UNIVERSITÀ DEGLI STUDI DI MILANO

DIPARTMENT OF ORGANIC AND INDUSTRIAL CHEMISTRY

DOCTORATE SCHOOL IN CHEMICAL SCIENCES AND TECHNOLOGIES

Curriculum

Chemical Science (XXIV Cycle)

Synthesis and conjugation of Neisseria Meningitidis X Capsular Polysaccharide Fragments

CHIM-06

PhD thesis of

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Tutor

Prof. LUIGI LAY

Coordinator

Prof. SILVIA ARDIZZONE

Academic Year 2010-2011

Preface

This PhD thesis is a part of a multidisciplinary project developed in collaboration with Dr. Paolo Costantino (Novartis Vaccine and Diagnostics).

An evaluation process for patent purpose is currently in progress. Thereafter, at least three manuscripts (concerning the synthesis of oligomers, NMR studies and glycoconjugations) will be submitted for publication.

Figures, tables, molecules, schemes, and literature numerations are not contiguous, each chapter has its own numbering.

The full structural characterization (MS, ^{1}H-NMR, ^{13}C-NMR and, when relevant ^{31}P-NMR) of new products are reported. Some NMR spectra are incorporated for a better understanding of the described results. All the products are completely characterized in ***CHAPTER 9*** (***Experimental Section***).

1st YEAR (A. Y. 2008-2009)

Attended and successfully accomplished courses

UNIMI

- ✓ "*Magnetic resonance techniques for the study of the interaction between ligands and protein receptors.*", Dr. drD. Potenza and Dr. C. Giannini, March 2009
- ✓ "*Discovery and development of neuraminidase inhibitors as therapeutic agents against influenza virus.*", Prof. A. Bernardi, July-September 2009
- ✓ "*Organic synthesis in non-conventional media.*", Prof. L. Lay and Dr. L. Poletti, July 2009

VISITING PROFESSORS

- ✓ "*Small peptides as organocatalysts.*", Prof. H. Wennemers, May 2009
- ✓ "*Modern reagents for chemical synthesis: 1) The problematic of plasticizer; 2) Green chemistry, renewable sources, gold catalysis; 3) Catalysis a key technology.*", Prof. A.S.K. Hashmi, May 2009
- ✓ "*Biological chemistry of carbohydrates and glycoproteins*.", Prof. B. Davies, July 2009

Department Seminars

- ♦ *Nuovi Orientamenti in Sintesi Organica,* Milano, November 23 2009
- ♦ *Dipartimento di Chimica Organica e Industriale: Incontro con l'Università, il CNR e l'Industria*, Milano, February 12 2009
- ♦ *Towards the Unification of the Doctoral Studies in the European Union*, Milano, May 20-22 2009

Attended schools

XXXIV Corso estivo "A. Corbella", Gargnano (BS), June 22-26 2009

2nd YEAR (A. Y. 2009-2010)

Attended and successfully accomplished courses

UNIMI

- ✓ "*Applications of chiral-pool compounds in stereoselective synthesis.*", Prof. G. Russo, March 2010
- ✓ "*Peptide nucleic acids (PNA) as DNA and RNA mimics: from traditional systems to new conjugates with magnetic nanoparticles.*", Prof. E. Licandro, March 2011
- ✓ "*G-Protein-coupled receptors: biological targets for the discovery of novel drugs.*", Prof. M. De Amici, April 2011

VISITING PROFESSORS

- ✓ "*Case studies in the discovery and development of drugs.*", Prof. K.H. Altmann, March 2010
- ✓ "*An enantiomeric scaffolding strategy for the synthesis of alkaloids: use of a chiral oxazolopiperidone lactams.*", Prof. M. Amat, May 2010
- ✓ "*Protein/protein interactions: modeling techniques and applications.*", Prof. N. Hirayama, June 2010

Department Seminars

- ♦ *Nuovi Orientamenti in Sintesi Organica,* Società Chimica Italiana, Milano, November 15 2010
- ♦ *Incontro con l'Università, il CNR e l'Industria*, Dipartimento di Chimica Organica e Industriale, Milano, February 11 2010
- ♦ *NMR and Molecular Recognition*, Prof. Jimenez-Barbero Milano, February 16 2010

Attended schools

- ♦ Summer Course of Glycoscience *"11th European training Course on Carbohydrate"*, Wageningen (NL), May 17-20 2010
- ♦ *XII Convegno-Scuola sulla Chimica dei Carboidrati*, Certosa di Pontignano (SI), June 20-23 2010

Poster presentations

Synthesis and conjugation of Neisseria Meningitidis X Capsular Polysaccharide fragments, Convegno-Scuola sulla Chimica dei Carboidrati, Certosa di Pontignano (SI), June 22 2010

Publications

Cheshev, P., Morelli, L., Marchesi, M., Podlipnik, Č., Bergström, M. and Bernardi, A. (2010), Synthesis and Affinity Evaluation of a Small Library of Bidentate Cholera Toxin Ligands: Towards Nonhydrolyzable Ganglioside Mimics. Chemistry - A European Journal, 16(6): 1951–1967.

3rd YEAR (A. Y. 2010-2011)

Attended and successfully accomplished courses

UNIMI

- ✓ "*Drug metabolism in medicinal chemistry*", Prof. Aldini, Vistoli, Dondio and Testa, March 2011

VISITING PROFESSORS

- ✓ "*Functionalized oligonucleotides for the creation of reactive nucleic acid duplexes: design and applications.*", Prof. A. Madder, February 2011
- ✓ "*Metal/RNA interactions.*", Prof. R. Sigel, March 2011
- ✓ "*The art and science of chemical synthesis: where to next?*", Prof. I. Baxendale, June 2011

Attended schools

- ♦ XXXVI "A. Corbella" Summer School, Gargnano (BS), Palazzo Feltrinelli, June 13-17 2011
- ♦ 16th European Carbohydrate Symposium, Sorrento (NA), July 3-7 2011

Oral communications

Synthesis and conjugation of Neisseria Meningitidis X Capsular Polysaccharide fragments, XXXVI "A. Corbella" Summer School, Gargnano (BS), Palazzo Feltrinelli, June 13-17 2011

Poster presentations

Synthesis and conjugation of Neisseria Meningitidis X Capsular Polysaccharide fragments, 16th European Carbohydrate Symposium, Sorrento (NA), July 3-7 2011

Stage as visiting scientist in Novartis

- *July* (1 month)
- *December* (1 week: 11-17)

Publications

Morelli, L., Poletti, L. and Lay, L. (2011), Carbohydrates and Immunology: Synthetic Oligosaccharide Antigens for Vaccine Formulation. European Journal of Organic Chemistry 2011(29), 5723–5777.

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CHAPTER 9: Experimental section

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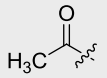
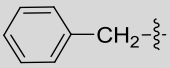
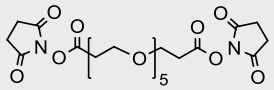
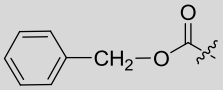
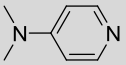
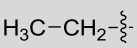
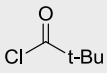
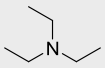
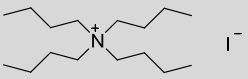
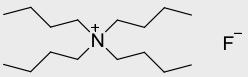
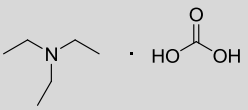
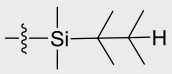
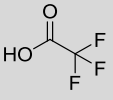
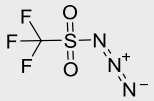
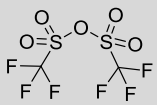
Acronyms and Abbreviations

APC	Antigen Presenting Cell
BCG	*Bacillus Calmette-Guérin*
BCR	B cell receptor
BSA	Bovine Serum Albumin
CDAP	1-Cyano-4-dimethylamino-pyridinium tetrafluoroborate
CPS	Capsular polysaccharide
CRM_{197}	Cross-Reacting Material 197
CTL	Citotoxic T Lymphocytes (*or* $CD8^+$ T cells)
DC	Dendritic Cell
DCC	Dicyclohexyl carbodiimide
DCM	Dichloromethane
ELISA	Enzyme-linked immunosorbent assay
Glc*p*	Glucopyranosyl
Hib	Haemophilus influenzae type b
HIV	Human Immunodeficiency Virus
HSA	Human Serum Albumin
IgG ab	high affinity antibodies
IgM ab	low affinity antibodies
KLH	Keyhole Limpet Hemocyanin
LPS	Lipopolysaccharide
MenA	*N. meningitidis* type A
MenX	*N. meningitidis* type X
MHC	Major Histocompatibility Complex
MPL A	Monophosphoryl Lipid A
NAc	N-Acetyl
NMR	Nuclear Magnetic Resonance

Acronyms and Abbreviations

PAMP	Pathogen-Associated Molecular Patterns
PRR	Pattern-Recognition Receptor
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
QS-21	saponin extracted from *Quillaja saponoria*
TACA	Tumor-Associated Carbohydrate Antigen
TCR	T-cell Receptor
Th	helper T cells (*or* $CD4^+$ T cells)
TLR	Toll-Like Receptor
TPS-Cl	2,4,6-Triisopropylbenzenesulfonyl chloride
TPS-NT	3-Nitro-1-(2,4,6-triisopropylbenzenesulfonyl)-1,2,4-triazole
TT	Tetanus Toxoid

Acronyms and Abbreviations

Ac	Acetyl	
Bn	Benzyl	
$BS(PEG)_5$	Bis-succinimidyl penta-ethylene glycol	
Cbz	Carbobenzyloxy	
DCM	Dichloromethane	
DMAP	4-Dimethylaminopyridine	
DMF	Dimethylformamide	
Et	Ethyl	
Me	Methyl	
Ph	Phenyl	
PivCl	Trimethylacetyl chloride, or Pivaloyl chloride	
Py	Pyridine	
TEA	Triethylamine	
TBAI	Tetra-*n*-butylammonium iodide	
TBAF	Tetra-*n*-butylammonium fluoride	
TEAB	Triethylammonium bicarbonate	
TDS	Thexyldimethylsilyl	
TFA	Trifluoroacetic acid	
TfN_3	Trifluoromethanesulfonyl Azide	
Tf_2O	Trifluoromethanesulfonic Anhydride	
THF	Tetrahydrofuran	

CHAPTER 1

Introduction to immunology and glycobiology

Glycoimmunobiology is an emerging field. Immunology and glycobiology are connected by a two-way street: the immune response is linked to glycobiology by recognizing carbohydrates as antigens, on the other hand in glycobiology glycans influence multiple levels of the immune response.[1]

THE IMMUNE SYSTEM

Before being adopted by scientific community, the term immunity was used in legal field. Immunity is an exemption from a burden, an obligation or a duty. The term *immune* derives from the Latin word *immunitas*, "*inmunitas*" from the archaic *munus*, meaning office, duty, assignment and performance. "Immunitas" was commonly used by Romans to suggest the dispensation from an activity, taxes or military service.

"Druides a bello abesse consuerunt neque tributa una cum reliquis pendunt. Militiae vacationem omniumque rerum habent immunitatem."

Gaius Julius Caesar

(*Commentaries on the Gallic War*, book 6, par.14).

In the middle of 19th century, its meaning was extended to persons "except from" or protected against something, so to be immune. The term *immune system* refers to a collection of mechanisms that protects against foreign substances by identifying and killing invading pathogens.

The immune system is amazingly complex: it is a network of cells (**Figure 1**), tissues, and organs that work together to defend the body against attacks by foreign invaders.

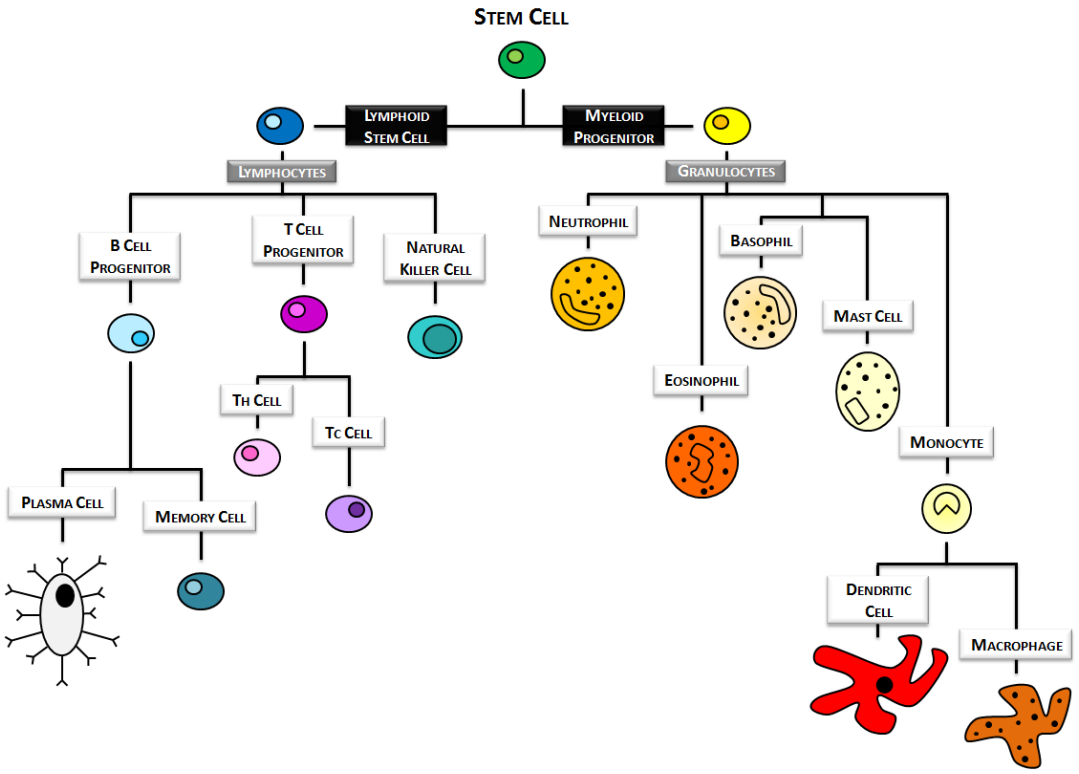


Figure 1_Cells of the Immune System.

These are primarily microbes—tiny organisms such as bacteria, parasites, and fungi that can cause infections. Viruses also cause infections, but are too primitive to be classified as living organisms. The human body provides an ideal environment for many microbes. It is the immune system's job to keep them out or, failing that, to seek out and destroy them.

IMMUNE RESPONSE

Antigen is any molecule perceived by the immune system as foreign invader or simply potentially dangerous for the host. The immune system responds to antigens by eliciting a proper immune response. More specifically, protective immunity against pathogen exposure is achieved by the integration of two distinct arms of the immune response, the innate and adaptive (antigen-specific) responses. The immune response comprises different steps. First, physical barriers, i.e. skin in humans, prevent pathogens from entering in the body.[2] But then, if a pathogen is able to cross them, the innate system, founded both in plants and animals, acts quite immediately in a non-specific manner.[3] A third level of protection, the adaptive system, can be activated if the pathogens impedes the innate system. In the case of adaptive system, the immune response retains a "memory" of the pathogen, so that the immune response works faster and stronger each time that a specific pathogen is encountered.

INNATE IMMUNITY AND ADAPTIVE IMMUNITY

The innate response is rapid and aspecific, it is mediated by antigen presenting cells (APCs) and establishes the first line of immune defence. It acts during the early stages of infection (within minutes), detecting and responding to pathogen-associated molecular patterns (PAMPs), which are structurally and chemically diverse compounds highly conserved in pathogens and absent in their multicellular host.

By contrast, the adaptive response, which is mediated by B and T lymphocytes, recognizes pathogens with high affinity, providing the fine antigenic specificity required for complete elimination of the infective agent and the generation of the immunological memory. However, the establishment of adaptive immunity typically takes days to weeks to become effective.

APCs (and in particular dendritic cells, DCs) provide a crucial bridge between the two responses. APC surface is plenty of pattern-recognition receptors (PRRs), including the recently discovered Toll-like receptor family (TLRs), that can recognize a huge variety of PAMPs. PRRs stimulation creates the necessary pro-inflammatory context (expression of costimulatory molecules and secretion of soluble cytokines and chemokines) leading to full maturation of DCs, antigen uptake and intracellular processing (**Figure 2a**). Mature DCs migrate to the draining lymph nodes where they prime naive T cells, thus triggering and amplifying the adaptive arm of the immune response. The crucial event of the T cells activation-differentiation process is the immunological synapse, initiated by formation of a ternary complex MHC (major histocompatibility complex, class I or II, on DC surface) antigen T cell receptor (TCR) on T cell surface, followed by further specific interactions. Depending on the antigen exposed on DC surface, the immunological synapse may induce the activation of cytotoxic T lymphocytes (CTL, or CD8+ T cells, in case the antigen is presented by MHC class I), effector T cells that destroy target cells infected by intracellular viruses or bacteria, and/or the proliferation of helper T cells (Th, or CD4+ T cells, in case the antigen is presented by

MHC class II). In turn, activated Th cells elicit a conventional T cell-dependent immune response by interacting with resting B cells via MHC class II and driving their proliferation and differentiation into plasma cells (antibody-forming cells, mainly producing low affinity IgM-type antibodies) and memory B cells. Contrary to plasma cells, memory B cells survive for a long time in the body and respond rapidly to subsequent exposures of antigen by secreting high affinity IgG antibodies.

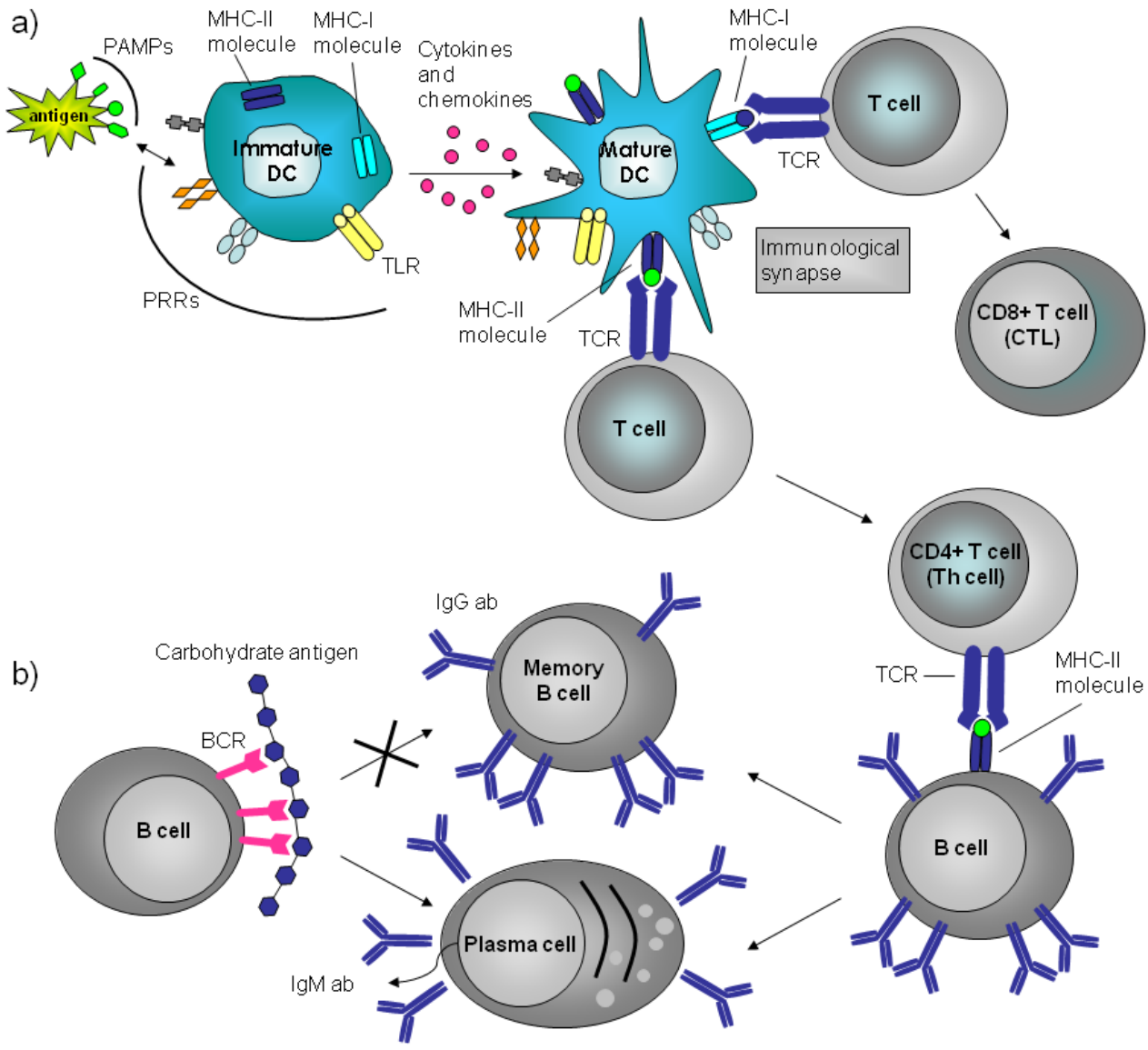


Figure 2_Diagramatic representation of the immune response.

GLYCOBIOLOGY

In 1988 the term glycobiology was first used to describe the merging of the traditional disciplines of carbohydrate chemistry, biochemistry, and cell biology.[4] However, almost a century earlier, the experiments on biologically important sugars performed by Emily Fischer predated the recognition of glycobiology as a distinctive field of investigation.

CARBOHYDRATE ANTIGENS

Amongst human pathogens, there is a large number of bacterial species which causes serious public health concerns. The surface of bacterial pathogens is covered with a dense array of oligo- and polysaccharides that, besides conferring mechanical stability to the cell membrane of microorganisms, are crucial protective antigens and virulence factors.
The polysaccharide surface can be in the form of capsules, glycoproteins or glycolipids. In Gram-negative bacteria, the lipopolysaccharide (LPS) also referred to as endotoxin, covers ca. 40% of the bacterial surface. The capsular polysaccharides (CPS) may be present in both Gram-negative bacteria such as *N. meningitidis*, *Haemophilus influenzae*, *E. coli* or *Salmonella typhi* and in Gram-positive such as S*treptococci* and *Staphylococci*, while, the glycolipid LPS is only present in Gram-negative bacteria and is part of the outer membrane. It is built of a lipid part and a polysaccharide part. The polysaccharide can be divided in a core oligosaccharide proximal to the lipid part and an O-polysaccharide. The O-polysaccharide is, like the CPS, either a homopolymer (*Vibrio cholerae* O1, *Brucella abortus*, *B. melitensis*) or made up from repeating units which may be di- to hexasaccharides.

HYSTORY

Historically, the first example of carbohydrate antigens dates back to 1900 in the form of ABO(H) blood group antigens.[5] The ABO blood group system is widely credited to have been discovered by the Austrian scientist Karl Landsteiner, who found three different blood types:[6] he was awarded the Nobel Prize in Physiology or Medicine in 1930 for his work.
In 1917 a "specific soluble substance" secreted by pneumococci during growth was discovered by Dochez and Avery.[7] They showed that there was present in the fluid media of pneumococcus cultures a substance which precipitated specifically in antipneumococcus serum of homologous type. Further work by Heidelberger and Avery has shown that two specific substances
could be isolated from Types I, II, and III pneumococci; namely, a species specific protein and a type specific polysaccharide.
These are the first examples in which any material other than protein have displayed antigenic properties.
Between 1920s and 1930s the immunological properties of bacterial CPSs became the target of several scientists: it was well known that an immune response against the surface polysaccharides confers protection against the disease.[8] In the mid-1940s it was evident that CPS elicited type-specific protective immune responses, and unfortunately infants and young children did not respond with type-specific antibodies; moreover that type-specific antibodies conferred

protection and vaccination with polysaccharides reduced the carrier rate of bacteria of the same types as in the vaccine. An important discovery regarded the coming of "glycoconjugates", using oligosaccharides covalently linked to a carrier protein.[9] On the basis of rabbits experiments, it was found that neo-glycoconjugates were able to induce high titred antibody responses which were, boostable and protective against challenge infection.

IMMUNE RESPONSE TO POLYSACCHARIDES

While most antigens, especially proteins and derivatives, are T dependent immunogens, *i.e.* they induce T cell activation *via* a MHC II-restricted pathway, the immune response to polysaccharides is typically T cell-independent. Owing to their polymeric structure, polysaccharides bind simultaneously several B cell receptors (BCR) leading to direct activation of B cells without T cells cooperation (**Figure 2b**).[10] As a result, the immune response to carbohydrates is hallmarked by an exclusive primary immune response with low affinity IgM production and no class switch to high affinity IgG antibodies. Moreover, pure polysaccharides cause immune responses of relatively short duration, and they do not induce immunological memory, *i.e.* they fail to result in a booster effect. T cell independent polysaccharides can be converted into T cell dependent immunogens by their covalent attachment to carrier proteins. The protein carrier incorporates T cell epitope peptides, which facilitate uptake and processing of the glycoconjugate by APC enhancing the presentation of the carbohydrate antigen for helper T cells activation. In this way immunological memory is established, raising a strong, durable and protective immune response from early childhood. Typical examples of immunogenic carrier proteins include bovine serum albumin (BSA) or its human variant HSA, keyhole limpet hemocyanin (KLH), bacillus Calmette-Guérin (BCG), CRM_{197} (Cross-Reacting Material 197), and tetanus toxoid (TT).

Furthermore, in order to achieve an optimal host protection, a vaccine setting should include a component (adjuvant) able to amplify the immune response. In particular, since saccharide antigens are often poorly immunogenic, carbohydrate-based vaccines need adjuvants to improve their efficiency and the quality and specificity of the immune response. Typical immunoadjuvants widely employed in vaccine settings are complete and incomplete Freund's adjuvants,[11] Detox, QS-21 (a saponin extracted from the bark of the *Quillaja saponoria* tree), and monophosphoryl lipid A (MPLA). However, the administration of these strong immunoactive species may cause undesired side effects, and milder and safer lipopeptide-based immunoadjuvants have been employed in many vaccine candidates.

Adjuvants are perceived as "danger signals" after binding to PRRs, and stimulate the activation and maturation process of APCs, thus enhancing the speed and duration of both the innate and adaptive immune response. In particular, adjuvants function as immune potentiators, providing the pro-inflammatory context necessary for optimal antigen-specific immune activation and amplifying the innate immune response. After the discovery of the TLR family, it has been shown that many PAMPs, as well as synthetic adjuvants, activate DCs upon stimulation of a specific TLR. These findings suggested that TLRs are essential in linking innate and adaptive immunity throughout the entire course of the host defence response, since they are involved in multiple immunostimulatory activities. Therefore, TLRs can be defined as general adjuvant receptors in the

body. On the other hand, adjuvants may also act as delivery systems to localize vaccine components and to target them to APCs.

CARBOHYDRATES AND PATHOGENS: ENCAPSULATED BACTERIA

BACTERIAL CELL STRUCTURE

Bacteria, despite their simplicity, contain a well-developed cell structure which is responsible for many of their unique biological properties. Because of the simplicity of bacteria relative to larger organisms and the ease which they can be manipulated experimentally with, the cell structure of bacteria has been well studied.

The most elemental structural property of bacteria is cell morphology (shape): typical examples (exemplified in **Figure 3**) are coccus (spherical), bacillus (rod-like), spirillum (spiral) and filamentous. Cell shape is often the first characteristics observed by a microbiologist to determine the identity of an unknown bacterial culture, moreover the Gram's method, based on chemical and physical properties of cell walls, allows to differentiate bacterial species as gram-positive and gram-negative.

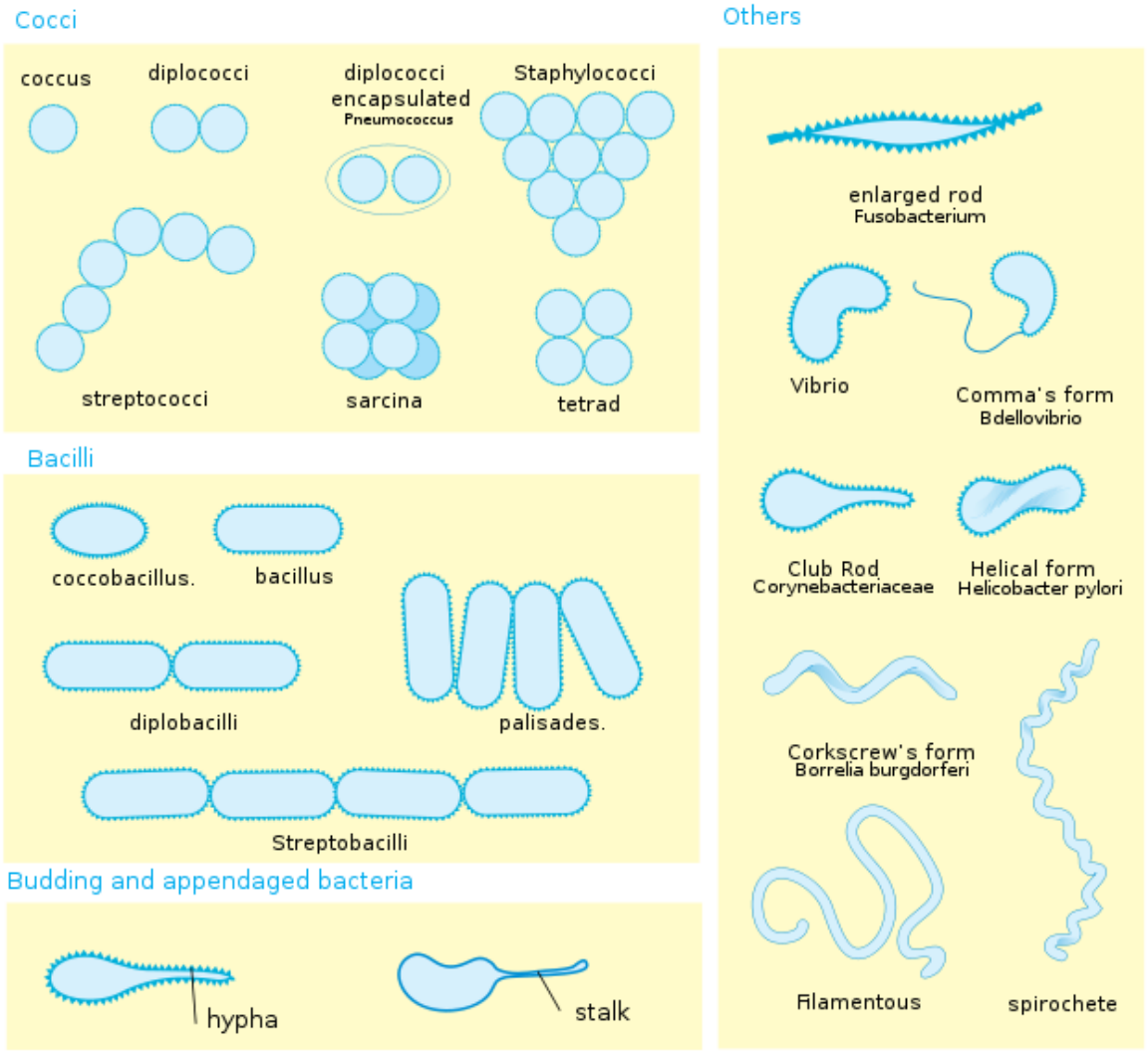


Figure 3_Bacterial cell morphology.

Gram staining is a laboratory technique used to detect peptidoglycan (cross-linked multilayered polysaccharide-peptide complex), which is present outside the lipid cell membrane: those that retains gram stain are called gram-positive, the others, that do not, are gram-negative. A gram-positive results in a purple/blue colour while a gram-negative in a pink/red colour.
In comparison to eukaryotes, the intracellular features of the bacterial cell are extremely simple. Bacteria do not contain *organelles* in the same sense as eukaryotes. Instead, the *chromosome* and perhaps *ribosomes* are the only easily observable intracellular structures found in all bacteria. Unlike eukaryotes, the bacterial chromosome is not enclosed inside of a membrane-bound nucleus but instead resides inside the bacterial cytoplasm. This means that the transfer of cellular information through the processes of translation, transcription and DNA replication all occur within the same compartment and can interact with other cytoplasmic structures, most notably ribosomes. In most bacteria the most numerous intracellular structure is the ribosome, the site of protein synthesis in all living organisms. They do exist, however, specialized groups of bacteria that contain more complex intracellular structures.

ENCAPSULATED BACTERIA

Encapsulated bacteria, both gram positive and gram negative, possess a polysaccharide coat (capsule, **Figure 4**) surrounding the bacterial cell and that is essential for their pathogenicity, exerting a protective function against the host's immune defence.

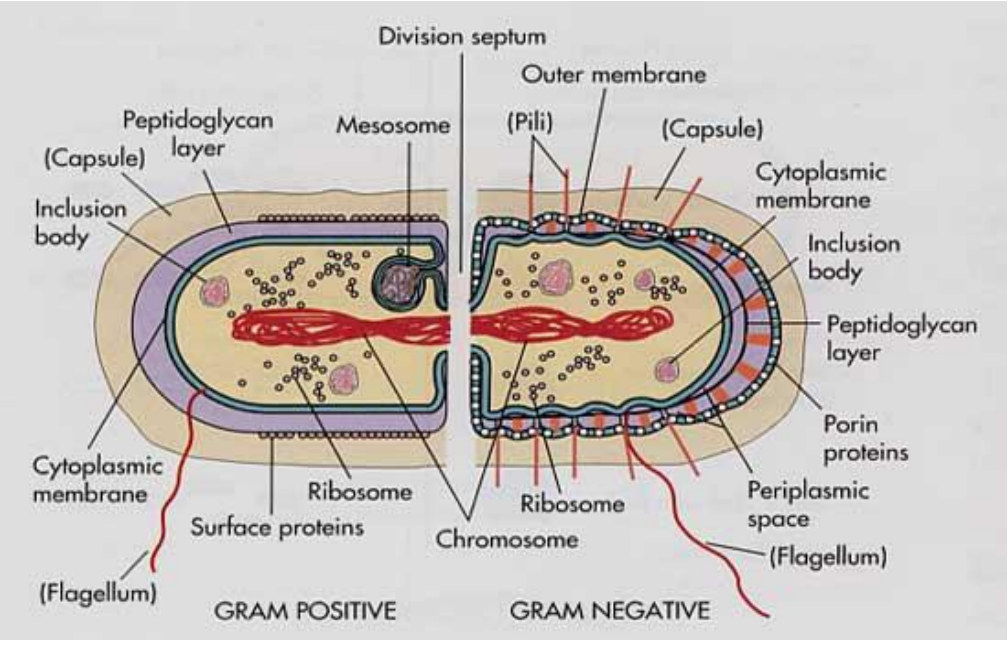


Figure 4_Bacterial, Gram-positive and Gram-negative, cells exemplification.

As a result, infectious diseases from encapsulated bacteria are still the third leading cause of death in the world. A large body of literature data indicates that carbohydrate-specific antibodies are predominantly responsible for protection against bacteria (*N. meningitidis*, *Haemophilus influenzae*, *Escherichia coli*, *Salmonella typhi*, and *Staphilococcus aureus*) with either a capsule or lipopolysaccharide on their surface, suggesting that vaccines consisting of purified pathogen-associated saccharide antigens may be effective to confer protection against infectious diseases.

In spite of the increased knowledge in immunology, there are several problems that remain to be solved:

- the base of serogrouping or serotyping systems is due to the structural differences in the CPS, so that carbohydrate antigens exhibit a large degree of antigenic variation.
- homology between carbohydrate structures present on bacterial surface and those of host cell membranes have been reported: i.e. the *N. meningitidis* serogroup B CPS, as well as the E. Coli K1 antigen, are antigenically similar to structures expressed on human foetal neuronal cells and consequently, poor immunogens in humans.[12] Therefore, the use of N. meningitidis serogroup B CPS in a vaccine has the potential risk of inducing autoantibodies.[10]
- polysaccharide antigens are mostly poor immunogens (T-independent antigens).[13] Children below 2 years of age and elderly respond poorly to olysaccharide antigens.

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CHAPTER 2

Carbohydrate-based vaccines

About plague of Athens, 430 BC

"Epidemic doesn't strike twice: or, at least, the potential relapse is not deadly".

Thucydides

(*History of the Peloponnesian War*, book 2, par.51)

INTRODUCTION

The *Plague of Athens* was a devastating epidemic which hit the city-state of Athens in ancient Greece during the second year of the Peloponnesian War (430 BC). Athens lost perhaps one third of the people sheltered within its walls. Historians have long tried to identify the disease behind the named *Plague* of Athens (plague, smallpox, or typhoid fever). Given the possibility that symptoms of a known disease may have mutated over time, or that the epidemic was caused by a disease which no longer exists, the exact nature of the Athenian epidemic may never be known. But, Thucydides himself suffered the illness, and survived: survivors could nurse sick person without falling again sick. This is the first example of *acquired immunity*.

HISTORY OF VACCINATION

Not only in Greece, but epidemics were well known in the ancient world. The smallpox virus (caused by either of two virus variant, *Variola major* and *Variola minor*) appeared in Africa during 10000 BC and is also known by the Latin name *Variola*, derived "*varius*", meaning spotted, or "*varus*", meaning pimple. The earliest physical evidence of smallpox is probably the pustular rash on the mummified body of Pharaoh Ramses V of Egypt.

During the whole human history, smallpox epidemics came one after the other with regularity and constancy. The earliest procedure used to prevent smallpox was inoculation (also known as variolation). Inoculation was possibly practiced in India as early as 1000 BC, and involved either nasal insufflation of powdered smallpox scabs, or scratching material from a smallpox lesion into the skin. However, the idea that inoculation originated in India has been challenged as few of the ancient Sanskrit medical texts described the process of inoculation. Accounts of inoculation against smallpox in China can be found as early as the late 10th century, and the procedure was widely practiced by the 16th century, during the Ming Dynasty. Lady Mary Wortley Montagu observed smallpox inoculation during her stay in the Ottoman Empire, writing detailed accounts of the practice in her letters, and enthusiastically promoted the procedure in England upon her return in 1718. In 1721, Cotton Mather and colleagues provoked controversy in Boston by inoculating hundreds.

In 1796, Edward Jenner[1], a doctor in Berkeley, Gloucestershire, rural England, discovered that immunity to smallpox could be produced by inoculating a person with material from a cowpox lesion. Cowpox is a poxvirus in the same family as variola. Jenner called the material used for inoculation *vaccine*, from the root word "vacca", which is Latin for cow.

Figure 1_ (James Gillray, 1802) Caricature of Jenner vaccinating patients.

The procedure was much safer than variolation, and did not involve a risk of smallpox transmission. Vaccination to prevent smallpox was soon practiced all over the world. In the end of 19th century other pathogenic diseases were rampant, but Jenner's method didn't work with them.

In 1858, the French chemist Louis Pasteur demonstrated that fermentation is caused by the growth of micro-organisms, and that the emergent growth of bacteria in nutrient broths is not due to spontaneous generation (*Omne vivum ex vivo* "all life is from life"): infections caused by germs came from outside into the human body. After that the deaths due to infection drastically decreased by washing with disinfectants hands and/or surgical instruments.

Pasteur's later work on diseases included work on chicken cholera. During this work, a culture of the responsible bacteria had spoiled and failed to induce the disease in some chickens he was infecting with the disease. Upon reusing these healthy chickens, Pasteur discovered that he could not infect them, even with fresh bacteria; the *weakened bacteria* (without feeding they were deadened) had caused the chickens to become immune to the disease, even though they had caused only mild symptoms.

The notion of a weak form of a disease causing immunity to the virulent version was not new; this had been known for a long time for smallpox and Jenner's immunization. In Pasteur experiment the novelty was that weakened disease can't be found in nature: human intervention had determined the restricted virulence of bacteria. Pasteur gave these artificially *weakened diseases* the generic name of *vaccines*, in honour of Jenner's discovery. Pasteur produced the first vaccine for rabies by growing the virus in rabbits, and then weakening it by drying the affected nerve tissue.

For a century, Vaccine developers have followed Pasteur's rules:

- Jonas Salk developed a vaccine containing a poliovirus (killed by treatment with formaldehyde);
- Albert Sabin used an attenuated, by in vitro purifications, poliovirus.
- vaccines against measles, mumps, and rubella were pioneered by Hilleman;
- Ramon (1924) and Glenny (1923) laid the fundamentals for the development of vaccines against diphtheria and tethanus, *N. meningitidis*, *Streptococcus pneumoniae*, *Haemophilus influenzae,* and others diseases: they isolated and inactivated essential components of bacteria and viruses.

At the end of 20th century, many vaccines have been designed and new technologies, such as recombinant DNA and chemical conjugation between sugars and carrier proteins, have contributed to the evolution of the traditional technologies with the aim of conquer the remaining pathogens. But an important new technology, reverse vaccinology, become available to scientists after the discovery of microbial genomes.

IMMUNISATION

Immunity to infectious microorganisms can be achieved by *active* or *passive* immunization, and acquired either by natural (transfer from mother to foetus or previous infection) or artificial (injection of antibodies or vaccine) processes.
Antibodies from humans or animals, specific for a pathogen or toxin, are transferred to non-immune individuals when there is a risk of infection and the body have no time to develop own immune response: passive immunization is induced artificially, but it can occur also naturally by transfer of maternal antibodies to the foetus through placenta. This method acts quickly but is short-lasting, because the antibodies are naturally broken down.
The introduction of a foreign molecule (microbe or parts of it) into the body causes the body itself to generate immunity against the target. Active immunization allows immunologic memory and can be *achieved* by infection with a microorganism or be *acquired* by administration of a vaccine. Active immunity is generally long-term and invokes B cells proliferation and T cells activation.

TYPE OF VACCINES

There are several approaches to design vaccines against microbes. These choice are based on information about the type of microbe, for instance how it infects cells or how the immune system responds to it, or on the regions of the world where it has to be used.
The type of vaccines currently in use are:

- ✓ live, attenuated vaccines;
- ✓ inactivated vaccines;
- ✓ subunit vaccines;
- ✓ toxoid vaccines;
- ✓ conjugated vaccines;
- ✓ DNA vaccines;
- ✓ recombinant vector vaccines.

Carbohydrate-based vaccines

Microbes can be attenuated and weakened in laboratory. Loosing their ability to cause a disease, live-attenuated vaccines are similar to a natural infection and they are "good teacher" of immune system: with only few doses they are able to confer long-life immunity by eliciting a strong immune response. The most important disadvantage of this kind of vaccines lie in their nature: they are still "alive" and so they could change or mutate so that could revert in virulent form and cause disease. Moreover people who have damaged or weakened immune system cannot receive this kind of treatment. Another limitations are:

- storage: they need to be freezing to maintain the efficacy and it's a problem for transport especially by long travel (in developing countries it could be not the best choice);
- easy to create for certain viruses (containing a small number of genes), but very difficult to create for bacteria (having thousands of genes they are much harder to control).

Chemicals, heating, or radiations are the common procedure to create inactivated vaccines. In this case the microbe can't mutate because it's dead. In contrast with live-attenuated vaccines, inactivated vaccines can be easily stored and transported because they don't need refrigeration, which make them suitable for developing countries. Unfortunately they are not able to stimulate a strong immune response, and several doses are required.

Subunit vaccines (i.e. *Hepatitis B virus*) are based on the essential antigens (and not on the entire microbe) that best stimulate the immune system. Essential antigens are identified by a time-consuming and tricky process consisting in:

- to grow the microbe in lab and then use chemicals to break it;

or

- to build antigen molecules from the entire microbe using a recombinant DNA approach (this vaccines are called *recombinant subunit vaccines*).

Toxoid vaccines are designed in the case of bacteria can secrete toxins that is the main cause of disease. Toxoids, inactivated toxins by formalin, are safe for use as vaccines (i.e. *diphtheria* and *tetanus*).

If the scientist knows and is able to analyze all the genes of a microbe, a DNA based vaccine can be a solution to the disease. DNA based vaccines are still in experimental stages, but to use codifying genes for important antigens show a great promise: in fact, they are easy and inexpensive to construct, moreover they couldn't cause disease because they are just copies of genes, not containing the microbe.

Recombinant Vector vaccines are similar to DNA vaccines: "vector" is the microbe used as carrier to introduce microbial DNA to cells into the body. This kind of vaccines are currently under investigation and by the moment are used for HIV (human immunodeficiency virus), rabies and measles.

The virulence of some pathogenic bacteria depends on the non-pathogenic properties of their outer coating, a *capsule* made of polysaccharides. Since capsular polysaccharides (CPSs) are the key virulence factors for encapsulated bacteria, various CPS-based antibacterial vaccines have been developed and introduced in the market. However, as polysaccharides are T-independent antigens, purified CPS-based vaccines are poorly immunogenic in infants and young children, and they fail to establish immunological memory. A key breakthrough in this field was the introduction of glycoconjugate vaccines, where the CPSs (or their synthetic fragments) are conjugated to carrier

proteins, thus eliciting a T-dependent response which enhances the immunogenic properties of the saccharide moiety. In this way, boosting of the antibody response occurs on reimmunisation and immunological memory is established. In 1945 the immunization with specific CPS, to prevent pneumococcal pneumonia, was the first example of polysaccharide vaccine.[2] Vaccines currently present on the market are based on this concept.

REVERSE VACCINOLOGY[3]

If the genome of a pathogen is accessible, the vaccine design could be rationalized by computer using the informations present in its genome, without the need to grow the specif microbe. This process is called "reverse vaccinology".

The genomic information was first used for the development of a vaccine against serogroups B meningococcus,[4] and moreover the ability to interrogate the entire antigenic repertoire has implemented the power of this technique. In principle the reverse vaccinology takes into account the entire protein repertoire of each pathogen to select the best candidate antigen.

In **Table 1** is reported a comparison between the conventional vaccination and the reverse vaccinology.

Table 1[a]_Traditional vaccinology *vs.* Reverse vaccinology.

	TRADITIONAL VACCINOLOGY	REVERSE VACCINOLOGY
ANTIGENS AVAILABLE	10-25 identified by biochemical or genetic tools.	Virtually all antigens encoded by the genome.
PROPERTY OF ANTIGENS	The most abundant antigens, the most immunogenic during disease, only from cultivable microorganism.	All antigens are available, even if not highly immunogenic during disease. Antigens from noncultivable microorganisms can be identified.
IMMUNOLOGY OF THE ANTIGENS	Highly immunogenic antigens, often variable in sequence, because of immune selective pressure. Some may contain domains mimicking self-antigens and may induce autoimmunity.	The most conserved protective antigens can be identified. Usually they are not the most immunogenic during infection. The novel antigens are screened against the human genome, and antigens with homology to self-antigens are removed upfront.
POLYSACCHARIDE ANTIGENS	A major target of traditional bacterial vaccines.	Cannot be identified by reverse vaccinology. But novel carbohydrate antigens can be discovered by the identification of operons coding for the biosynthesis of polysaccharides.
T CELL EPITOPES	Known epitopes limited to the known antigens.	Virtually every single T cell epitope is available. Screening of the total T cell immunity can be done by overlapping peptides.

[a] Extracted from *ref. 3*.

GLYCOCONJUGATE VACCINES[5,6]

As introduced in ***CHAPTER 1***, in 1929 Avery and Goebel found out the solution to the weaker sugar antigens: their chemo-immunological studies included the immunological specificity of synthetic sugar-protein antigens. They demonstrated that non-immunogenic derivatives of glucose and galactose were able to induce proliferation of antibodies in rabbit only if conjugated to proteins.[7] Indeed, the combination of a microbial poly- or oligosaccharide (weak antigen, B-cell epitopes) with a carrier protein (T-cell epitopes) generates a conjugate vaccine, thus increasing in quantity and quality the immunogenicity of sugar-based antigens.

Bacterial conjugate vaccines currently in use are based on three steps synthesis:

- fermentation, followed by purification, leads to pure bacterial polysaccharides;
- fermentation, and purification by formaldehyde lead to purified toxins (i.e. tetanus toxoid (TT) or diphtheria toxins are the most used);
- the conjugation of the pure bacterial polysaccharide with a toxin, by chemical methods, allows the formation of a covalent bond between the two components.

The functional groups used to create this bond (**Table 2**) could be intrinsic to the polysaccharide and the protein.[8] Protein-saccharide conjugation can be achieved by site selective chemical modification of the sugar chain, for example by introduction of a thiol or a carboxylic group at the reducing end.[9]

Alternatively, the efficiency and speed of conjugation can be improved by the insertion of a spacer arm on the sugar chain suitable for protein conjugation (*single point attachment*).[10] The introduction of a spacer can relieve the steric hindrance between the two moieties, and moreover it allow an easier coupling step. Then, the spacer has to be "immunologically silent": it means that no component of the linker should induce high titres of linker-specific antibodies.

CONSIDERATIONS ON THE IMMUNOGENICITY OF CONJUGATED VACCINES

Different issues can influence the immunogenicity of conjugated vaccines:[11]

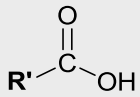
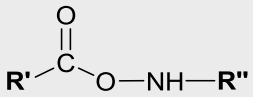
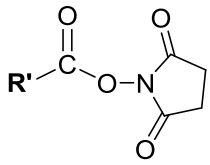
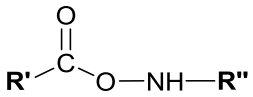
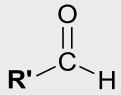
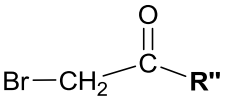
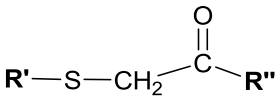
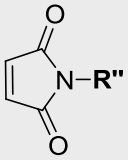
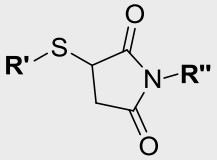
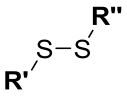
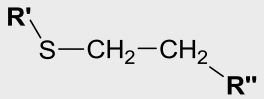
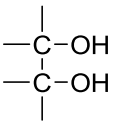
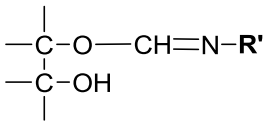
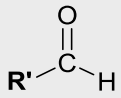
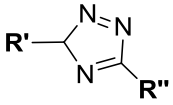
i. the size of the saccharide chain;
ii. *O*-acetylation degree of the carbohydrate;
iii. the loading: sugar/protein ratio;
iv. the nature and the length of the spacer;
v. the nature of the protein carrier.

SACCHARIDE MOIETY: SIZE AND SUGAR/PROTEIN RATIO

In 1989 the first evaluation of the size and chemistry effects on immunogenicity has been accomplished by Seppala and co-workers.[11e] There is not a rule that can determine what is the proper number of repeating units of saccharide, and the best sugar/protein ratio for a better conjugation. In principle, less than four repeating units are not enough to establish immune response. The influence of the sugar/protein ratio is different for antigens of variable length.

In general, the immunogenicity can increase with the molecular size of the polysaccharide and also of the conjugates.

Table 2_Examples of functional groups used in chemical conjugation.

CHEMISTRY	FUNCTIONAL GROUPS INVOLVED		PRODUCTS
Carbodiimide-mediated condensation		H_2N-R''	
Active ester		H_2N-R''	
Reductive amination		H_2N-R''	$R'-CH_2-NH-R''$
Thioalkylation[12]	$R'-SH$		
Thiol addition[13]	$R'-SH$		
Disulfide formation[14]	$R'-SH$	$HS-R''$	
Thiol-ene[15,16]	$R'-SH$	$CH_2=CH-R''$	
Cyanogen bromide or CDAP activation		H_2N-R'	
Oxime formation[17]		H_2N-O-R''	$R'-CH=N-O-R''$
"Click Chemistry"[18]	$R'-N=\overset{+}{N}=N^-$	$HC\equiv C-R''$	

CDAP = 1-Cyano-4-dimethylamino-pyridinium tetrafluoroborate

SPACER'S NATURE

Also the nature of the spacer is correlated to immunogenicity problems. Rigid constrained spacer, i.e. cyclic structure, such as maleimido, are known to drive the immune response away from the sugar target, because of the formation of considerable amounts of undesirable antibodies. A flexible spacer, i.e. alkyl type, resulted as non-immunogenic.

CARRIER PROTEINS COMMONLY USED

In first attempts, Diphtheria and TTs toxins families have been used for conjugate vaccines preparation, but in general a large number of protein carriers has been evaluated as suitable for conjugate vaccines.[19] Keyhole limpet hemocyanin (KLH) and the virus-like particle Qβ were also

used.[20] CRM_{197},[21] a non toxic (so it doesn't need detoxification by formaldehyde) mutant of diphtheria toxin, has been extensively used for preparation of licensed vaccines.
In principle, the preferable features for a carrier are:

- high level of purity;
- high level of safety;
- the possibility to be fully characterized by physicochemical methods;
- ability to induce a strong T-cell help;
- low antibody response to carrier-specific B-cell epitopes, in order to drive the response against sugar moiety.

SYNTHETIC APPROACHES

Since the synthesis of conjugate vaccins is commonly accomplished by extraction and subsequent purification of poly- or oligosaccharides from natural capsule of microbial culture, two significant drawbacks can't be avoided:

- microbial contamination: traces of bacterial contaminants, such as endotoxins;
- heterogeneity: coexistence of CPS fragments of different sizes.

Moreover, in particular cases (fungal antigens, viral oligosaccharides, tumor-associated carbohydrate antigens (TACAs)) it's not easy to extract carbohydrate antigens from biological sources, while in other cases they can't be obtained at all.
Thus, the overcoming of these disadvantages is constituted by the application of the fully synthetic approach.
In 1975 Lemieux's group[22] pioneered the first attempts on fully synthetic oligosaccharide conjugates, while Bencomo[13] and co-workers developed the first fully synthetic conjugate vaccine.
The chemical synthesis of CPS fragments of different length leads to:

- ✓ provide homogeneous, well defined and characterized fragments;
- ✓ obtain fragments that were not accessible by extraction from microbial cultures;
- ✓ understand the minimal structural requirements to induce immune response;
- ✓ fragments of high purity, that present a suitable linker apt to conjugation with carrier proteins.

In this context however, optimization procedures are required to:

- simplify and optimize the large scale synthesis of fragments containing challenging structural motifs;
- simplify and optimize the synthesis of fragments made up by several repeating units.

Thus, a conjugating reagent should possess sufficiently high reactivity in order to:

- ✓ maximize the yield of glycoconjugate;
- ✓ achieve, reproducibly, a proper number of sugars attached to protein;
- ✓ avoid the need for large excess of sugar or protein patterns.

Moreover, the activated intermediate, precursor of the glycoconjugate, has to tolerate the aqueous buffer conditions required in conjugation reaction.

FULLY SYNTHETIC GLYCOPEPTIDE VACCINES

The evolution of this concept deal with fully synthetic glycopeptide vaccines.[23] It's even more challenging developing a glycoconjugate by the chemical synthesis of the sugar moiety together with the chemical synthesis of the carrier protein. The carrier is constitute of synthetic peptides representing $CD4^+$ T-cell epitopes. MHC class II genetic restriction is the obstacle for human application.[24]

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CHAPTER 3

Neisseria meningitidis

Neisseria Meningitidis

The Gram-negative encapsulated organism Neisseria meningitidis is the leading cause of bacterial meningitis. This invasive infection affects mostly infants, children, and adolescents who do not possess specific antibodies.

INTRODUCTION

Meningococcal disease incidence in human population reaches its climax over 100000 population/year, and it is influenced by the virulence potential of circulating Meningococci by host or environmental factors.[1]

The first outbreaks of meningococcal meningitis were first described in Geneva in 1804 and in New England in 1806, while in 1887 Weichselbaum discovered the causative agent and almost 100 years ago is dated the beginning of epidemics in the sub-Saharan Africa.

The family *Neisseriaceae* consists of Gram-negative aerobic bacteria from fourteen genera, including *Neisseria*, *Chromobacterium*, *Kingella*, and *Aquaspirillum*. The genus *Neisseria* contains two important human pathogens, *N. gonorrhoeae* and *N. meningitidis*. *N. gonorrhoeae* causes gonorrhea, and *N. meningitidis* is the cause of meningococcal meningitis. *N. gonorrhoeae* infections have a high prevalence and low mortality, whereas *N. meningitidis* infections have a low prevalence and high mortality.

MENINGITIS

Bacterial meningitis causes approximately 170,000 annual deaths upon more than 1,200,000 cases, with at least a 5-10% of case fatality in industrialized countries and a 20% in the developing world.[2] Meningitis is an inflammation of the protective membranes covering the brain and spinal cord, known collectively as the meninges (**Figure 1**).

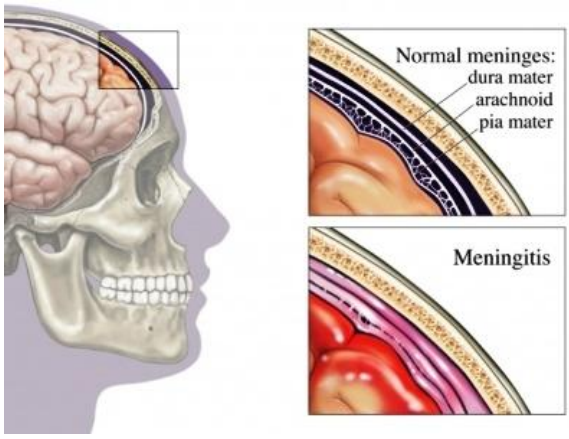

Figure 1_Meningitis inflammation.

The inflammation may be caused by infection with viruses, bacteria, or other microorganisms, and less commonly by certain drugs. Meningitis can be life-threatening because of the inflammation's proximity to the brain and spinal cord; therefore the condition is classified as a medical emergency.

The most common symptoms of meningitis are headache and neck stiffness associated with fever, confusion or altered consciousness, vomiting, and an inability to tolerate light (photophobia) or loud noises (phonophobia). Sometimes, especially in small children, only nonspecific symptoms may be present, such as irritability and drowsiness. If a rash is present, it may indicate a particular cause of meningitis; for instance, meningitis caused by meningococcal bacteria may be accompanied by a characteristic rash.
In addition, serious sequelae such as brain damage, hearing loss or a learning disability affect up to one third of survivors. *Streptococcus pneumoniae*, *Haemophilus influenzae* type b (Hib) and *Neisseria meningitidis*[3] are responsible for most of the cases of bacterial meningitis worldwide although, with the advent of conjugate vaccines for Hib and for the pneumococcus, the meningococcus is the remaining major bacterial pathogen causing meningitis in children and adults.

NEISSERIA MENINGITIDIS

In 1887 Weichselbaum was the first to isolate the causative agent of cerebrospinal meningitis, which he called *Diplococcus intracellularis meningitidis*. *N. meningitidis* is an encapsulated, diplococci, gram-negative bacterium (**Figure 2**): its outer membrane is surrounded by a polysaccharide coat that is essential for its pathogenicity.

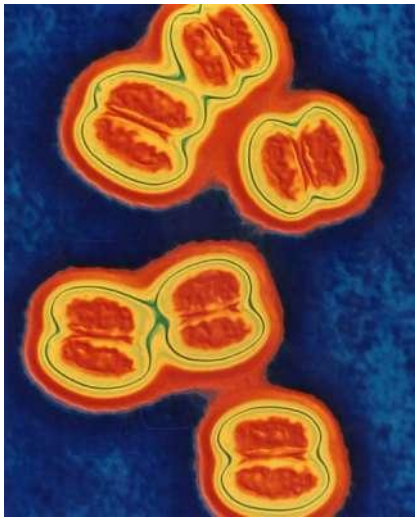

Figure 2_Diplococci encapsulated (green covering) bacterium of *N. meningitidis*.

N. meningitidis has been recognized as the cause worldwide of epidemics of meningococcal meningitis and meningococcemia.
N. meningitidis colonizes the upper respiratory tract of almost 10% of human population, and humans are the only known host. Individuals who are colonized are carriers of the pathogen who can transmit disease to nonimmune individuals. The bacterium is able to enter in the bloodstream, where it multiplies to high density and causes a form of sepsis characterized by a dramatic disruption of the endothelium and microvasculature. Through the bloodstream the bacterium can cross the blood-brain barrier and cause meningitis.

In general the meningococcus usually inhabits the human nasopharynx without causing detectable disease. This latency may last for a few days to months and is important because it not only provides a reservoir for meningococcal infection but also stimulates host immunity. Between 5 and 30% of normal individuals are carriers at any given time, yet few develop meningococcal disease. Carriage rates are highest in older children and young adults. Highest attack rates occur in infants from 3 months to 1 year old. Meningococcal meningitis occurs both sporadically (mainly groups B and C meningococci) and in epidemics (mainly group A meningococci), with the highest incidence during late winter and early spring. The invasive infection affects mostly infants, children, and adolescents who do not possess specific antibodies. After infection some of these subjects may develop the disease within a few hours and, of them, about 5-15% die while up to 25% develop permanent damages, such as epilepsy, mental retardation or sensorineural deafness.[4]

MENINGOCOCCAL SEROGROUPS

Based on the chemical composition of the polysaccharide capsule, 13 capsular serogroups (A, B, C, D, H, I, K, L, X, Y, Z, 29E, and W135) of *N. meningitidis* have been defined so far, but only six serotypes (A, B, C, Y, W135, and recently X) are currently associated with significant pathogenic potential.[5,6] The relative incidence of *N. meningitidis* serogroups is strictly dependent on geographic area. Serogroups B and C are responsible for the majority of cases of meningococcal disease in developed country, i.e. USA and Europe.

Although all *N. meningitidis* serotypes can cause epidemics, group A strains (MenA) are the main responsible for epidemics in the sub-Saharan Africa (the so-called "meningitis belt"), where the annual disease incidence ranges from 1 to 8‰ of the population.[7] Most of these infections are caused by serogroup A, but since 2002 serogroup W135 has been considered to be also a major threat. However, in the past 20 years sporadic cases or clusters of meningitis due to other *N. meningitidis* serogroups have emerged.

CONJUGATE VACCINES COMMERCIALLY AVAILABLE

Although there are two licensed vaccines (bivalent A/C and tetravalent A/C/Y/W135) which are effective for persons older than two years, the formulation of suitable glycoconjugate vaccines against *N. meningitidis* is required to improve immune responses in young children. Accordingly, three monovalent group C conjugate vaccines and a tetravalent meningococcal conjugate vaccine against groups A, C, Y and W135 are currently present on the market (**Table 1**).

Table 1_Commercially available meningococcal conjugate vaccines.

N. MENINGITIDIS SEROGROUPS	TYPE OF CONJUGATE	MANUFACTURER
A →6)-α-D-Man*p*NAc(3/4OAc)-(1→PO_3^-→ **C** →9)-α-D-Neu*p*5Ac(7/8OAc)-(2→ **W_{135}** →6)-α-D-Gal*p* (1→4)-α-D-Neu*p*5Ac(9OAc)-(2→ **Y** →6)-α-D-Gal*p* (1→4)-α-D-Neu*p*5Ac(9OAc)-(2→	MenA-TT	Serum Institute India
	MenC-CRM_{197}	Pfizer Novartis Vaccines
	MenC-TT	Baxter
	MenACWY-DT	Sanofi-Pasteur
	MenACWY-CRM_{197}	Novartis Vaccines

FULLY SYNTHETIC NEO-GLYCOCONJUGATES

The groups of Pozsgay[8] and Oscarson[9] reported the syntheses of fragments (up to the trimer) of the MenA CPS, consisting of (1→6)-linked 2-acetamido-2-deoxy-α-D-mannopyranosyl phosphate residues (**Figure 3**).

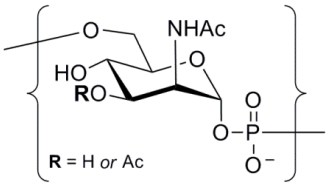

Figure 3_Repeating unit of MenA.

In particular, Pozsgay described also the conjugation of the synthetic fragments to HSA, showing that a polyclonal anti-*N. meningitidis* A antiserum can recognize a monosaccharide fragment of its CPS. However, the MenA CPS suffers from poor stability in water due to the inherent lability of the anomeric phosphodiester groups bridging two *N*-acetyl mannosamine units. This structural property makes the development of a fully synthetic glycoconjugate vaccine a challenging task.

The access to synthetic analogues endowed with both the immunological properties of the natural compounds (*i.e*., the ability to induce the production of antibodies that will cross-react with the bacterial capsule) and an increased stability in water, is therefore highly desirable. For this purpose, syntheses of phosphonoester-bridged fragments of the MenA CPS, where 1-*C*-phosphonates have been used as isosteric and nonhydrolyzable analogues of glycosyl 1-*O*-phosphates, have been reported by Oscarson[10] and our group[11]. We also investigated the relative affinities of the synthetic molecules (monomer, dimer and trimer, **Figure 4**) by using a competitive ELISA assay (Enzyme-Linked ImmunoSorbent Assay), showing that the synthetic fragments containing the unnatural interglycosidic phosphonoester linkage are recognized by a human polyclonal anti-MenA serum.[10b]

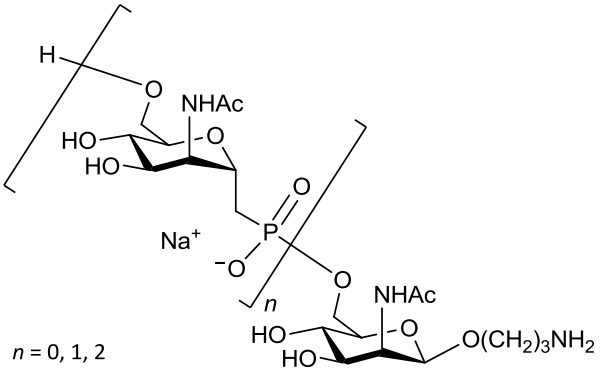

Figure 4_ Phosphonoester-linked oligomers of *Neisseria meningitidis* A capsular polysaccharide.

Following these encouraging results, the synthetic oligomers were conjugated to passivating thiols and employed for the fabrication of multivalent gold glyconanoparticles mimicking the bacterial capsule.[12] Interestingly, gold glyconanoparticles displaying the synthetic Men A CPS fragments bind to specific anti-MenA antibody with at least two orders of magnitude higher than the corresponding non-conjugated, monovalent oligomers.[13] Investigations are in progress to establish whether these gold glyconanoparticles are also able to induce immune cell responses.

N. MENINGITIDIS TYPE X

In 1963 Slaterus[14] isolated new types of Meningococci from patients in a non-epidemic period in Netherlands. The new types were X, Y, and Z: these three strains failed to react with antisera prepared against A, B, C, and D strains[15], first discovered by Branham. In 1973, Bundle, Kenny, and Jennings have improved the procedure for MenX isolation.[16]

MENX STRUCTURE

The structure of *N. meningitidis* type X (MenX) was first elucidated by Bundle and co-workers in 1974.[17] The Men X polysaccharide antigens consists of linear chains of 2-acetamido-2-deoxy-D-glucopyranose linked (1→4)-α by phosphodiester bonds.[18] That MenX polysaccharide was an homopolymer of 2-acetamido-2-deoxy-glucosyl phosphate was demonstrated by analytical data, shown in **Table 2**.

Table 2_Analysis of MenX polysaccharide.

Composition	% by weight[a]	Molar ratio
Carbon	29.2	
Hydrogen	4.3	
Nitrogen	4.3	
Phosphorous	9.3	1.0
Total acetyl	12.7	0.98
O-Acetyl	0.0	
Glucosamine	51.8	0.97
Amino acids[b]	<1	
Nucleic acids[c]	~2-3	

[a] corrected for moisture
[b] determined by Lowry's method[19] and Technicon AutoAnalyzer
[c] determined by ultraviolet absorption (260 and 280 nm)

Glucosamine, *N*-Acetyl, and phosphate are present in equimolar proportions in the polymer. Moreover, MenX homopolymer has an average chain length of 50 units, differently from its cognate *N. meningiditis* A CPS, it does not contain *O*-acetyl groups in the backbone.

REPORTED MENX CASES

MenX was responsible of rare cases of meningococcal diseases, meningitis in particular, in North America,[20] Europe,[21] Australia,[22] Africa[23] and the People's Republic of China.[24] More recently, MenX has emerged in Africa as pathogenic potential: it has caused increasing cases of meningitis. Men X outbreaks have been described in Ghana[5d] (9 cases over a 2-year period) and in Niger (134 cases between 1995 and 2000,[25] representing almost 4% of the meningococcal isolates from all cerebrospinal fluid samples).

It was in 2006, however, that WHO started to consider Men X as a substantial threat, when an unprecedented incidence of meningitis caused by Men X was observed in Niger: 51% of 1,139 confirmed cases of meningococcal meningitis were found to be caused by serogroup X.[26] In the same year, a meningococcal disease outbreak was notified in Western Kenya, bordering Uganda, with 74 cases reported between January and March and 27% ascribed to Men X infections.[27]

The meningitis cases due to Men X do not present any clinical or epidemiological difference from those due to serogroup A. Most cases (93%) were recorded during the dry season, with a mean age of the patients of 9.2 years and a fatality rate of 11.9%.[28]
Very recently, a case of invasive meningococcal disease caused by MenX was described in Italy: the patient was a 55-year-old Italian woman.[29]

MENX VACCINE

As mentioned above, serogroups A, B, C, Y and W135 are responsible for most meningococcal disease worldwide. However, recent outbreaks of Men X indicate that this serogroup also has considerable pathogenic potential. Vaccines currently available on the market against meningococcal disease, as well as those under licence, do not include antigenic components of Men X, and therefore they do not offer protection against infections caused by this emerging serogroup. Although serogroup X usually causes only a small proportion of meningococcal disease, it is possible that repeated vaccination against some serogroups (especially A and C) in many African countries has the potential to select meningococci of other serogroups (for example, Men X) and might result in a changed profile of meningococcal disease. This possibility should be considered when conjugate vaccines carrying limited ranges of serogroups are introduced.[30] The development of more comprehensive conjugate vaccines including Men X CPS fragments could therefore become an urgent issue in the near future.

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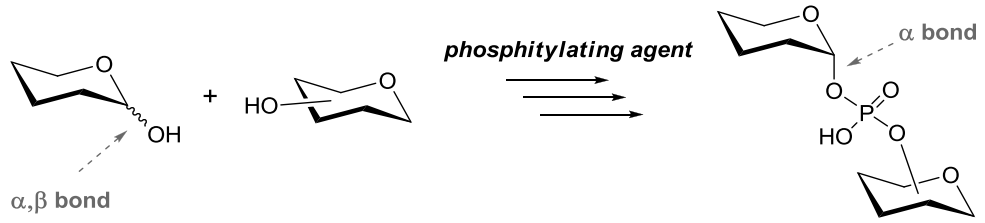
CHAPTER 4

Synthesis of glycosyl phosphodiesters

INTRODUCTION

The linkage of a glycosyl phosphate unit with an hydroxyl group of another saccharide acceptor provides a glycosyl phosphodiester. The cell wall, or capsule, of many bacteria and yeasts, as well as the extracellular surface of protozoan parasites, are composed by phosphoglycans with this type of linkage. It could be also found in animals glycoproteins. It is well known that the antigenic determinants, which define the immunogenical specificity, are in many case constituted by glycosyl phosphate units.

Natural phosphoglycans composed of glycosyl phosphate (or oligoglycosyl phosphate) repeating units could be chemically classified as poly(glycosyl phosphates). Since phosphoglycans are immunologically active components of the outer membrane of pathogenic microbes, it is important to develop a synthetic route for chemical preparation of fully synthetic biopolymers. The synthesis of anomeric phosphodiesters is a challenging task (**Scheme 1**), due to the stereochemical configuration of the hydroxyl group in anomeric position together with the lability of anomeric phosphodiester *O*-linkages.



Scheme 1_The stereochemical control in phosphodiester bridge synthesis.

In literature, there are three review papers describing the chemical preparation of fully synthetic glycosyl phosphodiester-linked oligomers: by Thiem and Franzkowiak in 1989,[1] by Hansson and Oscarson in 2000,[2] and by Nikolaev et al. in 2007.[3]

CHEMICAL SYNTHESIS OF GLYCOSYL PHOSPHOSACCHARIDES

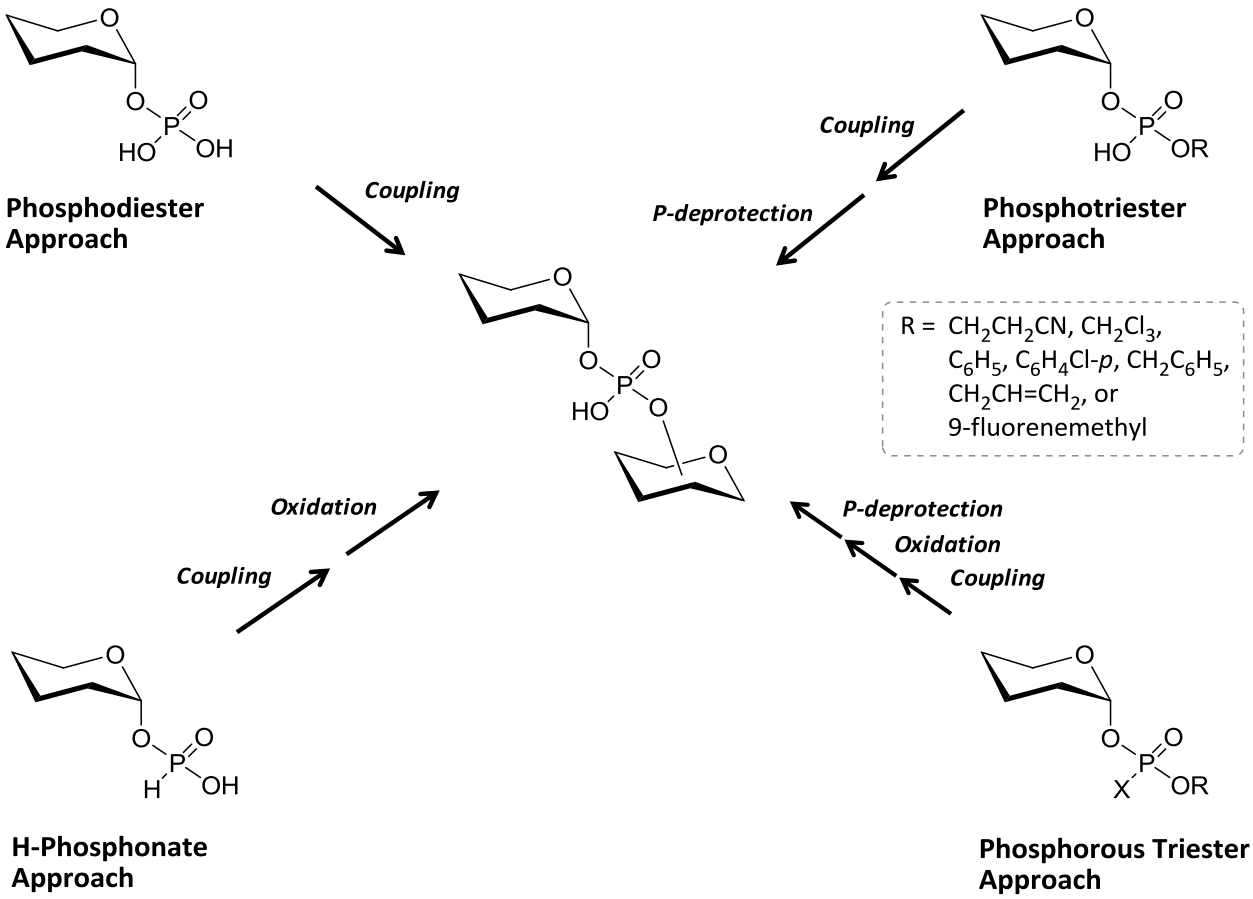
To date, there are examples of synthetic fragments containing the 1- phosphates of:

- ✓ α-D-mannopyranose, α-D-glucopyranose, α-D-galactopyranose, α-L-fucopyranose, α-D-rhamnopyranose, α-L-rhamnopyranose;
- ✓ 2-acetamido-2-deoxy-α-D-mannopyranose, 2-acetamido-2-deoxy-α-D-glucopyranose, 2-acetamido-2-deoxy-α-D-galactopyranose.

As far as the preparation of glycosyl phosphosaccharides is concerned, five basic synthetic methodologies can be found in literature:

i. Phosphodiester;
ii. Phosphotriester;
iii. Phosphorous (or Phosphite) triester [including phosphoramidite and phosphorochloridite versions];
iv. Hydrogen-phosphonate (H-phosphonate) approaches;
v. Others based on the glycosylation reaction.

Glycosyl phosphosaccharides



Scheme 2_Approaches to glycosyl phosphosaccharides synthesis.

The first four methods were originally designed for nucleotide chemistry: their mechanism is based on the condensation of various P-containing glycosyl components (electrophiles) with alcohol monosaccharide derivates (nucleophiles).

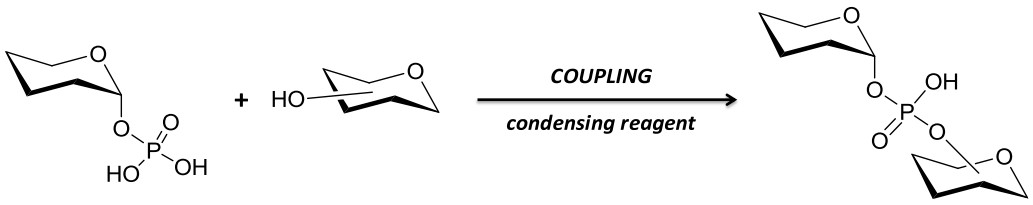
For 20 years the chemical synthesis of glycosyl phosphosaccharides was based on phosphodiester approach, developed in the mid-1950s.[4] The phosphotriester[5] and the H-phosphonate[6] methods were pioneered by Todd's group during 1950's, and optimized[7,8] later. In 1981 the phosphoramidite method[9] was discovered and after that, thanks to its simplicity and efficiency, it became the most used.

P(V) CHEMISTRY: PHOSPHODIESTER AND PHOSPHOTRIESTER METHODS

PHOSPHODIESTER METHOD

In 1971, Cawley and Letters published the first chemical synthesis of mannosyl phosphodiesters (1→4)- and (1→6)-linked.[10]

The phosphodiester method is historically the first method used, moreover it is conceptually the simplest: a protected glycosyl phosphate is coupled with an hydroxyl carbohydrate acceptor (i.e. **Scheme 3**) by condensing reagents.



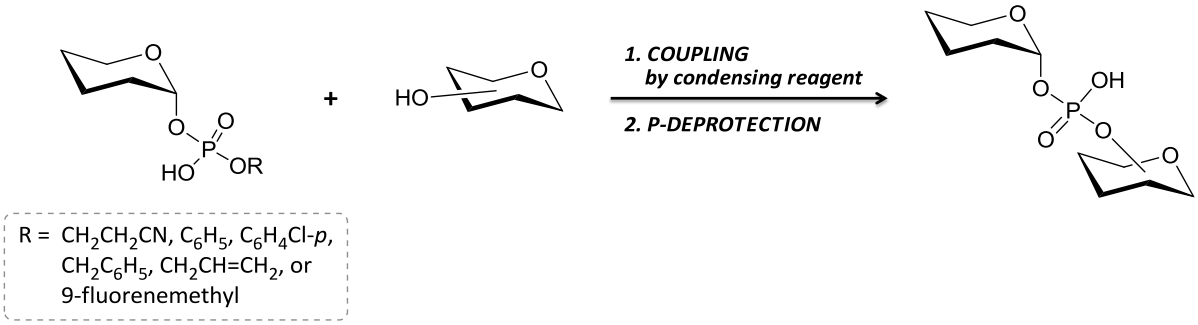
Scheme 3_Phosphodiester approach.

The most used condensing reagents are dicyclohexylcarbodiimide (DCC), 2,4,6-triisopropylbenzenesulfonyl chloride (TPS-Cl) or 3-nitro-1-(2,4,6-triisopropylbenzenesulfonyl)-1,2,4-triazole (TPS-NT). The glycosyl phosphate is synthesized by glycosylation reactions (*see below*) or by simple manipulations of commercially available glycosyl phosphates.

In 1978 the first application on *N*-acetyl-D-glucosamine substrate is showed.[11] In general, the most compounds synthesized with the present method contained mostly the (1→6)-linked phosphodiester linkages. Concerning the condensation/deprotection steps of α-D-Glc*p* 1-phosphate compounds, the yields are moderate and ranged from 69% to 15%, while in the case of α-D-Glc*p*NAc 1-phosphate derivatives the yields are constantly very low (<20%). The rate of coupling reaction is influenced by the condensing reagent used: reactions proceeded in 2-10 days using DCC or TPS-Cl, while the use of TPS-NT allowed to decrease the reaction time to 7 hours.

PHOSPHOTRIESTER METHOD

The Phosphotriester approach is a variant of the previous method. In this case the glycosyl phosphate is P-protected (see **Scheme 4**).



Scheme 4

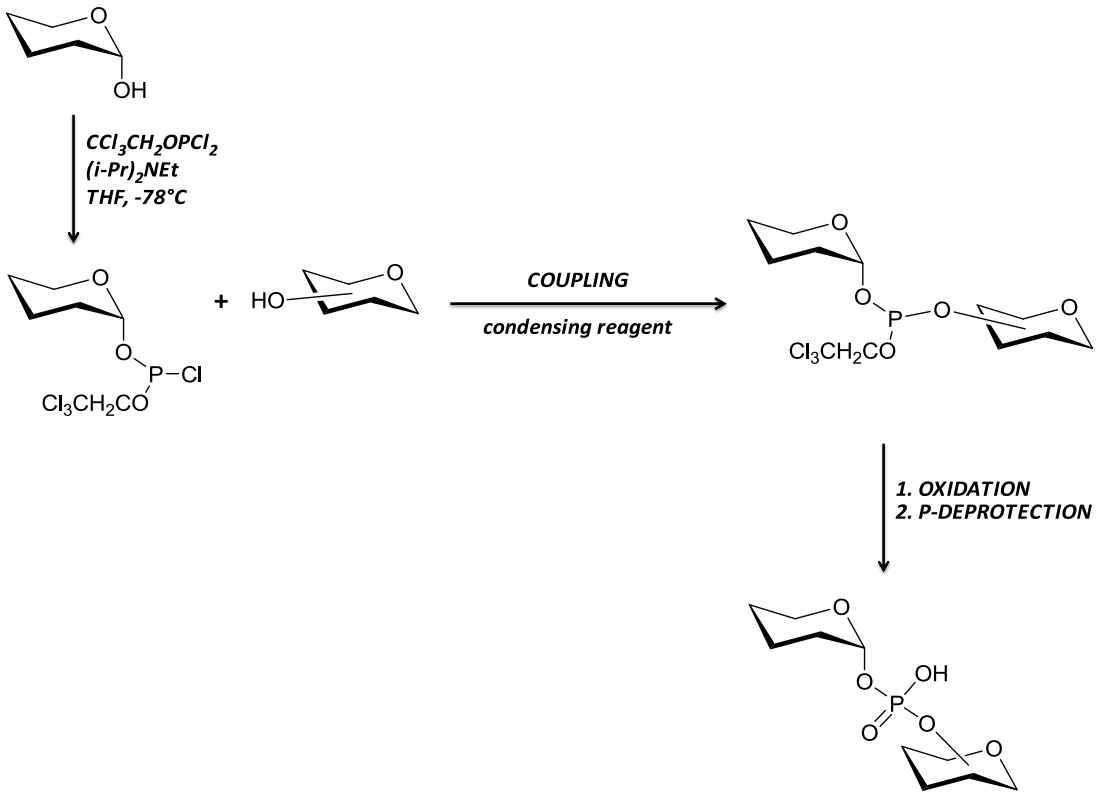
The phosphotriester method is suitable for nucleotide chemistry, moreover the yields resulted in increases by the use of solid-phase synthesis of oligodeoxyribonucleotides.[12] By contrast, this method is not efficient for the synthesis of glycosyl phosphosaccharides.

P(III) CHEMISTRY: PHOSPHOROUS TRIESTER AND H-PHOSPHONATE METHODS

PHOSPHOROUS TRIESTER (PHOSPHITE TRIESTER) METHODS

PHOSPHOROCHLORIDITE METHOD

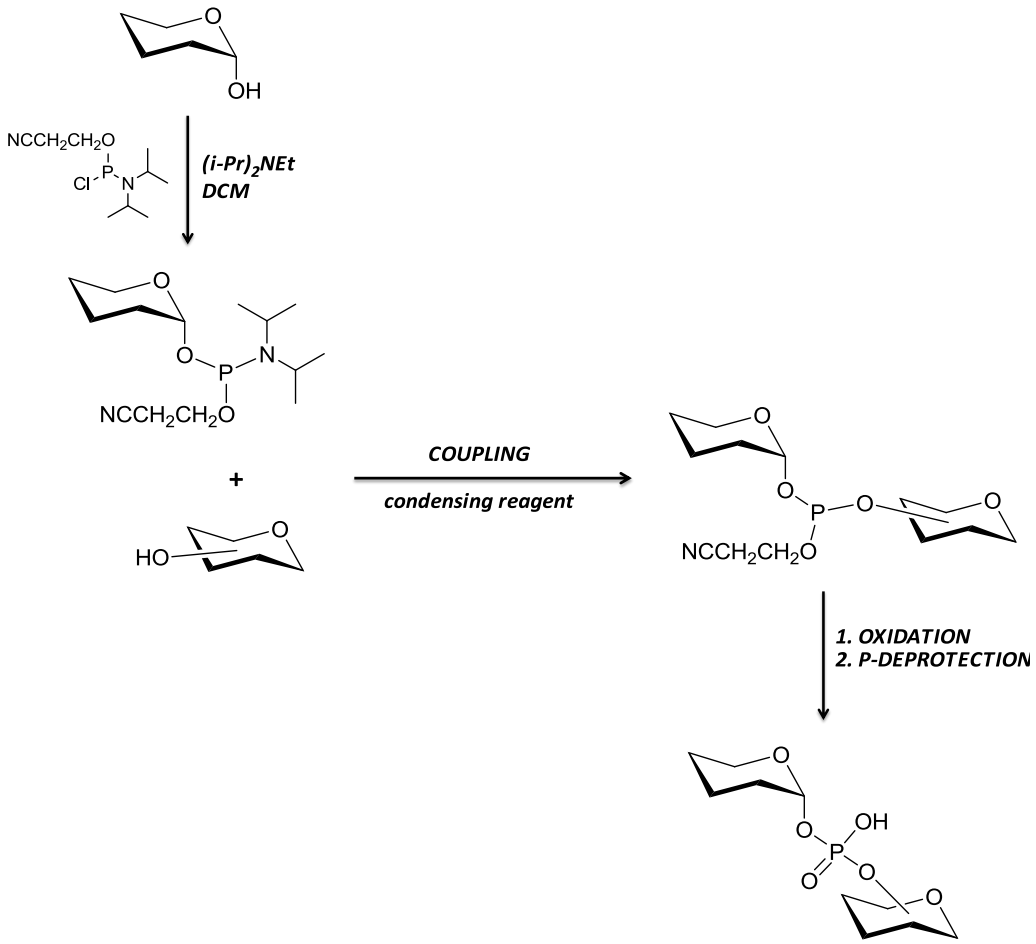
Ogawa and Seta[13] have first pioneered the use of P(III) compounds. The 2,2,2-trichloroethyl phosphorodichloride (**Scheme 5**) is used to phosphitylate the hemiacetal and form the glycosyl phosphorochloridite, which then is coupled to provide a phosphorous triester (generally in high yields): the desired phosphosaccharide is obtained after a step of oxidation followed by P-deprotection (Zn-Cu couple in the presence of 2,4-pentadione).



Scheme 5_Phosphorochloridite approach.

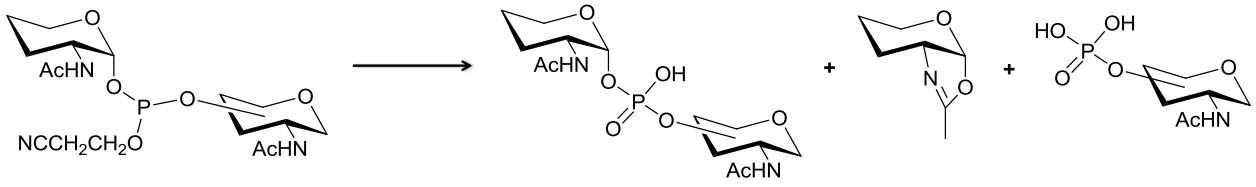
PHOSPHORAMIDITE METHOD

The van Boom's group developed the phosphoramidite method (**Scheme 6**).[14] The glycosyl phosphoramidite is achieved by the use of *N,N*-di-isopropylphosphoramidochloridite, and successively coupled with a suitable alcohol acceptor to give the phosphoroustriester. Again, a step of oxidation followed by P-deprotection (NH_3) provides the desired phosphodiester. The completion of the reaction has to be monitored by ^{31}P-NMR, and sometimes the ammonolysis of the cyanoethyl group is accompanied by partial cleavage of the glycosyl phosphate linkage.
The best reaction procedure has been optimized by Kajihara and co-workers.[15]



Scheme 6_Phosphoramidite approach.

The application of this method to *N*-acetyl-D-glucosamine is not without problems: the moderate yields are explained with the formation of oxazoline and monosaccharide phosphate by-products by 2-acetamido group participation (**Scheme 7**).

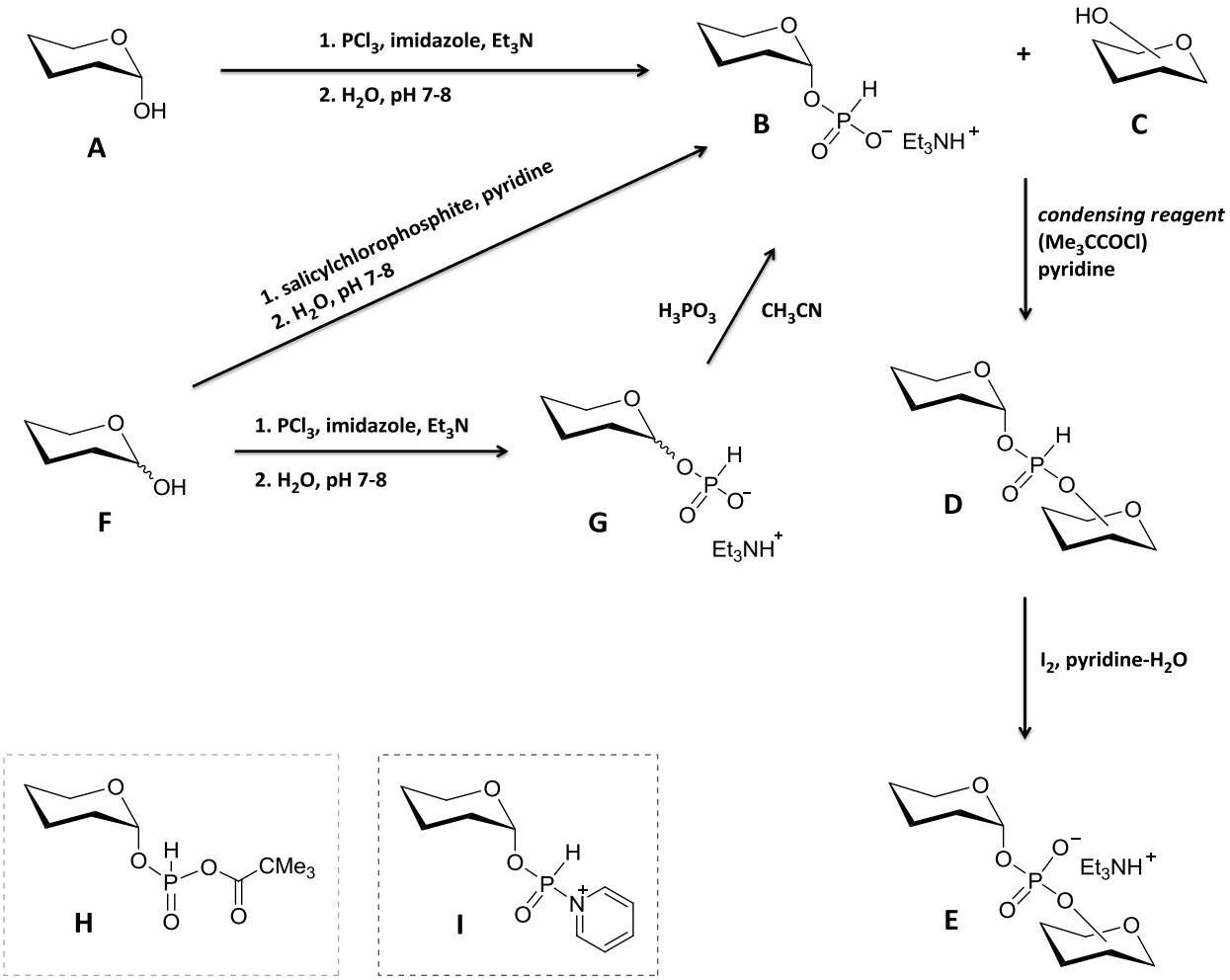


Scheme 7_Oxazoline and monosaccharide phosphate by-products.

HYDROGEN-PHOSPHONATE METHODS

In the mid-1980s the first applications of H-phosphonate method in carbohydrate chemistry were described by van Boom's group[16,17] and by Nikolaev's group[18].

A glycosyl H-phosphonate sugar-type **B** is coupled with an alcohol derivative **C** in the presence of a condensing reagent[17,19,20] to produce a H-phosphonate diester **D**, which forms the target **E** by *in situ* oxidation (**Scheme 4**). The most used condensing reagent is trimethylacetyl chloride[17] (Pivaloyl chloride, PivCl) and the oxidation step is normally performed *in situ* using iodine in aqueous pyridine.



Scheme 8_H-phosphonate approach: starting materials and intermediates.

The glycosyl H-phosphonate **B** can be obtained from the corresponding α-hemiacetal **A** or the α,β-hemiacetal **F**.

The H-phosphonate derived from **A** (pure α sugar) can be achieved using either

- tri-imidazolylphosphine (generated in from PCl_3, imidazole and Et_3N)[8b,19,20,21]
- 2-chloro-1,3,2-benzodioxaphosphorin-4-one (commonly named salicylchlorophosphite)[16,17]

or

- diphenyl phosphite[22,23]

for phosphonylation followed by hydrolysis.

The reaction of α,β-hemiacetals **F** with salicylchlorophosphite in pyridine[24] or H_3PO_3 in the presence of 2-chloro-5,5-dimethyl-2-oxo-1,3,2-dioxaphosphorinane[25,26] leads to the formation of the pure α-glycosyl H-phosphonate **B**.

By ^{31}P-NMR studies Nikolaev[27] and co-workers demonstrated that:

- during the coupling between H-phosphonate **B** and alcohol **C** the mixed anhydride **H** (**Scheme 4**) is the main reactive intermediate leading to the formation of H-phosphonate diester **D**: this is further oxidised to phosphodiester **E**;
- prolonged delay (more than 24 hours) of the oxidation step can lead to decomposition of the substrates thus resulting in low yield of **E** by partial transformation of **D** into C-phosphonate and phosphorous triester derivatives;

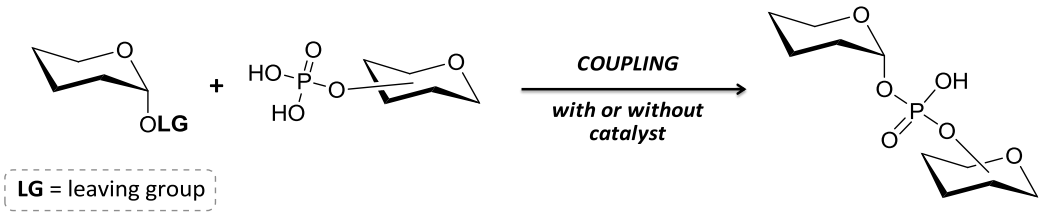
- the solvent can participate in the reaction as a nucleophilic catalyst to assist the transformation of **H** and **D** via activated pyridinium intermediate **I** (in according to data describing the behaviour of H-phosphonates in pyridine).

In contrast to both the phosphodiester and the phosphoramidite, H-phosphonate is the most efficient method for general preparation of glycosyl phosphosaccharides and in particular of glycosyl phosphodiester containing *N*-acetyl-α-D-glucosamine units. The most prominent characteristics of this method are:

- ✓ high effectiveness;
- ✓ high reaction rate for all the three steps involved;
- ✓ does not require a P-protecting group;
- ✓ high yields.

THE GLYCOSYLATION REACTION

The glycosylation reaction method is exclusive for carbohydrates. This approach, exemplified in **Scheme 9**, involves the reaction of a glycosyl donor (electrophile) with a P-containing glycosyl acceptor (nucleophile).



Scheme 9_Glycosylation reaction approach.

The substitution of a suitable leaving group at the anomeric position with a phosphoric acid derivative provides the phosphodiester linkage. The glycosyl trichloroacetimidates are the most used glycosyl donors.[28] The yields ranged from moderate to good and the stereochemical control of the reaction depends mainly on three factors: the stereochemistry of the glycosyl donor, the nature of the protecting groups of the patterns, and the reaction conditions.

Some examples of combination between the glycosylation and the H-phosphonate methods are reported in literature.[29]

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CHAPTER 5

Synthetic MenX fragments

RESEARCH METHODOLOGY

The aim of the present research project is the synthesis of phosphodiester-linked oligomers of the native Men X CPS (**Figure 1**).

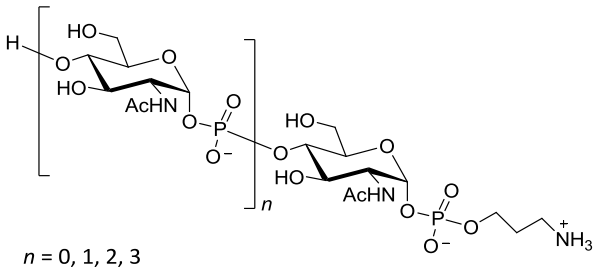


Figure 1_Synthetic MenX oligomers.

The synthetic plan is based on the Hydrogen-phosphonate approach (H-phosphonate).[1] The choice of Hydrogen-phosphonate (H-phosphonate) approach for the synthesis of phosphodiester-linked oligomers was due to the advantages of this method compared to the others (see ***CHAPTER 4***). Moreover, each oligomer contains a phosphodiester-linked aminopropyl spacer at its reducing end to allow protein conjugation and synthesis of neo-glycoconjugates capable to elicit a stronger and durable immune response (**Figure 2**).

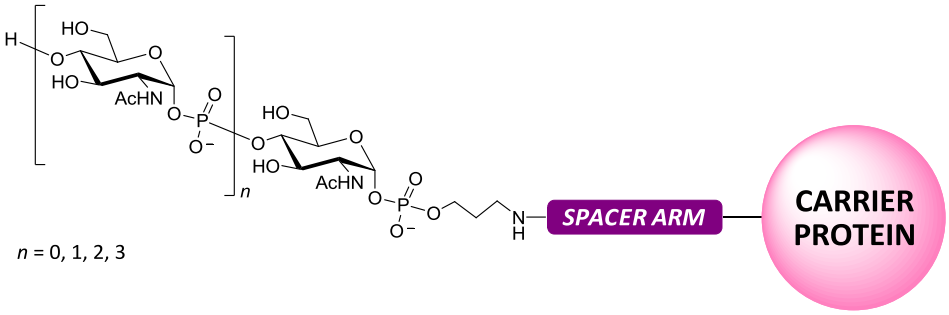
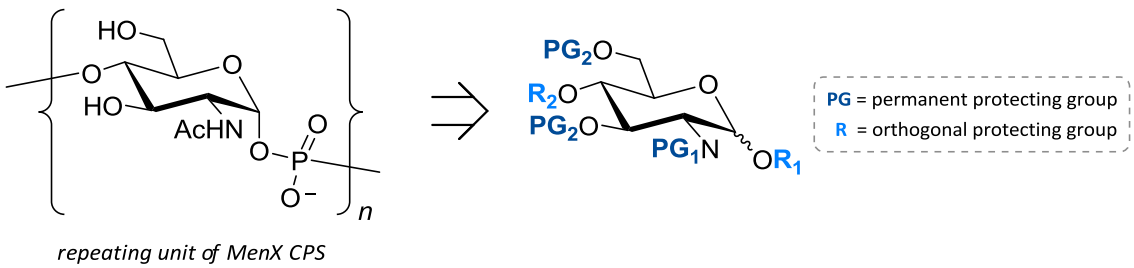


Figure 2_Men X neo-glycoconjugates.

The synthetic compounds have been first conjugated to HSA as a proof of principle, then to the more immunogenic carrier protein CRM_{197}.[2]

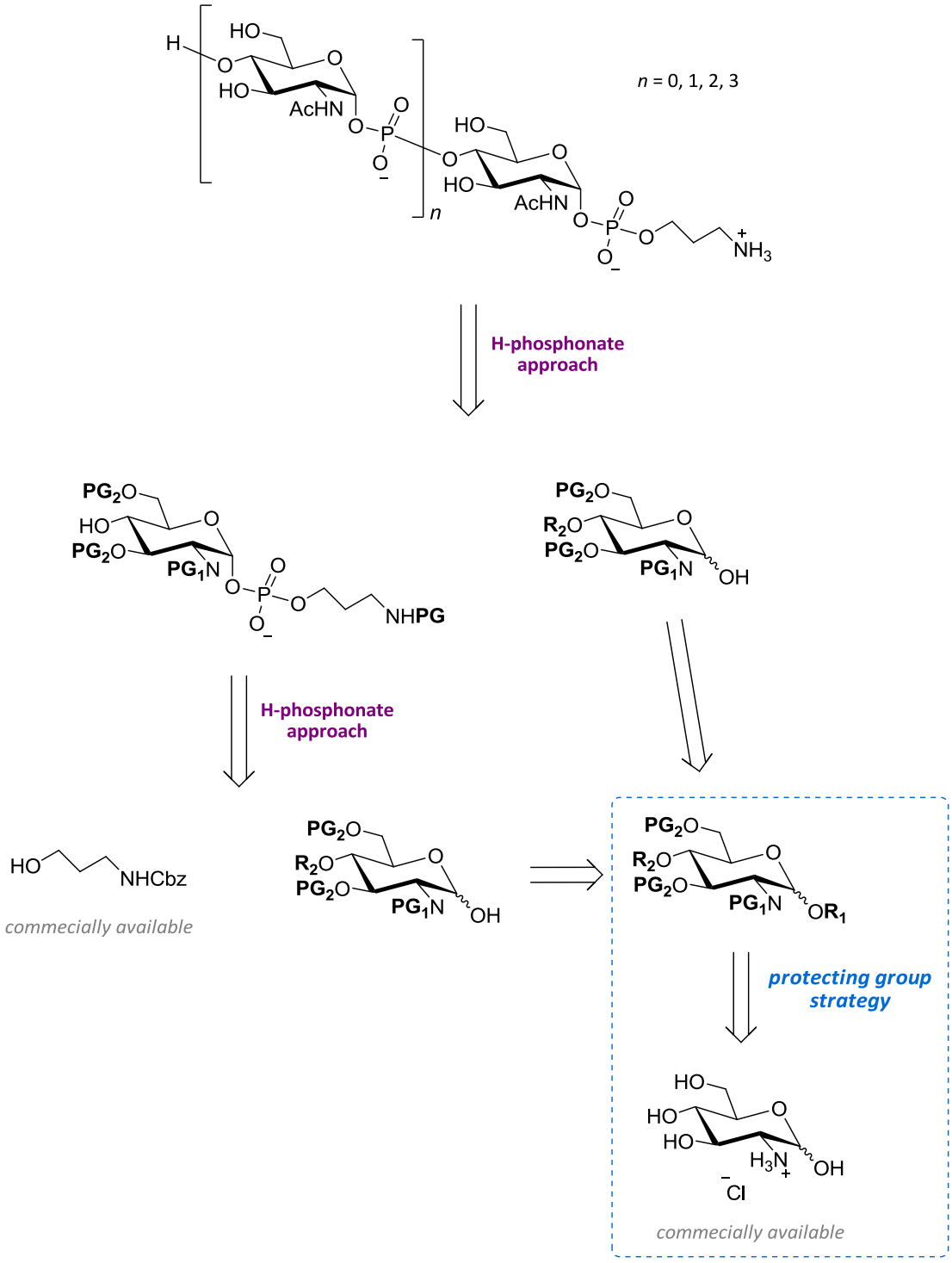
PROTECTING GROUP STRATEGY

The first part of the synthetic endeavour has been devoted to the preparation of suitably protected monosaccharide building blocks (**Scheme 1**), as key precursors of our synthetic route.



Scheme 1_Suitably protected monosaccharides: the key precursors of the synthetic route.

Protecting group strategy was crucial for a successful outcome of the project. Then, permanent protecting groups (PG_2) should be introduced on 3-OH and 6-OH, while temporary protecting groups (R_1 and R_2) should be selected in order to allow selective unblocking of the corresponding hydroxyls and to allow elongation of the oligomers. In addition, an azide (PG_1) was installed on C-2 in agreement with literature data, where nearly all syntheses of similar compounds relied on the 2-azido group as a non-participating precursor of the 2-acetamido moiety.
The stereochemical control in the synthesis of α-phosphodiester-linked oligomers was the first goal of the present work. The proposed synthetic strategy is illustrated in **Scheme 2**.

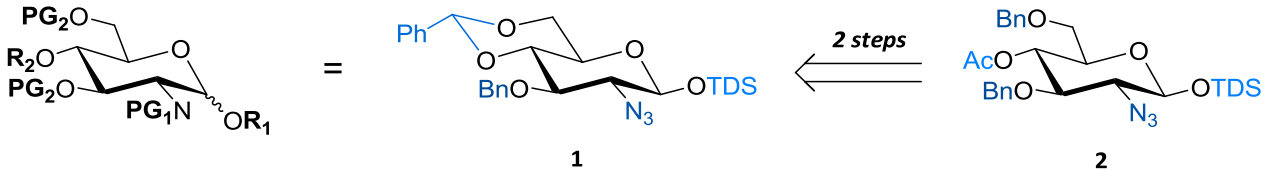


Scheme 2_The proposed synthetic strategy.

Starting from commercially available D-glucosamine hydrochloride, we were able to achieve a suitably protected key precursor of our route: its structure and synthesis is described in the next paragraph. The selective delivery either of the anomeric position or of 4-OH enabled the blockwise elongation. The unblocking of R_1 allowed to obtain an hemiacetal intermediate apt to be coupled via H-phosphonate with the 3-*N*-carbobenzyloxy aminopropyl spacer. The obtained monomer, after delivery of 4-OH, was the glycosyl acceptor to provide the dimer following the same approach with a second residue of hemiacetal intermediate. The iteration of the above protocol led to oligomers of different length.

SYNTHETIC STRATEGY

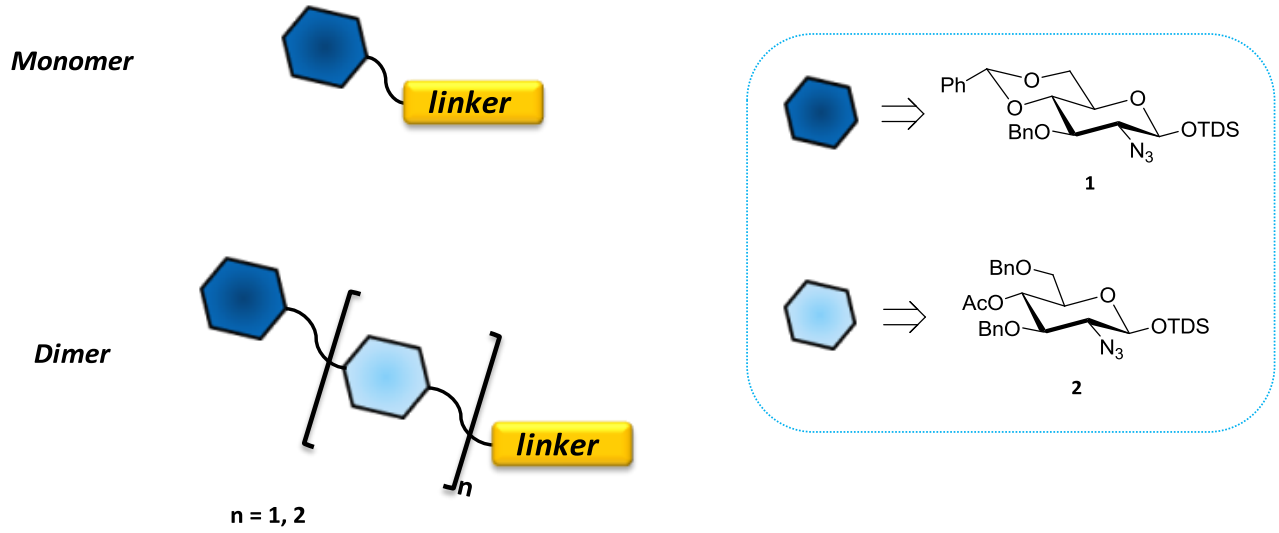
The first part of the synthetic endeavour has been devoted to the preparation of the suitably protected monosaccharides **1** and **2** (Scheme 3), in medium (up to 2 g) and large scale (5-30 g) synthesis.



Scheme 3_Key precursors **1** and **2**.

Why a second key precursor?

We reasoned that oligomer elongation based on **2** was a much reliable approach, since O-deacetylation is a milder and safer transformation in comparison with regioselective/reductive opening of benzylidene acetal. The latter required indeed strongly acidic conditions, which could lead to partial decomposition of longer oligomers. On the other hand, we envisaged **1** as a capping sugar (the non-reducing end) for the completion of the targets (Scheme 4).



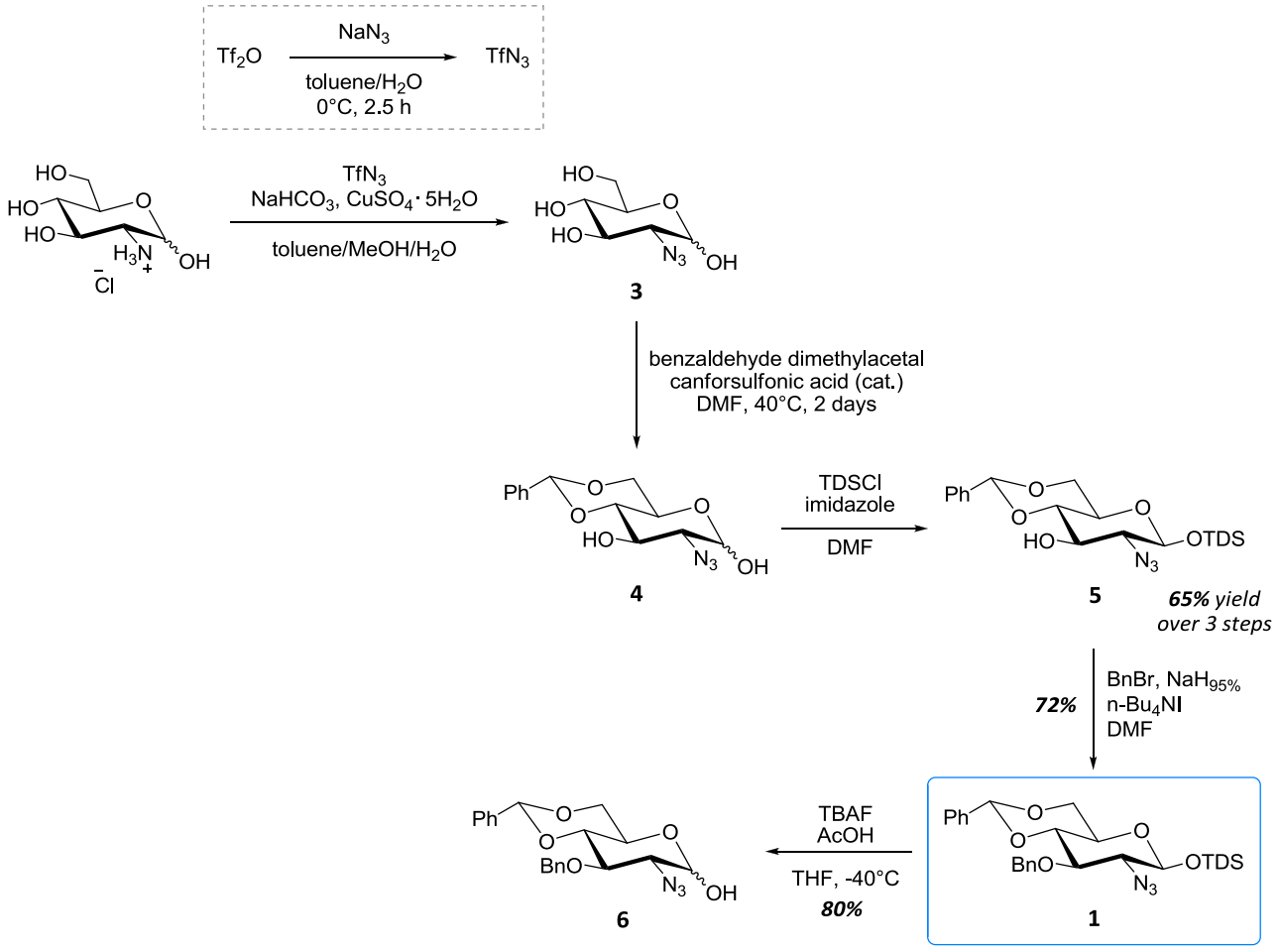
Scheme 4_The synthetic strategy for oligomer elongation.

SYNTHESIS OF KEY PRECURSORS 1 AND 2

Medium scale synthesis (up to 2g)

In principle, fully protected building block **1** can be synthesized just in 4 steps (37% overall yield). This route was however not reproducible when using large amounts of starting material.

Starting from the commercially available D-glucosamine (Scheme 5), the amino group was masked as an azide (**3**) by using the diazo-transfer reaction.[3] Without any further purification, the introduction of a 4,6-O-benzylidene acetal[4] (**4**) and the selective silylation of 1-OH with thexyldimethylsilylchloride[5] (TDSCl) led to compound **5** in 65% yield over three steps. The last free hydroxyl group was then protected as a benzyl ether[6] to achieve the key precursor **1** in 72% yield.

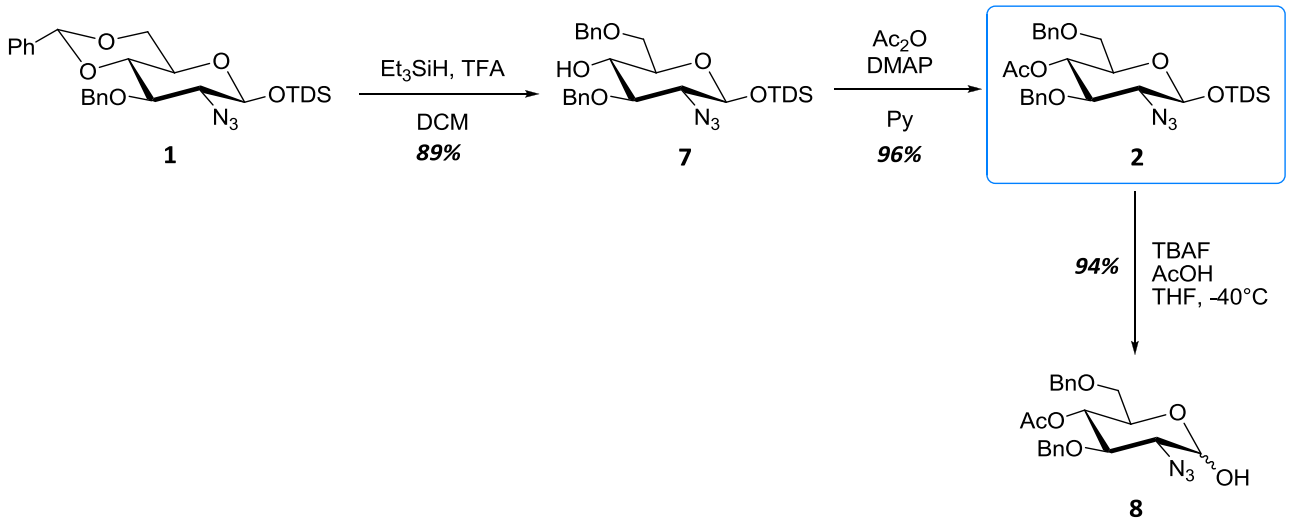


Scheme 5_Synthesis of key precursor **1** and hemiacetal **6**.

Building block **2** was obtained from **1** by regioselective reductive opening of the benzylidene acetal[7] (**7**, 89% yield) followed by acetylation at 4-OH (96% yield, Scheme 6).

Hemiacetals **6** (Scheme 5) and **8** (Scheme 6) were synthesized by desilylation of the corresponding building blocks using tetrabutylammonium fluoride (TBAF) 1M in THF and glacial acetic acid (80% and 94% yield, respectively).[19]

During the preparation of **5** (2-5 g), the overall yield was very low and we observed the formation of the bis-silylated by-product. Since the silylation was performed on crude diol **4** (derived in turn from crude **3**), we used a very large excess of TDSCl causing a substantial bis-silylation.

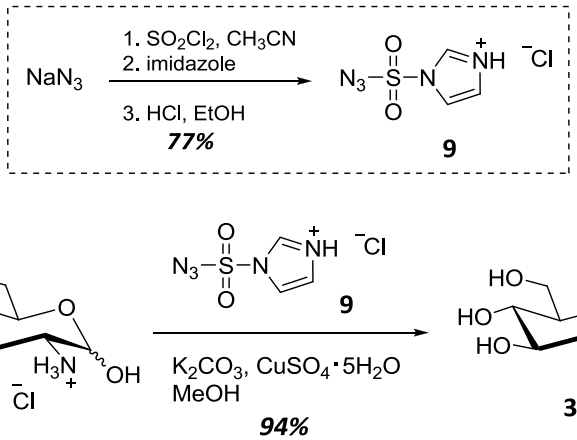


Scheme 6_Synthesis of key precursor **2** and hemiacetal **8**.

Moreover, the use of trifluoromethanesulfonyl azide (TfN_3, **Scheme 4**) as a "diazo donor" in the Cu(II)-catalyzed conversion of the amine into the azide led to high yields. However, this process included several drawbacks:

- the explosive nature of neat TfN_3 and its relatively poor shelf life;
- the high cost of Tf_2O;
- the removal of trifluoromethanesulfonamide from polar products required specialized work-up procedures;
- the laborious work-up procedure, particularly challenging in large-scale (> 10 g) preparations.

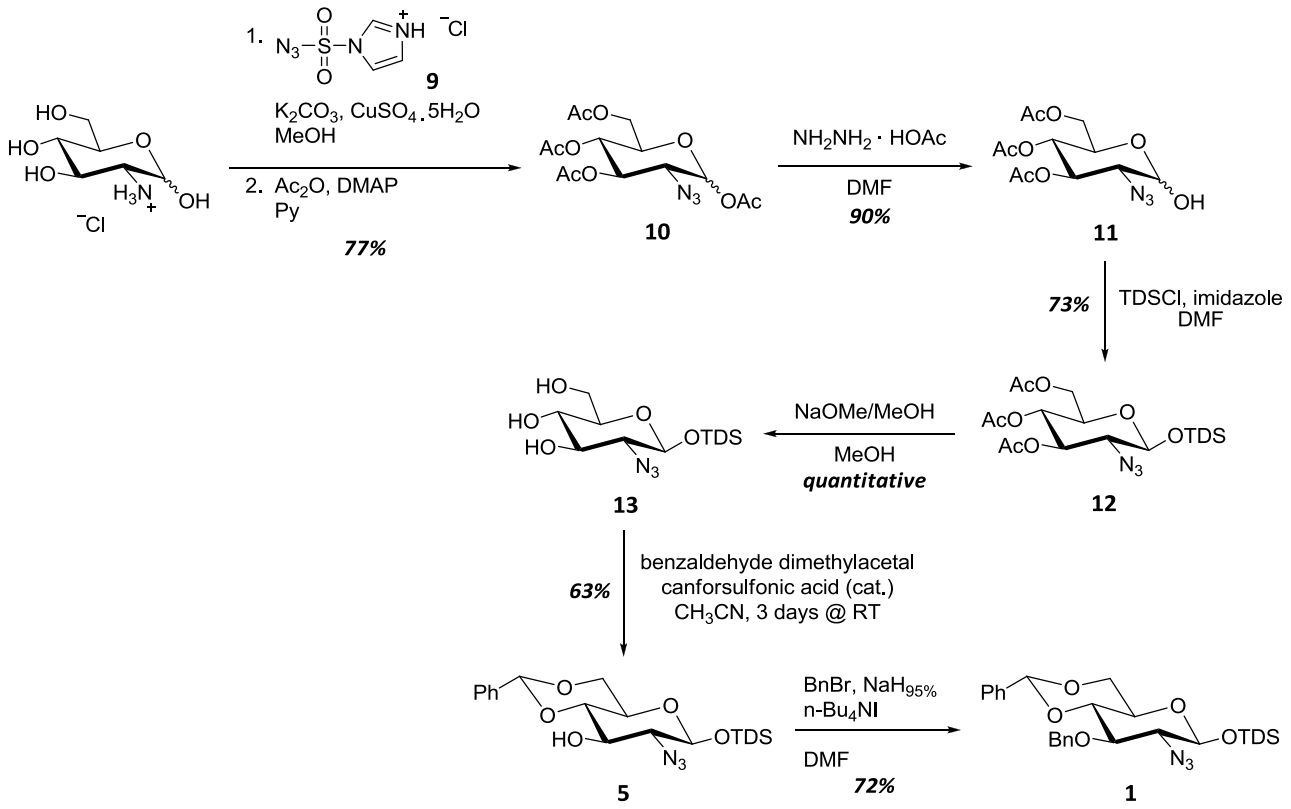
To circumvent these problems and establish the diazotransfer reaction as a commonplace and industrially useful synthetic transformation, a cheap, robust, and safe alternative to TfN_3 was required. In particular, we needed an electron-withdrawing group capable of replacing the trifluoromethanesulfonyl moiety, and we chose the imidazole-1-sulfonate (imidazylate)[8], which is known to exhibit very similar reactivity to trifluoromethanesulfonates but benefits by a longer shelf life: accordingly, imidazole-1-sulfonate hydrochloride **9** (77% yield) was employed for the preparation of azide **3** (quantitative yield) as described in the literature (**Scheme 7**).[20]



Scheme 7_Synthesis of imidazole-1-sulfonate hydrochloride **9** and glucopyranosyl azide **3**.

Large scale synthesis

In large scale synthesis (5-30 g), we applied a longer but more reliable approach for the synthesis of **1** (23% yield over 6 steps, **Scheme 8**): in this way we could achieve intermediate **1** in higher purity and yield.



Scheme 8_Large scale synthesis of building block **1**.

The diazotransfer reaction was followed by acetylation that enables the purification of **10** by flash chromatography. Although this reaction is described in the literature[9] in 94% yield, we got 77% yield as the best result. The next step consisted in the regioselective removal of the anomeric acetate by a freshly prepared 2M solution of hydrazine acetate in DMF: hemiacetal **11** was obtained in very high yield (90%). The silylation of the anomeric position with TDSCl (**12**, 73% yield) followed by Zemplen deacetylation[10] (**13**, *quantitative* yield) allowed the introduction of a 4,6-O-benzylidene acetal to get alcohol **5** (63% yield), that was eventually transformed into building blocks **1** by standard benzylation protocol.

APPROACHES TO THE CONSTRUCTION OF OLIGOMERS

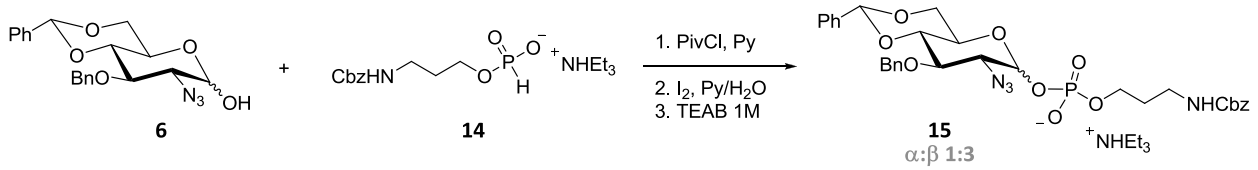
As far as the elongation of our oligomers is concerned (**Scheme 9**), we decided to install the phosphodiester linkages via the well-established H-phosphonate approach. In principle, there are two main approaches towards this task:

A - the coupling between an hemiacetal derivative and a stable non-anomeric H-phosphonate (4-H-phosphonate-glucoside or linker H-phosphonate);

B - the coupling between an anomerically pure α H-phosphonate and an acceptor (saccharide or spacer).

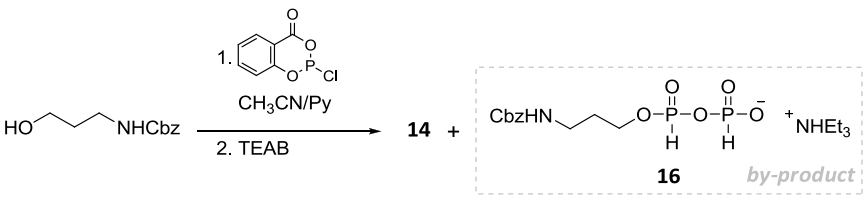
Scheme 9_Phosphodiester bridge formation: approach A and approach B.

The stereochemical control in the synthesis of phosphodiester-linked oligomers is a challenging task. First, we applied *approach A* based on the oxidative coupling between hemiacetal **6** and 3-*N*-carbobenzyloxy-propanolamine H-phosphonate **14** (Scheme 10).



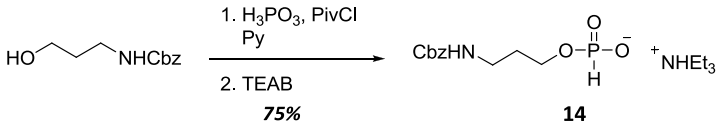
Scheme 10_Synthesis of **15**: first attempt of coupling.

Compound **14** was obtained by the treatment of 3-*N*-carbobenzyloxy-aminopropanol with salicylchlorophosphite in acetonitrile/pyridine mixture and then with 1M triethylammonium bicarbonate (TEAB) buffer leading to the formation of **14** (60% yield) in 2 hours (Scheme 11). In a scale of 2g the yield decreased because of the formation of by-product **16**.



Scheme 11_Small scale synthesis of H-phosphonate **14**.

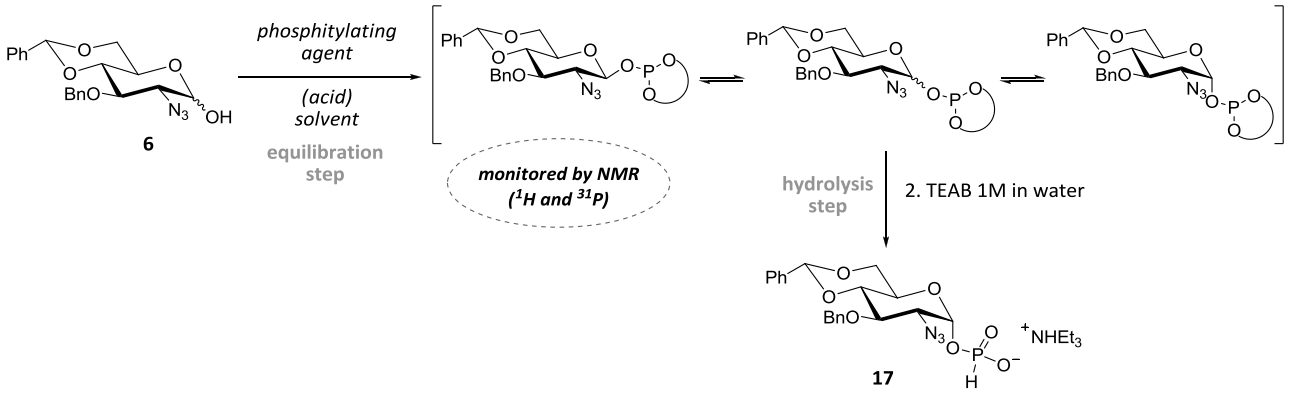
For large amount preparation, we changed the procedure using phosphorous acid (H_3PO_3) instead of salicylchlorophosphite as phosphilating agent (**Scheme 12**).



Scheme 12_Large scale synthesis of H-phosphonate **14**.

Next, compound **6** and H-phosphonate **14** were coupled under standard conditions (see the ***Experimental section***, ***CHAPTER 8***), and the resulting H-phosphonate diester was *in situ* oxidized with iodine in a pyridine/water mixture to provide the phosphodiester **15**. However, NMR analysis evidenced the predominant formation of the β anomer of **15**. Likewise, the synthesis of a phosphodiester-linked disaccharide according to *approach A* led to an inseparable mixture of anomers, suggesting that a different approach (*approach B*) must be employed to achieve our goal.

The application of *approach B* requires the obtainment of an anomerically pure α H-phosphonate intermediate. Since there are no examples in literature describing the synthesis of exclusively α-anomeric H-phosphonate from 2-azido-2-deoxy-glucopyranosyl derivatives, we decided to focus our attention on the synthesis of **17** by introducing the H-phosphonate moiety on **6** (**Scheme 13**).



Scheme 13_Synthesis of α-pure H-phosphonate **17**, following the approach B.

The leading concept is to achieve equilibration of the initially formed α,β mixture of the anomeric H-phosphonates into the pure, more thermodynamically stable α anomer **17**.

To this aim, we explored different protocols (**Table 1**):

I. treatment of **6** with a slight excess of salicylchlorophosphite in a mixture of dry dioxane and pyridine at r.t.;
II. treatment of **6** with salicylchlorophosphite in a mixture of dry acetonitrile and pyridine, from 0°C to r.t.;[11]
III. treatment of **6** with triimidazolylphosphine (prepared *in situ* from PCl_3, imidazole and triethylamine) in dry acetonitrile, from 0°C to r.t.;[12]
IV. treatment of **6** with H_3PO_3 and 2-chloro-5,5-dimethyl-2-oxo-1,3,2-dioxaphosphorinane in pyridine, from r.t. to 40°C;[14]
V. treatment of **6** with H_3PO_3 and salicylchlorophosphite in pyridine, from r.t. to 40°C.

In all cases, the reaction course was monitored by NMR analysis (^1H and ^{31}P).

Method V is our modification of method IV (described in the literature[14]) that gave the best results (Table 1, entries 6 and 7).

Table 1_Different reaction conditions explored..

entry	*amount of* **6** (**1eq**)	*phosphitylating agent*	*acid*	*solvent*	*temperature (°C)*	*α:β ratio*
1	55 mg	salicylchlorophosphite (**1.2 eq**)	-	dioxane/pyridine 3:1 **0.3 M**	r.t.	2:1
2	47 mg	salicylchlorophosphite (**1.2 eq**)	-	dioxane/pyridine 3:1 **0.3 M**	0°C to r.t.	2:1
3	53 mg	PCl_3 (**5 eq**) imidazole (**5.5 eq**) triethylamine (**5.7 eq**)	-	acetonitrile/pyridine 5:3 **0.3 M**	0°C to r.t.	3:1
4	88 mg	2-chloro-5,5-dimethyl-2-oxo-1,3,2-dioxaphosphorinane (**5 eq**)	H_3PO_3 (**10 eq**)	acetonitrile **0.3 M**	r.t. to + 40°C	-
5	200 mg	salicylchlorophosphite (**5 eq**)	H_3PO_3 (**10 eq**)	pyridine **0.4 M**	r.t. to + 40°C	4:1
6	48 mg - 1 g	salicylchlorophosphite (**5 eq**)	H_3PO_3 (**4 eq**)	pyridine **0.4 M**	r.t. to + 40°C	> 95:5
7	50 mg - 2 g	salicylchlorophosphite (**1.5 eq**)	H_3PO_3 (**3 eq**)	pyridine **0.4 M**	r.t. to + 40°C	> 95:5

After disappearance of the starting material **6**, a 1:1 α,β ratio was determined by ^{1}H- and ^{31}P-NMR of the crude sample (Figure 3). After 6 days at r.t., the ratio moved to 2:1. As the reaction temperature was raised up to 40°C, the α,β ratio increased progressively, from 7:1 (after 12 hours at 40°C) until 98:2 (after further 3 days at 40°C, 48% yield).

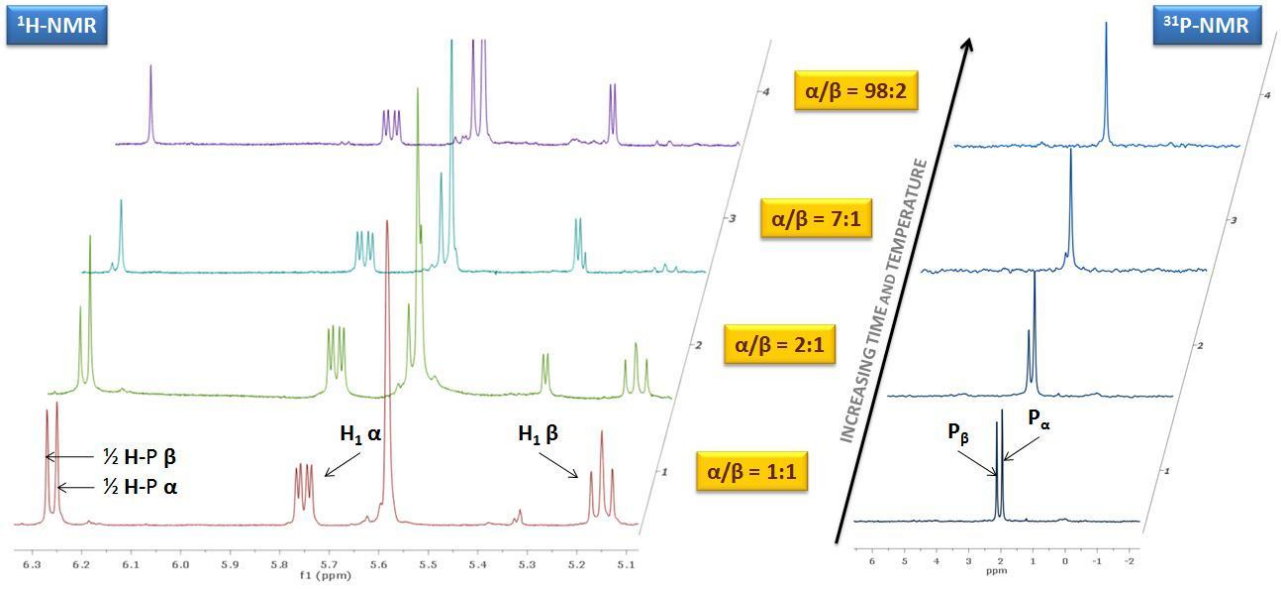
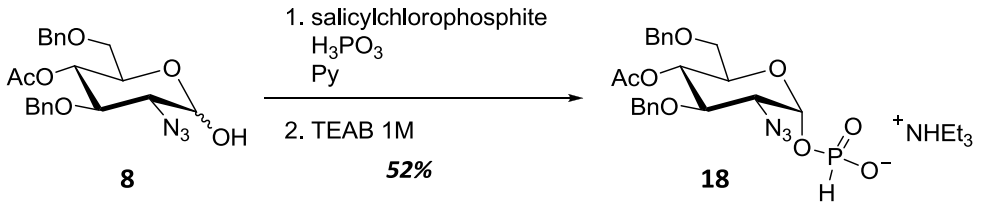


Figure 3_Synthesis of **17**: ^{1}H- and ^{31}P-NMR spectra of reaction's aliquots.

H-phosphonate **18** was also synthesized following procedure V (52% yield, Scheme 14). Unlike the case of **6**, we decided to increase the temperature up to 40°C as soon as the disappearance of the starting material **8** occurred (1.5 hour).



Scheme 14_Synthesis of α-pure H-phosphonate **18**.

After 1 day the 2:1 α,β ratio was determined by ^{1}H- and ^{31}P-NMR of the crude sample (**Figure 4**). After 3 days at 40°C the ratio moved to 7:1, and it increased progressively until 98:2 (after 2 days more at 40°C).

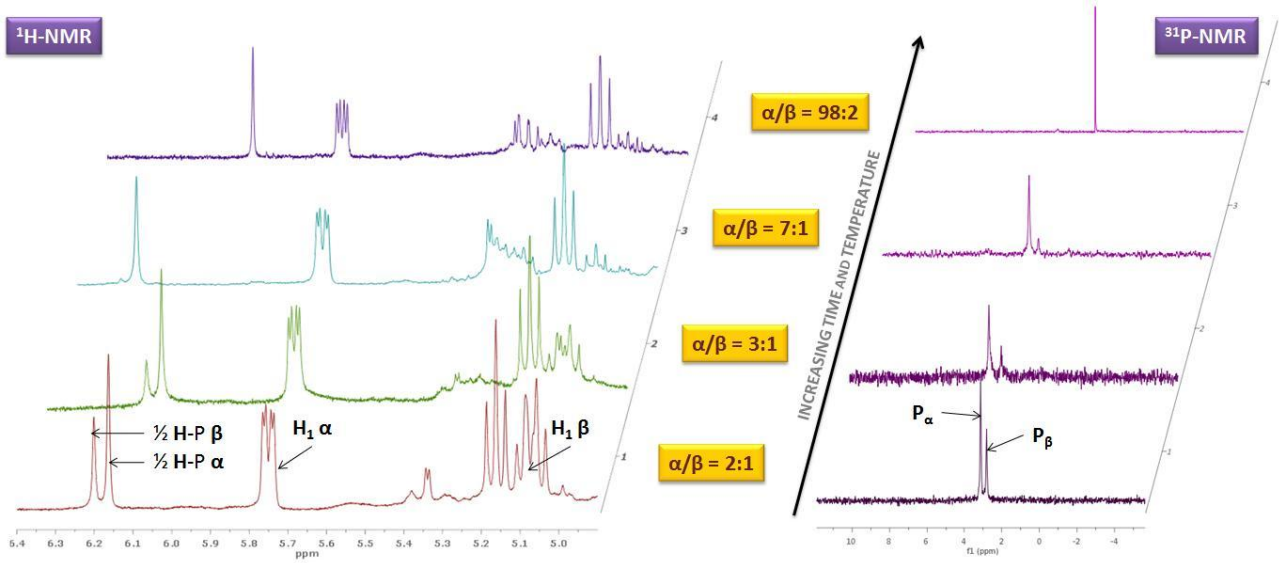


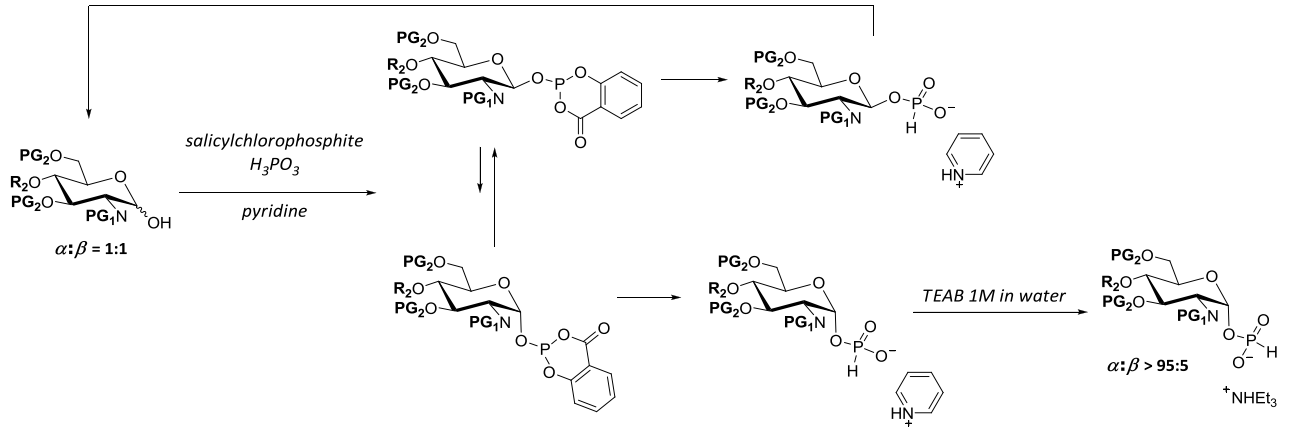
Figure 4_Synthesis of **18**: ^{1}H- and ^{31}P-NMR spectra of reaction's aliquots.

We also tried to reduce the reaction time by treatment of **8** with a slight excess of salicylchlorophosphite (1.2 eq) in a 1:1 mixture of dry dioxane and triethylamine at r.t. (20% yield). Disappointingly we observed the formation of unknown by-products in ^{31}P-NMR and the α,β ratio was 2:1.

After several attempts, some considerations can be assumed:

- ✓ in comparison with **17**, the reaction time needed for the synthesis of **18** is much shorter (10 days *vs*. 6 days, respectively), due to the higher reactivity of the starting hemiacetal;
- ✓ further addictions of reagents (H_3PO_3 and/or salicylchlorophosphite) leads to decomposition and it's not helpful to the reaction's progress;
- ✓ in large scale synthesis (up to 2 g), extended reaction times were noticed;
- ✓ in both cases, yields were moderated.

To explain the last issue, a reasonable mechanism of the H-phosphonate formation is displayed in **Scheme 15**.



Scheme 15_Mechanism of H-phosphonate formation.

The H_3PO_3 is responsible for the decomposition of the β adduct pyridinium salt, leading to the recovery of the starting material.

In agreement with this mechanism, we observed the formation of substantial amounts of hydrolysis by-product (starting material) by ^{1}H-NMR (**Figure 5**), which however can be easily recycled.

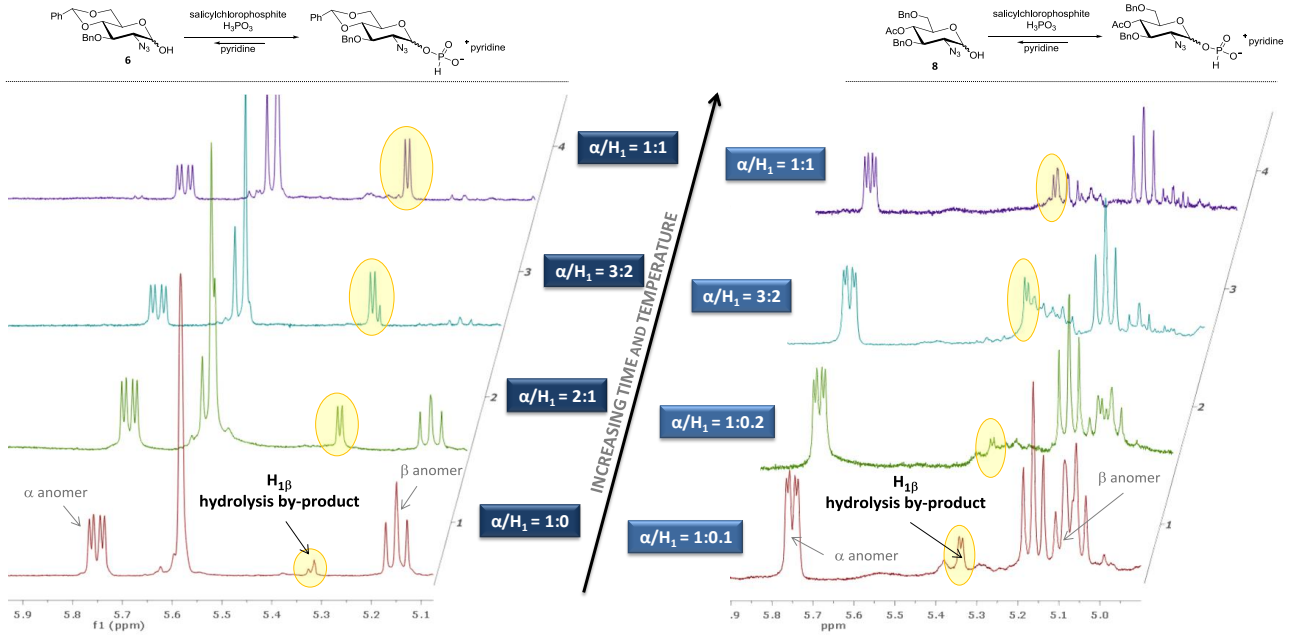
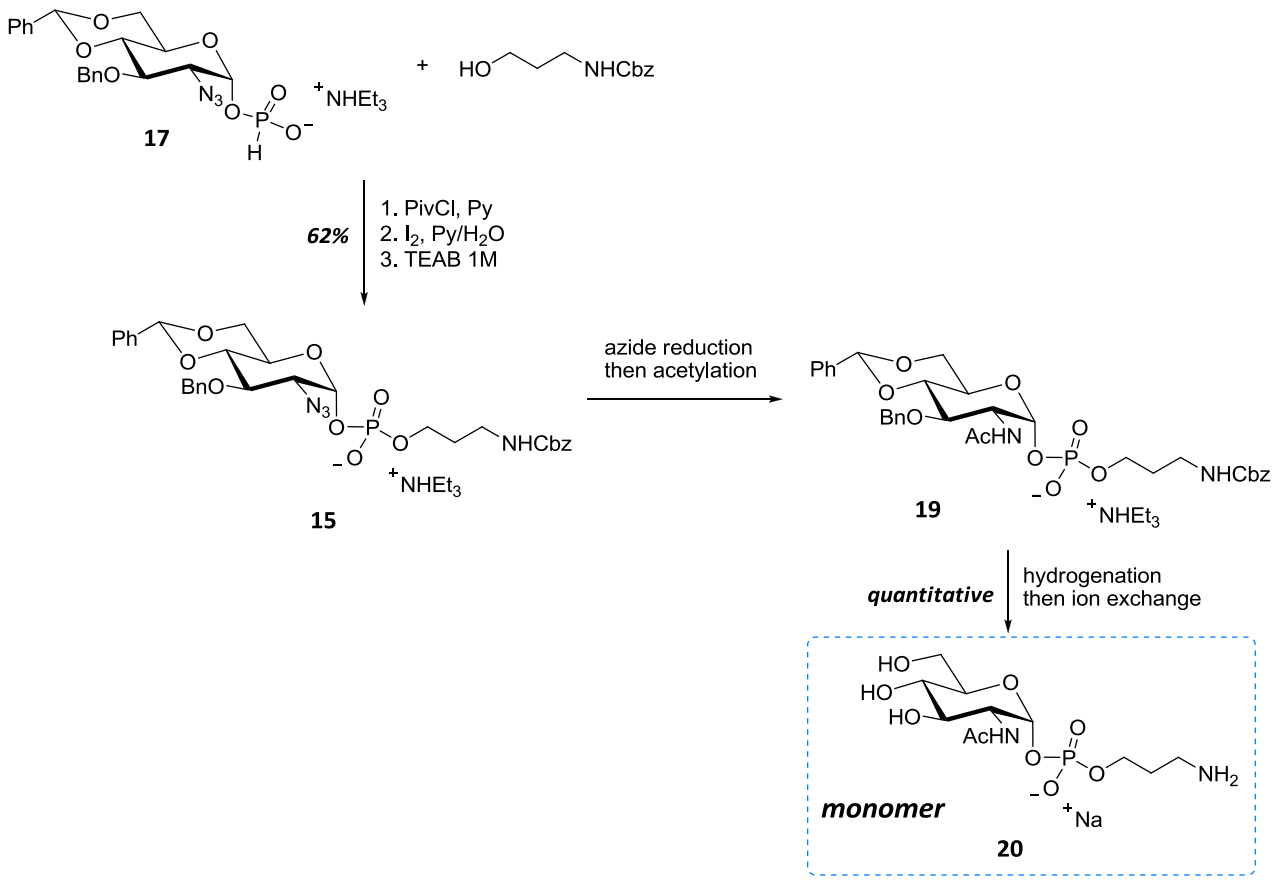


Figure 5_^{1}H-NMR spectra of H-phosphonates, **17** and **18**, reaction crudes: the by-product formation is highlighted in yellow.

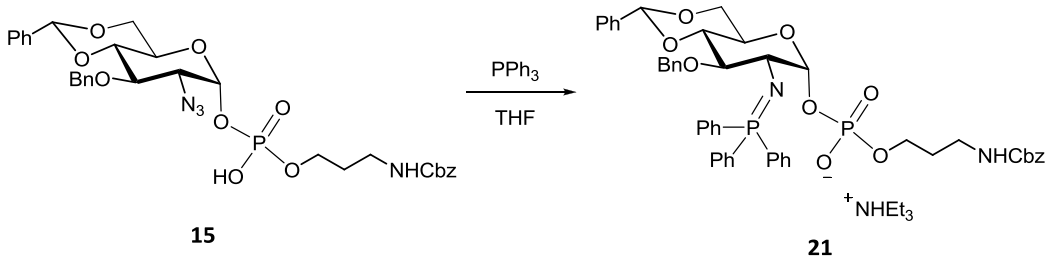
Starting from α-H-phosphonate **17**, the synthesis of the spacer-containing monomer was achieved in 3 steps (**Scheme 16**):

- coupling between **17** and 3-*N*-carbobenzyloxy-propanolamine to give **15** (62% yield);
- NH-acetamide **19** formation by azide reduction (low yield, see below);
- Pd/C catalyzed hydrogenation followed by ion exchange to achieve **20** in *quantitative* yield.



Scheme 16_Synthesis of monomer **20**.

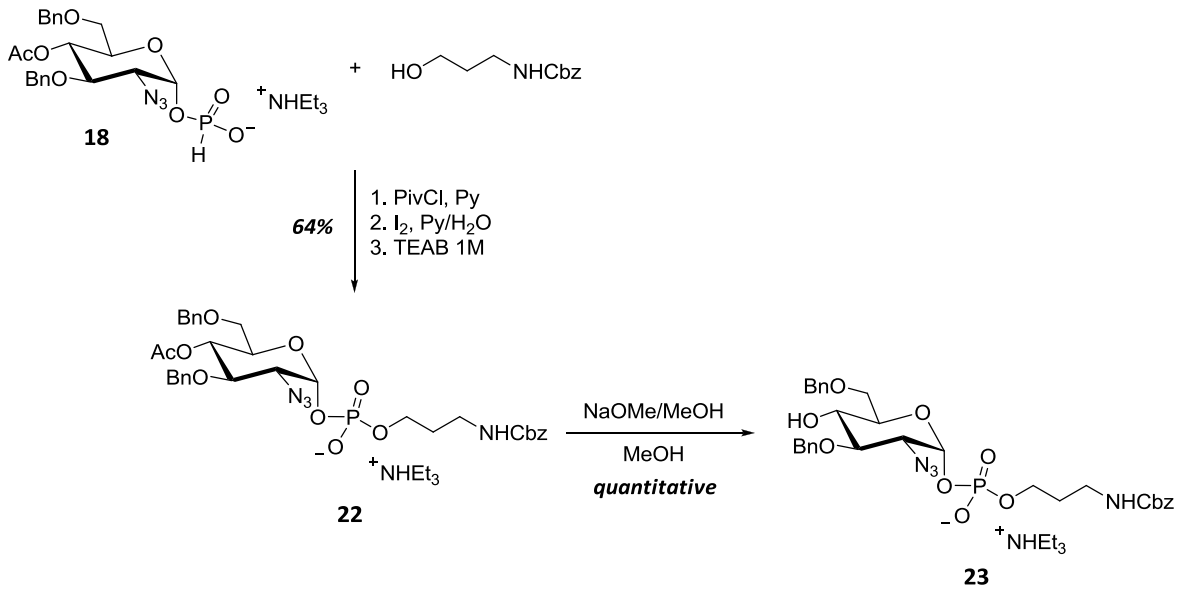
Disappointingly, the classical Staudinger reduction[13] of azide **15** did not work in our hands (Scheme 17). The iminophosphorane **21** (the typical Staudinger intermediate) exhibited unexpected stability towards water hydrolysis, even at high temperature (80°C).



Scheme 17_Azide reduction: the Staudinger reaction.

NH-acetamide **19** was therefore achieved by "$NiCl_2/NaBH_4$" protocol followed by *in situ* acetylation (13% yield).

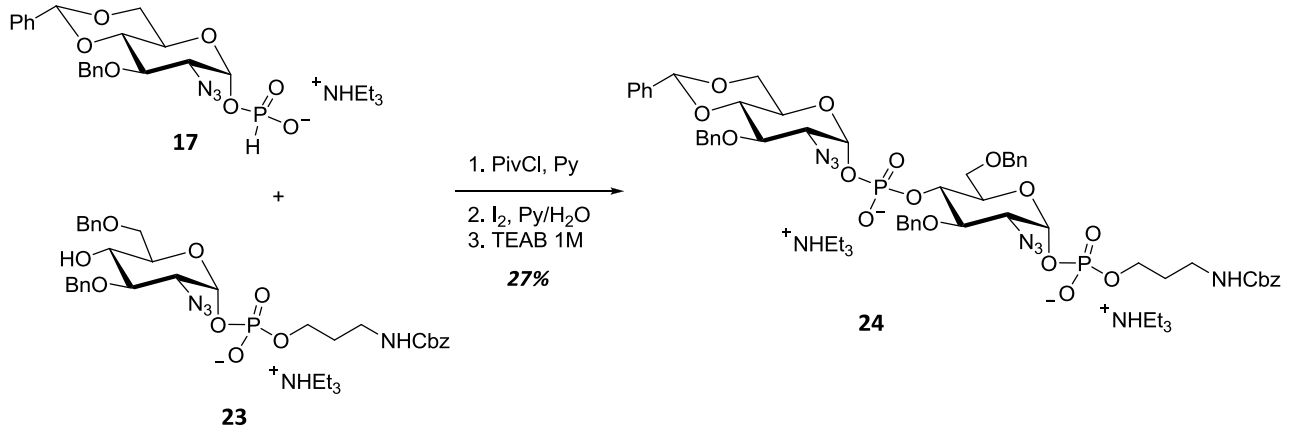
The coupling between H-phosphonate **18** and the linker was accomplished in standard conditions (Scheme 18).



Scheme 18_Synthesis of alcohol acceptor **23**.

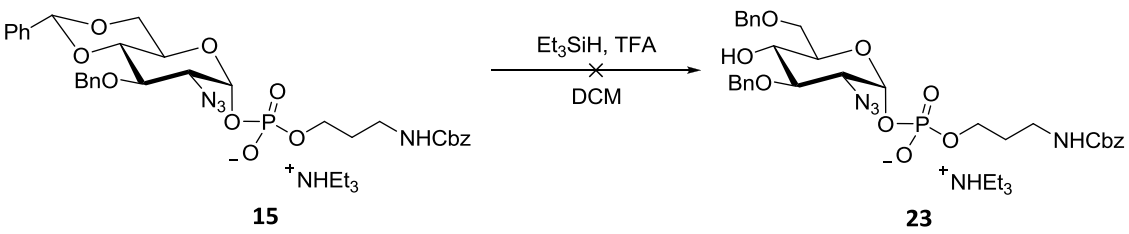
The 3-*N*-carbobenzyloxy-propanolamine was the limitating agent, while **18** and PivCl were used in excess.

Oligomers elongation was achieved by O-deacetylation (**23**, *quantitative* yield) of monomer **22** followed by the coupling with H-phosphonate **17**, obtaining smoothly dimer **24** (**Scheme 19**), even if in low yield.



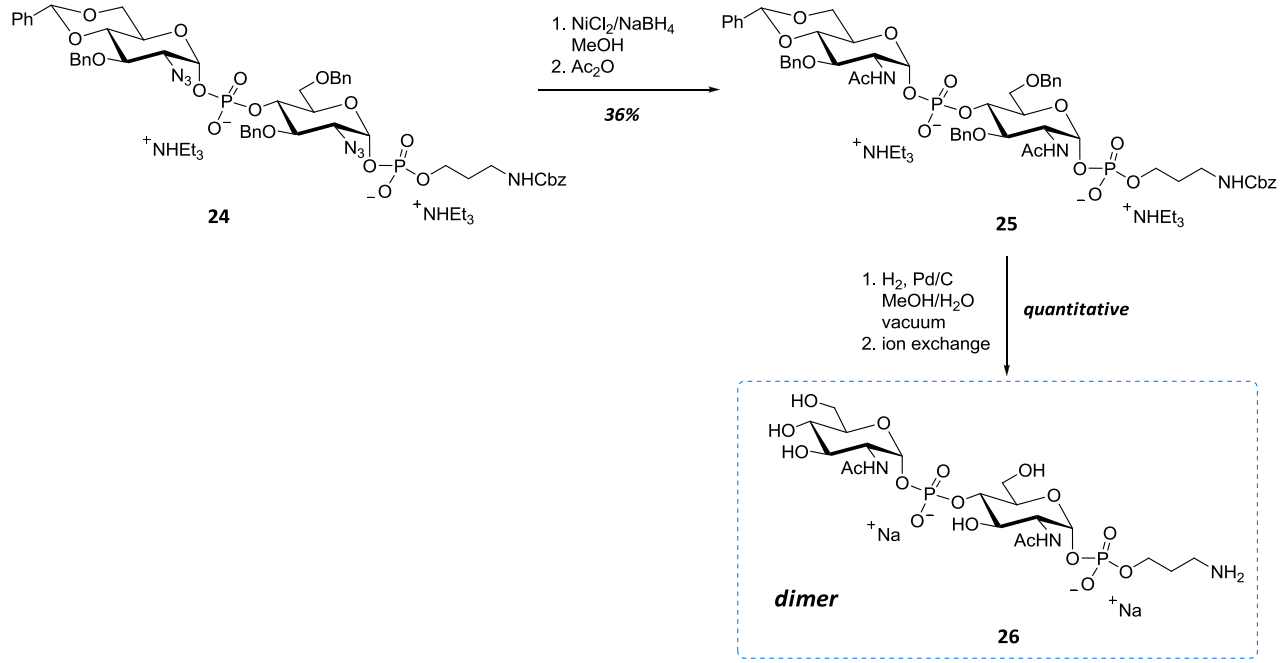
Scheme 19_Synthesis of dimer **24**: the coupling between acceptor **23** and H-phosphonate **17**.

Alcohol **23** is the precursor for the synthesis of both the dimer and the trimer. In order to confirm our previous assumption (i.e. the benzylidene acetal is not the optimal protecting group for blockwise oligomer chain elongation), we tried the regioselective/reductive opening of benzylidene **15** (**Scheme 20**), obtaining the decomposition of the product.



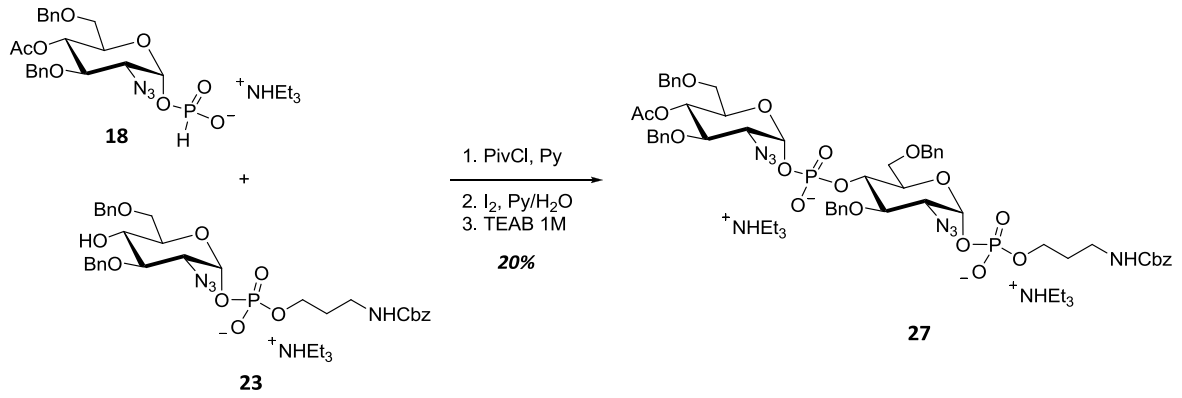
Scheme 20_Regioselective reducting opening of benzylidene acetal **15**.

NH-acetamide **25** was obtained by "$NiCl_2/NaBH_4$" protocol followed by *in situ* acetylation, then Pd/C catalyzed hydrogenation followed by ion exchange led to dimer **26** in *quantitative* yield (**Scheme 21**).



Scheme 3_Synthesis of dimer **26**.

Trimer formation needed 2 steps more, following the same strategy. Coupling between H-phosphonate **18** and alcohol **23**, followed by oxidation, provided dimer **27** (20% yield, **Scheme 22**).

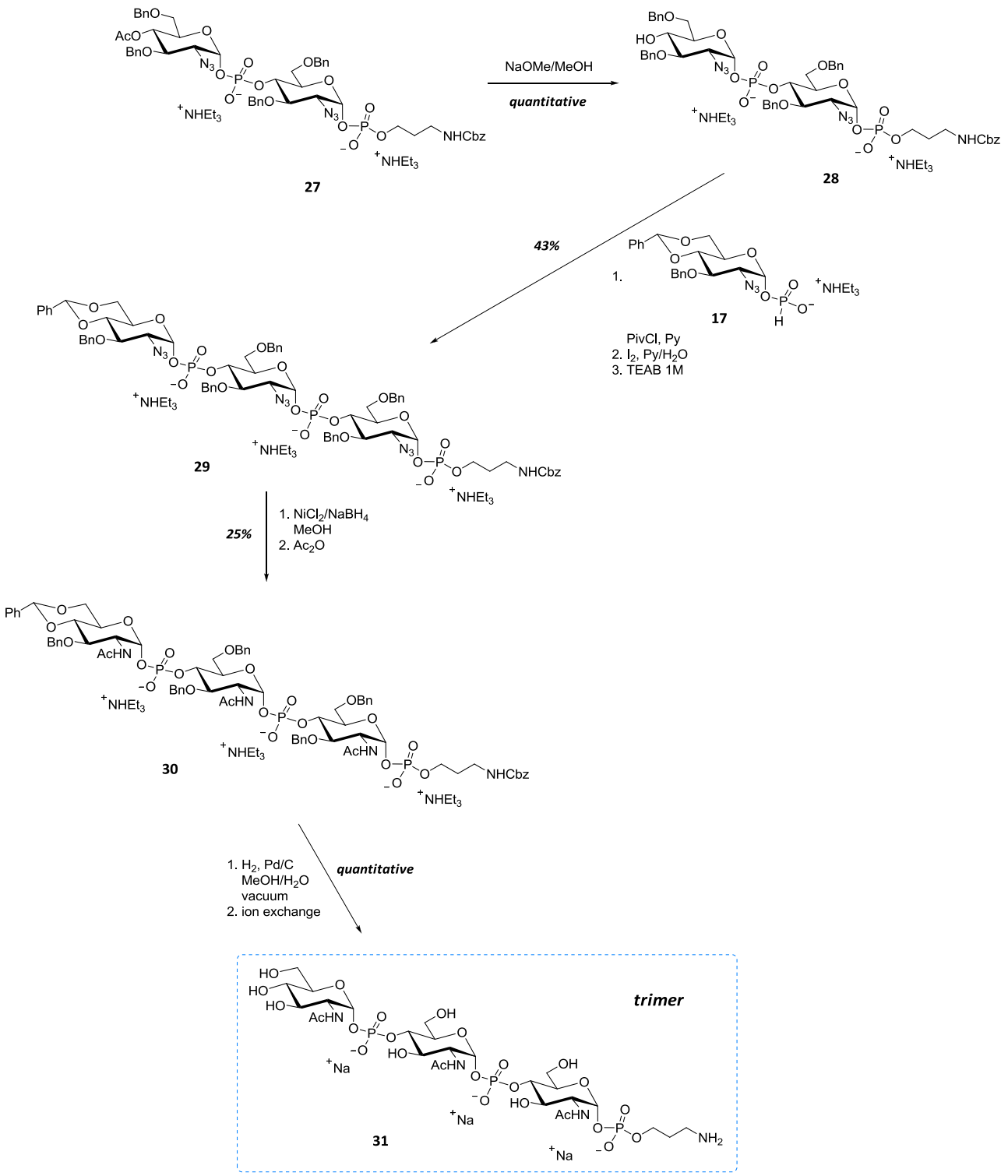


Scheme 4_Synthesis of dimer **27**: the coupling between acceptor **23** and H-phosphonate **18**.

Oligomer elongation was achieved by O-deacetylation of dimer **27** (**28**, *quantitative* yield), and reaction of **17** with **28** to get the phosphotrisaccharide **29** (43% yield, **Scheme 23**).

NH-acetamide **30** was obtained by "$NiCl_2/NaBH_4$" protocol followed by *in situ* acetylation (25% yield), then Pd/C catalyzed hydrogenation and ion exchange furnished trimer **31** (*quantitative* yield).

Synthetic MenX CPS fragments



Scheme 5_Synthesis of trimer **31**.

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CHAPTER 6

MenX neo-glycoconjugates

SYNTHESIS OF NEO-GLYCOCONJUGATES

NEO-GLYCOCONJUGATES FOR BIOLOGICAL ASSAYS

Further goal of the present thesis was the synthesis of neo-glycoconjugates based on fully-synthetic MenX fragments. The glycoconjugates will be employed for mice immunization and ELISA (Enzyme-Linked ImmunoSorbent Assay) assay at Novartis Vaccines and Diagnostics Research Centre in Siena.

To this aim, two different immunogenic proteins were employed: CRM_{197}[1] (Cross-Reacting Material 197, a non-toxic mutant of diphtheria toxin) was used for immunizations, while Human Serum Albumin (HSA) for ELISA tests. With conjugate vaccines, the first carrier proteins were diphtheria and tetanus toxoids, that require detoxification. CRM_{197}, first isolated in 1973, does not need chemical detoxification and maintains its native three-dimensional structure during processing. Lysine residues in CRM_{197} presented outwardly on the molecule can link with polysaccharides through a suitable linker molecule (i.e. adipic acid).

CRM_{197} is commonly used as carrier protein in carbohydrate based conjugate vaccines, especially in routine childhood vaccines.[2] Concerning meningococcal diseases, Menveo® (Novartis Vaccines, Switzerland) is a tetravalent meningococcal CRM_{197}-conjugate vaccine against meningococcal serogroups A, C, W-135, and Y that has been licensed for use.[3]

Moreover two chemically different spacers were used as molecular bridges, namely an adipate for CRM_{197} and a PEG-based spacer for HSA. In particular, ELISA test is performed to determine the antibody specificity.

This study was designed to better understand immunogenical profile of synthetic antigens, and to investigate whether synthetic MenX glycoconjugates can compete with neo-glycoconjugates obtained with purified CPS.

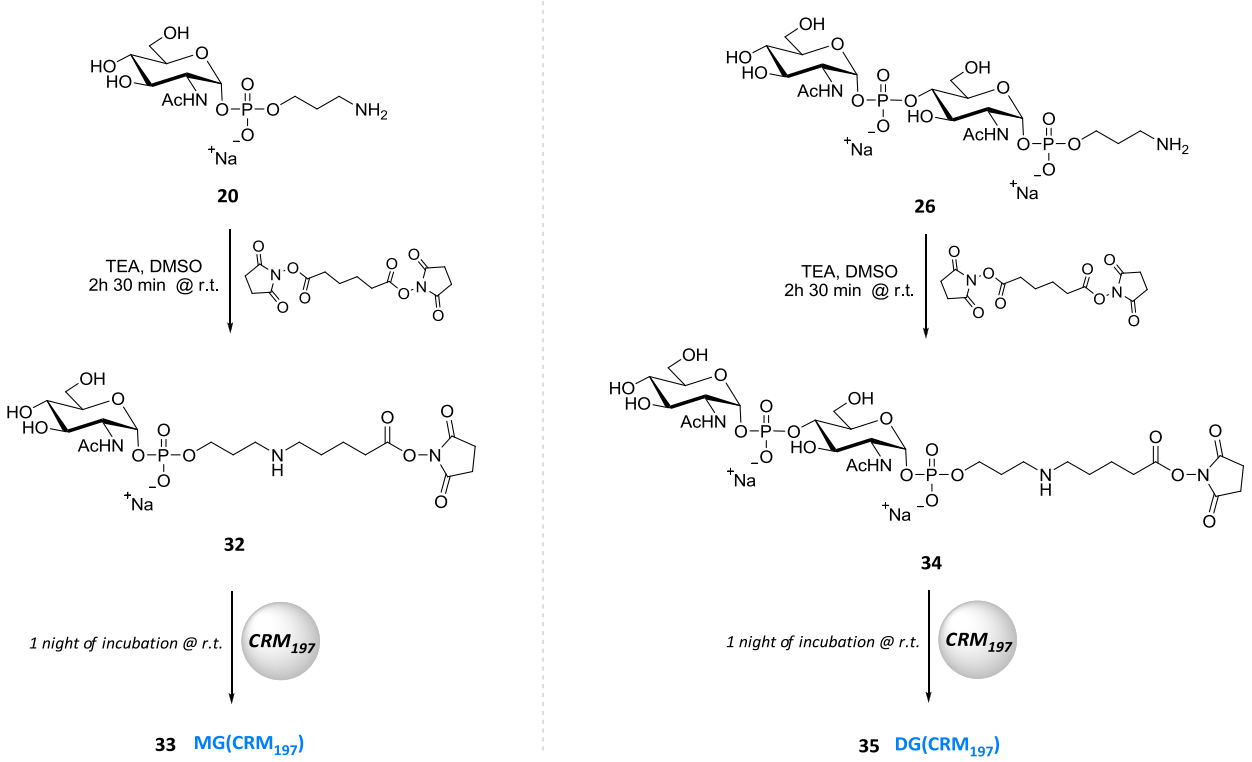
CONJUGATION OF FRAGMENTS TO CARRIER PROTEINS

During my stage as a visiting scientist in Novartis, the neo-glycoconjugates based on monomer and dimer fragments were synthesized.

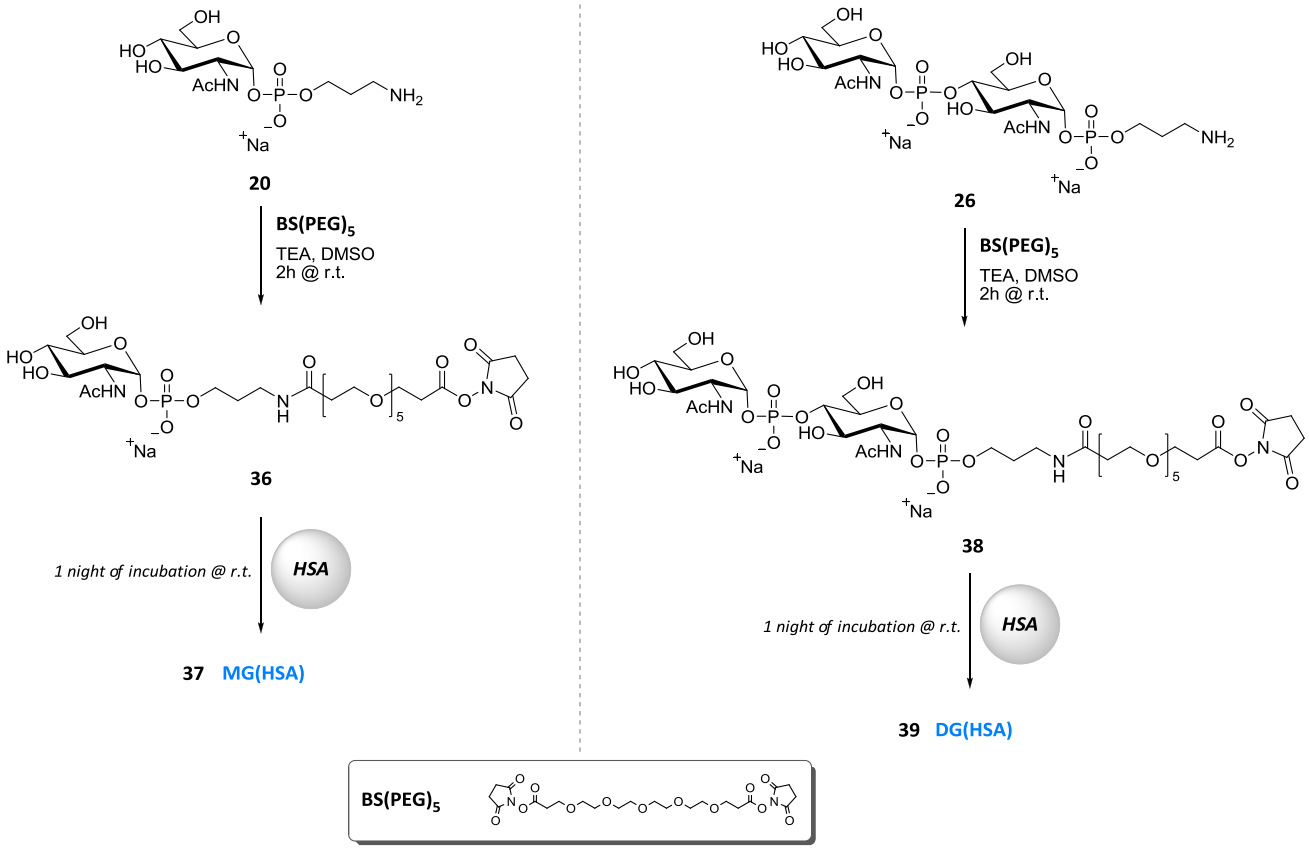
Disuccinimidyl adipate was used to activate synthetic fragments apt to conjugation with CRM_{197} (**Scheme 1**). After purification (see the ***Experimental section***, ***CHAPTER 8***), activated oligosaccharides **32** and **34** were then reacted with CRM_{197} (stoichiometry 100:1 $mol_{sugar}/mol_{protein}$).

On the other hand $BS(PEG)_5$ (bis-succinimidyl penta-ethylene glycol) was used as immunosilent spacer apt to conjugation with HSA (stoichiometry 100:1 $mol_{sugar}/mol_{protein}$, **Scheme 2**).

CRM_{197} and HSA glycoconjugates were analysed by SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis)[4] and MALDI-TOF analysis.



Scheme 1_Synthesis of monomer-CRM_{197} glycoconjugate **33** and dimer-CRM_{197} glycoconjugate **35**.



Scheme 2_Synthesis of monomer-HSA glycoconjugate **37** and dimer-HSA glycoconjugate **39**.

MenX neo-glycoconjugates

A fundamental parameter in the synthesis of neo-glycoconjugates is the loading on the carrier protein. The sugar/protein ratio was determined by MALDI spectra, while the SDS-PAGE allowed to confirm the occurrence of the conjugation. The profiles of the glycoconjugates were composed of polydispersions:

- ✓ MG(CRM_{197}) **33** was 62258.89 m/z, corresponding to approximately 7 sugar chains per protein molecule;
- ✓ DG(CRM_{197}) **35** was 61731.51 m/z, corresponding to approximately 4 sugar chains per protein molecule;
- ✓ MG(HSA) **37** was 68320.268 m/z, corresponding to approximately 3 sugar chains per protein molecule;
- ✓ MG(HSA) **39** was 67734.440 m/z, corresponding to approximately 1 sugar chain per protein molecule.

First attempts of glycoconjugations were not successful, but new attempts are currently in progress.

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[4] *Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis* is technique widely used in biochemistry to separate proteins (and/or glycoconjugates) according to their electrophoretic mobility

CHAPTER 7

“Acetamido route” to oligomers synthesis

SYNTHESIS OF THE TETRAMER

CONSIDERATIONS ABOUT THE SYNTHETIC STRATEGY

A significant drawback in the synthesis of monomer **20**, dimer **26**, and trimer **31** from H-phosphonates **17** and **18** was the low yield of some crucial steps. In particular:

- the condensation of the H-phosphonate with the proper alcohol acceptor (in contrast with the good results reported in literature);
- the azide reduction.

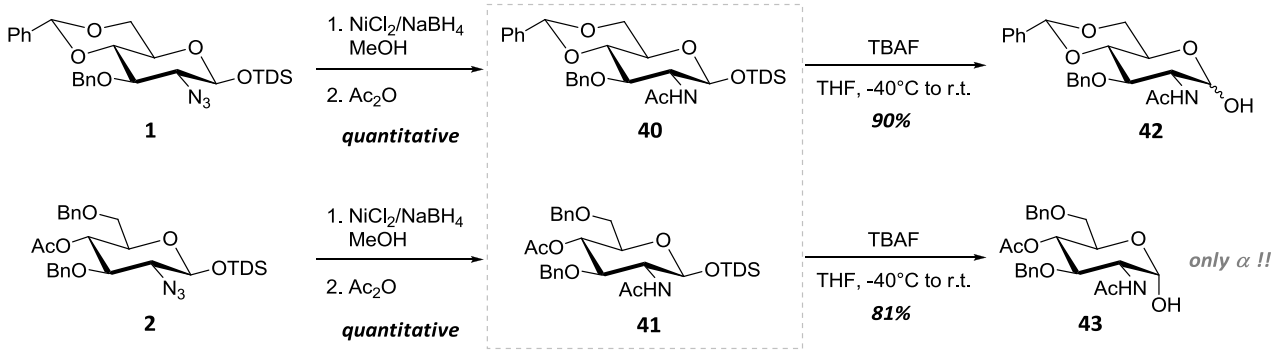
Concerning the coupling, we also explored the *inverse procedure*. Briefly, the donor was co-evaporated 3 times with dry pyridine and/or dry toluene and let it dry in vacuum pump overnight, then it was dissolved in pyridine and pivaloyl chloride was added: after 15 minutes a solution of the acceptor in dry pyridine was added. However this procedure did not lead to a significant improvement of the chemical yields. Likewise, the use of the H-phosphonate (instead of the alcohol) as a limitating agent was not beneficial, the only exception being the coupling of hemiacetals **6** or **8** with the spacer (70% and 75% yield, respectively).

The low yields are presumably due to the poor reactivity of H-phosphonates **17** and **18**. Then Oscarson's approach[1] seems to be not suitable for this kind of coupling.

Further elongation and synthesis of higher oligomers has to be performed using a different approach.

A NEW SYNTHETIC ROUTE FOR TETRAMER FORMATION

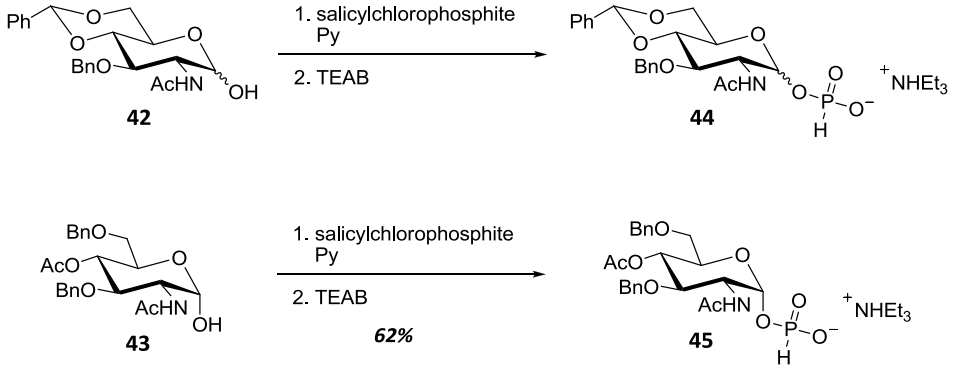
As far as the azide reduction is concerned, the "$NiCl_2/NaBH_4$" protocol led to very low yields when applied on pre-formed phosphodiester-linked oligomers. We therefore decided to reduce the azido function in a early stage of the synthesis, namely on building blocks **1** and **2** to provide *N*-acetamido derivates **40** and **41**, respectively, in *quantitative* yield (**Scheme 1**).



Scheme 1_Acetamido key precursors: synthesis of **40** and **41**, and of hemiacetals **42** and α-**43**.

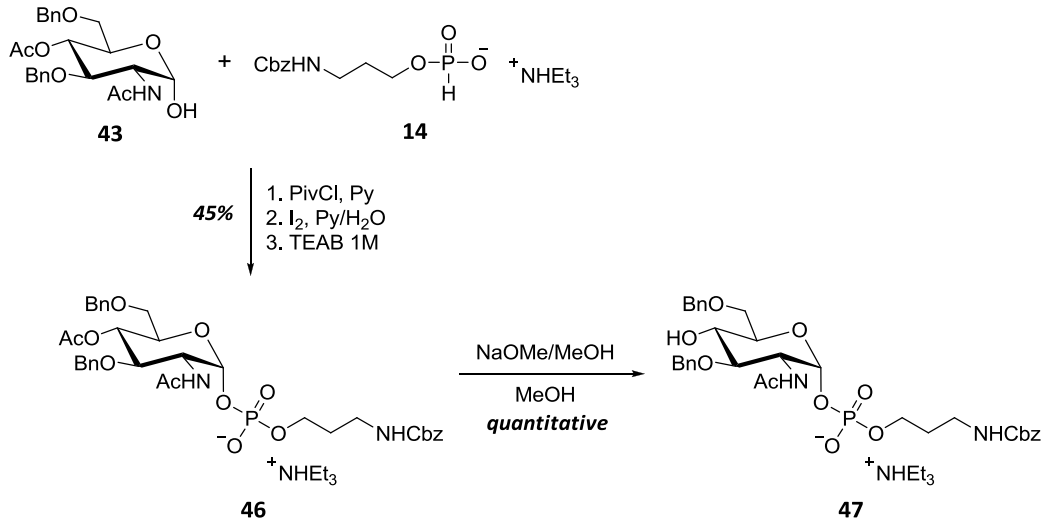
Hemiacetals **42** and **43** were synthesized by desilylation of corresponding building blocks using glacial acetic acid and tetrabutylammonium fluoride (TBAF) 1M in THF (90% and 81% yield, respectively). Quite surprisingly, NMR analysis showed that **42** was obtained as an α,β mixture, while **43** as a pure α hemiacetal (**Scheme 1**). Taking into account these results, first the synthesis of H-phosphonates **44** and **45** was tested. Only H-phosphonate **45** was obtained as an α pure

stereoisomer without the use of H_3PO_3 as equilibrating-agent, while **44** was achieved as an anomeric mixture (**Scheme 2**).



Scheme 2_Synthesis of acetamido H-phosphonates.

Since 2-*N*-acetamido H-phosphonates are less stable than the corresponding azido H-phosphonates, the coupling between **43** and a stable H-phosphonate (see ***CHAPTER 5***, ***"approach A"***), i.e. 3-N-carbobenzyloxy-propanolamine H-phosphonate **14**, was explored (**46**, 45% yield, **Scheme 3**).

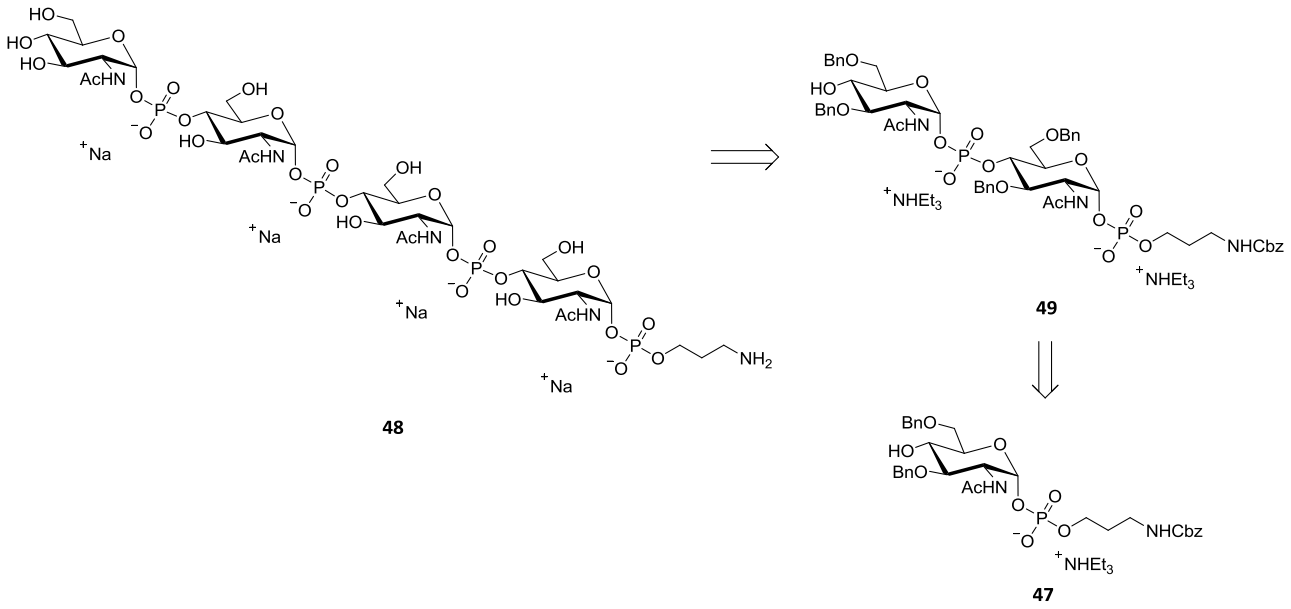


Scheme 3_Synthesis of alcohol acceptor **47**.

The coupling was accomplished in standard conditions, and NMR analysis confirmed the α configuration of the anomeric carbon. According to oligomer elongation, alcohol **47** was achieved by O-deacetylation of **46** (*quantitative* yield).

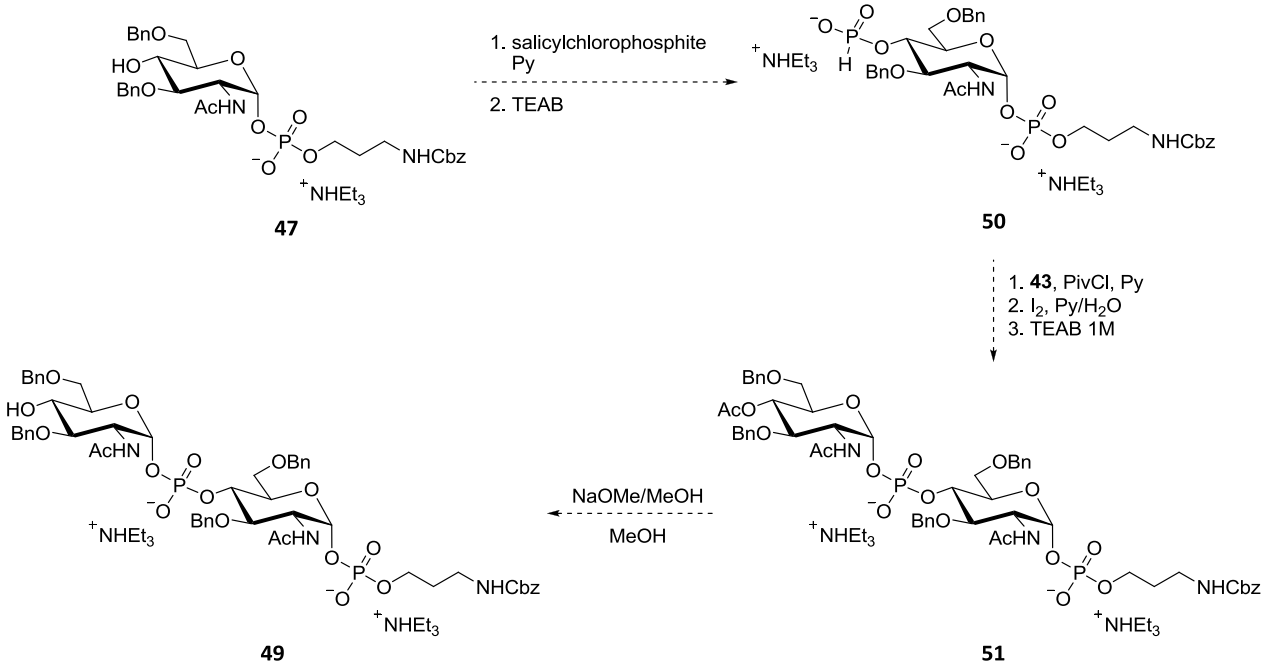
Acceptor **47** is the key building block for the synthesis of longer oligomer, i.e. the tetramer **46**: in **Scheme 4** is reported the synthetic 1+1+2 approach we envisaged for this goal.

"Acetamido route" to oligomers synthesis



Scheme 4_Proposed 1+1+2 synthetic approach for the synthesis of tetramer **48**.

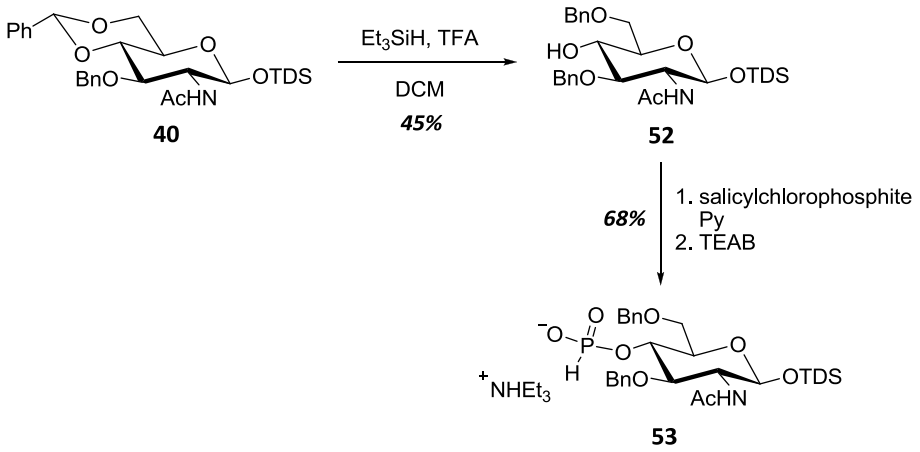
Acceptor **47** will be converted into the H-phosphonate **50**, with salicylchlorophosphite in pyridine (**Scheme 5**). Then the coupling between hemiacetal **43** and H-phosphonate **50** will be accomplished in standard conditions, providing (according to previous results) α,α dimer **51**. Dimer **51** will be submitted to O-deacetylation to obtain **49**.



Scheme 5_Reactions in progress.

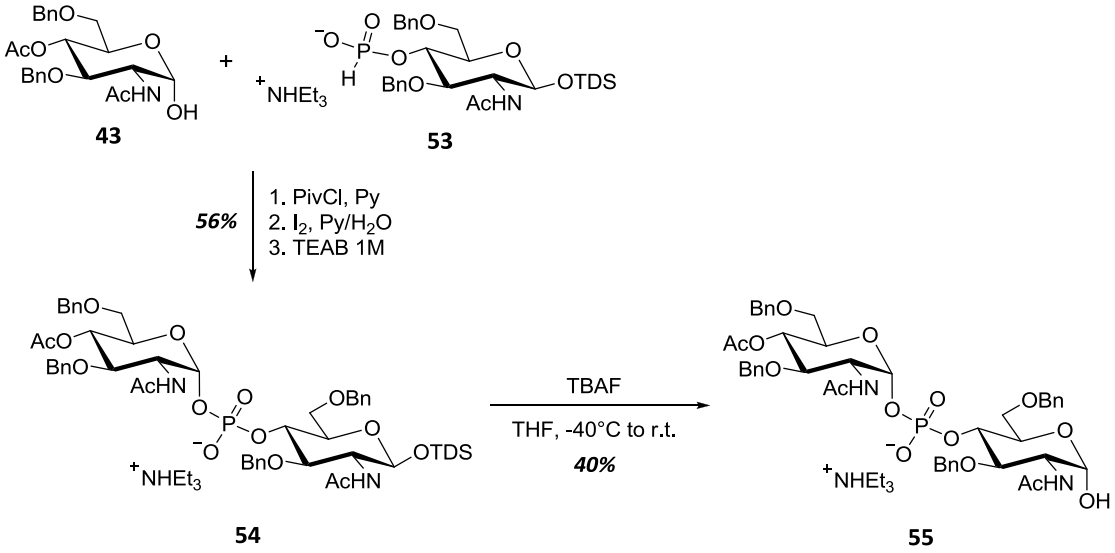
On the other hand, we synthesized acceptor **55**. The regioselective reductive opening of the benzylidene acetal in compound **40** afforded **52** (45%), followed by H-phosphonate formation at 4-OH to provide the stable H-phosphonate **53** in 68% yield (**Scheme 6**).

"Acetamido route" to oligomers synthesis



Scheme 6_Synthesis of H-phosphonate **53**.

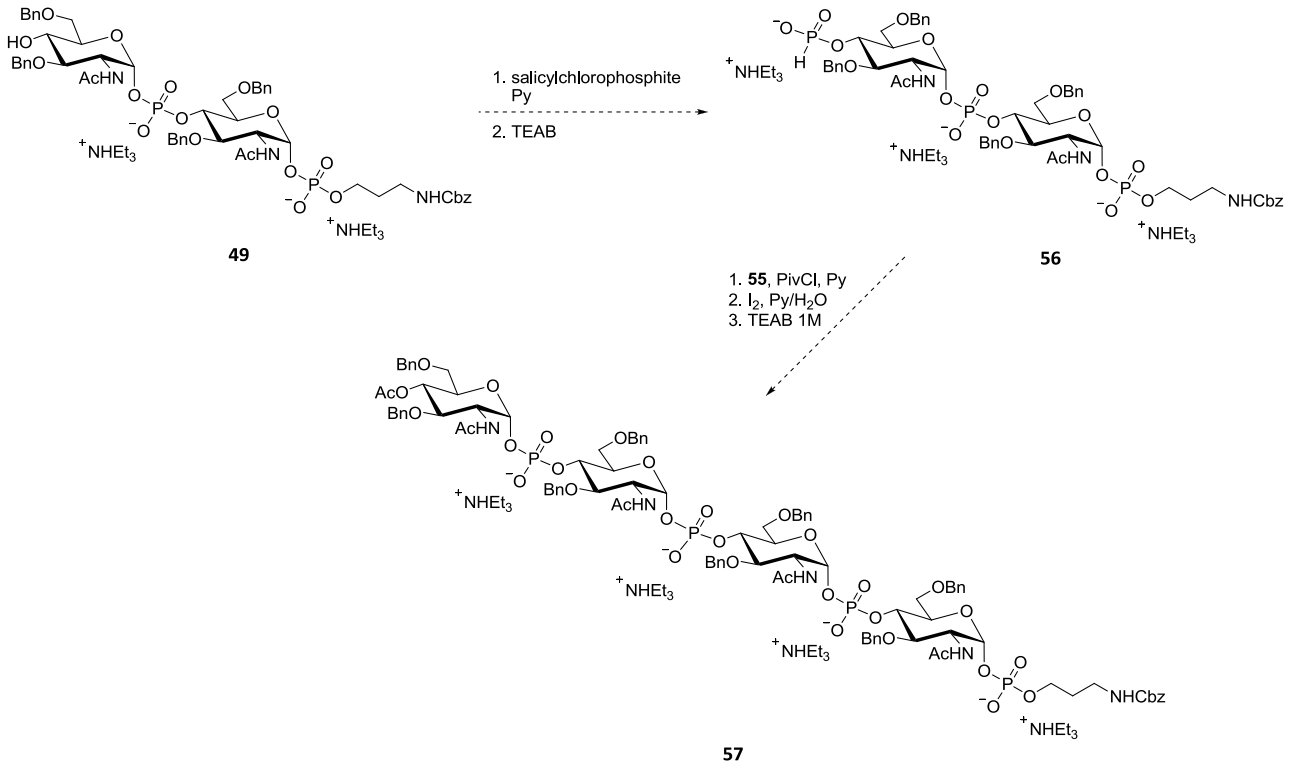
The coupling between hemiacetal **43** and H-phosphonate **53** was accomplished in standard conditions (56% yield, **Scheme 7**). NMR spectra confirmed the α configuration of the phosphodiester bridge of dimer **54**.



Scheme 7_Synthesis of dimer **55**.

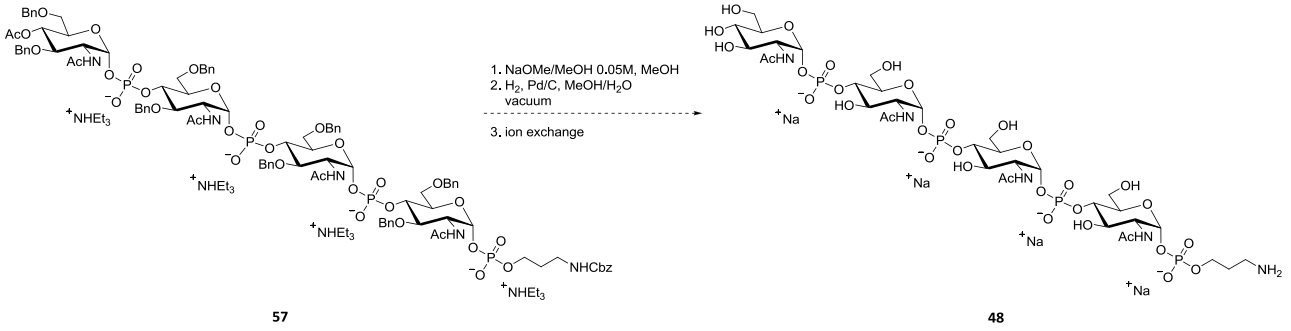
Dimer **54** was submitted to desilylation by TBAF 1M in dry THF to obtain **55** (40% yield, **Scheme 7**). NMR analysis highlighted the obtainment of only the α hemiacetal. This unexpected result will allow the coupling between **55** and H-phosphonate **56** (**Scheme 8**), to achieve tetramer **57**, containing exclusively α phosphodiesters bridges.

"Acetamido route" to oligomers synthesis



Scheme 8_Synthesis of full protected tetramer **57**: the coupling between acceptor **55** and H-phosphonate **56**.

Eventually, conventional protecting groups removal will lead to the tetramer **48** as a tetrasodium salt (**Scheme 9**).



Scheme 9_Protecting group removal leads to **48**.

BIBLIOGRAPHY

[1] R. Slättegård, P. Teodorovic, H. Hadgu Kinfe, N. Ravenscroft, D. W. Gammon, S. Oscarson, *Org. Biomol. Chem.* **2005**, *3*, 3782-3787

CHAPTER 8

Conclusions and future work

CONCLUSIONS

In summary, the synthesis of key precursors **1** and **2** was accomplished in medium (up to 2 g) and in large (up to 30 g) scale.

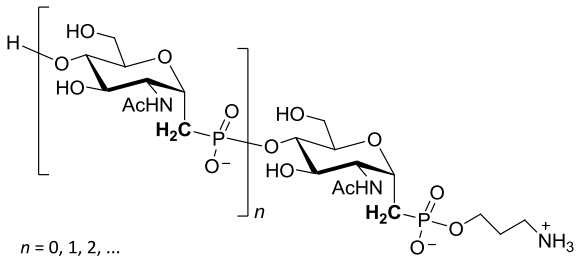
We have explored the synthesis of synthetic oligomers via H-phosphonate approach. Accordingly we were able to synthesize 2-azido-2-deoxy glycosyl α-anomeric H-phosphonates (**17** and **18**). In this way, we achieved an important goal, since pure alpha 2-azido-2-deoxy glycosyl H-phosphonates have never been reported in the literature. Following this route, we were able to synthesize monomer **20**, dimer **26**, and trimer **31**. These compounds have anomerically *O*-linked aminopropyl phosphate linker at their reducing end to allow conjugation to a protein carrier. A significant drawback, in their synthesis from H-phosphonates **17** and **18**, was the low yield of some crucial steps.

Neo-glycoconjugates of monomer and dimer have been synthesized during my stage at Novartis (Siena). The immunogenical profile of synthetic antigens will be elucidated as a result of mice immunization and ELISA (Enzyme-Linked ImmunoSorbent Assay, performed to determine the antibody specificity) assay, that will be performed at Novartis Vaccines and Diagnostics Research Centre. Trimer-glycoconjugate will be synthesized in the near future.

We tried to solve some problem of the synthetic strategy using the *NH-acetamido* route. The obtainment of alpha pure alcohols **35** and **40** was quite a surprise. On the basis of the last results, we envisaged the synthesis of the tetramer following a 1+1+2 approach. Work is in progress to achieve this goal.

FUTURE WORK

A problem often encountered in the formulation of fully synthetic carbohydrate-based vaccines is the poor chemical and/or enzymatic stability of many CPSs. This can greatly hamper the manufacture of conjugate vaccines, where chemical manipulation of the saccharide is an inevitable step so that it may be linked to the protein carrier. In the case of Men X, chemical lability might derive from the acetalic position of the phosphodiester bridges, as it occurs in *N. meningiditis* A CPS. Indeed, phosphodiesters are normally quite stable, but when they engage the anomeric position of a carbohydrate residue, the C1-O1 bond becomes much more labile due to the possibility of the electron pair on the ring oxygen displacing the phosphate or phosphodiester group. In case stability problems should emerge during natural fragments manipulation, a part of the present research project will be devoted to the synthesis of modified structures which, while keeping the immunological properties of the natural counterparts (*i.e*. to elicit antibodies cross-reacting with the bacterial capsule), are endowed with an increased stability. The stability of the phosphodiester bridges can be increased by applying a number of structural modifications on the phosphodiester group. Based on our recent experience acquired on the synthesis of stable analogues of *N. meningiditis* A CPS, the most promising candidates as possible vaccine components can be obtained by replacing the anomeric oxygen of the phosphodiester with a methylene group, i.e. by replacing the phosphodiester with a phosphonoester bridge. In that event, fragments such as those reported in **Figure 1** will be synthesised and tested.



Synthetic C-glycosyl oligomers

Figure 1

Finally, the synthetic oligomers endowed with the most promising biological profile will be conjugated to polyfunctional rigid scaffolds in order to amplify their immune response by exploiting the "multivalency effect". Synthetic antigens are indeed often too small to effectively stimulate the immune system. It is known that, in order to be immunogenic, the saccharide chain needs to be composed by several monosaccharide units. On the other hand, a chemical synthesis of such a kind of fragments is unpractical. A possible solution is the preparation of multivalent compounds in which several short saccharide units are linked to a core, thus generating a system containing a large number of antigenic determinants, which could enhance the immune response acting as a mimic of the natural polymer.

CHAPTER 9

Experimental section

GENERAL EXPERIMENTAL METHODS

CHARACTERISATION OF THE PRODUCTS

Thin Layer Chromatography

Thin Layer Chromatography (TLC) and High Performance Thin Layer Chromatography (HPTLC) were performed on Merck precoated $60F_{254}$ plates (0.25 mm and 0.2 mm thickness, respectively). Reactions were always followed by TLC-analysis; compounds were visualized, when appropriate, by UV light (254 nm) and always by spraying with:

- *Molybdic solution* [21 g of $(NH_4)_4Mo_4O_{24}$, 1g of $Ce(SO_4)_2$, 31 mL of H_2SO_4 98%, 970 mL water], or
- *Sulphuric acid* [50 mL of H_2SO_4 98%, 450 mL of MeOH (or EtOH), 450 mL water], or
- *Ninhydrin* [2.7g of 2,2-Dihydroxyindane-1,3-dione, 27 mL of AcOH, 900 mL of n-BuOH (or EtOH)],

with detection by charring at 196°C.

Unless otherwise indicated, especially during the chromatography purifications, the molybdic solution was the most used reagent.

Flash chromatography

According to Still[1] procedure, compounds were purified by flash chromatography, using Silica gel (SiO_2, high-purity grade (Merck Grade 9385), pore size 60 Å, 230-400 mesh particle size) by Sigma-Aldrich®.

In some cases it was also used a flash purification system, Biotage® SP1™:[2] SP1 means a single-column flash purification system [with Touch Logic Control™ and TLC-to-gradient feature designed to accelerate and simplify the purification process]. Available in sizes from 10 g to 340 g with standard (50 µm) silica, Biotage SNAP cartridges were used to purify our compounds.

NMR analysis

NMR spectra were recorded in a Bruker Avance 400 instrument (400, 101 and 162 MHz for ^{1}H, ^{13}C, and ^{31}P respectively), and all were run at room temperature (298K). The samples were prepared using deuterated solvents, as $CDCl_3$, D_2O, CD_3OD and $(CD_3)_2SO$, by Sigma-Aldrich®.

Chemical shifts (δ) are reported in ppm scale and the coupling constants (*J*) in Hz. The chemical shift displacement is based on the residual proton in the solvent (e.g. the $CHCl_3$, 0.01 % in 99.99 % $CDCl_3$), following the solvent's value established by Hugo E. Gottlieb and Abraham Nudelman.[3] The multiplicity of signals has been described as: *s* (singlet), *d* (doublet), *t* (triplet), *m* (multiplet), *dd* (doublet of doublets), *dt* (doublet of triplets), *sept* (septet).

^{1}H-NMR spectra were recorded for all the synthesized products. In the case of unknown structure, the characterization is here reported by ^{1}H-NMR, ^{13}C-NMR and, when relevant, by ^{31}P-NMR: the registration of bidimensional (2D-COSY, 2D-HETCOR) analyses was due to better assignment of peaks to the structure.

To better understand peaks assignments, two examples are reported in **Figure 1**: for instance, in the case of a dimer we called ring-A the sugar coupled with the spacer and ring-B the one at the non-reducing terminus.

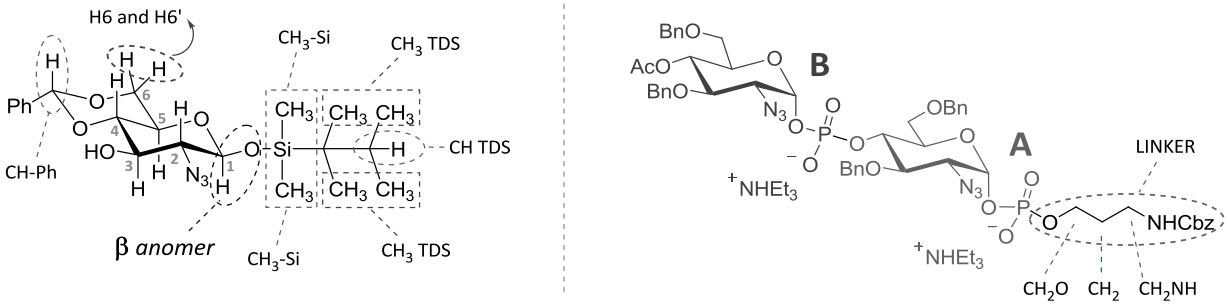


Figure 1

NMR experiments of the lyophilized fully deprotected final products [dissolved in deuterium oxide (D_2O, 99.9% atom D, Aldrich)], were recorded at 25°C on a Brucker Avance III 400 MHz spectrometer at Novartis Vaccines and Diagnostics Research Center.

Mass analysis

ESI mass were recorded in negative or positive modes on Jeol AX-505 and for high resolution on Bruker Daltonics APEX™ II (FT-ICR) instrument belonging to CIGA.[4]

MALDI-TOF mass spectra of CRM_{197}, HSA and glycoconjugates were recorded by UltraFlex III MALDI-TOF/TOF instrument (Bruker Daltonics).

Optical rotatory power

Optical rotations were measured at r.t. with Pelkin-Elmer 241 polarimeter (589 nm, D line from sodium lamp); the polarimeter tube was 100 mm thick and 1 ml of capacity. Optical rotatory powers were measured following the equation:

$$\text{specific rotation} = \frac{10000 \cdot \alpha}{l \cdot c}$$

where α is the observed rotation (on the average of 15 experimental values), l is the length of the observed layer in mm, c is the number of g of substance contained in 100 ml of solution. The optical rotation (α) can be expressed in angular degrees: values are given in 10^{-1} deg cm^2 g^{-1} unit.

SOLVENT CONDITIONS

ANHYDROUS ENVIRONMENT

Unless otherwise indicated, all the reaction were conducted under nitrogen atmosphere. When stirred overnight, the reaction were put in anhydrous atmosphere by an Argon-balloon.

SOLVENT CONDITIONS

Unless otherwise indicated, all the reactions were performed using dry solvents: DCM, MeOH, pyridine, toluene, DMF, CH_3CN and THF were purchased over molecular sieves from Sigma-Aldrich® and used without further purifications.

GENERAL PROCEDURES

A. DESILYLATION[5]

Glacial acetic acid (3 eq) and Tetrabutylammonium fluoride (3 eq) 1M in THF solution (10 mL/mmol) were sequentially added to a solution of the sugar in anhydrous THF at -40°C. After completion of the reaction, the mixture was warmed up to r.t., poured in DCM, and washed three times with brine. The organic layer was dried (Na_2SO_4), filtered and concentrated. The residue was purified by column chromatography.

B. REGIOSELECTIVE/REDUCTIVE OPENING OF BENZYLIDENE ACETAL[6]

The sugar was dissolved in dry DCM (5 mL/mmol) and Et_3SiH (5 eq) was added dropwise. After 30 min, the mixture is cooled to 0 °C, and TFA (5 eq) was slowly added. The reaction was stirred at r.t. and, after completion, diluted with DCM, quenched by addiction of a saturated aqueous solution of $NaHCO_3$. The aqueous phase was extracted with DCM, the combined organic phases were dried over Na_2SO_4 and the solvent removed under reduced pressure. The crude product was purified by flash chromatography.

C. ZEMPLEN REACTION[7]

The reactant was dissolved in dry methanol and stirred under nitrogen. A solution of NaOMe/MeOH 0.1 M (or 0.05 M) was added dropwise until basic pH. After deacetylation, confirmed by TLC, the reaction was neutralized by IRA120-H^+ ion-exchange resin (Amberlite® IR120, hydrogen form, 16-50 mesh, exchange capacity = 1.9 meq/mL, by Sigma-Aldrich®), filtered and concentrated.

D. α ANOMERIC H-PHOSPHONATE

H_3PO_3 was coevaporated three times with dry toluene, and dried by high vacuum pump. A 2M solution in pyridine of H_3PO_3 (3 eq) was dropped to a solution of the hemiacetal in dry pyridine (4 mL/mmol), and thereafter the solution was cooled to 0°C and salicyl chlorophosphite was slowly added. The reaction was stirred at r.t. and, after disappearance of the starting material (*over night*), the reaction temperature was raised up to +40°C and the mixture was stirred under argon until complete disappearance of the β anomer (verified by ^{1}H- and ^{31}P-NMR spectra). 1M solution of TEAB (4 mL/mmol) was added to the reaction at r.t. and, the mixture was diluted with CH_2Cl_2, washed three times with cold TEAB (0.5 M), dried (Na_2SO_4), filtered and concentrated. The crude was purified by flash chromatography (DCM:MeOH + 1% TEA). The H-phosphonate has to be stabilized, washing with 0.25M cold TEAB [then drying (Na_2SO_4), filtering and concentrating] the purified product.

E. STANDARD PROCEDURE FOR PHOSPHODIESTER SYNTHESIS

The donor (H-phosphonate, 1.2 eq) and the acceptor (alcohol, 1 eq) are first co-evaporated three times with dry pyridine or dry toluene, thereafter they are dried by high vacuum pump overnight. The reactants were dissolved in pyridine (10 mL/mmol), then pivaloyl chloride (2.5 eq) was added

dropwise and the reaction mixture was stirred under nitrogen. The reaction completion was monitored by TLC (or HPTLC). After cooling to -40°C, a freshly prepared 0.5 M solution of iodine (2.5 eq) in pyridine/water 19:1 was added. The oxidation was completed at 0°C and quenched by dropwise addition of a 0.5 M solution of $Na_2S_2O_3 \cdot 5H_2O$ (10% w/v). The mixture was diluted with CH_2Cl_2, washed two times with $Na_2S_2O_3 \cdot 5H_2O$ (0.5 M), then with cold TEAB (0.5 M), dried (Na_2SO_4), filtered and concentrated. The crude was purified by flash chromatography (DCM:MeOH + 1% TEA). The phosphate bridge has to be stabilized, washing with 0.25M cold TEAB [then drying (Na_2SO_4), filtering and concentrating] the purified product.

F. AZIDE REDUCTION BY $NaBH_4/NiCl_2 \cdot 6H_2O$[8]

To a mixture of azide-containing compound (1 eq) and $NiCl_2 \cdot 6H_2O$ (3 eq) in MeOH (10 mL/mmol), under stirring and N_2 atmosphere, $NaBH_4$ (8 eq) was added at 0 °C in small portion (1 hour). The formation of a black precipitate indicated the formation of a Ni-B species. The mixture was stirred at 0 °C and, after consumption of the starting material (amine formation is monitored by Ninhydrin-detection), Ac_2O (20eq) was added. The mixture was concentrated under reduced pressure, diluted with CH_2Cl_2 and washed three times with water. The organic phase was dried (Na_2SO_4), concentrated, and purified by flash chromatography to give the pure acetamide.

G.HYDROGENATION CATALYZED BY PALLADIUM

The sugar was dissolved in a 5:1 solution of methanol/water, then a catalytic amount of Pd/C (10%) was added. The reaction mixture was vigorously stirred under H_2 atmosphere at r.t. overnight. The solution was then filtered over a celite pad, and finally concentrated under reduced pressure.

H.ION EXCHANGE

The ion exchange (from $^+NHEt_3$ to ^+Na) of the final products was carried out using a strong resin, the commercially available DOWEX® 50WX8 (H^+ form, 50-100 mesh, exchange capacity = 1.7 meq/mL, by Sigma-Aldrich®).

Two distinct portions of resin were activated by washing with a 5% aqueous solution of HCl (^+H) and with a 5% aqueous solution of NaOH (^+Na).

The equation reported below was used to calculate the required volume of regenerant.

$$V(mL)_{regenerant} = \frac{x(mL)_{resin} \cdot exchangecapacity_{resin} \cdot MW_{regenerant} \cdot 100}{1000 \cdot \%\frac{w}{v}_{regenerant}} \cdot y$$

The variable y is equal to 4 for strong resin, like in this case, and 2 for weak resin.

Regenerants were introduced into the flask containing the resin, the contact time had to be at least 1 hour: the two portion of resin were incubated independently and mixed by a digital shaker (Heidolph© Promax 1020). The product was eluted continuously very slowly and directly was exchanged from $^+NHEt_3$ first to H^+ and then to Na^+, as depicted in (***Figure 2***).

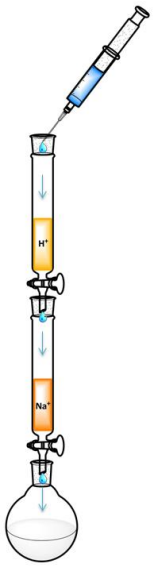
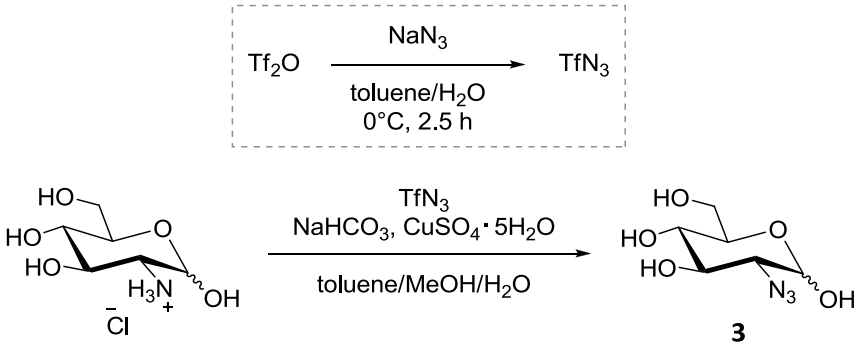


Figure 2

2-Azido-2-deoxy-α/β-D-glucopyranose (3)



Synthetic procedure

Preparation of TfN_3:

DCM (250 mL) was added to a solution of NaN_3 (59.5 g, 0.92 mol) in water (150 mL) at 0°C. The mixture was stirred vigorously and treated with trifluoromethanesulfonic anhydride (31.0 mL, 0.19 mol) over a period of 3 hours at 0°C. After the complete addition of Tf_2O, the reaction mixture was stirred at 0°C for 2.5 hours. The aqueous phase was extracted with DCM (2x100 mL) and the combined organic layers washed with saturated Na_2CO_3. The reaction volume was reduced until 100 mL and used directly in the diazotransfer step.

CAUTION: dry TfN_3 is explosive at high temperature!

Diazotransfer reaction:

$CuSO_4$ (140 mg, 0.88 mmol) and K_2CO_3 (19.2 g, 0.14 mol) were added to a solution of D-glucosamine hydrochloride (20.0 g, 0.092 mol) in water (300 mL). Methanol (600 mL) was added followed by the addition of the TfN_3 solution. The clear blue solution was allowed to stir for 24 hours at RT. The solvents were removed in vacuum to afford product **3** as a brown crude, without any further purification.

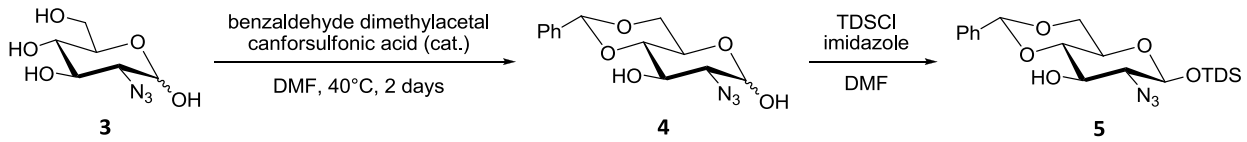
The reaction was performed also in large scale (up to 30 g).

Product characterization

Formula	$C_6H_{11}N_3O_5$
Molecular Weight	205.17 g/mol
TLC conditions	R_f (DCM/MeOH 7:3) 0.64

The spectroscopic data were in agreement with those reported in literature.[9]

Thexyldimethylsilyl 2-Azido-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside (5)



3 4 5

Synthetic procedure

Compound **3** (5 g, 24.37 mmol) was dissolved in dry DMF (100 mL) and benzaldehyde dimethylacetal (11 mL, 73.11 mmol) was added, followed by addition of camphorsulfonic acid (pH≈5). The reaction was heated at 40°C for 48 hours, then it was quenched by adding $NaHCO_3$ until neutral pH and solvents were removed under vacuum.

The resulting product, without any further purification, was dissolved in dry DCM (25 mL) and cooled to -10 °C. Imidazole (4.15 g, 60.92 mmol) and TDSCl (5.32 mL, 26.81 mmol) were added to the cooled solution and the mixture stirred 24 hours. The reaction, diluted with DCM, was quenched by adding water. The aqueous phase was extracted with DCM (3x200 mL), the combined organic phases were dried over Na_2SO_4 and the solvent removed under reduced pressure. The crude product was purified by flash chromatography (H/EA gradient) yielded **5** (yellow oil, 6.9 g, 15.84 mmol, 65% over three steps), only in β configuration.

Product 4 characterization

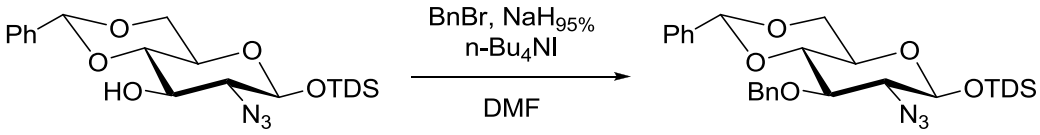
Formula	$C_{13}H_{15}N_3O_5$
Molecular Weight	293.28 g/mol
TLC conditions	R_f (DCM/MeOH 95:5) 0.35

Product 5 characterization

Formula	$C_{21}H_{33}N_3O_5Si$
Molecular Weight	435.59 g/mol
TLC conditions	R_f (H/EA 9:1) 0.25

The spectroscopic data were in agreement with those reported in literature.[10]

Thexyldimethylsilyl 2-Azido-3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside (1)



5 **1**

Synthetic procedure

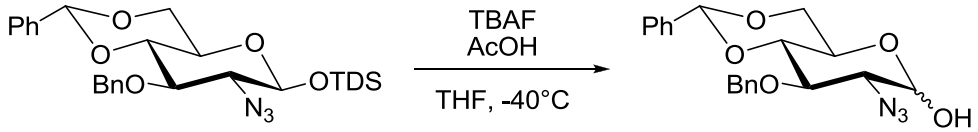
Compound **5** (11.79 g, 27.1 mmol) was dissolved in dry DMF (100 mL) and BnBr (6.4 mL, 54.1 mmol) and catalytic amount of TBAI (2.5 g, 6.8 mmol) were added to the solution. NaH 95% (974 mg, 40.6 mmol) was slowly added and reaction mixture was stirred at RT for 1 hour. The reaction was quenched by adding MeOH then the solvents were removed under vacuum. The residue was dissolved in EtOAc (200 mL) and washed two times with 5% solution of HCl (2x200 mL) and with brine (1x200 mL). The organic phase was dried over Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by flash chromatography (H/EA gradient) affording **1** (10.68g, 20.31 mmol,72% yield) as a white solid.

Product characterization

Formula	$C_{28}H_{39}N_3O_5Si$
Molecular Weight	525.71 g/mol
TLC conditions	R_f (H/EA 95:5) 0.30

The spectroscopic data were in agreement with those reported in literature.[11]

2-Azido-3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-α/β-D-glucopyranose (6)



1 **6**

Synthetic procedure

Compound **1** (5.47 g, 9.97 mmol) was treated as described in the ***General Procedure A***. Product **6** was obtained as an α:β mixture (3.06 g, 7.98 mmol, white solid, 80% yield).

Product characterization

Formula	$C_{20}H_{21}N_3O_5$
Molecular Weight	383.40 g/mol
TLC conditions	R_f (H/EA 7:3) 0.31

The spectroscopic data were in agreement with those reported in literature.[12]

Thexyldimethylsilyl 2-Azido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranoside (7)

1 **7**

Synthetic procedure

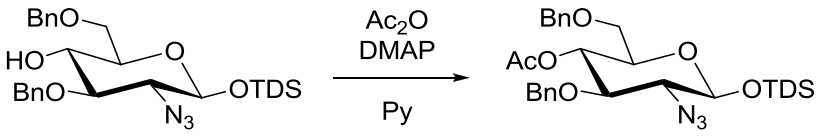
Compound **1** (4.07 g, 7.74 mmol) was treated as described in the ***General Procedure B***. Product **7** was obtained as white solid (3.49 g, 6.61 mmol, 85% yield).

Product characterization

Formula	$C_{28}H_{41}N_3O_5$
Molecular Weight	527.73 g/mol
TLC conditions	R_f (H/EA 9:1) 0.25

The spectroscopic data were in agreement with those reported in literature.[13]

Thexyldimethylsilyl 4-*O*-Acetyl-2-azido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranoside (2)



7 **2**

Synthetic procedure

Compound **7** (2.73 g, 5.18 mmol) was dissolved in dry pyridine (50 mL) and the solution was cooled to 0°C. Acetic anhydride (987 µL, 10.36 mmol) was added dropwise, followed by a catalytic addition of DMAP. The mixture was stirred at r.t. until completion. Then, the reaction was concentrated under reduced pressure and the obtained residue was diluted with EtOAc (100 mL) and washed with 5% solution of HCl (1x100 mL), saturated solution of $NaHCO_3$ (1x100 mL) and brine (1x100 mL). The first aqueous phase was extracted with DCM (1x100 mL) and the combined organic phases were dried over Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by flash chromatography (H/EA gradient) affording **2**, as a white solid (2.70 g, 4.74 mmol, white solid, 92% yield).

Product characterization

Formula	$C_{30}H_{43}N_3O_6$
Molecular Weight	569.76 g/mol
TLC conditions	R_f (H/EA 85:15) 0.45

Optical rotation

α_D^{25} = - 28.98 (c = 1 in $CHCl_3$)

HRMS (ESI)

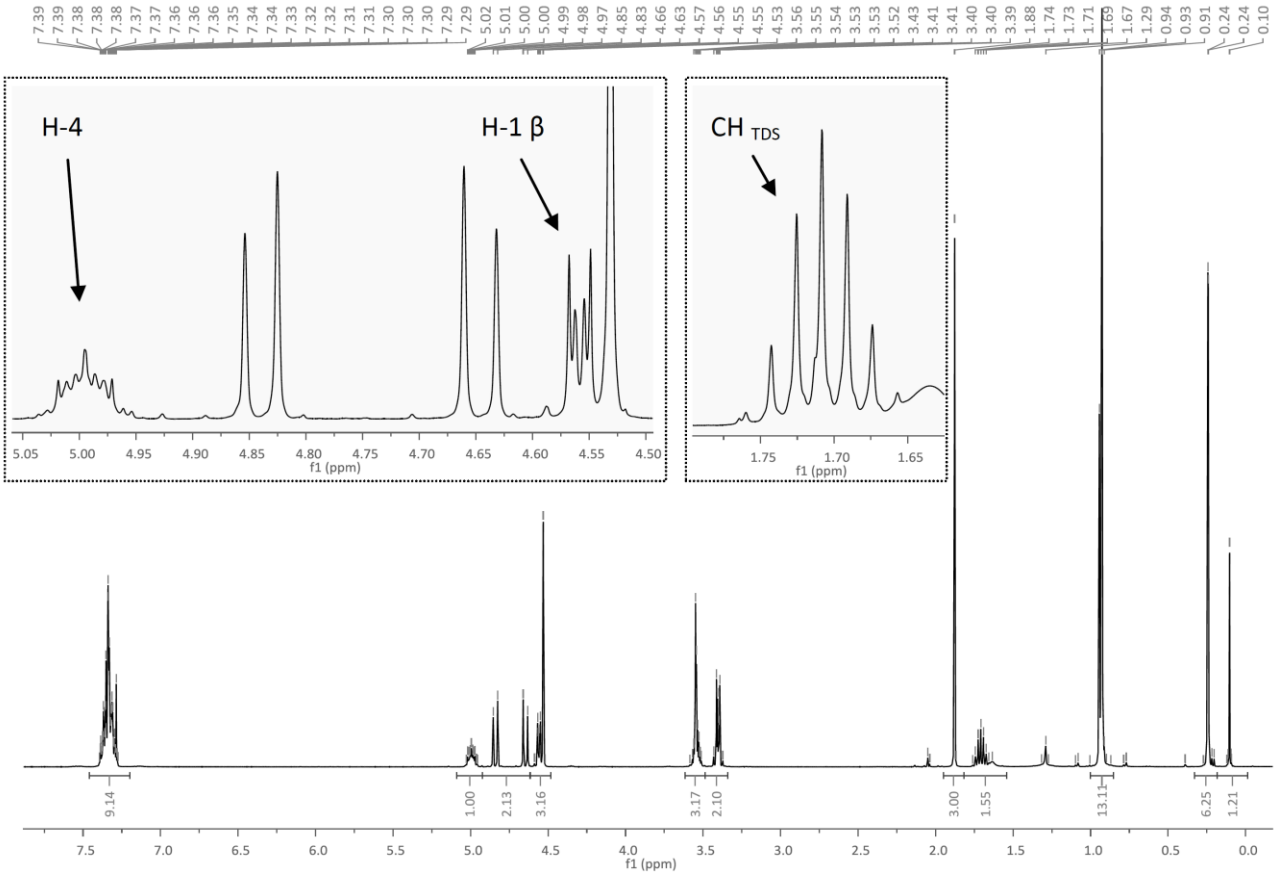
Experimental m/z: 592.28077 $[M+Na]^+$

NMR

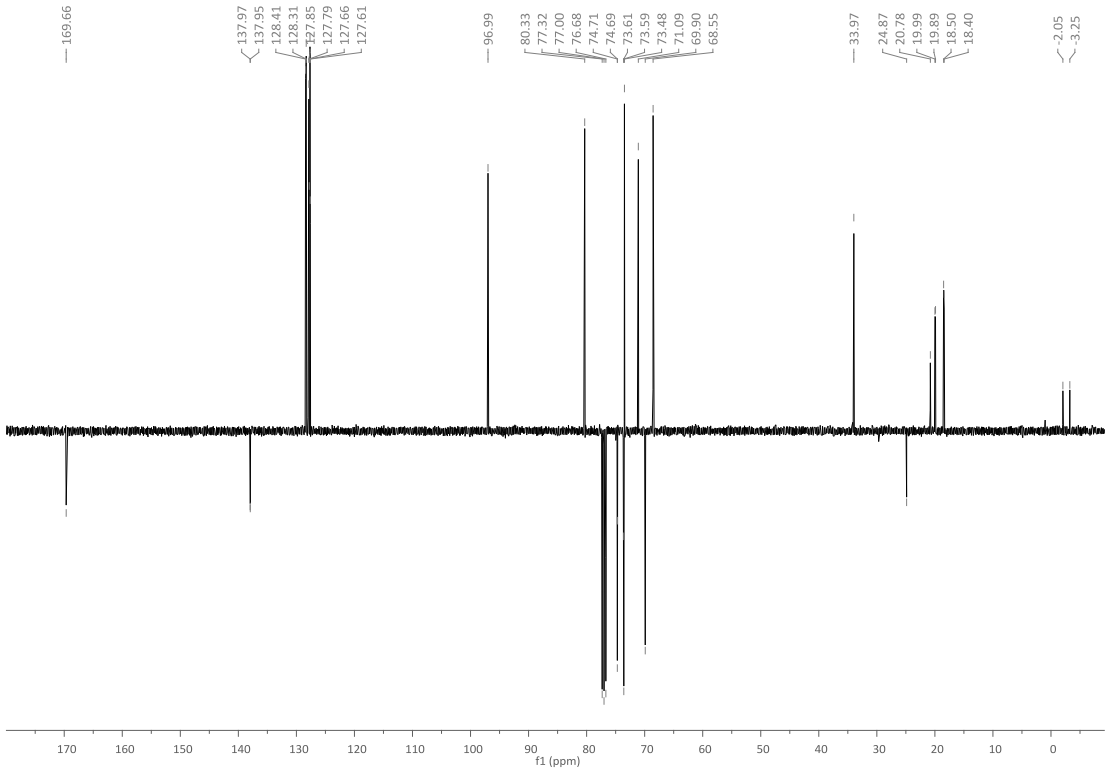
^{1}H NMR (400 MHz, $CDCl_3$) δ(ppm): 7.38 – 7.24 (m, 10H, Ar), 5.03 – 4.91 (m, 1H, H-4), 4.81 (d, *J* = 11.5 Hz, 1H, ½ C*H*$_2$Ph), 4.62 (d, *J* = 11.5 Hz, 1H, ½ C*H*$_2$Ph), 4.53 (dd, $J_{H\text{-}1,\ H\text{-}2}$ = 5.4 Hz, $J_{H\text{-}1,\ H\text{-}3}$ = 2.2 Hz, 1H, H-1 β), 4.51 (s, 2H, C*H*$_2$Ph), 3.57 – 3.47 (m, 3H, H-6, H-6', H-5), 3.41 – 3.34 (m, 2H, H-3, H-2), 1.85 (s, 3H, CH_3CO), 1.68 (ept, *J* = 7.0 Hz, 1H, CH TDS), 0.92 (s, 3H, CH_3 TDS), 0.90 (s, 9H, CH_3 TDS), 0.22 (s, 3H, CH_3Si TDS), 0.21 (s, 3H, CH_3Si TDS).

^{13}C NMR (101 MHz, $CDCl_3$) δ(ppm): 169.82 (CO), 138.13 (C_q Ar), 138.10 (C_q Ar), 128.57, 128.47, 128.01, 127.95, 127.82, 127.77 (CH Ar), 97.15 (C-1), 80.49 (C-3), 74.87 (CH_2Ph), 73.75 (CH_2Ph), 73.64 (C-5), 71.25 (C-4), 70.06 (C-6), 68.70 (C-2), 34.12 (CH TDS), 25.03 (C_q TDS), 20.94 (CH_3CO), 20.15, 20.05 , 18.66, 18.56 (4 CH_3 TDS), -1.89, -3.09 (2 CH_3Si TDS).

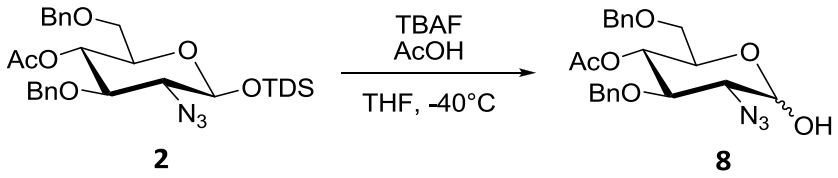
^{1}H (400 MHz, $CDCl_3$):



^{13}C (101 MHz, $CDCl_3$):



4-*O*-Acetyl-2-azido-3,6-di-*O*-benzyl-2-deoxy-α/β-D-glucopyranose (8)



2 **8**

Synthetic procedure

Compound **2** (2.70 g, 4.74 mmol) was treated as described in the ***General Procedure A***. Product **8** was obtained as an α:β (2.64:1) mixture (1.90 g, 4.44 mmol, white solid, 94% yield).

Product characterization

Formula	$C_{22}H_{25}N_3O_6$
Molecular Weight	427.45 g/mol
TLC conditions	R_f (H/EA 7:3) 0.33

MS (ESI)

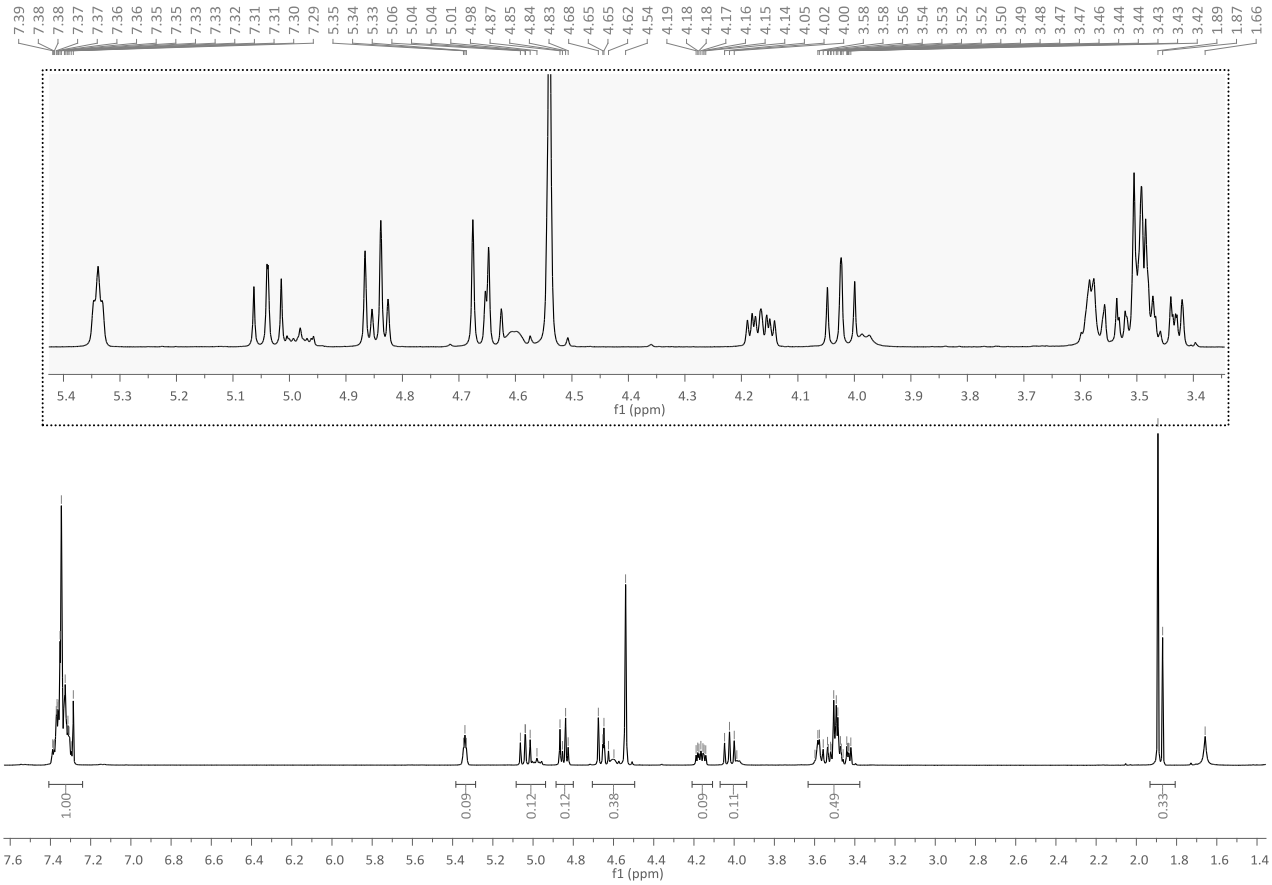
m/z (%): 450.5 (100) $[M+Na]^+$

NMR

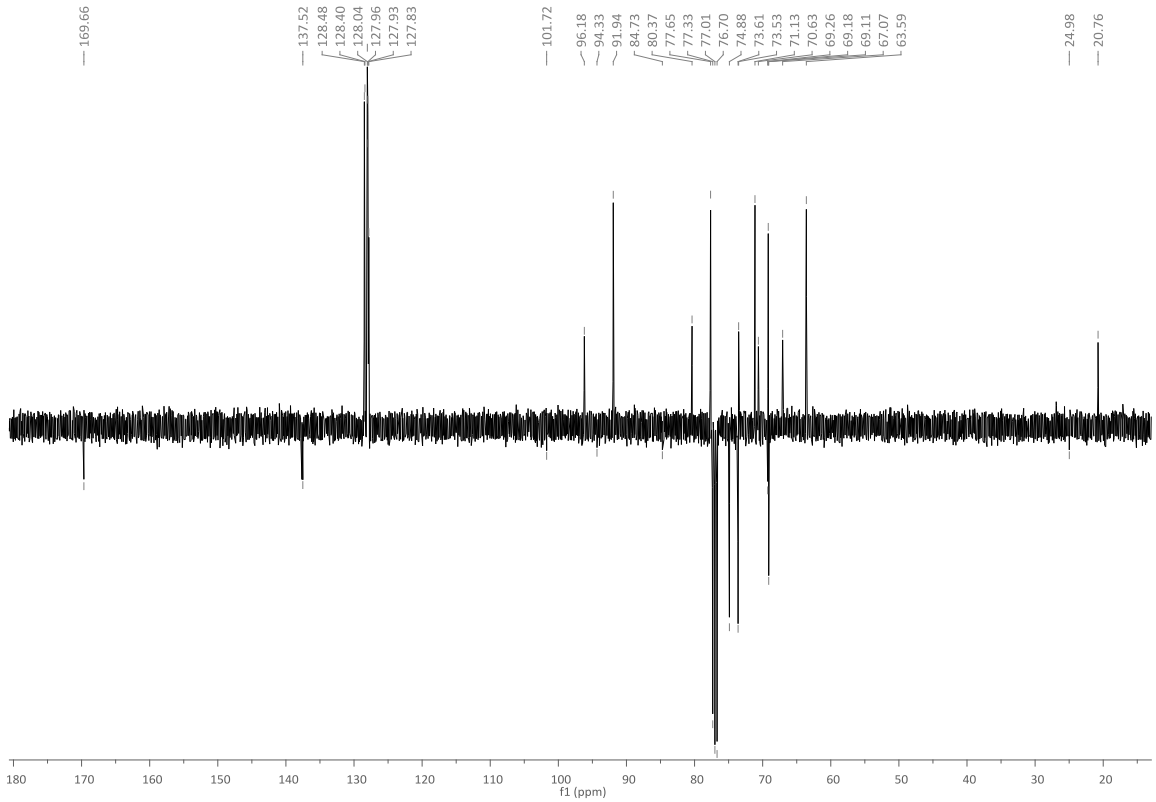
^{1}H NMR (400 MHz, $CDCl_3$) δ(ppm): 7.38 – 7.24 (m, Ar), 5.31 (t, $J_{H\text{-}1,\ H\text{-}2} = J_{H\text{-}1,\ H\text{-}3}$ 2.9 Hz, 1H, H-1 α), 5.01 (dd, $J_{H\text{-}4,\ H\text{-}3}$ = 10.1, $J_{H\text{-}4,\ H\text{-}5}$ = 9.3 Hz, 1H, H-4 α), 4.95 (m, 1H, H-4 β), 4.83 (d, *J* = 11.1 Hz, 1H, ½ C*H*$_2$Ph α), 4.81 (d, *J* = 11.4 Hz, 1H, ½ C*H*$_2$Ph β), 4.64 (d, *J* = 11.1 Hz, 1H, ½ C*H*$_2$Ph α), 4.61 (d, *J* = 11.4 Hz, 1H, ½ C*H*$_2$Ph β), 4.60 – 4.55 (m, 1H, H-1 β), 4.51 (s, 4H, C*H*$_2$Ph β, C*H*$_2$Ph α), 4.19 – 4.10 (m, 1H, H-5 α), 4.04 – 3.92 (m, 1H, H-3 α), 3.60 – 3.37 (m, 8H, H-5 β, H-6 β, H-6' β, 2 H-6 α, H-6' α, H-2 β, H-2 α, H-3 β), 1.87 (s, 3H, CH_3CO α), 1.84 (s, 3H, CH_3CO β).

^{13}C NMR (101 MHz, $CDCl_3$) δ 169.82 (CO β), 169.81 (CO α), 137.80, 137.66 (C_q Ar β, C_q Ar α), 128.67, 128.63, 128.57, 128.54, 128.24, 128.19, 128.11, 128.07, 128.02, 127.97 (CH Ar β, CH Ar α), 96.33 (C-1 β), 92.08 (C-1 α), 80.51 (C-3 β), 77.80 (C-3 α), 75.03, 73.73 (CH_2Ph β, CH_2Ph α), 73.67 (C-5 β), 71.28 (C-4 α), 70.77 (C-4 β), 69.33 (C-5 α), 69.26 (C-6 β, C-6 α), 67.21 (C-2 β), 63.73 (C-2 α), 20.90 (CH_3CO β, CH_3CO α).

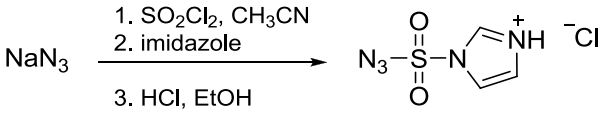
^{1}H (400 MHz, $CDCl_3$):



^{13}C (101 MHz, $CDCl_3$):



Imidazole-1-sulfonyl azide hydrochloride (9)



9

Synthetic procedure

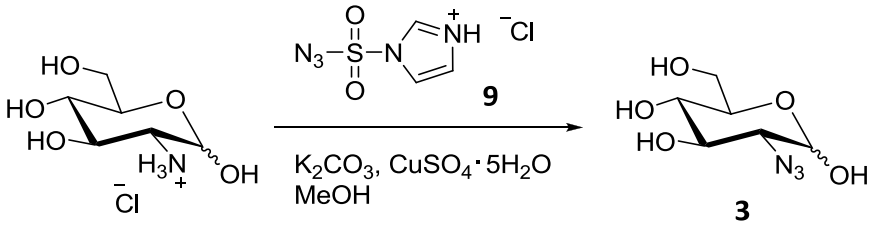
Sulfuryl chloride (16.2 mL, 200 mmol) was added dropwise to an ice-cooled suspension of NaN_3 (13.0 g, 200 mmol) in acetonitrile (200 mL) and the mixture stirred overnight at room temperature. Imidazole (25.9 g, 380 mmol) was added portion-wise to the ice-cooled mixture and the resulting slurry stirred at r.t. for 3 h. The mixture was diluted with EtOAc (400 mL), washed with H_2O (2×400 mL) then saturated aqueous $NaHCO_3$ (2×400 mL), dried over Na_2SO_4 and filtered. A solution of HCl in EtOH [obtained by the dropwise addition of AcCl (21.3 mL, 300 mmol) to ice-cooled dry ethanol (75 mL)] was added dropwise to the filtrate while stirring, the mixture chilled in an ice-bath, filtered and the filter cake washed with EtOAc (3×100 mL) to give **9** as white needles powder (32.26 g, 154 mmol, 77%).

Product characterization

Formula	$C_3H_4ClN_5O_2S$
Molecular Weight	209.61 g/mol

The spectroscopic data were in agreement with those reported in literature.[14]

2-Azido-2-deoxy-α/β-D-glucopyranose (3)



Procedure

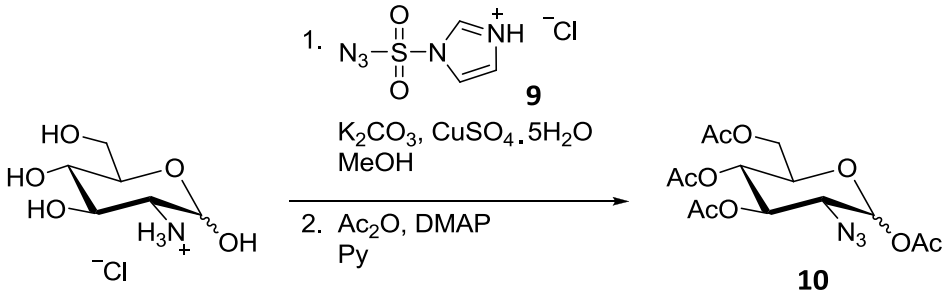
Imidazole-1-sulfonyl azide hydrochloride **9** (4.6 g, 22 mmol) was added at 0°C to the D-glucosamine hydrochloride (3.3 g, 18.3 mmol), K_2CO_3 (5.8 g, 42.09 mmol) and $CuSO_4$ pentahydrate (55 mg, 0.183 mmol) in MeOH (91 mL) and the mixture was stirred at room temperature for 3 hours. The solution was neutralized with conc. HCl (dropwise addition) and concentrated under reduced pressure. Flash chromatography gave the azide **3** (4.24 g, 20.68 mmol, 94%).

Product characterization

Formula	$C_6H_{11}N_3O_5$
Molecular Weight	205.17 g/mol
TLC conditions	R_f (DCM/MeOH 7:3) 0.64

The spectroscopic data were in agreement with those reported in literature.[15]

1,3,4,6-Tetra-*O*-acetyl-2-azido-2-deoxy-α/β-D-glucopyranose (10)



Procedure

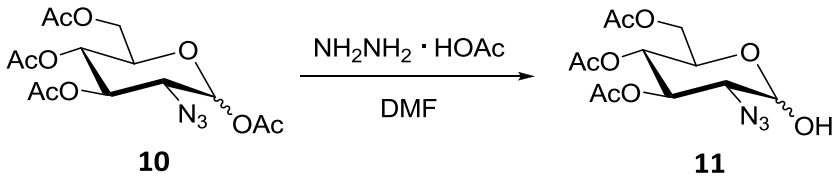
Imidazole-1-sulfonyl azide hydrochloride **9** (11.75 g, 56.04 mmol) was added at 0°C to the D-glucosamine hydrochloride (10.07 g, 46.7 mmol), K_2CO_3 (17.43 g, 126.1 mmol) and $CuSO_4$ pentahydrate (140 mg, 0.467 mmol) in MeOH (234 mL) and the mixture was stirred at room temperature for 3 hours. The solution was concentrated and co-evaporated with toluene (2x100 mL). Acetic anhydride (35.6 mL, 373.6 mmol) and a catalytic amount of DMAP were added to the residue in pyridine (120 mL) and the mixture was stirred for 3 hours, then concentrated, diluted with water (500 mL) and extracted three times with DCM (3x500 mL). The combined organic layers were dried (Na_2SO_4), filtered and concentrated. Flash chromatography gave the azide **10** (13.41 g, 35.92 mmol, 77%).

Product characterization

Formula	$C_{14}H_{19}N_3O_9$
Molecular Weight	373.32 g/mol
TLC conditions	R_f (H/EA 6:4) 0.53

The spectroscopic data were in agreement with those reported in literature.[15,16]

3,4,6-Tri-O-acetyl-2-azido-2-deoxy-α/β-D-glucopyranose (11)



10 **11**

Procedure

Freshly prepared 2M solution of hydrazine acetate [$NH_2NH_2 \cdot H_2O$ (1.5mL, 29.20 mmmol), AcOH (1.4 mL, 22.46 mmol) and dry MeOH (15.6 mL)] was dropwise added to a solution of **10** (8.38 g, 22.46 mmol) in dry DMF (100 mL) at 0°C. The mixture was stirred at r.t. for 2 hour. The mixture was concentrated, diluted with EtOAc (300 mL) and extracted with water (3x300 mL). The organic layer was dried (Na_2SO_4), filtered, concentrated and finally purified by flash chromatography. Product **11** was obtained as an α:β mixture (6.7 g, 20.21 mmol, 90% yield).

Product characterization

Formula	$C_{12}H_{17}N_3O_8$
Molecular Weight	331.28 g/mol
TLC conditions	R_f (H/EA 6:4) 0.43

The spectroscopic data were in agreement with those reported in literature.[17]

Thexyldimethylsilyl 3,4,6-Tri-*O*-acetyl-2-azido-2-deoxy-β-D-glucopyranoside (12)

11 **12**

Procedure

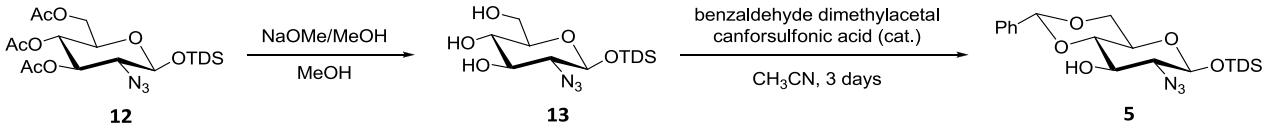
Hemiacetal **11** (10.37 g, 31.3 mmol) was dissolved in dry DMF (150 mL) and cooled to 0°C, then imidazole (6.39 g, 93.9 mmol) and TDSCl (9.2 mL, 46.9 mmol) were added. The mixture was stirred 24 hours. The reaction was concentrated to eliminate as much as possible the DMF. The crude was diluted by EtOAc (200 mL) and washed with water (2 x 300 mL) and brine (300 mL). The organic phase was dried over Na_2SO_4 and the solvent removed under reduced pressure. Flash chromatography gave **12** as a yellow solid (11.39 g, 21.11 mmol, 73%).

Product characterization

Formula	$C_{20}H_{35}N_3O_8Si$
Molecular Weight	473.59 g/mol
TLC conditions	R_f (H/EA 7:3) 0.32

The spectroscopic data were in agreement with those reported in literature.[18]

Thexyldimethylsilyl 2-Azido-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside (5)



Synthetic procedure

Compound **12** (6.11 g, 12.9 mmol) was treated as described in the ***General Procedure C***. Crude **13** (4.48 g, 12.9 mmol) was diluted in dry acetonitrile (130 mL) and benzaldheyde dimethylacetal (5.8 mL, 38.7 mmol) was added, followed by addition of camphorsulfonic acid (pH≈5). The reaction was vigorously stirred at r.t. for 3 days, then it was quenched by dropwise addition of TEA until neutral pH and solvents were removed under vacuum. The crude was purified by flash chromatography (H/EA gradient) yielded **5** (3.56 g, 8.17 mmol, 63% over two steps).

Product 13 characterization

Formula	$C_{14}H_{29}N_3O_5Si$
Molecular Weight	347.48 g/mol
TLC conditions	R_f (H/EA 1:1) 0.27

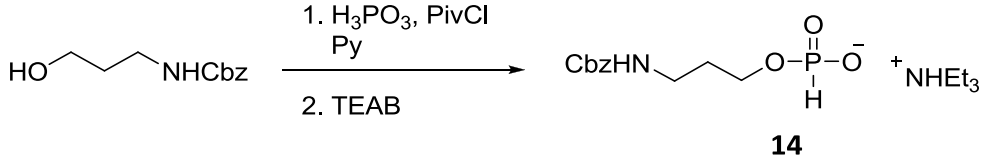
The spectroscopic data were in agreement with those reported in literature.[19]

Product 5 characterization

Formula	$C_{21}H_{33}N_3O_5Si$
Molecular Weight	435.59 g/mol
TLC conditions	R_f (H/EA 9:1) 0.25

The spectroscopic data were in agreement with those reported in literature.[10]

3-*N*-Carbobenzyloxy-aminopropyl Hydrogen-phosphonate (14), triethylammonium salt



14

Synthetic procedure

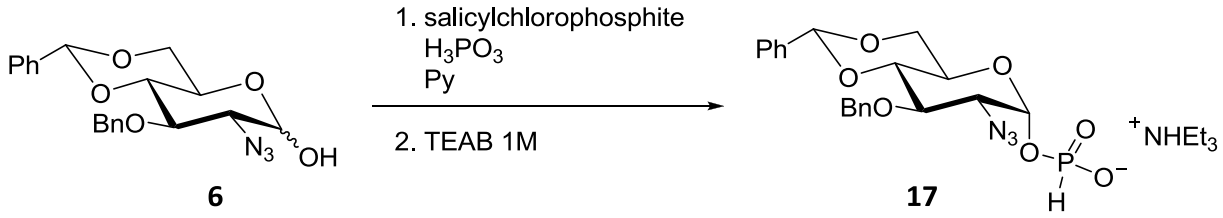
The H_3PO_3 (973 mg, 11.87 mmol) and the commercially available 3-*N*-Cbz-aminopropanol (2.07 g, 9.89 mmol) were mixed and co-evaporated three times with dry pyridine (10 mL), then dried in vacuum overnight. The reactants were dissolved in pyridine (50 mL), pivaloyl chloride (1.5 mL, 11.87 mmol) was added dropwise and the reaction is stirred for 30 min: then TEAB 1M (40 mL) was added. The solvents were removed under reduced pressure and the crude was purified by flash chromatography (2.77 g, 7.42 mmol, 75%).

Product characterization

Formula	$C_{17}H_{31}N_2O_5P$
Molecular Weight	374.41 g/mol
TLC conditions	R_f (DCM/MeOH 9:1) 0.34

The spectroscopic data were in agreement with those reported in literature.[20]

2-Azido-3-*O*-benzyl-4,6-*O*-benzilydene-2-deoxy-α-D-glucopyranosyl Hydrogen-phosphonate (17), triethylammonium salt



6 **17**

Synthetic procedure

Compound **6** (168 mg, 0.44 mmol) was treated as described in the ***General Procedure D***. After 12 days the NMR analysis (^{1}H- and ^{31}P-NMR) highlighted the disappearance of the β anomer. Product **17** α was obtained in 41% yield (98 mg, 0.18 mmol).

The reaction was performed also in medium-large scale (up to 1 g).

In large scale, the reaction time raised up and the yield of the by-product of hydrolysis increased.

Product characterization

Formula	$C_{26}H_{37}N_4O_7P$
Molecular Weight	548.57 g/mol
TLC conditions	R_f (DCM/MeOH 8:2) 0.38

Optical rotation

$\alpha_D^{25} = +10.42$ (c = 1 in $CHCl_3$)

MS (ESI)

Experimental m/z (%): 446.3 (35) $[M]^-$

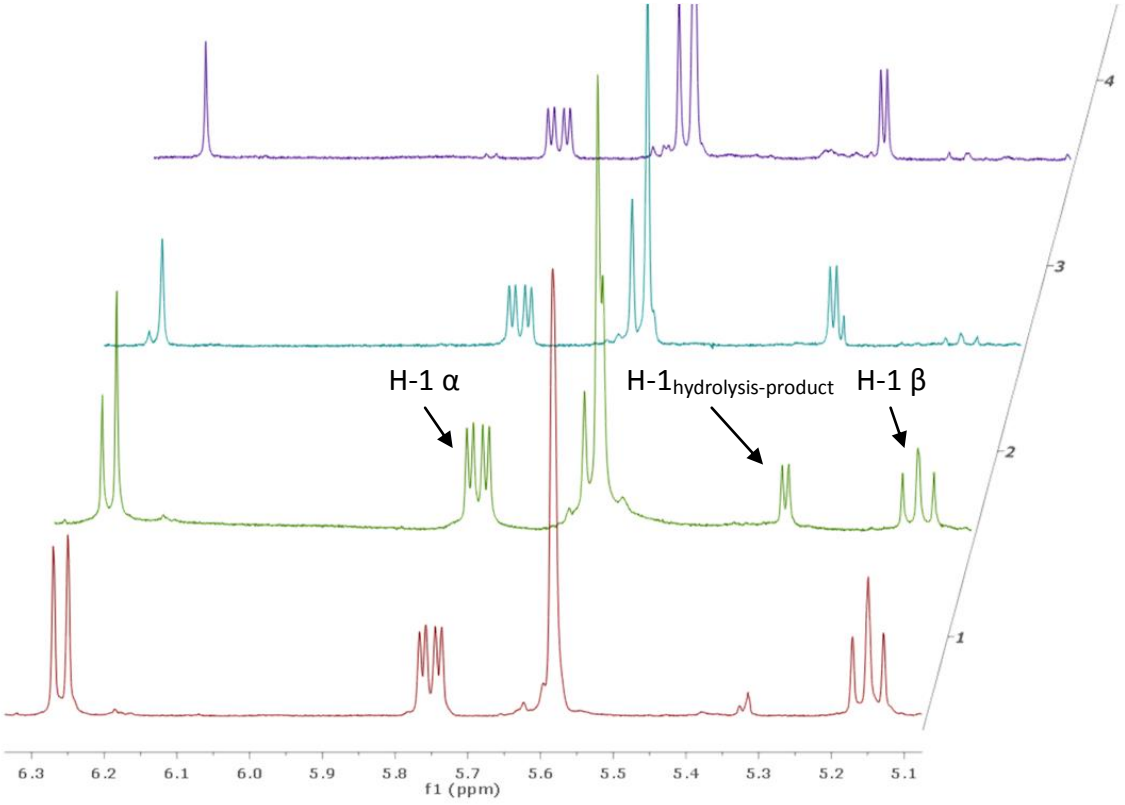
NMR

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.55-7.22 (m, 10H, Ar), 7.01 (d, $J_{H\text{-}P,P}$ = 640.4 Hz, 1H, *H*-P), 5.70 (dd, $J_{H\text{-}1,P}$ = 8.8, $J_{H\text{-}1,H\text{-}2}$ = 3.5 Hz, 1H, H-1), 5.55 (s, 1H, C*H*Ph), 4.92 (d, *J* = 11.1 Hz, 1H, ½ CH_2Ph), 4.76 (d, *J* = 11.1 Hz, 1H, ½ CH_2Ph), 4.25 (dd, $J_{H\text{-}6,\ H\text{-}6'}$ = 9.8, $J_{H\text{-}6',H\text{-}5}$ = 4.9 Hz, 1H, H-6), 4.21 – 4.14 (m, 1H, H-5), 4.11 (t, $J_{H\text{-}3,H\text{-}2} = J_{H\text{-}3,H\text{-}4}$ = 9.8 Hz, 1H, H-3), 3.71 (t, $J_{H\text{-}4,H\text{-}3} = J_{H\text{-}4,H\text{-}5} = J_{H\text{-}6',H\text{-}6} = J_{H\text{-}6',H\text{-}5}$ = 9.8 Hz, 2H, H-4, H-6'), 3.47 (ddd, $J_{H\text{-}2,H\text{-}3}$ = 9.8, $J_{H\text{-}2,H\text{-}1}$ = 3.5, $J_{H\text{-}2,P}$ = 1.3 Hz, 1H, H-2).

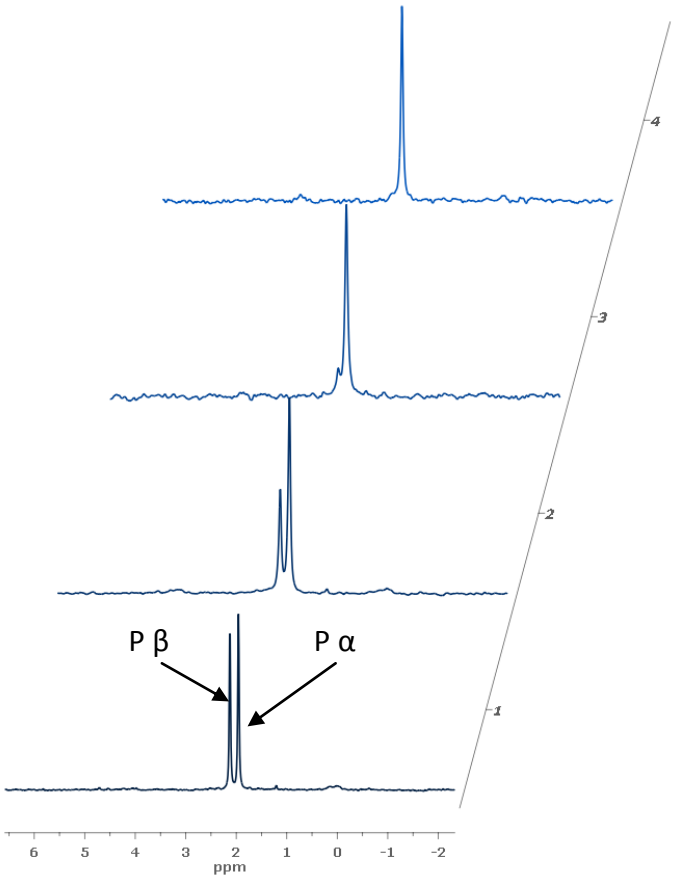
^{31}P NMR (162 MHz, $CDCl_3$) δ 1.84.

^{13}C NMR (101 MHz, $CDCl_3$) δ 138.12, 137.52 (C_q Ar), 129.09, 128.44, 128.34, 128.28, 127.86, 126.21 (CH Ar), 101.65 (*C*HPh), 93.77 (C-1), 82.89 (C-4), 76.34 (C-3), 74.92 (*C*H_2Ph), 69.01 (C-6), 63.65 (C-2), 63.55 (C-5).

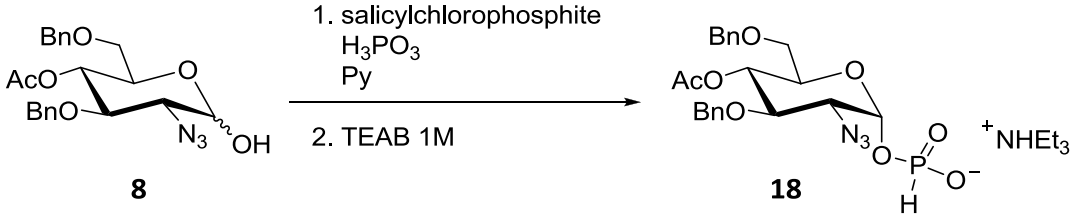
^{1}H (400 MHz, $CDCl_3$):



^{31}P (162 MHz, $CDCl_3$):



4-*O*-Acetyl-2-azido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl Hydrogen-phosphonate (18), triethylammonium salt



Synthetic procedure

Compound **8** (368.1 mg, 0.86 mmol) was treated as described in the ***General Procedure D***. The disappearance of the β anomer was monitored by NMR (^{1}H and ^{31}P) and was achieved in 6 days. Product **18** was obtained in 52% yield (265 mg, 0.44 mmol).

The reaction was performed also in medium-large scale (up to 2 g).

In large scale, the time reaction raised up and the yield of the by-product of hydrolysis increased.

Product characterization

Formula	$C_{28}H_{41}N_4O_8P$
Molecular Weight	592.62 g/mol
TLC conditions	R_f (DCM/MeOH 8:2) 0.44

Optical rotation

$\alpha_D^{25} = +9.67$ (c = 1.1 in $CHCl_3$)

MS (ESI)

Experimental m/z (%): 490.3 (35) $[M]^-$

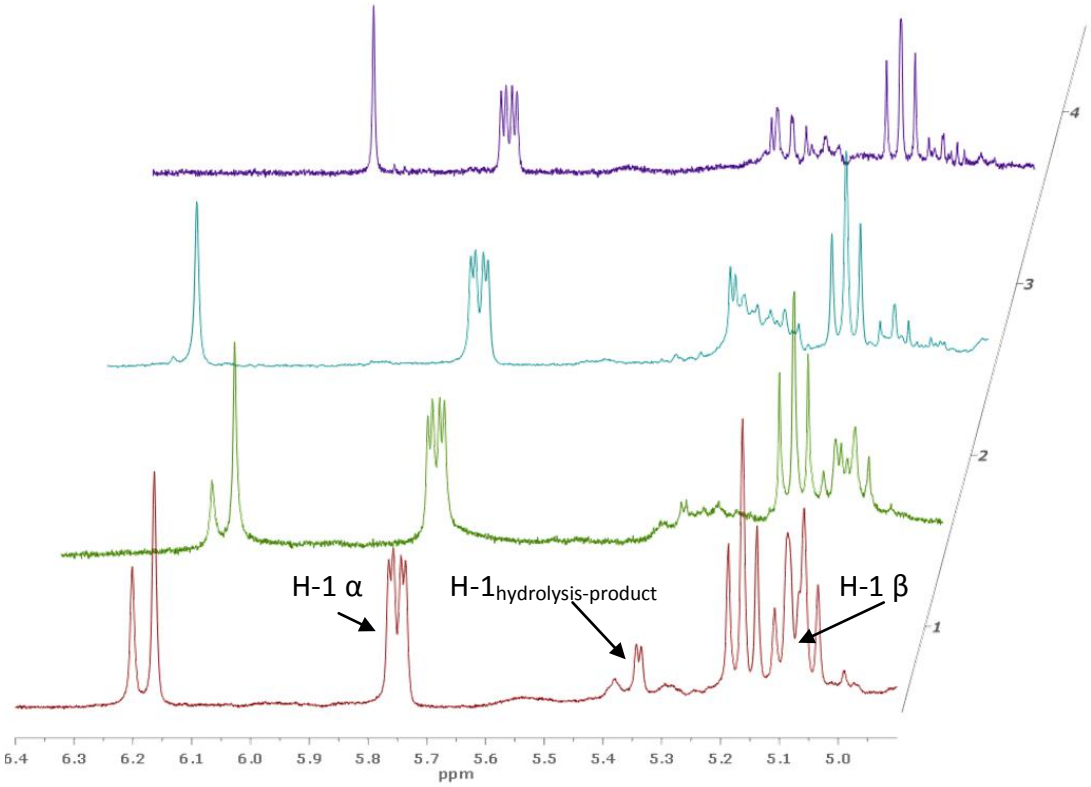
NMR

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38 – 7.19 (m, 10H, Ar), 7.03 (d, $J_{H\text{-}P,P}$ = 642.0 Hz, 1H, *H*-P), 5.73 (dd, $J_{H\text{-}1,P}$ = 8.7, $J_{H\text{-}1,H\text{-}2}$ = 3.0 Hz, 1H, H-1), 5.14 (t, $J_{H\text{-}4,H\text{-}3} = J_{H\text{-}4,H\text{-}5}$ = 9.7 Hz, 1H, H-4), 4.79 (d, *J* = 11.1 Hz, 1H, ½ C*H*$_2$Ph), 4.60 (d, *J* = 11.1 Hz, 1H, ½ C*H*$_2$Ph), 4.46 (q, *J* = 11.8 Hz, 2H, C*H*$_2$Ph), 4.19 (m, 1H, H-5), 4.05 (t, $J_{H\text{-}3,H\text{-}2} = J_{H\text{-}3,H\text{-}4}$ = 9.7 Hz, 1H, H-3), 3.57 – 3.38 (m, 3H, H-6, H-6', H-2), 1.83 (s, 3H, C*H*$_3$CO).

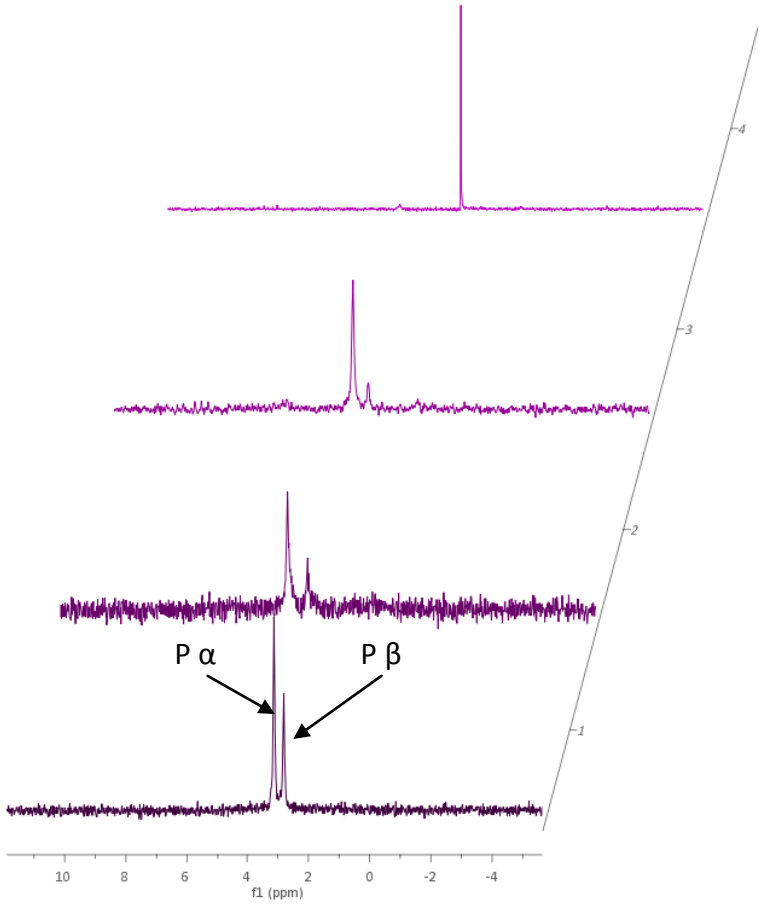
^{31}P NMR (162 MHz, $CDCl_3$) δ (ppm): 2.24.

^{13}C NMR (101 MHz, $CDCl_3$) δ 169.67 (CH$_3$*C*O), 137.95, 137.88 (C$_q$ Ar), 128.51, 128.37, 128.05, 128.03, 128.00, 127.89, 127.69 (CH Ar), 93.14, 93.11 (C-1), 78.00 (C-3), 74.77 (*C*H$_2$Ph), 73.59 (*C*H$_2$Ph), 70.96 (C-4), 70.21 (C-5), 68.98 (C-6), 63.60, 63.54 (C-2), 20.85 (*C*H$_3$CO).

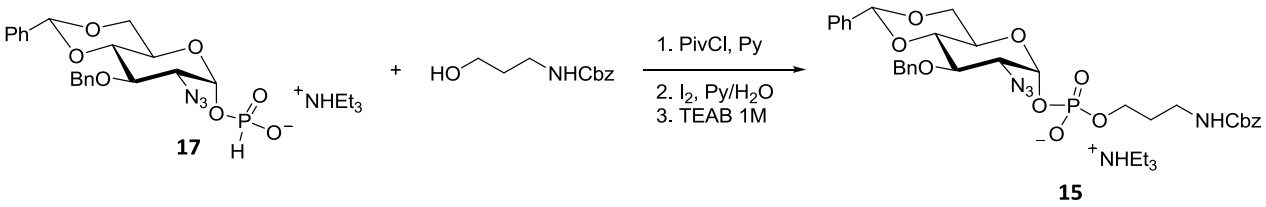
^{1}H (400 MHz, $CDCl_3$):



^{31}P (162 MHz, $CDCl_3$):



3-(*N*-Carbobenzyloxy)aminopropyl 1-*O*-(2-Azido-3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-α-D-glucopyranosyl phosphate) (15), triethylammonium salt



Synthetic procedure

H-phosphonate **17** (145.3 mg, 0.26 mmol, 1 eq) and 3-*N*-Cbz-propanolamine (136 mg, 0.65 mmol, 5 eq) are first co-evaporated three times with dry pyridine, thereafter they are dried by high vacuum pump overnight. The reactants were dissolved in dry pyridine (1.3 mL), then pivaloyl chloride (40 µL, 0.325 mmol, 2.5 eq) was added dropwise at 0°C and the reaction mixture was stirred under nitrogen at r.t.. The reaction completion was monitored by HPTLC (DCM:MeOH 9:1). After cooling to -40°C, a freshly prepared 0.5 M solution of iodine (2.5 eq) in pyridine/water 19:1 (2 mL) was added. The oxidation was completed at 0°C and quenched by dropwise addition of a 0.5 M solution of $Na_2S_2O_3 \cdot 5H_2O$ (10% w/v, 10 mL). The mixture was diluted with DCM (50 mL), washed two times with $Na_2S_2O_3 \cdot 5H_2O$ (0.5 M, 2x50 mL), then with cold TEAB (0.5 M, 1x25 mL), dried (Na_2SO_4), filtered and concentrated. The crude was purified by flash chromatography (DCM:MeOH + 1% TEA). The phosphate bridge has to be stabilized, washing with 0.25M cold TEAB [then drying (Na_2SO_4), filtering and concentrating] the purified product. Product **15** (122.4 mg, 0.16 mmol, 62% yield) is obtained as a pure-α anomer.

Product characterization

Formula	$C_{37}H_{50}N_5O_{10}P$
Molecular Weight	755.79 g/mol
TLC conditions	R_f (DCM/MeOH 8:2) 0.36

Optical rotation

$\alpha_D^{25} = +11.06$ (c = 1 in $CHCl_3$)

HRMS (ESI)

Experimental m/z: 653.20169 $[M]^-$

NMR

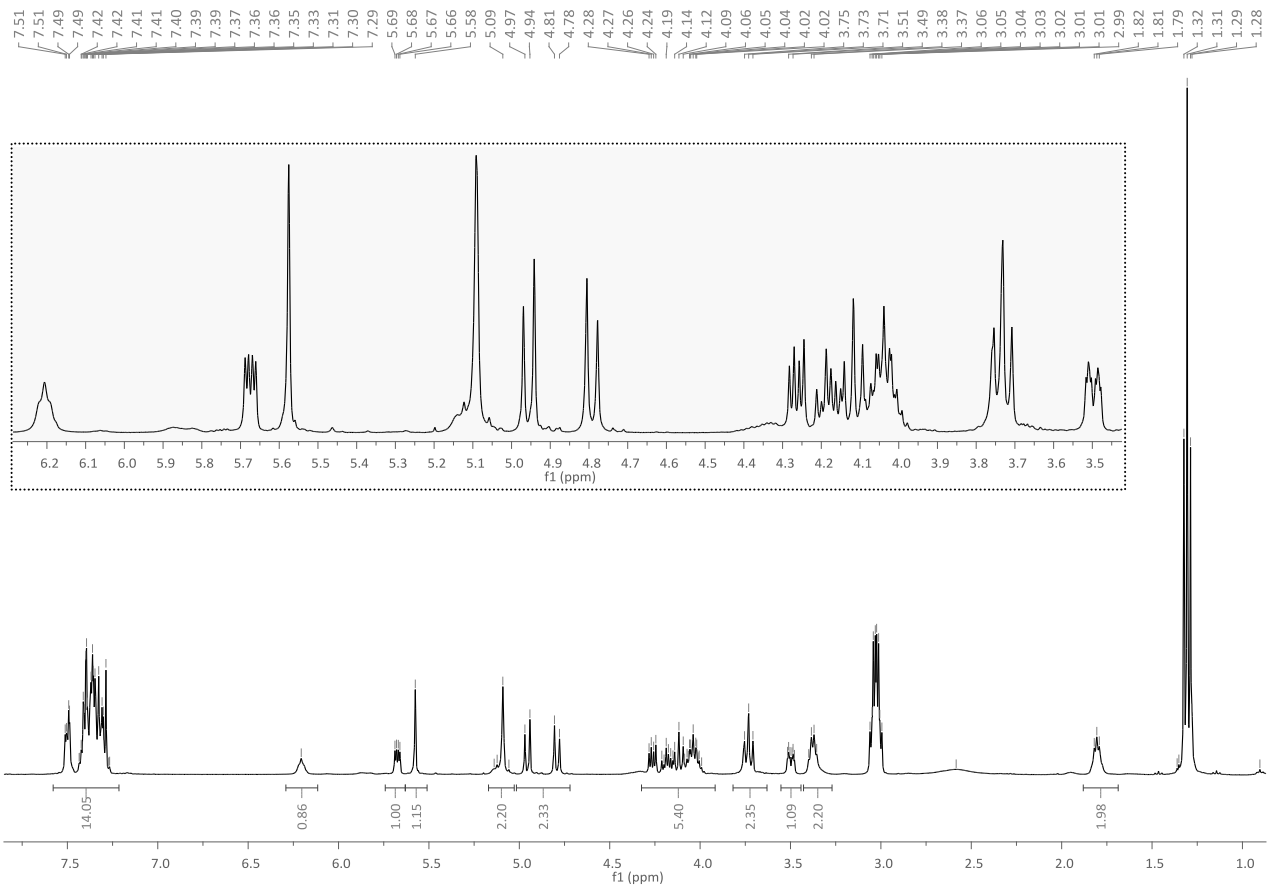
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.50 – 7.43 (m, 2H, Ar), 7.42 – 7.23 (m, 13H, Ar), 6.18 (t, *J* = 5.3 Hz, 1H, N*H*Cbz), 5.65 (dd, $J_{H\text{-}1,P}$ = 7.5, $J_{H\text{-}1,H\text{-}2}$ = 3.5 Hz, 1H, H-1), 5.55 (s, 1H, C*H*Ph), 5.06 (s, 2H, CH_2Ph), 4.93 (d, *J* = 11.1 Hz, 1H, ½ CH_2Ph), 4.76 (d, *J* = 11.1 Hz, 1H, ½ CH_2Ph), 4.24 (dd, $J_{H\text{-}6,H\text{-}6'}$ = 10.1, $J_{H\text{-}6',H\text{-}5}$ = 4.9 Hz, 1H, H-6), 4.20 – 3.94 (m, 4H, H-5, H-3, CH_2O $_{LINKER}$), 3.70 (t, $J_{H\text{-}4,H\text{-}3} = J_{H\text{-}4,H\text{-}5} = J_{H\text{-}6',H\text{-}6} = J_{H\text{-}6',H\text{-}5}$ =

9.3 Hz, 2H, H-4, H-6'), 3.51 – 3.43 (ddd, $J_{H\text{-}2,H\text{-}3}$ = 9.8, $J_{H\text{-}2,H\text{-}1}$ = 3.5, $J_{H\text{-}2,P}$ = 2.3 Hz, 1H, H-2), 3.35 (dd, *J* = 11.7, 5.9 Hz, 2H,C*H*$_2$NH $_{LINKER}$), 1.83 – 1.72 (m, 2H, C*H*$_2$ $_{LINKER}$).

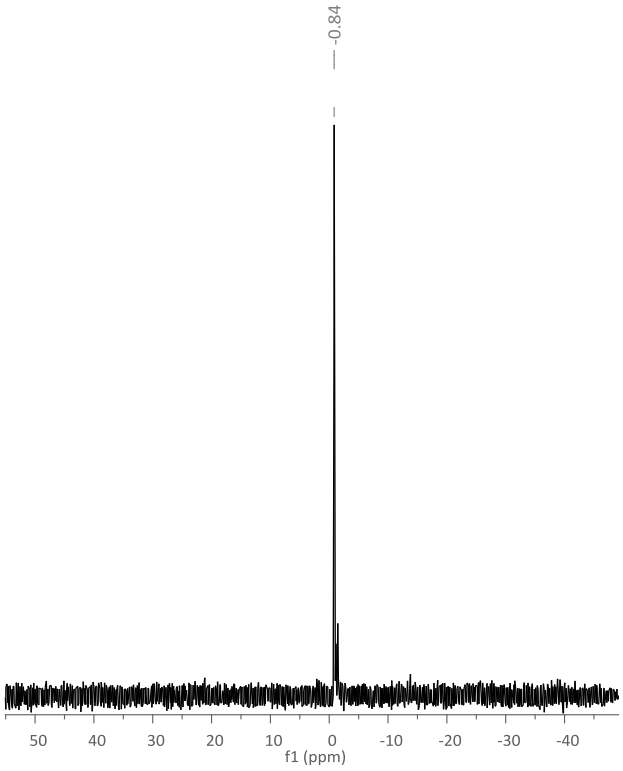
^{31}P NMR (162 MHz, $CDCl_3$) δ -0.84.

^{13}C NMR (101 MHz, $CDCl_3$) δ 156.71 (CO), 138.11, 137.49, 137.15 (C_q Ar), 129.13, 128.53, 128.47, 128.38, 128.33, 128.03, 127.90, 126.22 (CH Ar), 101.64 (*C*HPh), 94.65 (C-1), 82.85 (C-4), 76.30 (C-3), 74.95 (C-6), 69.07 (*C*H$_2$Ph), 66.49 (*C*H$_2$Ph), 63.72 (C-2), 63.56 (C-5), 63.04 (*C*H$_2$O $_{LINKER}$), 37.46 (*C*H$_2$NH $_{LINKER}$), 30.45 (*C*H$_2$ $_{LINKER}$).

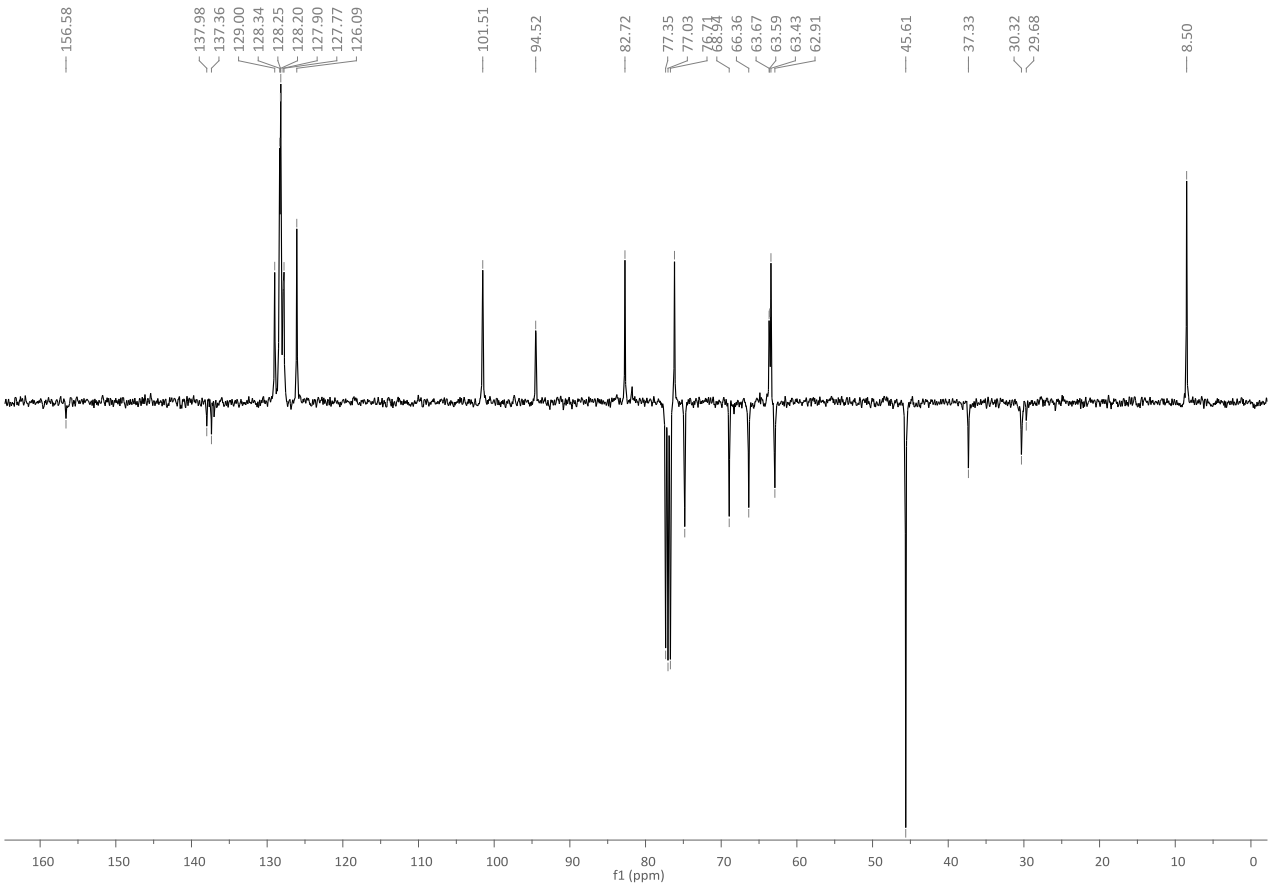
^{1}H (400 MHz, $CDCl_3$):



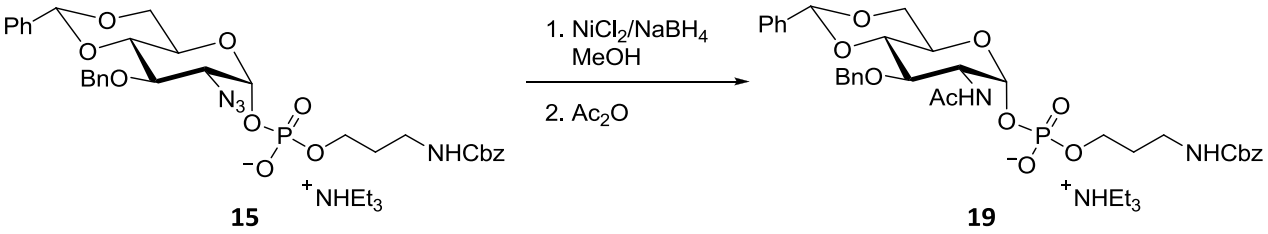
^{31}P NMR (162 MHz, $CDCl_3$):



^{13}C (101 MHz, $CDCl_3$):



3-(*N*-Carbobenzyloxy)aminopropyl 1-*O*-(2-Acetamido-3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-α-D-glucopyranosyl phosphate) (19), triethylammonium salt



15 **19**

Synthetic procedure

Azide **15** (97.1 mg, 0.13 mmol) was converted into acetamide **19** (13 mg, 0.017 mmol, 13% yield) as described in the ***General Procedure F***. The phosphate bridge has to be stabilized, washing with 0.25M cold TEAB [then drying (Na_2SO_4), filtering and concentrating] the purified product.

Product characterization

Formula	$C_{39}H_{54}N_3O_{11}P$
Molecular Weight	771.83 g/mol
TLC conditions	R_f (EA/MeOH 9:1) 0.11 R_f (EA/MeOH 8:2) 0.60 R_f (MeCN/MeOH/H_2O 10:1:1) 0.48

Optical rotation

$\alpha_D^{25} = +31.24$ (c = 0.4 in MeOH)

MS (ESI)

Experimental m/z: 699.4 $[M]^-$

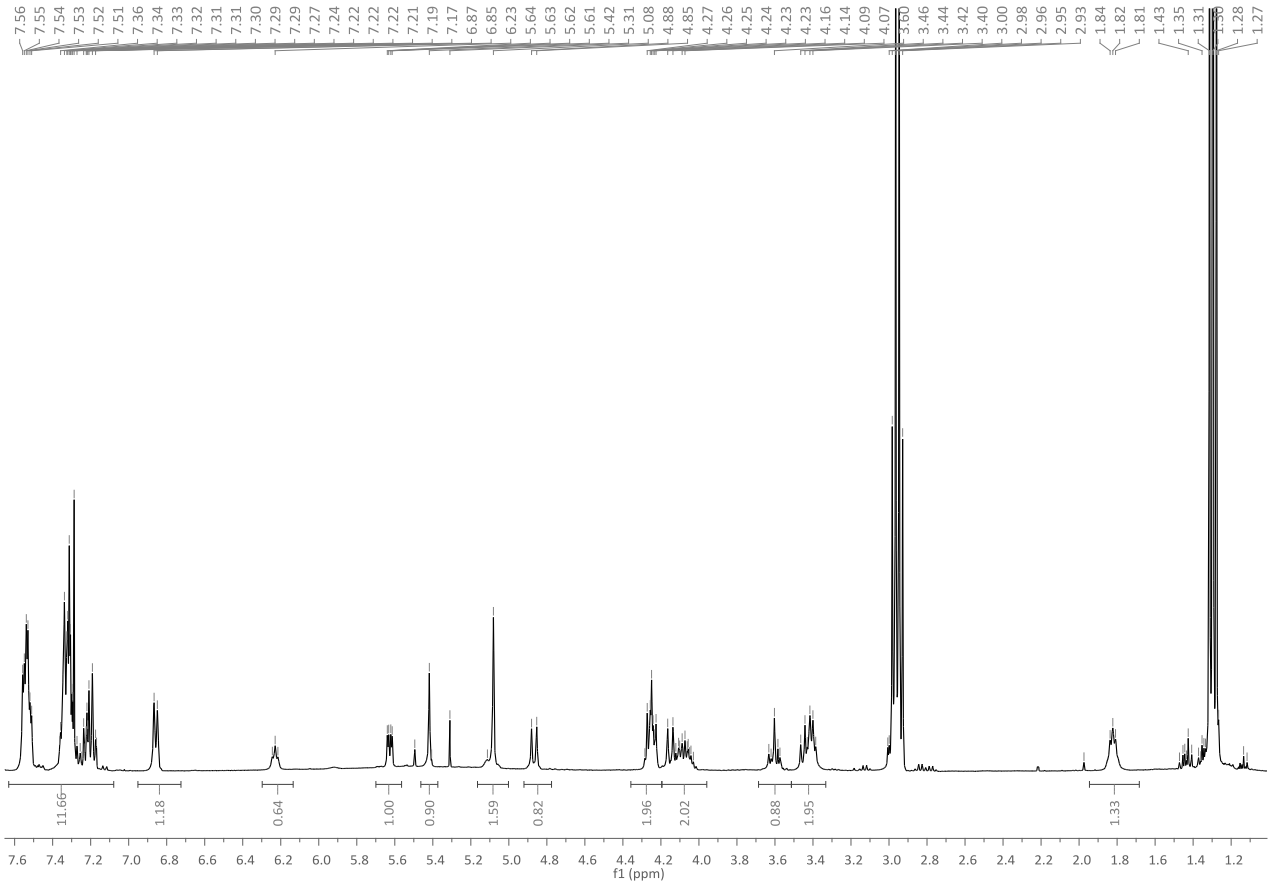
NMR

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.53 – 7.44 (m, 2H, Ar), 7.44 – 7.20 (m, 13H, Ar), 6.76 (d, *J* = 9.9 Hz, 1H, N*H*Ac), 5.99 (t, *J* = 5.5 Hz, 1H, N*H*Cbz), 5.56 (s, 1H, C*H*Ph), 5.47 (dd, $J_{H\text{-}1,P}$ = 7.2, $J_{H\text{-}1,H\text{-}2}$ = 3.5 Hz, 1H, H-1), 5.05 (s, 2H, CH_2Ph), 4.88 (d, *J* = 12.1 Hz, 1H, ½ CH_2Ph), 4.66 (d, *J* = 12.1 Hz, 1H, ½ CH_2Ph), 4.36 – 4.27 (m, 1H, H-2), 4.21 (dd, $J_{H\text{-}6,H\text{-}6'}$ = 10.2, $J_{H\text{-}6,H\text{-}5}$ = 4.9 Hz, 1H, H-6), 4.10 – 4.02 (m, 1H, H-4), 4.02 – 3.87 (m, 2H, CH_2O $_{LINKER}$), 3.86 – 3.68 (m, 3H, H-3, H-5, H-6'), 3.32 (dd, *J* = 12.3, 6.2 Hz, 2H, CH_2NH $_{LINKER}$), 1.91 (s, 3H, NHCOCH_3), 1.81 – 1.71 (m, 2H, CH_2 $_{LINKER}$).

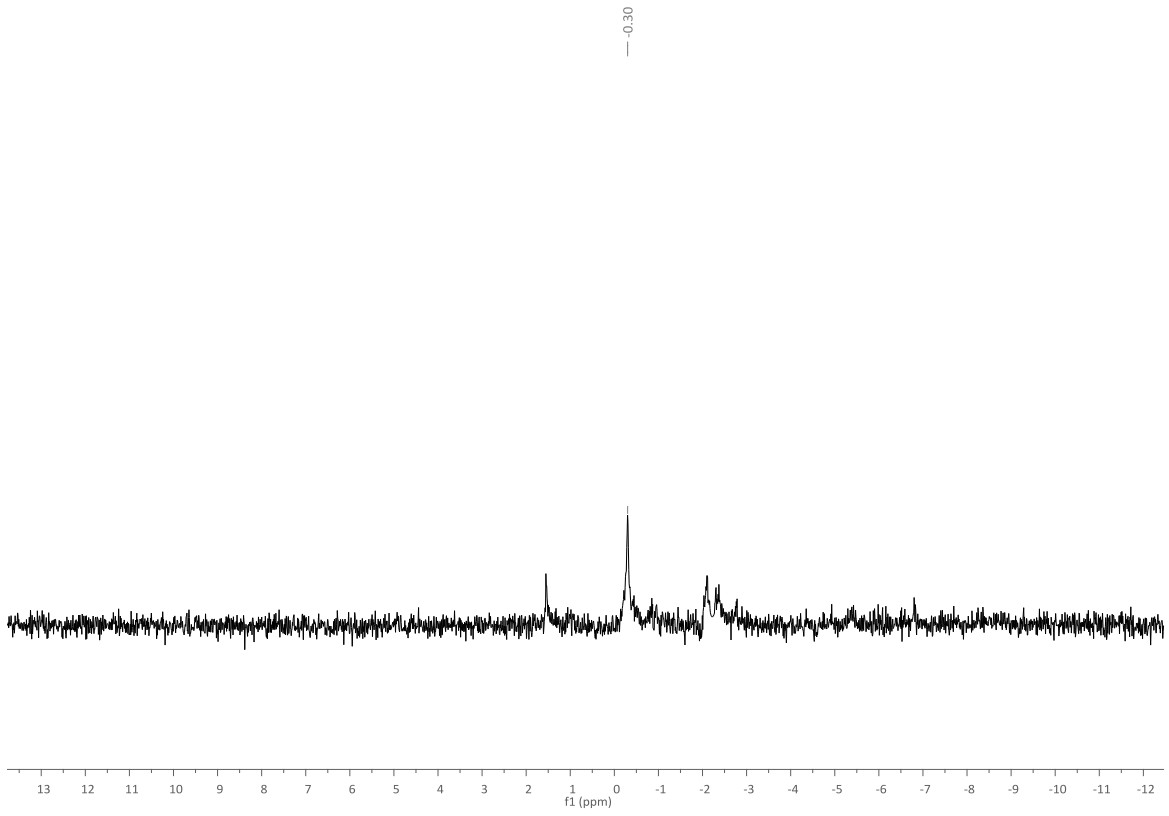
^{31}P NMR (162 MHz, $CDCl_3$) δ -0.49.

^{13}C NMR (101 MHz, $CDCl_3$) δ 156.71 (CO), 138.11, 137.49, 137.15 (C_q Ar), 129.13, 128.53, 128.47, 128.38, 128.33, 128.03, 127.90, 126.22 (CH Ar), 101.64 (*C*HPh), 94.65 (C-1), 82.85 (C-4), 76.30 (C-3), 74.95 (C-6), 69.07 (*C*H_2Ph), 66.49 (*C*H_2Ph), 63.72 (C-2), 63.56 (C-5), 63.04 (*C*H_2O $_{LINKER}$), 37.46 (*C*H_2NH $_{LINKER}$), 30.45 (*C*H_2 $_{LINKER}$).

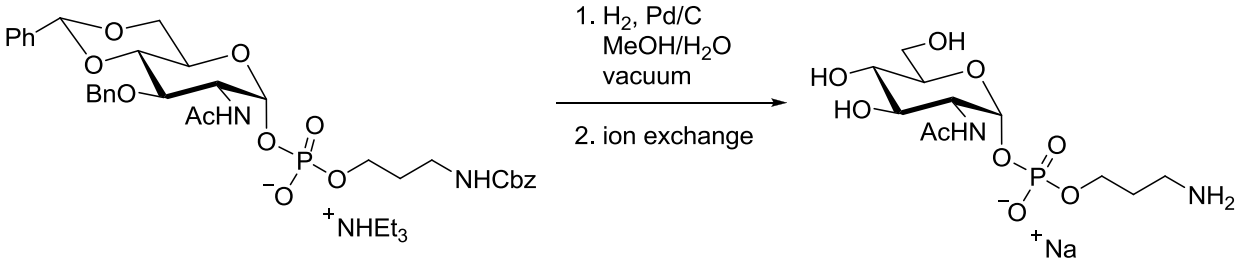
^{1}H (400 MHz, $CDCl_3$):



^{31}P NMR (162 MHz, $CDCl_3$):



3-aminopropyl 1-*O*-(2-Acetamido-2-deoxy-α-D-glucopyranosyl phosphate) (20), sodium salt



19 **20**

Synthetic procedure

Fully protected acetamide **19** (13 mg, 0.017 mmol) was submitted to hydrogenation as described in the ***General Procedure G***. Final monomer **20** (6.5 mg, 0.016 mmol, 62% yield) is obtained as sodium salt after ion exchange (see ***General Procedure H***).

Product characterization

Formula	$C_{11}H_{22}N_2NaO_9P$
Molecular Weight	380.3 g/mol

Optical rotation

$\alpha_D^{25} = +66.00$ (c = 0.11 in H_2O)

HRMS (ESI)

Experimental m/z (%):	357.10632 $[M]^-$
	381.10368 $[M+H]^+$
	403.08561 $[M+H+Na]^{2+}$

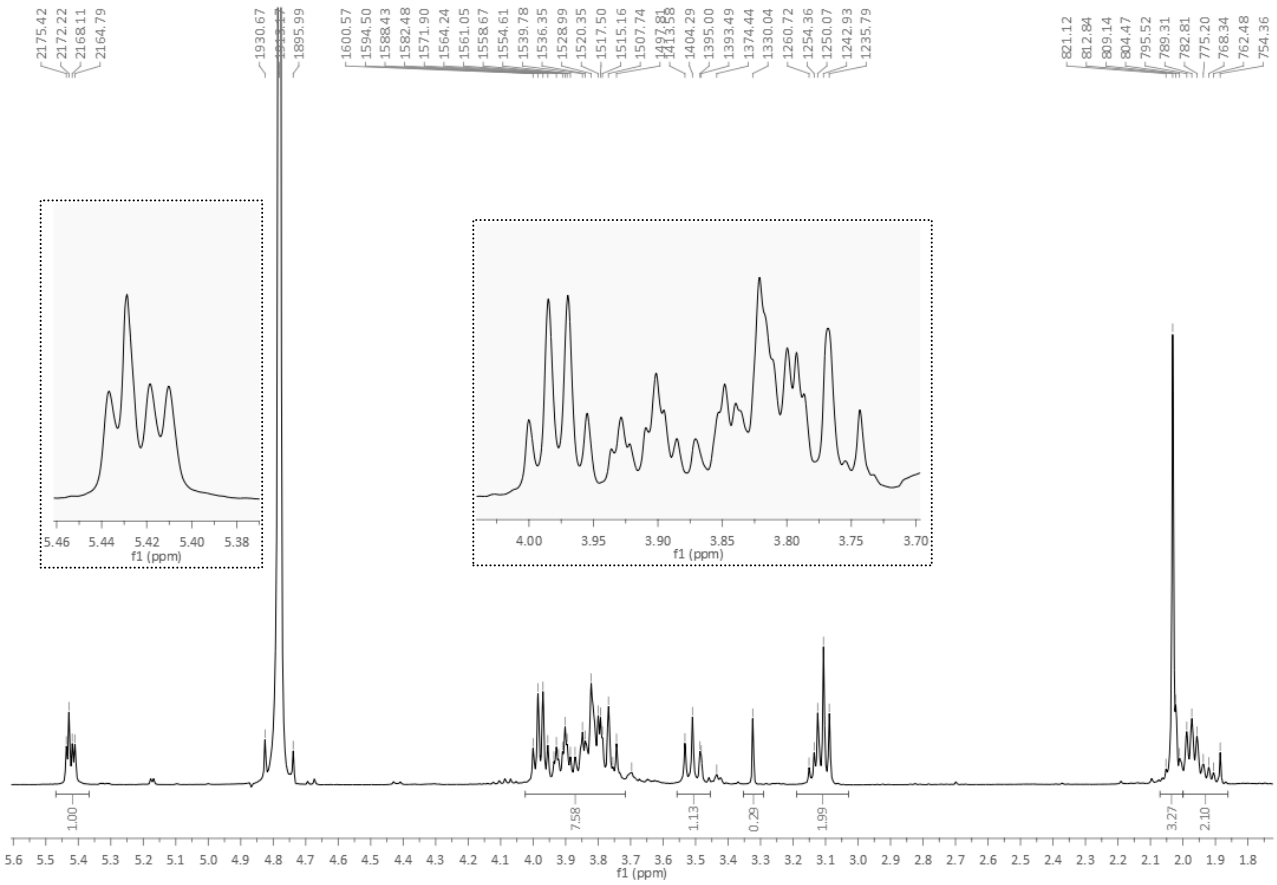
NMR

^{1}H NMR (400 MHz, D_2O) δ 5.43 (dd, $J_{H-1,P}$ = 7.4, $J_{H-1,H-2}$ = 3.3 Hz, 1H), 4.03 – 3.73 (m, 7H, C*H*$_2$O $_{LINKER}$, H-2, H-6, H-6', H-3, H-5), 3.51 (t, $J_{H-4,H-3}$ = $J_{H-4,H-5}$ = 9.3 Hz, 1H, H-4), 3.20 – 3.04 (m, 2H, C*H*$_2$NH $_{LINKER}$), 2.08 – 2.01 (m, 3H, NHCOC*H*$_3$), 2.01 – 1.87 (m, 2H, C*H*$_2$ $_{LINKER}$).

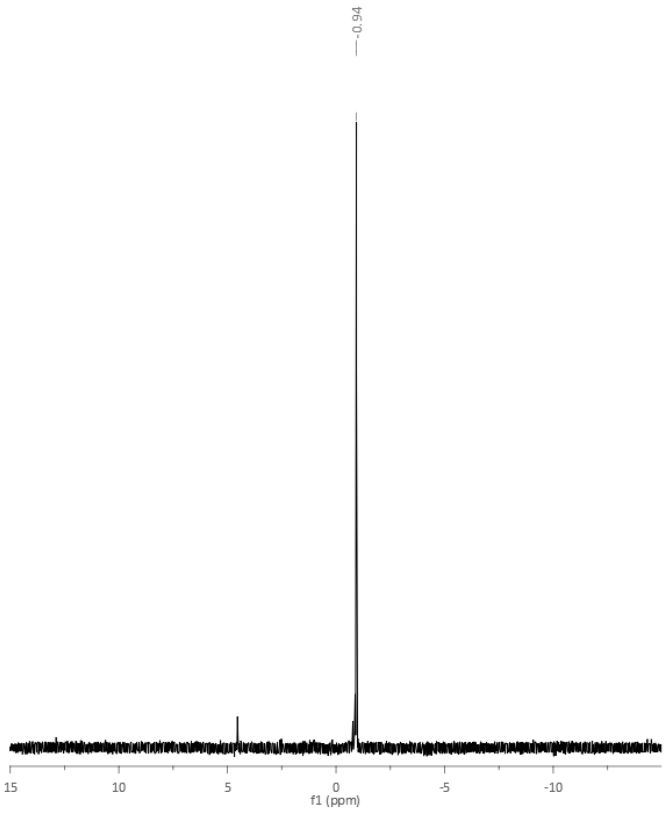
^{31}P NMR (162 MHz, D_2O) δ (ppm): - 0.94.

^{13}C NMR (101 MHz, D_2O) δ 93.69, 93.63 (C-1), 72.95 (C-3), 70.14 (C-5), 69.45 (C-4), 63.26, 63.20 (C*H*$_2$O $_{LINKER}$), 60.25 (C-6), 53.70, 53.62 (C-2), 36.93 (C*H*$_2$NH $_{LINKER}$), 27.65 (C*H*$_2$ $_{LINKER}$), 21.74 (NHCOC*H*$_3$).

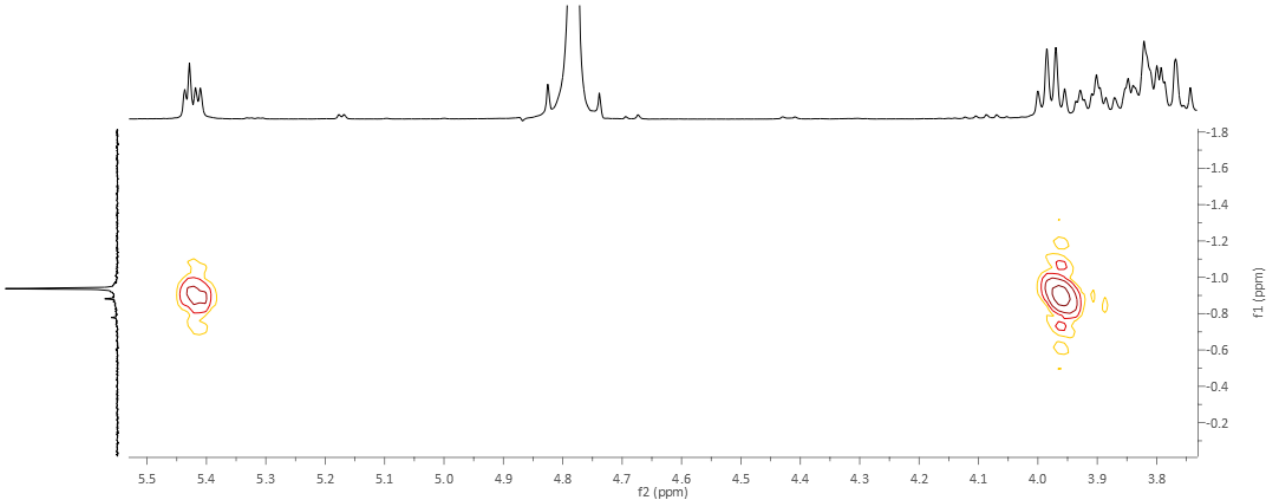
^{1}H (400 MHz, D_2O):



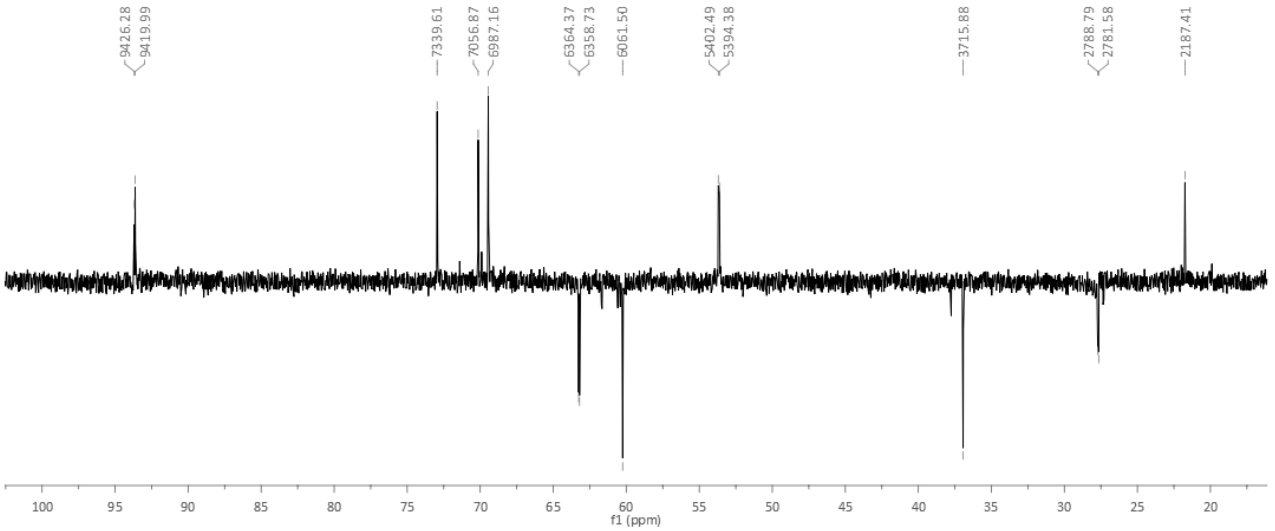
^{31}P NMR (162 MHz, D_2O):



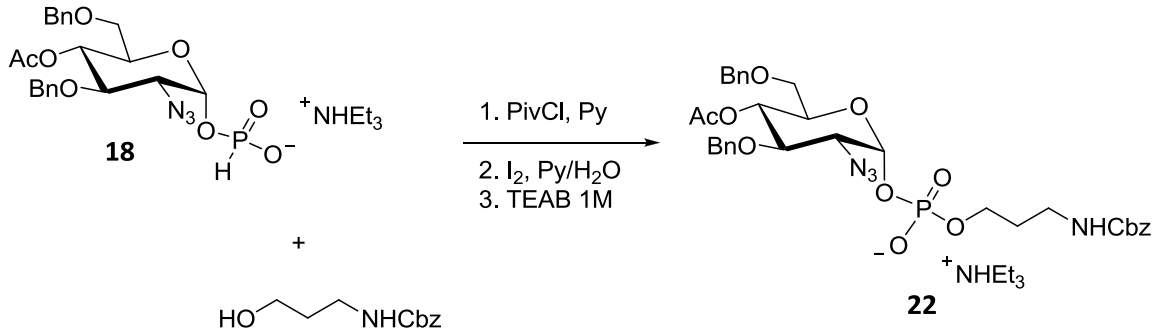
HETCOR ^{31}P-^{1}H (400 MHz, D_2O):



^{13}C – DEPT135 (101 MHz, D_2O):



3-(*N*-Carbobenzyloxy)aminopropyl 1-*O*-(4-*O*-Acetyl-2-azido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl phosphate) (22), triethylammonium salt



Synthetic procedure

H-phosphonate **18** (76.4 mg, 0.13 mmol, 1 eq) and 3-*N*-Cbz-propanolamine (136 mg, 0.65 mmol, 5 eq) are first co-evaporated three times with dry pyridine, thereafter they are dried by high vacuum pump overnight. The reactants were dissolved in dry pyridine (1.3 mL), then pivaloyl chloride (40 µL, 0.325 mmol, 2.5 eq) was added dropwise at 0°C and the reaction mixture was stirred under nitrogen at r.t.. The reaction completion was monitored by HPTLC (DCM:MeOH 9:1). After cooling to -40°C, a freshly prepared 0.5 M solution of iodine (2.5 eq) in pyridine/water 19:1 (2 mL) was added. The oxidation was completed at 0°C and quenched by dropwise addition of a 0.5 M solution of $Na_2S_2O_3 \cdot 5H_2O$ (10% w/v, 10 mL). The mixture was diluted with DCM (50 mL), washed with $Na_2S_2O_3 \cdot 5H_2O$ (0.5 M, 2x50 mL), then with cold TEAB (0.5 M, 1x25 mL), dried (Na_2SO_4), filtered and concentrated. The crude was purified by flash chromatography (DCM:MeOH + 1% TEA). The phosphate bridge has to be stabilized, washing with 0.25M cold TEAB [then drying (Na_2SO_4), filtering and concentrating] the purified product. Product **22** (66.3 mg, 0.083 mmol, 64% yield) was obtained as pure-α anomer.

Product characterization

Formula	$C_{39}H_{54}N_5O_{11}P$
Molecular Weight	799.80 g/mol
HP-TLC conditions	R_f (DCM/MeOH 9:1) 0.30
	R_f (DCM/MeOH 8:2) 0.60

Optical rotation

$\alpha_D^{25} = +35.76$ (c = 1.2 in $CHCl_3$)

HRMS (ESI negative)

Experimental m/z: 697.22780 $[M]^-$

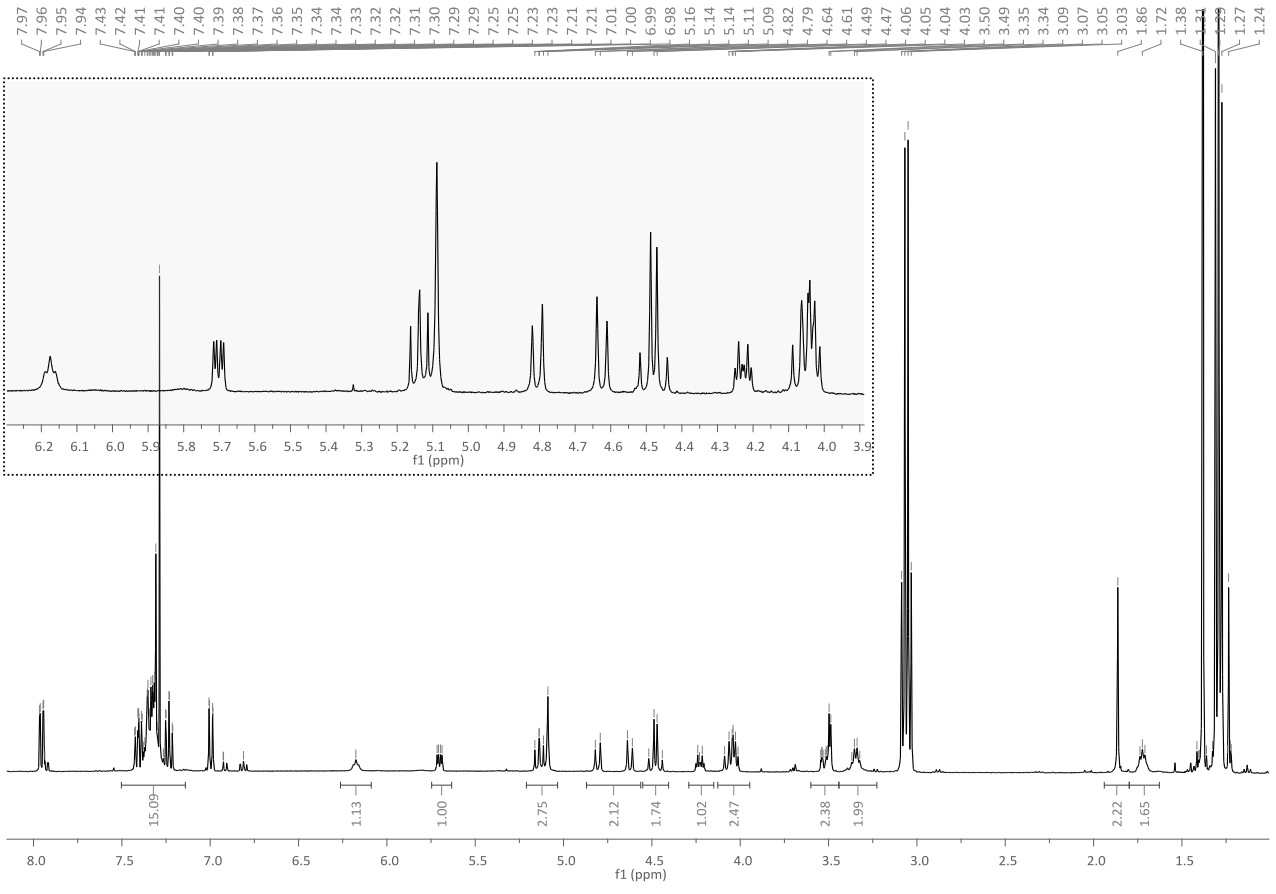
NMR

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.33 – 7.17 (m, 15H, Ar), 6.20 (t, *J* = 5.5 Hz, 1H, N*H*), 5.62 (dd, $J_{H\text{-}1,P}$ =7.8, $J_{H\text{-}1,H\text{-}2}$ = 3.1 Hz, 1H, H-1), 5.06 (t, $J_{H\text{-}4,H\text{-}3}$ = $J_{H\text{-}4,H\text{-}5}$ = 9.7 Hz, 1H, H-4), 5.02 (s, 2H, CH_2Ph), 4.74 (d, *J* = 11.1 Hz, 1H, ½ CH_2Ph), 4.56 (d, *J* = 11.1 Hz, 1H, ½ CH_2Ph), 4.41 (q, *J* = 11.8 Hz, 2H, CH_2Ph), 4.20 – 4.09 (m, 1H, H-5), 4.05 – 3.90 (m, 3H, H-3, CH_2O $_{LINKER}$), 3.56 – 3.46 (m, 3H, H-2, H-6, H-6'), 3.27 (dd, *J* = 11.5, 5.7 Hz, 2H, CH_2NH $_{LINKER}$), 1.81 (s, 3H, CH_3CO), 1.73 – 1.55 (m, 2H, CH_2 $_{LINKER}$).

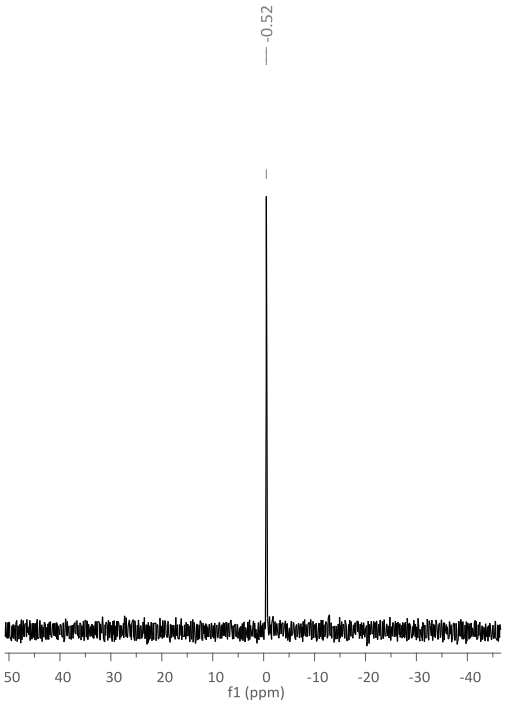
^{31}P NMR (162 MHz, $CDCl_3$) δ -0.52.

^{13}C NMR (101 MHz, $CDCl_3$) δ 169.67, 156.60 (CO), 137.70, 136.97(C_q Ar), 128.47, 128.43, 128.29, 128.15, 128.02, 127.98, 127.94, 127.84, 127.69 (CH Ar), 93.76, 93.71 (C-1), 77.73 (C-3), 74.70 (*C*H_2Ph), 73.48 (*C*H_2Ph), 70.88 (C-4), 69.90 (C-5), 69.05 (C-6), 66.30 (*C*H_2Ph), 63.69, 63.61 (C-2), 62.80 (*C*H_2O $_{LINKER}$), 37.28 (*C*H_2NH $_{LINKER}$), 30.21, 30.15 (CH_2 $_{LINKER}$), 20.79 (*C*H_3CO).

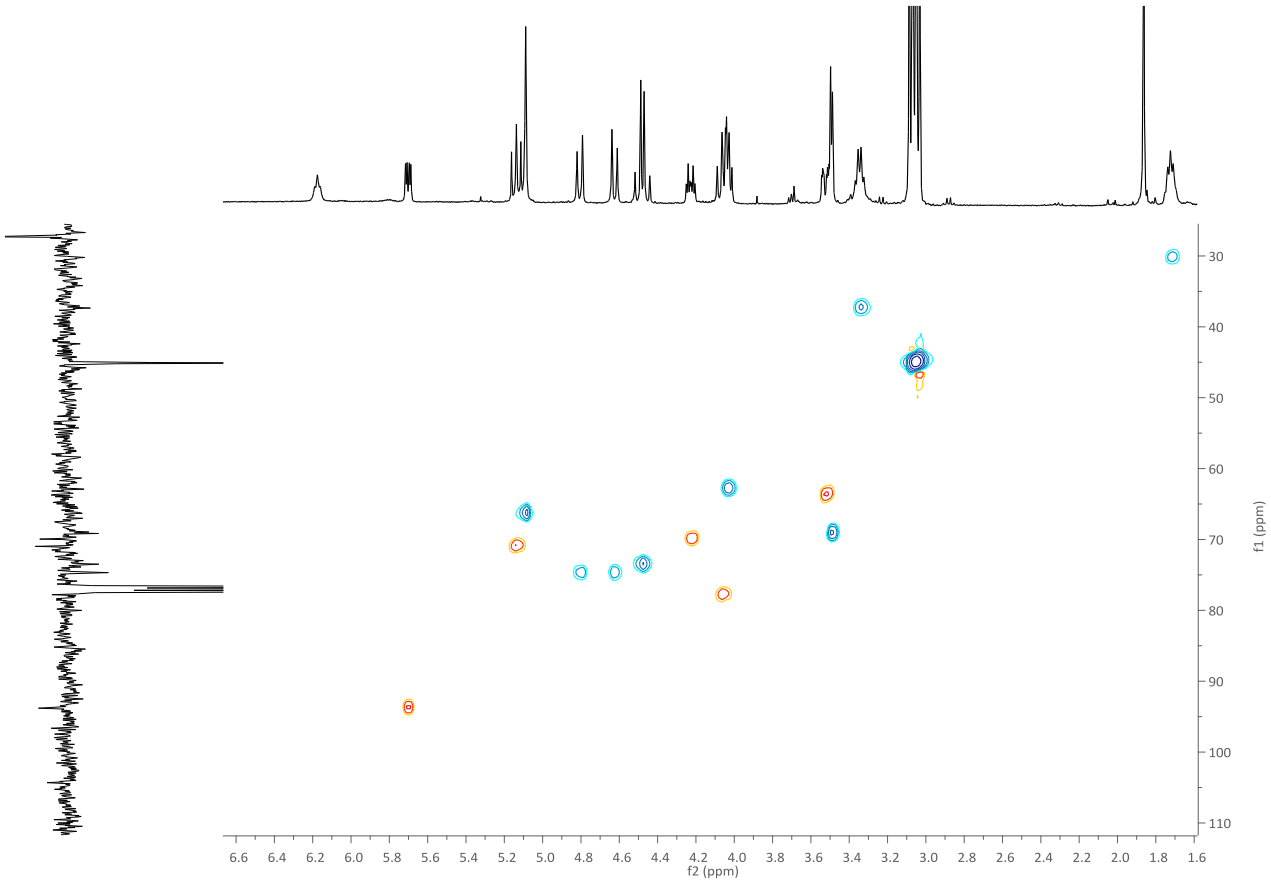
^{1}H (400 MHz, $CDCl_3$):



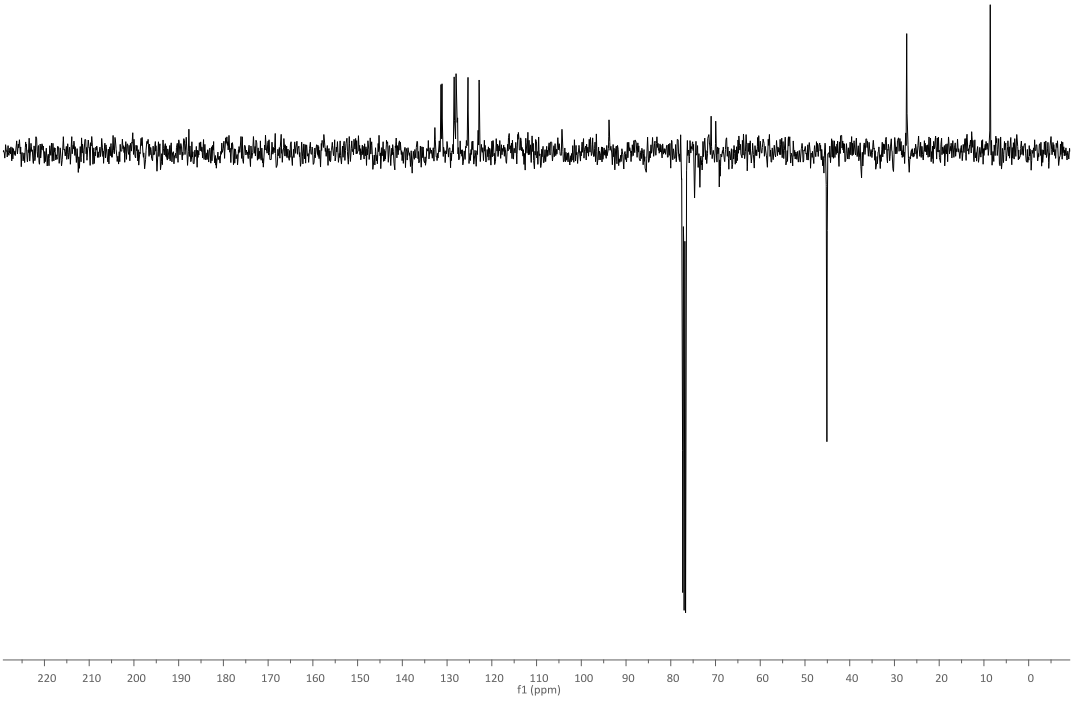
^{31}P NMR (162 MHz, $CDCl_3$):



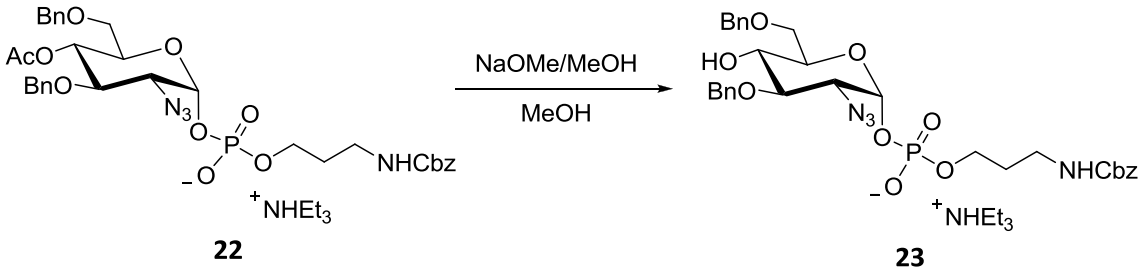
HETCOR ^{1}H-^{13}C (400 MHz, $CDCl_3$):



^{13}C (101 MHz, $CDCl_3$):



3-(*N*-Carbobenzyloxy)aminopropyl 1-*O*-(2-Azido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl phosphate) (23), triethylammonium salt



22 **23**

Synthetic procedure

Compound **22** (60.3 mg, 0.075 mmol) was treated as described in the ***General Procedure C***. Product **23** (55.32 mg, 0.073 mmol) was obtained in *quantitative* yield.

Product characterization

Formula	$C_{37}H_{52}N_5O_{10}P$
Molecular Weight	757.80 g/mol
HP-TLC conditions	R_f (DCM/MeOH 85:15) 0.29

Optical rotation

$\alpha_D^{25} = +18.50$ (c = 1.1 in $CHCl_3$)

MS (ESI)

Experimental m/z: 655.2 $[M]^-$

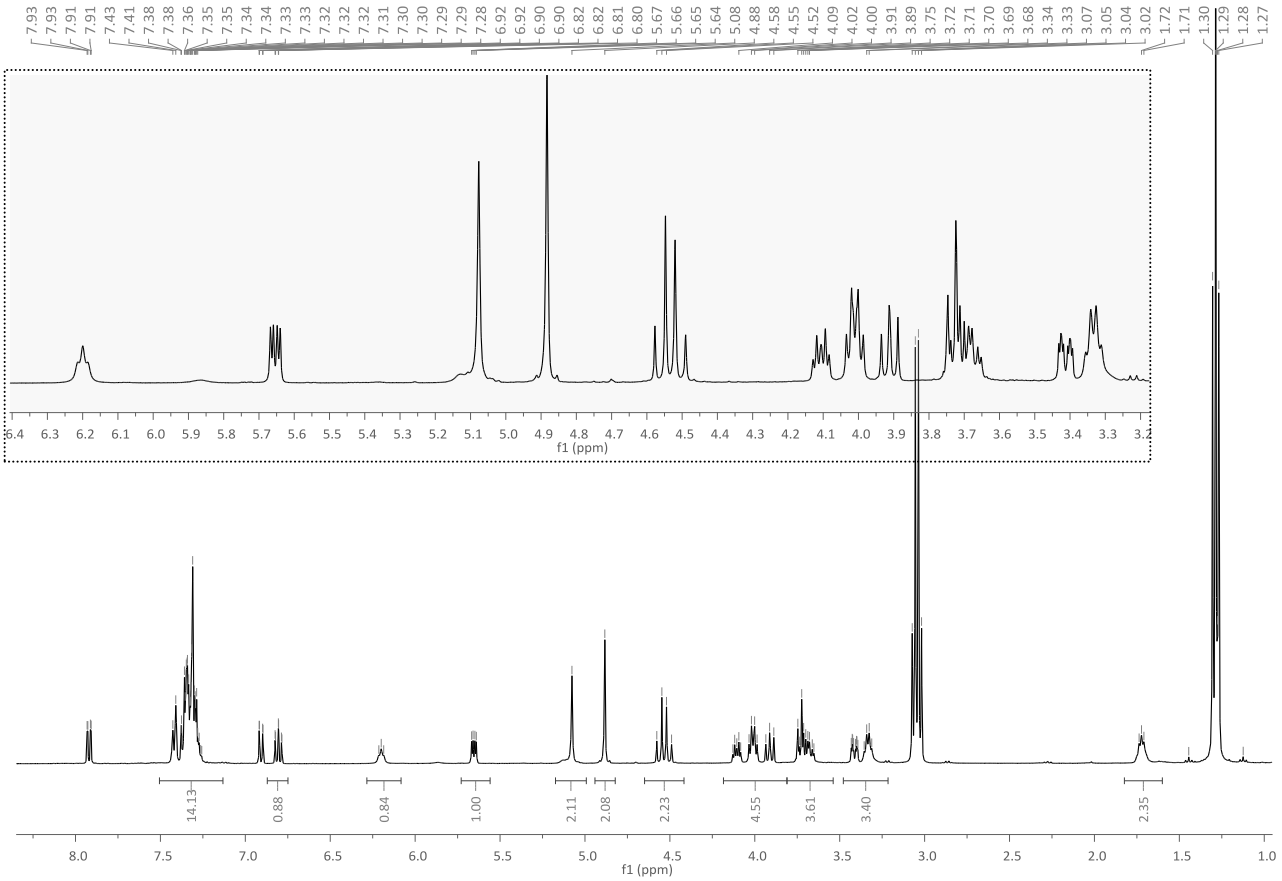
NMR

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.44 – 7.20 (m, 15H, Ar), 6.17 (t, *J* = 5.7 Hz, 1H, N*H*Cbz), 5.63 (dd, $J_{H\text{-}1,P}$ = 7.7, $J_{H\text{-}1,H\text{-}2}$ = 3.3 Hz, 1H, H-1), 5.05 (s, 2H, CH_2Ph), 4.86 (s, 2H, CH_2Ph), 4.51 (q, *J* = 12.0 Hz, 2H, CH_2Ph), 4.13 – 4.04 (m, 1H, H-5), 3.98 (dt, *J* = 7.4, 5.8 Hz, 2H, CH_2O $_{\text{LINKER}}$), 3.93 – 3.83 (t, $J_{H\text{-}3,H\text{-}2}$ = $J_{H\text{-}3,H\text{-}4}$ = 10.1, 1H, H-3), 3.76 – 3.59 (m, 3H, H-4, H-6, H-6'), 3.39 (ddd, $J_{H\text{-}2,H\text{-}3}$ = 10.1, $J_{H\text{-}2,H\text{-}1}$ = 3.2, $J_{H\text{-}2,P}$ = 2.2 Hz, 1H, H-2), 3.31 (dd, *J* = 11.9, 6.0 Hz, 2H, CH_2NH $_{\text{LINKER}}$), 1.76 – 1.63 (m, 2H, CH_2 $_{\text{LINKER}}$).

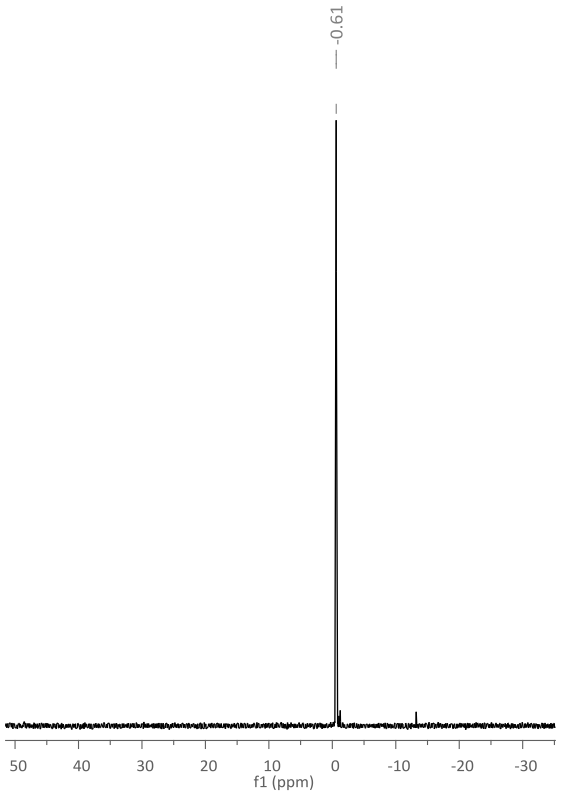
^{31}P NMR (162 MHz, $CDCl_3$) δ -0.61.

^{13}C NMR (101 MHz, $CDCl_3$) δ 156.70 (CO), 138.49, 137.98, 137.15 (C_q Ar), 128.58, 128.50, 128.48, 128.19, 127.99, 127.92, 127.83 (CH Ar), 94.20, 94.15 (C-1), 79.81 (C-3), 75.02 (*C*H_2Ph), 73.70 (*C*H_2Ph), 72.61 (C-4), 71.21 (C-5), 70.25 (C-6), 66.42 (*C*H_2Ph), 63.71, 63.63 (C-2), 62.99, 62.93 (*C*H_2O $_{\text{LINKER}}$), 37.50 (*C*H_2NH $_{\text{LINKER}}$), 29.78 (*C*H_2 $_{\text{LINKER}}$).

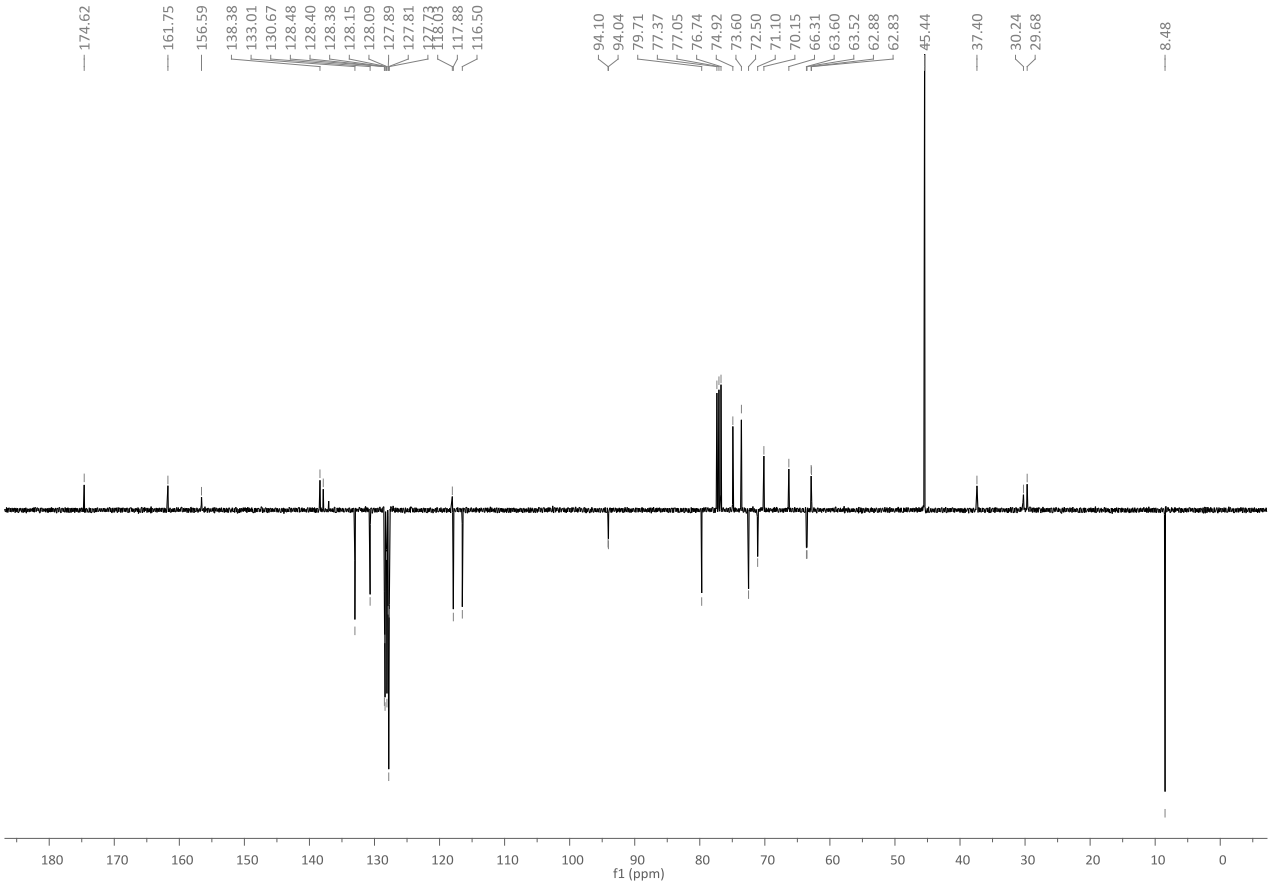
^{1}H (400 MHz, $CDCl_3$):



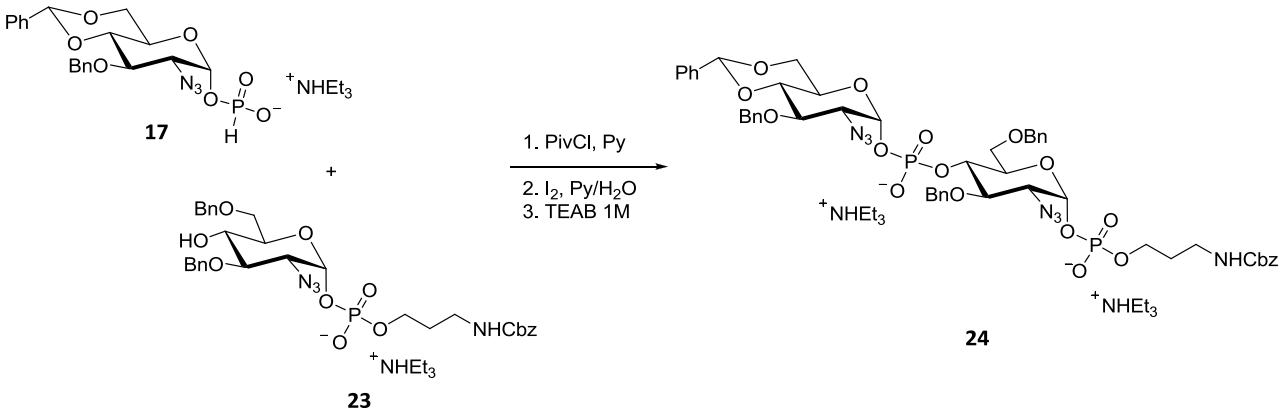
^{31}P NMR (162 MHz, $CDCl_3$):



^{13}C (101 MHz, $CDCl_3$):



3-(*N*-Carbobenzyloxy)aminopropyl 1-*O*-[2-Azido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl phosphate 4-(2-azido-3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-α-D-glucopyranosyl phosphate)] (24), triethylammonium salt



Synthetic procedure

H-phosphonate donor **17** (123.4 mg, 0.23 mmol, 1 eq) was coupled with acceptor **23** (202.6 mg, 0.27 mmol, 1.2 eq) as described in the ***General Procedure E***. Dimer **24** (94.7 mg, 0.073 mmol) was obtained in 27% yield.

Product characterization

Formula	$C_{63}H_{87}N_9O_{17}P_2$
Molecular Weight	1304.40 g/mol
TLC conditions	R_f (DCM/MeOH 8:2) 0.36

Optical rotation

$\alpha_D^{25} = +12.76$ (c = 1.15 in $CHCl_3$)

HRMS (ESI)

Experimental m/z (%):	1122.30177 $[M+Na]^-$
	549.65634 $[M]^{2-}$

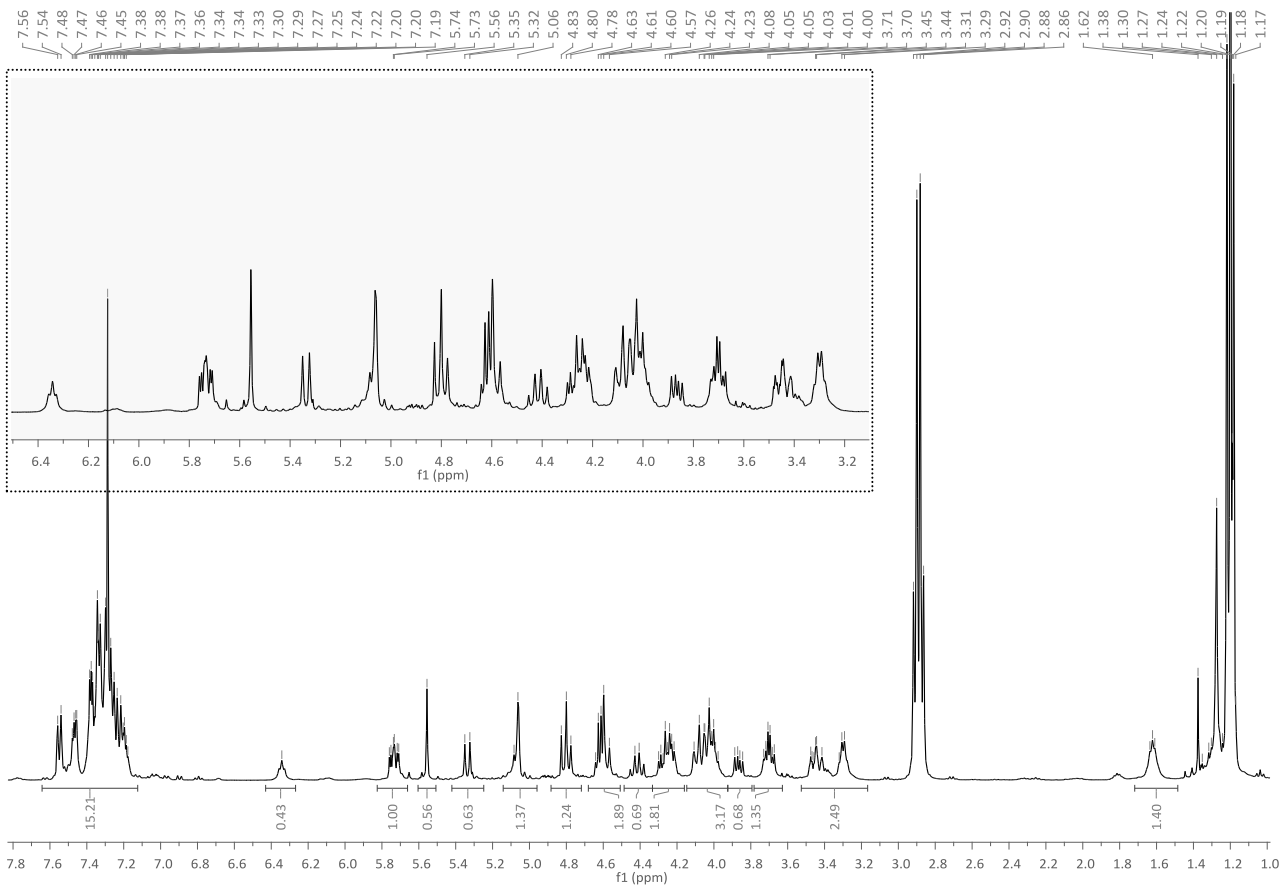
NMR

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.58 – 7.41 (m, 5H, Ar), 7.41 – 7.13 (m, 20H, Ar), 6.34 (t, *J* = 5.9 Hz, 1H, N*H*Cbz), 5.78 – 5.67 (m, 2H, H-1 A, H-1 B), 5.56 (s, 1H, C*H*Ph), 5.34 (d, *J* = 11.0 Hz, 1H, ½ CH_2Ph), 5.06 (s, J = 1.4 Hz, 2H, CH_2Ph), 4.80 (t, 2H, 2x ½CH_2Ph), 4.61 (dt, 3H, ½CH_2Ph, CH_2Ph), 4.42 (dd, $J_{H\text{-}4A,H\text{-}3A}$ = 19.2, $J_{H\text{-}4A,H\text{-}5A}$ = 10.0 Hz, 1H, H-4A), 4.34 – 4.16 (m, 3H, H-6B, H-5A,H-5B), 4.13 – 3.93 (m, 5H, H-6A, H-3B, H-3A, CH_2O $_{LINKER}$), 3.87 (dd, $J_{H\text{-}6'A,H\text{-}5A}$ = 10.9, $J_{H\text{-}6'A,H\text{-}6A}$ = 6.2 Hz, 1H, H-6'A), 3.77 –

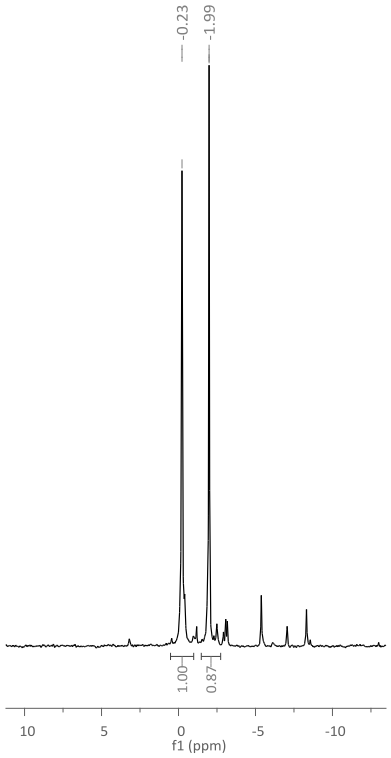
3.65 (m, 2H, H-6'B, H-4B), 3.52 – 3.35 (m, 2H, H-2A, H-2B), 3.30 (dd, J = 10.9, 5.2 Hz, 2H, CH_2NH $_{LINKER}$), 1.69 – 1.52 (m, 2H, CH_2 $_{LINKER}$).
^{31}P NMR (162 MHz, $CDCl_3$) δ -0.23, -1.99.

^{13}C NMR (101 MHz, $CDCl_3$) δ 139.22, 138.93, 138.09, 137.50 (C_q Ar), 128.90, 128.36, 128.28, 128.15, 128.08, 128.03, 128.01, 127.80, 127.72, 127.66, 127.18, 127.13, 126.17 (*C*H Ar), 101.48 (*C*HPh), 94.56, 94.50 (C-1B), 93.80, 93.74 (C-1A), 82.83 (C-4B), 79.05, 79.02 (C-3B), 76.53 (C-3A), 74.79 (*C*H_2Ph), 74.74 (C-4A), 74.36 (*C*H_2Ph), 73.36 (*C*H_2Ph), 72.22, 72.19 (C-5A), 69.87 (C-6A), 68.97 (C-6B), 66.19 (*C*H_2 Cbz), 64.00, 63.92 (C-2B), 63.71, 63.62 (C-2A), 63.29 (C-5B), 62.74, 62.68 (*C*H_2O $_{LINKER}$), 37.25 (*C*H_2NH $_{LINKER}$), 30.17 (*C*H_2 $_{LINKER}$).

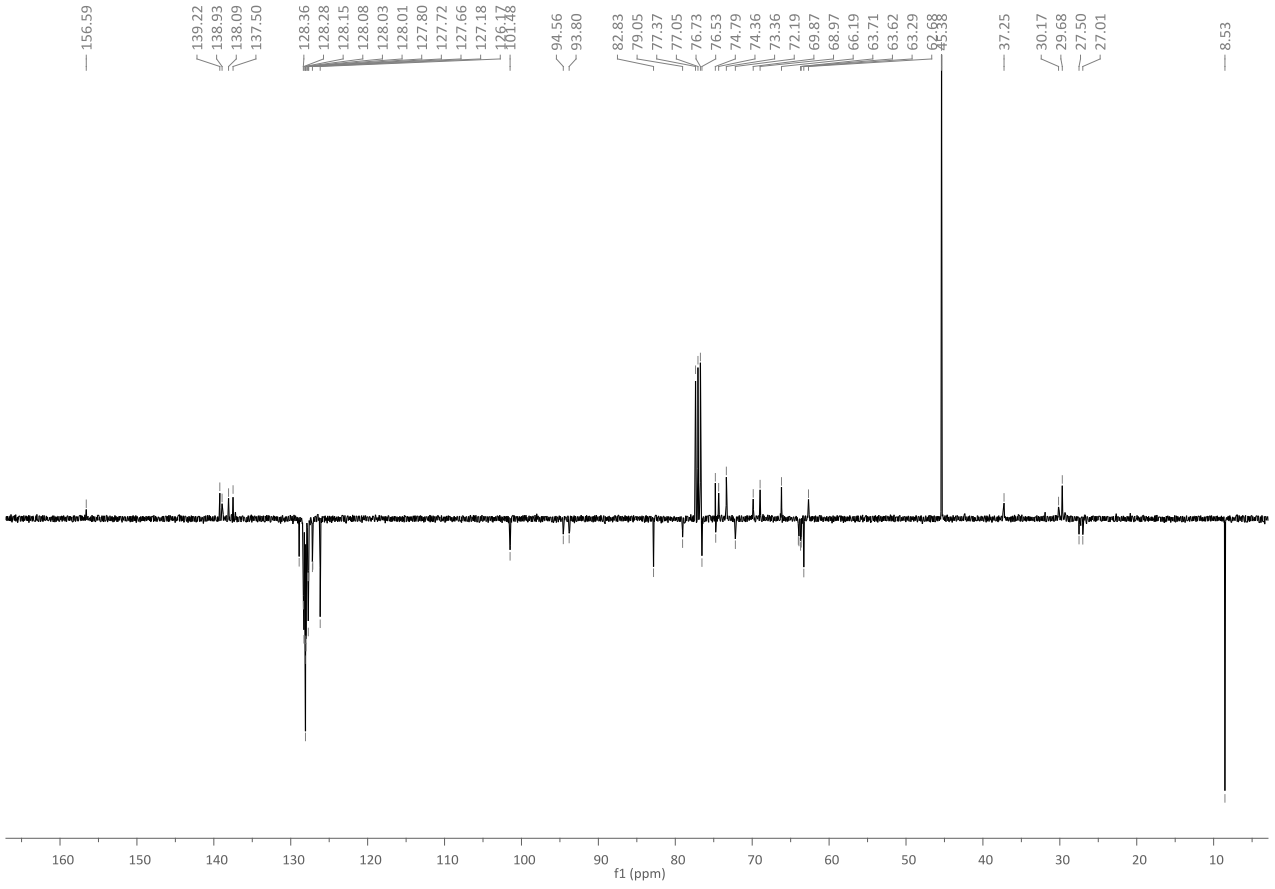
^{1}H (400 MHz, $CDCl_3$):



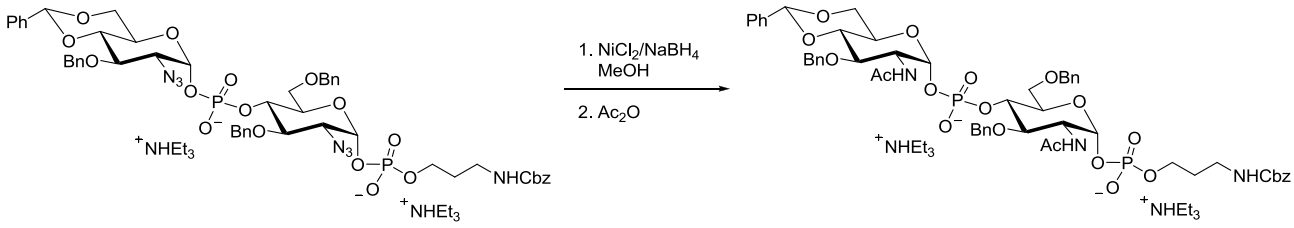
^{31}P NMR (162 MHz, $CDCl_3$):



^{13}C (101 MHz, $CDCl_3$):



3-(*N*-Carbobenzyloxy)aminopropyl 1-*O*-[2-Acetamido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl phosphate 4-(2-acetamido-3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-α-D-glucopyranosyl phosphate)] (25), triethylammonium salt



24 **25**

Synthetic procedure

Dimer **24** (94.7 mg, 0.073 mmol) was converted into the corresponding acetamide **25** (34.7 mg, 0.026 mmol, 36% yield) as described in the ***General Procedure F***. The phosphate bridge has to be stabilized, washing with 0.25M cold TEAB [then drying (Na_2SO_4), filtering and concentrating] the purified product.

Product characterization

Formula	$C_{67}H_{95}N_5O_{19}P_2$
Molecular Weight	1336.4 g/mol
TLC conditions	R_f (DCM/MeOH 7:3) 0.41

Optical rotation

$\alpha \,{}^{25}_{D} = +\,22.03$ (c = 0.3 in MeOH)

MS (ESI)

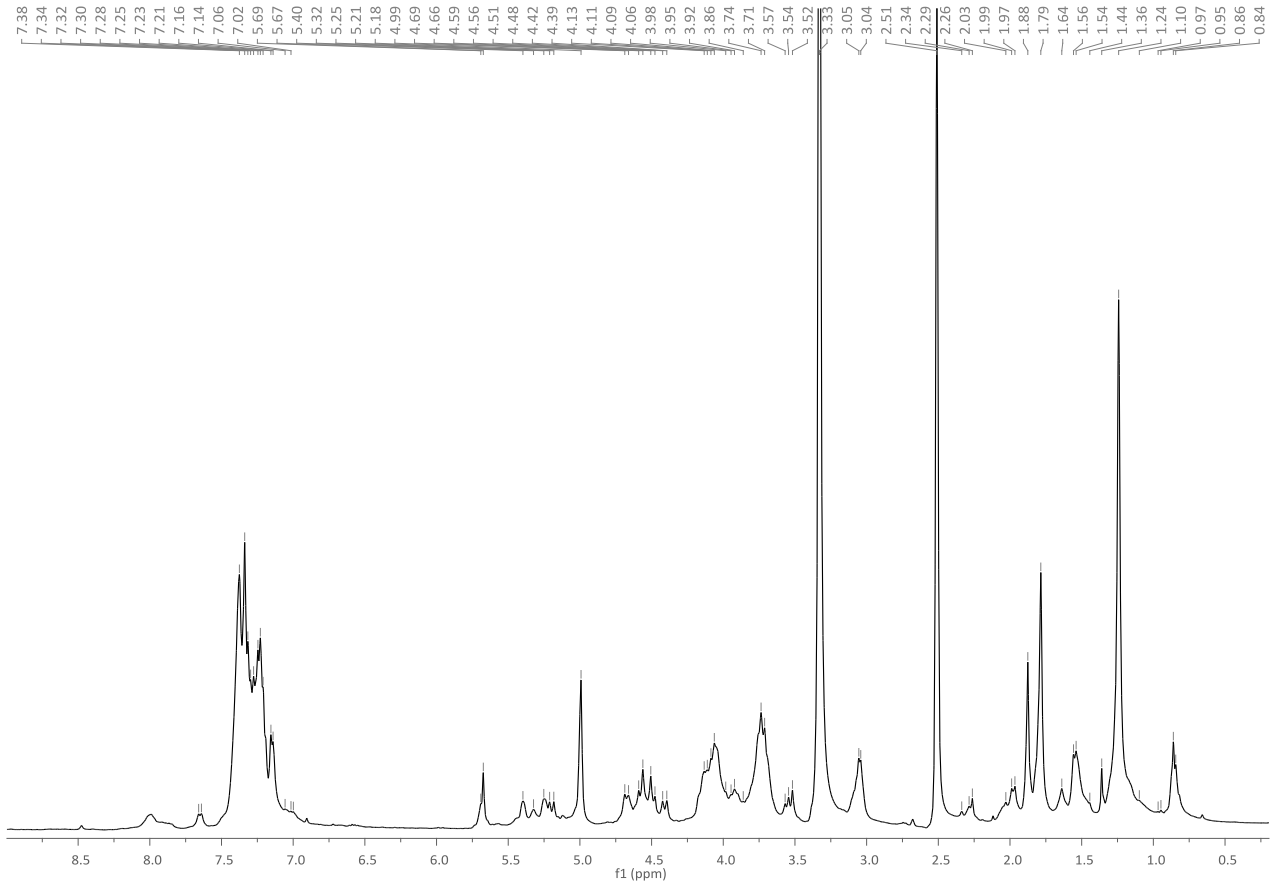
Experimental m/z (%):	1132.4 (100) $[M]^-$
	1154.5 (70) $[M+Na]^-$

*Fully NMR characterization of product **25** wasn't possible.*

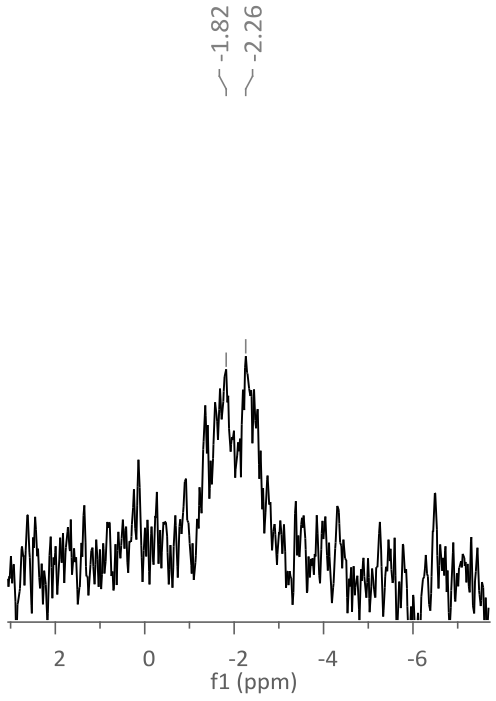
The NMR analysis has been conducted in different solvent ($CDCl_3$, CD_3OD, and $(CD_3)_2S$) and temperature conditions.

The spectra reported below are the best for resolution, and correspond to ^1H, ^{31}P and HETCOR in $(CD_3)_2SO$ at +60°C.

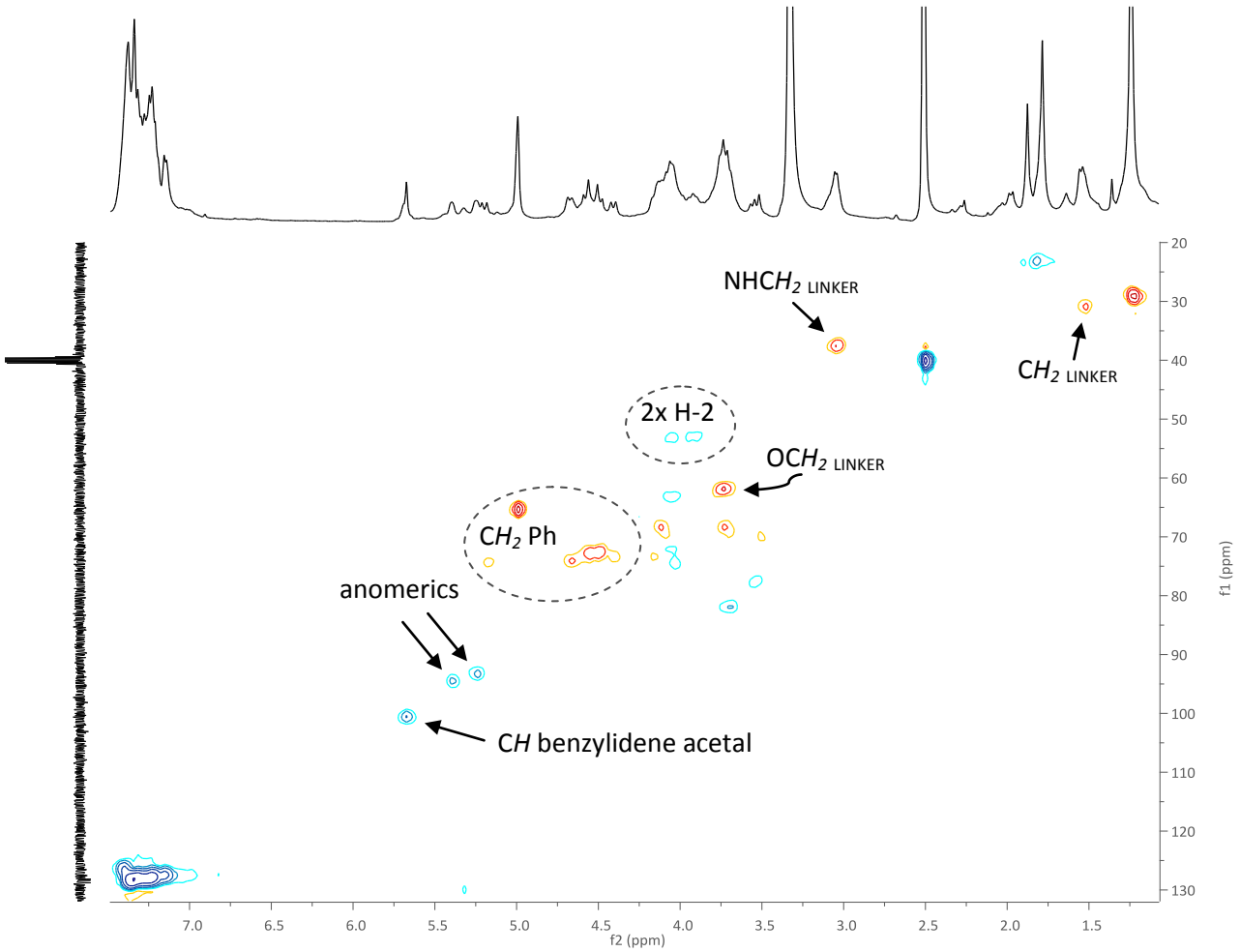
^{1}H (400 MHz, $(CD_3)_2SO$):



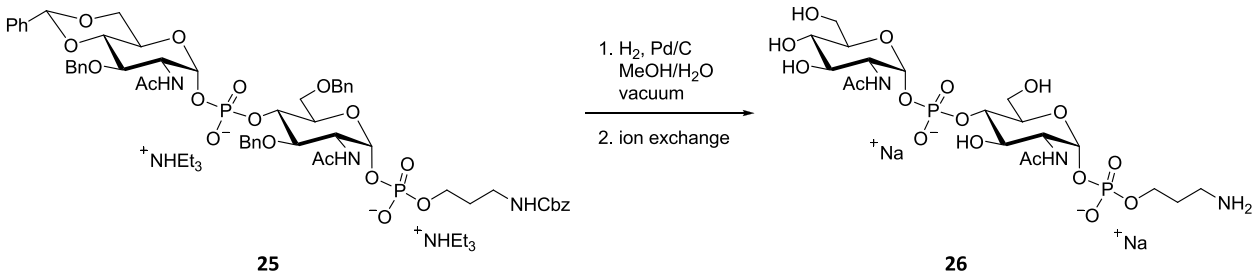
^{31}P NMR (162 MHz, $(CD_3)_2SO$):



HETCOR ^{1}H-^{13}C (400 MHz, $(CD_3)_2SO$):



3-aminopropyl 1-*O*-[2-Acetamido-2-deoxy-α-D-glucopyranosyl phosphate 4-(2-Acetamido-2-deoxy-α-D-glucopyranosyl phosphate)] (26), disodium salt



25 **26**

Synthetic procedure

Fully protected dimer **25** (13 mg, 0.017 mmol) was submitted to hydrogenation as described in the ***General Procedure G***. Final dimer **26** (6.5 mg, 0.016 mmol, 62% yield) was obtained as disodium salt after ion exchange (see ***General Procedure H***).

Product characterization

Formula	$C_{19}H_{35}N_3Na_2O_{17}P_2$
Molecular Weight	685.4 g/mol

Optical rotation

$\alpha_D^{25} = +19.00$ (c = 0.07 in H_2O)

HRMS (ESI)

Experimental m/z: 661.77595 $[M+Na]^-$

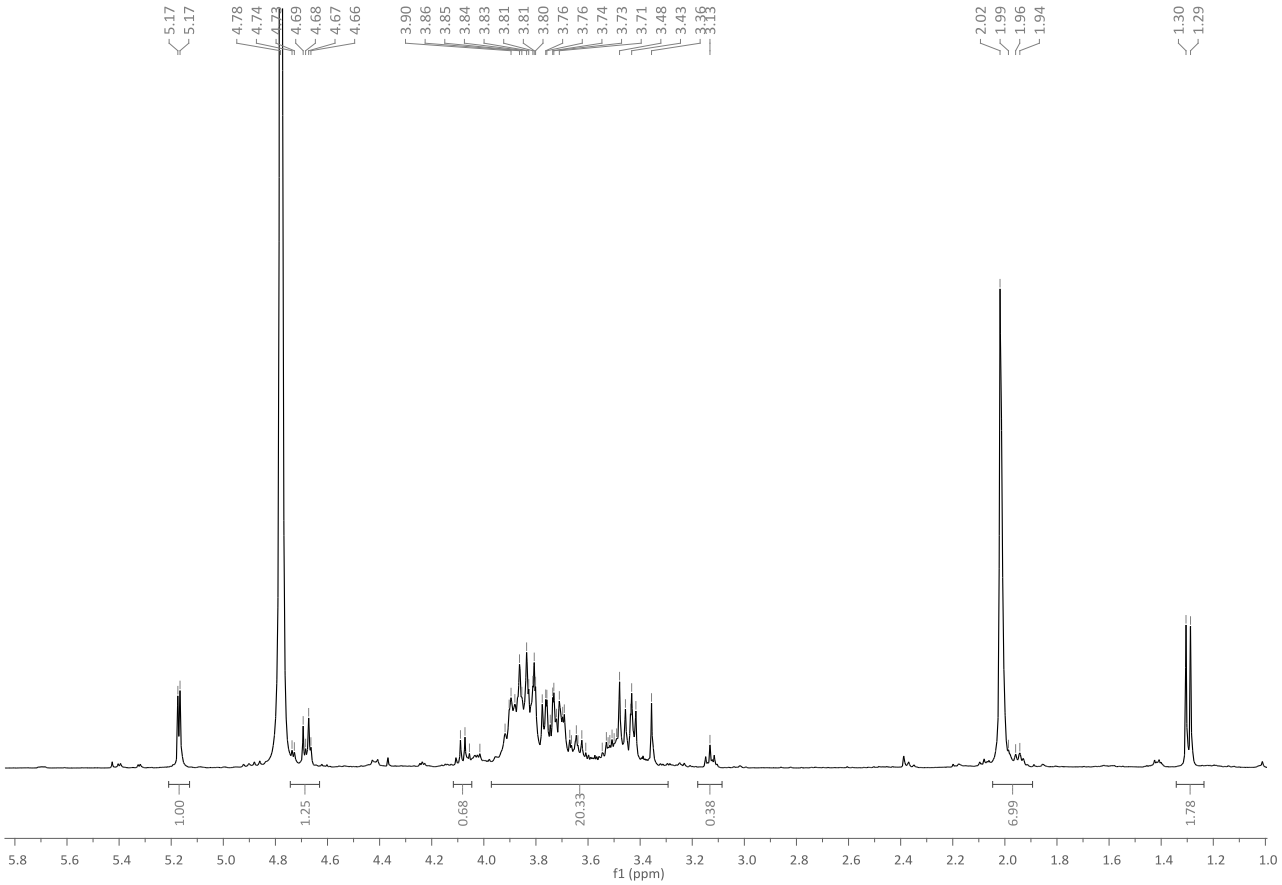
NMR

^{1}H NMR (400 MHz, H_2O+D_2O) δ 5.17 (d, *J* = 3.3 Hz, 1H), 4.70 (dt, *J* = 8.2, 3.5 Hz, 1H), 4.12 – 4.05 (m, 1H), 3.97 – 3.29 (m, 20H), 3.13 (s, 1H), 2.05 – 1.89 (m, 7H), 1.30 (d, *J* = 6.9 Hz, 2H).

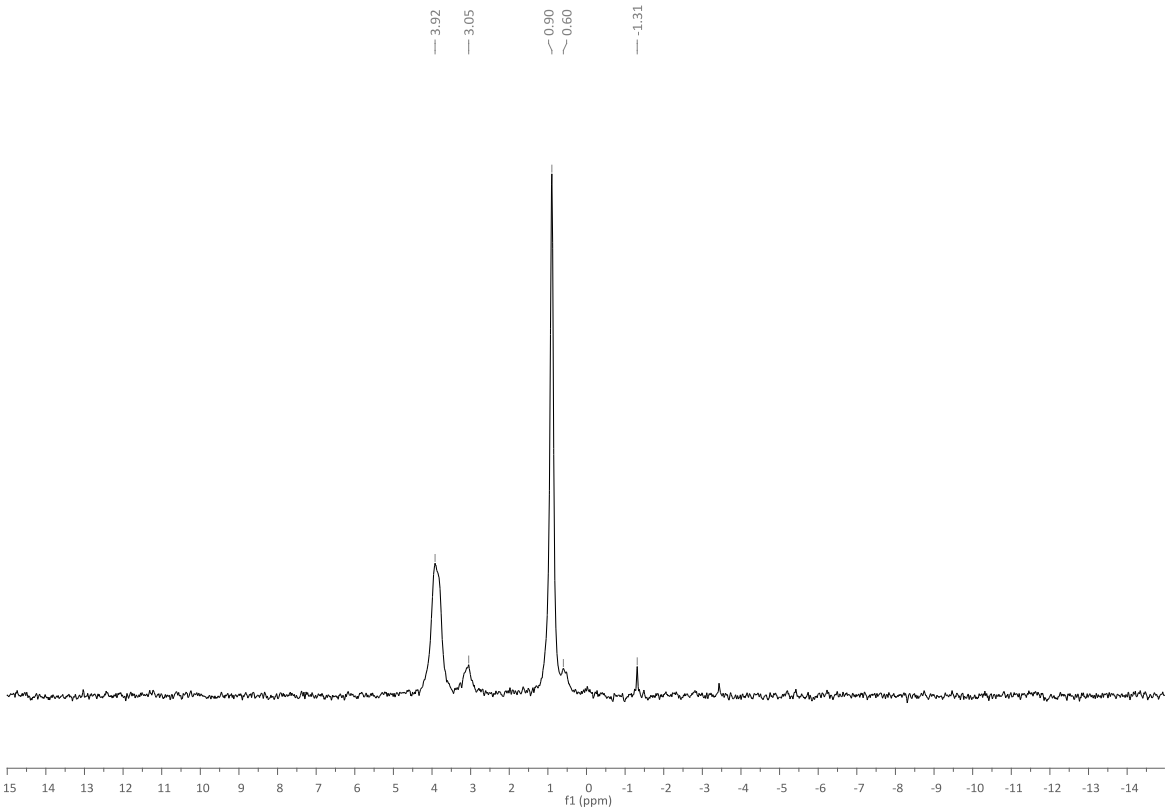
^{31}P NMR (162 MHz, $CDCl_3$) δ: +3.92, +0.90.

^{13}C NMR (101 MHz, D_2O) δ 94.83, 94.77, 90.68 (C-1B), 90.41 (C-1A), 75.79 (C-3A), 73.74 (C-3B), 71.40, 70.51 (C-5B), 70.36 (C-4B), 69.89, 69.66 (C-4A), 68.34 (CH_2O_{LINKER}), 60.40 (C-6A, C-6B), 56.52, 56.26 (C-2B), 53.93, 53.88 (C-2A), 22.02 ($NHCOCH_3$), 21.74 (CH_2NH_{LINKER}), 19.91 ($CH_{2\ \text{LINKER}}$).

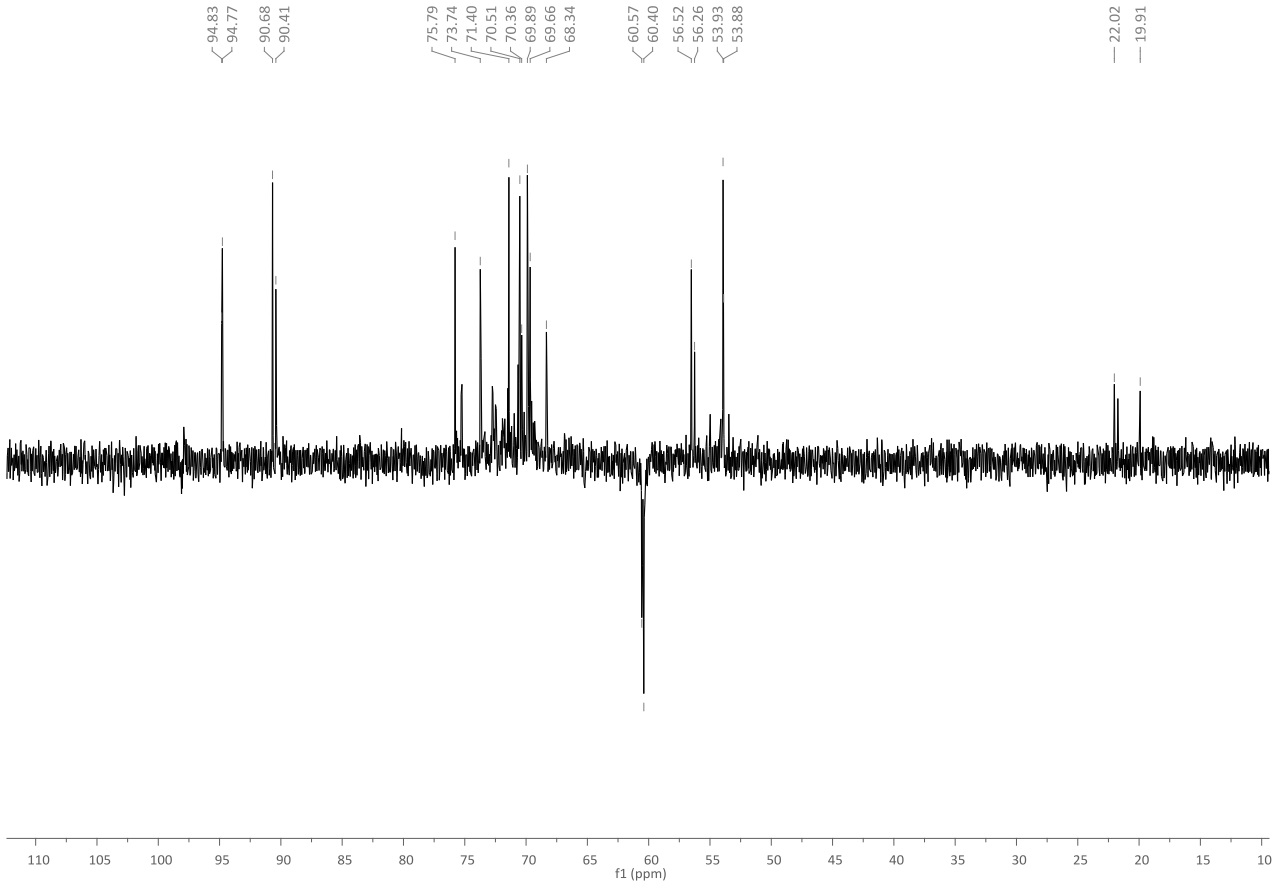
^{1}H (400 MHz, D_2O):



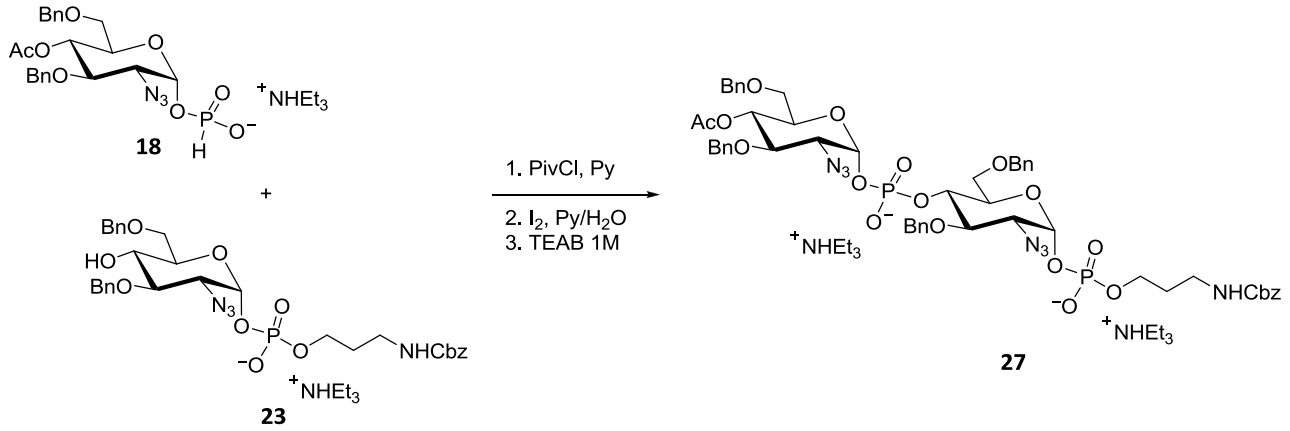
^{31}P NMR (162 MHz, D_2O):



^{13}C – DEPT135 (101 MHz, D_2O):



3-(*N*-Carbobenzyloxy)aminopropyl 1-*O*-[2-Azido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl phosphate 4-(4-*O*-Acetyl-2-azido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl phosphate)] (27), bis-triethylammonium salt



Synthetic procedure

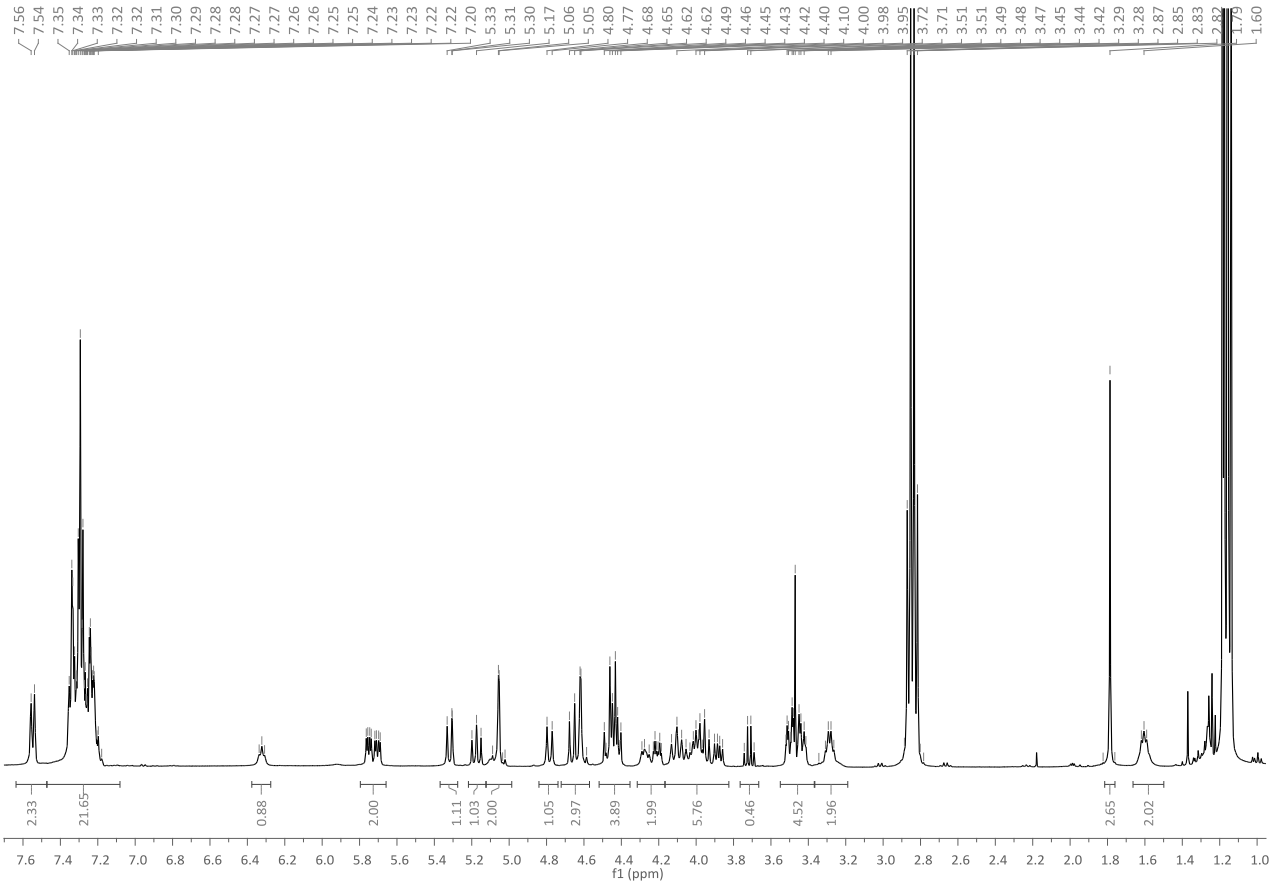
H-phosphonate **18** (366 mg, 0.62 mmol, 1.1 eq) and acceptor **23** (425.5 mg, 0.56 mmol, 1 eq) are first co-evaporated three times with dry pyridine, thereafter they are dried by high vacuum pump overnight. The reactants were dissolved in dry pyridine (2.5 mL), then pivaloyl chloride (140 µL, 1.11 mmol, 1.5 eq) was added dropwise at 0°C and the reaction mixture was stirred under nitrogen at r.t. for half an hour. The reaction completion was monitored by HPTLC (DCM:MeOH 85:15). After cooling to -40°C, a freshly prepared 0.5 M solution of iodine (470 mg, 1.85 mmol, 2.5 eq) in pyridine/water 19:1 (2 mL) was added. The oxidation was completed at 0°C (10 min) and quenched by dropwise addition of a 0.5 M solution of $Na_2S_2O_3 \cdot 5H_2O$ (10% w/v, 10 mL). The mixture was diluted with DCM (50 mL), washed two times with $Na_2S_2O_3 \cdot 5H_2O$ (0.5 M, 2x50 mL), then with cold TEAB (0.5 M, 1x25 mL), dried (Na_2SO_4), filtered and concentrated. The crude was purified by flash chromatography (DCM:MeOH + 1% TEA). The phosphate bridge has to be stabilized, washing with 0.25M cold TEAB [then drying (Na_2SO_4), filtering and concentrating] the purified product **22** (145 mg, 0.11 mmol, 20% yield).

Product characterization

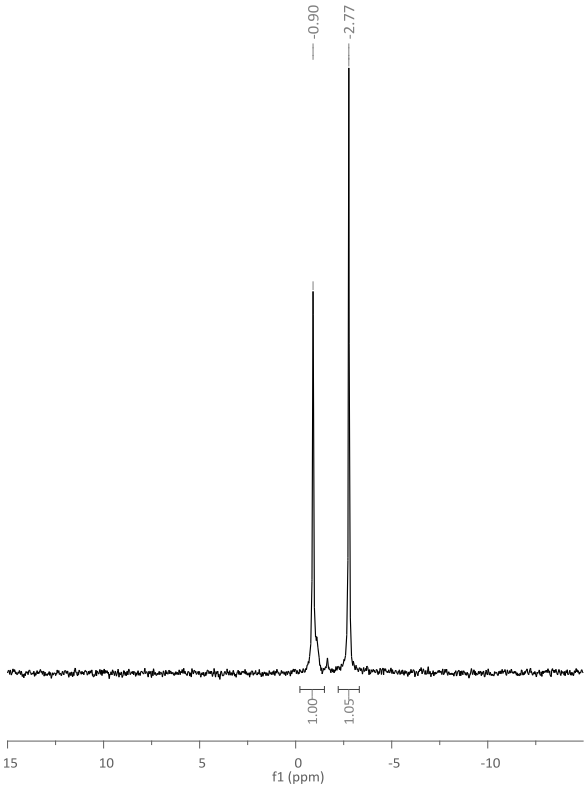
Formula	$C_{65}H_{91}N_9O_{18}P_2$
Molecular Weight	1348.4 g/mol
HP-TLC conditions	R_f (DCM/MeOH 85:15) 0.26

*Fully spectroscopic characterization of product **27** is currently in progress.*

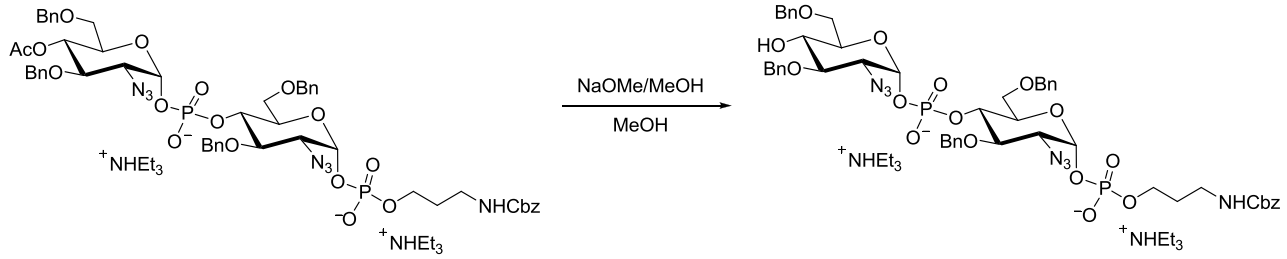
^{1}H (400 MHz, $CDCl_3$):



^{31}P NMR (162 MHz, $CDCl_3$):



3-(*N*-Carbobenzyloxy)aminopropyl 1-*O*-[(2-azido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl phosphate) 4-(2-azido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl phosphate)] (28), bis-triethylammonium salt



27 **28**

Synthetic procedure

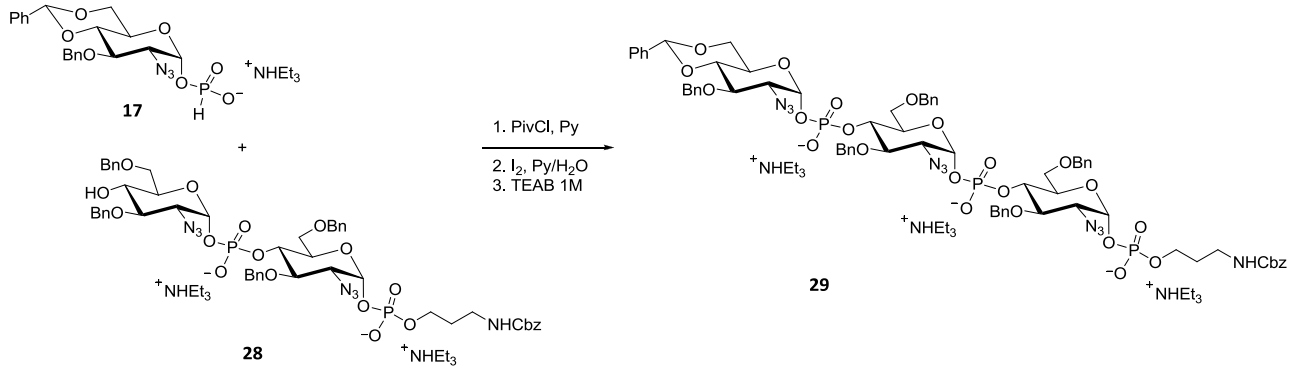
Compound **27** (145 mg, 0.11 mmol) was treated as described in the ***General Procedure C***. Product **28** (114 mg, 0.08 mmol) was obtained in 80% yield as crude.

Product characterization

Formula	$C_{63}H_{89}N_9O_{17}P_2$
Molecular Weight	1306.4 g/mol
HP-TLC conditions	R_f (DCM/MeOH 85:15) 0.26

*Fully spectroscopic characterization of product **28** is currently in progress.*

3-(*N*-Carbobenzyloxy)aminopropyl 1-*O*-{2-Azido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl phosphate 4-[2-azido-3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-α-D-glucopyranosyl phosphate 4-(2-azido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl phosphate)]} (29), tris-triethylammonium salt



Synthetic procedure

H-phosphonate donor **17** (71 mg, 0.12 mmol, 1.5 eq) was coupled with acceptor **28** (114 mg, 0.08 mmol, 1 eq) as described in the ***General Procedure E***. Trimer **29** (95 mg, 0.051 mmol) was obtained in 43% yield.

Product characterization

Formula	$C_{89}H_{124}N_7O_{27}P_3$
Molecular Weight	1851.9 g/mol
HP-TLC conditions	R_f (DCM/MeOH 8:2) 0.19

Optical rotation

$\alpha_D^{25} = +12.81$ (c = 1 in $CHCl_3$)

HRMS (ESI)

Experimental m/z (%):	773.21635 $[M]^{2-}$
	784.20616 $[M+Na]^{2-}$
	1591.39910 $[M+2Na]^{-}$

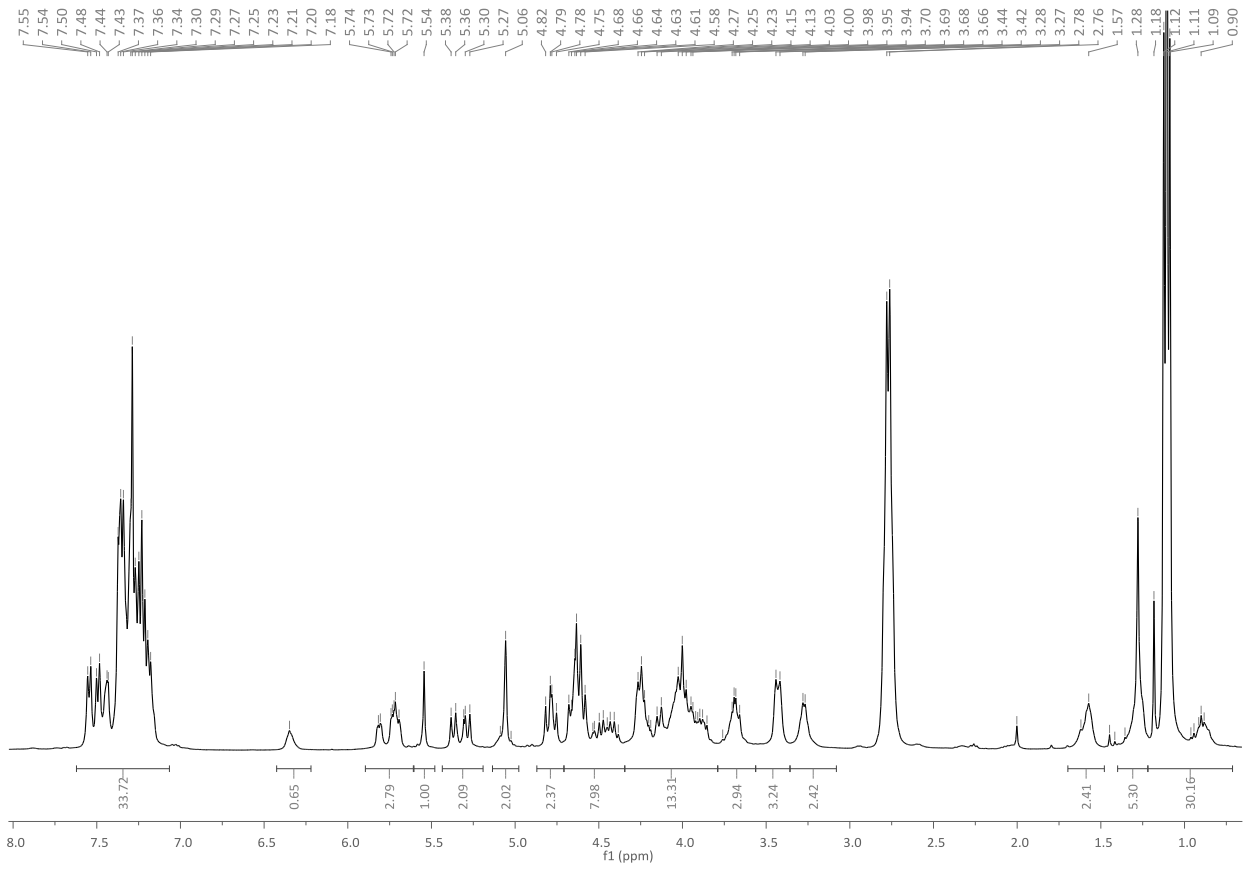
NMR

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.59 – 7.04 (m, 35H, Ar), 6.32 (s, 1H, N*H*Cbz), 5.78 (dd, $J_{H\text{-}1A,P}$ = 7.0, $J_{H\text{-}1A,H\text{-}2A}$ =2.4 Hz, 1H, H-1A), 5.74 – 5.58 (m, 2H, H-1B, H-1C), 5.52 (s, 1H, C*H*Ph), 5.41 – 5.16 (m, 2H, 2x ½ CH_2Ph), 5.03 (s, 2H, CH_2Ph), 4.83 – 4.70 (m, 2H, 2x ½ CH_2Ph), 4.68 – 4.52 (m, 5H, 3X ½ CH_2Ph, CH_2Ph), 4.52 – 4.32 (m, 2H, H-4B, H-4A), 4.32 – 3.76 (m, 12H, H-6C, H-5A, H-5B, H-6A, H-3B, H-3A,

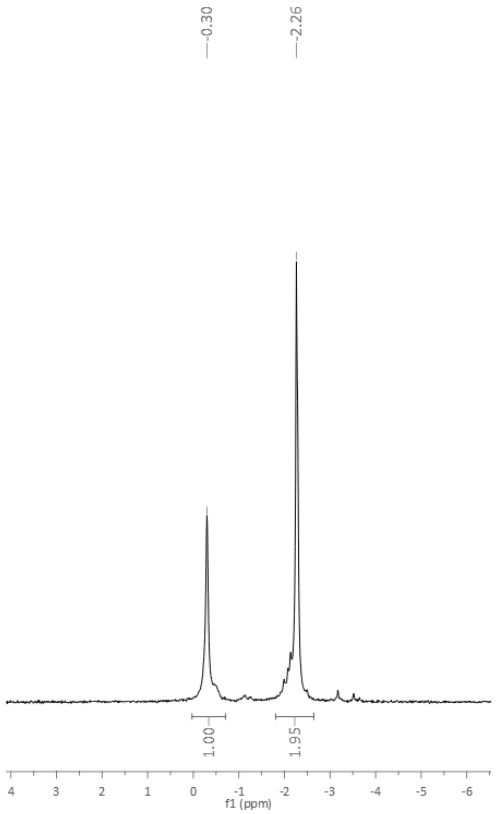
H-6B, H-3C, C*H*$_2$O $_{LINKER}$, H-6'A, H-6'B), 3.76 – 3.53 (m, 3H, H-6'C, H-4C), 3.40 (d, *J* = 9.6 Hz, 3H, H-2A, H-2B, H-2C), 3.25 (d, *J* = 5.2 Hz, 2H, C*H*$_2$NH $_{LINKER}$), 1.62 – 1.45 (m, 2H, C*H*$_2$ $_{LINKER}$).
^{31}P NMR (162 MHz, $CDCl_3$) δ -0.30 (s, 1P, *A*), -2.26 (s, 2P, *B*, *C*).

^{13}C NMR (101 MHz, $CDCl_3$) δ 128.45, 128.38, 128.25, 128.23, 128.16, 128.10, 128.05, 127.95, 127.91, 127.74, 127.20, 127.14, 126.27, 126.24 (*C*H Ar), 101.56 (*C*H Ph), 94.61, 94.56 (C-1C), 94.08, 94.02, 93.89, 93.84 (C-1A, C-1B), 82.90 (C-4C), 79.46, 79.00 (C-3A, C-3B), 76.68 (C-3C), 75.11, 75.05 (C-4A), 74.86 (*C*H_2 Ph), 74.77, 74.71 (C-4B), 74.51 (*C*H_2 Ph), 73.52, 73.42 (*C*H_2 Ph), 72.29 (C-5A), 72.27 (C-5B), 70.04 (C-6B), 69.70 (C-6A), 69.05 (C-6C), 66.27 (*C*H_2 Ph), 64.11, 64.03 (C-2A, C-2B), 63.80, 63.71 (C-2C), 63.35 (C-5C), 62.79 (*C*H_2O $_{LINKER}$), 37.33 (*C*H_2NH $_{LINKER}$), 29.78 (*C*H_2 $_{LINKER}$).

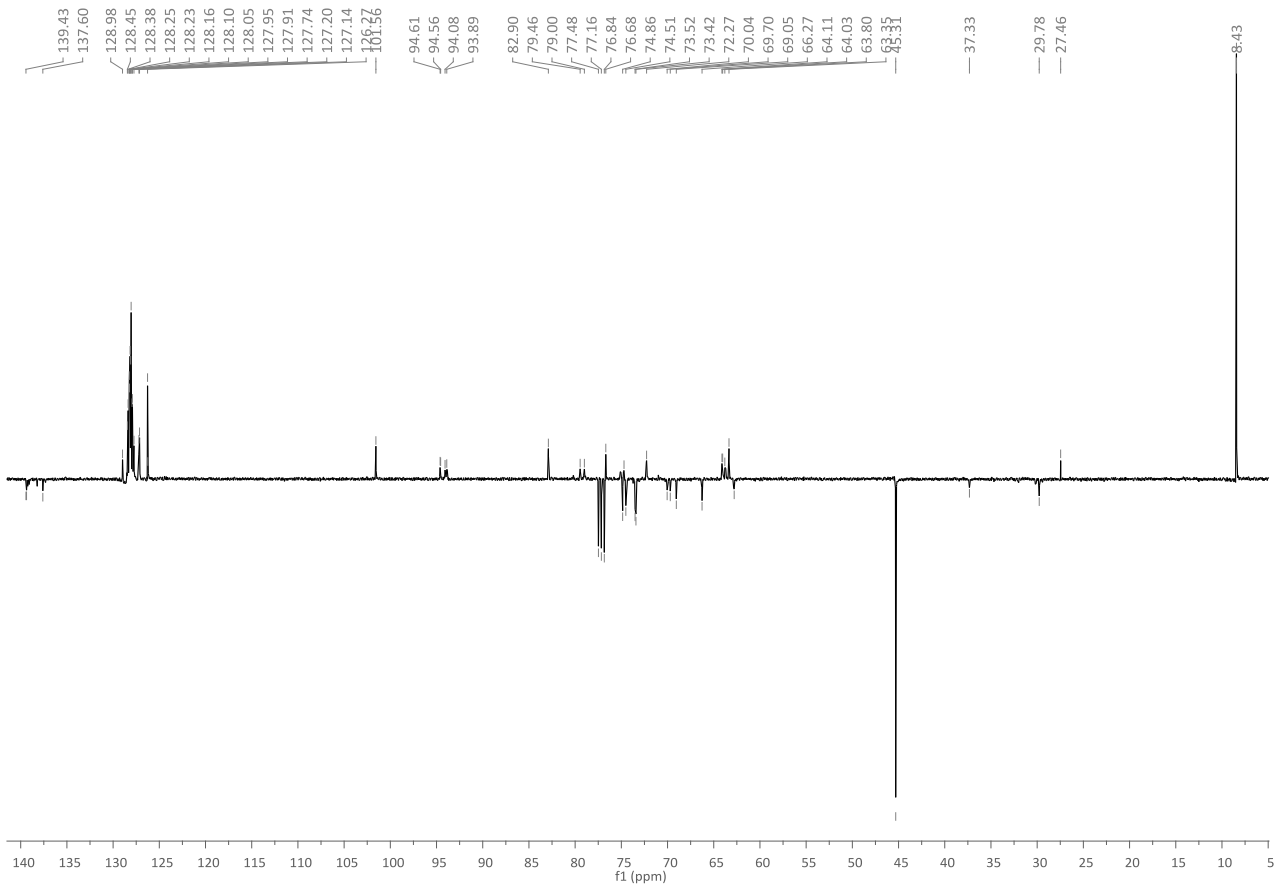
^{1}H (400 MHz, $CDCl_3$):



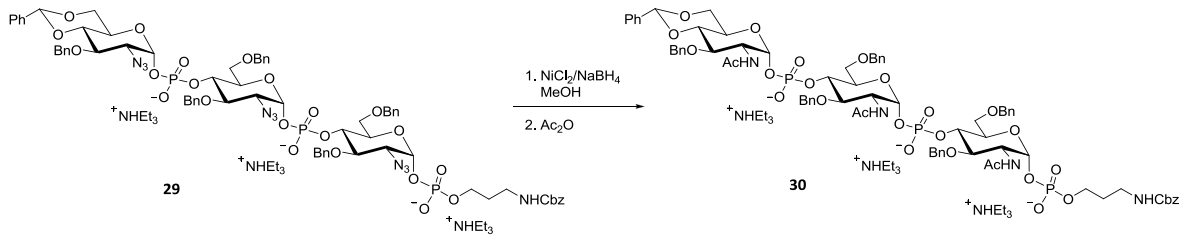
^{31}P NMR (162 MHz, $CDCl_3$):



^{13}C (101 MHz, $CDCl_3$):



3-(*N*-Carbobenzyloxy)aminopropyl 1-*O*-{2-Acetamido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl phosphate 4-[2-acetamido-3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-α-D-glucopyranosyl phosphate 4-(2-acetamido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl phosphate)]} (30), tris-triethylammonium salt



Synthetic procedure

Trimer **29** (80 mg, 0.043 mmol) was converted into the corresponding acetamide **30** (22.14 mg, 0.011 mmol, 25% yield) as described in the ***General Procedure F***.

Product characterization

Formula	$C_{95}H_{136}N_7O_{27}P_3$
Molecular Weight	1901.1 g/mol
HP-TLC conditions	R_f (DCM/MeOH 7:3) 0.27

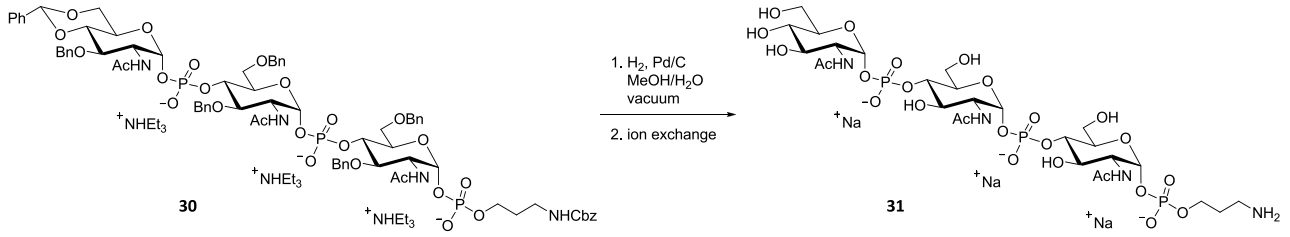
HRMS (ESI negative)

Experimental m/z (%):	1639.47585 $[M+2Na]^-$
	808.24062 $[M+Na]^{2-}$
	531.16428 $[M]^{3-}$

*Fully NMR characterization of product **30** wasn't possible.*

The NMR analysis has been conducted in different solvent ($CDCl_3$, and CD_3OD) and temperature conditions.

3-aminopropyl 1-*O*-{2-Acetamido-2-deoxy-α-D-glucopyranosyl phosphate 4-[2-acetamido-2-deoxy-α-D-glucopyranosyl phosphate 4-(2-acetamido-2-deoxy-α-D-glucopyranosyl phosphate)]} (31), tris-sodium salt



Synthetic procedure

Fully protected trimer **30** (20 mg, 0.010 mmol) was submitted to hydrogenation as described in the ***General Procedure G***. Final trimer **31** (8.6 mg, 0.009 mmol, 87% yield) was obtained as disodium salt after ion exchange (see ***General Procedure H***).

Product characterization

Formula	$C_{26}H_{48}N_4Na_3O_{25}P_3$
Molecular Weight	990.6 g/mol

Optical rotation

$\alpha_D^{25} = +86.61$ (c = 0.4 in H_2O)

HRMS (ESI)

Experimental m/z:	967.16133 $[M+2Na]^-$
	945.17800 $[M+H+Na]^-$
	472.08681 $[M+Na]^{2-}$
	461.09538 $[M+H]^{2-}$

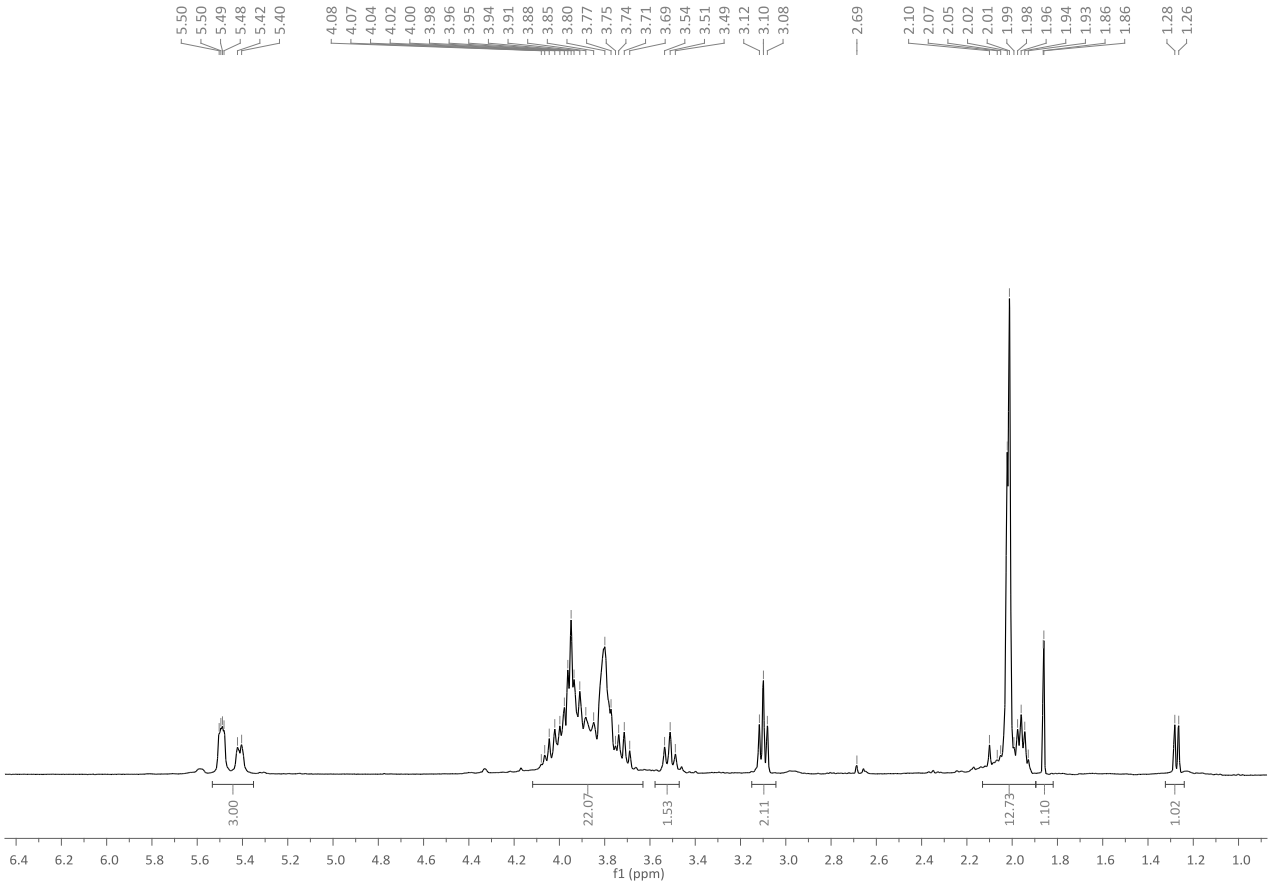
NMR

^{1}H NMR (400 MHz, D_2O) δ 5.55 – 5.45 (m, 2H, H-1A, H-1B), 5.45 – 5.35 (m, 1H, H-1C), 4.16 – 3.61 (m, 16H, C*H*$_2$O $_{LINKER}$, 3x H-2 ABC, 3x H-6 ABC, 3x H-6' ABC, 3x H-3 ABC,3x H-5 ABC, 2x H-4 AB), 3.51 (t, $J_{H-4,H-3} = J_{H-4,H-5}$ = 9.5 Hz, 1H, H-4C), 3.10 (t, *J* = 7.0 Hz, 1H, C*H*$_2$NH $_{LINKER}$), 2.07 – 1.88 (m, 11H, NHCOC*H*$_3$, C*H*$_2$ $_{LINKER}$).

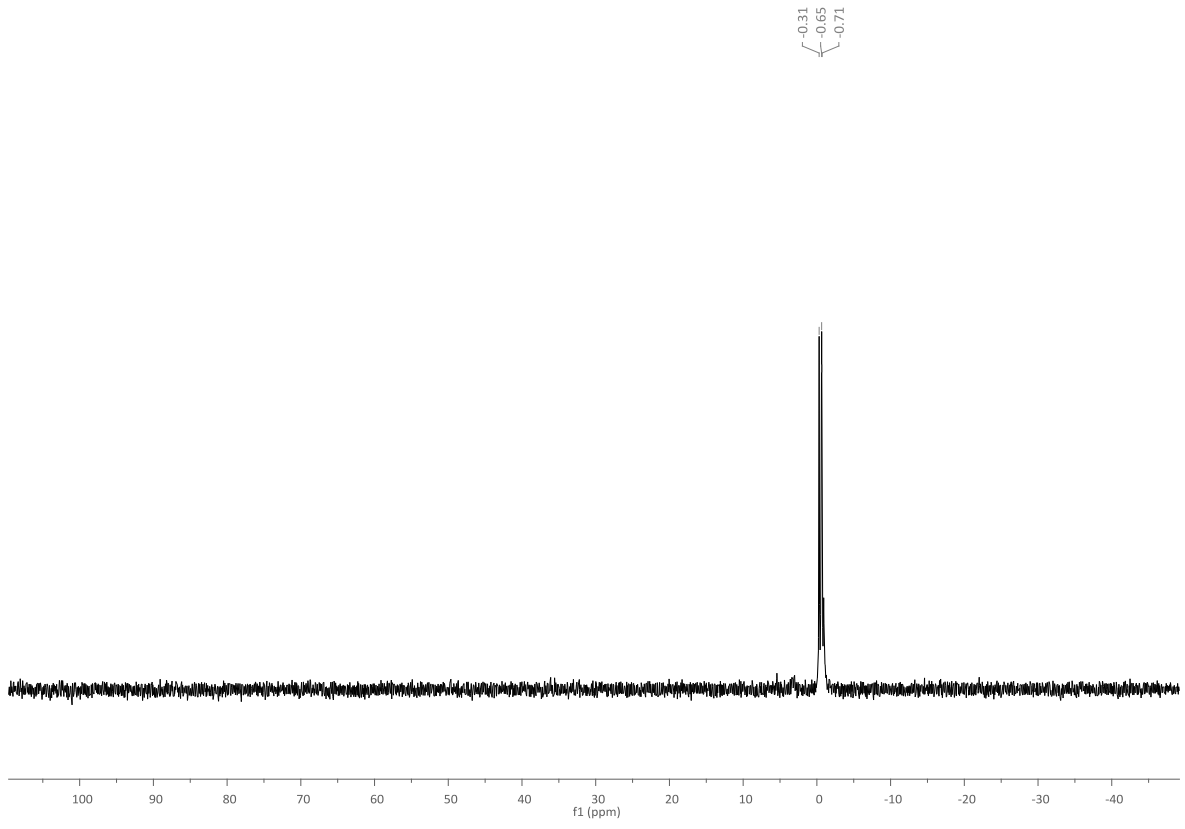
^{31}P NMR (162 MHz, D_2O) δ (ppm): - 0.31, -0.65, -0.71.

^{13}C NMR (101 MHz, D_2O) δ 174.61 (*C*O), 94.54, 94.49 (C-1B), 94.25, 94.18 (C-1C), 93.60, 93.55 8 (C-1), 74.04 (2x C-3 AB), 73.08 (C-3C), 72.20 (2x C-5 AB), 70.87 (C-5C), 70.14, 69.58 (C-4C), 63.41 (C*H*$_2$O $_{LINKER}$), 60.35 (3x C-6), 53.74 (3x C-2), 37.17 (C*H*$_2$NH $_{LINKER}$), 27.69 (C*H*$_2$ $_{LINKER}$), 22.16 (NHCO*C*H$_3$).

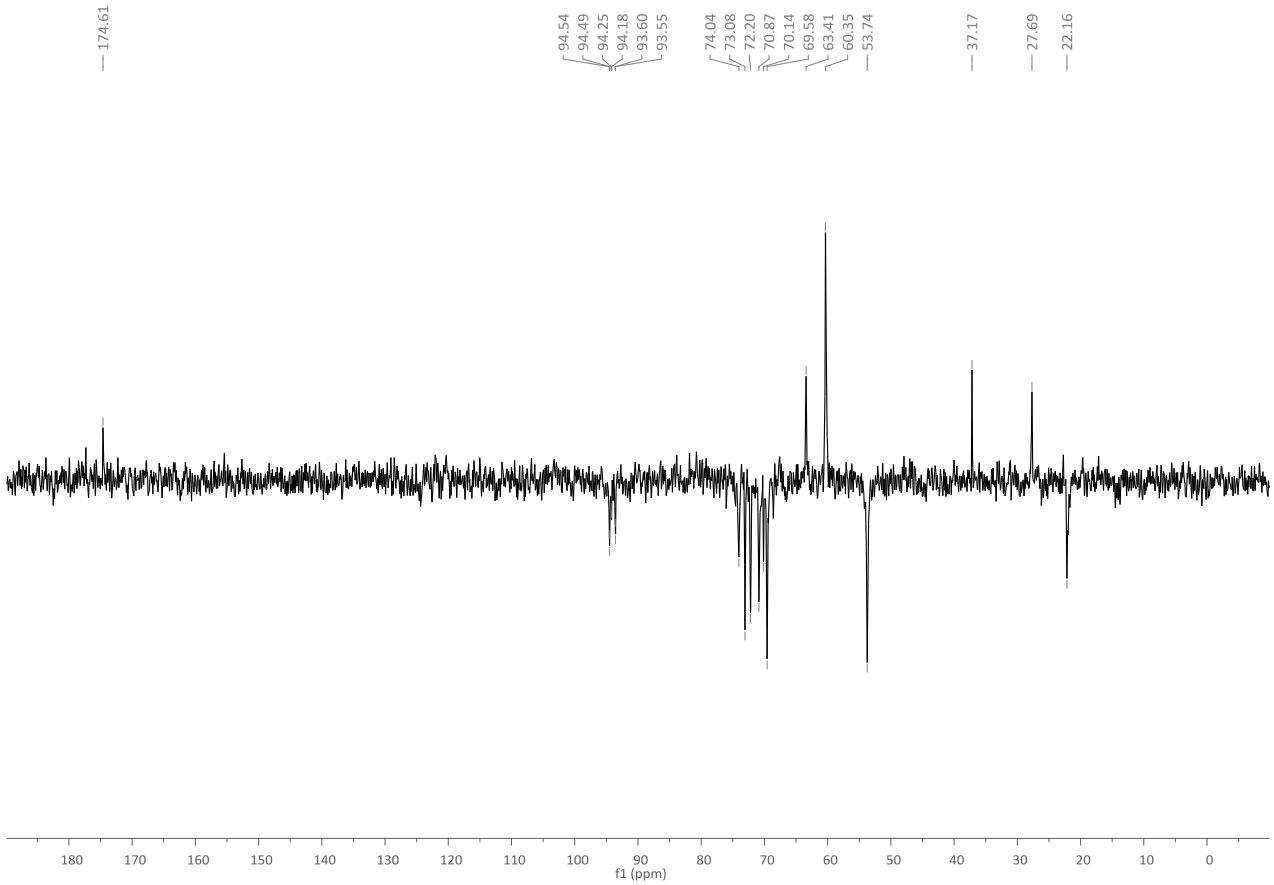
^{1}H (400 MHz, D_2O):



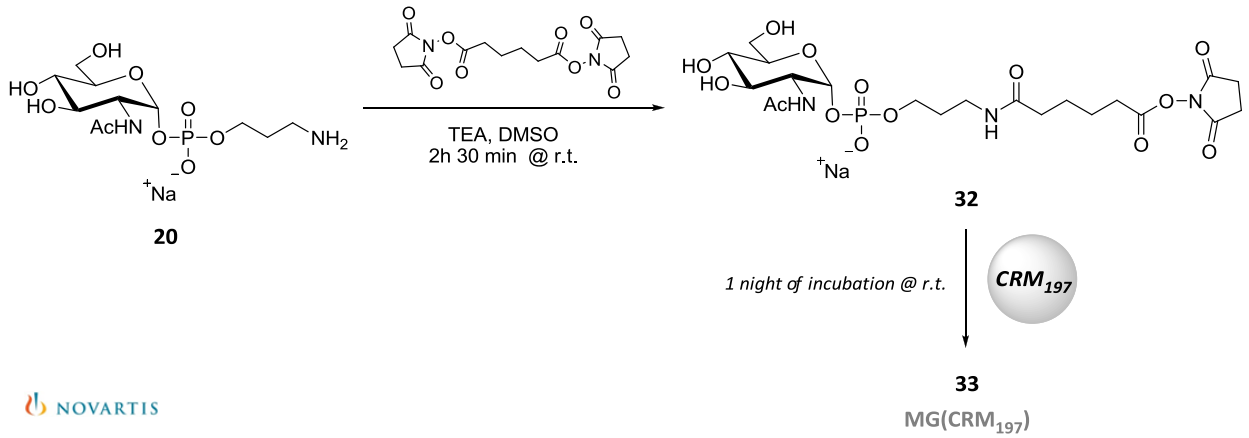
^{31}P NMR (162 MHz, D_2O):



^{13}C – DEPT135 (101 MHz, D_2O):



Monomer-Glycoconjugate(CRM_{197}) (33)



Synthetic procedure

reagents	M.W. (g/mol)	d (g/cm^3)	mmol	amount	eq
20	380.26	-	0.015	5.65 mg	1
disuccinimidyl adipate	340.29	-	0.15	51 mg	10
triethylamine	101.19	0.726	0.3	42 μL	20
DMSO	78.13	1.1	11.26	800 μL	-

Monomer **20** (15 μmol), dissolved in 200 μL of DMSO containing triethylamine (20 eq), was slowly dropped into a murky mixture of disuccinimidyl adipate (10 eq) in DMSO (400 μL). After 2.5 hours of vigorous stirring the activated oligosaccharide was purified from the excess of linker by precipitation of the reaction mixture in nine volumes (9 mL) of ethyl acetate. The pellet obtained by subsequent centrifugation was washed 10 times with 3 mL (1/3 of the volume) of ethyl acetate and then dried under vacuum (4 hours of lyophilisation).

Compound **32** (5.35 mg) was reacted with 2.5 mg of CRM_{197} (77 μL of a solution 32.5mg/mL of protein in NaPi 10 mM +10% saccharosium, pH 7.2) in 77 μL of 100 mM NaPi buffer (pH 7.2): the conjugation stoichiometry was about 100:1 sugar/protein (mol/mol). The mixture was incubated over night at room temperature, mixing very gently with a magnetic stirrer. The glycoconjugate was purified from the excess of unconjugated carbohydrate using precipitation by $(NH_4)_2SO_4$.

Product characterization

MALDI-TOF mass spectrum of **33** was recorded (Figure 3): the found value for the glycoconjugate **33** was 61523.32 m/z, corresponding to approximately 5 oligosaccharide chains per CRM_{197} molecule.

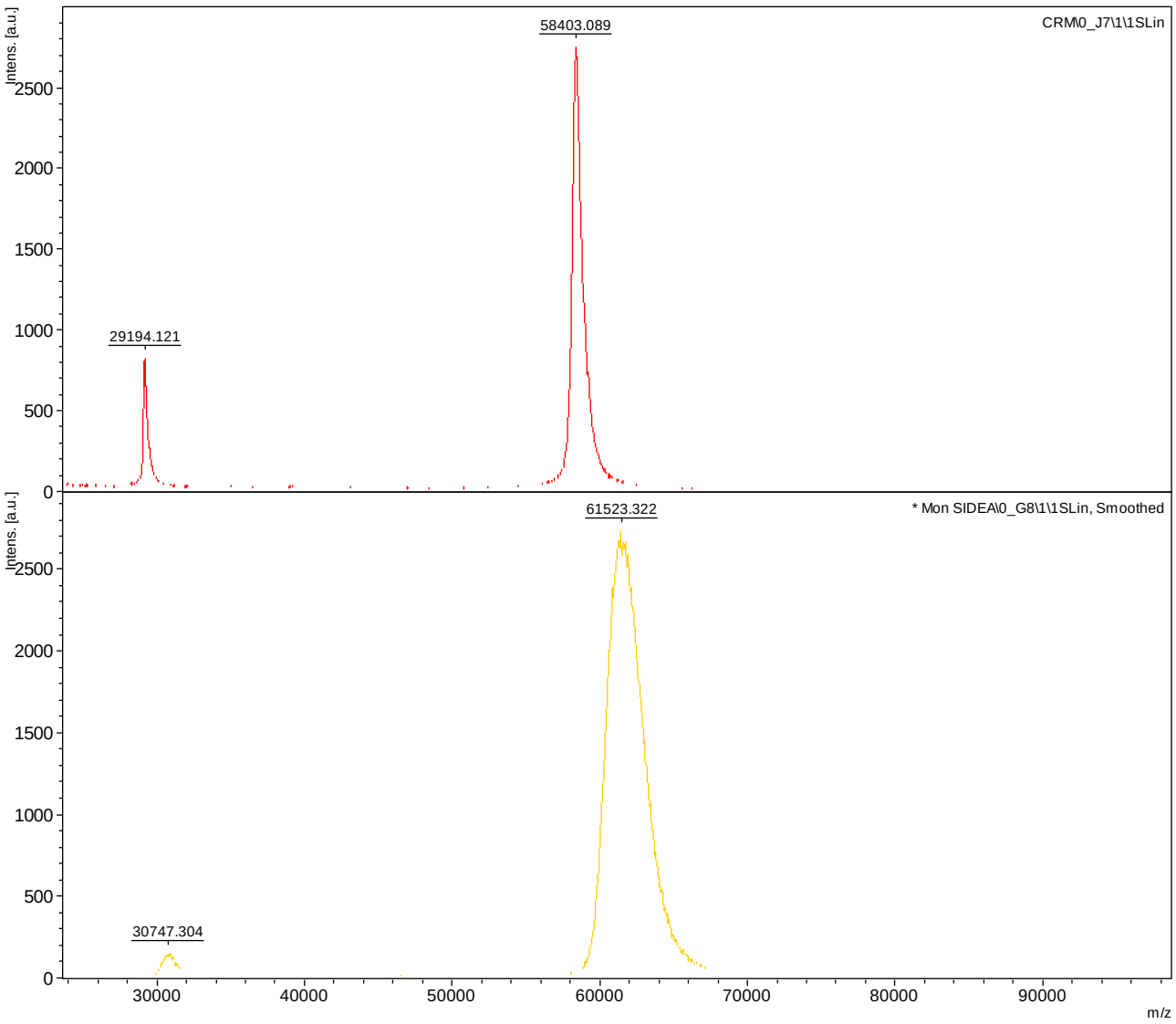


Figure 3

We made a second attempt of glycoconjugation: the pellet **32** (1.95 mg) was reacted, with a conjugation stoichiometry of 150:1 sugar/protein (mol/mol), with CRM_{197} (1.3 mg, 40 μL) in 100 μM NaPi buffer pH 7.2 (10 μL). After 6 hours the glycoconjugate was analysed by SDS-PAGE in 4-12% Bis-Tris Gel. The mixture was incubated over night at room temperature, mixing very gently with a magnetic stirrer. CRM_{197} glycoconjugate was analysed by SDS-PAGE in 4-12% Bis-Tris Gel (**Figure 4**), and MALDI-TOF mass spectrum was recorded (**Figure 5**): the found value for the glycoconjugate MG(CRM)_02 was 62258.89 m/z, corresponding to approximately 7 oligosaccharide chains per CRM_{197} molecule.

The glycoconjugate was not purified and left in the freezer (-20°C).

Experimental Section

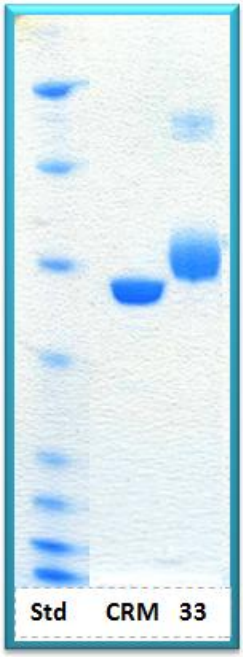


Figure 4

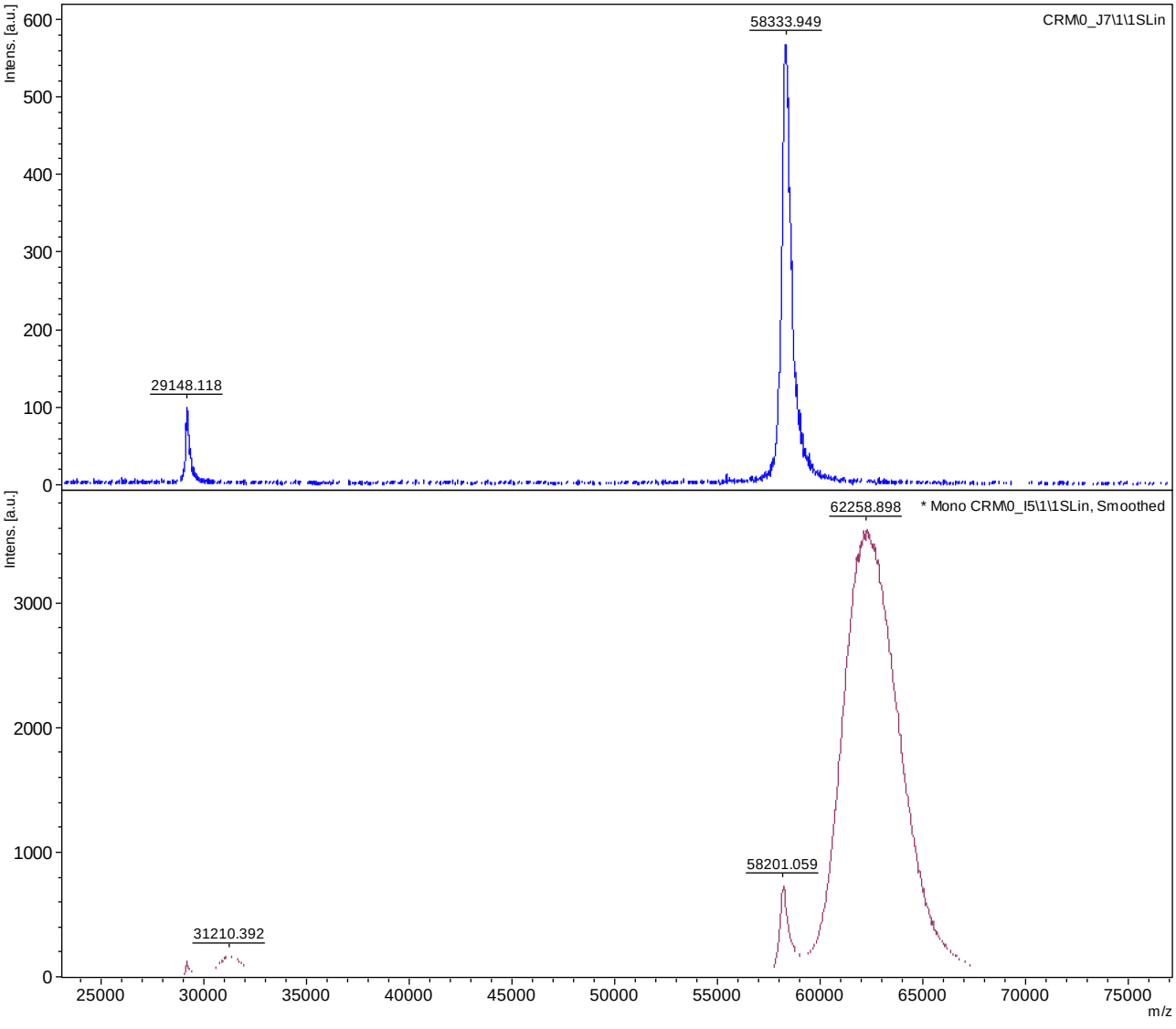
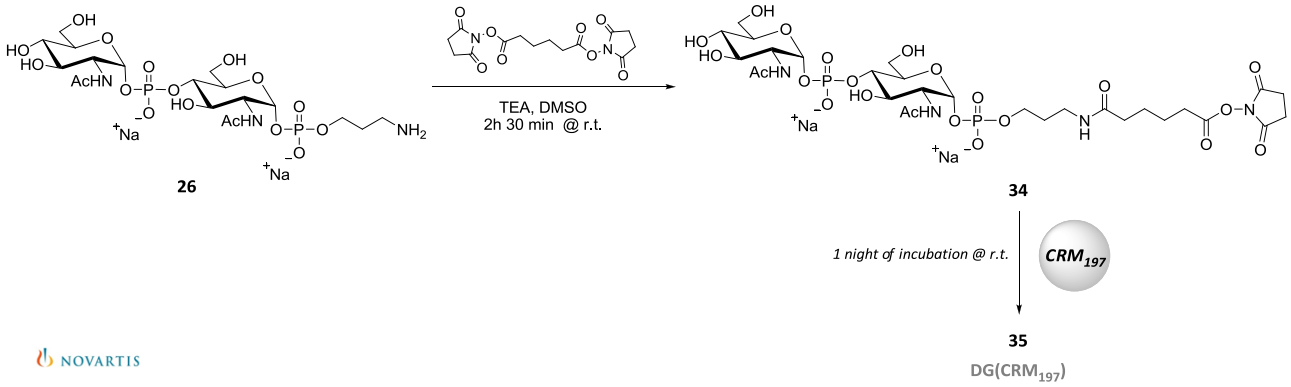


Figure 5

Dimer-Glycoconjugate(CRM$_{197}$) (35)



Synthetic procedure

reagents	M.W. (g/mol)	d (g/cm^3)	mmol	amount	eq
26	685.42	-	0.008	5.5 mg	1
disuccinimidyl adipate	340.29	-	0.08	28 mg	10
triethylamine	101.19	0.726	0.16	20 μL	20
DMSO	78.13	1.1	8.45	600 μL	-

Dimer **26** (8 μmol), dissolved in 200 μL of DMSO containing triethylamine (20 eq), was slowly dropped into a murky mixture of disuccinimidyl adipate (10 eq) in DMSO (200 μL). After 3 hours of vigorous stirring the activated oligosaccharide was purified from the excess of linker by precipitation of the reaction mixture in nine volumes (9 mL) of ethyl acetate. The pellet obtained by subsequent centrifugation was washed 10 times with 3 mL (1/3 of the volume) of ethyl acetate and then dried under vacuum (lyophilisation over night). We failed to determine the active ester groups.

Compound **34** (4 mg) was reacted with 2.5 mg of CRM$_{197}$ (76 μL of a solution 32.5mg/mL of protein in 10 mM NaPi buffer +10% saccharosium, pH 7.2) in 76 μL of 100 mM NaPi buffer (pH 7.2): the conjugation stoichiometry was about 100:1 sugar/protein (mol/mol). The mixture was incubated over night at room temperature, mixing very gently with a magnetic stirrer. The glycoconjugate **35** was purified from the excess of unconjugated carbohydrates using precipitation by $(NH_4)_2SO_4$.

Product characterization

35 was analysed by SDS-PAGE in 4-12% Bis-Tris Gel (Figure 6), and MALDI-TOF mass spectrum was recorded (Figure 7): the profile of the glycoconjugate **35** was composed of a polydispersion centered at 61731.51 m/z, corresponding to approximately 4 oligosaccharide chains per CRM$_{197}$ molecule.

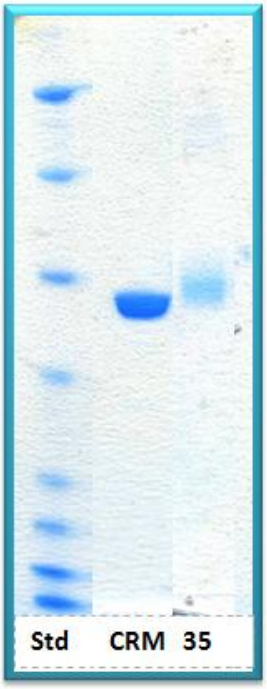


Figure 6

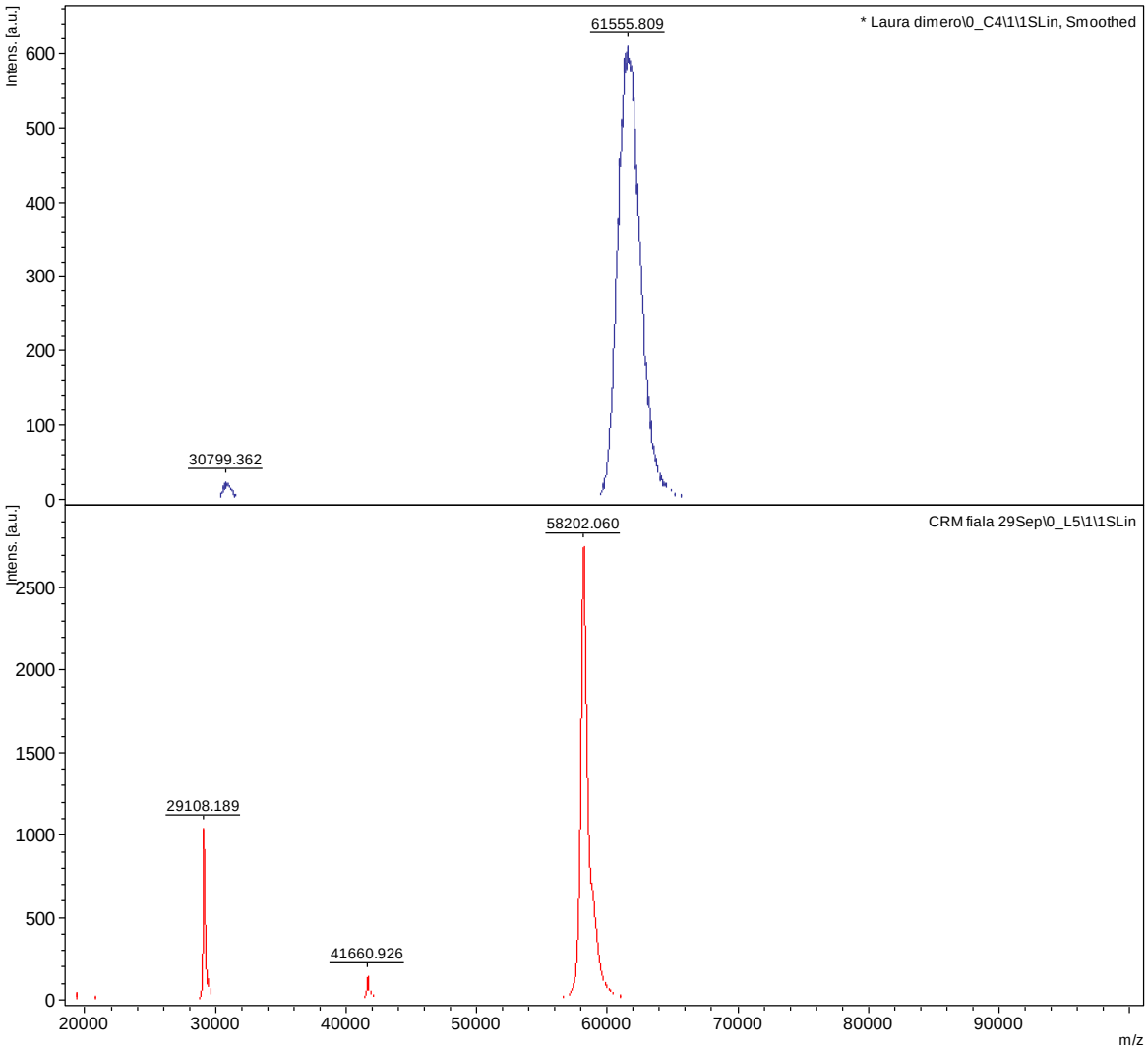
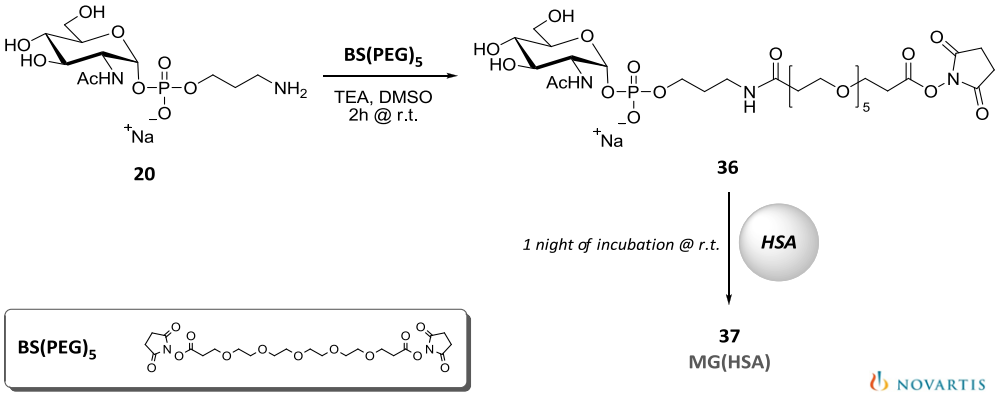


Figure 7

Monomer-Glycoconjugate(HSA) (37)



Synthetic procedure

reagents	M.W. (g/mol)	d (g/cm^3)	mmol	amount	eq
20	380.26	-	0.008	3.1 mg	1
bis(succinimidyl) penta(ethylene glycol)	535.5	-	0.08	42.84 mg 425 μL of a 100mg/mL solution	10
triethylamine	101.19	0.726	0.16	23 μL	20
DMSO	78.13	1.1	11.61	400 μL	-

Monomer **20** (8 μmol), dissolved in 200 μL of DMSO containing triethylamine (20 eq), was slowly dropped into a solution of BS(PEG)5 (10 eq) in DMSO. After 3 hours of vigorous stirring the activated oligosaccharide was purified from the excess of linker by precipitation of the reaction mixture in nine volumes (9 mL) of acetone. The pellet obtained by subsequent centrifugation was washed 10 times with 3 mL (1/3 of the volume) of acetone and then dried under vacuum (4 hours of lyophilisation). Compound **36** (1.95 mg) was reacted with HSA (1.3 mg, 40 μL) in 100 μM NaPi buffer pH 7.2 (10 μL), with a conjugation stoichiometry of 150:1 sugar/protein (mol/mol).

Product characterization

After 6 hours of incubation, mixing very gently with a magnetic stirrer, the glycoconjugate **37** was analysed by SDS-PAGE in 4-12% Bis-Tris Gel (Figure 8) and MALDI-TOF mass spectrum was recorded (Figure 9): the found value for **37** was 68320.268 m/z, corresponding to approximately 3 oligosaccharide chains per HSA molecule. The glycoconjugate was stored at -20°C for future purification.

Experimental Section

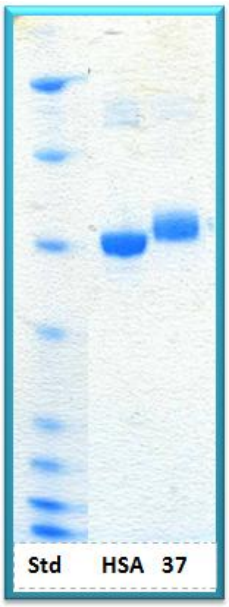


Figure 8

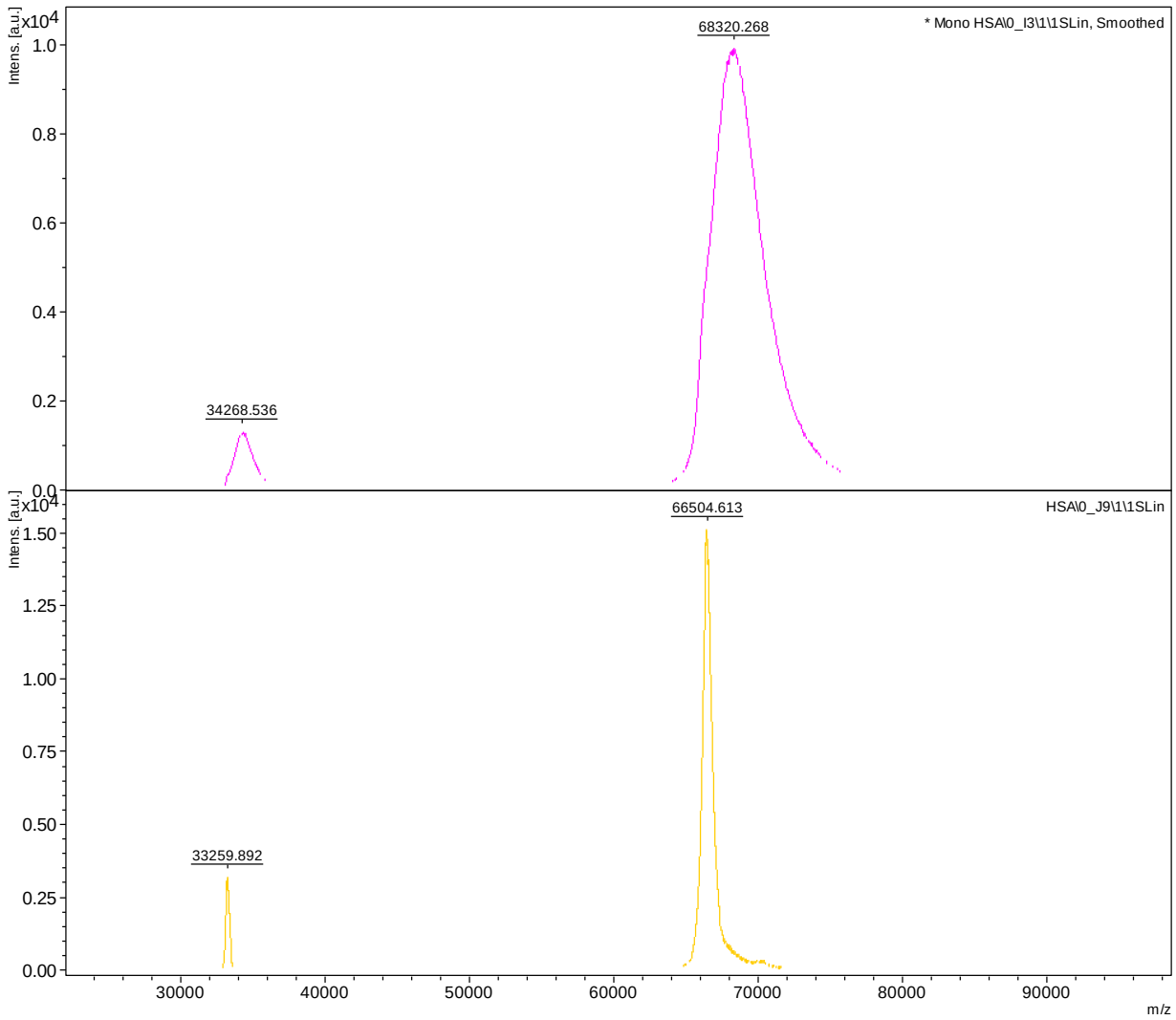
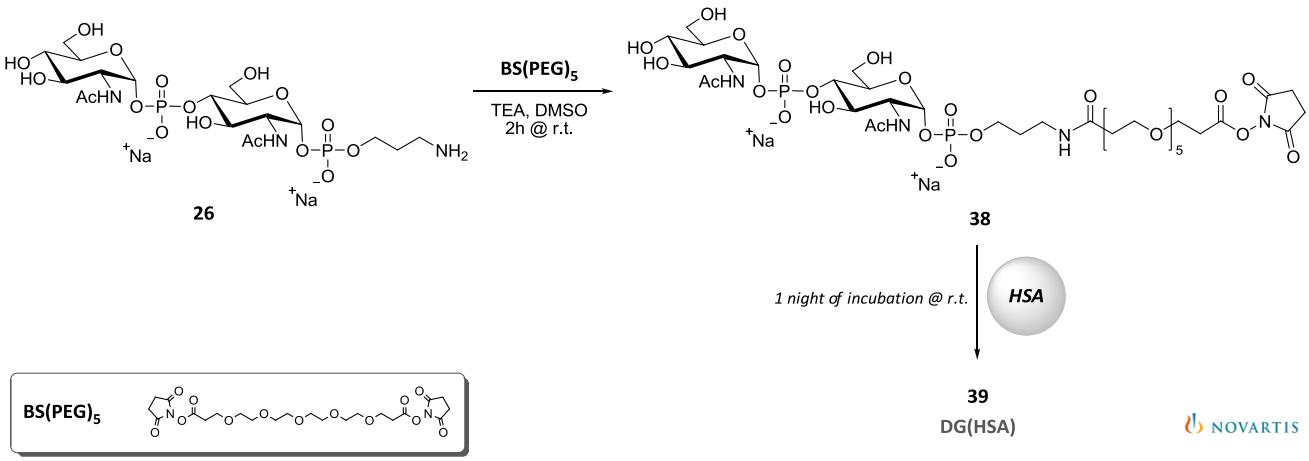


Figure 9

Dimer-Glycoconjugate(HSA) (39)



Synthetic procedure

reagents	M.W. (g/mol)	d (g/cm^3)	mmol	amount	eq
26	685.42	-	0.0047	3.23 mg	1
bis(succinimidyl) penta(ethylene glycol)	535.5	-	0.047	(25.17 mg) 250 μL of a 100mg/mL solution	10
triethylamine	101.19	0.726	0.094	17 μL	20
DMSO	78.13	1.1	11.61	300 μL	-

Dimer **26** (4.7 μmol), dissolved in 150 μL of DMSO containing triethylamine (20 eq), was slowly dropped into a solution of BS(PEG)5 (10 eq) in DMSO. After 3 hours of vigorous stirring the activated oligosaccharide was purified from the excess of linker by precipitation of the reaction mixture in nine volumes (9 mL) of acetone. The pellet obtained by subsequent centrifugation was washed 10 times with 3 mL (1/3 of the volume) of acetone and then dried under vacuum (4 hours of lyophilisation). Compound **38** (1.71 mg) was reacted with CRM_{197} (1 mg, 33 μL) in 100 μM NaPi buffer pH 7.2 (33 μL), with a conjugation stoichiometry of 100:1 sugar/protein (mol/mol). The mixture was incubated over night at room temperature, mixing very gently with a magnetic stirrer.

Product characterization

CRM_{197} glycoconjugate was analysed by SDS-PAGE in 4-12% Bis-Tris Gel (**Figure 10**), and MALDI-TOF mass spectrum was recorded (**Figure 11**). Both the characterizations highlighted the lack of conjugation: the found value for **39** corresponded to just 1 oligosaccharide chain per HSA molecule.

Experimental Section

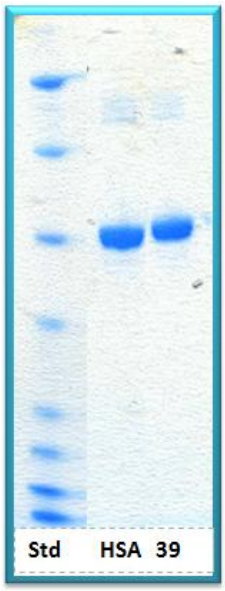


Figure 10

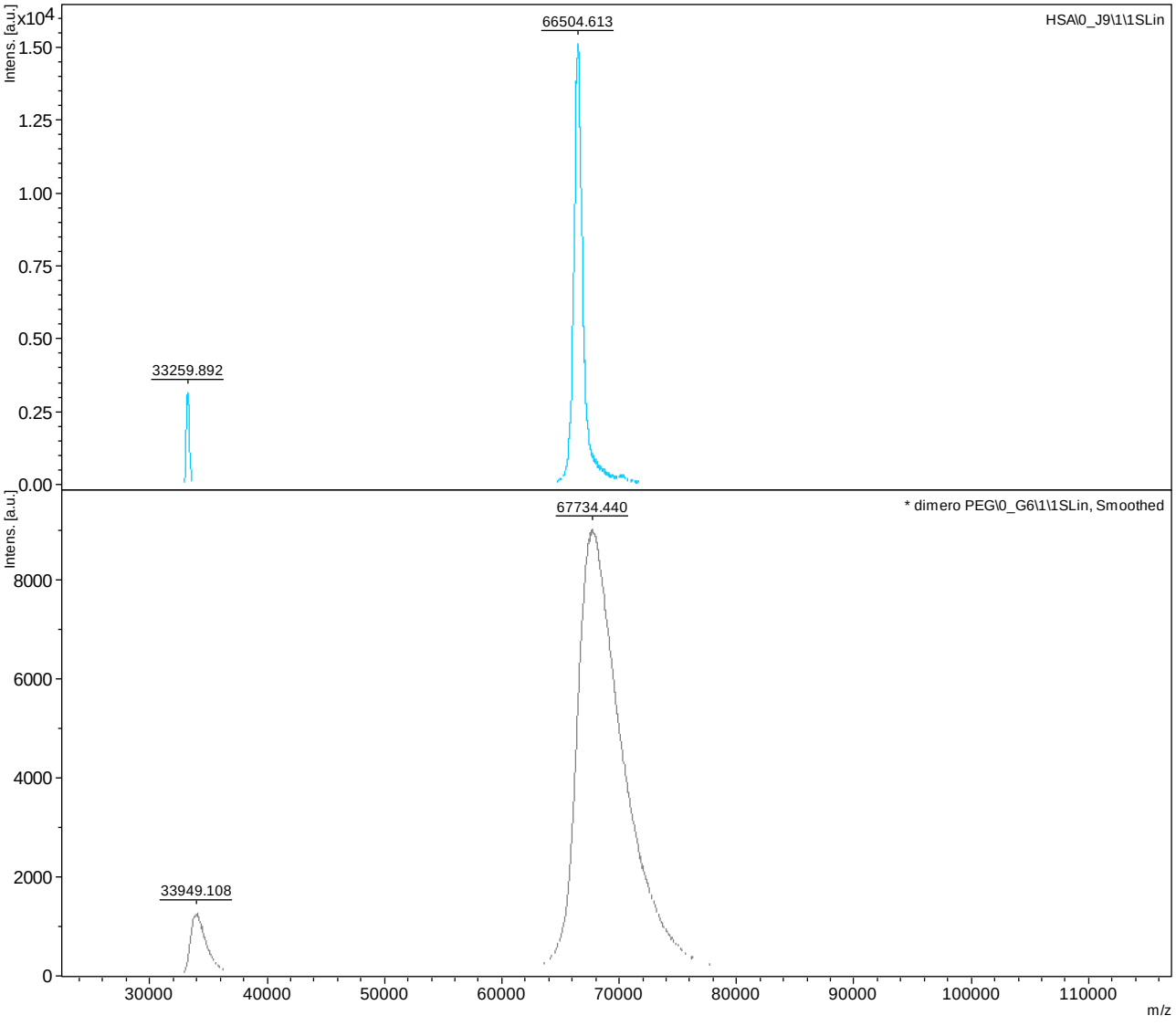
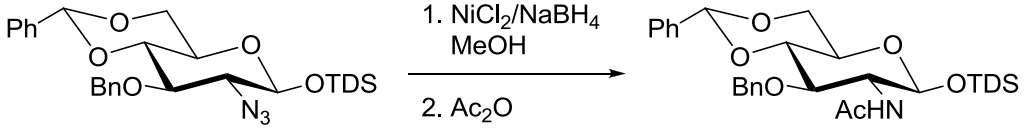


Figure 11

Thexyldimethylsilyl 2-Acetamido-3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside (40)



1 **40**

Synthetic procedure

Compound **1** (1.4 g, 2.7 mmol) was treated as described in the ***General Procedure F***. Product **40** is obtained in *quantitative* yield (1.45 g, 2.6 mmol).

Product characterization

Formula	$C_{30}H_{43}NO_6Si$
Molecular Weight	541.75 g/mol
TLC conditions	R_f (H/EA 9:1) 0.13

Optical rotation

$\alpha_D^{25} = -44.28$ (c = 1 in MeOH)

HRMS (ESI positive)

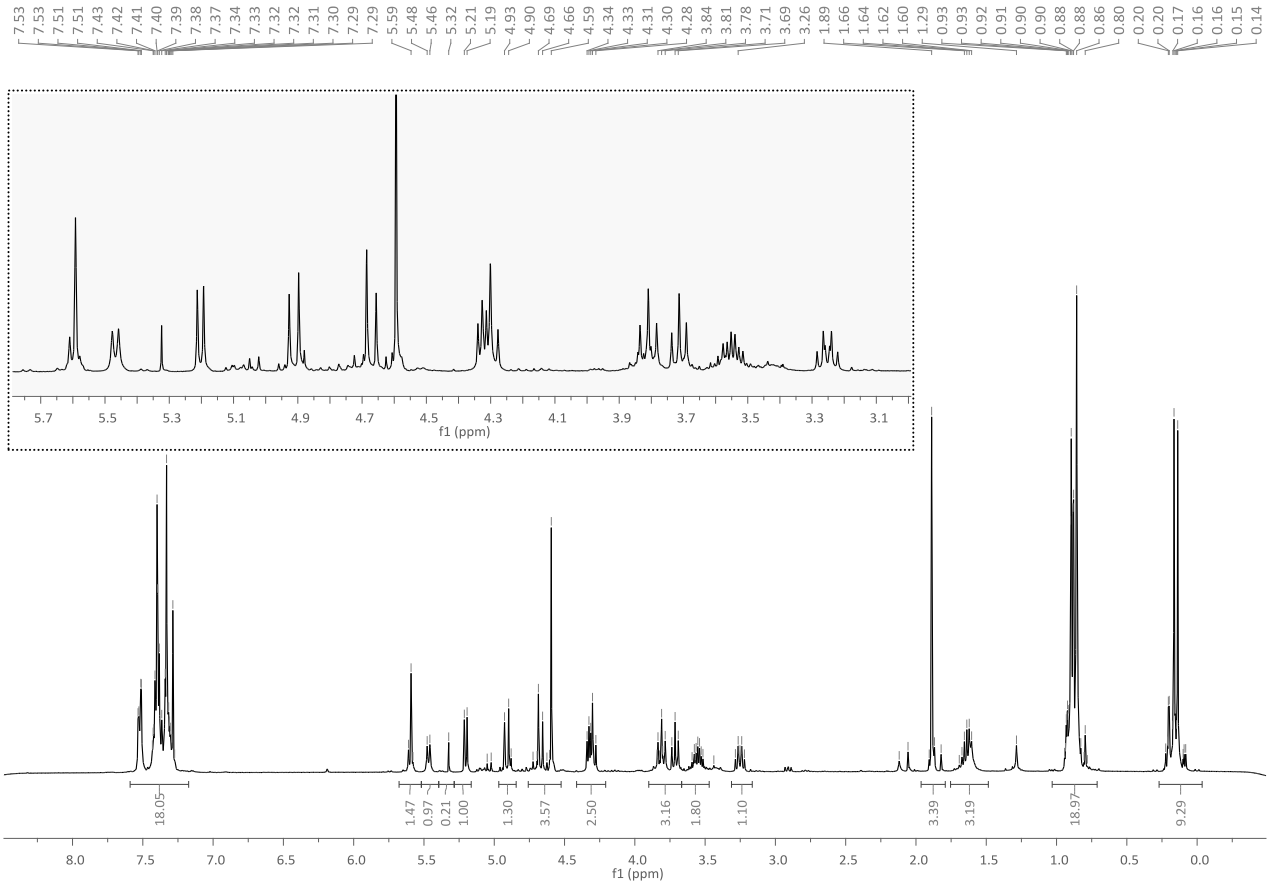
m/z (%): 541.9 (20) $[M]^+$

NMR

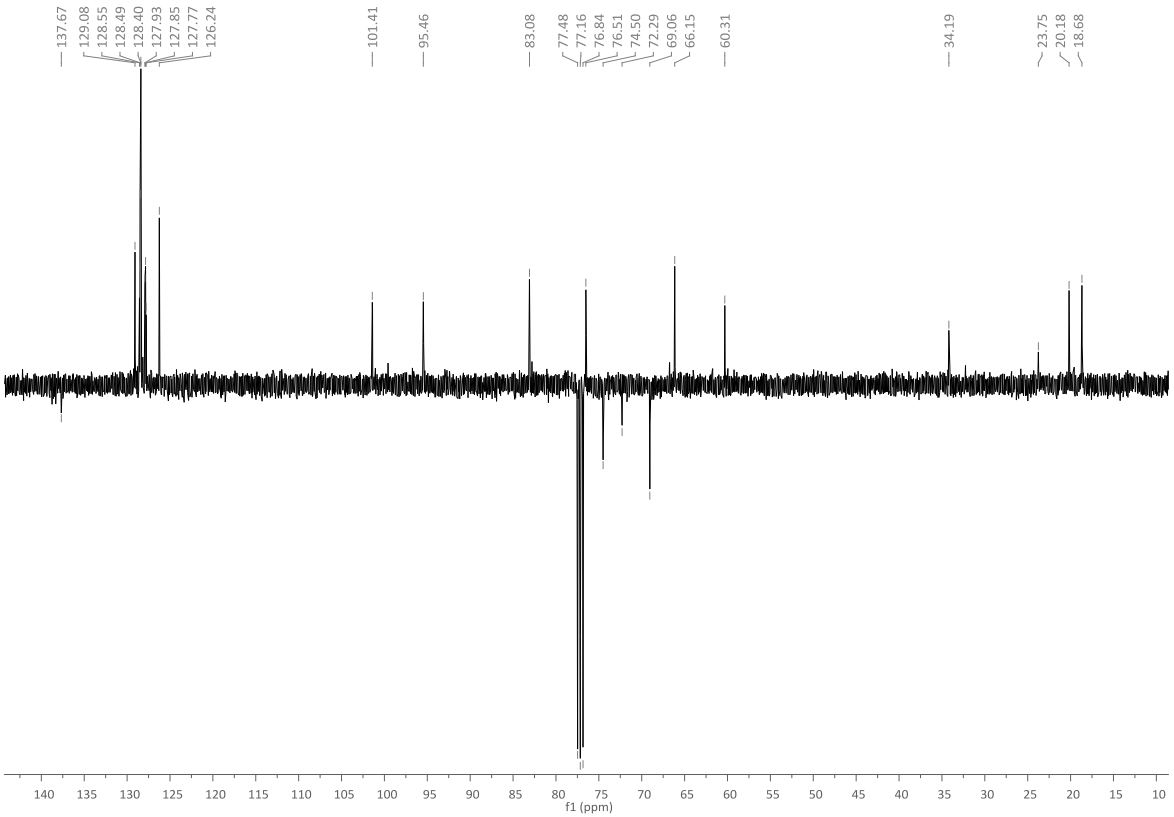
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.53 – 7.23 (m, Ar), 5.56 (s, 1H, C*H*Ph), 5.44 (d, *J* = 7.7 Hz, 1H, NH), 5.18 (d, $J_{1,2}$ = 7.8 Hz, 1H, H-1), 4.89 (d, *J* = 11.8 Hz, 1H, ½ CH_2Ph), 4.64 (d, *J* = 11.9 Hz, 1H, ½ CH_2Ph), 4.57 (s, 2H, CH_2Ph), 4.33 – 4.24 (m, 2H, H-6, H-3), 3.78 (t, $J_{6',6} = J_{6',5}$ = 10.3 Hz, 1H, H-6'), 3.69 (t, $J_{4,3} = J_{4,5}$ = 9.2 Hz, 1H, H-4), 3.60 – 3.45 (m, 1H, H-5), 3.23 (dt, $J_{2,3}$ = 10.1, $J_{2,1}$ = 7.8 Hz, 1H, H-2), 1.86 (s, 3H, CH_3CO), 1.71 – 1.50 (m, 1H, CH TDS), 0.88 – 0.85 (m, 3H, CH_3 TDS), 0.83 (s, 2H), 0.14 (s, *J* = 3.2 Hz, 1H), 0.11 (s, 1H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 170.14 (CO), 137.67, 137.64 (C_q Ar), 129.08, 128.55, 128.49, 128.40, 127.93, 127.85, 127.77, 126.24 (CH Ar), 101.39 (*C*HPh), 95.46 (C-1), 83.08 (C-4), 76.51(C-3), 74.50 (*C*H_2Ph), 72.29 (*C*H_2Ph), 69.06 (C-6), 66.15 (C-5), 60.31 (C-2), 34.22 (CH TDS), 24.96 (C_q TDS), 23.75 (*C*H_3CO), 20.18, 18.68 (CH_3 TDS), -1.07, -2.29 (CH_3Si TDS).

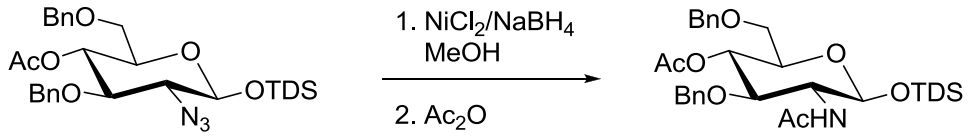
^{1}H (400 MHz, $CDCl_3$):



^{13}C (101 MHz, $CDCl_3$):



Thexyldimethylsilyl 2-Acetamido-4-*O*-acetyl-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranoside (41)



2 **41**

Synthetic procedure

Compound **2** (1.69 g, 2.98 mmol) was treated as described in the ***General Procedure F***. Product **41** was obtained in *quantitative* yield (1.7 g, 2.9 mmol).

Product characterization

Formula	$C_{32}H_{47}NO_7Si$
Molecular Weight	585.80 g/mol
TLC conditions	R_f (H/EA 9:1) 0.18

Optical rotation

α_D^{25} = - 2.92 (c = 1.3 in MeOH)

MS (ESI positive)

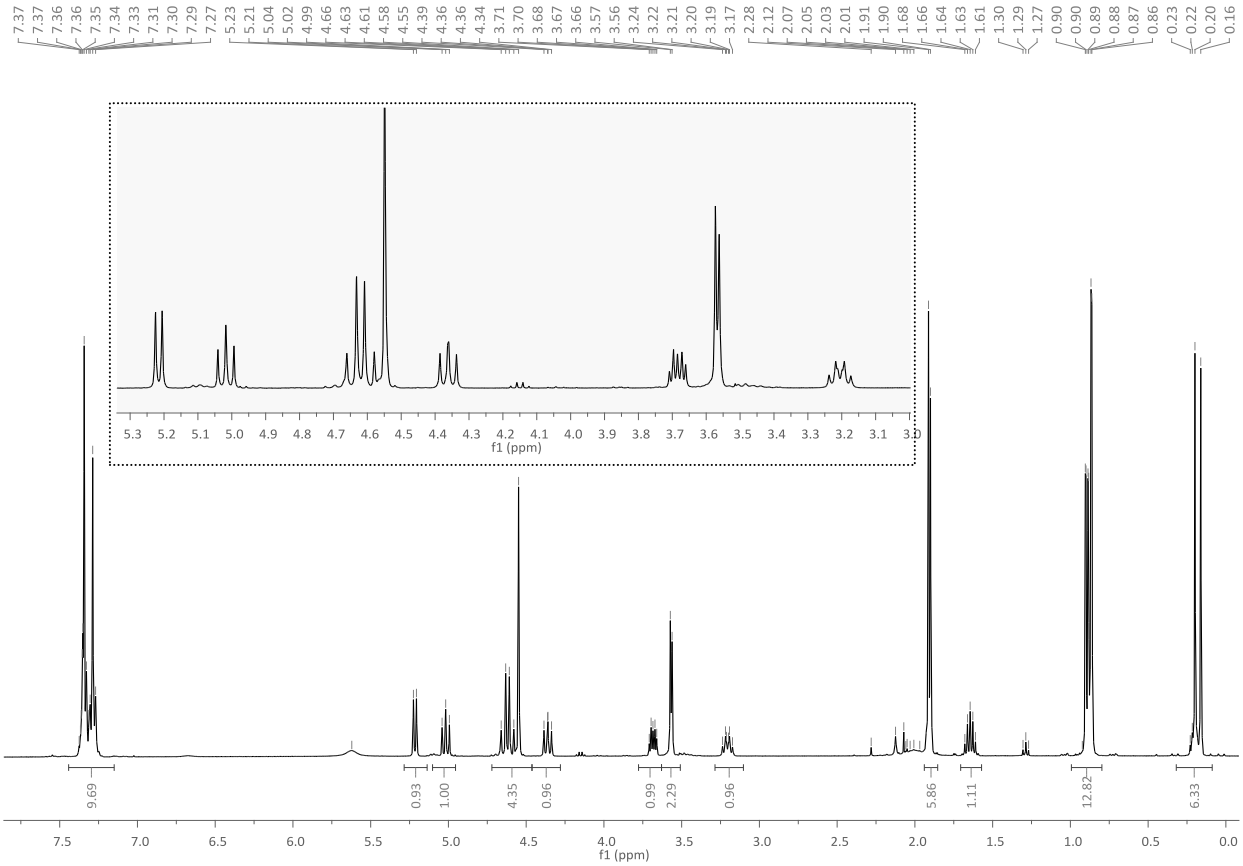
m/z (%): 608.5 (100) $[M+Na]^+$

NMR

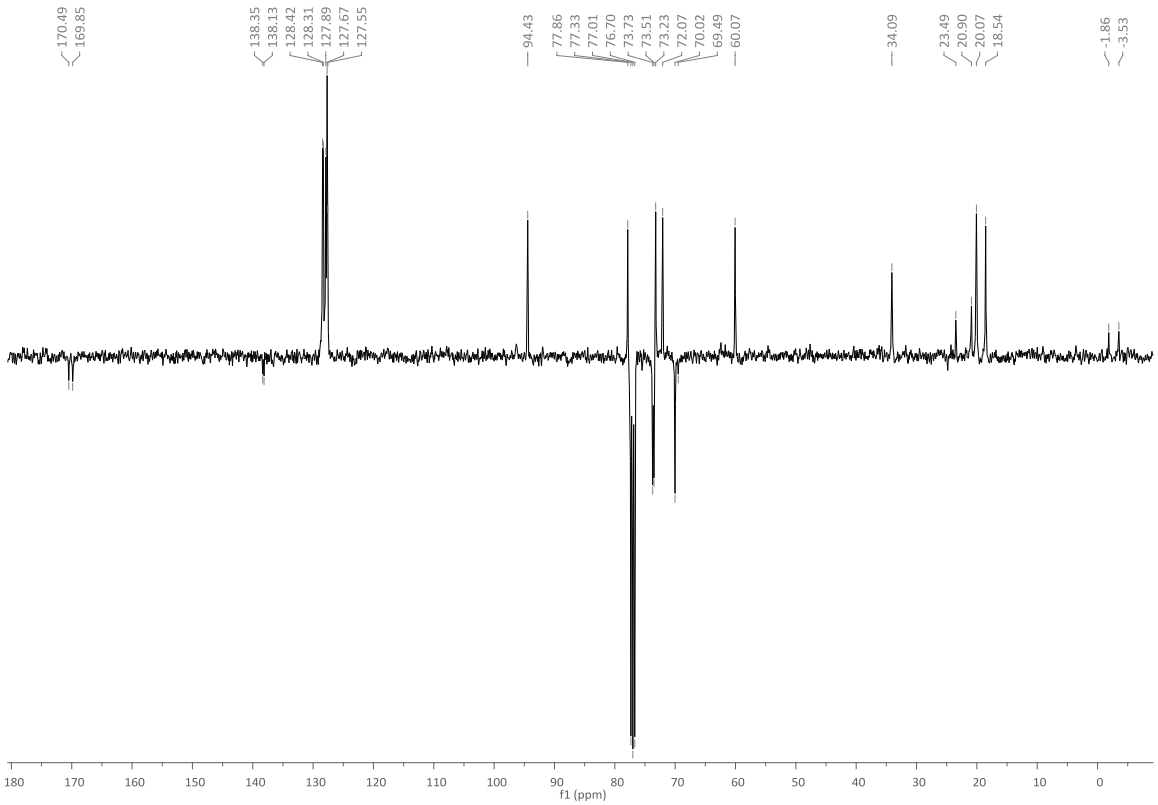
^{1}H NMR (400 MHz, $CDCl_3$) δ(ppm): 7.37 – 7.20 (m, 10H, Ar), 5.60 (s, 1H, NH), 5.19 (d, $J_{1,2}$ = 7.8 Hz, 1H, H-1), 4.99 (t, $J_{4,5}$ = $J_{4,3}$ = 9.5 Hz, 1H, H-4), 4.59 (q, *J* = 11.5 Hz, 2H, CH_2Ph), 4.52 (s, 2H, CH_2Ph), 4.33 (dd, $J_{3,2}$ = 10.3, $J_{3,4}$ = 9.5 Hz, 1H, H-3), 3.70 – 3.62 (m, 1H, H-5), 3.54 (d, *J* = 4.5 Hz, 2H, H-6, H-6'), 3.18 (dt, $J_{2,3}$ = 10.3, $J_{1,2}$ = 7.8 Hz, 1H, H-2), 1.88 (s, 3H, CH_3CO), 1.87 (s, 3H, CH_3CO), 1.62 (ept, *J* = 7.0 Hz, 1H, CH TDS), 0.87 (d, *J* = 1.8 Hz, 3H, CH_3 TDS), 0.86 (d, *J* = 1.7 Hz, 3H, CH_3 TDS), 0.84 (d, *J* = 2.2 Hz, 6H, 2 CH_3 TDS), 0.17 (s, 3H, CH_3Si TDS), 0.13 (s, 3H, CH_3Si TDS).

^{13}C NMR (101 MHz, $CDCl_3$) δ(ppm): 170.63, 169.99 (CO), 138.49, 138.27 (C_q Ar), 128.89, 128.76, 128.57, 128.45, 128.03, 127.82, 127.70 (CH Ar), 94.58 (C-1), 78.00 (C-3), 73.87 (CH_2Ph), 73.65 (CH_2Ph), 73.37 (C-5), 72.21 (C-4), 70.16 (C-6), 60.21 (C-2), 34.23 (CH TDS), 24.96 (C_q TDS), 23.63 (CH_3CO), 21.04, 20.22 , 18.69 (CH_3 TDS), -1.71, -3.39 (CH_3Si TDS).

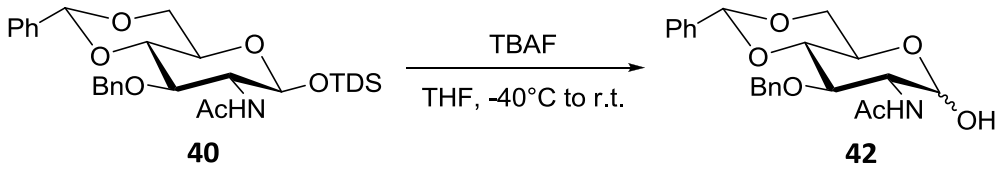
^{1}H (400 MHz, $CDCl_3$):



^{13}C (101 MHz, $CDCl_3$):



2-Acetamido-3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-α/β-D-glucopyranose (42)



40 **42**

Synthetic procedure

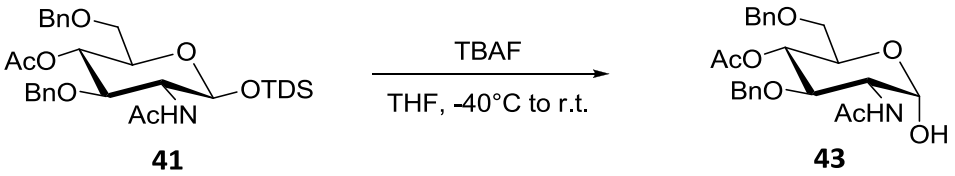
Compound **40** (1.4 g, 2.7 mmol) was treated as described in the ***General Procedure A***; but without the addition of AcOH. Product **42** was obtained in 85% yield (540 mg, 2.6 mmol).

Product characterization

Formula	$C_{22}H_{25}NO_6$
Molecular Weight	339.44 g/mol
TLC conditions	R_f (H/EA 1:1) 0.10

The spectroscopic data were in agreement with those reported in literature.[21]

2-Acetamido-4-*O*-acetyl-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranose (43)



41 **43**

Synthetic procedure

Compound **41** (1.3 g, 2.2 mmol) was treated as described in the ***General Procedure A***; but without the addition of AcOH. Product **43** is obtained in 81% yield (789.5 mg, 2.6 mmol).

Product characterization

Formula	$C_{24}H_{29}NO_7$
Molecular Weight	443.49 g/mol
TLC conditions	R_f (DCM/MeOH 9:1) 0.49

Optical rotation

$\alpha_D^{25} = +38.46$ (c = 1.1 in MeOH)

MS (ESI positive)

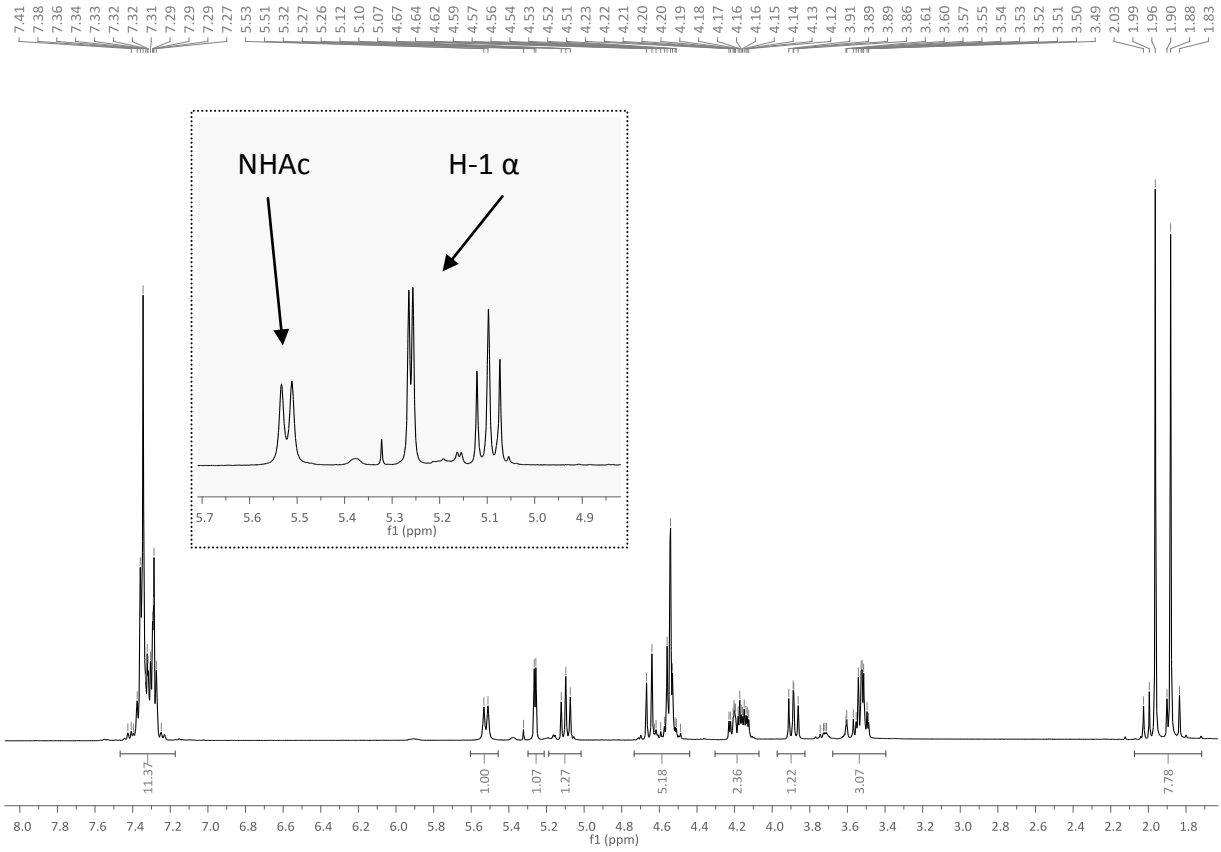
m/z (%): 466.4 (100) $[M+Na]^+$

NMR

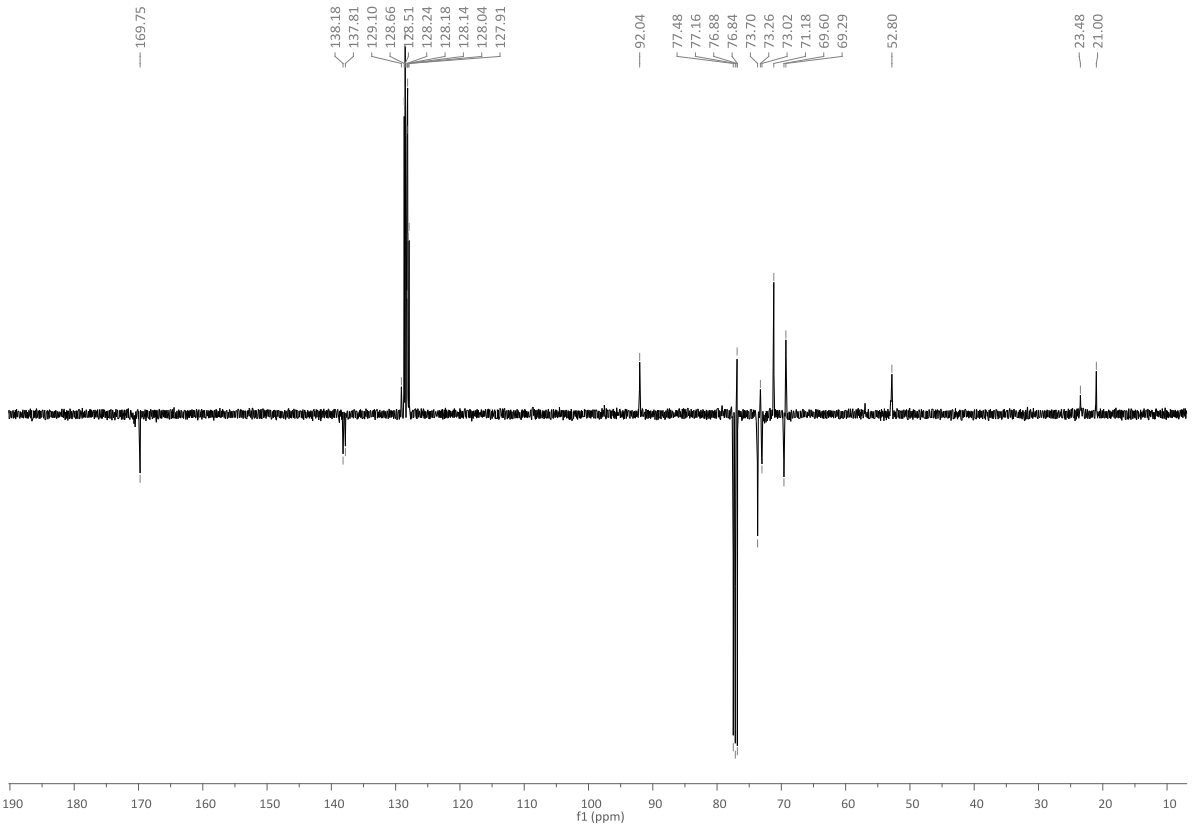
^{1}H NMR (400 MHz, $CDCl_3$) δ(ppm): 7.36 – 7.23 (m, 10H, Ar), 5.49 (d, *J* = 8.7 Hz, 1H, NH), 5.23 (d, $J_{1,2}$ = 3.5 Hz, 1H, H-1), 5.07 (t, $J_{4,5} = J_{4,3}$ = 9.5 Hz, 1H, H-4), 4.63 (d, *J* = 11.5 Hz, 1H, ½ CH_2Ph), 4.52 (d, *J* = 11.5 Hz, 1H, ½ CH_2Ph), 4.51 (s, 2H, CH_2Ph), 4.23 – 4.05 (m, 2H, H-2, H-5), 3.86 (dd, $J_{3,2}$ = 10.5, $J_{3,4}$ = 9.5 Hz, 1H, H-3), 3.53 – 3.45 (m, 2H, H-6, H-6'), 1.94 (s, 3H, CH_3CO), 1.85 (s, 3H, CH_3CO).

^{13}C NMR (101 MHz, $CDCl_3$) δ 170.52, 169.75 (CO), 138.18, 137.81 (C_q Ar), 128.66, 128.51, 128.24, 128.18, 128.14, 128.04, 127.91 (CH Ar), 92.04 (C-1), 76.84 (C-3), 73.70 (CH_2Ph), 73.02 (CH_2Ph), 71.23 (C-4), 69.60 (C-6), 69.56 (C-5), 52.96 (C-2), 23.45 (CH_3CO), 21.02 (CH_3CO).

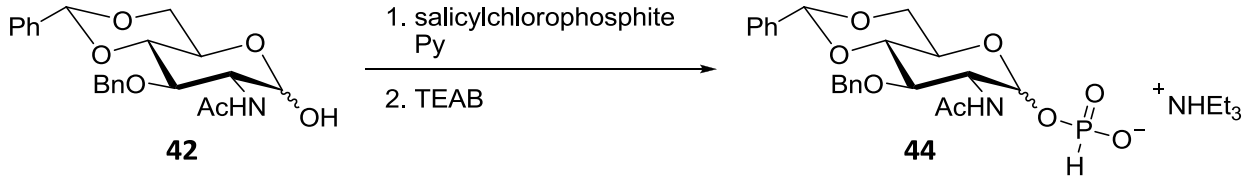
^{1}H (400 MHz, $CDCl_3$):



^{13}C (101 MHz, $CDCl_3$):



2-Acetamido-3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-D-glucopyranosyl Hydrogen-phosphonate, triethylammonium salt (44), α/β mixture



42 **44**

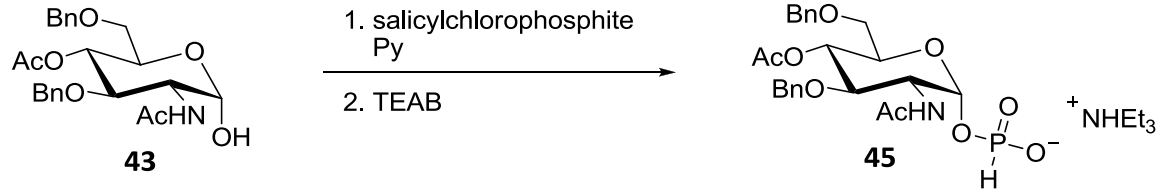
Synthetic procedure

Salicylchlorophosphite (146 mg, 0.72 mmol) was slowly added to a solution of the alcohol **42** (192 mg, 0.48 mmol) in dry pyridine (1.9 mL) at 0°C. The reaction was stirred at r.t. for 4 hours. Then a 1M solution of TEAB (4 mL/mmol) was added to the reaction at r.t., and the mixture was diluted with CH_2Cl_2, washed three times with cold TEAB (0.5 M), dried (Na_2SO_4), filtered and concentrated. The crude was purified by flash chromatography. The H-phosphonate has to be stabilized, washing with 0.25M cold TEAB [then drying (Na_2SO_4), filtering and concentrating] the purified product **44** (144 mg, 0.255 mmol, 75% yield).

Product characterization

Formula	$C_{28}H_{41}N_2O_8P$
Molecular Weight	564.61 g/mol
TLC conditions	R_f (DCM/MeOH 9:1) 0.12

2-Acetamido-4-*O*-acetyl-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl Hydrogen-phosphonate, triethylammonium salt (45)



Synthetic procedure

Salicylchlorophosphite (96 mg, 0.473 mmol) was slowly added to a solution of the alcohol **43** (140 mg, 0.315 mmol) in dry pyridine (1 mL) at 0°C. The reaction was stirred at r.t. for 3.5 hours. Then a 1M solution of TEAB (4 mL/mmol) was added to the reaction at r.t., and the mixture was diluted with CH_2Cl_2, washed three times with cold TEAB (0.5 M), dried (Na_2SO_4), filtered and concentrated. The crude was purified by flash chromatography. The H-phosphonate has to be stabilized, washing with 0.25M cold TEAB [then drying (Na_2SO_4), filtering and concentrating] the purified product **45** (119 mg, 0.195 mmol, 62% yield).

Product characterization

Formula	$C_{30}H_{45}N_2O_9P$
Molecular Weight	608.66 g/mol
TLC conditions	R_f (DCM/MeOH 9:1) 0.19

MS (ESI)

Experimental m/z (%): 506.2 (70) $[M]^-$

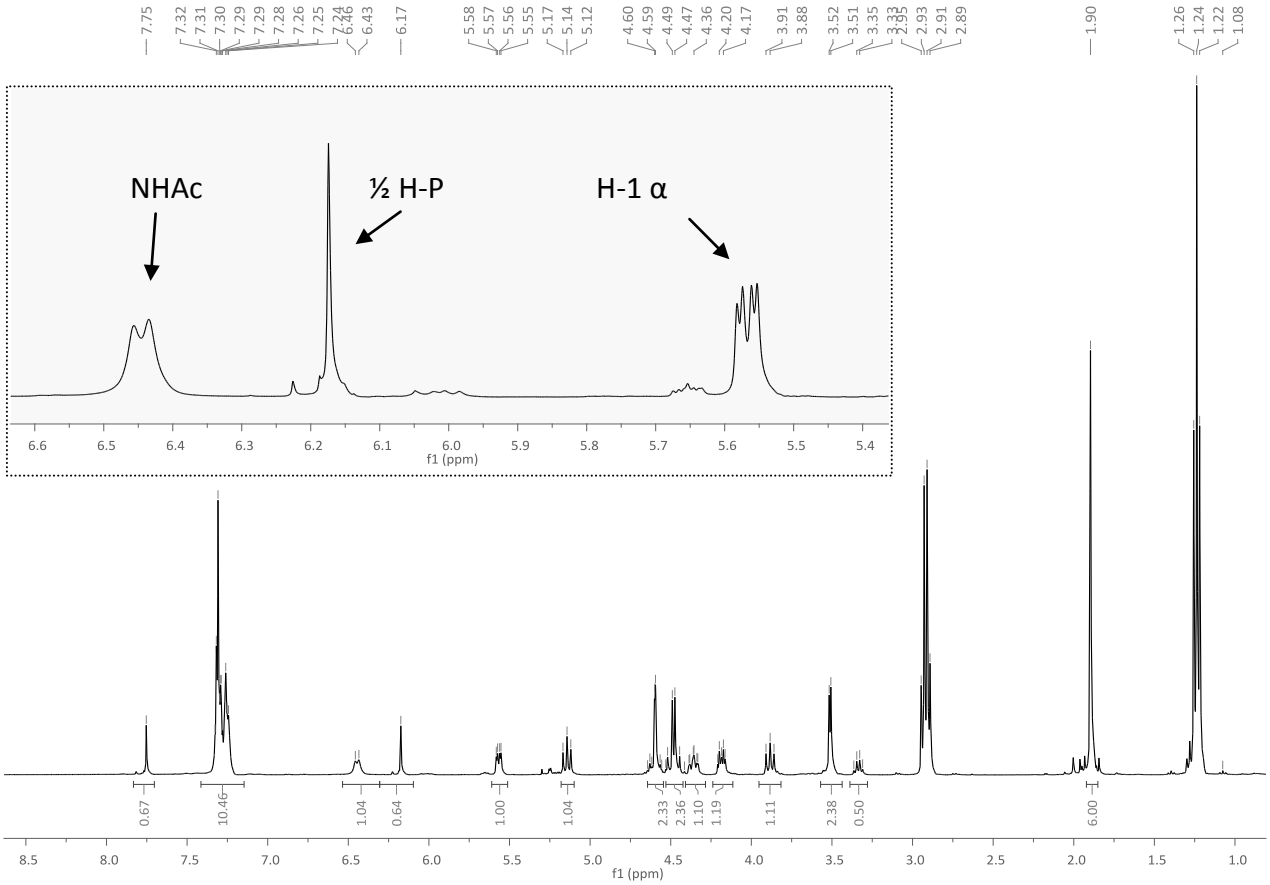
NMR

^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 7.33 – 7.15 (m, 10H, Ar), 6.93 (d, *J* = 631.8 Hz, 1H, *H*-P), 6.41 (d, *J* = 8.8 Hz, 1H, N*H*), 5.53 (dd, $J_{1,P}$ = 8.4, $J_{1,2}$ = 3.2 Hz, 1H, H-1), 5.11 (t, $J_{4,3} = J_{4,5}$ = 9.7 Hz, 1H, H-4), 4. 57 (s, 1H, ½ CH_2Ph), 4.56 (s, 1H, ½ CH_2Ph), 4.50 – 4.40 (m, 2H, CH_2Ph), 4.37 – 4.26 (m, 1H, H-2), 4.19 – 4.11 (m, 1H, H-5), 3.85 (t, $J_{3,4}$ = 9.7 Hz, 1H, H-3), 3.50 – 3.43 (m, 2H, H-6, H-6'), 1.86 (s, 6H, CH_3CO).

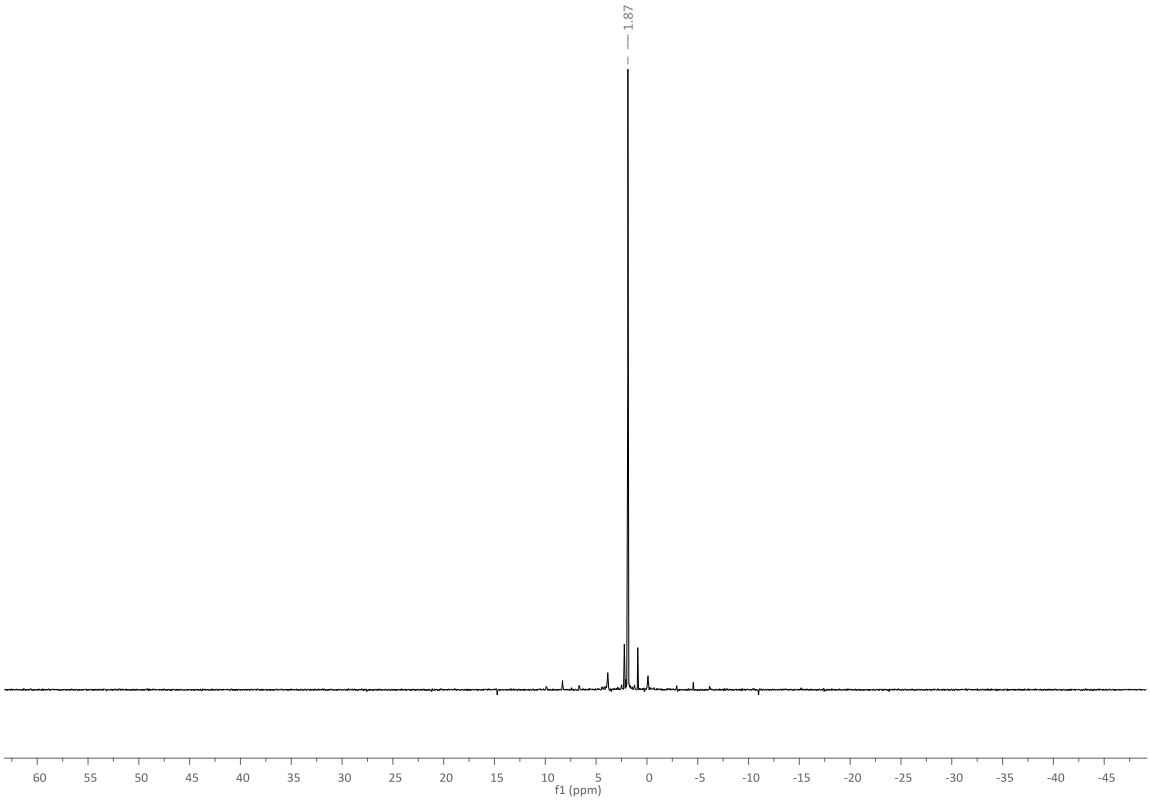
^{31}P NMR (162 MHz, $CDCl_3$) δ (ppm) 1.87.

^{13}C NMR (101 MHz, $CDCl_3$) δ 170.22, 169.67 (CO), 138.46, 138.07 (C_q Ar), 128.44, 128.36, 127.99, 127.69 (CH Ar), 93.35, 93.29 (C-1), 77.96 (C-3), 73.52 (*C*H_2Ph), 73.39 (*C*H_2Ph), 71.00 (C-4), 70.39 (C-5), 69.65 (C-6), 52.44, 52.39 (C-2), 23.35 (*C*H_3CO), 20.91 (*C*H_3CO).

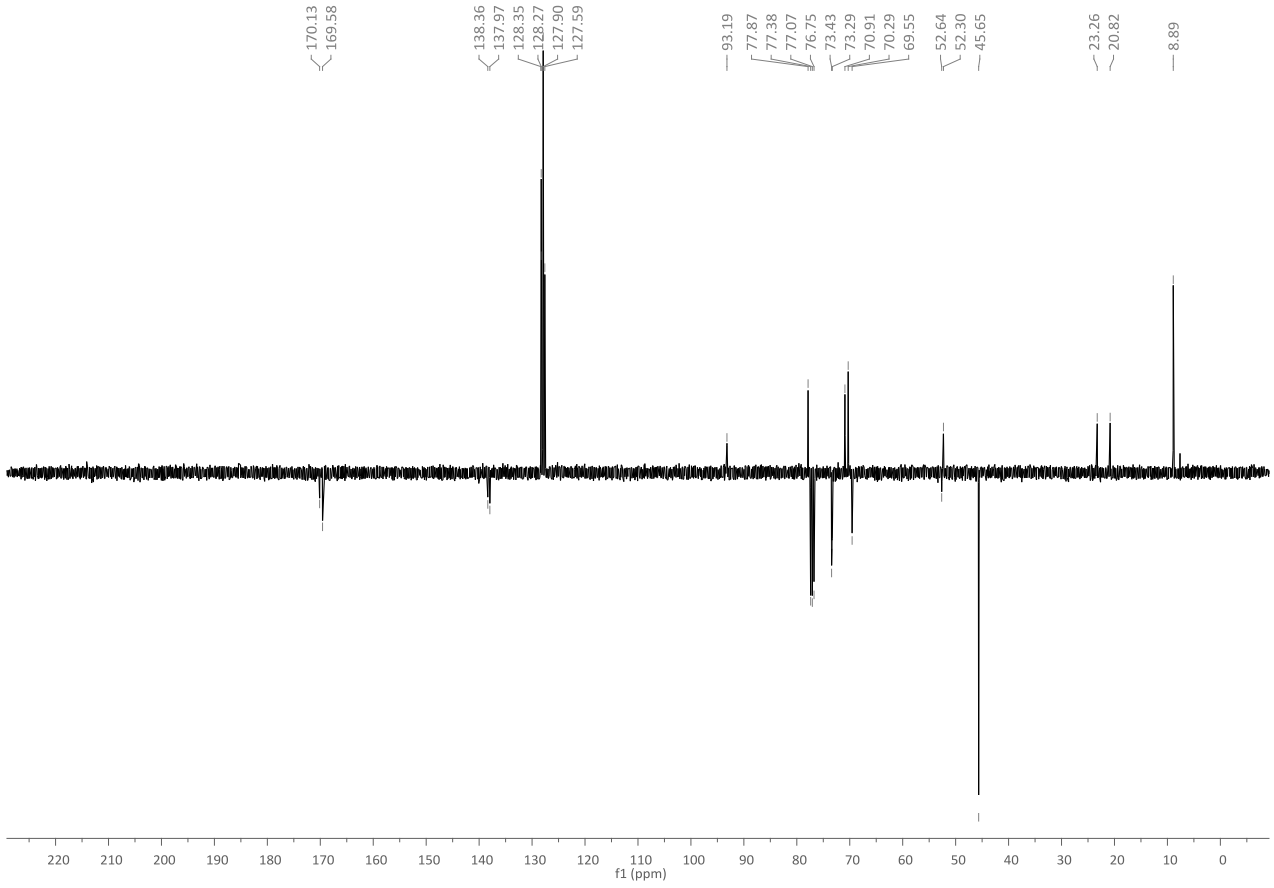
^{1}H (400 MHz, $CDCl_3$):



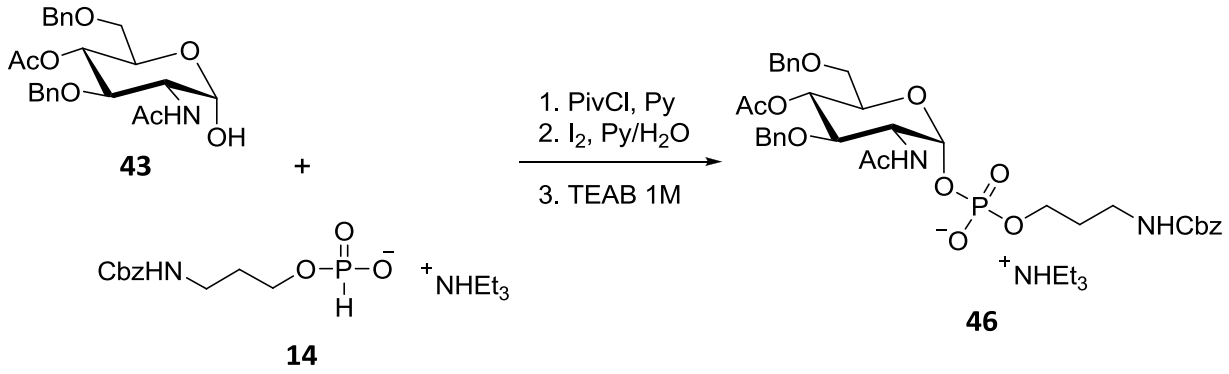
^{31}P NMR (162 MHz, $CDCl_3$):



^{13}C (101 MHz, $CDCl_3$):



3-(*N*-Carbobenzyloxy)aminopropyl 1-*O*-(2-acetamido-4-*O*-acetyl-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl phosphate), triethylammonium salt (46)



Synthetic procedure

Donor **14** (320 mg, 0.85 mmol) and acceptor **43** (310 mg, 0.69 mmol) were treated as described in the ***General Procedure E***. Compound **46** was obtained in 45% yield (253.3 mg, 0.31mmol).

Product characterization

Formula	$C_{41}H_{85}N_3O_{12}P$
Molecular Weight	815.89 g/mol
TLC conditions	R_f (DCM/MeOH 8:2) 0.31

Optical rotation

$\alpha_D^{25} = +39.47$ (c = 1.4 in $CHCl_3$)

MS (ESI)

Experimental m/z (%): 713.3 (100) $[M]^-$

HRMS (ESI)

Experimental m/z (%): 713.24653 (100) $[M]^-$

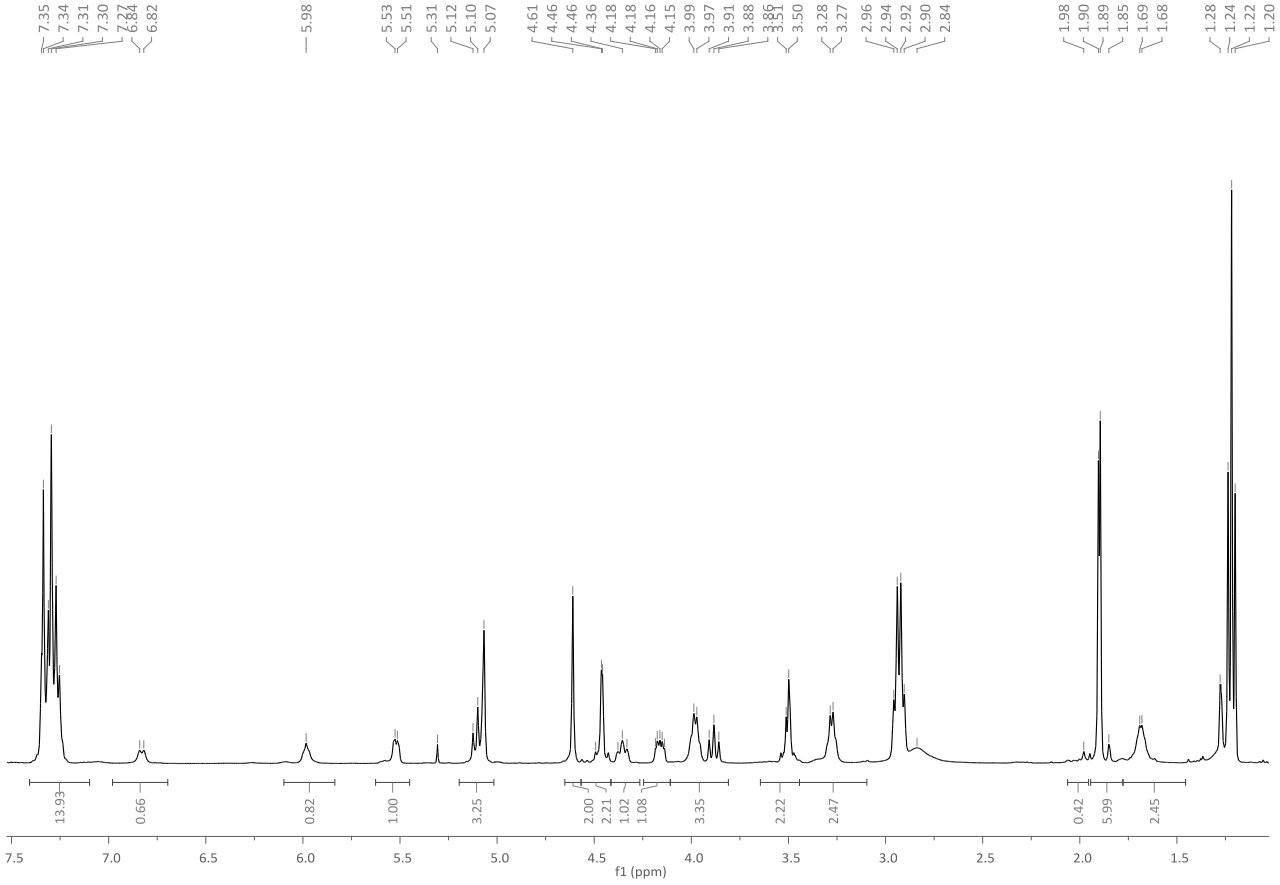
NMR

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.34 – 7.18 (m, 15H, Ar), 6.79 (d, *J* = 8.5 Hz, 1H, N*H*Ac), 5.95 (t, *J* = 5.4 Hz, 1H, N*H*Cbz), 5.49 (dd, $J_{1,P}$ = 7.5, $J_{1,2}$ = 3.5 Hz, 1H, H-1), 5.12 – 4.99 (m, 3H, H-4, C*H*$_2$Ph), 4.58 (s, 2H, C*H*$_2$Ph), 4.47 – 4.37 (m, 2H, C*H*$_2$Ph), 4.36 – 4.27 (m, 1H, H-2), 4.18 – 4.08 (m, 1H, H-5), 4.00 – 3.90 (m, 2H, C*H*$_2$O*linker*), 3.85 (t, $J_{3,4}$ = $J_{3,2}$ = 9.9 Hz, 1H, H-3), 3.52 – 3.39 (m, 2H, H-6, H-6'), 3.30 – 3.18 (m, 2H, C*H*$_2$NH), 1.87 (s, 3H, C*H*$_3$CO), 1.86 (s, 3H, C*H*$_3$CO), 1.65 (m, 2H, C*H*$_2$*linker*).

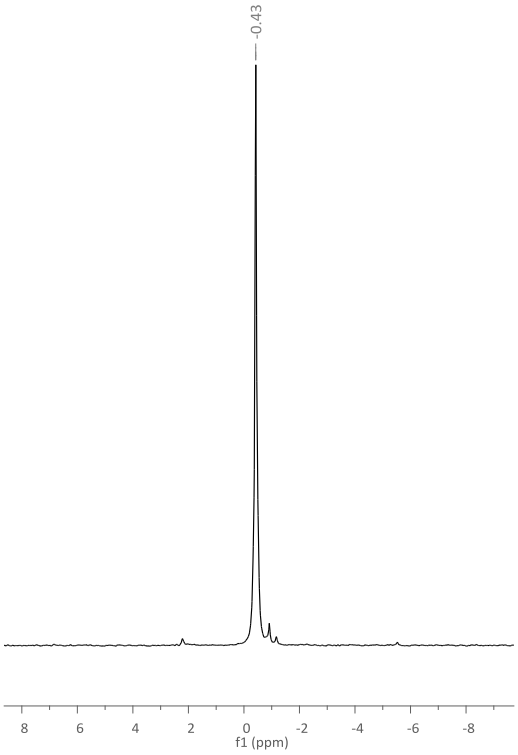
^{31}P NMR (162 MHz, $CDCl_3$) δ -0.43.

^{13}C NMR (101 MHz, $CDCl_3$) δ 170.60, 169.75 (CO), 156.66 (C_q Ar-NH), 138.45, 137.89, 136.95 (C_q Ar), 128.56, 128.44, 128.40, 128.10, 128.08, 128.01, 127.79, 127.69 (CH Ar), 94.66 (C-1), 77.59 (C-3), 73.56 (CH_2Ph), 73.39 (CH_2Ph), 70.95 (C-4), 70.47 (C-5), 69.79 (C-6), 66.51 (CH_2Ph), 63.19 (*C*H_2O*linker*), 52.69 (C-2), 37.51 (*C*H_2NH), 30.47 (*C*$H_{2\ LINKER}$).

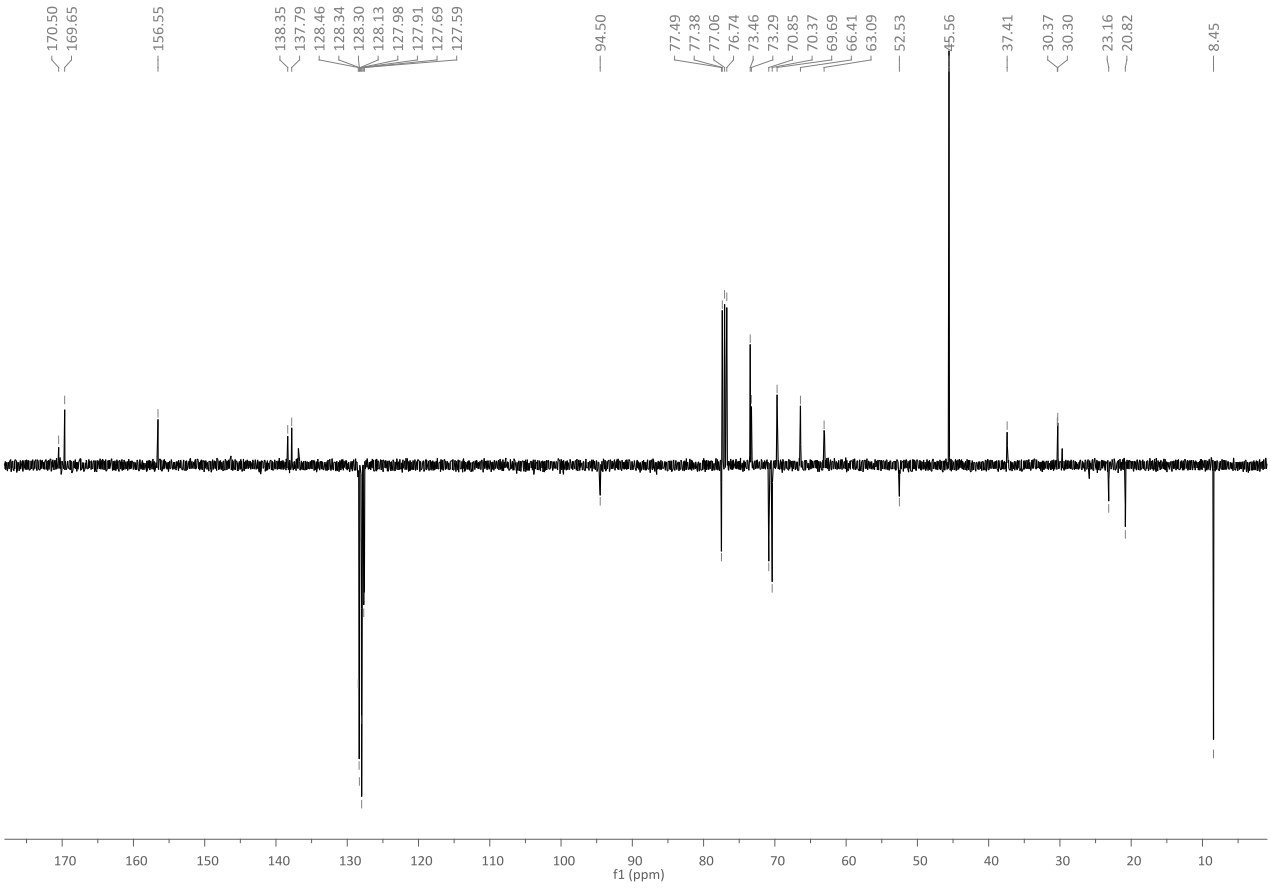
^{1}H (400 MHz, $CDCl_3$):



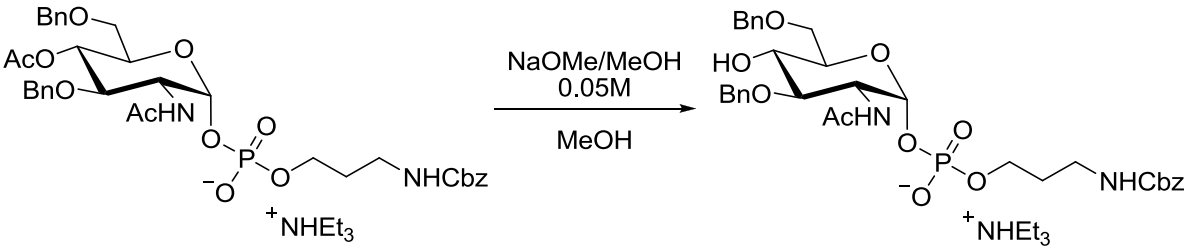
^{31}P NMR (162 MHz, $CDCl_3$):



^{13}C (101 MHz, $CDCl_3$):



3-(*N*-Carbobenzyloxy)aminopropyl 1-*O*-(2-acetamido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl phosphate), triethylammonium salt (47)



46 **47**

Synthetic procedure

Compound **46** (60.3 mg, 0.075 mmol) was treated as described in the ***General Procedure C***. Product **47** (55.32 mg, 0.073 mmol) was obtained in *quantitative* yield.

Product characterization

Formula	$C_{39}H_{56}N_3O_{11}P$
Molecular Weight	773.80 g/mol
TLC conditions	R_f (DCM/MeOH 8:2) 0.31

MS (ESI)

Experimental m/z (%): 671.3 (100) $[M]^-$

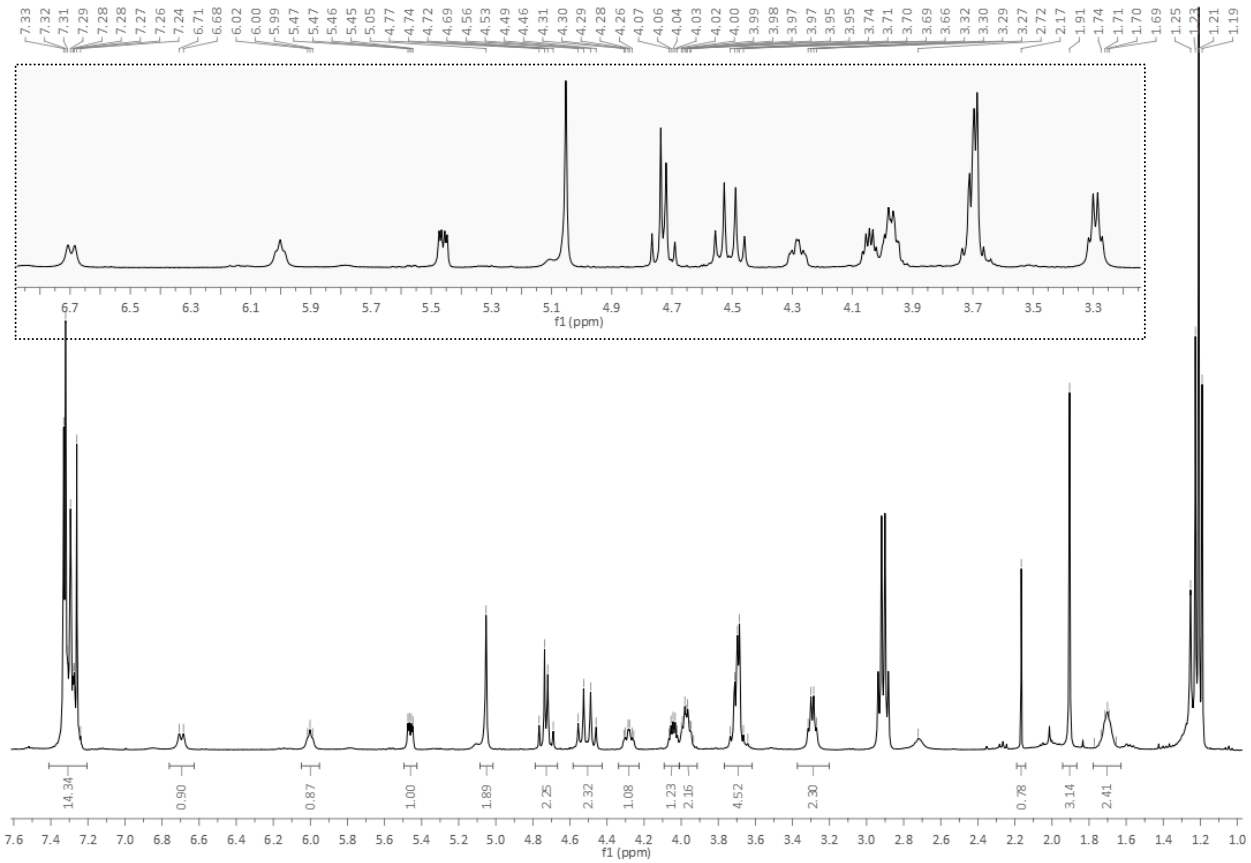
NMR

^{1}H NMR (400 MHz, MeOD) δ 7.41 – 7.20 (m, 15H, Ar), 6.70 (d, *J* = 9.2 Hz, 1H, N*H*Ac), 6.00 (t, *J* = 5.5 Hz, 1H, N*H*Cbz), 5.46 (dd, $J_{H\text{-}1,P}$ =7.5, $J_{H\text{-}1,H\text{-}2}$ = 3.2 Hz, 1H, H-1), 5.05 (s, 2H, CH_2Ph), 4.73 (q, *J* = 11.7 Hz, 2H, CH_2Ph), 4.51 (dd, *J* = 26.8, 11.8 Hz, 2H, CH_2Ph), 4.28 (td, $J_{H\text{-}2,H\text{-}3}$ = 9.1, $J_{H\text{-}2,H\text{-}1}$ = 3.2 Hz, 1H, H-2), 4.04 (dt, $J_{H\text{-}4,H\text{-}5}$ = 8.9, $J_{H\text{-}4,H\text{-}3}$ = 4.3 Hz , 1H, H-4), 4.01 – 3.91 (m, 1H, CH_2O $_{LINKER}$), 3.75 – 3.62 (m, 4H, H-5, H-3, H-6, H-6'), 3.29 (dd, *J* = 12.1, 6.0 Hz, 2H, CH_2NH $_{LINKER}$), 1.91 (s, 3H, CH_3CO), 1.78 – 1.63 (m, 2H, C$H_{2\ LINKER}$).

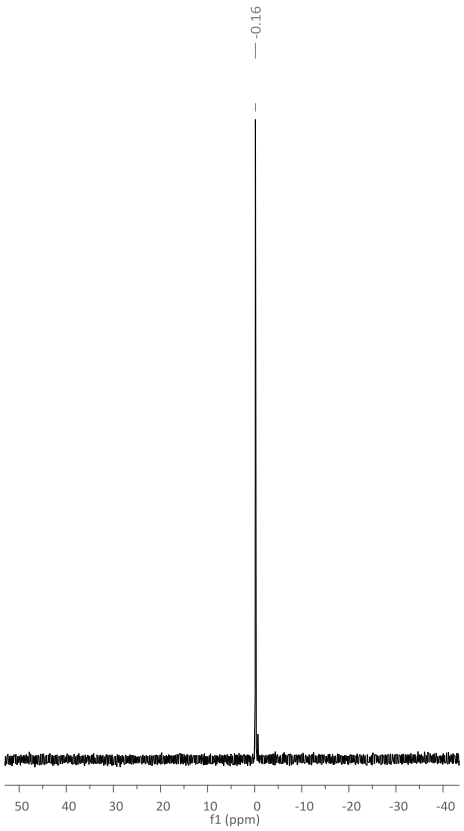
^{31}P NMR (162 MHz, $CDCl_3$) δ -0.16.

^{13}C NMR (101 MHz, $CDCl_3$) δ 170.60 (CO), 156.66 (C_q Ar-NH), 138.79, 138.10, 137.02 (C_q Ar), 128.64, 128.51, 128.33, 128.16, 127.95, 127.87 (CH Ar), 95.06 (C-1), 80.13 (C-3), 74.16 (CH_2Ph), 73.72 (CH_2Ph), 71.76 (C-4, C-5), 70.58 (C-6), 66.57 (CH_2Ph), 63.22 (*C*H_2O*linker*), 52.75 (C-2), 37.59 (*C*H_2NH), 30.53 (*C*$H_{2\ LINKER}$).

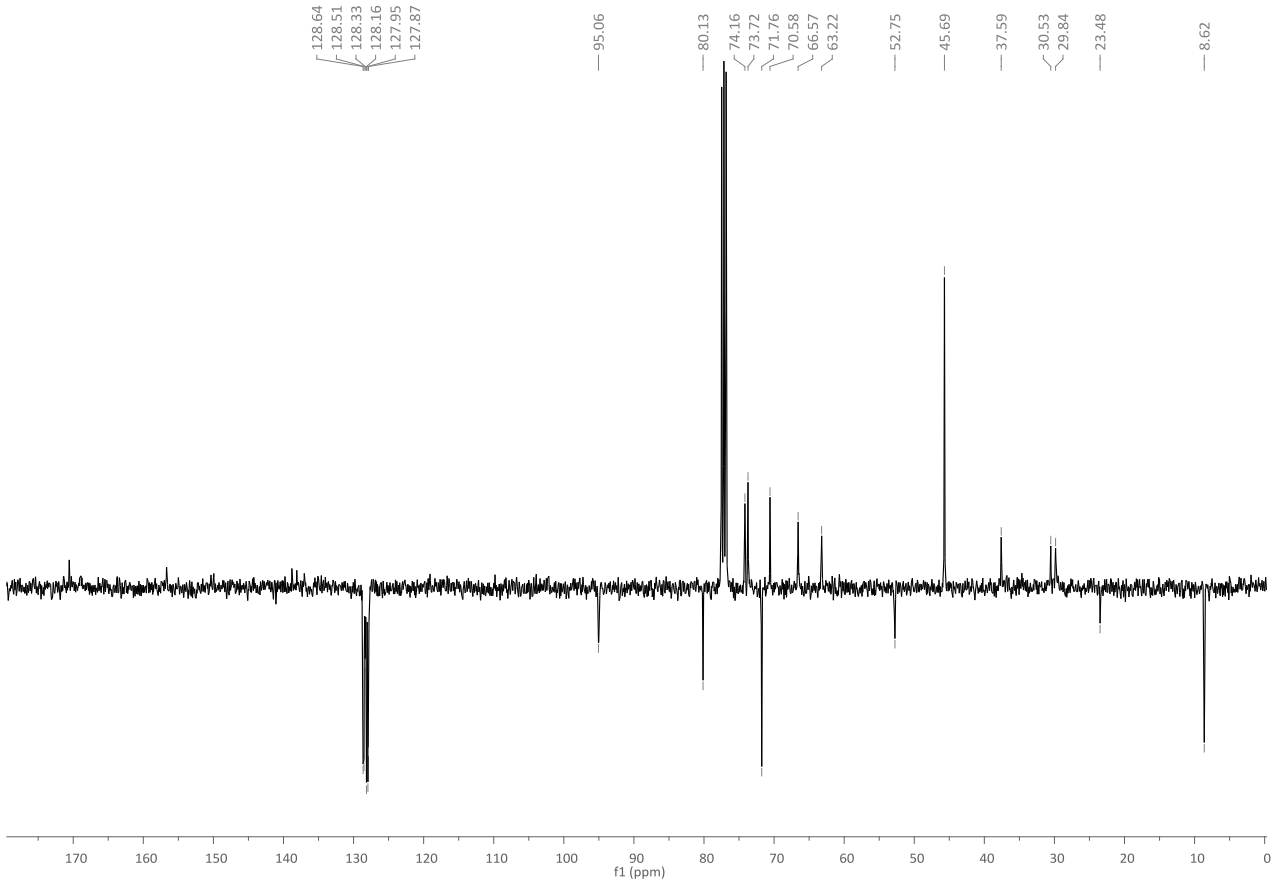
^{1}H (400 MHz, $CDCl_3$):



^{31}P NMR (162 MHz, $CDCl_3$):



^{13}C (101 MHz, $CDCl_3$):



Thexyldimethylsilyl 2-Acetamido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranoside (52)

40 **52**

Synthetic procedure

Compound **40** (437 mg, 0.81 mmol) was treated as described in the ***General Procedure B***. Product **52** was obtained as a white solid (197 mg, 0.363 mmol, 45% yield).

Product characterization

Formula	$C_{30}H_{45}NO_6PSi$
Molecular Weight	543.77 g/mol
TLC conditions	R_f (H/EA 1:1) 0.41

Optical rotation

$\alpha_D^{25} = +5.35$ (c = 1.1 in MeOH)

HRMS (ESI)

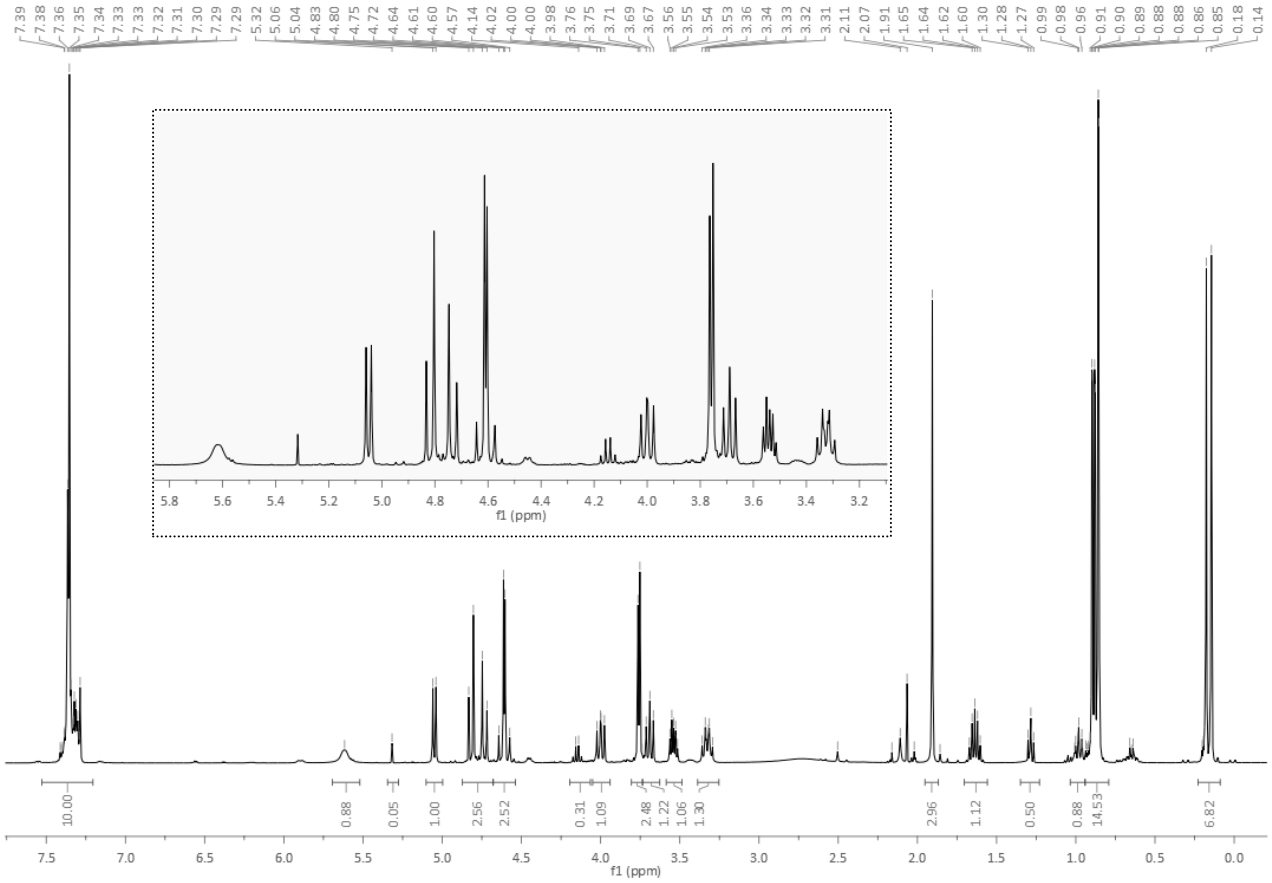
Experimental m/z: 566.29067 $[M+Na]^+$

NMR

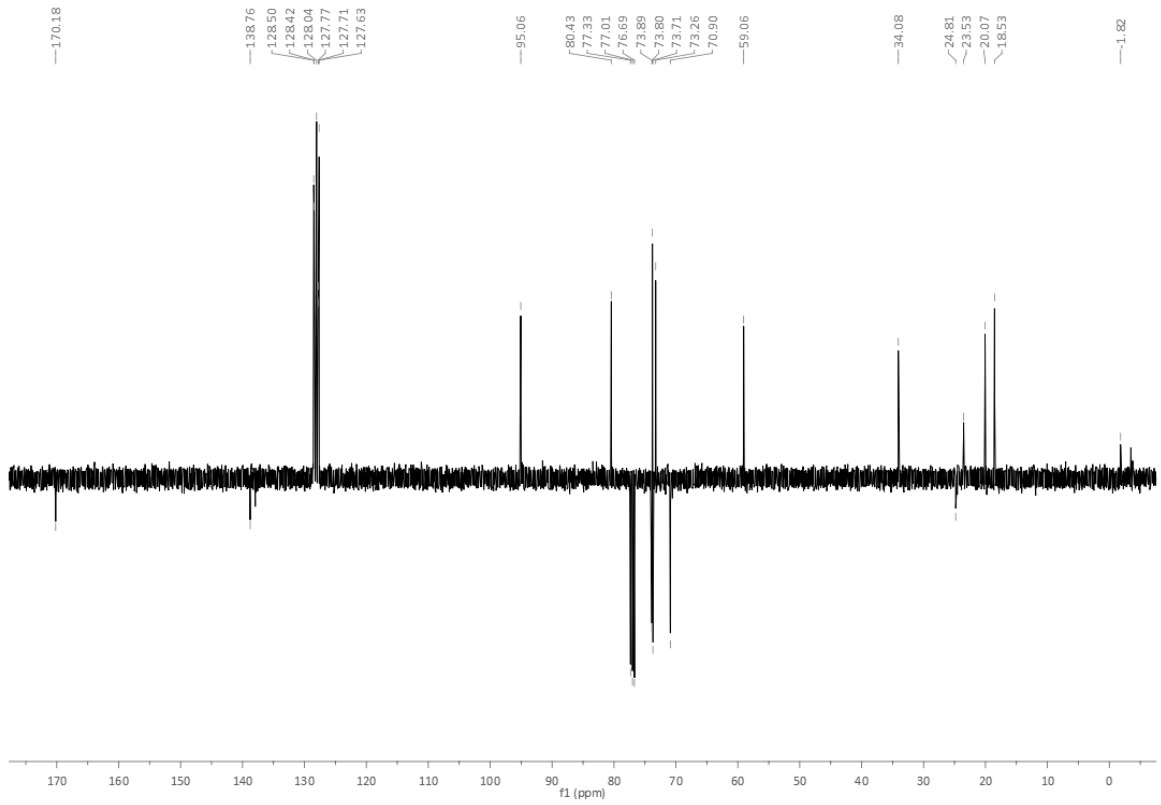
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40 – 7.24 (m, 10H, Ar), 5.60 (s, 1H, N*H*Ac), 5.02 (d, $J_{H-1,H-2}$ = 7.8 Hz, 1H, H-1), 4.79 (d, *J* = 11.7 Hz, 1H, ½ CH_2Ph), 4.71 (d, *J* = 11.7 Hz, 1H, ½ CH_2Ph), 4.60 (d, *J* = 12.0 Hz, 1H, ½ CH_2Ph), 4.56 (d, *J* = 12.0 Hz, 1H, ½ CH_2Ph), 3.97 (dd, $J_{H-3,H-2}$ = 10.2, $J_{H-3,H-4}$ = 9.1 Hz, 1H, H-3), 3.73 (d, $J_{H-6,H-5} = J_{H-6',H-5}$ = 4.9 Hz, 2H, H-6, H-6'), 3.66 (t, $J_{H-4,H-3} = J_{H-4,H-5}$= 9.1 Hz, 1H, H-4), 3.51 (dt, $J_{H-5,H-4}$ = 9.1, $J_{H-5,H-6} = J_{H-5,H-6'}$ = 4.9 Hz, 1H, H-5), 3.30 (dt, $J_{H-2,H-3}$ = 10.2, $J_{H-2,H-1}$ = 7.8 Hz, 1H, H-2), 1.88 (s, 3H, C*H*$_3$CO), 1.69 – 1.54 (ept, *J* = 6.8 Hz, 1H, CH TDS), 0.87 (d, *J* = 1.7 Hz, 3H, CH_3 TDS), 0.85 (d, *J* = 1.7 Hz, 3H, CH_3 TDS), 0.83 (d, *J* = 1.2 Hz, 6H, 2 CH_3 TDS), 0.15 (s, 3H, CH_3Si TDS), 0.12 (s, 3H, CH_3Si TDS).

^{13}C NMR (101 MHz, $CDCl_3$) δ 170.33 (CO), 138.91, 138.09 (C_q Ar), 128.65, 128.57, 128.19, 127.91, 127.86, 127.77 (CH Ar), 95.21, 95.17 (C-1), 80.58 (C-3), 74.03 (*C*H_2Ph), 73.95 (C-5), 73.86 (*C*H_2Ph), 73.41 (C-4), 71.05 (C-6), 59.21 (C-2), 34.23 (*C*H TDS), 24.96 (C_q TDS), 23.68 (CH_3CO), 20.21 (CH_3 TDS), 18.68 (CH_3 TDS), -1.67, -3.33 (CH_3Si TDS).

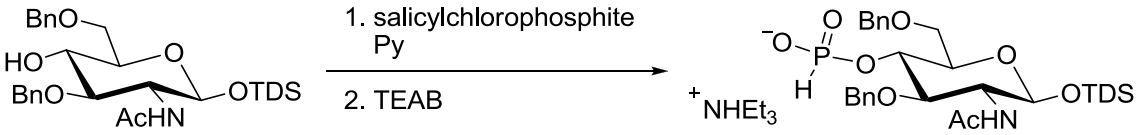
^{1}H (400 MHz, $CDCl_3$):



^{13}C (101 MHz, $CDCl_3$):



Thexyldimethylsilyl 2-Acetamido-3,6-di-*O*-benzyl-2-deoxy-4-hydrogen-phosphonate-β-D-glucopyranosyl (53), triethylammonium salt



52 **53**

Synthetic procedure

Salicylchlorophosphite (110 mg, 0.543 mmol) was slowly added to a solution of the alcohol **52** (197 mg, 0.363 mmol) in dry pyridine (1.5 mL) at 0°C. The reaction was stirred at r.t. for 1 hour. Then a 1M solution of TEAB (4 mL/mmol) was added to the reaction mixture at r.t., and the mixture was diluted with CH_2Cl_2, washed three times with cold TEAB (0.5 M), dried (Na_2SO_4), filtered and concentrated. The crude was purified by flash chromatography. The H-phosphonate has to be stabilized, washing with 0.25M cold TEAB [then drying (Na_2SO_4), filtering and concentrating] the purified product **53** (154.3 mg, 0.22 mmol, 68% yield).

Product characterization

Formula	$C_{36}H_{61}N_2O_8PSi$
Molecular Weight	708.94 g/mol
TLC conditions	R_f (DCM/MeOH 9:1) 0.16

Optical rotation

$\alpha_D^{25} = +23.19$ (c = 1.3 in MeOH)

MS (ESI)

Experimental m/z: 606.4 $[M]^-$

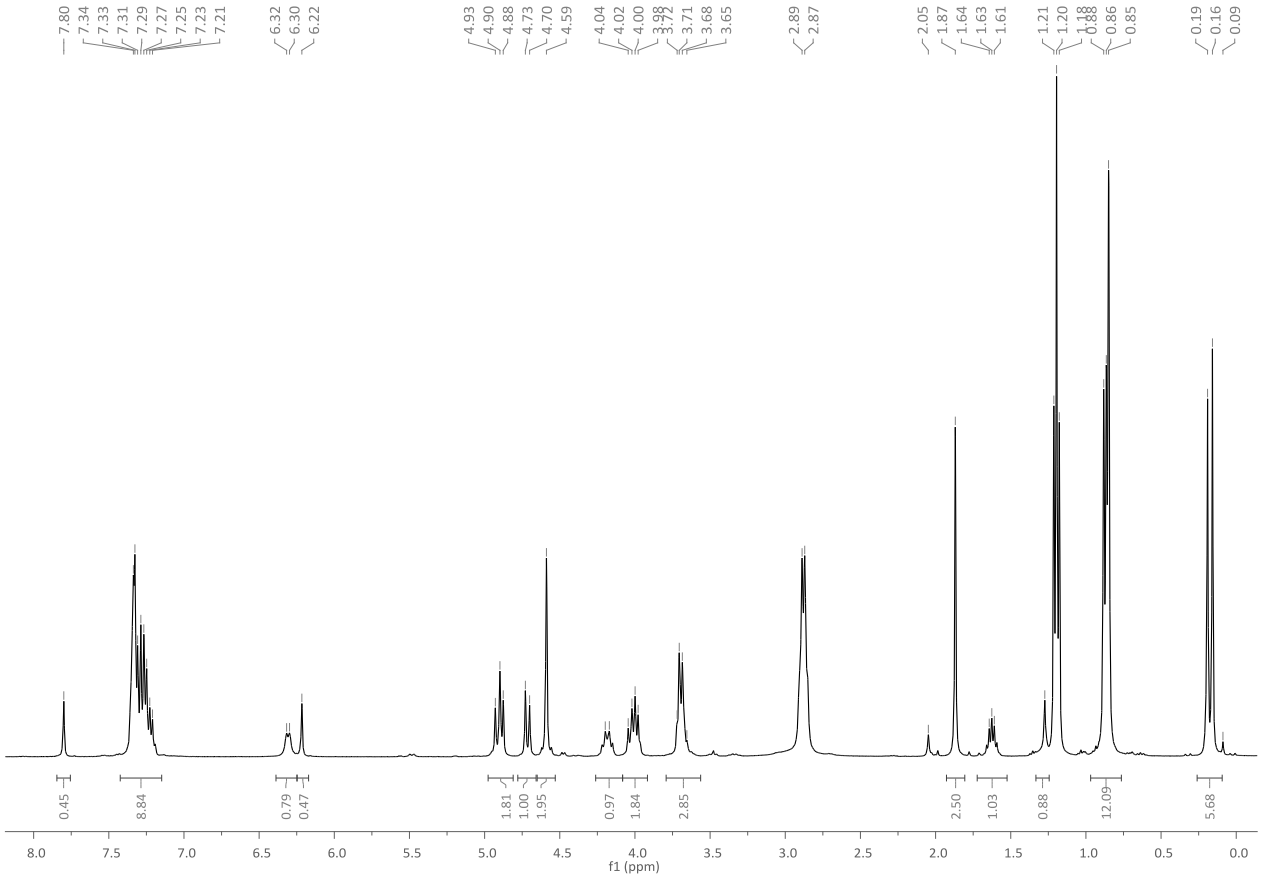
NMR

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 (s, 1H), 7.38 – 7.14 (m, 9H), 6.98 (d, $J_{H\text{-}P,P}$ = 633.6 Hz, 1H, *H*P), 6.28 (d, *J* = 7.8 Hz, 1H, N*H*Ac), 4.93 – 4.83 (m, 2H, ½ CH_2Ph, H-1), 4.69 (d, *J* = 11.3 Hz, 1H, ½ CH_2Ph), 4.63 – 4.50 (m, 2H, CH_2Ph), 4.23 – 4.09 (m, 1H, H-4), 4.06 – 3.90 (m, 2H, H-3, H-6), 3.75 – 3.58 (m, 3H, H-2, H-5, H-6'), 1.84 (s, 3H, C*H*$_3$CO), 1.66 – 1.52 (ept, *J* = 6.8 Hz, 1H, CH TDS), 0.85 (s, 3H, CH_3 TDS), 0.84 (s, 3H), 0.82 (s, 6H, 2 CH_3 TDS), 0.16 (s, 3H, CH_3Si TDS), 0.13 (s, 3H, CH_3Si TDS).

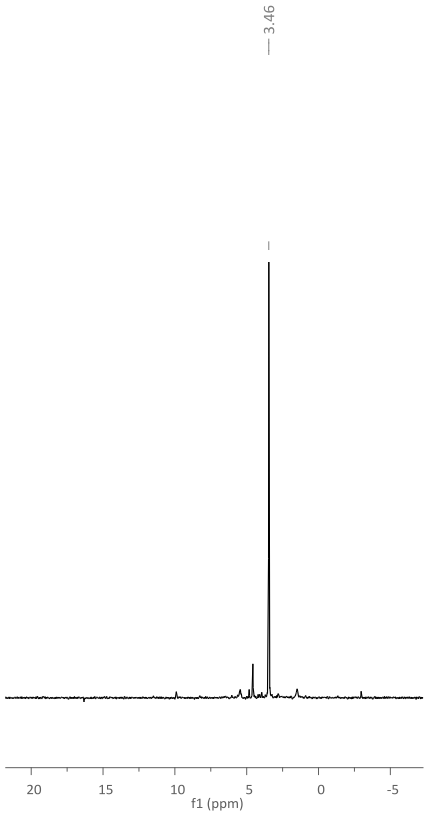
^{31}P NMR (162 MHz, $CDCl_3$) δ (ppm) 3.46.

^{13}C NMR (101 MHz, $CDCl_3$) δ 170.17 (CO), 139.17, 139.07 (C_q Ar), 128.29, 128.21, 127.56, 127.41, 127.35 (CH Ar), 95.91 (C-1), 80.79, 80.78 (C-3), 75.34, 75.30 (C-5), 74.00 (*C*H_2Ph), 73.46 (*C*H_2Ph), 73.37, 73.31 (C-4), 70.30 (C-6), 58.01 (C-2), 34.19 (*C*H TDS), 24.94 (C_q TDS), 23.60, 23.59 (*C*H_3CO), 20.20, 20.19, 18.69, 18.67 (CH_3 TDS), -1.67, -3.25 (CH_3Si TDS).

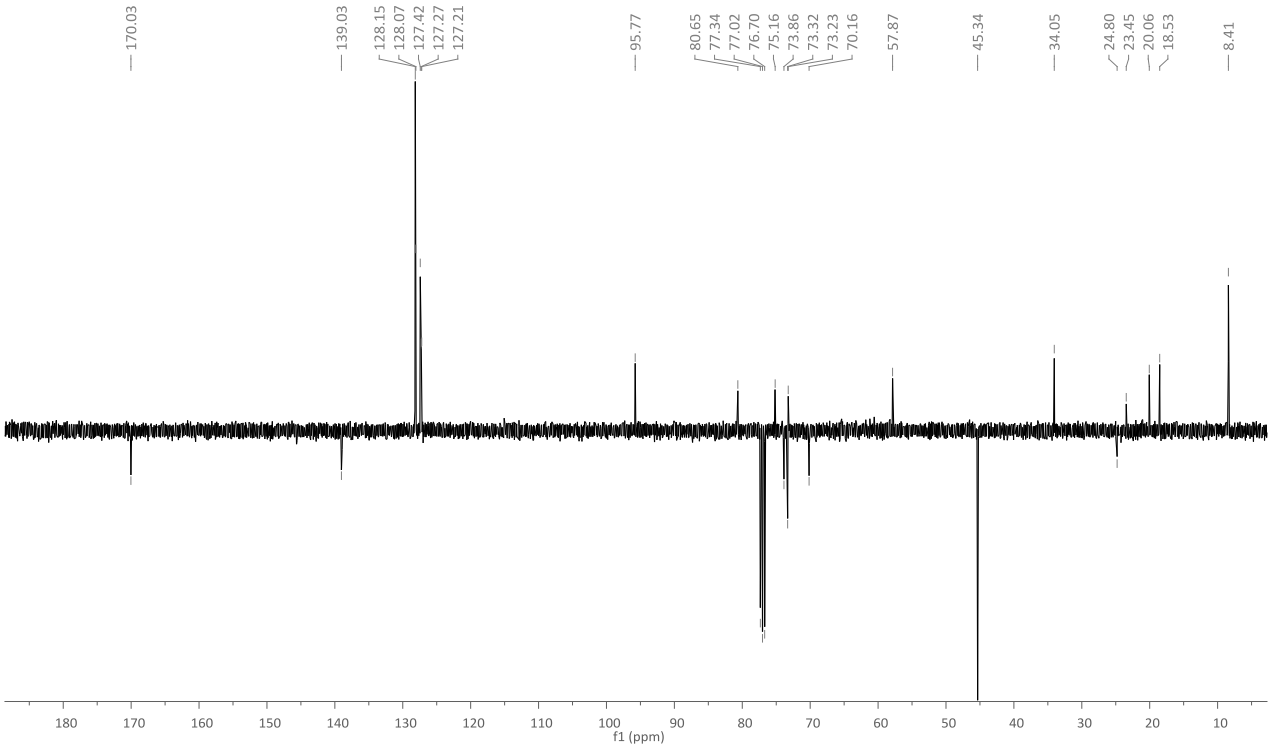
^{1}H (400 MHz, $CDCl_3$):



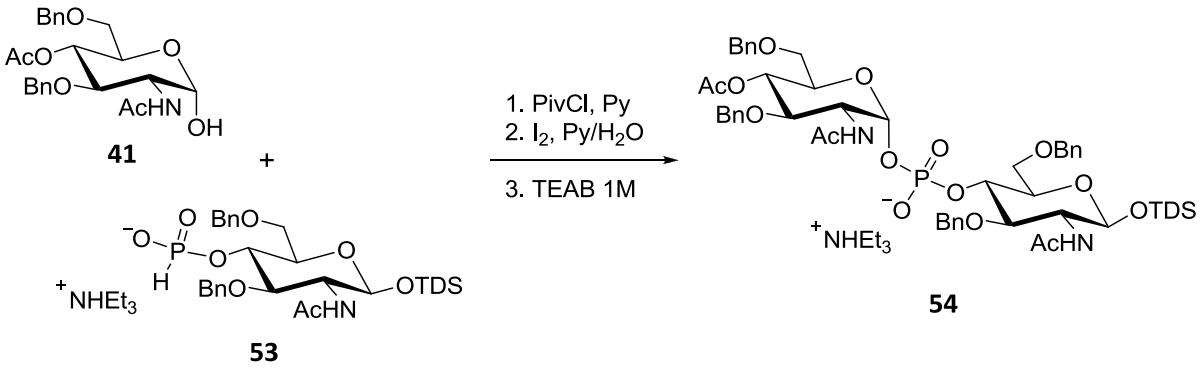
^{31}P NMR (162 MHz, $CDCl_3$):



^{13}C (101 MHz, $CDCl_3$):



Thexyldimethylsilyl 2-Acetamido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranose 4-(2-acetamido-4-*O*-acetyl-3,6-di-*O*-benzyl-α-D-glucopyranosyl phosphate) (54), triethylammonium salt



Synthetic procedure

Donor **53** (154.3 mg, 0.22 mmol) and acceptor **43** (82 mg, 0.185 mmol) were treated as described in the ***General Procedure E***. Compound **54** was obtained in 56% yield (124 mg, 0.123 mmol).

Product characterization

Formula	$C_{60}H_{88}N_3O_{15}PSi$
Molecular Weight	1150.4 g/mol
TLC conditions	R_f (DCM/MeOH 9:1) 0.25

Optical rotation

$\alpha_D^{25} = +33.98$ (c = 0.55 in MeOH)

HRMS (ESI)

Experimental m/z: 1047.44387 $[M]^-$

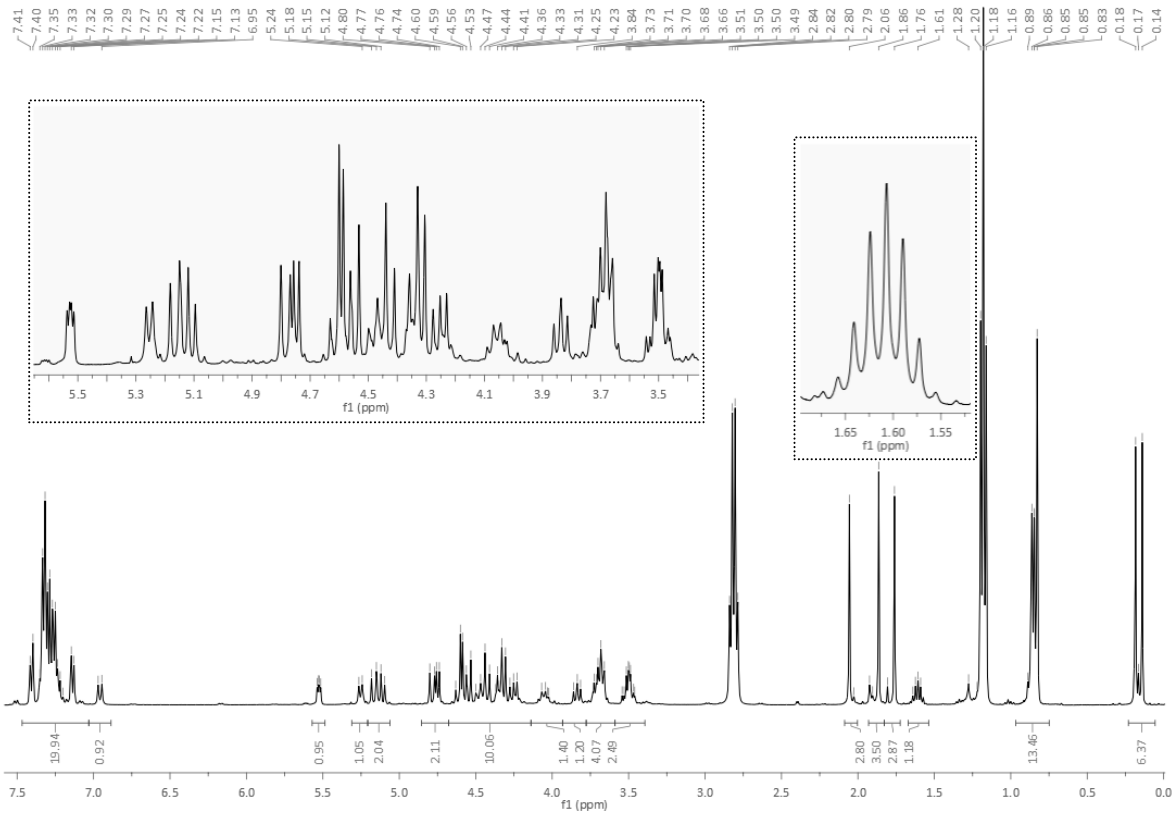
NMR

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.42 – 7.02 (m, 20H, Ar), 6.93 (d, $J_{NH,H-2B}$ = 9.7 Hz, 1H, N*H*Ac-B), 5.50 (dd, $J_{H-1B,P}$ = 5.8, $J_{H-B,H-2B}$ = 3.4 Hz, 1H, H-1B), 5.23 (d, $J_{NH,H-2A}$ = 8.7 Hz, 1H, N*H*Ac-A), 5.18 – 5.05 (m, 2H, ½ CH_2Ph, H-4B), 4.79 – 4.70 (m, 2H, ½ CH_2Ph, H-1A), 4.65 – 4.13 (m, 9H, 3 C*H*$_2$Ph, H-2B, H-5B, H-6A), 4.09 – 3.97 (m, 1H, H-4A), 3.86 – 3.77 (m, 1H, H-3A), 3.72 – 3.59 (m, 4H, H-3B, H-2A, H-6'A, H-5A), 3.53 – 3.42 (m, 2H, H-6B, H-6'B), 2.03 (s, 3H, C*H*$_3$CO), 1.84 (s, 3H, C*H*$_3$CONH), 1.73 (s, 3H, C*H*$_3$CONH), 1.58 (ept, *J* = 6.82 Hz, 1H, CH TDS), 0.83 (s, 3H, CH_3Si TDS), 0.82 (s, 3H, CH_3Si TDS), 0.80 (s, 6H, CH_3Si TDS), 0.16 (s, 3H, CH_3Si TDS), 0.11 (s, 3H, CH_3Si TDS).

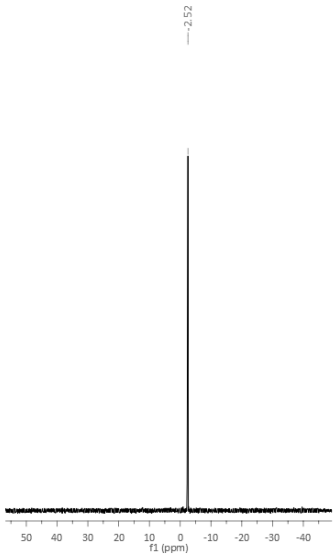
^{31}P NMR (162 MHz, $CDCl_3$) δ (ppm) -2.52.

^{13}C NMR (101 MHz, $CDCl_3$) δ 170.78, 169.74, 169.67 (CO), 139.03, 138.88, 138.61, 138.22 (C_q Ar), 128.58, 128.46, 128.38, 128.27, 128.16, 128.11, 127.83, 127.69, 127.57, 127.44 (CH Ar), 96.00 (C-1_A), 95.50, 95.46 (C-1_B), 81.47, 81.44 (C-3_A), 78.71 (C-3_B), 75.83, 75.80 (C-5_A), 75.19, 75.12 (C-4_A), 75.03, 73.49, 73.34, 72.99 (*C*H_2Ph), 70.86 (C-4_B), 70.66 (C-6_A), 70.00 (C-6_B), 69.70 (C-5_B), 57.50 (C-2_A), 52.31, 52.23 (C-2_B), 34.16 (*C*H TDS), 24.91 (C_q TDS), 23.64, 23.59 (*C*H_3CONH), 20.93(*C*H_3CO), 20.16, 18.64 (CH_3 TDS), -1.65, -3.30 (CH_3Si TDS).

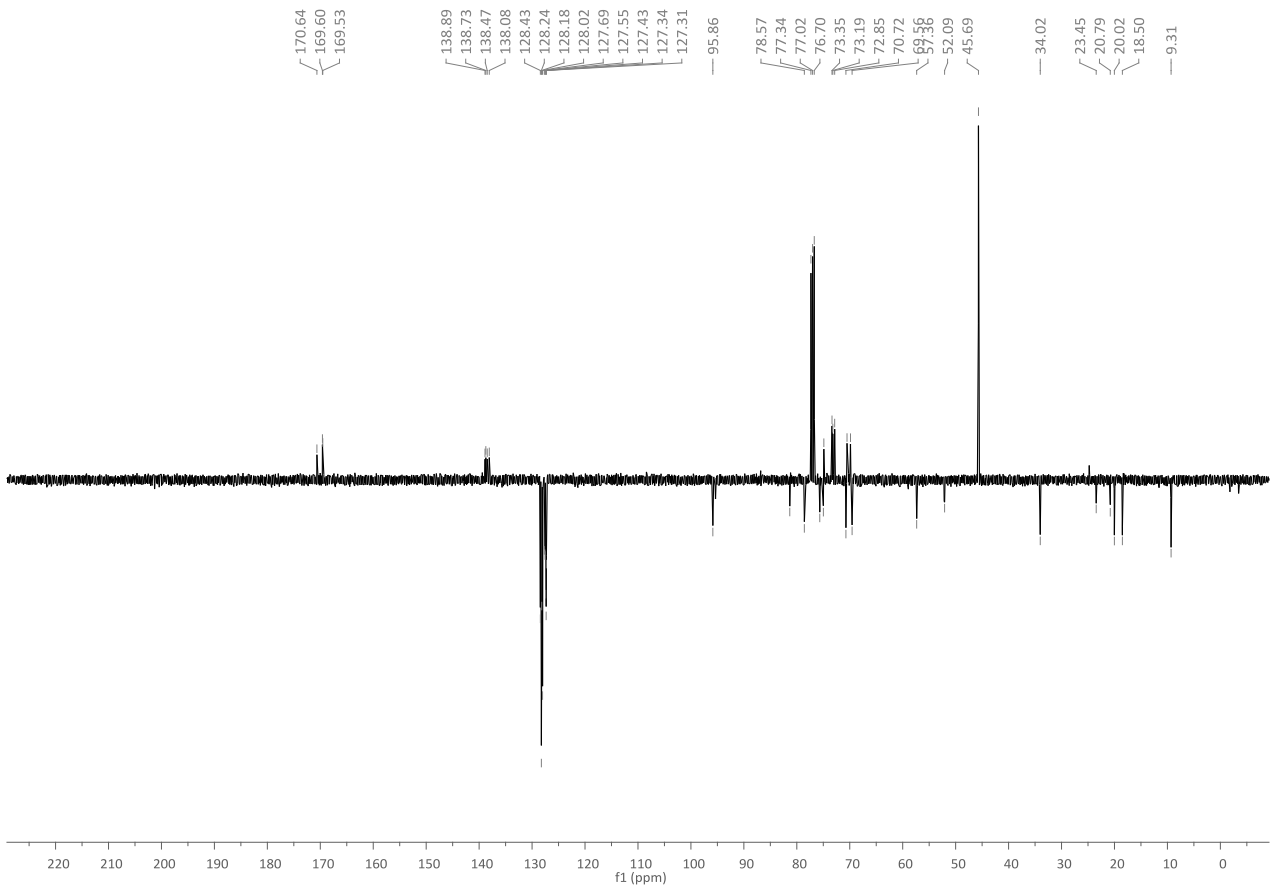
^{1}H (400 MHz, $CDCl_3$):



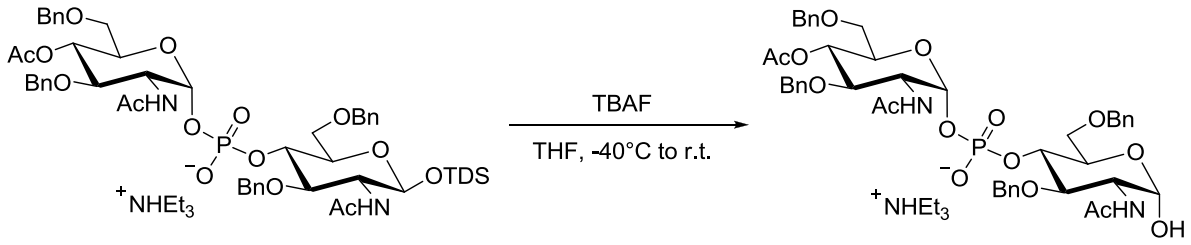
^{31}P NMR (162 MHz, $CDCl_3$):



^{13}C (101 MHz, $CDCl_3$):



2-Acetamido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranose 4-(2-acetamido-4-*O*-acetyl-3,6-di-*O*-benzyl-α-D-glucopyranosyl phosphate) (55), triethylammonium salt



54 **55**

Synthetic procedure

Compound **54** (120 mg, 0.12 mmol) was treated as described in the ***General Procedure A***. Product **55** was obtained as a pure-α anomer (50 mg, 0.05 mmol, white solid, 40% yield).

Product characterization

Formula	$C_{52}H_{70}N_3O_{15}P$
Molecular Weight	1008.10 g/mol
TLC conditions	R_f (DCM/MeOH 8:2) 0.18

HRMS (ESI)

Experimental m/z: 905.32394 $[M]^-$

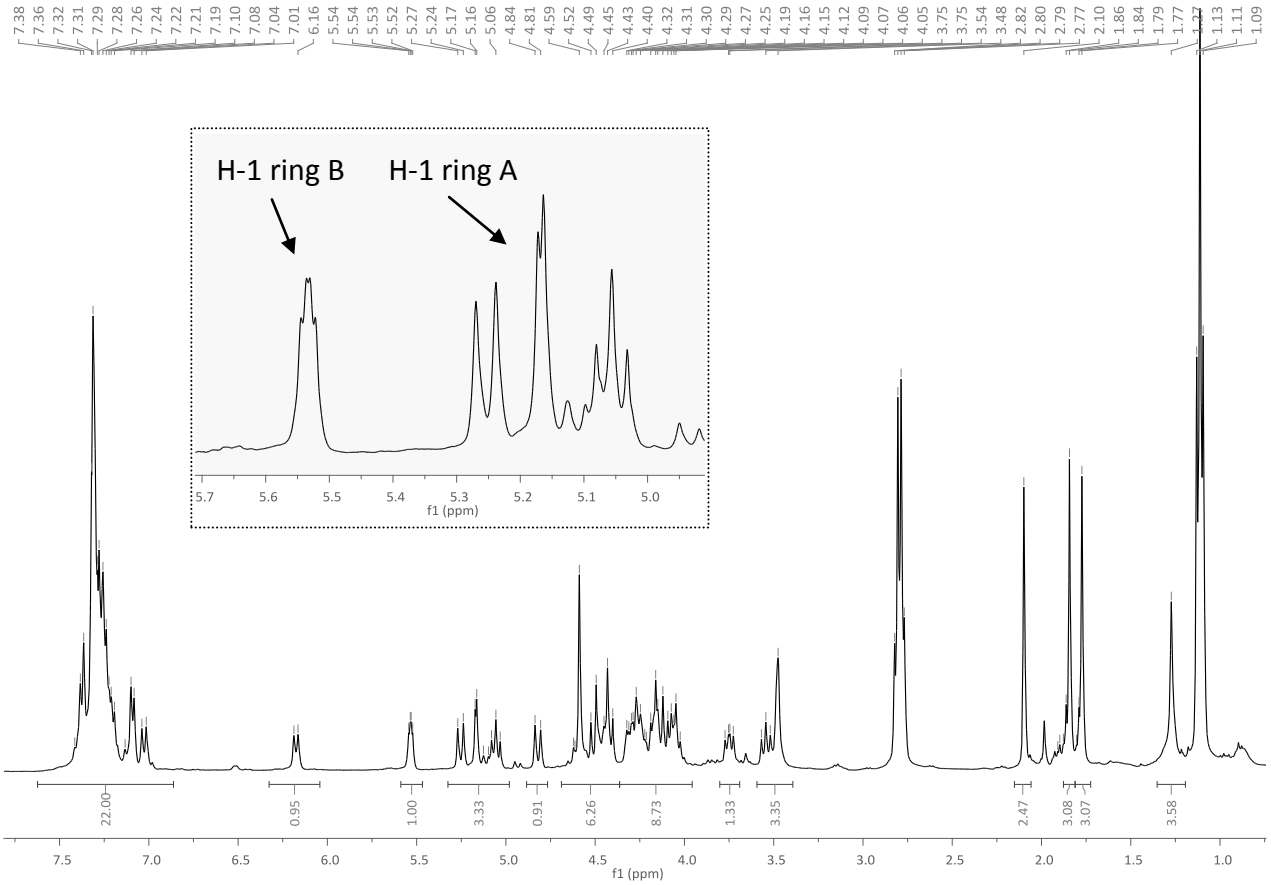
NMR

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.50 – 7.03 (m, 20H, Ar), 7.00 (d, $J_{NH,H\text{-}2B}$ = 9.8 Hz, 1H, N*H*Ac-B), 6.15 (d, $J_{NH,H\text{-}2A}$ = 9.2 Hz, 1H, N*H*Ac-A), 5.51 (dd, $J_{H\text{-}1B,P}$ = 5.5, $J_{H\text{-}1B,H\text{-}2B}$ = 3.5 Hz, 1H, H-1B), 5.28 – 4.97 (m, 3H, ½ C*H*$_2$Ph, H-1A, H-4B), 4.79 (d, *J* = 12.6 Hz, 1H, ½ C*H*$_2$Ph), 4.61 – 4.51 (m, 2H, C*H*$_2$Ph), 4.51 – 4.33 (m, 3H, ½ C*H*$_2$Ph, ½ C*H*$_2$Ph, H-2B), 4.33 – 3.92 (m, 8H, H-5B, H-5A, H-2A, ½ C*H*$_2$Ph, H-6A, ½ C*H*$_2$Ph, H-3A, H-4A), 3.72 (dd, $J_{H\text{-}6A,H\text{-}5A}$ = 10.8, $J_{H\text{-}6'A,H\text{-}6A}$ = 7.6 Hz, 1H, H-6'A), 3.56 – 3.38 (m, 3H, H-3B, H-6B, H-6'B), 2.07 (s, 3H, C*H*$_3$CO), 1.82 (s, 3H, C*H*$_3$CONH), 1.75 (s, 3H, C*H*$_3$CONH).

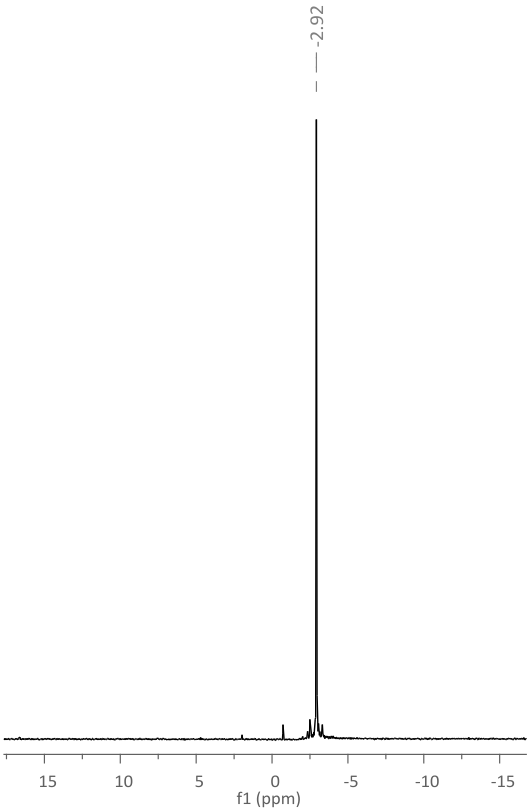
^{31}P NMR (162 MHz, $CDCl_3$) δ (ppm) -2.92.

^{13}C NMR (101 MHz, $CDCl_3$) δ 170.98, 170.23, 169.65 (CO), 138.90, 138.55, 137.94 (C_q Ar), 128.67, 128.41, 128.25, 128.09, 127.84, 127.80, 127.76, 127.49, 127.37 (CH Ar), 95.50, 95.45 (C-1_B), 91.82 (C-1_A), 80.58, 80.55 (C-3_A), 78.69 (C-3_B), 75.88 (*C*H$_2$Ph), 75.37, 75.30 (C-4_A), 73.37 (*C*H$_2$Ph), 73.36 (*C*H$_2$Ph), 73.12 (*C*H$_2$Ph), 70.99 (C-4_B), 70.91, 70.89 (C-5_A), 70.40 (C-6_A), 69.97 (C-6_B), 69.61 (C-5_B), 53.10 (C-2_A), 52.30, 52.22 (C-2_B), 23.55, 23.54 (*C*H$_3$CONH), 23.37, 23.36 (*C*H$_3$CONH), 20.91(*C*H$_3$CO).

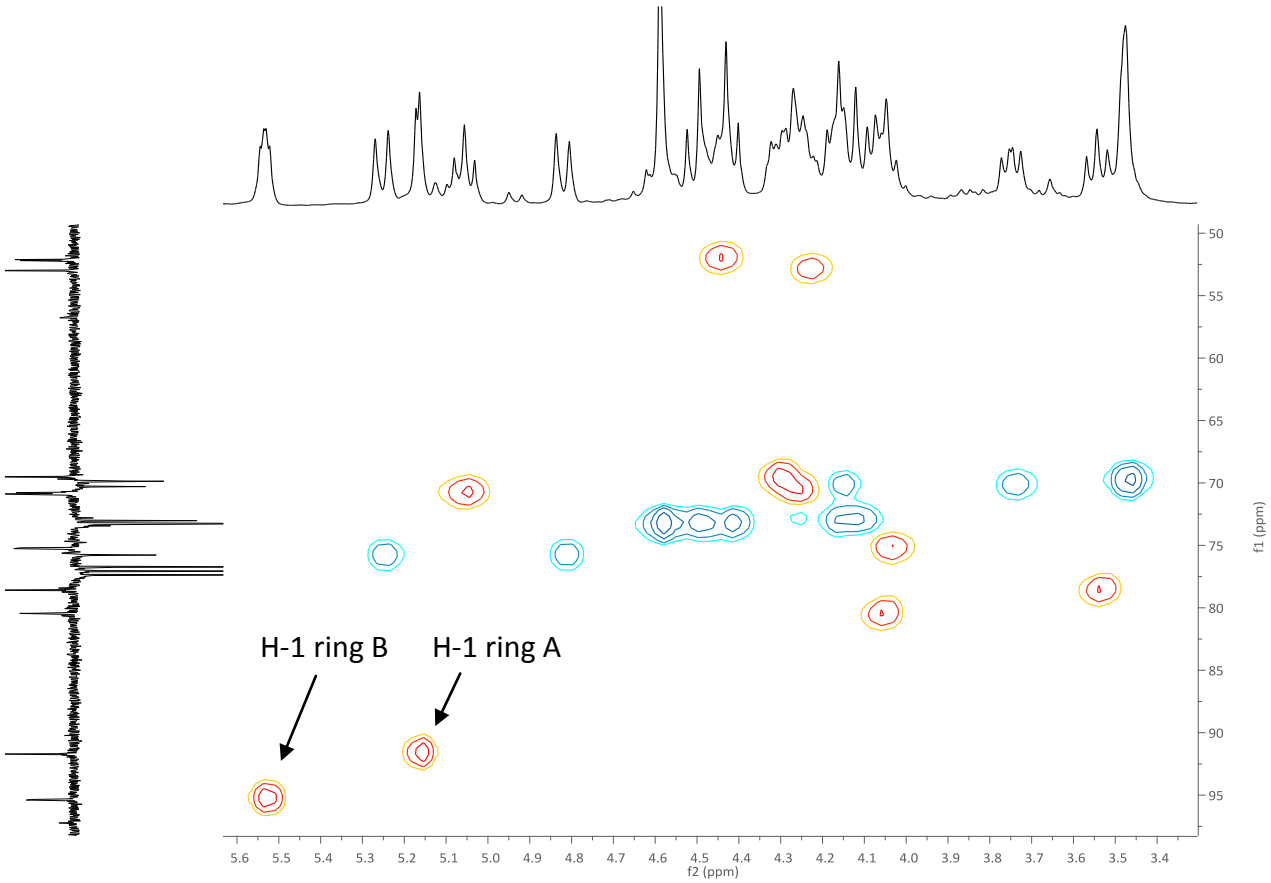
^{1}H (400 MHz, $CDCl_3$):



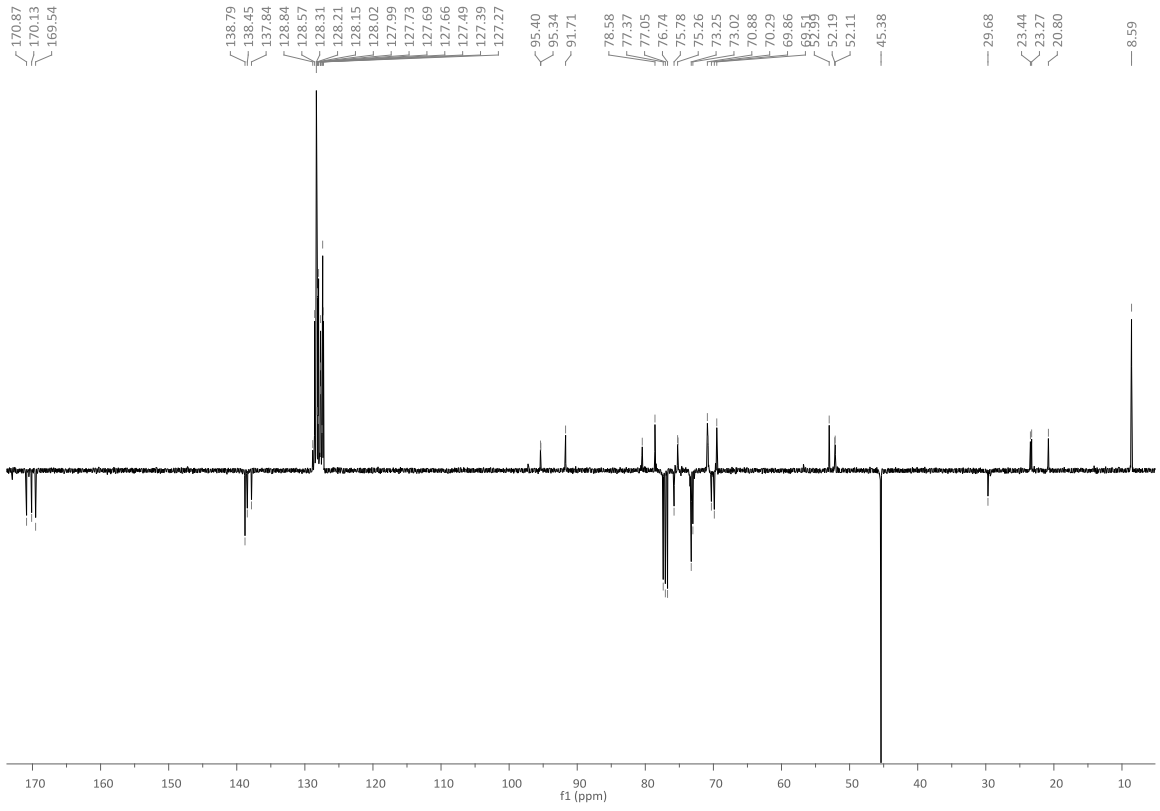
^{31}P NMR (162 MHz, $CDCl_3$):



HETCOR ^{1}H-^{13}C (400 MHz, $CDCl_3$):



^{13}C (101 MHz, $CDCl_3$):



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