioxaphosphorin-4-one or diphenyl phosphite und then can be either coupled with a linker (e.g. ethanolamine) or can be hydrolysed to give in the end a monoester. The next step is the coupling to another hydroxyl group (e.g. carbohydrate) and then an in situ oxidation by treatment with aqueous pyridine and iodine. The H-Phosphonate can be coupled also first to a glycoside and then to a linker, so that two different approaches lead to the desired phosphorylated glycoside. High effectiveness and high reaction rates in all three steps make this methodology favoured[32]. Additional advantages are that the linker on the H-Phosphonate can be exchanged and that there is no additional protecting group on the phosphorus. The lack of the P- protecting group decreases also the ability of acting as leaving group and therefore the yield can be increased.

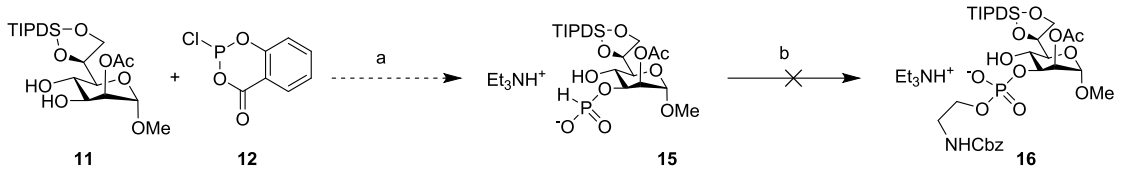


Scheme 8 Introducing the H-Phosphonate **13** in the heptoside **11** to receive **14**. a) Cbz-ethanolamine, TEAB (1 M, pH 8)

b) PivCl, **14**

According to Lay, the following synthesis was carried out (**Scheme 8**)[45]. The H-Phosphonate was coupled at first to the Cbz-protected ethanolamine and then to the heptoside **11** to receive **14**. Treatment of 2-chloro-1,2-benzodioxaphosphorin-4-one (salicyl chlorophosphite, **12**) with Cbz-protected ethanolamine gave the desired H-Phosphonate as triethylammonium salt **13** with the significant coupling constant of ~620 Hz in the ^{1}H coupled ^{31}P spectrum[45]. The next step was the phosphorylation with the heptoside **11** in which PivCl (1.2 equiv.) was used as activation reagent of the

leaving group and the following oxidation with iodine in aqueous pyridine were unsuccessful. No coupling with the heptoside **11** was detected by NMR spectra of the crude product.



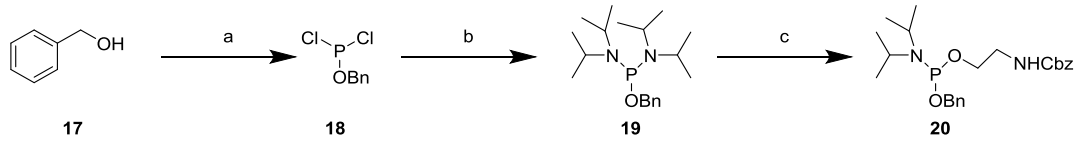
Scheme 9 Coupling of the H-Phosphonate at first to **13** and then to ethanolamine. a) TEAB (1 M, pH 8)

b) PivCl, Cbz-ethanolamine

The other possibility was the introduction of H-Phosphonate first to the heptoside **11** by using salicyl chlorophosphite **12** and then coupling to ethanolamine as linker (Scheme 9). The first step was successful (coupling constant ~620 Hz in the ^{1}H coupled ^{31}P spectrum) but also here the final coupling of the ethanolamine with PivCl (1.2 equiv.) remained unsuccessful[45]. Due to the lack of purification of **15**, the final conclusion whether the H-Phosphonate at **15** was located at 3-OH or 4-OH position cannot be drawn.

3.1.2.3.2 Phosphoramidite approach

Besides the H-Phosphonate methodology van Boom applied another approach for the introduction of phosphor moieties to a glycoside- the phosphoramidite approach[46]. Amidites show a strong p_{π}-d_{π} interaction of the N atom of the diisopropylamine group and the P-atom, so weak acids (e.g. 1*H*-tetrazole) are able to protonate and can so activate the amidite for the coupling with the glycoside[33].



Scheme 10 Phosphoramidite synthesis, a) PCl_3, b) diisopropylamine c) Cbz-ethanolamine, 1*H*-tetrazole

The intermediate **18** is very prone to hydrolysis, so the intermediate was not purified and the reactions were carried out in a one pot reaction. The reaction rate was checked by ^{31}P-NMR, so the reaction can be monitored easily (**Figure 12**). The synthesis of the desired phosphoramidite **20** was carried out starting from benzyl alcohol **18** (1 equiv.) and phosphor trichloride (1.3 equiv.). After 3.5 h the conversion to **18** was complete and showed the desired ^{31}P-NMR shift at 177 ppm. Treatment of **18** with diisopropylamine led to the stable intermediate **19** (~123 ppm). Coupling of **19** with Cbz-ethanolamine afforded the desired product **20** (~148 ppm). The shifts around 10 ppm showed hydrolysed material, in which it cannot be verified whether the material hydrolysed during the NMR

experiments or in the reaction. But the small amount of hydrolysed material can be disregarded. All measured shifts of the ^{31}P-NMRs were according to published data (**Figure 12**)[47].

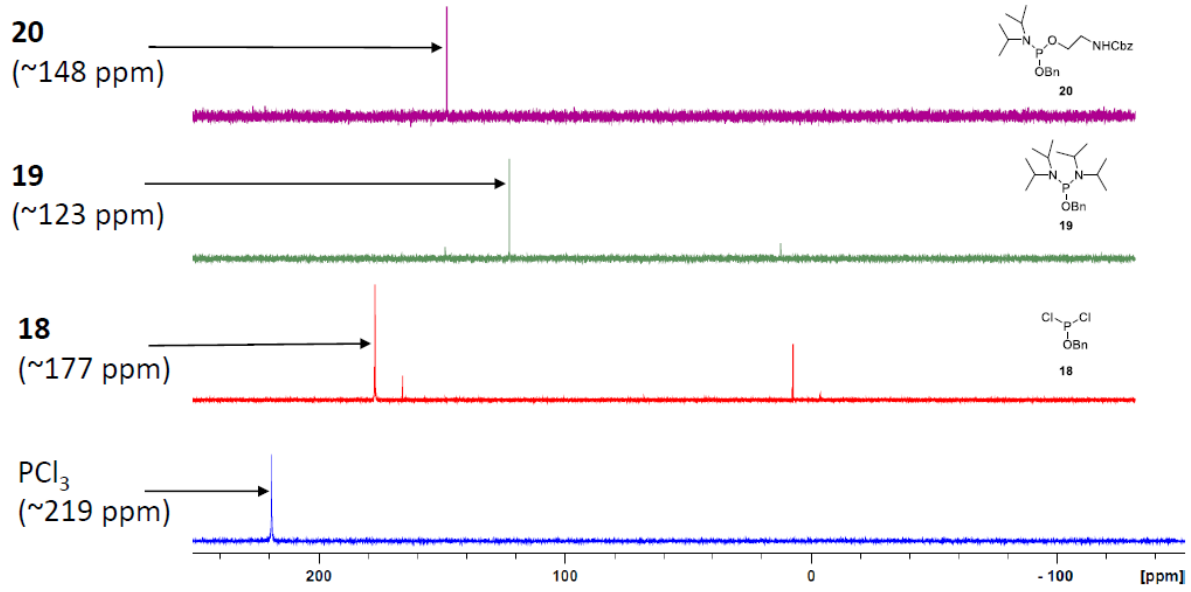
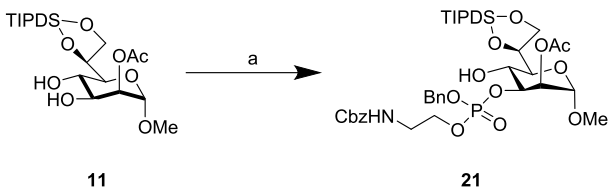


Figure 12 Comparison of the shifts during the synthesis of phosphoramidite **20**

The desired phosphorylation was carried out with molecular sieves (4 Å), 1*H*-tetrazole solution (0.45 M in ACN, 2 equiv.) and **20** (2 equiv.) to give **21** in 20 – 30 % after oxidation with *m*CPBA (**Scheme 11**).



Scheme 11 Phosphorylation by using phosphoramidite approach. A) 1*H*-tetrazole (0.45 M in ACN), **23** then *m*CPBA, -78°C

NMR analysis showed the successful formation of **21** (**Figure 13** and **Figure 14**). The ^{1}H-shift of H3 indicates a substitution and the ^{31}P-^{1}H HMBC spectrum shows the coupling of the single phosphorus to H-3 and to the benzyl protecting group of the phosphotriester.

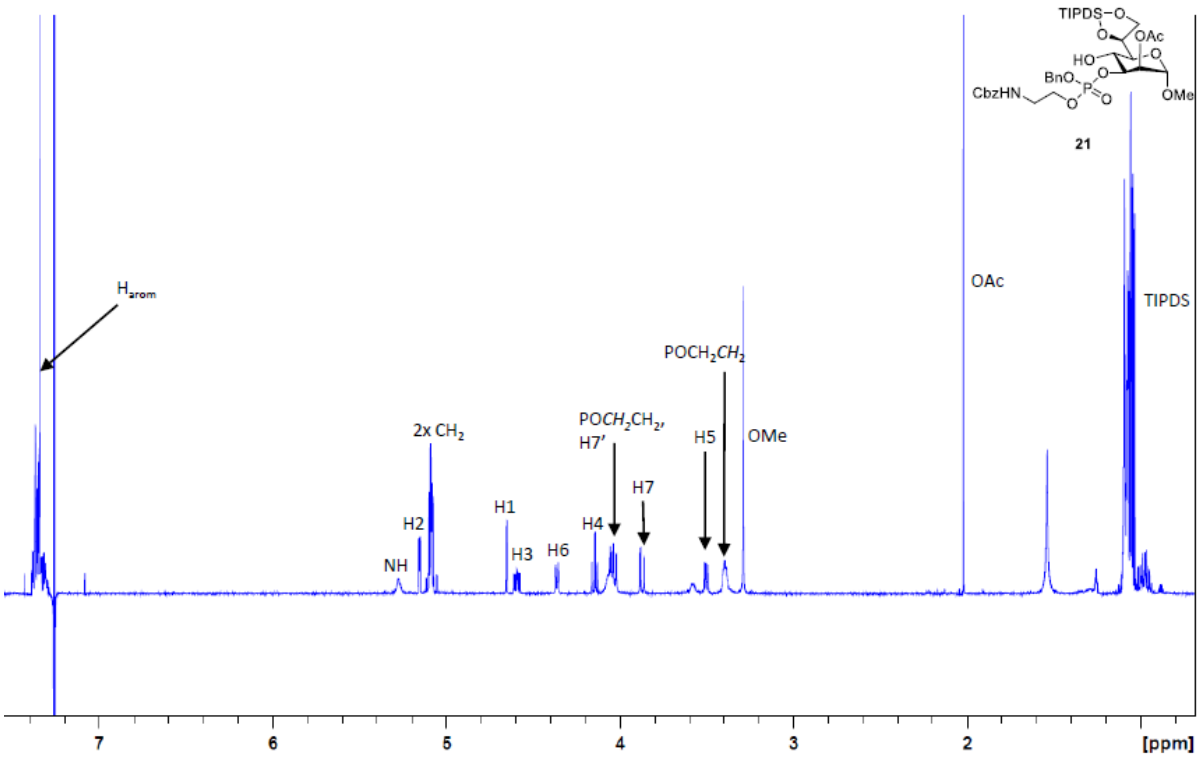


Figure 13 ^{1}H-NMR spectrum of monophosphorylated product **21**

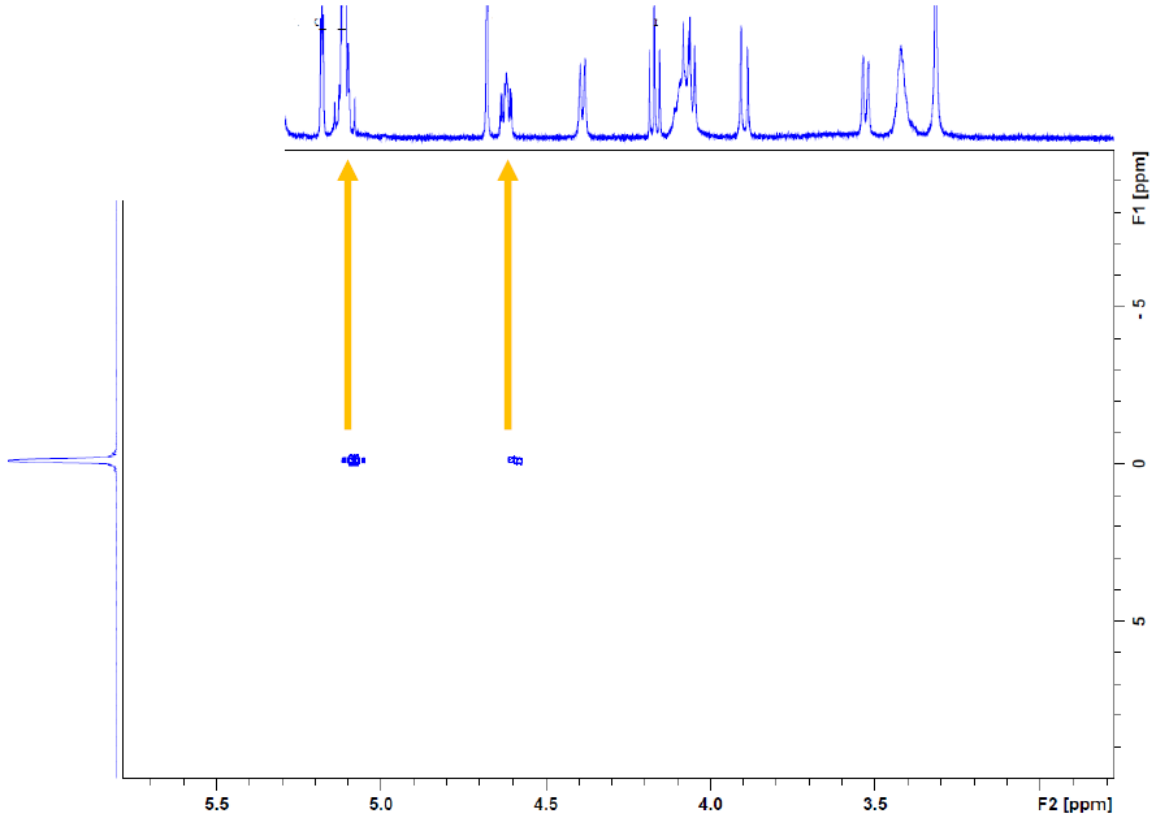
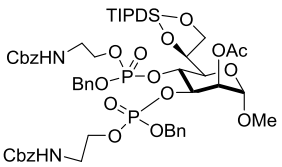


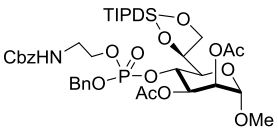
Figure 14 ^{31}P-^{1}H HMBC spectrum of the successful synthesis of **21**

Separation of the crude mixture with HPLC gave 30 % (over two steps) yield, whereas separation with gel column led to 20 % (over two steps). Nevertheless for starting material of more than 50 mg amount the gel column (Sephadex Bio-Beads SX-1) should be favoured, because the product decomposed on silica, although a two dimensional TLC showed stability. The yield could not be increased by using different equivalents of phosphoramidite, so the side products were analysed. The amount of side products was significant, but unfortunately the side products could not be isolated and purified by flash chromatography even after acetylation.



Exact Mass: 1202,44

22

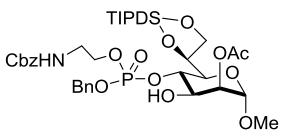


Exact Mass: 897,36

23

Scheme 12 List of side products according to LC-MS measurements

Due to unsuccessful purification of the side products no NMR was measured and the analysis after acetylation was performed *via* LC-MS, which showed two significant masses: 1203.4 and 898.4. The first mass would fit perfectly to a double phosphorylated heptoside **22.** The second one would could indicate a monophosphorylated heptoside, which is acetylated at the other free hydroxyl group. As the desired product **21** had already been separated from the side products, **23** could be the other regioisomeric side product (Rf values: **21**: 0.45, **24**: 0.19, hexane:ethylacetate 1:1). If these observations were correct, it would give the impression that 3-OH and 4-OH were phosphorylated by the phosphoramidite approach to give **22** and **24**.



Exact Mass: 855,34

24

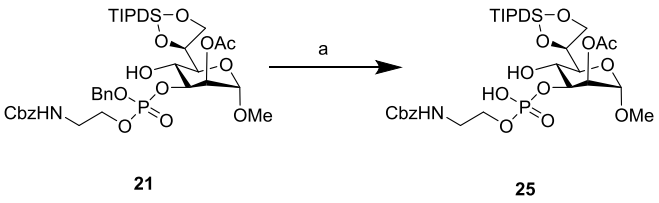
As LC-MS analysis gave only limited structural insights, no final proof of **22** and **24** as side products is given.

3.1.2.4 Deprotection of the monosaccharide

Two different deprotection approaches were carried out.

1) Beginning with the selective deprotection of the benzyl group to give a phosphodiester
2) Beginning with the deprotection of the bifunctional silyl protecting group.

At first the selective deprotection of the benzyl group will be discussed.



Scheme 13 Selective deprotection of the phosphate. a) Conditions summarised in Table 6

There are different strategies to deprotect a benzyl group. The most common one is by hydrogenolysis with Pd/C as catalyst[48]. The solvent effect on hydrogenation is remarkable and can influence the relative rate of the reaction. Additionally, the substitution pattern and the level of steric hindrance affect the outcome of hydrogenolysis. Furthermore, oxidative methods or Lewis acid- based deprotection is possible[36]. The challenge is the Cbz as protecting group of the amine. Benzyl carbamates are often protecting groups for amides. The formation is easy and the protection is effective under basic and neutral conditions.

1995 Sajiki described methods to cleave Cbz groups, olefin, benzyl esters and azides whereas the protecting group pattern of benzyl ethers were not changed[49]. Now our aim was the selective debenzylation, in which the Cbz protecting group should stay intact. As the Cbz can be cleaved during hydrogenolysis, no selective deprotection of the benzyl group is possible with Pd/C as catalyst[50].

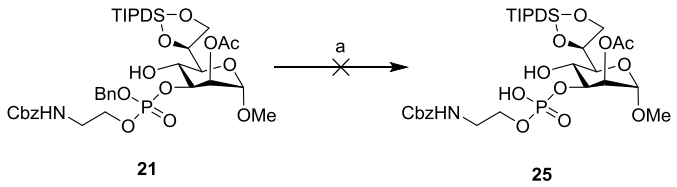
The planned deprotection of a benzyl in the presence of a Cbz group was not literature known, so the deprotection was carried out with two different reagents (**Table 6**).

Table 6 Selective debenzylation conditions

Strategy	*reagent*	*equiv.*	*Temp.*	*solvent*	*Reaction time*
1	TFA	1.5	r.t.	DCM	7 days
2a	*Tert*- $BuNH_2$	As solvent	r.t.	-	10 d days
2b	*Tert*- $BuNH_2$	As solvent	40°C	-	4 days

1st strategy:

In 1989 Perich and Johns described a mild deprotection of the benzyl group by treatment of an P-Tyr-Leu-Gly peptide with TFA[51]. Additionally, it is not literature known that TFA could cleave the Cbz protecting group. Now TFA is a well proven reagent for debenzylation in protected phosphate chemistry[52,53].

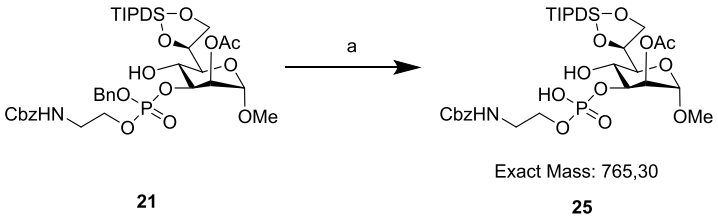


Scheme 14 Selective deprotection of benzyl group. a) TFA

During the reaction of one equivalent TFA to **21** no change of starting material could be observed. The 2-aminoethylphosphate linker as well the TIPDS and acetyl group showed stability according to MS measurements and TLC observations.

2nd strategy:

Gray and Smith showed that benzyl groups can be deprotected under basic conditions with *tert*-butyl amine and especially the volatility of the reagent/solvent eases the removal[54]. Additionally, Ellie et al described a selective removal of P(V)-benzyl with *tert*-butyl amine in the presence of other O-benzyl groups[55]. Besides it is not literature known whether Cbz groups were cleaved by *tert*-butylamine.



Scheme 15 Selective deprotection of the benzyl group. a) *tert*-butyl amine

Treatment of **21** with *tert*-butyl amine indicated slow conversion at r.t. in 7 days (strategy 2a) and better conversion after 4 days at 40°C (strategy 2b). In both cases the reaction was monitored by LC-MS and indicated a full conversion to a molecule with the mass of 766.5. NMR spectra (^{1}H, COSY, HSQC, ^{31}P) of the crude product were measured and indications for the successful conversion were found. It should be noticed, that the product was not pure, so the interpretation was difficult. In all spectra only the main product was further investigated. On the basis of the ^{1}H-NMR spectrum the protecting group pattern (OAc, TIPDS) was still consistent as well as the 2-aminoethylphosphate linker (**Figure 15**). Signals in the aromatic sector point either to Cbz and/or to benzyl group. The shift of H3 designates

the substitution of the 2-aminoethylphosphate. Additionally, the ^{31}P-^{1}H HMBC showed a coupling of the phosphorus with two signals, which can be identified as H3 and the ethanolamine linker (**Figure 16**, marked in yellow). The HSCQ spectrum (**Figure 17**) indicates only one O*CH*$_2$Ph signal either of the Cbz or the benzyl group (pointed out in red circle).

As there is no literature known by using *tert*-butylamine to deprotect the Cbz group and the mass fits to **25**, the desired product **25** might have been formed.

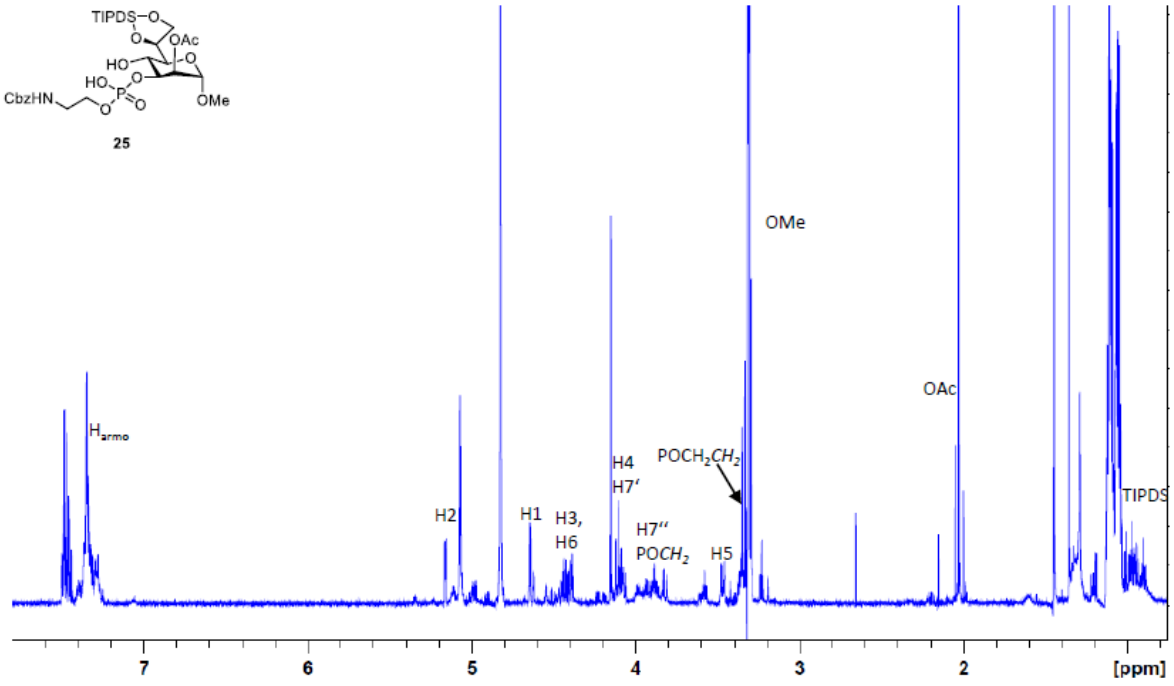


Figure 15 ^{1}H spectrum of **25** indicates all protecting group pattern

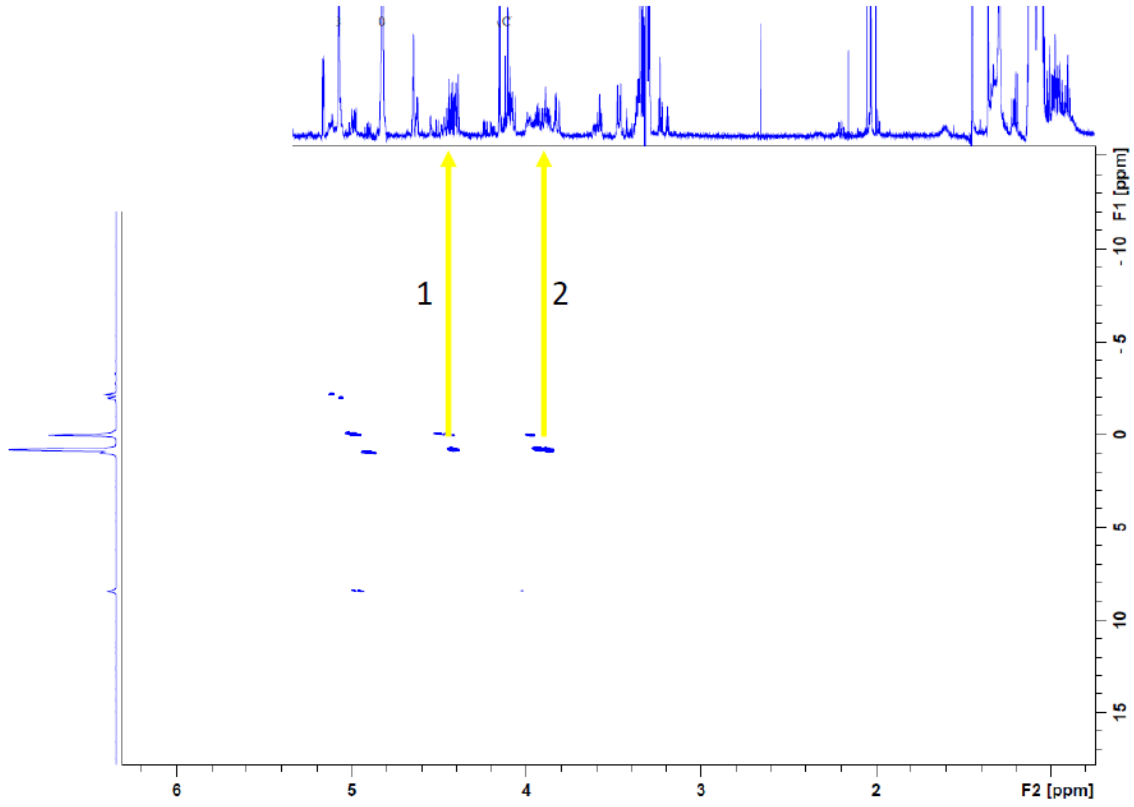


Figure 16 ^{31}P-^{1}H HMBC of crude **25**

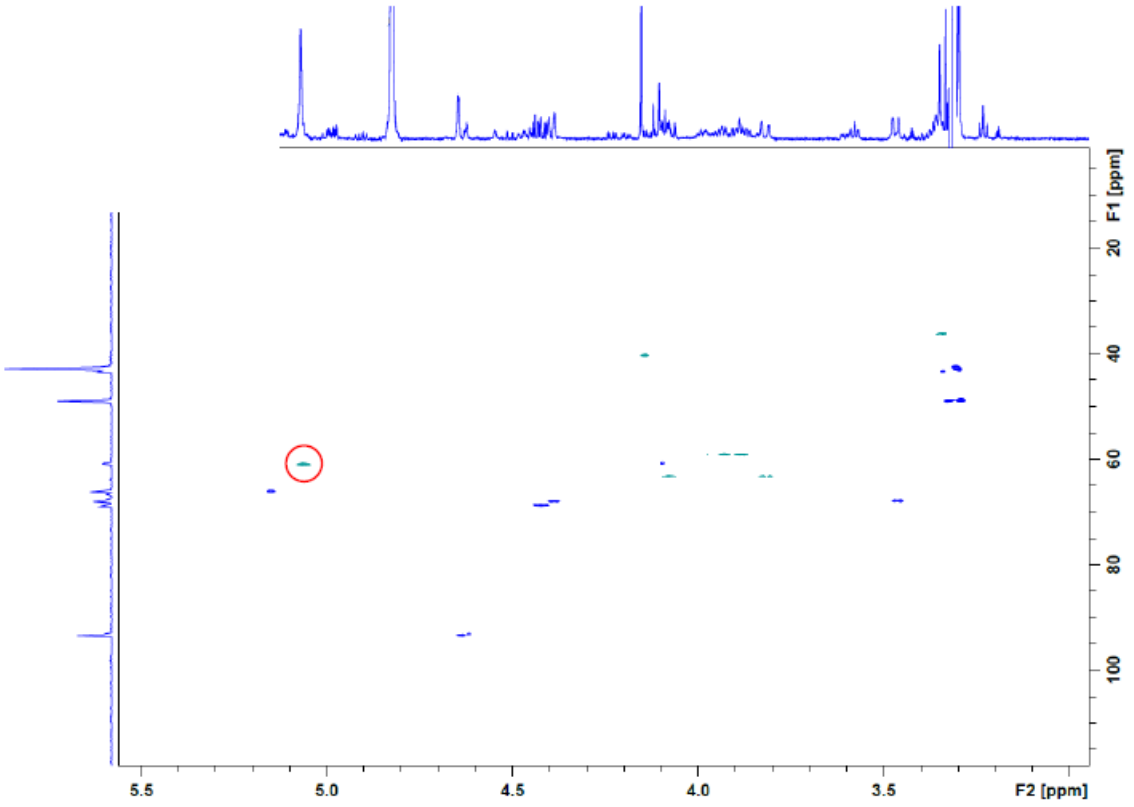
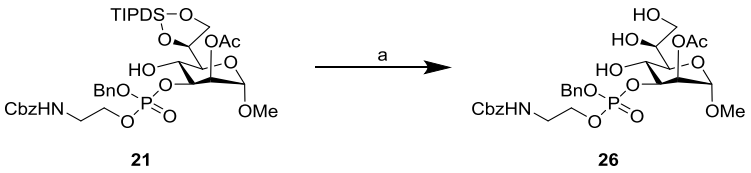


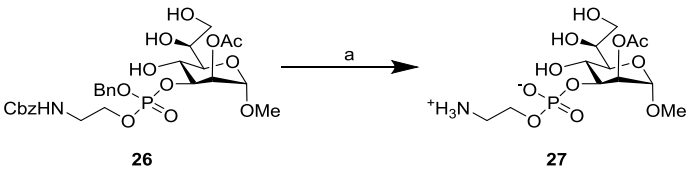
Figure 17 HSQC of **25** shows only one CH_2 group either of Cbz- or OBn- group

But due to the lack of pure product, the structure of **25** could only be assumed and was not fully proven. So the 2nd deprotection approach was carried out. This method dealt with the deprotection sequence starting with the TIPDS group.

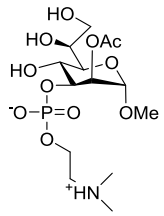


Scheme 16 Deprotection of the silyl group. a) TREAT, 0°C-r.t.

Treatment of **21** with TREAT (40 equiv.) over 17 h, after which the reaction was quenched with basic Dowex AG1X8 led to **26** in over 81 % yield after purification by column chromatography. Interestingly, quenching of TREAT with aqueous bicarbonate solution is not the way to go, because **26** was soluble in the water phase and could not be reextracted into the organic phase.



Scheme 17 Deprotection of the Bn and Cbz group to give **27**. a) Pd/C (10 %), H_2



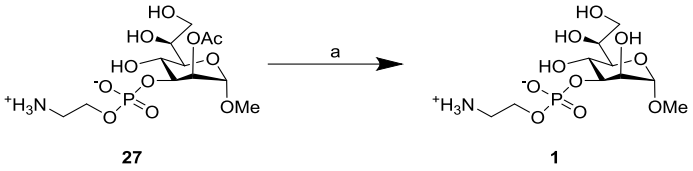
28

Removal of the Cbz and the benzyl group simultaneously was carried out by hydrogenation with Pd/C (10 %) as catalyst. When MeOH (100 %) was employed as solvent for the hydrogenation, LC-MS analysis showed mainly formation of a product with the mass of 418 which fits to the dimethylated product **28**. It seems that the formaldehyde, which is even in traces present in HPLC grade methanol, could act as source for N-methylation[56]. In 1992 Wakselman[57] also reported methylated side products by using MeOH as solvent for hydrogenation. Protonation with acetic acid in MeOH (1 %) of the resulting amine could overcome the formation of the side product and could increase the yield up to 93 % for the desired zwitter ion **27**.

The last deprotection step was carried out under two different conditions:

1) Zemplen saponification[58]

2) Saponification with TEA and water[59]



27 1

Scheme 18 Deprotection of OAc. a) TEA, H_2O

The Zemplen O-deacetylation *via* transesterification is a common reaction to remove esters under catalytic basic conditions[60]. The reaction was monitored *via* LC-MS analysis and LC-MS showed fast conversion to the desired product **1**. After 3 h MS data showed full conversion and the solution was neutralized with Dowex 50WX8. Apparently the product got stuck onto the Dowex WX8 and even after washing with basic ammonia solution (0.1 M, pH~9-10) no material was obtained. While the basic deprotection proved to be successful, the neutralization turned out to be more cumbersome than expected. As an alternative approach, a deprotection system that would allow the mild removal of the basic components by e.g. evaporating was tested. Treatment of **27** with TEA and water in MeOH showed full conversion after 7 days and the desired monosaccharide could be obtained in 62 % yield after purification on a Sephadex G10 column. The ^{1}H-NMR spectrum showed the successful synthesis of **1** as a triethylammonium salt (**Figure 18**).

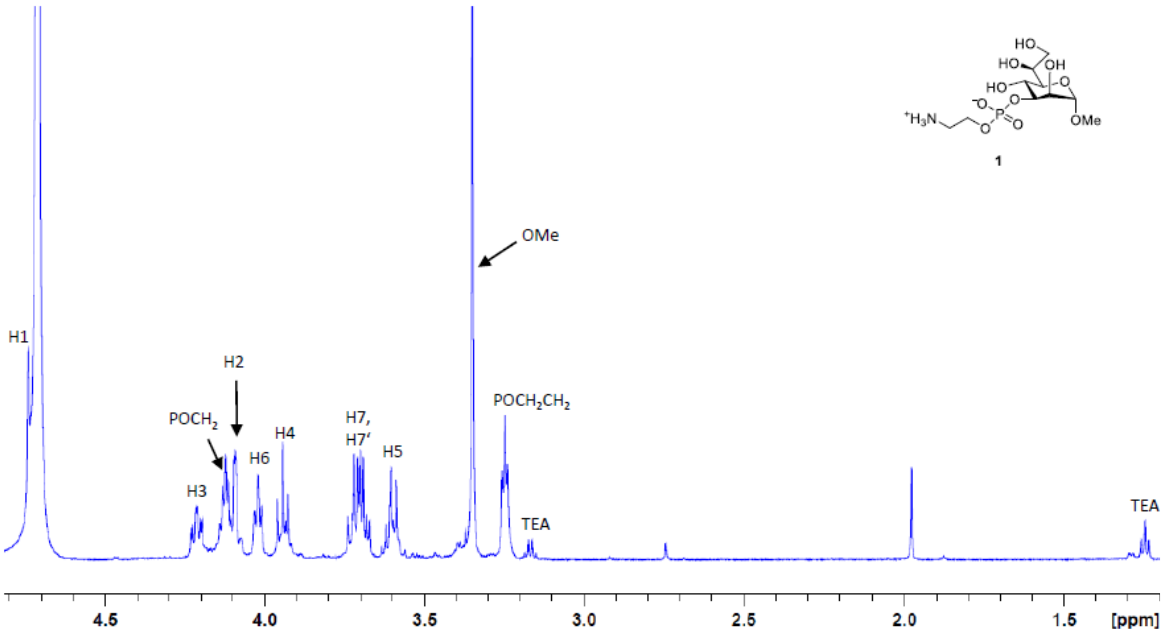
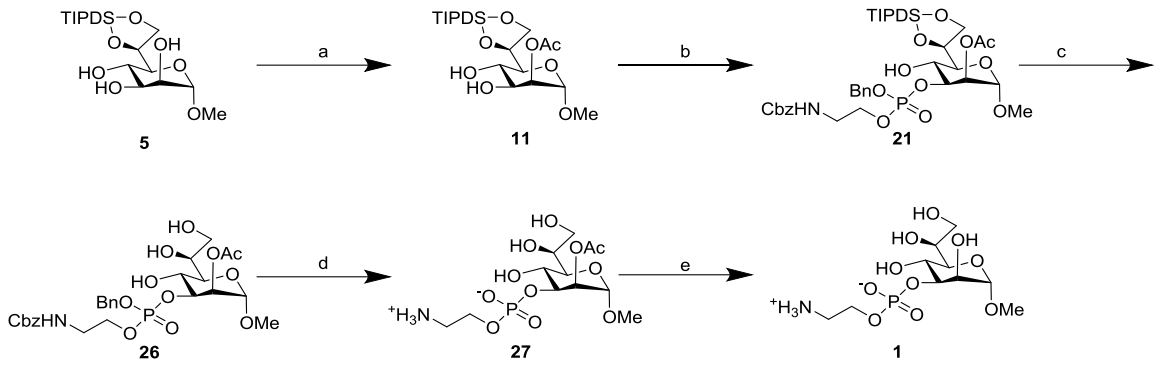


Figure 18 ^{1}H-NMR spectrum of **1** as triethylammonium salt

3.1.3 Overview of the final Synthesis



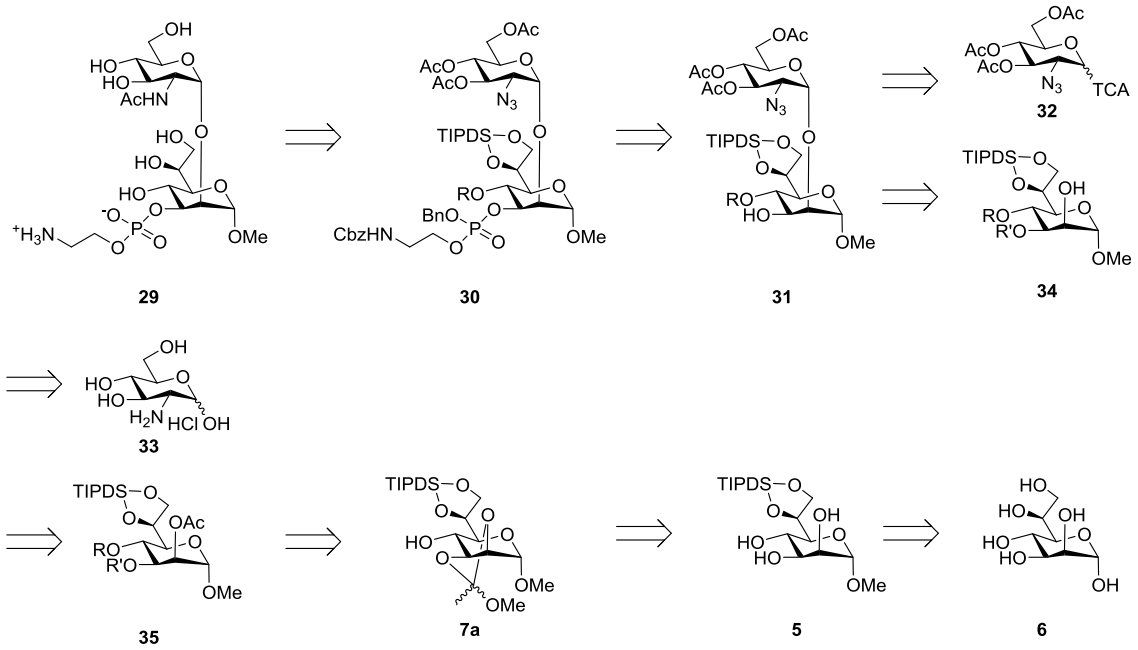
Scheme 19 Synthesis of Methyl-L-*glycero*-D-*manno*-heptopyranoside substituted at 3-OH with 2-aminoethylphosphate. Reagents and conditions: (a) i) trimethyl orthoacetate, CSA, DCM ii) H_2O, 71% (over two steps) (b) **20**, 1*H*-tetrazole (0.45M in ACN), *m*CPBA -78°C –r.t, 4 A molecular sieves, 30% (c) TREAT, 0°C –r.t., 81%, (d) H_2, Pd/C, MeOH + 1% Acetic acid, 93% (e) H_2O, TEA, 62%

The starting point of the synthesis was the TIPDS protected heptoside **5**, which is already protected at 6-OH and 7-OH with a silyl protecting group. The starting material was prepared in a two steps synthesis starting from L-*glycero*-D-*manno*-heptose[61]. According to Stanetty et al[34] the formation of an orthoester led to selective simultaneous protection of 2-OH and 3-OH, which can be opened by hydrolysis to receive **11** in good yields (71 % over two steps). The next step was the phosphorylation with **20** and the subsequent oxidation with *m*CPBA to obtain the desired phosphotriester **21**. The following deprotection was straightforward beginning by the silyl deprotection with TREAT to achieve **26**. Treatment with H_2 and Pd/C as catalyst in MeOH and 1 % acetic acid led to deprotection of Cbz and Bn groups, to form the zwitterion **27**. The final step was a saponification using TEA and H_2O in MeOH to give **1**.

3.2 Synthesis Approach towards the Disaccharide

First it should be stated, that during this thesis only preliminary studies for the disaccharide **30** were carried out and the main focus remained on the monosaccharide **1**.

3.2.1 Retrosynthesis of the Disaccharide



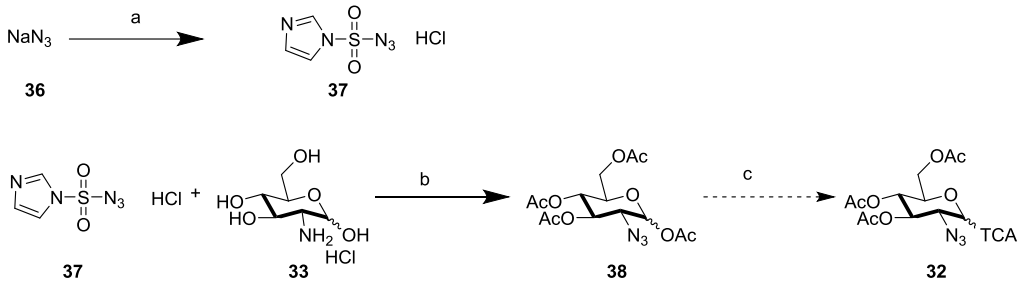
Scheme 20 Retrosynthesis scheme for the disaccharide **30**

29 shows the α-1,2 linkage of a phosphorylated heptoside and *N*-acetyl-glucosamine. *N*-acetyl protecting groups influence the glycosidic bonds due to neighboring group participation, so they favour a ß-linkage and not a α-1,2 linkage[62]. This is why the azide as non-participation neighboring group was chosen for the glycosylation to get the α-gycosidic bond in **29**[63]. Additionally, the azide is a temporary protecting group, which can be reduced easily to an amine. The glycosyl donor is a trichloroacetimidate **33** and can be prepared *via* established procedures over two step synthesis form **32**[64]. The synthesis of the acceptor should be based on the successful synthesis of the monosaccharide **1**. The formation of the 2,3 orthoacetate in **7a** allows the introduction of a protecting group at the 4-hydroxyl group. Furthermore, with the regioselective opening of the orthoester to 2-OAc another protecting group can be introduced at 3-OH group. The phosphorylation with 2-aminoethylphosphate linker should be carried out after glycosylation by using the phosphoramidite method. A closer look at the protecting group pattern of **35** gives information about the choice for the protecting groups for the 3-OH and the 4-OH group.

The 4- hydroxyl group should be easy to introduce during 2,3 orthoester formation. Additionally, the protecting group should be stable during the reaction sequence as well as the glycosylation with the *N*-acetyl-glucosamine donor. As the protecting group at the 3-OH group is replaced with the 2-aminoethylphosphate linker, this group should be easy to remove. Besides to listed qualities the 3- and 4- hydroxyl groups have to be orthogonal to each other. So for the 4- OH protecting group the benzyl group or a silyl group would be suitable and for the 3-hydroxyl group the PMBO group for example.

3.2.2 Donor synthesis

The azide in **32** should be introduced by using diazotransfer reaction. Different diazotransfer reactions are described in the literature. Cavender and Shiner reported in 1972 that trifluoromethanesulfonyl showed promising electron-withdrawing effects so a diazotransfer as triflyl azide is successful[65]. Vasella et al. described first such a diazo transfer onto an amine and later the transfer was improved by using catalytic amounts of copper sulfate under basic conditions[66,67]. The advantages of the method are the mild reaction conditions, high yields and a forecasted stereochemistry[64]. However the danger of an explosion with TfN_3 and the low shelf life point out the drawbacks of this procedure.



Scheme 21 Synthesis scheme of the donor **32** by using imidazole chlorosulfonyl azide as diazotranfer reagent. a) i) SO_2Cl_2, ii) imidazole, ii) HCl in EtOH; b) i) $CuSO_4$ x $5H_2O$, K_2CO_3, ii)Ac_2O, DMAP, pyridine

Goddard-Borger and Stick found the efficient and shelf-stable reagent **37** for a diazo transfer[64]. The synthesis starts with sodium azide and sulfuryl chloride to give chlorosulfonyl azide, which can be converted with imidazole in a one pot reaction. Treatment with HCl in EtOH gives **37** in 86 % yield[64]. The diazotransfer is carried out with D-glucosamine hydrochloride followed by acetylation to give **38** in 27 % yield over two steps. **32** as activated donor shows a higher reactivity and should be synthesized prior to glycosylation to avoid decomposition. As no glycosylation was performed in this thesis the last step is missing, but the activated donor is literature known and could be carried out according to Lee[68].

4 Summary and Outlook

Up to 10 % of adults are carrier of the gram negative bacterium *Neisseria meningitis* in their nasopharynx. The genus can be classified into 13 serogroups whereas six of the serogroups (=A, B, C, W-135, X, Y) can cause meningococcal infections like meningococcemia and meningitis. The therapy is hindered, because the symptoms like headache and stiffness of the neck are not specific. Worldwide these infections cause a high number of deaths especially of people from the meningitis belt in Africa. Furthermore, the highest rate appears in infants. Different strategies for immunisation for development of vaccines against disease-causing strains were studied. The licensed vaccines still have disadvantages, as they are not compatible for infants or their protection is just for a few strains.

A novel approach uses the inner core structure of LOS of *N.meningitidis*, as the structure is similar in all serogroups. Furthermore monoclonal antibody binding on the inner core were studied. Parker et al. showed that the disaccharide α-D-GlcNAc-(1→2)- α-HepII of the inner core structure is highly important for the interaction with mAb LPT3-1. In 70 % of all serogroups HepII is substituted with 2-aminoethylphosphate at the 3- hydroxyl group.

Within this thesis the monosaccharide HepII substituted 3-PEtn was successfully synthesized. Formation of a 2,3 orthoester and the selective opening allowed the introduction of the 2-aminoethylphosphate linker at position 3 *via* the phosphoramidite approach. However the results of the phosphorylation indicated no selectivity for the 3-hydroxyl group.

Besides the monosaccharide preliminary studies for the disaccharide α-D-GlcNAc-(1→2)- α-Hep with substitution at 3-PEtn should be considered. The donor with an azide as temporary protecting group was synthesized successfully. But due to unsuccessful introduction of the benzyl group in the acceptor, the synthesis of the acceptor could not be accomplished and other protecting group strategies have to be considered.

To study the interaction of the substituted 3-PEtn heptoside X-ray crystallography of the antigen-binding fragment (Fab) will be performed with cooperation partners. Furthermore with STD-NMR analysis of the surfactant protein D (SP-D) and the monosaccharide investigations of the interaction with the innate immune system can be studied.

5 Experimental part

5.1 General methods

Thin layer chromatography (TLC) was performed on silica gel F254 pre-coated glass plates (Merck) and (HPTLC) silica gel 60 F254 pre-coated glass plates with 2.5 cm concentration zone (Merck) were used. For detection the plates were dipped in anisaldehyde H_2SO_4- acetic acid or ninhydrin solution followed by heating on a hot plate.

Silica gel (230-400 mesh, Merck) was used for column chromatography. Size exclusion chromatography was performed on Sephadex Bio-Beads SX-1 and Sephadex G10 column.

Optical rotation was measured with a Perkin Elmer 243 B polarimeter and with a Modular Circular Polarimeter: Anton Paar MPP100. $[\alpha]_D^{20}$ values are given in unit deg dm^{-1} $cm^{-3}g^{-1}$.

HPLC-HRMS measurements were performed from H_2O/ACN solutions (concentration 1 mg/l) using a HPC PAL system autosampler (CTC Analytics AG), an Agilent 110/1200 HPLC with binary pump system, degasser and column thermostat (Agilent Technologies, Waldbronn, Germany) and Agilent 6210 ESI-TOF mass spectrometer (Agilent Technologies, Palo Alto, U.S.). Data analysis was carried out from Mass Hunter software (Agilent Technologies).

HPLC-MS analysis was measured with a SHIMADZU system and ELS detector.

Pumps	SHIMADZU LC 10 AD VP
Mass	LCMS-2020 SHIMADZU
ELSD	Alltech 3300 ELSD
Column	Jupiter 5 µm C4 300 Å, 150 x 2 mm
Solvent	ACN, H_20
Method	5 % - 100 % ACN, 20min, 0.5 ml/ min

Lyophilisation was performed using Christ Beta 1-8 LD Freeze dryer.

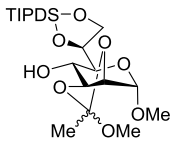
Nuclear magnetic resonance (NMR) spectra were measured either on a Bruker DPX 300 or a Bruker Avance III 600 spectrometer. All proton spectra were referenced to δ=7.26 ppm ($CDCl_3$), δ=3.31 ppm (methanol-d4) as internal standards or DSS (δ=0 ppm) in D_2O as external standard. ^{13}C-NMR spectra were referenced to δ=77.16 ppm ($CDCl_3$) and δ=49.00 ppm (methanol-d4) as internals standards and 1,4 dioxane in D_2O (δ= 67.4 ppm) as external standard[69]. ^{31}P-NMR spectra were referenced to H_3PO_4 in D_2O (δ= 0 ppm) as external standard or to their proton spectra *via* the SR value. The coupling constants are given in Hz.

All reaction were performed under Argon atmosphere. All chemicals were purchased from chemical suppliers.

DCM was used after being distilled from CaH_2 and stored over molecular sieves (4 Å). ACN was stored over molecular sieves (3 Å). The dryness of the solvents was checked by Karl-Fischer titration on a Mitsubishi Karl Fischer moisture meter model CA-21.

5.2 Experiments

5.2.1 Methyl-2,3-O-(methoxyethylidene)-6,7-O-(1,1,3,3-tetraisopropyl-1,3-disiloxane-1,3-diyl)-L-glycero-α-D-manno-heptopyranoside (7a)



7a

The heptoside **5** (0.107 mmol, 50 mg) was dissolved in dry DCM (2 ml) and afterwards trimethyl orthoacetate (0.128 mmol, 26 mg) and camphor sulfonic acid (0.012 mmol, 3 mg) were added. After 60 min the reaction showed complete conversion to the orthoester and the reaction mixture was quenched with TEA (20 µl) and diluted with DCM. The organic layer was washed with sat. $NaHCO_3$ (3x 20 ml) and the aqueous layers were rextracted with DCM (3 x 20 ml). The combined organic layers were washed with brine (1 x 30 ml), dried with Na_2SO_4 and concentrated. The crude labile product was not further purified (47 mg).

Orthoacetate: endo:exo 0.22/0.78

Rf $_{exo}$= 0.53 (3:1 hexane:ethylacetate)

Rf $_{endo}$= 0.49 (3:1 hexane:ethylacetate)

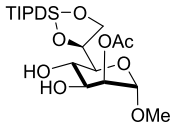
Endo: ^{1}H-NMR ($CDCl_3$): δ= 4.93 (dd, 1H, $J_{1,2}$ = 2.5 Hz, H1), 4.30- 4.25 (m, 2H, H4 or H5 or H6), 4.21 (t, 1H, $J_{3,2}$= $J_{3,4}$= 7.3 Hz, H3), 4.12 (dd, 1H, $J_{2,1}$= 2.5 Hz, $J_{2,3}$= 7.1, H2), 4.10- 3.94 (m, 3H, H4 or H5 or H6, H7, H7'), 3.40 (s, 3H, OMe), 3.34 (s, 3H, $OMe_{orthoester}$), 1.55 (s, 3H, CH_3), 1.14-0.93 (m, 28H, TIPDS)

Exo: ^{1}H-NMR ($CDCl_3$): δ= 4.89 (dd, 1H, $J_{1,2}$ = 0.9 Hz, H1), 4.30- 4.25 (m, 3H, H2, H3, H6), 4.10- 3.94 (m, 2H, H7, H7'),3.82 (m, 1H, H4), 3.59-3.54 (m, 1H, H5), 3.37 (s, 3H, OMe), 3.30 (s, 3H, $OMe_{orthoester}$), 1.61 (s, 3H, CH_3), 1.14-0.93 (m, 28H, TIPDS)

Endo: ^{13}C-NMR ($CDCl_3$): δ= 98.63 (C1), 79.87 (C3), 75.78 (C2), 75.13 (C6), 69.27, 69.14 (C4, C5), 67.79 (C7), 55.16 ($OMe_{orthoester}$), 50.53 (OMe), 22.82 (Me), 17.59, 17.46, 17.53, 17.52, 17.50, 17.48, 17.47, 17.42, 17.40, 17.31, 13.21, 13.14, 12.85, 12.76, 12.71, 12.54, 12.51 (TIPDS)

Exo: ^{13}C-NMR ($CDCl_3$): δ= 98.60 (C1), 79.16 (C3), 76.59 (C2), 74.5 (C6), 71.87 (C5), 70.72 (C4) 67.57 (C7), 55.42 ($OMe_{orthoester}$), 50.31 (OMe), 21.00 (Me), 17.59, 17.46, 17.53, 17.52, 17.50, 17.48, 17.47, 17.42, 17.40, 17.31, 13.21, 13.14, 12.85, 12.76, 12.71, 12.54, 12.51 (TIPDS)

5.2.2 Methyl 2-O-acetyl-6,7-O-(1,1,3,3-tetraisopropyl-1,3-disiloxane-1,3-diyl)- L-glycero- α-D-manno-heptopyranoside (11)



11

Trimethyl orthoacetate (0.291 mmol, 35 mg) and camphor sulfonic acid (0.012 mmol, 3 mg) were added to a solution of heptoside **5** (0. 242 mmol, 113 mg) in dry DCM (1 ml). After 60 min the reaction showed complete conversion to the orthoester. Then water (0.01 ml) and TEA (0.01 ml) were added. After 30 min the reaction mixture showed complete conversion and the mixture was diluted with DCM and the layers were separated. The organic layer was washed with sat. $NaHCO_3$ (3x 50 ml). The aqueous layers were reextracted with DCM (3 x 50 ml) and the combined organic layers were washed with brine (1 x 50 ml), dried with Na_2SO_4, and concentrated. The product was purified *via* flash chromatography (Isolute Flash Si II 2 g/ 6 ml, fraction size 2 ml, 2:1 hexane:ethylacetate) and HPLC (column YMC-Pack-Sil-06 250 x 20 mm, flow rate 15 ml/min, fraction size 15 ml, 4:1 → 2:1 hexane:ethylacetate) to give **11** (88 mg, 71 %).

Physical properties:

Yield: 88 mg (71%) as a white syrup/solid

Rf= 0.59 (1:1 hexane: ethylacetate)

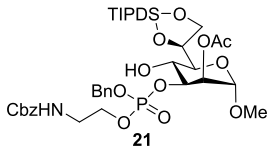
Optical rotation α_D^{21} = + 22.6 ° (c = 1.2, $CHCl_3$)

HRMS (ESI): *m/z* calculated ($C_{22}H_{44}O_9Si_2 + H^+$) 509.2597, found ($C_{22}H_{44}O_9Si_2 + H^+$) 509.2614

^{1}H-NMR ($CDCl_3$): δ= 5.02 (dd, 1H, $J_{2,3}$= 3.3 Hz, $J_{2,1}$= 1.7 Hz, H2), 4.69 (d, 1H, $J_{1,2}$= 1.6 Hz, H1), 4.33-4.30 (m, 1H, H6), 4.06- 4.02 (m, 1H, H7) 4.00- 3.91 (m, 3H, H-3, H-4, H7'), 3.57 (dd, 1H, $J_{5,6}$= 2.5 Hz, $J_{5,4}$= 9.2 Hz, H5), 3.33 (s, 3H, OMe), 2.10 (s, 3H, OMe), 1.13- 0.93 (m, 28H, TIPDS)

^{13}C-NMR ($CDCl_3$): δ= 170.91 (C=O), 98.63 (C-1), 74.83 (C-6), 71.95, 71.80 (C-2,C-5), 70.53, 68.09 (C-4, C-3), 67.75 (C-7), 55.17 (OMe), 20.99 (OAc), 17.63, 17.56, 17.53, 17.51, 17.49, 17.43, 17.42, 17.37, 13.20, 12.85, 12.51 (TIPDS)

5.2.3 Methyl 2-O-acetyl-3-O-[benzyloxy-2-(N-benzyloxycarbonylamino)ethylphosphoryl] 6, 7-O-(1, 1, 3, 3-tetraisopropyl-1,3-disiloxane-1,3-diyl)- L-glycero-α-D-manno-heptopyranoside (21)



21

Phosphoramidite **20** (0.059 mmol, 26 mg) and **11** (0.029 mmol, 15 mg) were dissolved in dry DCM (200 µl) and suspended with powdered molecular sieves (4 Å) for 1 h. Then 1*H*-tetrazole solution (0.45 M in ACN, 0.059 mmol, 0.131 µl) was added and the mixture was stirred at r.t. After 2 h the mixture was cooled to -78 °C and mCPBA (0.059 mmol, 10 mg) was added. After 1 h the mixture was quenched with TEA (10 µl) and slowly warmed up to r.t.. The solution was diluted with DCM. The organic layer was washed with sat. $NaHCO_3$ (3x 50 ml) and the aqueous layers were reextracted with DCM (3 x 50 ml). The combined organic layers were washed with brine (1x 20 ml), dried with Na_2SO_4 and concentrated. The product **21** was purified by HPLC to afford **21** (8.2 mg, 30 % yield) (YMC-Pack-Sil-06 250 x 10 mm, flow rate 5 ml/min, fraction size 5 ml, 2:1 to 1:2 hexane: ethylacetate)

Physical properties

Yield: 8 mg (30 %) as a yellow syrup

Rf: 0.45 (1:1 hexane: ethylacetate)

HRMS (ESI): *m/z* calculated ($C_{39}H_{62}NO_{14}PSi_2 + H^+$) 856.3519, found ($C_{39}H_{62}NO_{14}PSi_2 + H^+$) 856.3528

^{1}H-NMR ($CDCl_3$) for diastereomer a: δ= 7.38-7.28 (m, 10H, 2x Ph-a), 5.38 (bs, 1H, NH), 5.19 (dd, 1H, $J_{2,1}$= 1.9 Hz, $J_{2,3}$= 3.5 Hz, H2-a), 5.10- 5.08 (m, 4H, 2x CH_2), 4.66-4.63 (m, 1H, H1-a), 4.66- 4.61 (m, 1H, H3-a), 4.36 (m, 1H, H6-a), 4.16- 4.09 (m, 1H, H4-a), 4.16-4.09 (m, 1H, PO*CH_2*CH_2-a), 4.07- 4.00 (m, 2H, PO*CH_2*CH_2-a, H7-a), 3.85 (d, J = 11.9 Hz, H7-a‘), 3.53-3.39 (m, 3H, H5-a, POCH$_2$*CH_2-a*), 3.28 (s, 3H, OMe-a), 2.01 (s, 3H, OAc-a), 1.13-0.86 (m, 28H, TIPDS-a)

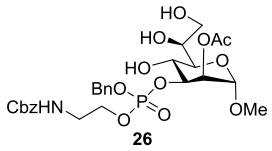
^{1}H-NMR ($CDCl_3$) for diastereomer b: δ=7.41-7.28 (m, 10H, 2x Ph-b), 5.28 (bs, 1H, NH-b), 5.16 (dd, 1H, $J_{2,1}$= 1.9 Hz, $J_{2,3}$= 3.5 Hz, H2-b), 5.12-5.05 (m, 4H, 2x CH_2), 4.65 (d, 1H, $J_{1,2}$= 1.5 Hz, H1-b), 4.59 (ddd, 1H, $J_{3,2}$= 3.33 Hz, $J_{H,P}$= 7.53 Hz, $J_{3,4}$= 9.6 Hz, H3-b), 4.36 (d, 1H, $J_{6,5}$= $J_{6,7}$= 8.88 Hz, H6-b), 4.15 (t, 1H, $J_{4,5}$ = $J_{4,3}$ = 9.5 Hz, H4-b) 4.11-4.01 (m, 3H, PO*CH_2*CH_2‘, H7-b), 3.87 (dd, 1H, J= 1.05 Hz, J= 12.15 Hz, H7-b‘), 3.50 (dd, 1H, $J_{5,4}$= 9.5 Hz, $J_{5,6}$= 1.7 Hz, H5-b), 3.42- 3.36 (bs, 2H, POCH$_2$*CH_2-b*), 3.29 (s, 3H, OMe-b), 2.02 (s, 3H, OAc-b), 1.34- 0.93 (m, 28H, TIPDS-b)

^{13}C-NMR ($CDCl_3$): δ= 170.26 (C=O), 128.98, 128.92, 128.86, 128.81, 128.66, 128.35, 128.28, 128.25, 128.19, 128.16 (C_{arom}), 98.44 (C1-a), 98.39 (C1-b), 77.74 (m, C3), 73.74 (C6-a), 73.66 (C6-b), 72.68 (C5-a), 72.63 (C5-b), 70.25 (CH_2-Ph), 70.19 (m, C2), 68.19 (C7), 67.55 (m, PO*CH_2*CH_2), 66.97 (CH_2-Cbz), 66.23 (C4), 55.08 (OMe), 41.42 (m, POCH$_2$*CH_2*), 20.84 (OAc), 17.59, 17.57, 17.47, 17.43, 17.41, 13.27, 13.00, 12.83, 12.56 (TIPDS)

a: ^{31}P-NMR ($CDCl_3$): δ= 0.52

b: ^{31}P-NMR ($CDCl_3$): δ= 0.45

5.2.4 Methyl 2-O-acetyl-3-O-[benzyloxy-2-(N-benzyloxycarbonylamino)ethylphosphoryl]- L-glycero-α-D-manno- heptopyranoside (26)



26

To a solution of **21** (0.010 mmol, 12 mg) in dry DCM (200 µl) TREAT (0.234 mmol, 38 mg) was added at 0°C. Then the mixture was stirred for 2 h at r.t. before adding TREAT (0.234 mmol, 38 mg) at 0°C once more. After 17 h the mixture was neutralized by adding Dowex 1X8 (HCO_3^--form)and filtered. The filtrate was purified by flash chromatography (Isolute Flash Si II 2 g/6 ml, fraction size 3 ml, 1:7 hexane:ethylacetate to 100 % ethylacetate to 9:1 ethylacetate:EtOH) to give **26** as product (6 mg, 81 %).

Physical properties

Yield: 6 mg (81%) as a yellow solid

Rf 0.35 (9:1 ethylacetate: ethanol)

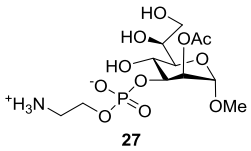
HRMS (ESI): *m/z* calculated ($C_{27}H_{36}NO_{13}P + H^+$) 614.1997, found ($C_{27}H_{36}NO_{13}P + H^+$) 614.1995

^{1}H-NMR ($CDCl_3$): δ= 7.39-7.29 (m, 10H, 2x Ph), 5.42 (bs, 1H, NH-a), 5.34 (bs, 1H, NH-b), 5.24 (dd, 1H, $J_{2,1}$= $J_{2,3}$= 3.5, H2-a), 5.20-5.18 (m, 1H, H2-b), 5.11-5.06 (m, 4H, 2 x CH_2), 4.68- 4.66 (m, 1H, H1), 4.63 (dddd, 1H, $J_{3,2}$=3.7 Hz, $J_{3,P}$= 7.6 Hz, $J_{3,4}$= 9.4 Hz, H3), 4.12 (t, 1H, $J_{4,5}$ =$J_{4,3}$= 9.8 Hz, H4), 4.14-4.02 (m, 3H, H6, OP*CH*$_2$CH$_2$), 3.85-3.78 (m, 1H, H7), 3.73- 3.68 (m, 1H, H7‘), 3.63- 3.58 (m, 1H, H5), 3.45- 3.67 (m, 2H, POCH$_2$*CH*$_2$), 3.34 (s, 3H, OMe-a), 3.32 (s, 3H, OMe-b), 2.56 (s, 3H, OAc-a), 2.05 (s, 3H, OMe-a)

^{13}C-NMR ($CDCl_3$): δ= 170.20 (C=0), 129.03, 129.01, 128.89, 128.87, 128.71, 128.69, 128.35, 128.33, 128.30, 128.20 8 (C_{arom}), 99.82 (C1-a), 98.78 (C1-b), 77.47 (m, C3), 72.46 (C5), 70.37 (m, *CH*$_2$Ph), 70.24 (m, C6), 70.10 (m, C2), 67.62 (PO*CH*$_2$CH$_2$), 67.06 (CH_2-Cbz), 66.54 (C4), 64.42 (C7), 55.35 (OMe), 41.43 (POCH$_2$*CH*$_2$), 20.94 (OAc)

^{31}P-NMR ($CDCl_3$): δ= 0.59, 0.44

5.2.5 Methyl 2-O-acetyl-3-O-(2-aminoethylphosphoryl)- L-glycero-α-D-manno- heptopyranoside (27)



27

26 (0.015 mmol, 9 mg) was dissolved in dry MeOH + 1 % acetic acid (400 µl) and the atmosphere was exchanged to Ar. Then 10 % Pd/C (4 mg) was added quickly and the atmosphere was exchanged to H_2. After 2 h the suspension was filtered through Celite and the filtrate was concentrated. Product **27** was obtained as a light yellow syrup (5.3 mg, 93 %).

Physical properties

Yield: 5.3 mg (93 %) as a light yellow syrup

Rf 0.51 (10:10:3 MeOH: $CHCl_3$: H_2O)

Optical rotation: α_D^{20} = + 23.2 ° (c = 0.53, MeOH)

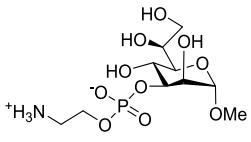
HRMS (ESI): *m/z* calculated ($C_{12}H_{24}NO_{11}P + H^+$) 390.1160, found ($C_{12}H_{24}NO_{11}P + H^+$) 390.1168

^{1}H-NMR (MeOD): δ= 5.22 (dd, 1H, $J_{2,3}$=3.6 Hz, $J_{2,1}$= 1.7 Hz, H2), 4.69 (d, 1H, $J_{1,2}$ = 1.7 Hz, H1), 4.47-4.42 (m, 1H, H3), 4.11-3.99 (m, 4H, H4, H6, PO*CH*$_2$CH$_2$), 3.70- 3.61 (m, 3H, H5, H7,H7'), 3.37 (s, 3H, OMe), 3.16-3.11 (POCH$_2$*CH*$_2$), 2.09 (s, 3H, OAc)

^{13}C-NMR (MeOD): δ= 172.16 (C=O), 99.90 (C1), 75.62 (d, J_{C-P}= 5.7 Hz, C3), 72.72 (d, J_{C-P}=2.1 Hz, C2), 72.54 (C5), 70.35 (C6), 67.17 (d, J_{C-P}= 4.5 Hz, C4), 64.15 (C-7),63.23 (d,J_{C-P}=5.5 Hz, PO*CH*$_2$CH$_2$),55.37 (OMe), 41.76 (d, J_{C-P}=6.6 Hz, POCH$_2$*CH*$_2$), 20.86 (OAc)

^{31}P-NMR (MeOD): δ= 0.81

5.2.6 Methyl -3-O-(2-aminoethylphosphoryl)- L-glycero-α-D-manno-heptopyranoside (1)



1

29 (5 mg, 0.013 mmol) was dissolved in a mixture of MeOH, TEA and water (8:1:1, 500 µl). After 3 days 40 µl of water were added once more. After 7 days the solvent was evaporated (water bath at r.t.) and the product was purified by Sephadex G10 column (solvent: H_2O dest, 5 % EtOH, 0.8 % NaCl, 0.2 % NaN_3, fraction size 2 ml). After lyophilization **1** was afforded as a yellow solid (2.8 mg, 62 %).

Physical properties

Yield: 2.8 mg (62 %) as a yellow solid

Rf 0.32 (10:8:2 MeOH: $CHCl_3$: H_2O)

Optical rotation: α_D^{20} = + 18.2 ° (c = 0.28, H_2O)

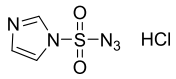
HRMS: *m/z* calculated ($C_{10}H_{22}NO_{10}P + K^+$) 386.0613, found ($C_{10}H_{22}NO_{10}P + K^+$) 386.0614

^{1}H-NMR (D_2O): δ= 4.74 (s, 1H, H1), 4.22 (dd, 1H, $J_{3,P}$= 3.3 Hz, $J_{3,4}$= $J_{3,2}$= 13.2 Hz, H3), 4.16- 4.10 (m, 2H, PO*CH*$_2$$CH_2$), 4.09 (dd, 1H, $J_{2,3}$=3.1 Hz, $J_{2,1}$=1.7 Hz, H2), 4.04- 4.00 (m, 1H, H6), 3.94 (t, 1H, $J_{4,3}$= $J_{4,5}$= 9.9 Hz, H4), 3.71 (dd, 1H, $J_{7,7'}$= 23.4, $J_{7,6}$= 11.5 Hz, H7), 3.70 (dd, 1H, $J_{7',7}$= 21.3 Hz, $J_{7',6}$= 11.3 Hz, H7'), 3.64-3.56 (m, 1H, H5), 3.45 (s, 3H, OMe), 3.25 (t, 2H, POCH$_2$*CH*$_2$), 3.17 (q, 6H, $J_{CH2-CH3}$= 7.3 Hz, N*CH*$_2$CH$_3$), 1.25 (t, 9H, $J_{CH3-CH2}$= 7.3 Hz, NCH$_2$*CH*$_3$)

^{13}C-NMR (D2O): δ= 101.55 (C1), 77.13 (d, J_{C-P}= 6.1 Hz, C3), 71.89 (C5), 69.94 (C2), 69.61 (C6), 65.82 (d, JC-P= 6.3 Hz, C4), 63.66 (C7), 62.78 (m, PO*CH*$_2$), 55.57 (OMe), 40.88 (d, J_{C-P} =7.27 Hz, PO*CH*$_2$)

^{31}P-NMR (D_2O): δ= -0.29 ppm

5.2.7 1H- imidazole-1-sulfonyl azide hydrochloride (37)

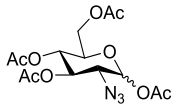


37

Sodium hydride (2.5 mmol, 163 mg) was dissolved in ACN (3 ml) and cooled to 0 °C. Afterwards sulfurylchloride (2.5 mmol, 337 mg, dissolved in 1 ml ACN) was added slowly. The mixture was stirred for 17 h at r.t.. Then the mixture was cooled to 0 °C and imidazole (5 mmol, 340 mg, dissolved in 2 ml ACN) was added. The solution was stirred for 4 h r.t. and then diluted with EtOAc (10 ml) and water (10 ml). The organic layers were washed with water (2x 20 ml) and sat. $NaHCO_3$ (1x 20 ml) and dried over $MgSO_4$. Then HCl in EtOH (preparation: AcCl [300 mol, 23.5 g] was added slowly to EtOH [30 ml]) was added slowly to the mixture to give a white precipitate. The precipitate was filtered and washed with EtOAc (2 x 10 ml) to give **37** (2.2 mmol, 0.451 g, 86 %). The product was dried and stored in a -20 °C freezer.

NMR data match to published data[64].D

5.2.8 1, 3, 4, 6-Tetra-O-acetyl-2-azido-2-deoxy-D-glucopyranose (38)

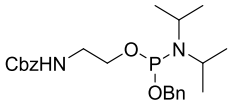


38

Imidazole-1-sulfonyl-azide-hydrochloride **37** (0.577 mmol, 100 mg) was added to a mixture of glucosamine (0.481 mmol, 104 mg), $CuSO_4$ x 5 H_2O (0.0048 mmol, 1.2 mg), K_2CO_3 (1.1 mmol, 160 mg) in dry MeOH (3 ml). The mixture was stirred for 15 h at r.t.. Afterwards the mixture was concentrated and coevaporated with toluene (2 x 10 ml). Then the residue was dissolved in pyridine (3 ml) and Ac_2O (3.848 mmol, 393 mg) was added. After 3 h the mixture was concentrated and diluted with water (5 ml) and ethylacetate (10 ml). The organic layer was extracted with water (2 x 10 ml), brine (10 ml) and dried over $MgSO_4$ to afford **38** (0.129 mmol, 48 mg, 27 %).

NMR data are identical to published data[66].

5.2.9 [2-(Benzyloxy-diisopropylamino-phosphanyloxy)-ethyl]-carbamic acid benzyl ester (20)



20

Preparation: PCl_3 was distilled, benzyl alcohol was dried over Molecular sieves (3 Å), diisopropylamine was distilled with NaOH. The glass equipment was heated to 250°C

PCl_3 (52 mmol, 7.22 g, freshly distilled) was dissolved in Et_2O and cooled to -20°C in a three-necked flask. Then benzyl alcohol (40 mmol, 4.24 g, dissolved in 40 ml Et_2O) was added slowly during 2.5 h. After 3.5 h the ^{31}P-NMR showed complete conversion. The unreacted PCl_3 and BnOH were removed *via* vacuum distillation. The intermediate **18** was dissolved in Et_2O and diisopropylamine (240 mmol, 33.73 g, dissolved in 30 ml Et_2O) was added slowly over night at 0°C. After 17 h the ^{31}P-NMR showed the product **19**. The mixture was diluted with Et_2O and water. The organic layer was washed with water, dried with Na_2SO_4 and concentrated to get **19** (10.48 g of crude product). **19** (2.12 g of crude product) and Cbz-ethanolamine (6.66 mmol, 1.34 g) were coevaporated with toluene and then dissolved in dry DCM (5 ml). 1*H*-tetrazole (3.13 mmol, 0.22 g) was added fast as a solid. After 20 min the ^{31}P-NMR showed complete conversion and the mixture was evaporated. The product **20** was purified by flash chromatography (6:1 hexane:ethylacetate 1 % TEA, fraction size 7 ml, Biotage Si 25 + M catridge) to give **20** (2.17 mmol, 0.94 g).

NMR data are identical to published data[70].

6 Literature

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