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Synthesis of glycoconjugates by Staudinger reactions

(Novel analogues of glycopeptides)

Ph.D. thesis submitted by

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Preface

I, DA'SAN M. M. JARADAT, formally submit my thesis:
''*Synthesis of glycoconjugates by Staudinger reactions (Novel analogues of glycopeptides)*''
in fulfillment of the requirements set forth by the doctoral program "Dahlem International Postgraduate School Chemistry" at the Institute of Chemistry of the Freie Universität Berlin.

This Ph.D thesis describes the work done between July 2006 and July 2010 at the Institute of Chemistry and Biochemistry at the Free University Berlin (Freie Universität Berlin), Berlin, under the supervision of Dr. Christian P. R. Hackenberger and within his research group.

I hereby certify that all the work described in this thesis was done by me, unless specified otherwise in the text. This thesis has not been submitted in whole or in part for any degree or diploma at this or any other university.

DA'SAN M. M. JARADAT

Berlin, July 2010

Dedicated to
my Family

1. Reviewer: Prof. Dr. Rainer Haag, Freie Universität Berlin, Berlin, Germany.

2. Reviewer: Dr. Christian P. R. Hackenberger, Freie Universität Berlin, Berlin, Germany.

Day of defense: 17.08.2010

List of abbreviations

Fmoc	9-Fluorenylmethoxycarbonyl
HBTU	*O*-(benzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium
HOBt	1-hydroxybenzotriazol
HPLC	High-Performance Liquid Chromatography
Hz	Hertz
NMR	Nuclear Magnetic Resonance
ppm	Parts per million
SPPS	Solid Phase Peptide Synthesis
TFA	Trifluoroacetic acid
TIS	Triisopropyl silane
TLC	Thin Layer Chromatography
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
PG	Protecting group
GPI	Glycosylphosphatidyl inositol
NCL	Native Chemical Ligation
EPL	Expressed protein ligation
TSL	Traceless Staudinger ligation
SPR	Staudinger phosphite reaction
TBAHS	Tetrabutylammonium hydrogen sulphate
Equiv	Equivalent
THF	Tetrahydrfuran
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DCM	Dichloromethane
OAc	Acetate
DMA	Dimethylacetamide
DIC	Diisopropylcarbodiimide
DMAP	Dimethylaminopyridine
DIPEA	Diisopropylethylamine

DABCO	1,4 diazabicyclo[2.2.2]octane
Trt	Trityl
Ph	Phenyl
Me	Methyl
Bn	Benzyl
tBu	tert-butyl
SPR	Surface Plasmon resonance
NMM	*N*-methylmorpholine
Alloc	Allyloxycarbonyl

Table of Contents

Abstract

Glycoconjugates are desirable targets for synthesis and investigation of the biological properties. In this Ph.D thesis, the utilization of Staudinger-type reactions for the synthesis of natural and unnatural glycoconjugates is presented.

The traceless Staudinger ligation is utilized to create amide bonds which allows the access of glycosyl amides. The key steps in this transformation are the synthesis of phosphino(thio)esters as well as their reactions with glycosyl azides.

Diphenyl-phosphinophenol and borane-protected phosphinomethanethiol, which have been demonstrated to be stable against oxidation, were synthesized and further utilized to prepare phosphinothioesters as well as phosphinophenolesters in moderate to good overall yield.

The acid-induced traceless Staudinger ligation using borane-protected phosphino-methanethiol showed a useful applicability to deliver glycosyl amides at small molecule and amino acid level, whereas it was not applicable to access neither iminophosphorane intermediates nor their corresponding *N*-linked glycopeptides.

On the other hand, the utilization of diphenyl-phosphinophenol as a linker in the traceless Staudinger ligation, at peptide level, allowed the acquisition of iminophosphorane (aza-ylide) intermediates which underwent a very slow N⟶O acyl shift into the corresponding amidophosphonium salts providing subsequently the desired *N*-linked glycopeptides in very low yields upon hydrolysis.

Unlike the traceless Staudinger ligation, the utilization of the Staudinger phosphite reaction allowed the acquisition of a novel glycopeptide mimetic by solid-phase synthesis. The key elements in this synthetic procedure were the synthesis of dimethyl phosphite containing peptides by global phosphitylation of an unprotected Ser residue as well as their reaction with glycosyl azides.

The phosphoramidate-linked glycoconjugates were furnished upon TFA-mediated cleavage in high yields and under high retention of the anomeric linkage. These novel artificial glycopeptides showed excellent stability under acidic and physiological conditions.

The Staudinger phosphite reaction allowed also the access to biotinylated mono- and divalent glycopeptides as interesting targets for Lectin binding studies.

Zusammenfassung

Glykokonjugate sind wünschenswerte Ziele für die Synthese und Untersuchung von biologischen Eigenschaften. In dieser Doktorarbeit wird die Anwendung der Staudinger Reaktionen auf die Synthese von natürlichen und unnatürlichen Glykokonjugaten vorgestellt.

Die spurlose Staudinger Ligation wird zur Bildung von Amidbindungen für den Zugang zu Glycosylamiden genutzt. Die wichtigsten Schritte in dieser Transformation sind die Synthese von Phosphino(thio)estern sowie deren Reaktionen mit Azidozuckern.

Diphenyl-phosphinophenol und Boran-geschütztes Phosphinomethanethiol, deren Stabilität gegenüber Oxidation gezeigt wurde, wurden synthetisiert und weiter genutzt, um Phosphinothioester und Phosphinophenolester in mäßigen bis guten Ausbeuten darzustellen.

Die Säure-induzierte spurlose Staudinger Ligation mit Boran-geschütztem Phosphino-methanthiol wies eine sinnvolle Anwendbarkeit auf Glycosylamide auf der Ebene kleine Moleküle und Aminosäuren auf, während sie weder auf Peptidische Iminophosphoran-Zwischenprodukte noch auf die entsprechenden *N*-Glykopeptide anwendbar war.

Andererseits, erlaubte die Verwendung von Diphenyl-phosphinophenol als Linker in der spurlosen Staudinger Ligation, auf Peptid-Ebene, die Darstellung von Iminophosphoran (Aza-Ylid) Zwischenprodukten, die eine sehr langsame N $\longrightarrow$ O Acyl Verschiebung zu den entsprechenden Amidophosphonium Salzen eingingen, und damit die Darstellung der gewünschten *N*-Glycopeptide in sehr geringen Ausbeuten durch Hydrolyse.

Anders als die spurlose Staudinger Ligation erlaubt die Anwendung der Staudinger Phosphit Reaktion die Darstellung eines neuen Glycopeptid-Mimetikums durch Festphasensynthese. Die Schlüsselelemente in diesem synthetischen Verfahren waren die Synthese von Peptiden mit Dimethylphosphit durch globale Phosphitylierung eines ungeschützten Serin Restes, sowie deren Reaktion mit Azidozuckern.

Die Phosphoramidat-Glykokonjugaten wurden durch TFA-vermittelte Spaltung in hohen Ausbeuten und mit hohen Stereoselektivität am anomere Zentrum dargestellt. Diese neuartigen künstlichen Glycopeptide zeigten ausgezeichnete Stabilität unter sauren und physiologischen Bedingungen.

Die Staudinger Phosphit Reaktion ermöglichte auch den Zugang zu biotinylierten Mono- und Di-glycosylierten Peptiden als interessante Zielstrukturen für Lektinbindungsstudien.

Chapter 1

Introduction

1 Introduction

1.1 Origin of nitrogen-containing compounds in living organisms

Atmospheric nitrogen, which constitutes 78% (by volume) of the Earth atmosphere,[1] is the essential source of nitrogen present in proteins, nucleic acids, and other vital nitrogen-containing compounds of the living cells, but atmospheric nitrogen must be modified before it can be utilized by most living systems.

Such a modification occurs in the course of nitrogen fixation by which N_2 is reduced to ammonia which is subsequently available for the biosynthesis of the essential building blocks of life such as amino acids for proteins and nucleotides for nucleic acids.[2]

Proteins are biopolymers that consist of many amino acid residues linked together in a long chain via amide bonds. These large biomolecules occur in every living organism and they are the most abundant of cellular components, including hormones, antibodies, enzymes, and transport molecules.[2,3] The structure of proteins is encoded by deoxy ribonucleic acid (DNA) and the code is transferred via ribonucleic acid (RNA) to the proteomic level.

1.2 Protein modifications

The total number of proteins in a cell (proteomes) is larger than the number predicted by DNA coding, for instance, the coding capacity in Humans is 30,000 genes whereas the proteome constitutes over 1000,000 molecular species of proteins.[4]

Humans have a lower number of genes than lower organisms but they have much more complex cellular functions. This complexity results not only from the combination of the common 20 amino acids in proteins, but also from protein modifications which can be classified according to the time of their occurrence into two main categories: co- and post-translational modifications.[5]

Protein modifications include amino acid side chain modifications as well as backbone modifications in which a cleavage at a specific peptide bond occurs by

[1] D. R. Lide, *CRC Handbook of Chemistry and Physics*, Taylor and Francis press, Boca Raton, FL., **2010**, *90th edition*.
[2] Arabic translation of N. A. Edwards, K. A. Hassall, *Biochemistry and physiology of the cell: An introductory text*, McGRAW-HILL Book Company (UK) Limited, London, **1980**, pp 95-139, pp 535-584. (By Dr. Elias Baydoun, The Jordan Academy of Arabic, Amman, Jordan, **1986**).
[3] J. McMurry, *Organic Chemistry*, THOMSON BROOKS/COLE, Belmont, USA, **2004**, pp 985-1020.
[4] C. T. Walsh, S. Garneau-Tsodikova, G. J. Gatto, Jr., *Angew. Chem. Int. Ed.*, **2005**, *44*, 7342-7372.
[5] L. L. Kiessling, *Chem. Eng. News*, **2001**, *79*, 246-246.

proteases.[4] In addition, head-to-tail protein cyclization[6] and protein splicing[7,8,9] occur posttranslationally.

As the name implies, these modifications occur during or after RNA has been translated into proteins. Therefore, it is worth understanding the process translation before going into detail on protein modifications, which then will lead us to the topic of protein biosynthesis.

Protein biosynthesis

Genetic information of cell determines cell nature, controls cell division and cell growth, and directs the biosynthesis of proteins and enzymes required for all cellular functions.[10] For the genetic information stored in DNA to be preserved and passed on, identical copies of DNA are made by *replication* followed by DNA *transcription*. By this process the genetic information is read and transferred by messenger RNA (mRNA) from the cell nucleus to the *ribosome*, where protein synthesis takes place. Finally, the genetic messages are decoded, translated and implemented to biosynthesize proteins necessary for cell functions by means of an enzymatic process in the presence of tRNA and rRNA. This process is referred to as *translation* (Figure 1).[11]

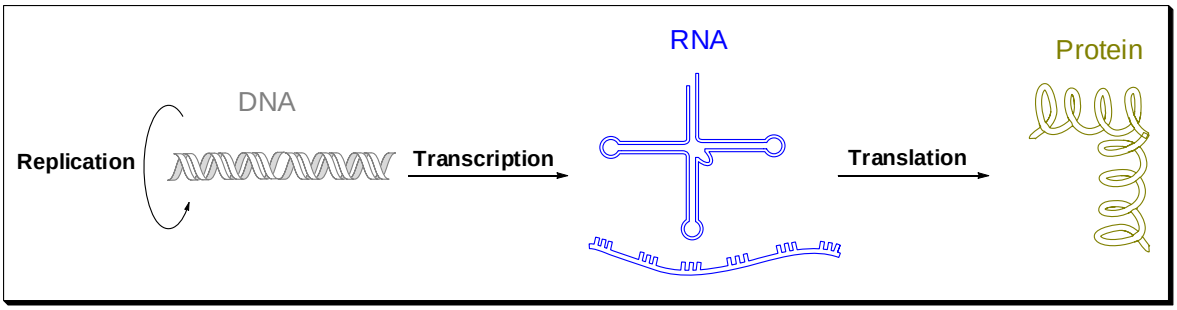


Figure 1. The three fundamental processes to transfer the genetic information and biosynthesize proteins.

DNA is a double helix containing a coding and a template strands, the latter being read from 3`to 5` and transcribed to create the complementary RNA in the direction from 5`to 3`. The sequence of the newly created RNA is the same as the coding strand of DNA

[6] M. Trabi, D. J. Craik, *Trends Biochem. Sci.* **2002**, *27*, 132-138.
[7] T. C. Evans, Jr., J. Benner,M. Q. Xu, *Protein Sci.* **1998**, *7*, 2256-2264.
[8] T. W. Muir, *Annu. Rev. Biochem.* **2003**, *72*, 249 -289.
[9] C. J. Noren, J.Wang, F. B. Perler, *Angew. Chem. Int. Ed.* **2000**, *39*, 450 – 466.
[10] B. Alberts, A. Johnson, J. Lewis, M. Raff, K. Roberts, P. Walter, *Molecular Biology of the Cell*, Garland Science, **2002**, *4th edition*.
[11] J. M. Berg, J. L. Tymoczko, L. Stryer, *Biochemistry*, W. H. Freeman, **2002**, *5th edition*.

except for the substitution of uracil for thymine. This preliminary RNA is then edited to the actual mRNA.

mRNA is a biopolymer of ribonucleotides, every three nucleotides form a ‘‘word’’ or codon that is specific for a given amino acid. The combination of these codons determines the order in which different amino acid residues are to be joined. There are 64 codons; 61 for specific amino acids and three for peptide chain termination. mRNA is translated in *ribosomes* by aminoacyl-tRNA molecules, each molecule contains an anticodon, a sequence of three nucleotides complementary to the codon sequence.

For instance, the codon sequence (5`) AUG (3`) found on mRNA is read by a methionine-bearing tRNA containing the complementary anticodon sequence (5`) UAC (3`). Successive reading of all codons on mRNA by different tRNAs delivers a polypeptide chain which is then released from the ribosome.

The polypeptide chain may fold into its final form without further modifications. Frequently though the folded protein is subjected to post-translational modifications, namely the addition of different groups to specific amino acid side chains or the removal of part of the polypeptide chain; this is what is known as post-translational modifications since it takes place after the translation process has been over as well as after a possible chaperone-assisted protein folding (Figure 2).

Co-translational modifications, e.g. *N*-glycosylation, occur during the translation process and before the polypeptide is released from the ribosome.

In general, amino acid side chain modifications include glycosylation, phosphorylation, alkylation,[4,12] acylation,[4,13,14] disulfide bond formation,[4,15,16] sulfation and some other rare modifications.[4,17]

[12] R. Roskoski, Jr., *Biochem. Biophys. Res. Commun.* **2003**, 303, 1-7.
[13] M-J. Bijlmakers, M. Marsh, *Trends Cell Biol.* **2003**, *13*, 32-42.
[14] C. L. Brooks, W. Gu, *Curr. Opin. Cell Biol.* **2002**, *15*, 164-171.
[15] N. M. Giles, A. B. Watts, G. I. Giles, F. H. Fry, J. A. Littlechild, C. Jacob, *Chem. Biol.* **2003**, *10*, 677-693.
[16] C. Jacob, G. I. Giles, N. M. Giles, H. Sies, *Angew. Chem. Int. Ed.* **2003**, *42*, 4742-4758.
[17] J. A. Vranka, L. Y. Sakai, H. P. Bächinger, *J. Biol. Chem.* **2004**, *279*, 23615-23621.

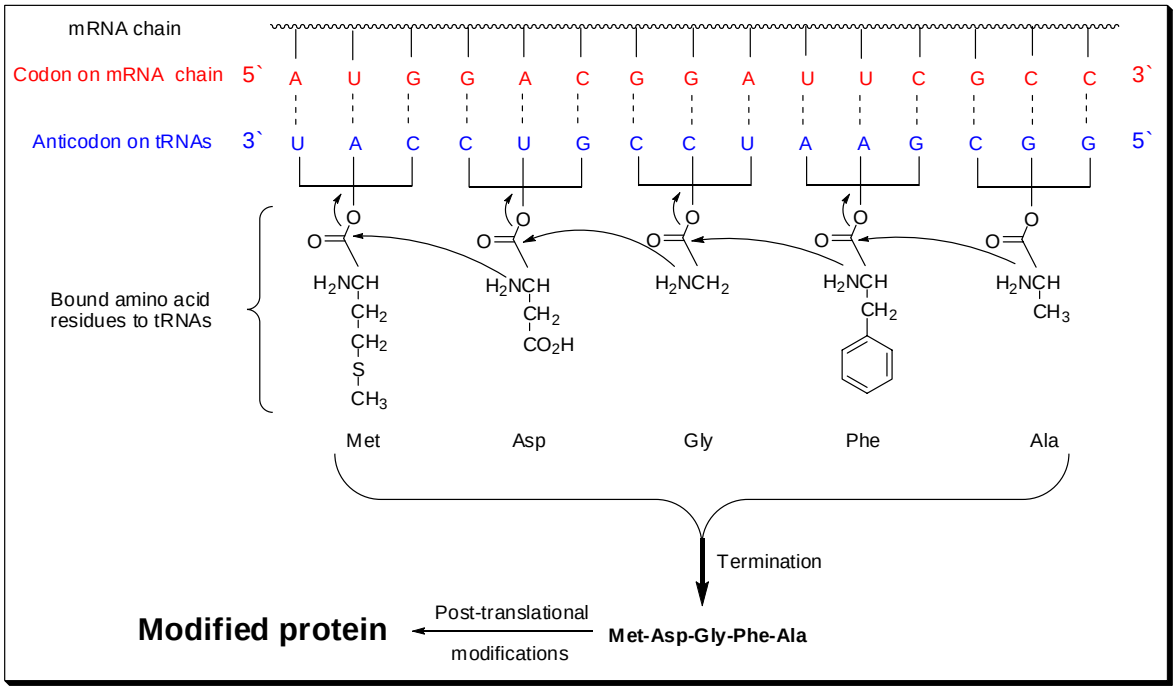


Figure 2. A schematic representation of protein biosynthesis and post-translational modifications.

Based on the scope of this thesis, protein phosphorylation and glycosylation will be described in more details.

1.2.1 Phosphorylation

Protein phosphorylation is a post-translational modification that introduces a phosphate group (PO_4^{3-}) to a protein. Phosphoserine (pS), phosphotyrosine (pY), and phosphothreonine (pT) are present in eukaryotic phosphoproteomes whereas prokaryotes additionally exhibit phosphorylation of aspartic acid (pAsp), glutamic acid (pGlu) and histidine (pHis) (Scheme 1A).[18]

Protein phosphorylation is a kinase-catalyzed process transferring a phosphate moiety from adenosine triphosphate (ATP) to the acceptor residue, thereby generating a negative charged phosphoprotein and adenosine diphosphate (ADP).[19] There are over 500 human protein kinases as biocatalyst for the phosphorylation.[20]

The reversed reaction is called dephosphorylation by which phosphate group removal from phosphoproteins is catalyzed by protein phosphatases. Many enzymes and

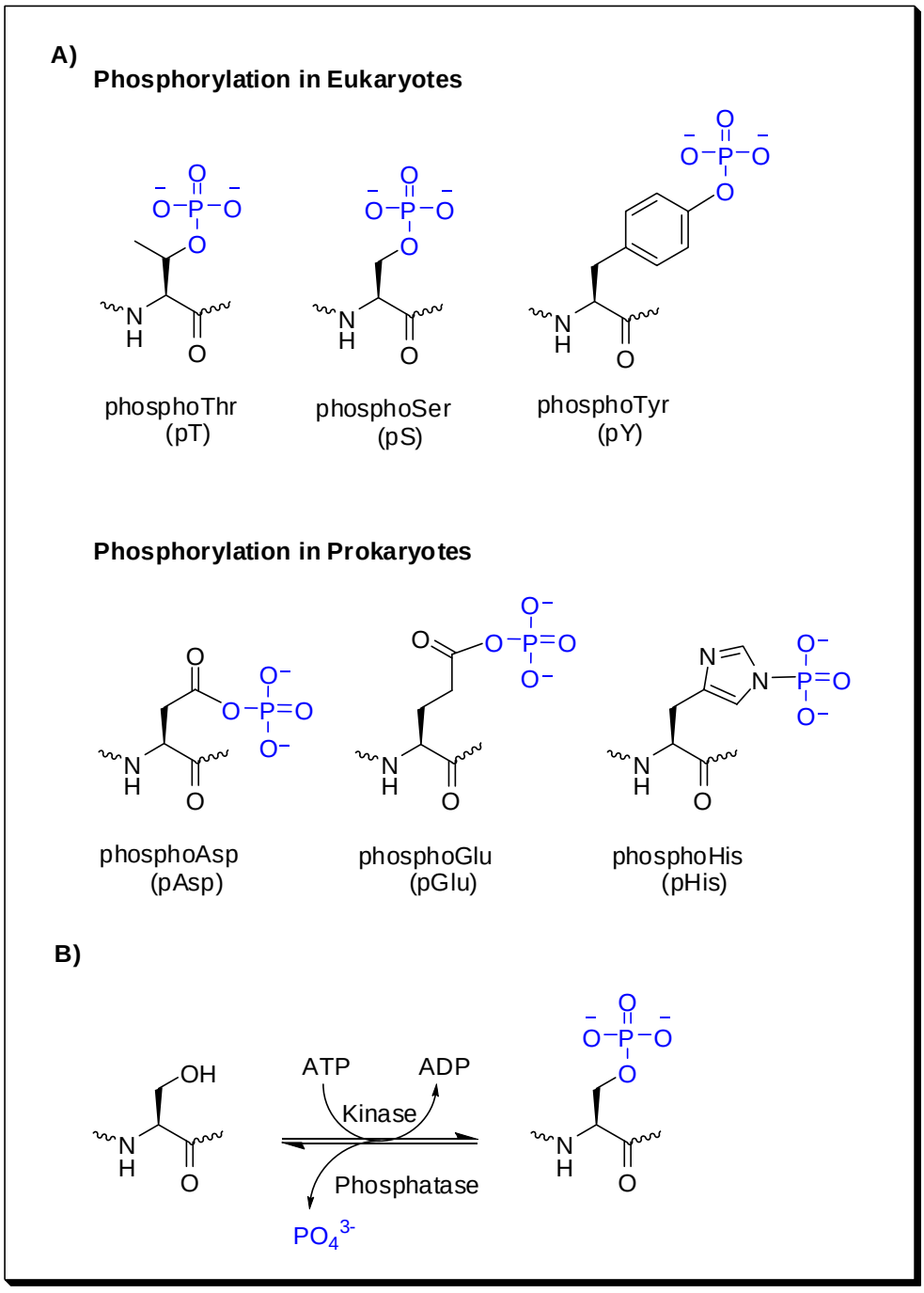
[18] M. Mann, S. E. Ong, M. Gronborg, H. Steen, O. N. Jensen, A. Pandey, *Trends Biotechnol.* **2002**, *20*, 261-268.
[19] N. Blom, T. Sicheritz-Pontén, R. Gupta1, S. Gammeltoft, S. Brunak, *Proteomics* 2004, *4*, 1633–1649.
[20] G. Manning, D. B. Whyte, R. Martinez, T. Hunter, S. Sudarsanam, *Science* 2002, *298*, 1912-1934.

receptors are activated or deactivated by phosphorylation and dephosphorylation (Scheme 1B).[2]

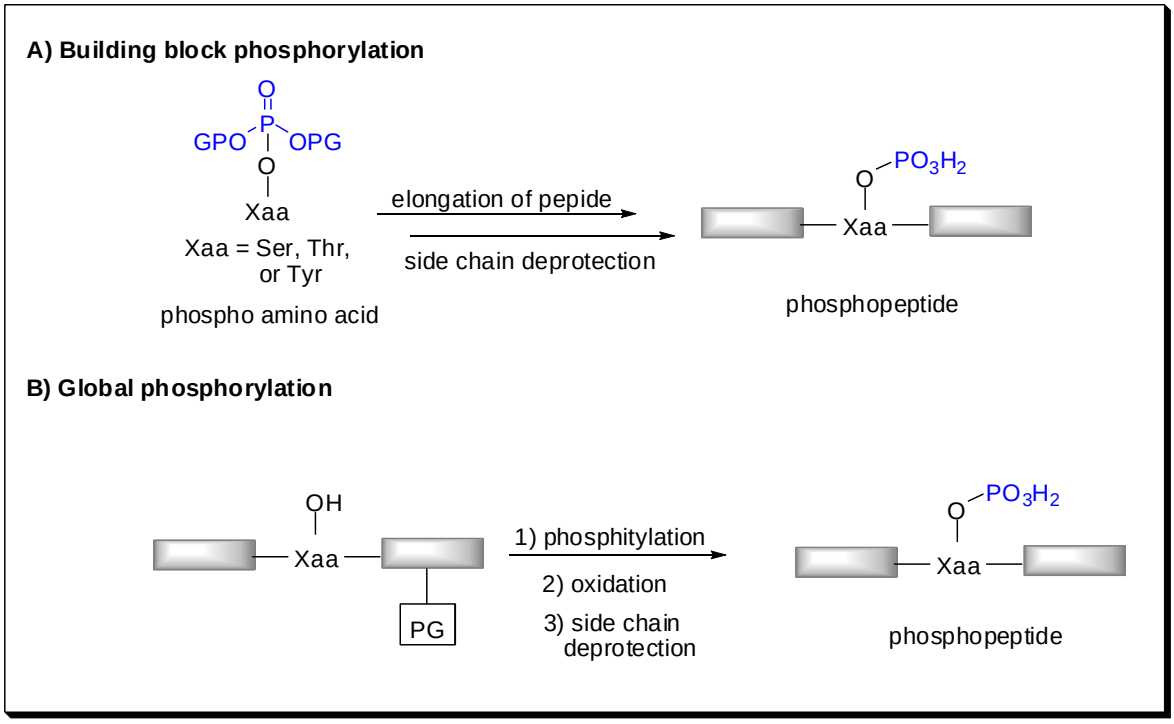
Apart from detailed phosphopeptide biosynthesis, various synthetic strategies for accessing phosphopeptides have been developed. Phosphopeptides can be accessed by either of two methods; building blocks or global phosphorylation (also known as postassembly phosphorylation). The Building blocks approach involves a sequential installation of amino acid and phosphoamino acid residues into the growing peptide chain. The protecting group of the phosphate groups (if present) is removed after the synthesis is accomplished.

On the other hand, global phosphorylation involves the synthesis of a peptide in which tyrosine, threonine, or serine at the latter phosphorylation site is incorporated with unprotected hydroxyl groups or with orthogonal protecting group relative to the other protecting groups of the side chains involved. After the removal of the orthogonal protecting group (if present), the free hydroxyl group is phosphitylated to yield peptide phosphite that is then oxidized to generate the desired phosphopeptide (Scheme 2).[21,22,23]

[21] J. S. McMurray, D. R. Coleman IV, W. Wang, M. L. Campbell, *Biopolymers*, **2001**, *60*, 3-31.
[22] J. W. Perich, *Lett. Pep. Sci.*, **1996**, *3*, 127-132.
[23] W. Bannwarth and A. Trzeciak, *Helv. Chim. Acta*, **1987**, *70*, 175.



Scheme 1. Protein phosphorylation. A) Phosphorylated forms of amino acid side chains in proteins of Eukaryotes and Prokaryotes; B) Kinase-catalyzed phosphorylation and Phosphatase-catalyzed dephosphorylation.



Scheme 2. Phosphopeptide preparation. A) Building block approach; B) Postassembly or global phosphorylation. PG = protecting group.

1.2.2 Glycosylation

Protein glycosylation, the covalent attachment of carbohydrates to protein, is one of the most important co- or post-translational modification steps in the synthesis of membrane proteins as well as secreted proteins. The resulting glycosylated biopolymeric materials play central roles in the organization of cellular processes and possess therefore important impact on human health and disease.[24] Generally, three different types of protein glycosylation patterns can be found in nature, which include the glycosylphosphatidyl inositol GPI-anchors as well as side-chain glycosylation occurring either on Ser or Thr in the *O*-glycosylation or on Asn in the *N*-glycosylation.[24,25]

In addition to these common modes of glycosylation other linkages have been identified as well, e.g. oligosaccharides linked to Thr or Ser by phosphodiester bridge which have been referred to as phosphoglycosylation.[26,27]

[24] (a) A. Varki, *Glycobiology* **1993**, *3*, 97-130; (b) C. R. Bertozzi, L. L. Kiessling, *Science* **2001**, *291*, 2357-2364; (c) P. M. Rudd, T. Elliott, P. Cresswell, I. A. Wilson, R. A. Dwek *Science* **2001**, *291*, 2370-2376. d) E. Weerapana, B. Imperiali, *Glycobiology* **2006**, *16*, 91R-101R.

[25] For a recent review on glycoprotein synthesis, see: D. P. Gamblin, E. M. Scanlan, B. G. Davis, *Chem. Rev*. **2009**, *109*, 131-163.

[26] a) T. Ilg, *Parasitol. Today*, **2000**, *16*, 489-497; b) G. A. Elsayed, G-J Boons, *Synlett.*, **2003**, *9*, 1373-1375; c) D. Majumdar G. A. Elsayed, T.Buskas, G-J Boons, *J. Org. Chem.*, **2005**, *70*, 1691-1697; d) D. P. Mehta, M. Ichikawa, P. V. Salimath, J. R. Etchison, R. Haak, A. Manzi, H. H. Freeze, *J. Biol. Chem.*, **1996**, ***271***, 10897-10903.

N-glycosylation

N-glycosylation occurs co-translationally and the oligosaccharide is attached onto an asparagine residue of the marker Asn-Xaa-Thr(Ser), where Xaa can be any amino acid except proline.[28] There are three main classes of *N*-linked oligosaccharides found in mature glycoproteins: high mannose, complex, and hybrid types. The core structure of all types is the same consisting of the pentasaccharide (α-Man(1-6)-)(α-Man(1-3)-)β-Man(1-4)-β-D-GlcNAc(1-4)-β-D-GlcNAc with terminal *N*-acetyl glucosamine residue attached to asparagine, whereas the antennary structure varies (Scheme 3).[29] Section 1.3 describes the biosynthesis of *N*-linked glycoproteins.

O-glycosylation

It is a post-translational event that does not require a consensus sequence and the carbohydrate moiety is glycosidically bound to the hydroxyl group of a serine or threonine.[30,31] Unlike *N*-linked glycoproteins, *O*-linked glycoproteins do not have a common core region but the most common type of *O*-linked glycans contain an initial α-*N*-acetylgalactoseamine residue (α-GalNAc) linked to Ser/Thr which is first added then other sugars are added successively (Scheme 4A).[32]

GPI anchored proteins

The *C*-terminus of the protein is modified by glycosylphosphatidyl inositol (GPI) post-translationally to yield a membrane bound protein. In this class of glycoproteins the carbohydrate moiety is linked to the protein *C*-terminus via a phosphodiester linkage of ethanolamine phosphate. The glycan core is conserved in all known examples, consisting of α-Man(1-2)-α-Man(1-6)-α-Man(1-4)-$GlcNH_2$, whereas the inositol phospholipid portion of the GPI moiety is variable (Scheme 4B).[32,33]

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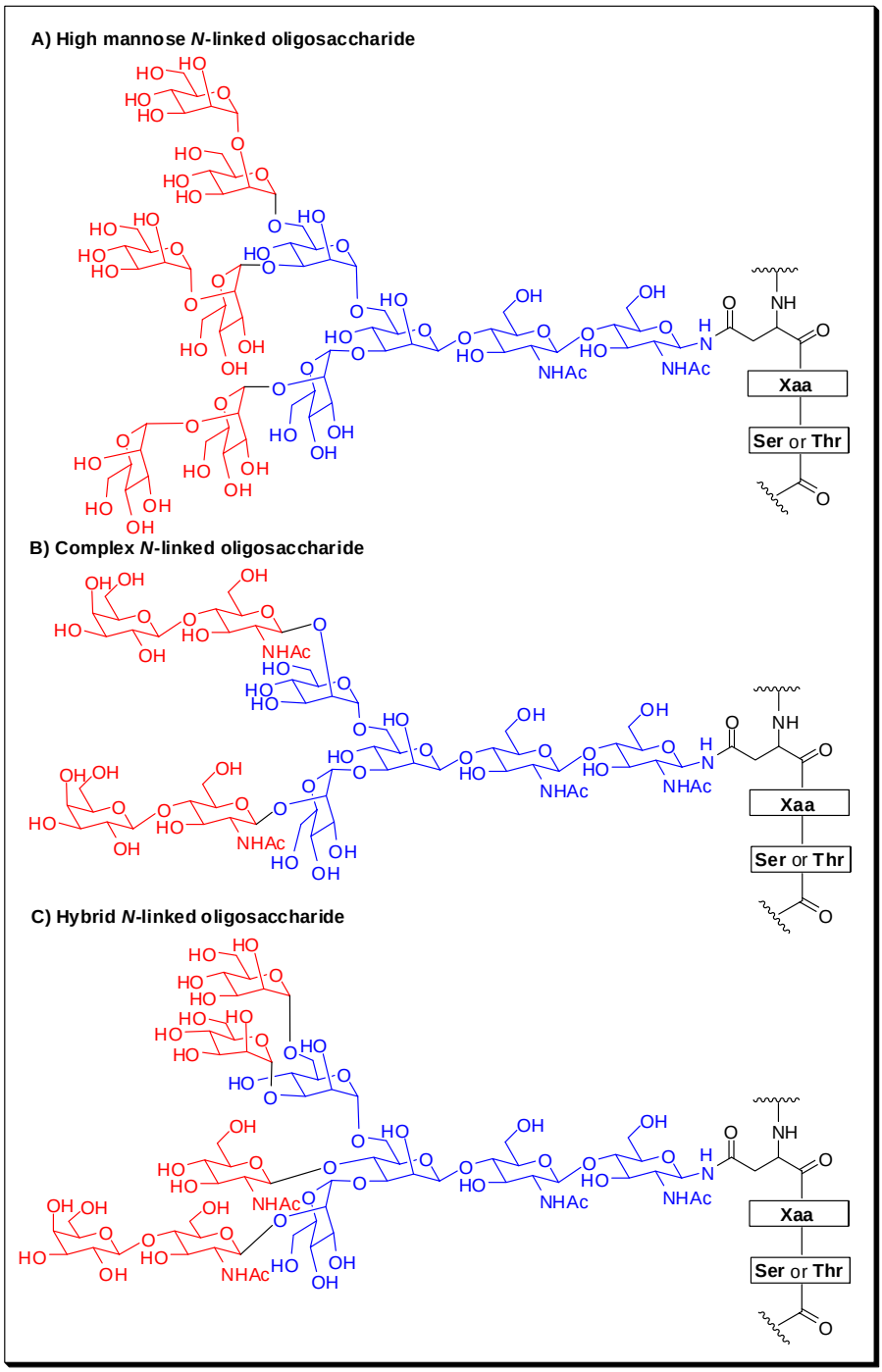
[27] For a mini review on phosphoglycosylation, see: P. A. Haynes, *Glycobiology* **1998**, *8*, 1–5
[28] I. Nilsson, G. von Hiejne, *J. Biol. Chem.* **1993**, *268*, 5798-5801.
[29] R. A. Dwek, *Chem. Rev.* **1996**, *96*, 683-720.
[30] P. E. Van den Steen, P. M. Rudd, R. A. Dwek, G. Opdenakker, *Crit. Rev. Biochem. Mol. Biol.* **1998**, *33*, 151-208
[31] P. E. Van den Steen, P. M. Rudd, M. R. Wormald, R. A. Dwek, G. Opdenakker, *Trends Glycos. Glycotech.* **2000**, *12*, 35-49.
[32] T. K. Lindhorst, *Essentials of carbohydrate chemistry and biochemistry*, 2007, WILEY-VCH, Germany, pp 213-236.
[33] P. Gerold, V. Eckert, R. T. Schwarz, *Trends Glycos. Glycotech.* **1996**, *8*, 265-277.

Phosphoglycosylation

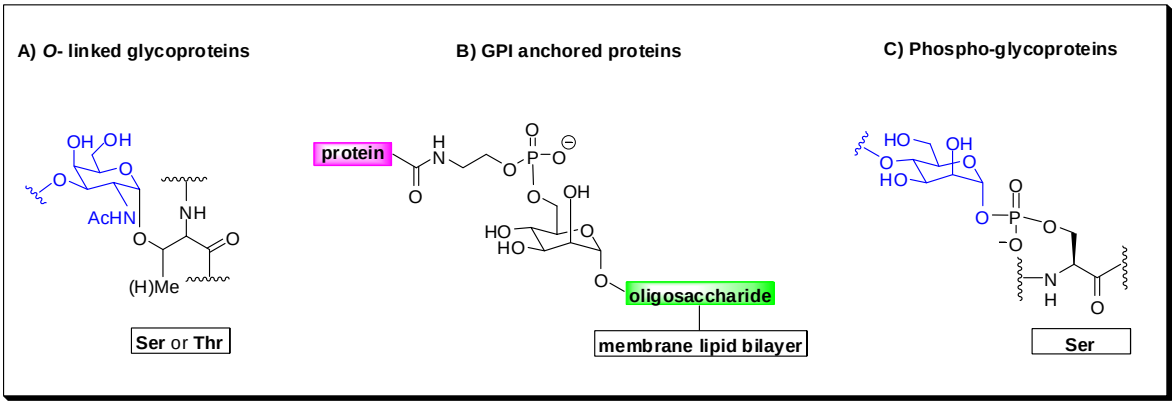
The human body faces everyday glycoproteins and glycoconjugates from food, pollen, as well as from microbial organisms. For instance, glycoconjugates of parasites play significant roles in their host interactions and pathology.[34]

Unfamiliar glycoproteins known as phosphoglycoproteins were identified in the phosphoglycans secreted during the insect and mammalian life cycle of several species of *Leishmania*.[26] For example, the secreted acid phosphate (sAP) of the parasite *Leishmania mexicana* is a phosphoglycoprotein in which the terminal sugar of the glycan moiety is attached to serine residue by a phosphodiester linkage (α-Man-1-PO_4-Ser) (Scheme 4C). The rest of the glycan moiety consists of monomeric mannose and a series of phosphorylated and neutral sugars.

[34] R. M. Bill, L. Revers, I. B. H. Wilson, *Protein glycosylation*, Kluwer Acadamic Publishers, USA, **1998**, pp 410.



Scheme 3. Types of *N*-linked oligosaccharides present in mature glycoproteins. A) High mannose type; B) Complex type; C) Hybrid type. Oligosaccharides in blue represent the pentameric core whereas the ones in red represent the antennary structures.



Scheme 4. Post-translational protein glycosylation. A) *O*-linked glycoproteins, α-GalNAc is the first sugar residue linked to Ser or Thr; B) GPI-anchored proteins via ethanolamine phosphate; C) Phosphoglycoprotein of *Leishmania mexicana*, α-Man-PO_4-Ser is the linkage unit.

1.3 Biosynthesis of *N*-linked glycopeptides

N-glycosylation is a co-translational event that begins in the rough endoplasmic reticulum (ER) and involves three stages; the phosphodolichol cycle, glycosylation of an asparagine side chain of a nascent polypeptide, and finally the elaboration of the glycan moiety.

The initial glycosylation step involves the biosynthesis of the precursor for all the various oligosaccharides present in *N*-linked glycoproteins. It takes place in the rough endoplasmic reticulum (ER) through the phosphodolichol cycle that involves sequential addition of sugars to a lipid carrier embedded in the ER membrane, dolicholphosphate, providing consequently a lipid-bound oligosaccharide as a donor for glycosylation, with $Glc_3Man_9(GlcNAc)_2$. Then the dolichol-bound tetradecasaccharide is transferred from the dolichol carrier to an asparagine side chain of a nascent polypeptide as it emerges into the ER lumen.[35] This process is catalyzed by an enzyme called oligosaccharyl transferase[36] that is embedded within the ER membrane and recognizes asparagine as a sugar acceptor in the consensus sequence Asn-Xaa-Ser/Thr, where Xaa is any amino acid residue except proline.

After the immature glycopolypeptide chain is released from the ribosome, trimming of the glycans starts within the ER by the removal of the three glucose molecules and a terminal mannose residue. Further glycan processing takes place after the glycoprotein is transferred to Golgi apparatus yielding mature glycoproteins which are sorted and transported to their final destination (Figure 3).[37,38]

[35] C. B. Hirschberg, M. D. Snider, *Annu. Rev. Biochem.* **1987**, *56*, 63-87.
[36] S. Silberstein, R. Gilmore, *FASEB J.* **1996**, 849-858.
[37] A. Helenius, M. Aebi, *Science* **2001**, *291*, 2364-2369.
[38] S. E. O'Connor, B. Imperiali, *Chem. Biol.* **1996**, *3*, 803-812. .

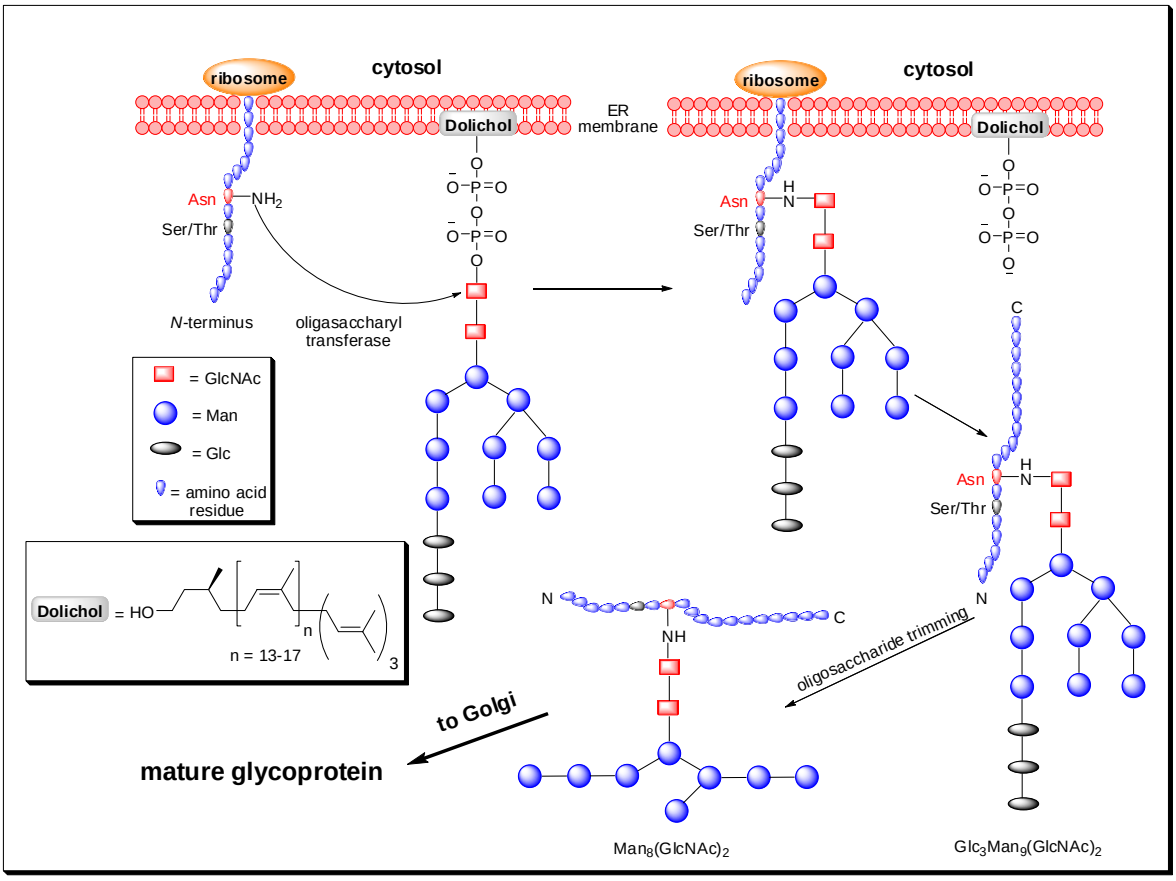


Figure 3. Schematic representation of co-translational biosynthesis of *N*-linked glycoproteins.

1.4 Synthetic strategies for *N*-linked glycopeptides

Although glycopeptides and glycoproteins are of great scientific importance for life sciences, the full elucidation of their biological functions has been severely hindered due to the difficulty of isolating them from natural sources in homogeneous forms as many of these proteins contain complex modifications with different glycoforms present.[39] In general, access of pure material is required for functional investigations.[40] Therefore, various synthetic strategies for accessing glycopeptides have been developed,[41] including the combination of various chemoselective ligation strategies upon expressed proteins.[42]

In principle, three synthetic routes can be envisioned for the synthesis of *N*-linked glycopeptides, which contain an amide bond linkage between the carbohydrate moiety and the polypeptide backbone via an Asn residue.

In the first route, suitable protected asparagine with a carbohydrate or oligosaccharide attached to the side chain is employed as building blocks during standard solid phase peptide synthesis (Scheme 5A). Although longer peptide sequences with simple carbohydrate modifications can be accessed, this strategy suffers occasionally from the final acidic deprotection conditions required for the protecting group removal of the individual amino acid protecting groups, since some of the glycosidic bonds in complex oligosaccharides are not stable under these conditions.[43]

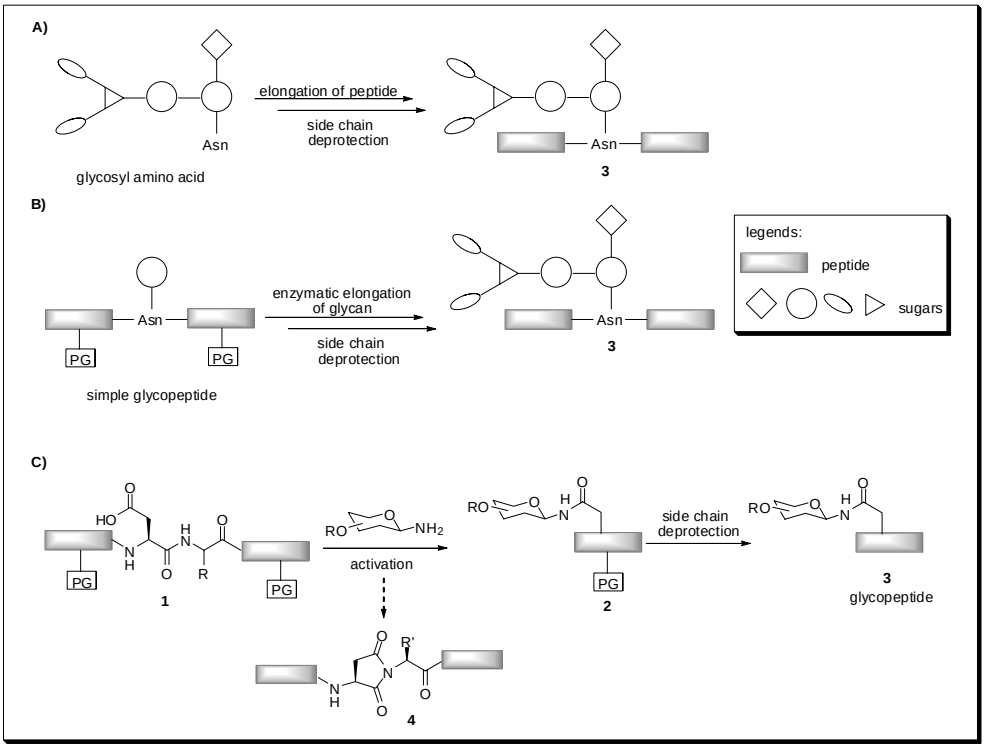
The second strategy utilizes sequential enzymatic glycosylation by glycosyltransferases which act upon a glycopeptide with at least one carbohydrate residue attached to the peptide side-chain (Scheme 5B).[44]

The third and from a synthetic point of view probably most attractive strategy is convergent approach, in which oligosaccharides are linked to full-length polypeptides, thereby resembling the natural co-translation process of *N*-glycosylation to a Asn-Xaa-Ser/Thr glycosylation site.

Several methodologies for the attachment of oligosaccharide branches to the peptide backbone by unnatural linkages exist, such as carbohydrate conjugation to Cys residues by disulfide or selenylsulfides linkages or to unnatural amino acids.[45] In contrast there are

[39] T. W. Rademacher, R. B. Parekh, R. A. Dwek, *Annu. Rev. Biochem.*, **1988**, *57*, 785-838.
[40] P. H. Seeberger, D. B. Werz, *Nature*, 2007, **446**, 1046-1051.
[41] M. J. Grogan,M. R. Pratt, L. A. Marcaurelle, C. R. Bertozzi, *Annu. Rev. Biochem*. **2002**, *71*, 593 – 634.
[42] C. P. R. Hackenberger, D. Schwarzer, *Angew. Chem. Int. Ed*. **2008**, *47,* 10030-10074.
[43] L. J. Otvos, L. Urge, M. Hollosi, K. Wroblewski, G. Graczyk, G. D. Fasman, J. Thurin, *Tetrahedron Lett*. **1990**, *31,* 5889-5892.
[44] Z. Guo, N. Shao, *Med. Res. Rev*. **2005**, *25,* 655-678.
[45] a) B. G. Davis, M. A. T. Maughan, M. P. Green, A. Ullman, J. B. Jones, *Tetrahedron: Asymmetry* **2000**, *11*, 245 – 262; b) D. P. Gamblin, P. Garnier, S. J. Ward, N. J. Oldham, A. J. Fairbanks, B. G. Davis, *Org. Biomol. Chem.* **2003**, *1*, 3642 – 3644; c) D. P. Gamblin, P. Garnier, S. van Kasteren, N. J. Oldham, A. J.

only few approaches for a convergent assembly of a glycopeptide with a natural (amide) *N*-linkage. These routes rely on the condensation of a glycosyl amine to an appropriately activated Asp-side chain in a protected peptide **1**, either by classic activation reagents in a direct activation of free Asp,[46] or by photolysis of a photoreactive Asp-amide side chain (Scheme 5C).[47]



Scheme 5. Synthetic strategies for *N*-linked glycopeptides. A) Building blocks approach; B) Enzymatic elongation of simple glycan; C) Convergent approach.

Although this convergent route has been applied to impressive syntheses of various complex glycopolypeptides **3** with great potential for vaccine development,[48] it still does not utilize a chemoselective amide bond formation thus requiring the protection of nucleophilic or carboxylic acid peptide side chains during the glycosyl amide-bond

Fairbanks, B. G. Davis, *Angew. Chem.* **2004**, *116*, 846 – 851; *Angew. Chem. Int. Ed.* **2004**, *43*, 828 – 833; d) G. J. L. Bernardes, D. P. Gamblin, B. G. Davis, *Angew. Chem.* **2006**, *118*, 4111 – 4115; *Angew. Chem. Int. Ed.* **2006**, *45*, 4007 – 4011.

[46] S. T. Ainsfeld, P. T. Lansburry *J. Am. Chem. Soc.* **1993**, *115*, 10531.

[47]C. M. Kaneshiro, K. Michael, *Angew. Chem.* **2006**, *118*, 1094-1098.

[48] a) V. Y. Dudkin, J. S. Miller, A. S. Dudkina, C. Antczak, D. A. Scheinberg, S. J. Danishefsky, *J. Am. Chem. Soc.* **2008**, *130*, 13598 – 13607; b) J. D. Warren, X. Geng, S. J. Danishefsky, *Top. Curr. Chem.* **2007**, *267*, 109 – 141.

coupling and an additional final deprotection step of **2**. Furthermore, aspartimides **4**[49] are potential by-products which can be formed upon activation of the Asp side-chain.

[49] S. T. Cohen-Anifeld, P. T. Lansbury, Jr., *J. Am. Chem. Soc.*, **1993**, *115*, 10531-10537.

1.5 Chemoselective ligation strategies for peptides

The utilization of synthetic strategies described in section 1.4 allows the access of glycopeptides. However, in order to access large glycoproteins, chemoselective ligation strategies for the installment of unprotected (glyco)peptide segments are required.[42]

1.5.1 Native Chemical Ligation (NCL)

This ligation strategy was introduced by Kent and co-workers.[50] The basis of this approach was shown in an early experiment in 1953 by Wieland et al.[51]

Native chemical ligation is carried out in mild reaction conditions, normally, in neutral buffered aqueous solutions. The first step of this chemoselective ligation approach is based on a reversible thioester exchange process between *C*-terminal peptide thioester and *N*-terminal Cys peptide to yield a thioester intermediate which undergoes spontaneous, irreversible rapid intramolecular rearrangement to form a native peptide bond at the ligation site (Scheme 6).

Although native chemical ligation enables the total chemical synthesis of proteins, but it suffers from the necessity of having a cystein residue at the ligation junction. However, to overcome this drawback, removable auxiliaries that act as cystein surrogates have been utilized to mediate the chemoselective ligation of peptide fragments.[52,53]

In 2005 Imperiali and co-workers utilized NCL in the semisynthesis of a glycosylated Im7 Analogue.[54] In the same year Unverzagt and co-workers reported another interesting example of *N*-linked glycopeptide synthesis by means of NCL in the course of the synthesis of a glycosylated fragment of RNase B.[55] Recently, they also reported an impressive total synthesis of the 124-amino acid Enzyme RNase C.[56,57,58]

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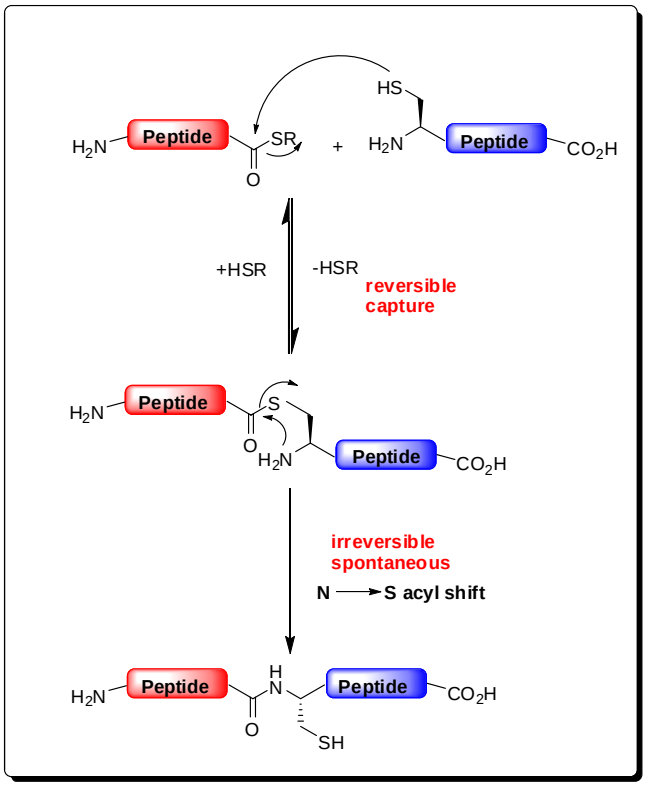
[50] P. E. Dawson, T. W. Muir, I. Clark-Lewis, S. B. H. Kent *Science* **1994**, *266*, 776-779.
[51] T. Wieland, E. Bokelmann, L. Bauer, H. U. Lang, W. Schafer *Liebigs Ann. Chem.* **1953**, *583*, 129-149.
[52] C. Marinzi, J. Offer,R. Longhi, P. E. Dawson, *Bioorg. Med. Chem.*, **2004**, *12*, 2749.
[53] J. Offer, C. N. C. Boddy, P. E. Dawson, *J. Am. Chem. Soc.*, **2002**, *124*, 4642.
[54] C. P. R. Hackenberger, C. T. Friel, S. E. Radford, B. Imperiali, *J. Am. Chem. Soc.* **2005**, *127*, 12883-12889.
[55] S. Mezzato, M. Schaffrath, C. Unverzagt, *Angew. Chem., Int. Ed.*, **2005**, *44*, 1650–1654.
[56]C. Piontek, P. Ring, O. Harjes, C. Heinlein, S. Mezzato, N. Lombana, C. Pohner, M. Puettner, D. V. Silva, A. Martin, F. X. Schmid and C. Unverzagt, *Angew. Chem., Int. Ed.*, **2009**, *48*, 1936–1940.
[57]C. Piontek, D. V. Silva, C. Heinlein, C. Pohner, S. Mezzato, P. Ring, A. Martin, F. X. Schmid and C. Unverzagt, *Angew. Chem., Int. Ed.*, **2009**, *48*, 1941–1945.
[58] For a recent review on the synthesis of glycopeptides by means of NCL, see: R. J. Payne, C-H. Wong *Chem. Commun.*, **2010**, *46*, 21–43.



Scheme 6. Proposed mechanism of Native Chemical Ligation (NCL).

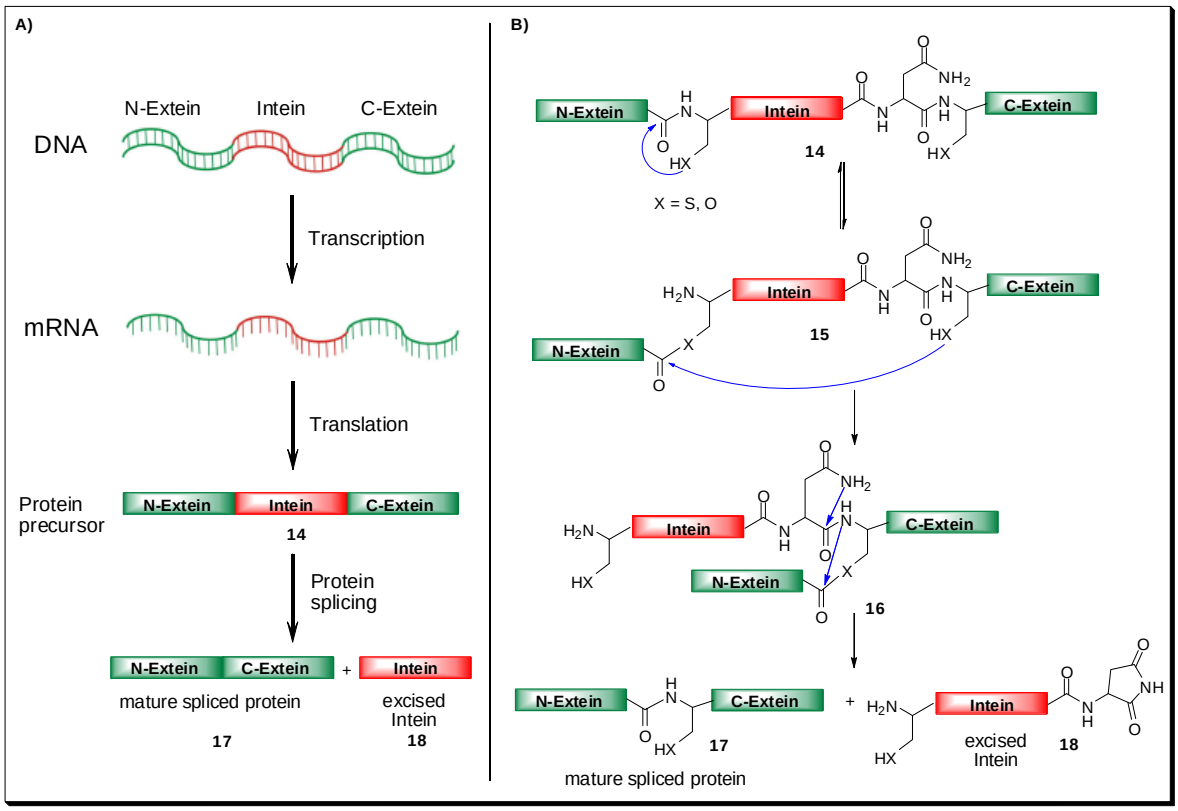
1.5.2 Protein splicing

As previously mentioned in section 1.2, protein biosynthesis involves DNA transcription into RNA which is then translated into protein. It was noted that there are many genes that encode amino acid sequences that are absent in the mature protein. These sequences are removed either from RNA prior to translation via RNA splicing or from a precursor protein by protein splicing.[9]

Protein splicing is a naturally occurring enzymatic process that occurs posttranslationally and results in excision of an internal precursor protein domain termed an intein and ligation of its N and C flanking sequences termed N- and C-exteins, respectively, yielding a mature protein (Scheme 7A).

Protein splicing is initiated by a nucleophilic attack by the side chain of the first intein residue (Cys, Ser, or Thr) at an amide bond in **14**, resulting in N⟶X acyl shift to form a (thio)ester **15** which undergoes a transesterification reaction where the thiol or the hydroxyl group of the first C-extein residue (Cys, Ser, or Thr) attacks the (thio)ester linkage, resulting in linking C-extein to N-extein through a new (thio)ester bond in **16**. Spontaneous rearrangement of the (thio)ester linkage between the ligated exteins to the

more stable native peptide linkage initiated by Asn residue at the C terminus of the intein provides a mature spliced protein **17** and a succinimide excised intein **18** (Scheme 7B).



Scheme 7. Protein splicing. A) Schematic description of protein splicing; B) Proposed mechanism of protein splicing, X represents either the oxygen present in the side chain of Ser or Thr or the sulfur present in Cys. In some inteins, Asn is replaced by Gln.

The concept of protein splicing has been utilized in protein semisynthesis which involves the ligation between synthetic and recombinant peptides by either ‘‘expressed protein ligation’’ (EPL)[59] or ‘‘protein trans-splicing’’.

EPL involves the utilization of protein splicing to overcome the limitations of converting the recombinant proteins into thioesters via fusing a protein of interest as an N-extein with an intein at the genetic level. After the first step and before the second step of protein splicing, the addition of excess thiol leads to the conversion of the N-extein into a thioester, thus, it can be used in NCL. Again, the thiol addition should be before Asn residue at the C terminus of the intein provides a mature spliced protein and a succinimide excised intein.

[59] R. David, M. P. O. Richter, A. G. Beck-Sickinger, *Eur. J. Biochem.* **2004**, *271*, 663-677.

1.5.3 Traceless Staudinger Ligation (TSL)

The chemical basis of the traceless[60,61,62] and the non-traceless Staudinger[63] ligation was discovered in 1919 by Staudinger et al.[64] The reaction between an azide and a phosphine results in an iminophosphorane which, in the presence of water, hydrolyzes spontaneously into a primary amine and the corresponding phosphine oxide.[65]

Scheme 8A shows the mechanism of Staudinger reaction in which a nucleophilic addition of the phosphine at the terminal nitrogen of the azide takes place to form a phosphazide **5** which, in turn, loses dinitrogen (N_2) via the 4-membered-ring transition state **6** to generate an iminophosphorane which is hydrolyzed in the presence of water to yield a primary amine and phosphine oxide.

The non-traceless Staudinger ligation was introduced by Bertozzi and co-workers[63] in 2000. An electrophilic trap is placed on the triaryl phosphine so that the nucleophilic nitrogen of the iminophosphorane attacks the carbonyl group of the ester (the electrophilic trap) to produce an amide linkage and the phosphine oxide upon hydrolysis with water.

The classical Staudinger reaction results in two products which are not covalently bound after hydrolysis whereas the Staudinger ligation links two molecules together by an amide bond with phosphine oxide still present in the product. Therefore this ligation is known as non-traceless Staudinger ligation (Scheme 8B).

The traceless Staudinger ligation was developed by Bertozzi and Raines and their co-workers[60,61,62]. As the name implies, this methodology ligates two molecules by an amide linkage to generate a product which does not contain phosphine oxide, therefore it is known as ‘‘traceless’’. Like the non-traceless Staudinger ligation, the aza-ylide **12** rearranges to amidophosphonium salt **13** which in turn generates an amide linkage in **11** and phosphine oxide upon hydrolysis (Scheme 8C).

Recently, the traceless Staudinger ligation could be applied to synthesize a cyclic peptide of 11 amino acid residue derived from the circular protein J25.[66]

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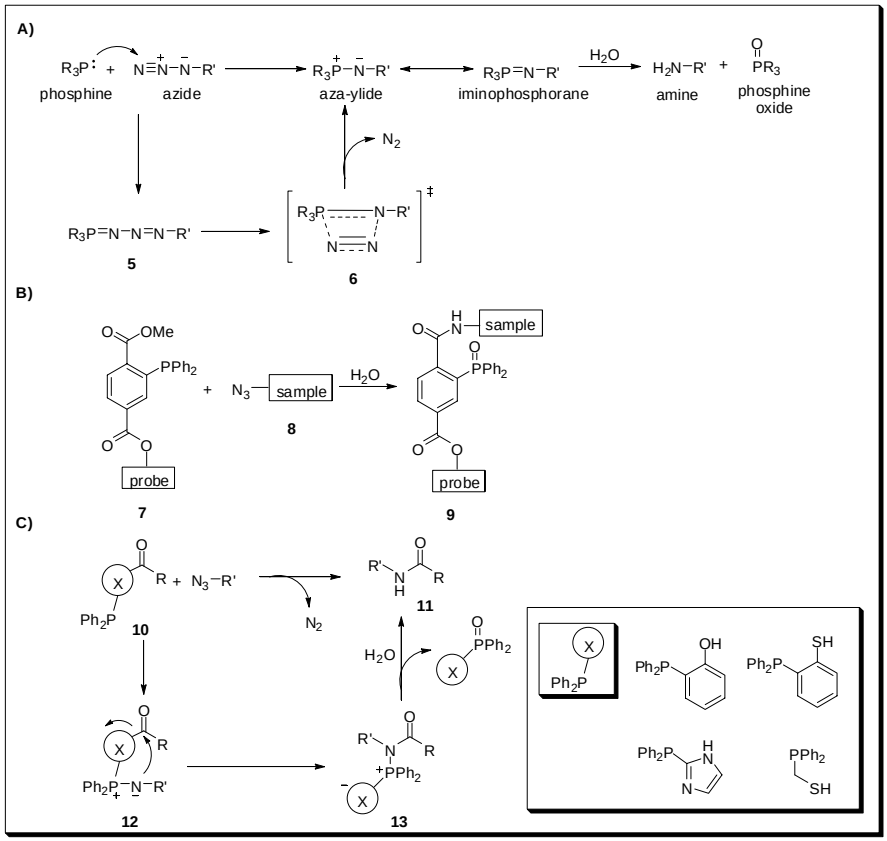
[60] B. L. Nilsson, L. L. Kiesling, R. T. Raines, *Org. Lett.* **2001**, *3*, 9 – 12.
[61] E. Saxon, J. I. Armstrong, C. R. Bertozzi, *Org. Lett.* **2000**, *2*, 2141 – 2143.
[62] B. L. Nilsson, L. L. Kiessling, R. T. Raines, *Org. Lett.* **2000**, *2*,1939 – 1941.
[63] E. Saxon, C. R. Bertozzi, *Science* **2000**, *287*, 2007 – 2010.
[64] H. Staudinger, J. Meyer, *Helv. Chim. Acta* **1919**, *2*, 635-646.
[65] a) M. Köhn, R. Breinbauer *Angew. Chem. Int. Ed.* **2004**, *43*, 3106-3116; b) M. B. Soellner, B. L. Nilsson, R. T. Raines, *J. Am. Chem. Soc.* **2006**, *128*, 8820-8828.
[66] R. Kleineweischede, C. P. R. Hackenberger, *Angew. Chem. Int. Ed.* **2008**, *47*, 5984 –5988.



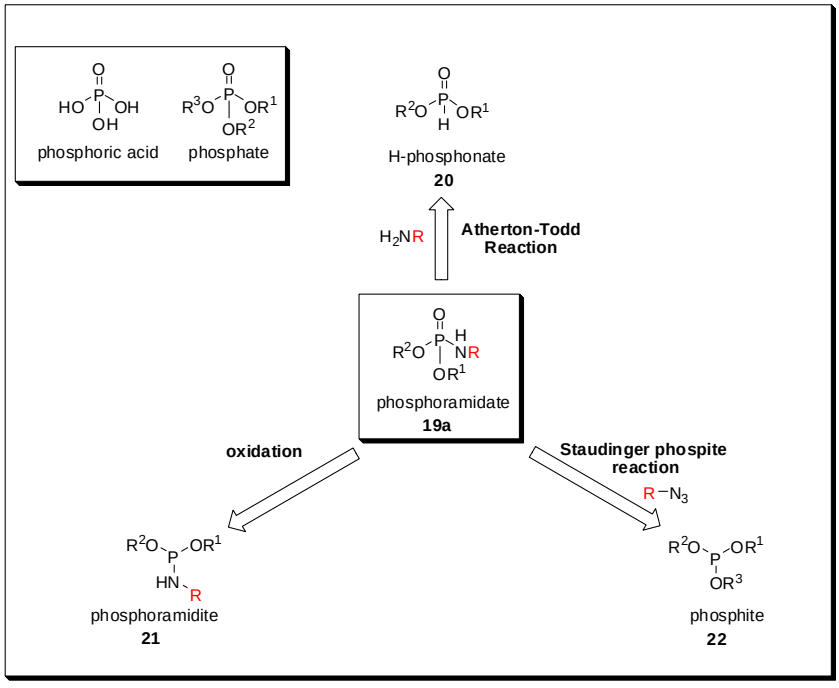
Scheme 8. Staudinger ligation. A) Classical Staudinger reaction and proposed mechanism; B) Non-traceless Staudinger ligation; C) Traceless Staudinger ligation. The scheme was adapted from reference [65].

1.6 Some aspects of phosphoramidate chemistry

Phosphoramidates **19a** belong to the family of amides of phosphoric acid H_3PO_4. More precisely, a phosphoramidate is a phosphate ester derivative that has one of its *OR* group substituted by NR_2.

Phosphoramidates, as P(V) compounds, are frequently synthesized by utilizing either P(V) or P(III) precursors (Scheme 9).

The Atherton-Todd reaction discovered in 1945 is a synthetically valuable approach for the preparation of phosphoramidates starting from P(V) precursors.[67,68,69,70] It proceeds under basic conditions involving the proton abstraction from the *H*-phosphonate **20** to form an active species **23** which reacts further with tetrachloromethane to generate phosphorochloridate **26** in situ via compound **24**.



Scheme 9. Synthetic approaches for phosphoramidates.

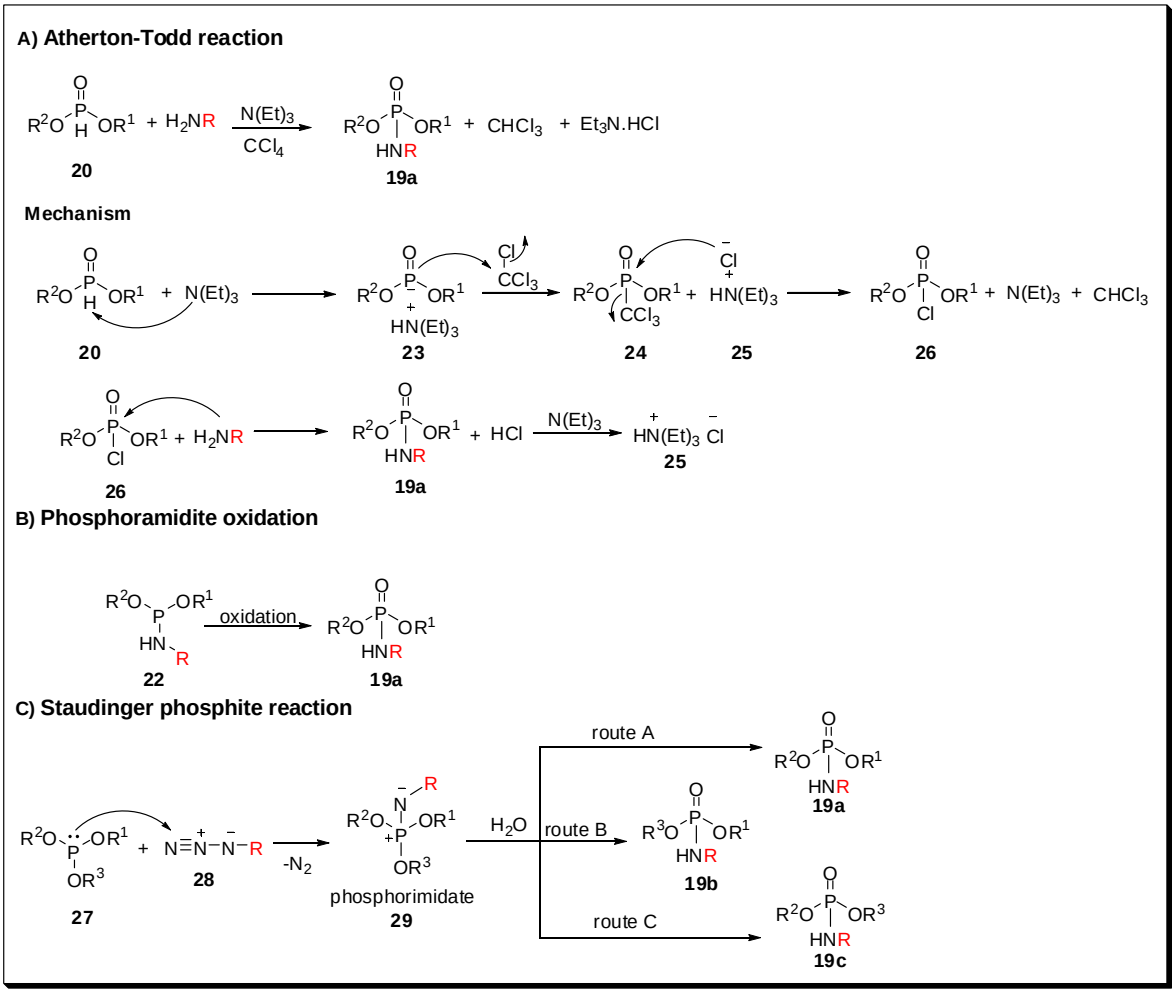
A subsequent nucleophilic addition of a primary amine at the phosphorous atom of **26** provides the corresponding phosphoramidate **19a** (Scheme 10A).

[67] F. R. Atherton, H. T. Openshaw, A. R. Todd, *J. Chem. SOC.*, **1945**, 660-663.
[68] F. R. Atherton, **A.** R. Todd, *J. Chem. SOC.*, 1947, 674-678.
[69] L. Liu, G. Li, X. Zeng, L. Fu, R. Cao, *Heteroatom Chemistry* **1996**, *7*, 131-138.
[70] K. Troev, E. M. G. Kirilov, D. M. Roundhill. *Bull. Chem. Soc. Jpn.*, **1990**, *63*, 1284-1285.

This reaction can be utilized to prepare phosphoramidate quickly, easily, and in good overall yields. For instance, the work up involves the removal of the insoluble salt **25** by filtration and the residue can be recrystallized to access the pure product.

The drawback of this reaction is the necessity for basic conditions which can be harmful for some basic labile adducts involved in this reaction. Consequently, it can not be generalized to access peptide phosphoramidate starting from *H*-phosphonate peptide since the nascent formed product can undergo β-elimination under the basic conditions of the reaction generating a dehydroalanine peptide.

Another limitation of this reaction is the non-chemoselectivity. Therefore, all the nucleophilic moieties involved in the reaction should be protected.



Scheme 10. Phosphoramidate synthesis. A) Atherton-Todd reaction and proposed mechanism; B) Phosphoramidite oxidation; C) Staudinger phosphite reaction.

In another approach, phosphoramidates can be delivered directly from phosphoramidite **22** (P(III) precursors) by oxidation with e.g. *t*-BuOOH (Scheme 10B)[71] or other oxidizing agent (Scheme 10B). This method can not be generalized either, as it relies on the oxidation step which will interfere with residues that are prone to oxidation.

Another approach relying on P(III) precursors is the Staudinger phosphite reaction (SPR) in which the first step involves the nucleophilic attack of the phosphorous atom of phosphite **27** on the azide moiety of **28** forming a phosphorimidate intermediate **29**. Unlike Staudinger reaction, the hydrolysis of **29** does not lead to a cleavage of the P─N bond, instead it leads to the formation of phosphoramidate **19**.

Based on mechanistic studies using O^{18}-enriched water, it was proposed that the hydrolysis proceeds via the nucleophilic attack of water at either the phosphorous atom of **29** releasing one of the OR groups or at the tetrahedral sp^3 hybridized α-carbon (if present).[72,73]

Regardless the mechanism of hydrolysis, one of the OR groups will be removed from the target molecule, being unproblematic for symmetric phosphite ($OR^1 = OR^2 = OR^3$). But in the case of an asymmetric phosphite, the hydrolysis of **29** can proceed via three possible routes (Scheme 10C) which may lead to unfunctionalized by-products.

In general, the Staudinger phosphite reaction can overcome the problems encountered in the previously mentioned strategies for accessing phosphoramidates, as it is chemoselective and does not involve an oxidation step. [74,75]

In a very recent publication, the relative propensities of simple substituents (OR) to be released during the phosphorimidate hydrolysis have been investigated.[76]

In addition, Staudinger phosphite reaction was applied for a chemoselective transformation of azido phenylalanine in peptides and proteins to generate analogues of phosphorylated Tyr residues in protein.[77]

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[71] J. Nielsen, M. H. Caruthers, *J. Am. Chem. SOC.* **1988**, *110*, 6275-6276.
[72] R. K. Chaturvedi, T. C. Pletcher, C. Zioudrou and G. L. Schmir, *Tetrahedron Lett.*, **1970**, *11*, 4339-4342.
[73] K. E. DeBruin and L. L. Thomas, *J. Chem. Soc., Chem. Commun.*, **1977**, 33-34.
[74] For previous work see: M. I. Kabachnik, V. A. Gilyarov, *Bull. Acad. Sci. USSR Div. Chem. Sci. (Engl. Transl.)* **1956**, *5*, 809-816. See also reference 75.
[75] R. Letsinger, G. A. Heavner, *Tetrahedron Lett.* **1975**, 16, 147-150.
[76] V. Böhrsch, R. Serwa, P. Majkut, E. Krause, C. P. R. Hackenberger, *Chem. Commun.*, **2010**, *46*, 3176-3178.
[77] R. Serwa, I. Wilkening, G. Del Signore, M. Mühlberg, I. Claußnitzer, C. Weise, M. Gerrits, C. P. R. Hackenberger, *Angew. Chem. Int. Ed.* **2009**, *48*, 8234–8239.

Chapter 2

Scope of the Thesis

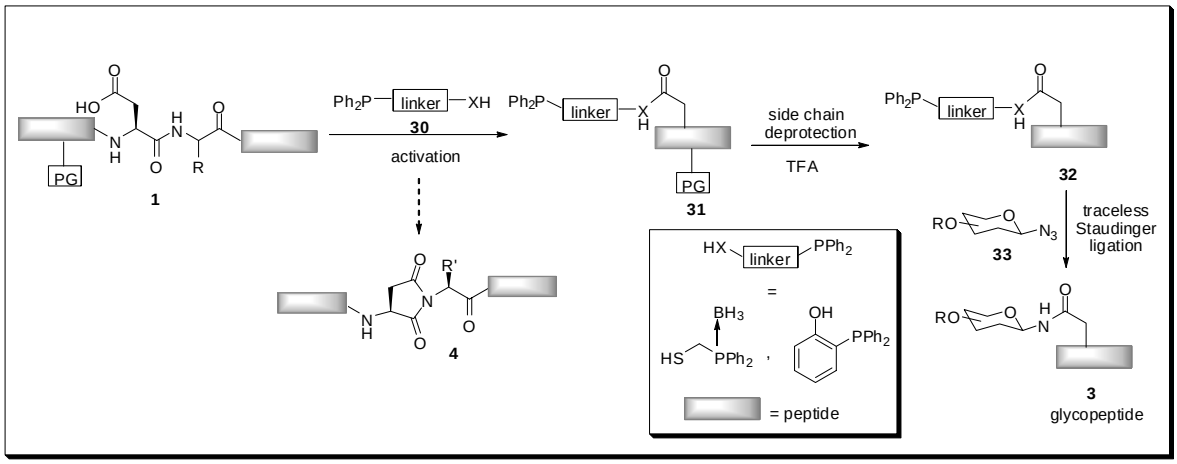
2 Scope of the Thesis

Glycopeptides are, as previously mentioned, desirable targets for synthesis and for the investigation of the biological properties. The aim of this thesis was to synthesize natural and unnatural glycoconjugates by the means of Staudinger-type reactions and to evaluate some of their biological properties.

The main concern was to synthesize *N*-linked glycopeptides by acid-induced Staudinger ligation which allows a convergent and possibly chemoselective approach overcoming the limitations involved in the convergent approach relying on the condensation of a glycosyl amines[78] with an appropriately activated Asp-side chain in a protected peptide (see chapter 1.4, scheme 5C).

An advantage of acid-induced traceless Staudinger ligation is that it allows the access to unprotected peptide-phosphinoesters **32** by acylation of a diphenylphosphine linker **30** with the preactivated Asp-side chain in a protected peptide yielding peptide-phosphinoesters **31**, where upon treatment with TFA the side chain protecting groups as well as the phosphine protecting group (if present) are removed, providing subsequently **32**. The latter can be reacted with (un)protected glycosyl azides **33** to form *N*-linked glycopeptides **3** upon hydrolysis with water.

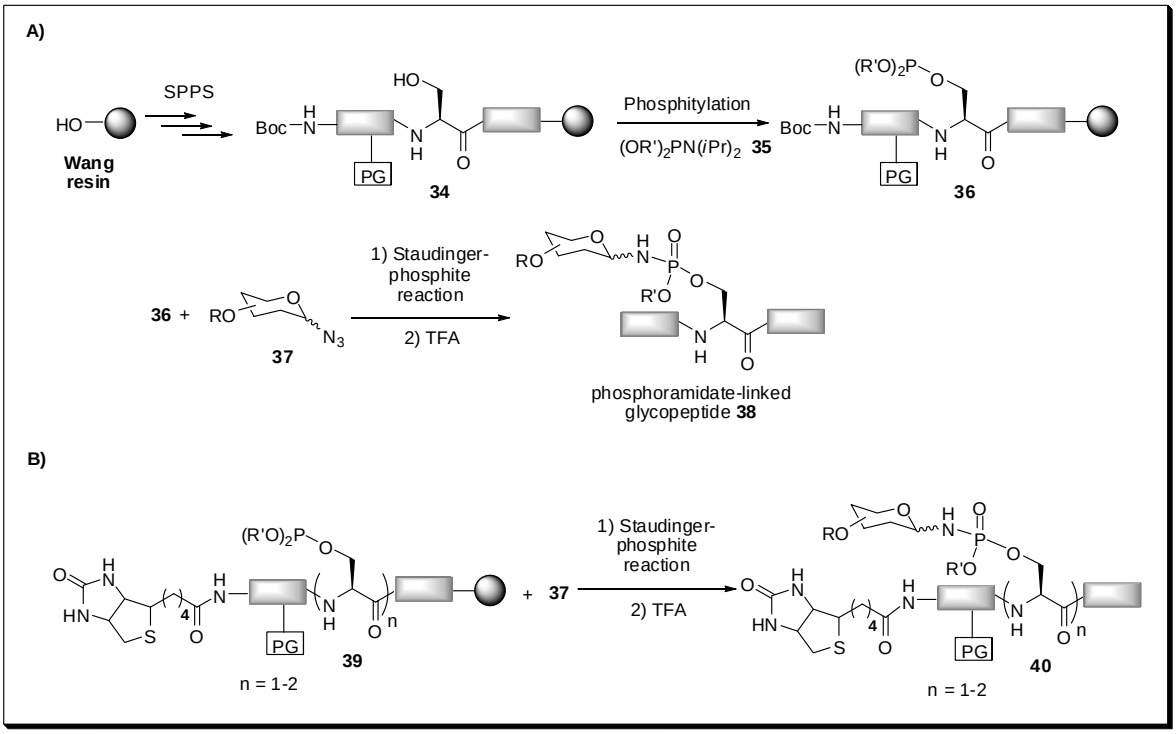
The main potential advantage of this strategy is that the peptide glycosylation is performed after removing all the protecting groups of amino acid residues. Thus, no acid treatment is required after the carbohydrate moiety has been attached to peptide segment which subsequently allows the attachment of oligosaccharide with acid-labile glycosidic bonds to peptides (Scheme 11).



Scheme 11. Our proposed strategy for *N*-linked glycopeptide synthesis. TFA = trifluoroacetic acid.

[78] S. Nakabayashi, C. D. Warren, R. W. Jeanloz, *Carbohydr. Res.* **1988**, *174*, 279-289.

The second aim of this research was to conduct conjugation of readily available carbohydrates with peptides on solid support and combine it with standard Fmoc-based solid-phase peptide chemistry (SPPS) to prepare glycoconjugate mimetics by the Staudinger phosphite reaction. Our synthetic strategy involves a two-step approach in which first a Ser-containing peptide **34** is converted into the phosphite-peptide **36** by global phosphitylation with phosphoramidites **35** on solid support.



Scheme 12. Our proposed Synthetic route to phosphoramidate-linked glycopeptides **38** and **40**. SPPS = solid-phase peptide synthesis, TFA = trifluoroacetic acid.

Then phosphite **36** is reacted with glycosyl azides **37** rendering the phosphoramidate-linked glycopeptide **38** after TFA cleavage from the solid support (Scheme 12A) [79].

A particular aim of this study was to access mono- and divalent biotinylated phosphoramidate-linked glycopeptides **40** to be utilized in recognition studies with other glycoproteins such as Lectins. **40** can be synthesized in analogy to glycopeptides **38** by blocking the *N*-terminus with biotin instead of a Boc protecting group (Scheme 12B).

[79] D. M. M. Jaradat, H. Hamouda, C. P. R. Hackenberger, *Eur. J. Org. Chem.* **2010**, DOI: 10.1002/ejoc.201000627.

Chapter 3

Results and Discussions:
Traceless Staudinger Ligation (TLS)

3 Results and Discussions: Traceless Staudinger Ligation (TSL)

3.1 Synthesis of glycosyl azides and phospinolinkers required for TSL

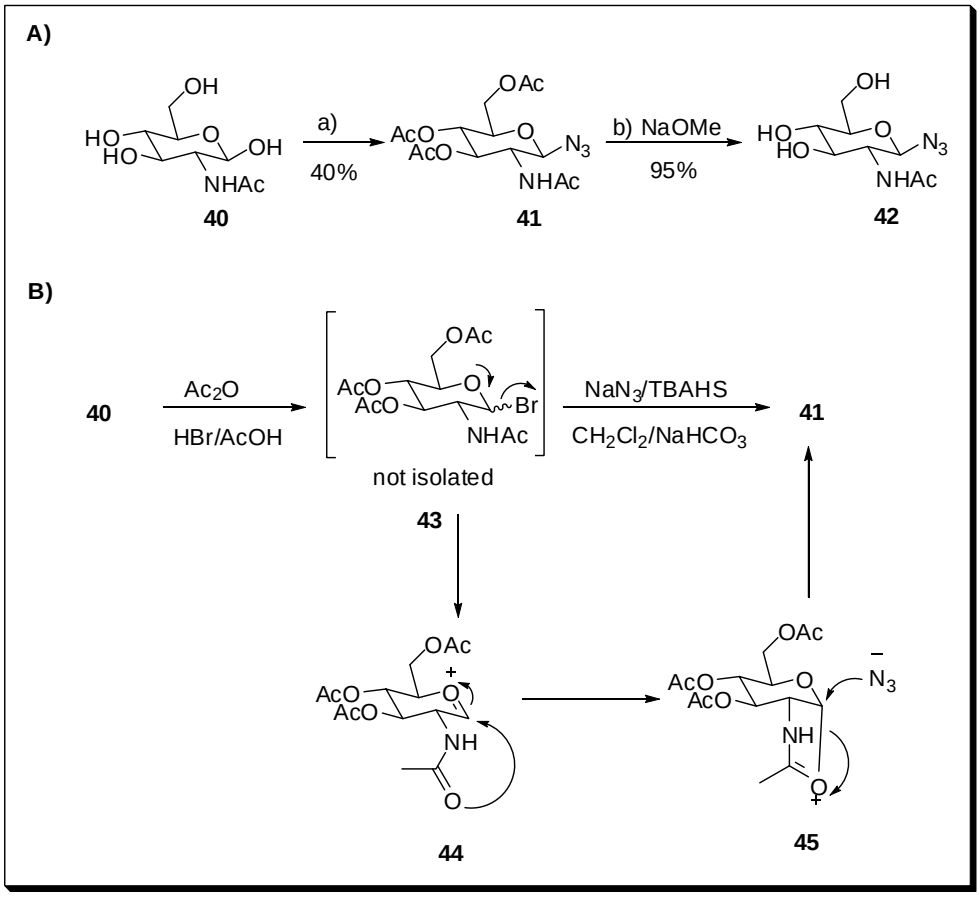
In order to carry out the traceless Staudinger ligation, different synthetic steps towards glycosyl azides and functional phosphinolinkers were performed.

β-*N*-acetyl-*D*-glucosamine azides (β-GlcNAc—N_3) **41** and **42** were chosen since β-GlcNAc represents the first carbohydrate moiety in the naturally occurring *N*-linked glycoproteins.

Acetylated β-GlcNAc—N_3 **41** was obtained from a two-step one-pot synthetic protocol in 40% overall yield,[80,81] which upon treatment with sodium methoxide in methanol afforded the unprotected β-GlcNAc—N_3 **42** in almost quantitative yield (Scheme 13A).[82,83,84]

In more details, GlcNAc **40** was treated with anhydrous hydrobromic acid in acetic acid to provide glycosyl bromides **43** via S_N1 mechanism which was then used without further purification due to its low stability. **43** (either α or β anomer) undergoes a spontaneous S_N1-like loss of Br^- to form **44**, followed by an internal reaction with the acetamide group at C2 to generate an oxonium ion **45** which then reacts with the azide ion via S_N2 yielding β-GlcNAc—N_3 **41** exclusively. The stereochemistry is determined by the neighboring group effect.[85] Tetrabutylammonium hydrogen sulphate (TBAHS) was used as a phase-transfer catalyst in the two phase reaction (Scheme 13B).

[80] R. Kumar, P. Tiwari, P. R. Maulik, A. K. Misra, *Eur. J. Org. Chem.*, **2006,** 74-79.
[81] M. A. Maier, C. G. Yannopoulos, N. Mohamed, A. Roland, H. Fritz, V. Mohan, G. Just, M. Monoharan, *Bioconjugate. Chem.*, **2003,** *14,* 18-29.
[82] S. Y. Hong, G. Tobias, B. Ballesteros, F. El Oualid, J. C. Errey, K. J. Doores, A. I. Kirkland, P. D. Nellist, M. L. H. Green, B. G. Davis, *J. Am. Chem. Soc.*, **2007**, *129*, 10966-10967.
[83] For a review on glycosyl azides, see: Z. Györgdeák, J. Thiem, *Adv. Carb. Chem. Biochem.*, **2006**, *60*, 103-163.
[84] Although the azides reported here did not show any instability, we strongly recommend caution and the use of appropriate protection during the handling of azides, especially with compounds of low molecular weight and during heating and/or concentrating steps. See also: S. Bräse, C. Gil, K. Knepper, V. Zimmermann, *Angew. Chem. Int. Ed.*, **2005**, *44*, 5188-5240.
[85] J. McMurry, *Organic Chemistry*, THOMSON BROOKS/COLE, Belmont, USA, **2004**, pp 956-960.



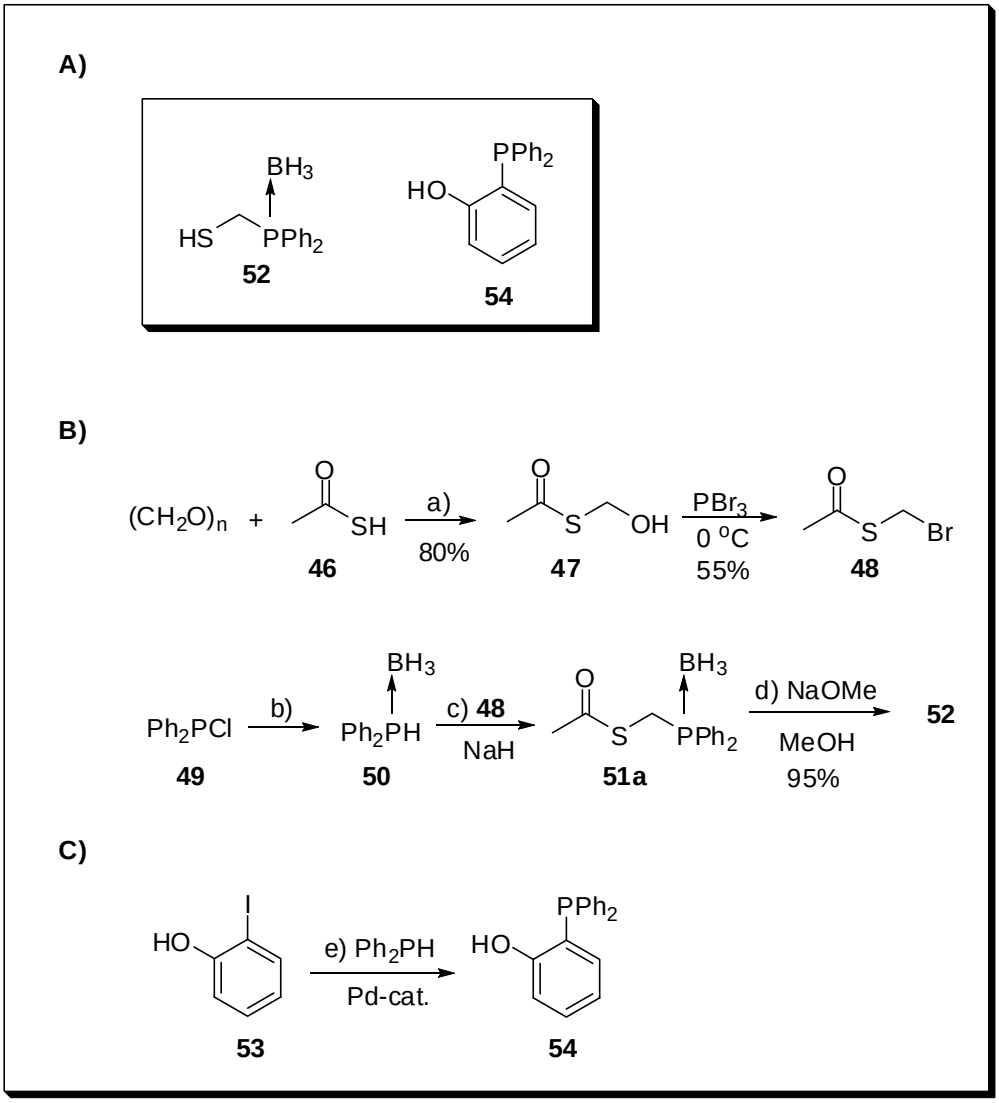
Scheme 13. A) Synthesis of β-GlcNAc-azides **41** and **42**; B) The neighboring group effect of a nearby acetamide. Reagents and Conditions: a) i) Ac_2O (5 equiv.), 33% HBr in AcOH, 0 °C to 25 °C; ii) NaN_3, TBAHS, CH_2Cl_2 then sat. $NaHCO_3$; b) NaOMe, MeOH. TBAHS = tetrabutylammonium hydrogen sulphate.

Next we intended to access phosphinolinkers **52** and **54** required for the traceless Staudinger ligation (Scheme 14A).

For the synthesis of borane-protected phosphinothiol **52**, hydroxymethyl acetyl sulfide **47** and bromomethyl acetyl sulfide **48** were prepared based on a reported protocol[86] by heating paraformaldehyde to 100 ^oC with thiolacetic acid **46** for three hours to form **47** which was further reacted with phosphorus tribromide at 0 ^oC to yield **48** as an alkylating agent for the synthesis of **52**. Next chlorodiphenylphosphine **49** was converted into diphenylphosphine-borane **50** in the presence of $LiAlH_4$ and the complexing agent BH_3.THF.[87] **50** was then alkylated by **48** in the presence of sodium hydride (NaH) affording diphenylphosphine(borane) methanethiol acetate **51a** in 36% overall yield.[88]

[86] G. K. Farrington, A. Kumar, F. C. Wedler, *Org. Prep. Proced. Int.* **1989**, *21*, 390- 392.
[87] T. Imamoto, T. Oshiki, T. Onozawa, T. Kusumoto, K. Sato, *J. Am. Chem. Soc.* **1990**, *112*, 5244-5252.
[88] M. B. Soellner, B. L. Nilsson, R. T. Raines, *J. Org. Chem.* **2002**, *67*, 4993-4996.

The alkylation proceeds via proton abstraction of **50** forming a compound with nucleophilic phosphorous which, in turn, attacks **48**. Treatment of **51a** with sodium methoxide in methanol provided the desired phosphinolinkers **52** almost quantitatively[89] (Scheme 16B). Compounds **51a** and **52** were found to be very stable against oxidation since they showed no sign of oxidation upon air exposure as revealed by ^{1}H-NMR and ^{31}P-NMR measurements.[90]



Scheme 14. Synthesis of phosphinolinkers. A) Phosphinolinkers **52** and **54**; B) Synthesis of borane-protected phosphinothiol **52**; C) Synthesis of diphenyl-phosphinophenol **54**. Reagents and Conditions: a) 100 °C on oil for 3 h; b) BH_3.THF (1.5 equiv.), $LiAlH_4$ (1.5 equiv.), 0 °C, 2 h; c) NaH (1.5 equiv.), **48** (1.5 equiv.), DMF, 0°C to 25°C, 15 h; e) KOAc (1.2 equiv.), $Pd(OAc)_2$ (cat.), Ph_2PH (1.0 equiv.), DMA, 130 °C, 16 h. DMF = dimethylformamide, DMA = dimethylacetamide.

[89] Y. He, R. J. Hinklin, J. Chang, L. L. Kiessling, *Org. Lett.*, **2004**, *6*, 4479-4482.
[90] M. Mühlberg , D. M. M. Jaradat, R. Kleineweischede , I. Papp, D. Dechtrirat, S. Muth, M. Broncel, C. P. R. Hackenberger, *Bioorg. Med. Chem.*, **2010**, *18* ,3679–3686.

Diphenyl-phosphinophenol **54** was synthesized by a palladium-catalyzed P—C cross coupling reaction between 2-iodophenol **53** and diphenylphosphine (Ph_2PH)[91] as outlined in scheme 14C in an overall yield of 65%. A major by-product of this reaction was the oxidized form of **54** which could be separated from **54** by flash chromatography.

Compound **54** showed excellent stability against oxidation in pure form since it could be stored at room temperature for an extended period of time with no apparent oxidation as revealed by ^{31}P-NMR measurements. Ph_2PH is very prone to oxidation, therefore, the major oxidized by-product can be attributed to the fact that Ph_2PH is oxidized partially during the reaction and it is then coupled with 2-iodophenol forming the oxidized form of **54**.

Recently, Marx and co-workers reported the synthesis of a water soluble phosphinophenol which enabled the traceless Staudinger ligation in water. [92]

[91] O. Herd, A. Heβler, M. Hingst, M.Tepper, O. Stelzer, *J. Organomet. Chem.* **1996**, *522*, 69-76.
[92] S. H. Weisbrod, A. Marx, *Synlett* **2010**, 787-789.

3.2 Acid-induced traceless Staudinger ligation with phosphinothiol 52

We began our investigations by employing the acid-induced traceless Staudinger glycosylation on small molecules to access glycosyl amides which was then compared to base-induced traceless Staudinger glycosylation.

Compound **51b** was obtained from acylation of phosphinothiol **52** with DIC-preactivated benzoic acid in 80% yield. Treatment of compounds **51** with neat TFA and subsequent removal of the volatiles under high vacuum afforded the phosphonium salts **55a** and **55b**. After addition of DIPEA, phosphines **55c** were delivered in-situ which reacted with **41** to give exclusively the desired β-glycosyl amides **56a** and **56b** in overall yields of 62% and 60%, respectively.

An alternative basic deprotection strategy with DABCO at 40 °C in a base-induced traceless Staudinger ligation furnished amide **56a** and **56b** in comparable yields of 60% and 55%, respectively (Scheme 15A).

Similarly, the acidic deprotection of the Asp-phosphinothioester **58**, which was obtained in high yields by standard DIC coupling, gave the phosphonium salt **59** and finally after traceless Staudinger ligation in the presence of DIPEA glycoamino acid **61** from **41** in 45% yield based on **58**, thereby implying potential for a similar glycopeptide transformation. As monitored by LC/MS analysis the aza-ylide (iminophosphorane) **60a** was formed initially, which rearranged partially to amidophosphonium salt **60b** which upon hydrolysis furnished **61**. Phosphinoxide **63** was observed as a by-product, which originated from either the hydrolysis of unrearranged **60a** or the oxidation of unreacted **59** (Scheme 15B).

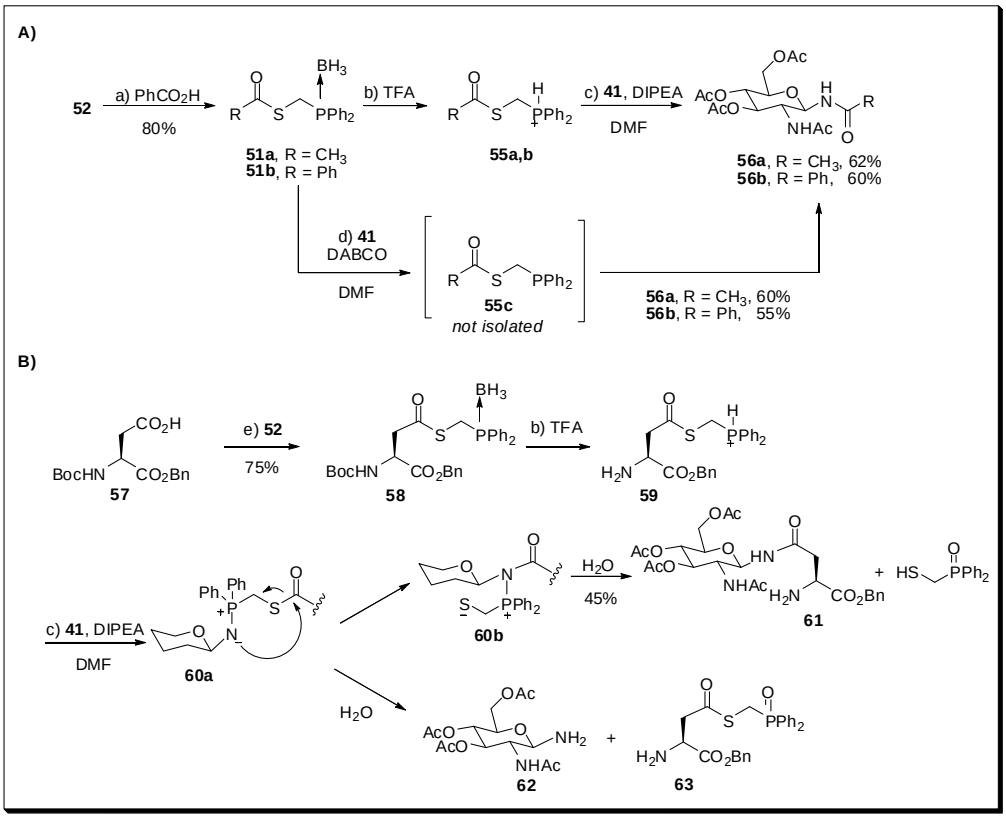
For glycopeptide synthesis we focused next on the synthesis of the aspartic acid side chain activated peptides **66**. Since we envisioned the formation of aspartimide[93] **67** as a significant side reaction during the activation of the Asp side-chain, in particular if the Asp residue is *N*-terminal linked to a small residue like alanine or glycine. We first investigated easily accessible model peptide **64** with only one functional Asp residue and thereby using phenylmethanethiol **65** and **52** for peptide thioester synthesis. The investigations were carried out with peptide concentration of 11 mM.

Indeed, we observed aspartimide formation under HBTU/DIPEA activation conditions, whereas DIC with catalytic amounts of DMAP provided peptide thioesters **66a**

[93] S. T. Anisfeld and P. T. Lansbury, Jr., *J. Org. Chem.* **1990**, *55*, 5560-5562.

and **66b** with only traces amounts of aspartimide present (Scheme 16A, table 1, figure 4).[94]

Borane-deprotection of **66b** into the phosphonium salt **68a** was accomplished upon TFA treatment, which proceeded in 65% conversion after one hour as monitored by LC/MS analysis (λ = 300 nm).

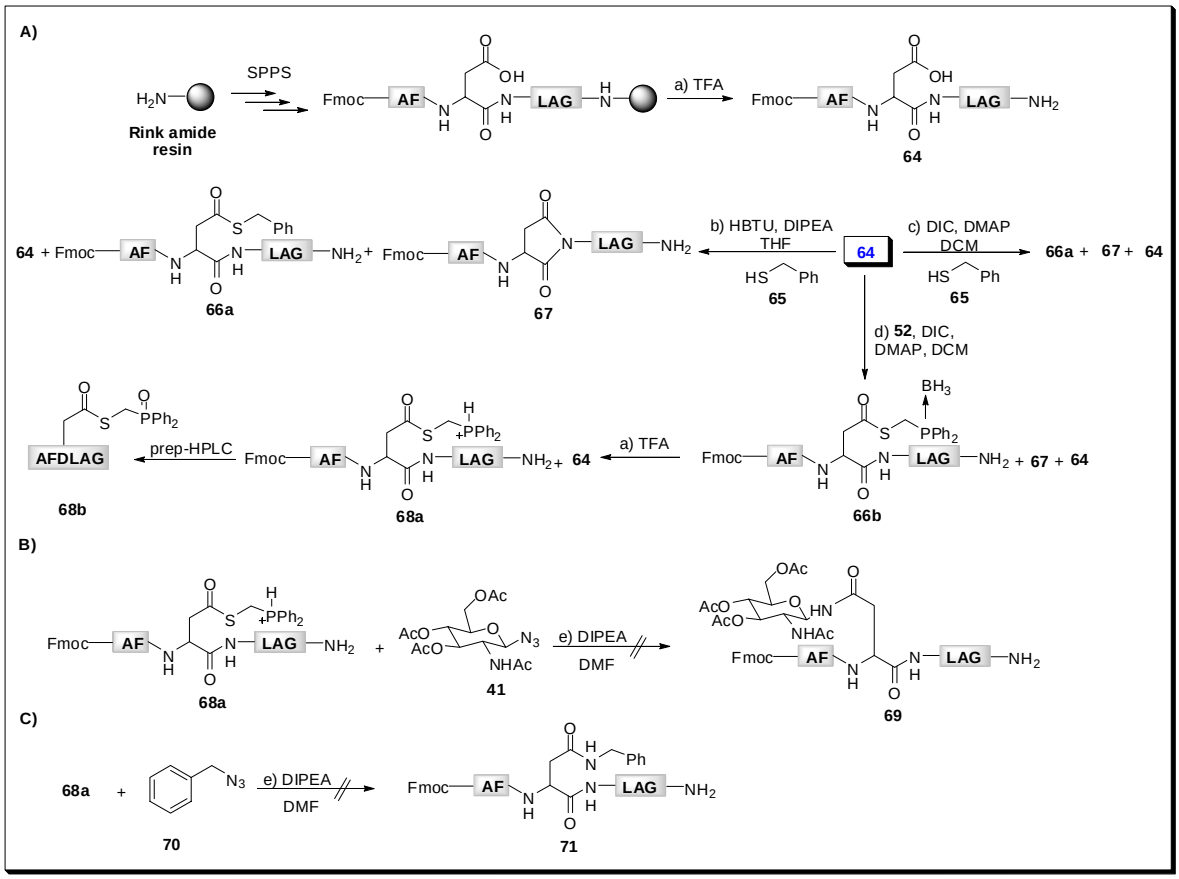


Scheme 15. Model studies: the acylation of β-GlcNAc-azide **41** by the traceless Staudinger ligation. A) Acidic and basic deprotection of borane-protected phosphinothiols for induced glycosylation reactions; B) Asp side-chain glycosylation by acidic deprotection strategy. Reagents and Conditions: a) $PhCO_2H$ (0.5 equiv), DIC, (3 equiv), DMAP (cat.), CH_2Cl_2; b) 99% TFA, 1h, then TFA removal under high vacuum; c) **41**, DIPEA (12 equiv), DMF, 40 °C, 18h; d) **41**, DABCO (3 equiv), DMF, 40 °C, 18h; e) **52** (2.0 equiv), DIC, (3 equiv), DMAP (cat.), CH_2Cl_2. DIC = diisopropylcarbodiimide, DMAP = dimethylaminopyridine, TFA = trifluoroacetic acid, DIPEA = diisopropylethylamine, DABCO = 1,4 diazabicyclo[2.2.2]octane, DMF = dimethylformamide.

In addition, the hydrolyzed by-product **64** was observed under acidic conditions (Figure 5). Since HPLC purification of **68a** delivered significant amounts of phosphine-

[94] The model peptide containing an Fmoc-moiety was chosen to ensure a specific and strong UV-absorption (300 nm) in the starting material and products for easy conversion analysis.

oxidized **68b**, the crude peptide was dissolved in DMF directly after TFA removal and the previous glycosylation conditions were applied for the glycosylation with azide **41** (Scheme 16B). Unfortunately, neither glycopeptide **69** nor an intermediate iminophosphorane were formed at 40 °C after 48 h.



Scheme 16. Acid-induced traceless Staudinger glycosylation. A) Asp side-chain activation and thioester synthesis; B) Acid-induced traceless Staudinger glycosylation of **68**; C) Acylation of benzyl azide **70**. Reagents and Conditions: a) 99% TFA; b) HBTU (4 equiv), DIPEA (8 equiv),10 min, then **65** (4 equiv), THF; c) DIC, (3 equiv), DMAP (cat.), 10 min, then **65** (4 equiv), CH_2Cl_2; d) DIC, (3 equiv), DMAP (cat.), 10 min, then **52** (4 equiv), CH_2Cl_2; e) DIPEA (12 equiv), DMF, 40 °C, 18h. DIC = diisopropylcarbodiimide, DMAP = dimethylaminopyridine, TFA = trifluoroacetic acid, DIPEA = diisopropylethylamine, DMF = dimethylformamide, THF = tetrahydrofuran, HBTU = *O*-Benzotriazole-*N*,*N*,*N*',*N*'-tetramethyl-uronium-hexafluoro-phosphate, Fmoc = fluorenylmethoxycarbonyl.

Additional optimization studies, which included a temperature screen (25 to 70 °C) as well as a solvent screen (DMF, DMA, $CHCL_3$, toluene, THF), increasing the glycosyl azide amount or probing the steric demand around the glycosylation sites in peptides **72**, **73** and **74** (Scheme 17A) by using different peptide sequences or using benzyl azide **70** (Scheme 16C) did not induce the traceless Staudinger ligation in the reaction mixture.

Instead, hydrolyzed and oxidized by-products from the phosphonium salt **68a** as well as aspartimide **67** were observed.

Table 1. Conversion rates for an aspartic acid side chain activation and thioester synthesis.

Entry[a]	64	66a	66b	67	Thiol	Method[b]	Time [h]
1	-	70%	-	30%	65	a	1
2	60%	-	-	40%	65	a	7
3	50%	47%	-	< 3%	65	b	1
4	5%	> 92%	-	< 3%	65	b	3
5	34%	-	65%	< 2%	52	b	3
6	8%	-	89%	3%	52	b	4

[a] Conversions are determined by LC-MS analysis (λ = 300 nm).
[b] Method a: HBTU (4 equiv), DIPEA (8 equiv), 10 min, then **65** (4 equiv), THF;
Method b: DIC (3 equiv), DMAP (cat.), 10 min, then **52** or **65** (4 equiv), CH_2Cl_2.

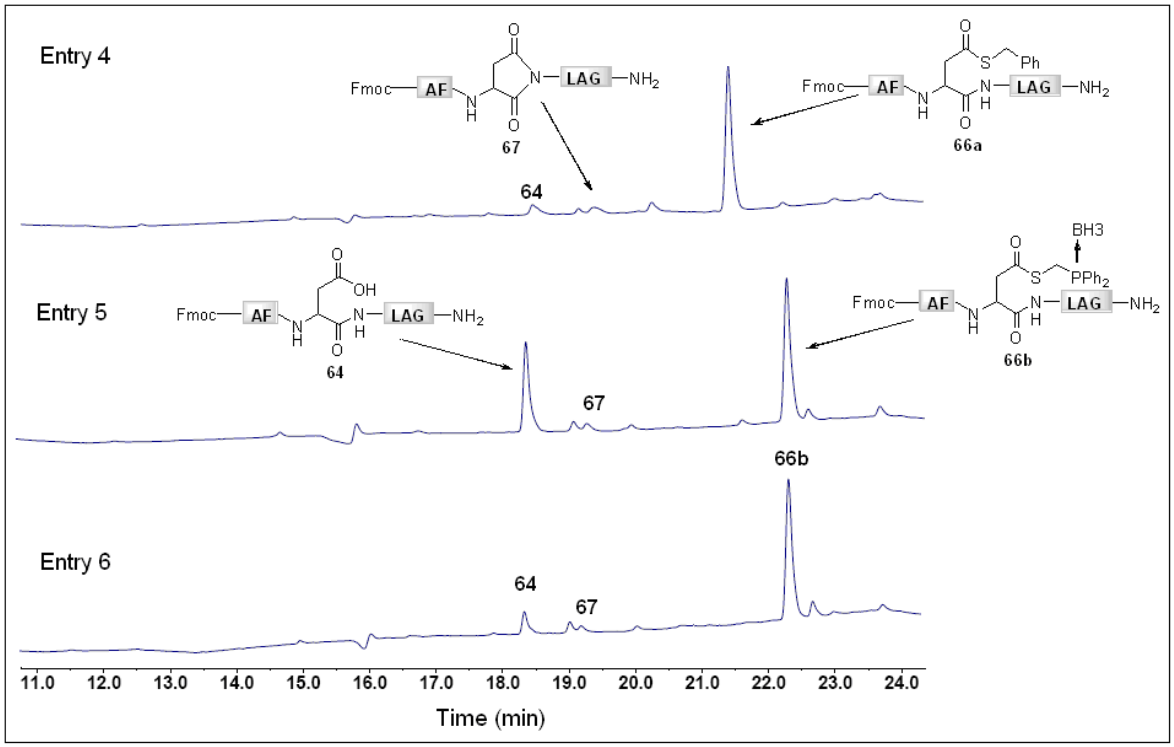


Figure 4. HPLC-UV profiles (λ = 300 nm) of Entries 4-6 of table 1.

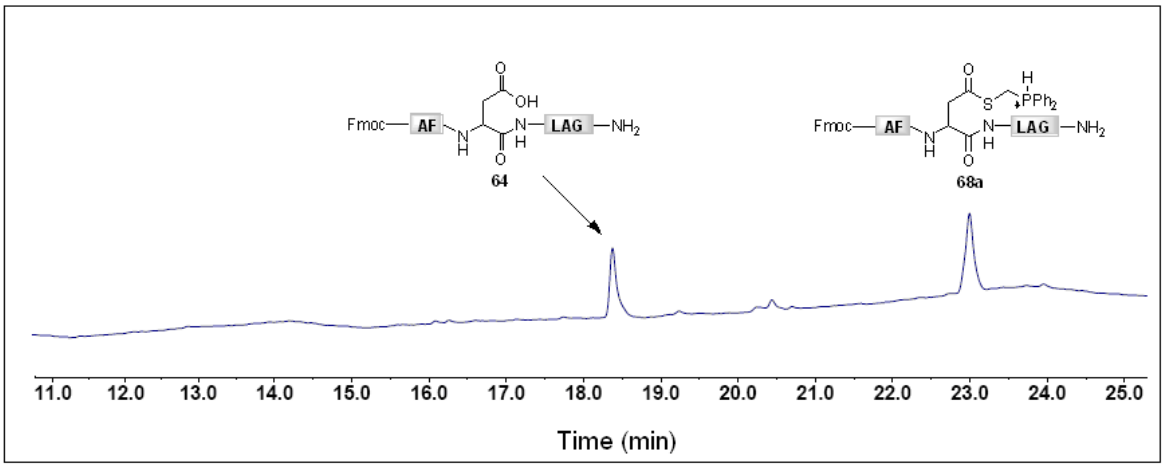


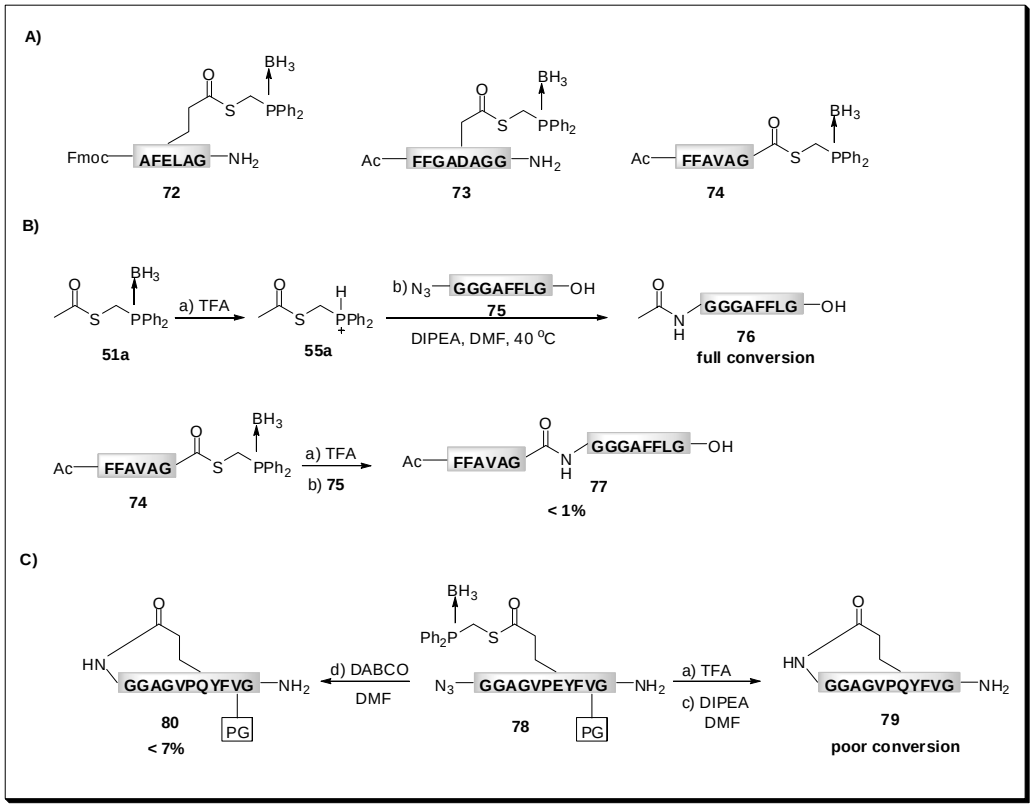
Figure 5. HPLC-UV profile (λ = 300 nm) of the formation of phosphonium salt **68a**.

After these unsuccessful glycosylation-attempts with an Asp side-chain phosphinothioester we focused on investigating the utility of **52** in other applications; indeed, in peptide segment ligation as well as head-to-side chain peptide cyclization.

As described before, **51a** was first converted to **55a** and then directly employed for the traceless Staudinger ligation with azidopeptide **75** in DMF in the presence of DIPEA at 40 ^{o}C. Full conversion of **75** into **76** was achieved after 40 hours as revealed by LC-MS analysis. Encouraged by these results, **51a** was next replaced with peptide phosphinothioester **74** which was first converted into the corresponding phosphonium salt and then reacted directly with **75** under the same reaction conditions. The desired product **77** was obtained in a very poor conversion (< 1%) (Scheme 17B).

For an intramolecular application in peptide cyclization, we focused on the solid phase peptide synthesis (SPPS) of a bifunctional azidopeptide phosphinothioester **78**. The amino acid residues of **78** were chosen based on the microbial peptide Microcin J25 (residues 1-11).[95]

[95] M. J. Bayro, J. Mukhopadhyay, G. V. T. Swapna, J. Y. Huang, L-C. Ma, E. Sineva, P. E. Dawson, G. T. Montelione, R. H. Ebright, *J. Am. Chem. Soc.* **2003**, *125*, 12382 -12383.



Scheme 17. Acid-induced traceless Staudinger glycosylation. A) Peptides used for acid-induced traceless Staudinger glycosylation; B) Peptide segment ligation by TSL; C) Cyclic peptide synthesis by TSL. Reagents and Conditions: a) 99% TFA; b) **75**, DIPEA (12 equiv), 40 ^{o}C, 40 h, DMF; c) DIPEA (12 equiv), 40 ^{o}C, 48 h, DMF; d) DABCO (3 equiv), DMF, 40 °C, 48h. TFA = trifluoroacetic acid, DIPEA = diisopropylethylamine, DABCO = 1,4 diazabicyclo[2.2.2]octane, DMF = dimethylformamide.

The acid-induced Staudinger cyclization was initiated upon treatment **78** with TFA followed by DIPEA (12 mM). The cyclized peptide **79** was obtained in a very low conversion (< 5%) after 48 hours as revealed by LC-MS analysis. In addition, hydrolyzed by-products were observed in the reaction mixture.

Furthermore, we decided to investigate the base-induced Staudinger cyclization which was carried out in DMF in the presence of DABCO at 40 ^{o}C. The desired cyclized product was obtained in low conversion (< 7%) with significant amount of hydrolyzed products and glutimide formation.

Despite the very low conversion to cyclized peptide, it seemed that the acid-induced traceless Staudinger cyclization worked out to some extent in comparison with the traceless Staudinger glycosylation. This might be attributed to the proximity of the azide and phosphine moiety, since they are located on the same molecule.

However, the peptide folding may influence the TSL, so that the phosphinothioester, upon treatment with TFA, may fold in such away that makes the phosphine moiety inaccessible for the glycosyl azides or other organic azides, thus, no reaction between the azide and the phosphine moiety. So, the lack of forming even the iminophosphorane-peptides can be attributed to this.

3.3 Traceless Staudinger ligation with phosphinophenol 54

After these unsuccessful glycosylation attempts with an Asp side-chain phosphinothioester we turned our attention to phosphinophenolester-peptides.

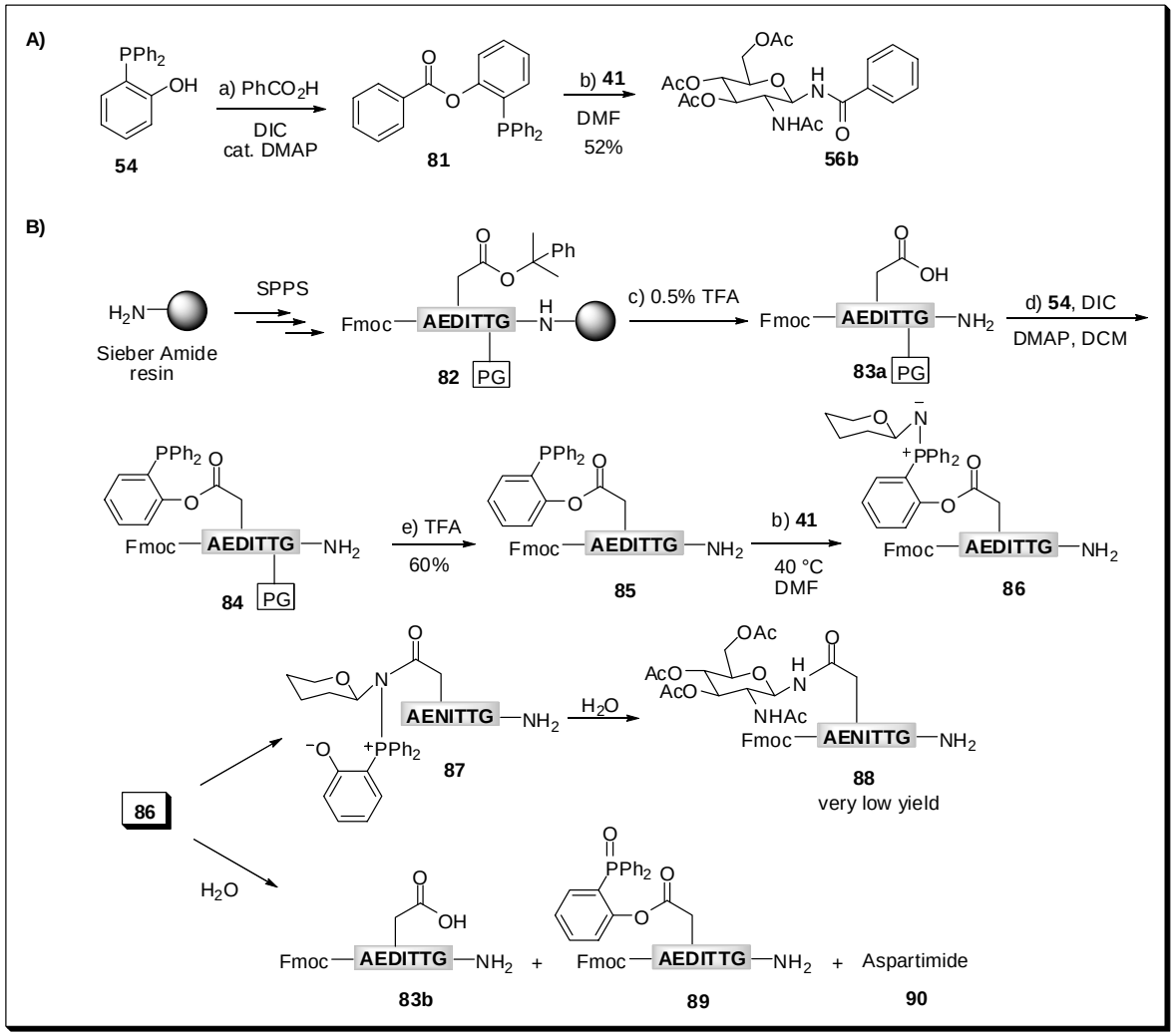
O-diphenyl-phosphino-phenol **54** showed a similar acylation yield for azide **41** as in the phosphinothiol studies[96,97] (Scheme 18A). Next we intended to investigate the utility of **54** in the synthesis of *N*-linked glycopeptides by TSL. Peptide with amino acid residues 31-37 of the glycoprotein erythropoietin (EPO) was chosen as a model sequence where Asp33 represents the glycosylation site.[98]

Fmoc-based solid phase peptide synthesis (SPPS) on an acid-labile Sieber Amide resin with an orthogonally protected Asp(O-2-PhiPr) residue delivered peptide **82** on the solid support which was cleaved from the resin to partially protected peptide **83a** with 0.5% TFA in CH_2Cl_2 (Scheme 18B). **83a** was transformed into the protected phosphinophenolester **84** under the optimized DIC activation conditions and directly deprotected with TFA for 3h to afford the unprotected phosphinophenolester **85** in an isolated yield of 60%.

The traceless Staudinger ligation between phosphinophenolester **85** and β-GlcNAc—N_3 **41** in DMF was then conducted in which **85** was fully consumed after 4 hours as revealed by LC-MS analysis (Figure 6). Since pure **85** did not show any sign of oxidation when it was exposed to air for a couple of hours as revealed by LC-MS analysis, we assume that **85** was converted quantitatively into aza-ylide (iminophosphorane) intermediate **86** which underwent a very slow N⟶O acyl shift to form the corresponding amidophosphonium salt **87** in very low conversion rate which upon hydrolysis with water provided the desired *N*-linked glycopeptide **88** in a very low yield.

The hydrolysis of the unrearranged intermediate **86** resulted in the formation of the phosphinoxide-peptide **89** and free acid peptide **83b** (Scheme 18B).

[96] A. Bianchi, A. Russo, A. Bernardi, *Tetrahedron: Asymmetry* **2005**, *16*, 381-386.
[97] A. Bianchi, A. Bernardi, *Tetrahedron Lett.* **2004**, *45*, 2231-2234.
[98] B. Wu, Z. Tan, G. Chen, J. Chen, Z. Hua, Q. Wan, K. Ranganathan, S. J. Danishefsky, *Tetrahedron Lett.* **2006**, *47*, 8009-8011.



Scheme 18. *N*-linked glycosylation by the traceless Staudinger ligation via phosphinophenolester activation. A) Benzoylation of β-GlcNAc-azide **41**; B) Synthesis of unprotected Asp side-chain activated peptide **85** and *N*-linked glycosylation. Reagents and conditions: a) $PhCO_2H$ (0.5 equiv), DIC, (3 equiv), DMAP (cat.), CH_2Cl_2; b) **41** (3 equiv), DMF, 40 °C, 18h; c) 0.5% TFA in CH_2Cl_2, 15 min; d) **54** (1.1 equiv), DIC, (3 equiv), DMAP (cat.), CH_2Cl_2; e) 99% TFA, 3h, then TFA removal under high vacuum.

Although no base was added, minimal aspartimide formation was observed which can be attributed to the slight elevation in pH due to the formation of iminophosphorane **86** which is regarded as a strong base.

Figure 6 shows the HPLC traces of pure phosphinophenolester **85** before TSL as well as the reaction mixture after TSL. LC-MS analysis showed the formation of **86** and **89** after 4 hours in conversion rates of 30% and 70%, respectively whereas **89** was the main product after 18 hours.

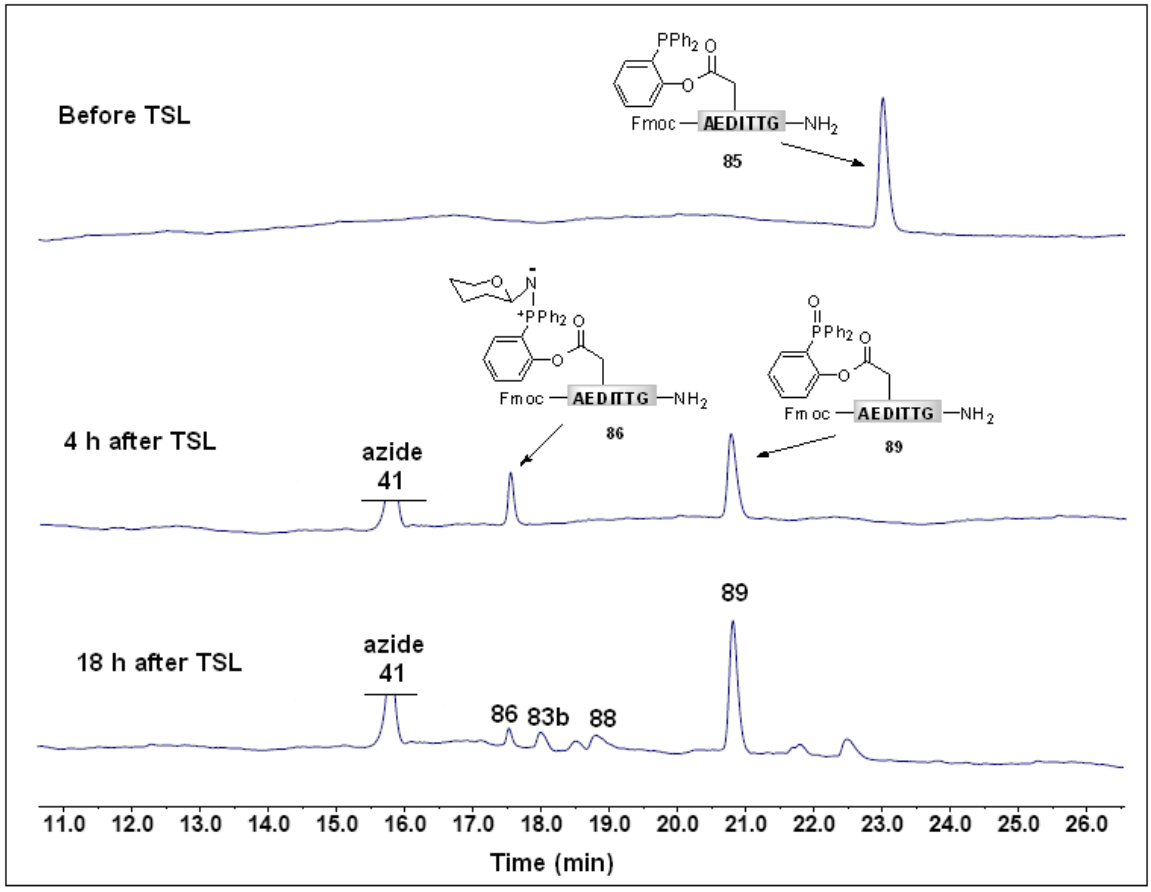


Figure 6. HPLC-UV profile (λ = 300 nm) of traceless Staudinger ligation of **85** with glycosyl azide **41**.

Next, we tested further model peptides to probe the presence of amino acid residues which could speed up the N⟶O acyl shift within the iminophosphorane by altering the polarity of the peptide under investigation.

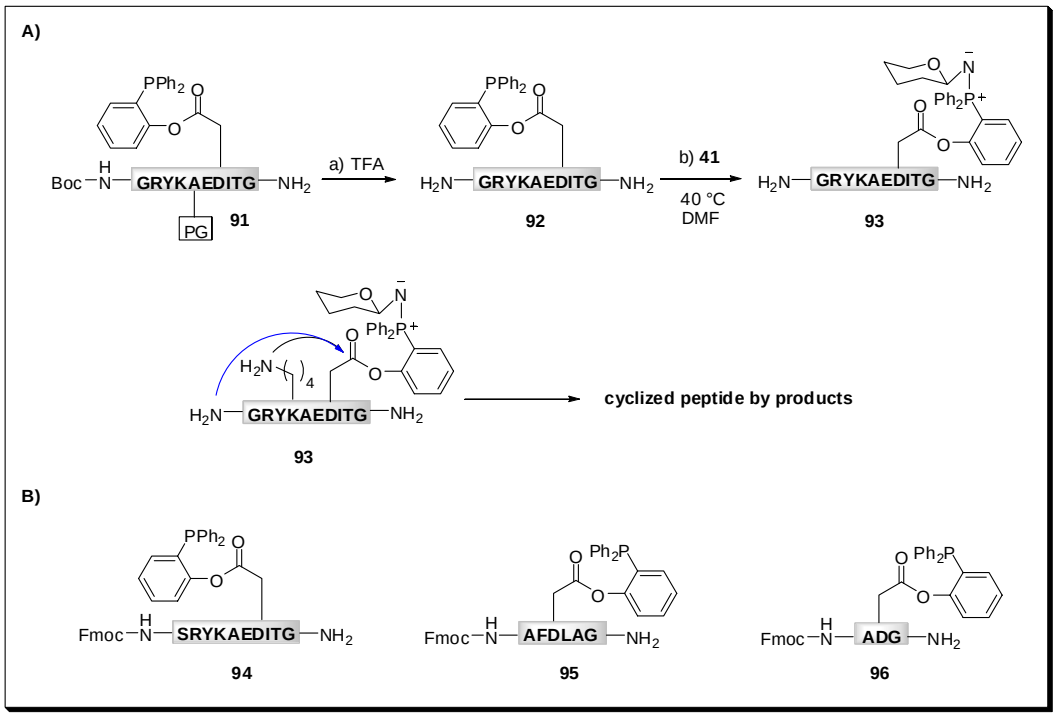
The polar phosphinophenolester **92** was synthesized in analogy to **85** incorporating a Boc protecting group instead of Fmoc group.

The traceless Staudinger ligation for **92** with **41** was conducted under the same reaction conditions yielding the desired *N*-linked glycopeptide in a very poor conversion rate.

Like the TSL for **85**, phosphinoxide-peptide and free acid-peptide were observed as by-products. In addition, intramolecular lactam formation occurred with the amino nucleophiles of a Lys and/or the N-terminus present in the peptide sequence (Scheme 19A).

The phosphinophenolester peptides **94-96** were subjected to the TSL and they showed similar trends to previously discussed phosphinophenolester peptides (Scheme

19B). The use of unprotected β-GlcNAc—N_3 **42** did not help speed up the N⟶O acyl shift either.



Scheme 19. A) Possible by-products encountered in TSL of peptide **92** with azide **41**; B) Other phosphinophenolester peptides that were used for traceless Staudinger glycosylation. Reagents and conditions: a) 99% TFA, 3h, then TFA removal under high vacuum; b) **41** (3 equiv), DMF, 40 °C, 18h.

In summary, the borane-protected phosphinothiol **52** and diphenyl-phosphinophenol **54** were synthesized successfully and further utilized to prepare phosphinothioesters as well as phosphinophenolesters in moderate to high overall yield and were subsequently employed for traceless Staudinger ligation with glycosyl azides.

The acid-induced TSL with **52** showed a useful applicability to generate glycosyl amides at small molecule and at amino acid level, whereas it was not applicable to access neither iminophosphorane intermediates nor their corresponding *N*-linked glycopeptides.

Like **52**, phosphinophenol **54** could be utilized to access glycosyl amides at a small molecule level. On the other hand, at peptide level, we believe that phosphinophenolester-peptides were converted quantitatively into aza-ylide (iminophosphorane) intermediates which underwent a very slow N⟶O acyl shift into the corresponding amidophosphonium salts.

Upon hydrolysis with water the desired *N*-linked glycopeptides were provided in very poor yields. Thus, various by-products were formed due to the hydrolysis of the unrearranged iminophosphorane intermediates.

Chapter 4

Results and Discussions:

Staudinger Phosphite Reaction (SPR)

4 Results and Discussions: Staudinger Phosphite Reaction (SPR)

The development of mimetics such as triazole-glycoconjugates has become an attractive approach in glycoscience,[99,100] since it can provide a simple modular synthetic access to glycoconjugates. Improved stability, resistance to hydrolysis by glycosidases or increased biological activity can be additional advantageous properties.[101]

Our interest in glycopeptides led us to the idea to test the Staudinger phosphite reaction (SPR)[76,77,102] for establishing a linkage between glycosyl moieties and peptide segments forming glycopeptide mimetics.

In order to explore the applicability and scope of Staudinger phosphite reaction to access glycopeptide mimetics, several substrates were synthesized.

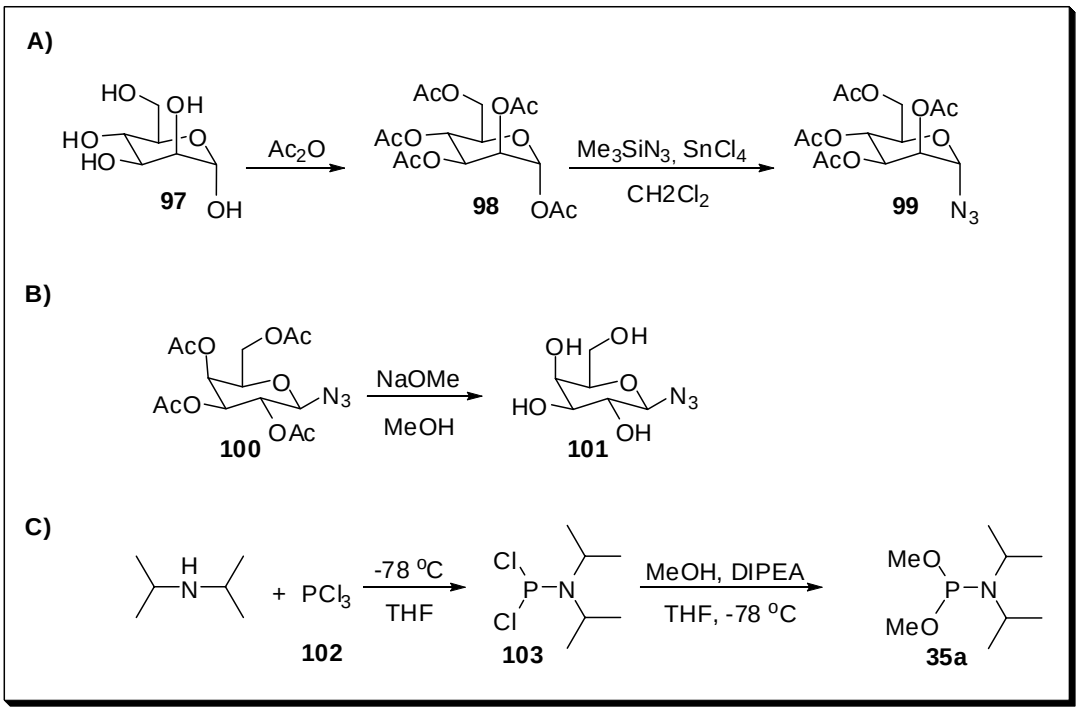
4.1 Synthesis of glycosyl azides and phosphoramidates required for SPR

Acetylated α-*D*-mannose azide (α-Man—N_3) **99** was obtained by reported protocols.[103,104] α-*D*-mannose **97** was acetylated with acetic anhydride in the presence of catalytic amount of sulphuric acid to give protected α-*D*-mannose **98**[105] which was converted into the target molecule **99** with trimethylsilyl azide in the presence of stannic tetrachloride in 83% overall yield (Scheme 20A).

Acetylated β-*D*-galactose azide (β-Gal—N_3) **100** was synthesized in analogy to **41** in 39%. Upon treatment with sodium methoxide in methanol **100** provided the unprotected β-Gal—N_3 **101** in 90% (Scheme 20B).

Dimethyl-*N*,*N*-diisopropylphosphoramidite **35a** was synthetically accessible according to a published procedure.[106] Dichloro-*N*,*N*-diisopropylphosphinamine **103** was obtained from the reaction between trichlorophosphine **102** and diisopropylamine in 47% overall yield.[107] Chlorides of **103** were replaced with methoxy groups upon treatment with methanol under basic conditions to afford **35a** in an isolated yield of 55% (Scheme 20C).

[99] W. Huang, S. Groothuys, A. Heredia, B. H. M. Kuijpers, F. P. J. T. Rutjes, F. L. van Delft, L.-X. Wang, *ChemBioChem* **2009**, *10*, 1234-1242.
[100] B. H. M. Kuijpers, S. Groothuys, C. Hawner, J. ten Dam, P. J. L. M. Quaedflieg, H. E. Schoemaker, F. L. van. Delft, F. P. J. T. Rutjes, *Org. Proc. Res. Dev.* **2008**, *12*, 503-511.
[101] D. Macmillan, A. M. Daines, M. Bayrhuber, S. L. Flitsch, *Org. Lett.*, 2002, **4**, 1467-1470.
[102] Y. G. Gololobov, I. N. Zhmurova, L. F. Kasukhin, *Tetrahedron* **1981**, *37*, 437-472.
[103] J. Geng, J. Lindqvist, G. Mantovani, G. Chen, C. T. Sayers, G. J. Clarkson, D. M. Haddleton, *QSAR Comb. Sci.* **2007**, *26*, 1220-1228
[104] M. M. Ponpipom, R. L. Bugianesi, T. Y. Shen, *Carbohydr. Res.* **1980**, *82*, 141-148.
[105] J. A. Watt, S. J. Williams, Org. Bio. Chem. **2005**, *3*, 1982-1992.
[106] M. Mag, J. W. Engels, *Nucleic Acids Res.* **1989**, *17*, 5973-5988.
[107] G. F. Ruda, V. P. Alibu, C. Mitsos, O. Bidet, M. Kaiser, R. Brun, M. P. Barrett, I. H. Gilbert, *ChemMedChem* **2007**, *2*, 1169-1180.



Scheme 20. A) Synthesis of α-Man—N_3 **99**; B) Synthesis of β-Gal—N_3 **101**; C) Synthesis of **35a**.

4.2 Staudinger phosphite reaction as a motive binding in bioorganic chemistry

In order to apply the Staudinger phosphite reaction to the glycosylation of peptides we first investigated the synthesis of different phosphite-containing peptides **106** on solid support which differ in the phosphite alkyl substituents.[108,109,110]

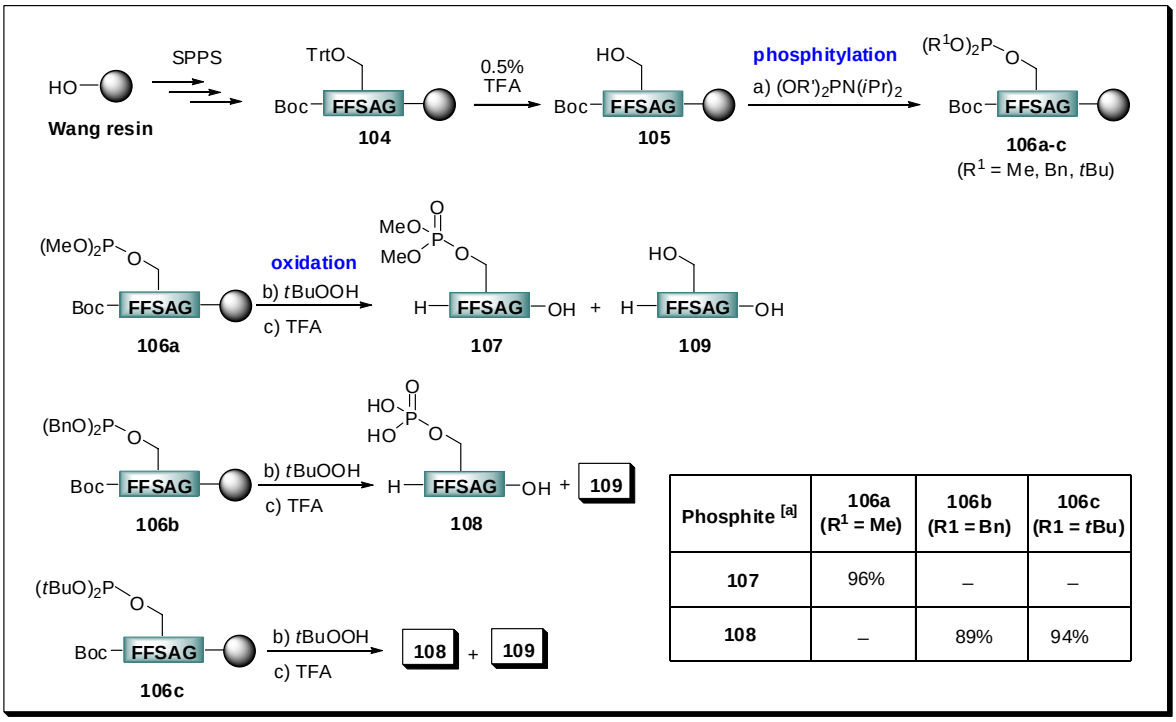
Solid phase peptide synthesis was performed by standard Fmoc-couplings on a Wang resin, in which the serine at the latter glycosylation site was trityl protected in **104**. After trityl removal with 0.5% TFA in CH_2Cl_2 the free hydroxy group was phosphitylated with either dimethyl-, dibenzyl-, or di-*tert*-butyl-*N,N*-diisopropylphosphoramidite.[106] This reaction was performed in dry DMF in the presence of 1*H*-tetrazole (Scheme 21).[111]

The conversion to the corresponding dimethyl-, dibenzyl-, and di-*tert*-butyl peptidyl phosphites **106a-c** was determined by oxidation of the nascent phosphitylated peptide with *t*BuOOH, subsequent cleavage from the resin with 95% TFA and final HPLC-MS analysis, in which phosphates **107** or **108** reflected the phosphite conversion of **106a-c**. In all reactions high conversions to the P(V) compounds were observed (89-96%), in which the benzyl and *tert*-butyl phosphites **106b** and **106c** led to the formation of phosphate monoester **108** due to the acid lability of the preceding phosphate triester (Scheme 21).

It proved to be advantageous to carry out the phosphitylation in dry DMF since 1*H*-tetrazole possesses a very high solubility in DMF relative to other solvents such as acetonitrile, in which lower conversion rates to the desired phosphites were observed.

We also observed that an extended time for phosphitylation reaction resulted in partial hydrolysis of the nascent phosphite-peptide to form the corresponding H-phosphonate-peptide[112] which could be attributed to the acidic conditions of phosphitylation. To confirm this assumption, trimethyl phosphite **110** was treated with an excess of 1*H*-tetrazole and the reaction was monitored by ^{31}P NMR spectroscopy which after 4 hours showed a full conversion of **110** into dimethyl phosphonate **111** (Scheme 22A).

[108] W. Bannwarth, E. A. Kitas, *Helv. Chim. Acta.* **1992**, *75*, 707-714.
[109] E. A. Kitas, R. Knorr, A. Trzeciak, W. Bannwarth, *Helv. Chim. Acta.* **1991**, *74*, 1314-1328.
[110] W. Bannwarth, A. Trzeciak. *Helv. Chim. Acta.* **1987**, *70*, 175-186.
[111] MERCK, *Novabiochem Peptide Synthesis*, 2008/2009, protocol for global phosphorylation was carried out with slight modification.
[112] J. W. Perich, *Lett. Pept. Sci.* **1998**, *5*, 49-55.

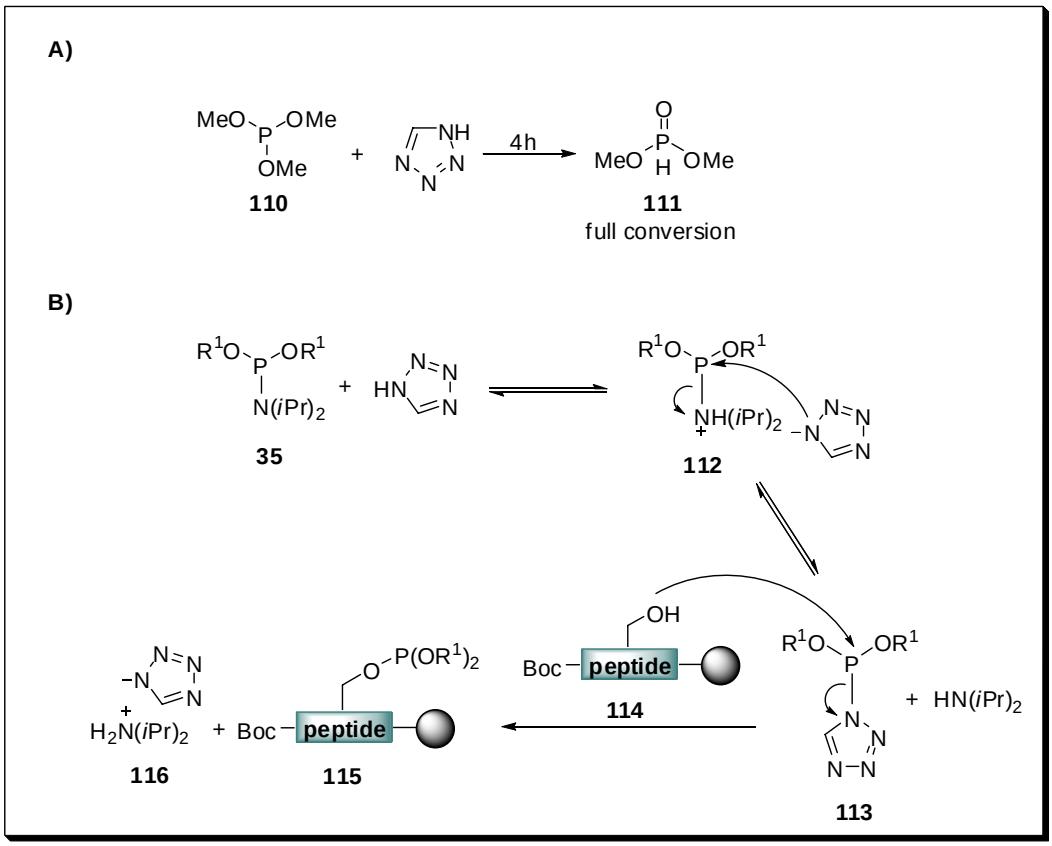


Phosphite [a]	106a (R^1 = Me)	106b (R1 = Bn)	106c (R1 = *t*Bu)
107	96%	–	–
108	–	89%	94%

Scheme 21. Phosphitylation of immobilized peptides. Reagents and conditions: a) $(MeO)_2PN(iPr)_2$, $(BnO)_2PN(iPr)_2$ or $(tBuO)_2PN(iPr)_2$ (20 equiv.), 1*H*-tetrazole (50 equiv), 25 °C, 1.5h, dry DMF; b) *t*BuOOH, 30 min, 25 °C, DMF; c) 95% TFA, 3h. [a] Conversions are determined by LC-MS analysis. Trt = trityl, DMF = dimethylformamide.

The 1*H*-tetrazole involved in the phosphitylation reaction has a dual role. First, it acts as an acid to protonate the nitrogen of diisopropylamine of **35** to deliver **112** and secondly, it acts as a nucleophile to displace the amine leaving group of the protonated amidite **112** to provide a highly reactive tetrazolide intermediate **113** which in turn undergoes a nucleophilic attack by a free hydroxy group on peptide **114** to form a phosphite-peptide **115** and salt **116** (Scheme 22B).[113,114]

[113] M. Russell, A. P. Laws, J. H. Atherton, M. I. Page, *Org. Biomol. Chem.* **2008**, *6*, 3270-3275.
[114] E. J. Nurminen, J. K. Mattinen, H. Lönnberg, *J. Chem. Soc., Perkin Trans. 2*, **1998**, 1621-1628.



Scheme 22. A) Hydrolysis of trimethyl phosphite **110** into H-phosphonate **111**; B) Proposed mechanism for peptide phosphitylation.

Next we probed a solid supported Staudinger phosphite reaction, in which the immobilized phosphite-containing peptides **106a-c** were reacted in dry CH_2Cl_2 with acetylated β-GlcNAc—N_3 **41** to afford different glycosyl phosphoramidate esters **117** and **118** (Scheme 23). Afterwards, water was added to ensure phosphorimidate hydrolysis and the peptides were cleaved with 95% TFA from the solid support.

It was found that the dimethyl-phosphite-containing peptide **106a** showed a conversion of 69% to the glycosyl-phosphoramidate methylester **117**. The analogous glycosyl-benzyl ester **118a** was only formed in moderate rates (< 25%), whereas the glycosyl-*tert*-butyl ester **119** was not obtained at all, and in both transformations the phosphate monoester **108** was identified as the major reaction product (Scheme 23).

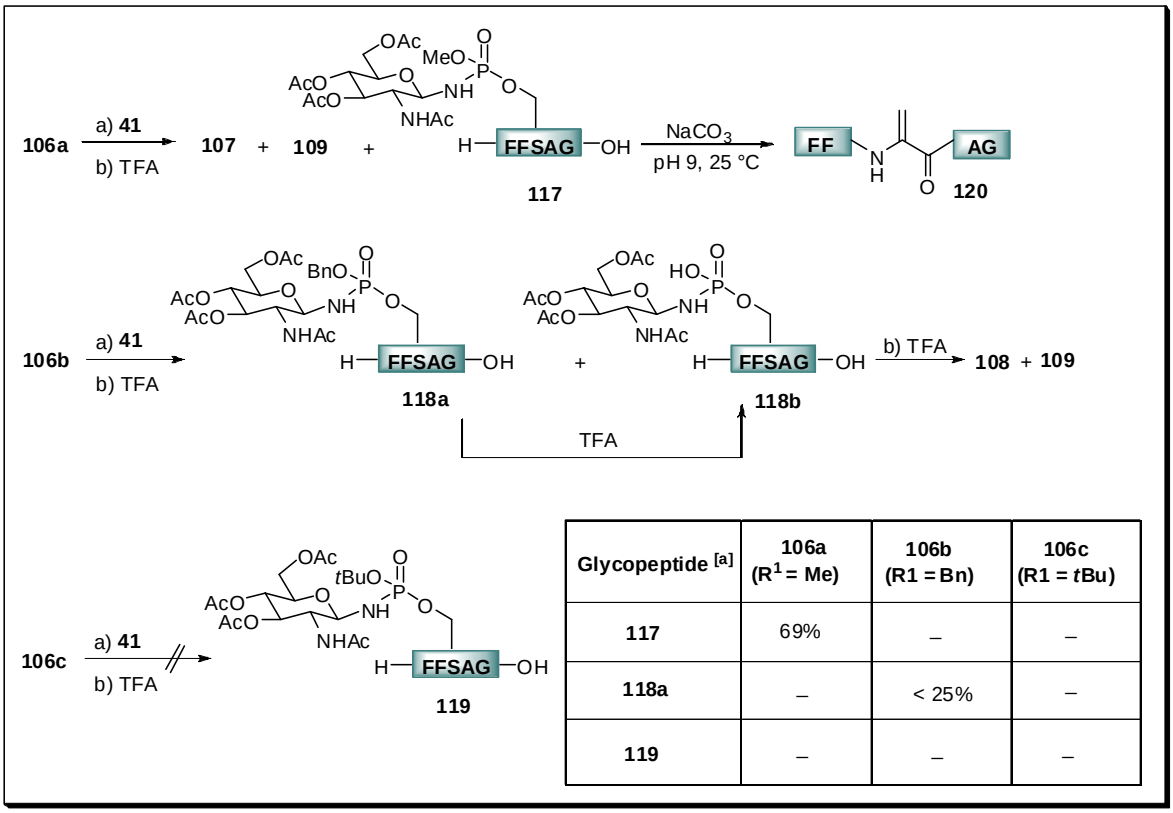
This difference in the formation of **117**, **118** and **119** can be rationalized by the acid lability of the P—N bond in saponified phosphoramidate **118b**,[115,116] which forms by the cleavage of benzyl or *tert*-butyl esters upon TFA exposure for extended time. However, the

[115] S. Suwal, M. K. H. Pflum, *Angew. Chem. Int. Ed.* **2010**, *49*, 1627 –1630.
[116] A. Zwierzak, J. Brylikowska-Piotrowicz, *Angew. Chem. Int. Ed.* **1977**, *16*, 107.

lack of forming **119** can be also attributed to increased steric hindrance about the phosphorous atom of **106c** so that it is not accessible for glycosyl azide **41**.

In contrast, glycosyl-phosphoramidate **117** showed excellent stability under neutral and also under acidic conditions when it was treated with TFA for 2 h and no P—N bond cleavage was observed.

Further stability tests under basic conditions revealed the occurrence of β-elimination of the phosphorylated Ser derivative upon treatment with Na_2CO_3 in MeOH to form dehydroalanine-peptide **120** (Scheme 23).



Glycopeptide [a]	106a (R^1 = Me)	106b (R1 = Bn)	106c (R1 = *t*Bu)
117	69%	–	–
118a	–	< 25%	–
119	–	–	–

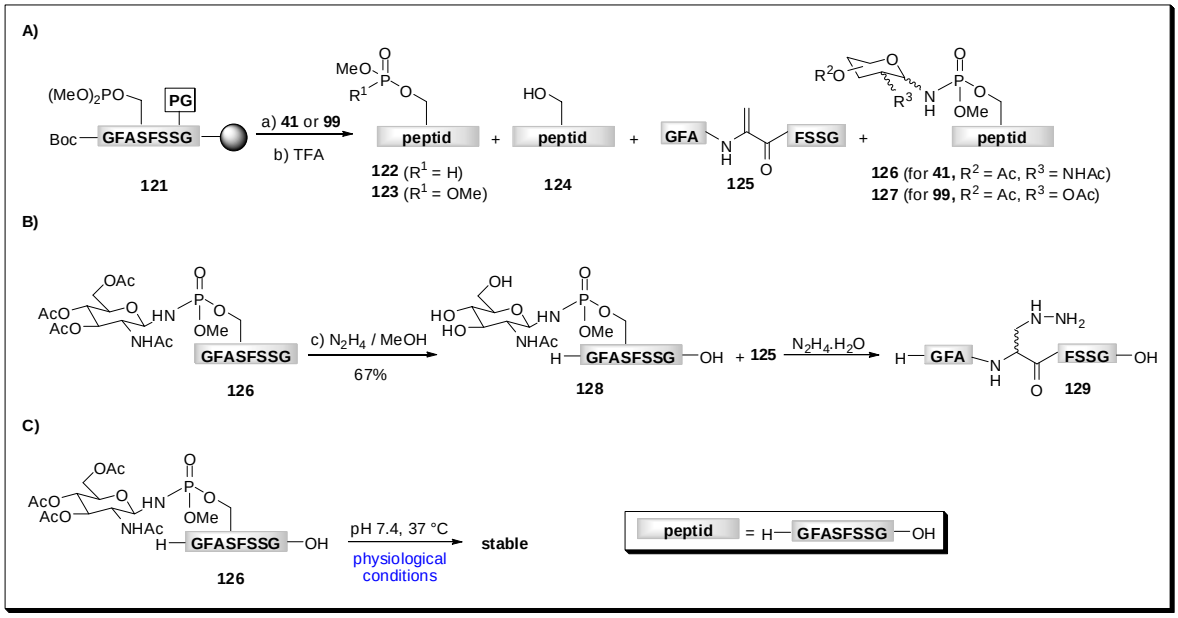
Scheme 23. Staudinger phosphite reaction to **117** and **118b**. Reagents and Conditions: a) i) **41** (7 equiv), dry CH_2Cl_2, 25 °C, 45 h; ii) H_2O (110 equiv), 15 h; b) 95% TFA, 3 h. [a] Conversions are determined by LC-MS analysis.

Consequently, these initial investigations pointed towards the use of dimethyl-*N*,*N*-diisopropylphosphoramidite **35a** as phosphitylating agent in further optimization and stability studies in which we used a more challenging octapeptide, in which the Ser at the desired glycosylation site was orthogonally protected in contrast to other Ser residues in the sequence (Scheme 24).

The Staudinger phosphite reaction of the dimethyl-phosphite-containing peptide **121** with **41** was performed at room temperature as well as at 40 °C in different dry or wet

solvents (DMF, DMSO, CH_2Cl_2) and monitored by LC-MS (Table 2, entries 1-9). The optimal reaction conditions were 45h in dry CH_2Cl_2 at 40°C providing glycopeptide **126** in 57% conversion after hydrolysis (based on HPLC analysis) and in 49% overall isolated yield after semipreparative HPLC purification.

Afterwards, the stability of the isolated glycopeptide **126** under physiological conditions (pH 7.4, 37 °C) was analyzed by LC-MS, in which no signs of decomposition were observed after 24h (Scheme 24C).



Scheme 24. A) Staudinger phosphite reaction to **126** and **127**; B) Deprotection of **126** to **128**; C) Stability test for **126**. Reagents and conditions: a) i) **41** (7 equiv) or **99** (7 equiv), dry solvent, 25 or 40 °C, 45 h; ii) H_2O (110 equiv), 15 h; b) 95% TFA, 3 h; c) $NH_2NH_2.H_2O$, MeOH, 20 °C, pH = 11, 1 h.

In addition to the desired glycopeptide **126**, the H-phosphonate **122**, phosphortriester **123**, and alcohol **124** were identified as by-products, which can be rationalized either by different hydrolysis pathways of the phosphorimidate **130** (Scheme 25) or by oxidation or hydrolysis of non-converted **121** as illustrated in Scheme 26.

These pathways were further analyzed by $H_2{}^{18}O$-labeling experiments and conversion studies, which indicate that only the hydrolysis routes A and B occur, since exclusively the ^{18}O-labeled products **126** and **132** could be detected (Scheme 25, see also Appendix scheme A1). In addition, since **123** did not contain the heavy oxygen isotope, it is likely to be formed by oxidation of **121** under the reaction conditions. Thus, it can be concluded that once the phosphorimidate **130** is formed, no P—N bond cleavage occurs during the hydrolysis *via* hydrolysis route C.

Another by-product in the reaction mixture included the dehydroalanine **125**, which was observed as an elimination product at 40 °C in DMSO and DMF (Table 2, entries 2,3,5 and 6).

Table 2. Conversion rates (in %) for the Staudinger-phosphite reaction of peptide **121** with glycosyl azides **41** and **99**.

Entry[a]	Glycosyl azide	**126** or **127**	**122**	**124**	**123**	**125**	Solvent	Water added	T [°C]
1	**41**	20	30	39	11	-	DMF	–	25
2	**41**	37	2	37	8	16	DMF	–	40
3	**41**	40	1	28	19	12	DMF	+	40
4	**41**	53	27	9	11	-	DMSO	–	25
5	**41**	31	-	14	37	18	DMSO	–	40
6	**41**	44	-	22	21	13	DMSO	+	40
7	**41**	38	7	49	6	-	CH_2Cl_2	–	25
8	**41**	54	8	34	4	-	CH_2Cl_2	+	40
9	**41**	57	14	25	4	-	CH_2Cl_2	–	40
10	**99**	77	10	4	9	-	DMSO	–	25
11	**99**	77	2	14	7	-	DMSO	–	40
12	**99**	6	82	6	6	-	CH_2Cl_2	–	25
13	**99**	26	48	22	4	-	CH_2Cl_2	–	40
14	**99**	5	-	4	91	-	DMF	–	25
15	**99**	12	-	5	83	-	DMF	–	40

[a] Conversions are determined by LC-MS analysis, see also Appendix part I.

Since the phosphitylation to peptidyl phosphite **121** was quantitative as verified by *t*BuOOH oxidation and HPLC-MS analysis, we assume that peptide alcohol **124** is formed by an unselective hydrolysis of **130** via the hydrolysis route B and/or by the acidic hydrolysis route B of non-converted **121** via **131**, whereas **122** is formed via the acidic hydrolysis route A (Scheme 26B) [117,118].

To validate the acidic hydrolysis pathway, **121** was treated in a separate experiment with H_2O for 15 h followed by cleavage from the resin with TFA for 3 h, which furnished **124** and the H-phosphonate **122** in conversion rates of 37% and 62%, respectively, with less than 1% of **123** present (Scheme 26A).

Finally, in order to remove the acetyl protecting groups, glycopeptide **126** was treated with hydrazine hydrate in methanol (1:5) for 1 hour to deliver the deprotected glycopeptide **128** in an isolated yield of 67 % as well as hydrazine-peptide **129** derived

[117] A. Meyer, F. Morvan, J.-J. Vasseur, *Tetrahedron Lett.* **2004**, *45*, 3745-3748.
[118] F. Ferreira, A. Meyer, J.-J. Vasseur, F. Morvan, *J. Org. Chem.* **2005**, *70*, 9198-9206.

from Michael addition of hydrazine to the nascent dehydroalanine-peptide **125** (Scheme 24B, Figure 7).[119,120,121] With Na_2CO_3 in methanol (6 mM) the de-*O*-acetylation of **126** proceeded in a lower conversion to provide **128** in 15% conversion rate after 1 hour along with significant amount of dehydroalanine-peptide **125**.

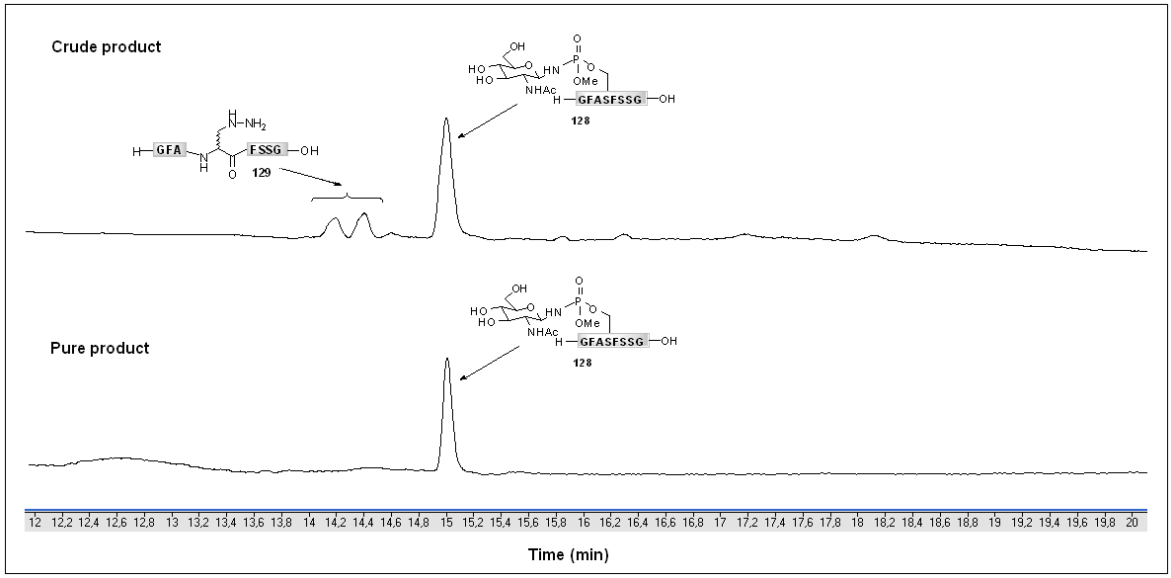
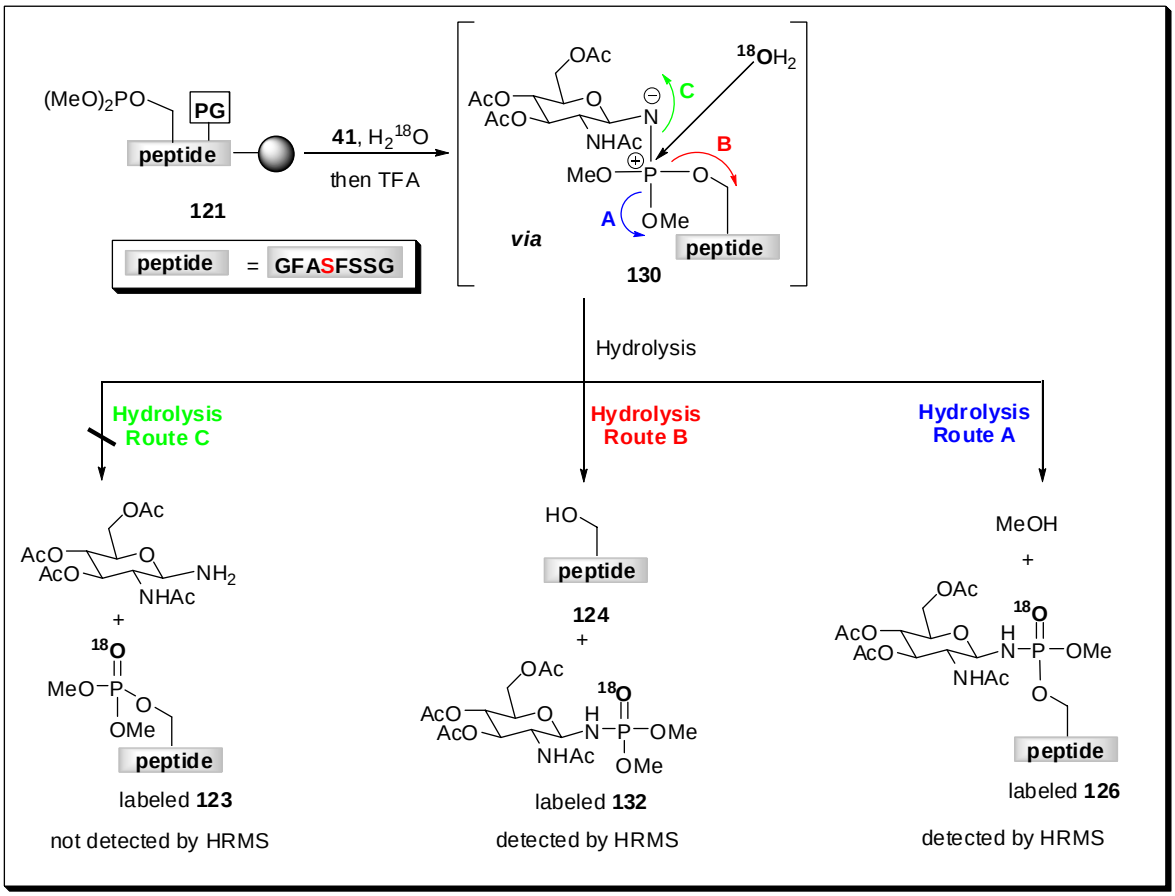


Figure 7. HPLC traces of crude and pure glycopeptide **128**.

In addition to the solid phase glycosylation of **121** with β-GlcNAc—N_3 **41** we also intended to access a mannosylated phosphoramidate-linked peptide as an analogue of the natural phosphodiester-linked glycopeptide (see scheme 4C), in which mannose contains an α-linkage to the phosphorylated peptide.

Upon reaction of the synthetically accessible acetyl α-Man—N_3 **99** with **121** the conversion to the desired glycopeptide **127** was found to be optimal in DMSO at either 25 or 40 °C (Table 1, entries 10-11), whereas the reaction in CH_2Cl_2 and DMF proceeded in significantly lower conversion. Under the optimal conditions **127** was formed in 77% conversion and in an isolated yield of 64% (Table 2, entry 11). However, a partial epimerization to the β-anomer was observed (Scheme 24A).

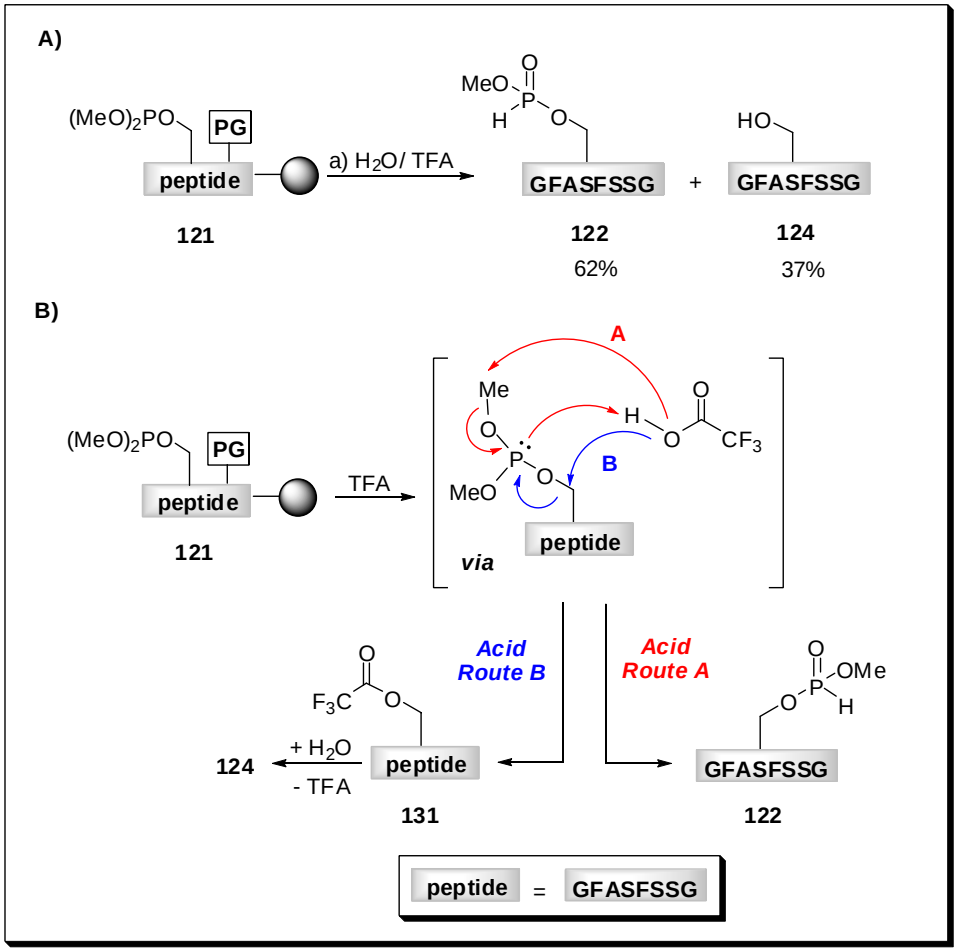
[119] P. Schultheiss-Reimann, H. Kunz, *Angew. Chem. Int. Ed. Engl.*, **1983**, *22*, 62-63.
[120] P. Sjölin, M. Elofsson, J. Kihlberg, *J.Org.Chem.*, **1996**, *61*, 560-565.
[121] H. Kunz, S. Birnbach, P. Wernig, *Carbohydr. Res.*, **1990**, *202*, 207-223.



Scheme 25. Hydrolysis pathways of phosphorimidate **130** by nucleophilic attack of $H_2{}^{18}O$ at phosphorous; (See also Appendix, scheme A1).

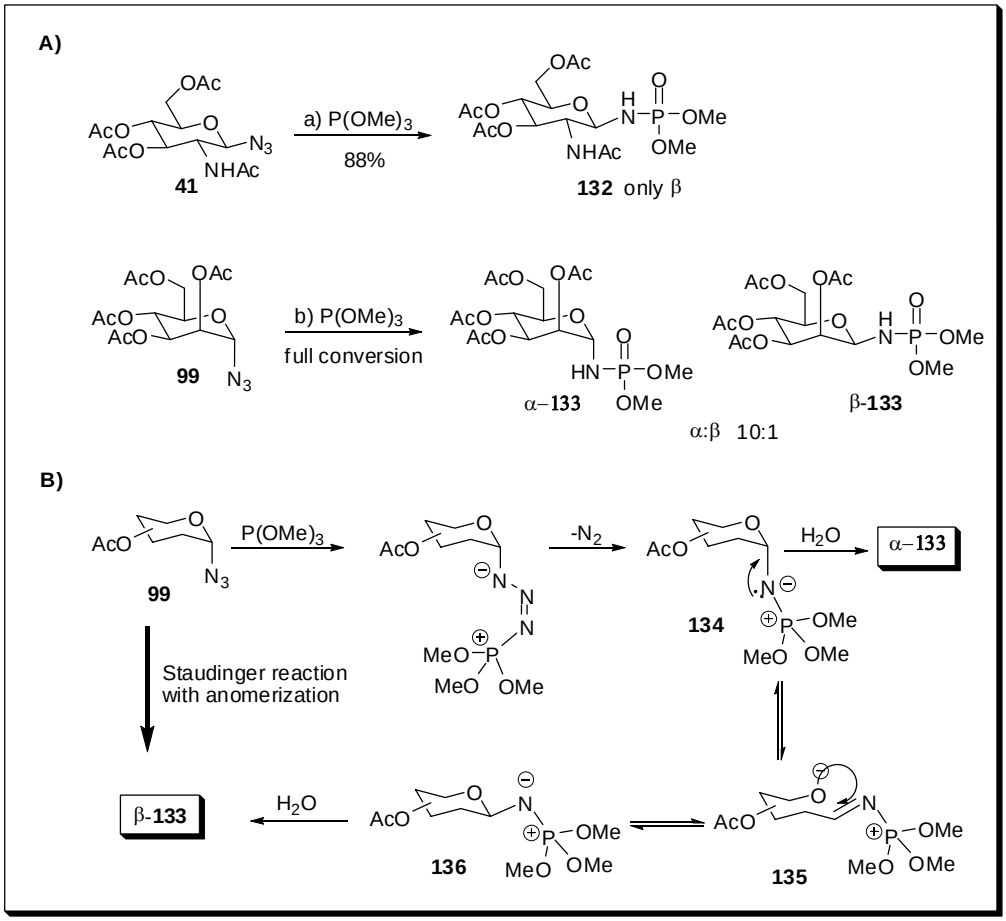
In order to further investigate the anomerization of glycosyl azides in Staudinger phosphite reactions with small molecules, we converted α-mannosyl azide **99** in DMSO with trimethlyphosphite into the corresponding phosphoramidate **133** (Scheme 27A). After full conversion to **133** a ratio of 10:1 for the α- to β-anomer was determined by NMR-measurements.[122,123,124] Analogously, for β-GlcNAc—N_3 **41** the stereochemical integrity at the anomeric centre was retained providing β-glycosyl phosphoramidate **132** exclusively at 25 °C in CH_2Cl_2 in 88% isolated yield (Scheme 27A).[125]

[122] A. Schierholt, H. A. Shaikh, J. Schmidt-Lassen, T. K. Lindhorst, *Eur. J. Org. Chem.* **2009**, 3783-3789.
[123] Y. He, R. J. Hinklin, J. Chang, L. L. Kiessling, *Org. Lett.* **2004,** *26*, 4479-4482.
[124] L. Kovács, E. Ősz, V. Domokos, W. Holzer, Z. Györgdeák, *Tetrahedron* **2001**, *57*, 4609-4621.
[125] T. Kannan, S. Vinodhkumar, B. Varghese, D. Loganathan, *Bioorg. Med. Chem. Lett.* **2001**, *11*, 2433-2435.



Scheme 26. Acidic hydrolysis pathways of non-converted **121** by trifluoroacetic acid.

The anomerization during Staudinger phosphite reaction of trimethyl phosphite with α-glycosyl azide **99** proceeds through the cleavage of the C1—O bond of the α-glycosyl phosphorimidate **134** to provide the open-chain form **135** which undergoes a reversible ring closure to either α-glycosyl phosphorimidate **134** or β-glycosyl phosphorimidate **136** yielding subsequently α-**133** and β-**133**, respectively (Scheme 27B).



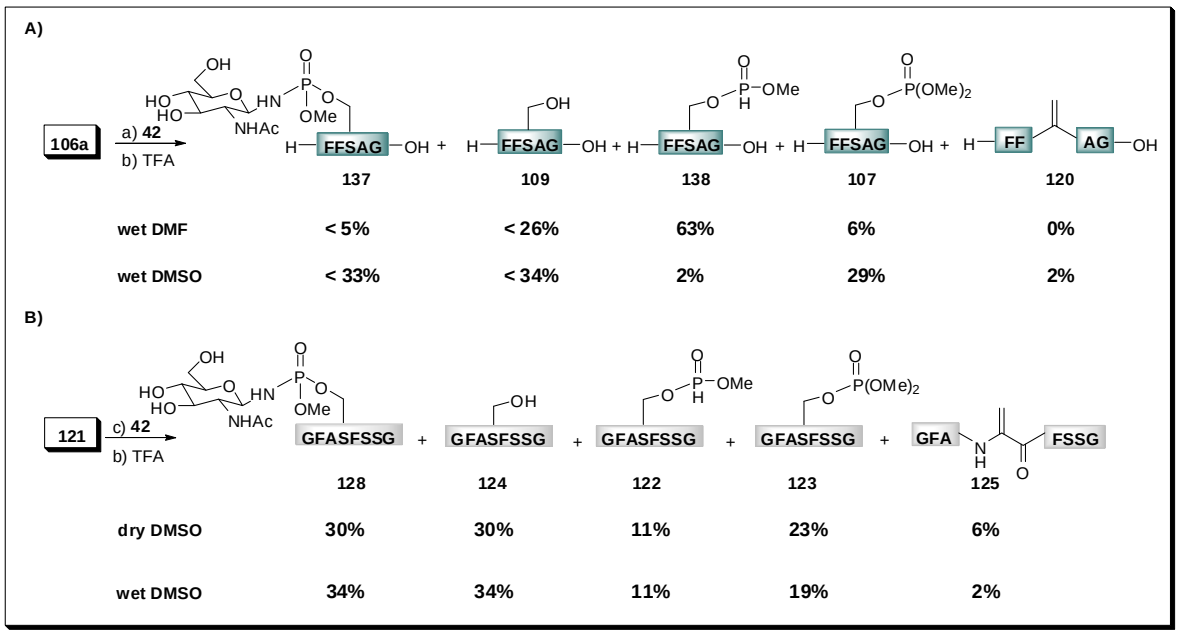
Scheme 27. Anomerization during Staudinger phosphite reaction of α-glycosyl azide **99** with trimethyl phosphite.

4.3 Chemoselective Staudinger phosphite reaction of phosphite-peptides with unprotected glycosyl azides

Encouraged by the previous results we intended next to explore and investigate the chemoselectivity of the Staudinger phosphite reaction (SPR) between phosphite-peptides and unprotected azido sugars β-GlcNAc—N_3 **42** and β-Gal—N_3 **101** in order to avoid the de-*O*-acetylation step by which some of the glycopeptide can be lost in dehydroalanine-peptide form.

According to our mechanistic study of the Staudinger phosphite reaction (see scheme 25), the phosphorimidate intermediate is attacked by water to give finally the corresponding phosphoramidate. Therefore we thought that the SPR could be interfered by the free hydroxy groups of the unprotected sugar at the phosphorimidate stage if the reaction would be done in dry solvent, therefore wet solvents as well as dry solvents have been utilized to explore this effect.

We began our investigations with phosphite-peptide **106a** which was reacted with **42** in either wet DMF or wet DMSO at 35 °C for 40 hours. The reaction in DMSO proceeded much better than in DMF, where the conversion rate to **137** was less than 33% in DMSO compared to less than 5% in DMF. The major by-product in wet DMF was H-phosphonate **138** indicating that the hydrolysis of phosphite **106a** proceeds faster than the Staudinger phosphite reaction. These initial results pointed towards the use of wet DMSO (Scheme 28A).

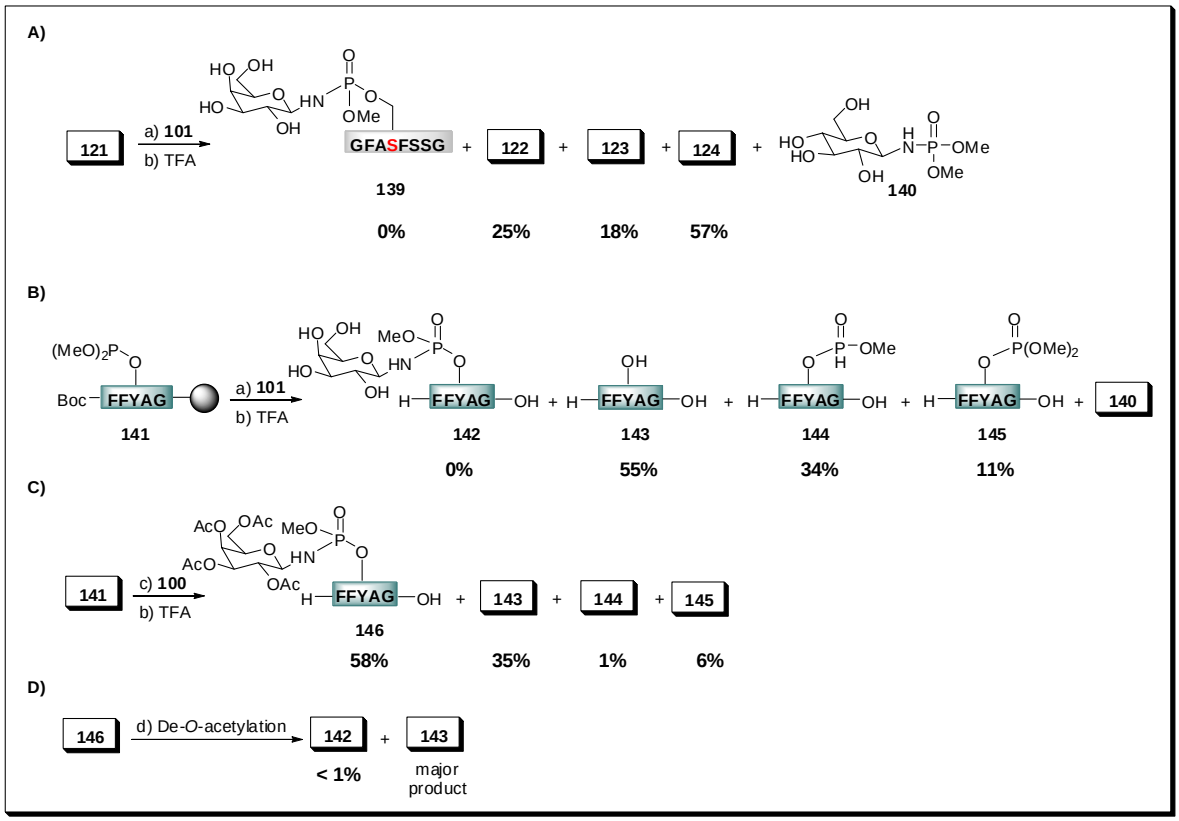


Scheme 28. Staudinger-phosphite reaction A) to **137;** and B) to **128**. Reagents and conditions: a) **42** (7 equiv), wet DMF or DMSO, 35 °C, 40 h; b) 95% TFA, 3 h; c) i) **42** (7 equiv), wet or dry DMSO, 35 °C, 40 h; ii) H_2O (110 equiv). Conversions are determined by LC-MS analysis.

In order to probe our assumption of free hydroxy group interference with phosphorimidate in dry solvent, we investigated next the reaction between **42** and phosphite-peptide **121** in either dry or wet DMSO applying the same reaction conditions as for **106a**. We found that the reaction in both cases provided the desired unprotected glycopeptide **128** in comparable conversion rates of 30% in dry DMSO and with slight increase in wet DMSO in 34% (Scheme 28B).

In addition to the desired glycopeptides, some by-products were formed whereas the expected by-products producing from free hydroxy group interference in dry solvents were not observed.

From these findings we can conclude that the free hydroxy groups of **42** do not influence the phosphorimidate hydrolysis.



Scheme 29. Staudinger-phosphite reaction A) to **139**; B) to **142;** and C) to **146**; D) De-*O*-acetylation of **146**. Reagents and conditions: a) **101** (7 equiv), wet DMSO, 35 °C, 40 h; b) 95% TFA, 3 h; c) **100** (7 equiv), wet DMSO, 35 °C, 40 h; d) Na_2CO_3, NaOMe or $N_2H_4.H_2O$ in methanol. Conversions are determined by LC-MS analysis.

Next we intended to prepare glycopeptides with galactose moiety to be utilized in Lectin binding studies. To achieve that, β-Gal—N_3 **101** was reacted with **121** in analogy to the reactions of **42**. Unlike **42**, the reaction did not furnish the desired glycopeptide **139** but

alcohol **124** was the major by-product. The high conversion rate of **124** as well as the formation of **140** indicate that the reaction between **101** and **121** took place and formed the corresponding phosphorimidate intermediate which upon hydrolysis with water underwent unselective hydrolysis to release **124** and form **140** (Scheme 29A).

Then we employed the phosphite-peptide **141** in Staudinger phosphite reaction with **101** to explore whether the amino acid residue at the glycosylation site is involved in the unselective hydrolysis.

141 was prepared in analogy to **106a** incorporating tyrosine (Y) instead of serine (S) at the latter glycosylation site. The reaction of azide **101** with phosphite-peptide **141** did not proceed and resulted in comparable results to those with phosphite-peptide **121** indicating that the amino acid residue at the glycosylation site does not play a major role in the unselective hydrolysis. Rather, the glycosyl azide involved plays a significant role (Scheme 29B).

To confirm this assumption **141** was reacted with acetylated β-Gal—N_3 **100**. The reaction proceeded very well and furnished the desired protected glycopeptide **146** in 58% conversion (Scheme 29C).

We thought also that the utilization of tyrosine at the latter glycosylation site would be an advantage to prepare glycopeptides with an acetylated galactose moiety and deprotect them later without any concern of dehydroalanine-peptide formation.

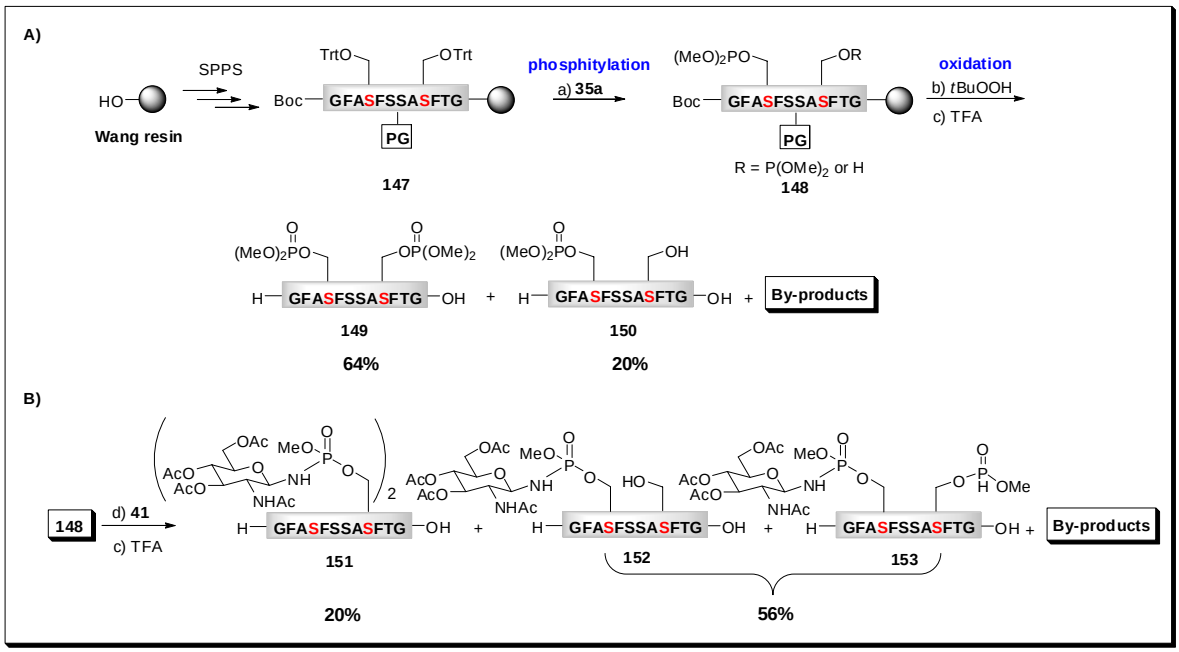
Unfortunately, the de-*O*-acetylation of **146** with various reagents (Na_2CO_3, $NaOCH_3$ or $N_2H_4.H_2O$ in methanol) provided **142** with very low yield (<1%) with peptide **143** as a major by-product. Thus, it can be concluded that Tyr is a good leaving group under basic conditions.

Further efforts and investigations devoted to tackle these limitations and access glycopeptide with unprotected galactose moiety required for Lectin binding studies are described in the upcoming pages.

4.4 Synthesis of biotinylated phosphoramidate-linked mono- and divalent glycopeptides

The aim of this part was to synthesize phosphoramidate-linked glycopeptides containing unprotected galactose as well as *N*-acetylglucoseamine with biotin attached to the N-terminus. The utility of the biotin tag is to immobilize the glycopeptide to be investigated on a sensor chip containing covalently bound streptavidin through the strong non-covalent interaction between biotin and streptavidin.[126,127] Thus the immobilized glycopeptide can be utilized in recognition study with glycoproteins such as Lectins by Surface Plasmon resonance (SPR) measurements. [128]

We started our investigation with the synthesis of divalent glycopeptide **151** with a free N-terminus. For that, peptide **147** was synthesized on a Wang resin with two potential glycosylation sites which are distant from each other by four amino acid residues.



Scheme 30. A) Phosphitylation of immobilized peptide to **148**; B) Staudinger-phosphite reaction to **151**. Reagents and conditions: a) i) 0.5% TFA in CH_2Cl_2 ii) **35a** $(MeO)_2PN(iPr)_2$ (40 equiv.), 1*H*-tetrazole (100 equiv), 25 °C, 2h, dry DMF; b) *t*BuOOH, 30 min, 25 °C, DMF; c) 95% TFA; d) i) **41** (14 equiv), dry CH_2Cl_2, 40 °C, 45 h; ii) H_2O (220 equiv), 15 h. Conversions are determined by LC-MS analysis.

[126] M. González, L. A. Bagatolli, I. Echabe, J. L. R. Arrondo, C. E. Argaraña, C. R. Cantor, G. D. Fidelio, *J. Biol. Chem.* **1997**, *272*, 11288-11294.
[127] S. Enders, G. Bernhard, A. Zakrzewicz, R. Tauber, *Biochimica et Biophysica Acta* **2007**, *1770*, 1441-1449.
[128] S. Endersa, S. B. Riesea, G. Bernharda, J. Derneddea, W. Reutterb, R. Tauber, *Biochem. Eng. J.* **2010**, *48*, 253-259.

Trityl groups were removed with 0.5 % TFA in CH_2H_2 and the free hydroxy groups were phosphitylated with **35a** to form phosphite-peptide **148**. Subsequent oxidation of **148** with *t*BuOOH followed with TFA-mediated cleavage reflected the phosphitylation reaction performance.

We found that the phosphitylation of the two free hydroxy groups did not proceed to completion since the divalent phosphate-peptide **149** as well as the monovalent phosphate-peptide **150** were formed in conversion rates of 64% and 20%, respectively. We believe that **150** was formed due to the incomplete phosphitylation of the closer serine residue to the solid support which could be partially steric hindered by the resin (Scheme 30A).

Next we probed the Staudinger phosphite glycosylation by reacting **148** with azido sugar **41** in dry CH_2Cl_2. Taking into account the phosphitylation experiment (Scheme 30A) we expected that the glycosylation reaction of **148** would proceed to provide the desired divalent glycopeptide **151** in comparable conversion rate to **149** but we found that divalent glycopeptide **151** was achieved in 20% conversion rate whereas the monovalent glycopeptide **152** as well as H-phosphonate-monovalent glycopeptide **153** were formed in 56% conversion rate.

These differences in conversion rates can be rationalized by the increasing steric hindrance of the glycosyl azide **41** whereas the formation of **153** can be attributed to the incomplete glycosylation of phosphite-serine residue closer to the C-terminus which subsequently undergoes acidic hydrolysis to the corresponding H-phosphonate.

Next we intended to prepare biotinylated mono- and divalent glycopeptides **154** and **155** to evaluate and compare their binding properties to Lectins (Scheme 31A).[129,130]

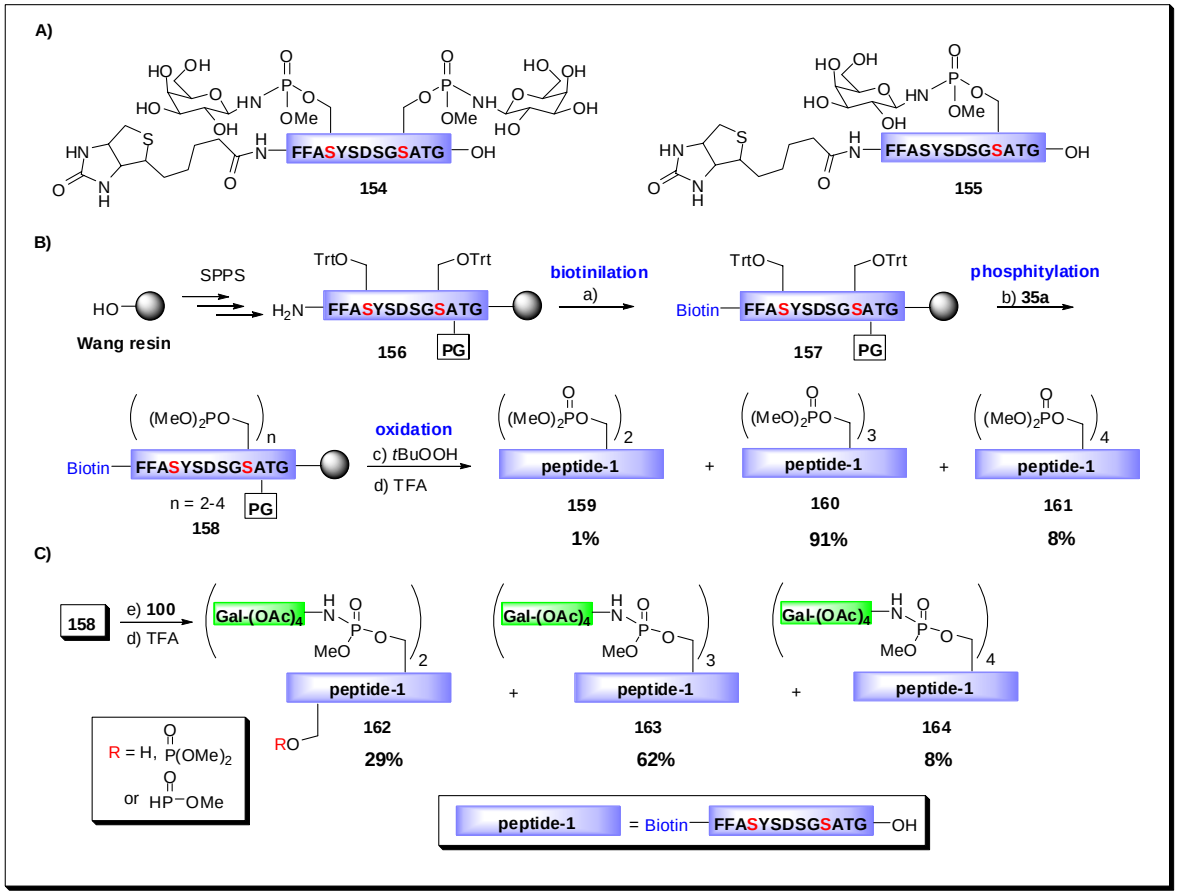
We started our investigation with synthesizing peptide **156** which was obtained in analogy to previously described protocols. **156** contained two trityl serine residues separated by five amino acid residues. Its free N-terminus was biotinylated[131] with pre-activated biotin with HBTU/HOBt in DMF in the presence of DIPEA. The biotinylation proceeded to completion after double coupling for 2.5 hours for each coupling.

Next, trityl groups were removed and the peptide was phosphitylated with **35a** followed with oxidation. We expected after the TFA-mediated cleavage to achieve a biotinylated divalent phosphate-peptide **159** as the major product. On the contrary, **159** was formed in a very low conversion rate (1%) whereas the unexpected trivalent phosphate-peptide **160** was achieved as the major reaction product with conversion rate of 91%. These

[129] J. Hirabayashi, *J. Biochem.* **2008**, *144*, 139-147.
[130] N. Sharon, *J. Biol. Chem.* **2007**, *282*, 2753-2764.
[131] For amino acid biotinylation see: S. Bhuniya, S. M. Park, B. H. Kim, *Org. Lett.*, **2005**, *7*, 1741-1744.

unexpected results showed that there was another potential phosphitylation site other than the expected ones on serine residues marked in red (Scheme 31B).

However, phosphite-peptide **158** was reacted in a separate experiment with unprotected azido galactose **101** in either wet or dry DMSO. Like phosphite-peptide **121**, the reaction did not afford the expected glycopeptide but resulted in comparable by-products.



Scheme 31. A) Glycopeptides **154** and **155**; B) Biotinylation and phosphitylation of immobilized peptide to **158**; C) Staudinger phosphite reaction. Reagents and conditions: a) Biotin (5 equiv), HBTU (5 equiv), HOBt (5 equiv), DIPEA (5 equiv), dry DMF. 5 h; b) i) 0.5% TFA in CH_2Cl_2 ii) **35a** $(MeO)_2PN(iPr)_2$ (40 equiv.), 1*H*-tetrazole (100 equiv), 25 °C, 2h, dry DMF; c) *t*BuOOH, 30 min, 25 °C, DMF; d) 95% TFA, 3h; e) i) **100** (14 equiv), dry DMSO, 40 °C, 45 h; ii) H_2O (220 equiv), 15 h. Conversions are determined by LC-MS analysis. Serine residues marked in red show the expected glycosylation site.

Next, the phosphite-peptide **158** was subjected to Staudinger phosphite reaction with protected azido galactose **100** in which trivalent glycopeptide **163** was formed in conversion rate of 62% (Scheme 31C). The formation of **163** as a major product was in agreement with the results revealed from the phosphitylation experiment with peptide **157.**

The formation of glycopeptides **162** can be rationalized by the same explanation as phosphite-peptide **148** (see scheme 30).

Next we focused on accessing the monovalent glycopeptide **155**; therefore peptide **165** was intended to be prepared. Peptide **165** has a similar sequence as **156** and differs only at the protecting group of the serine residue closer to the N-terminus which is *t*Bu. Peptide **165** was biotinylated, phosphitylated and glycosylated in analogy to **156** (Scheme 32).

Again peptide **165** had a similar trend as **156** since the divalent phosphopeptide **169** was formed in 90% conversion rate upon oxidation of phosphite-peptide **167**, whereas the monovalent phosphopeptide **168** was expected to be the major product.

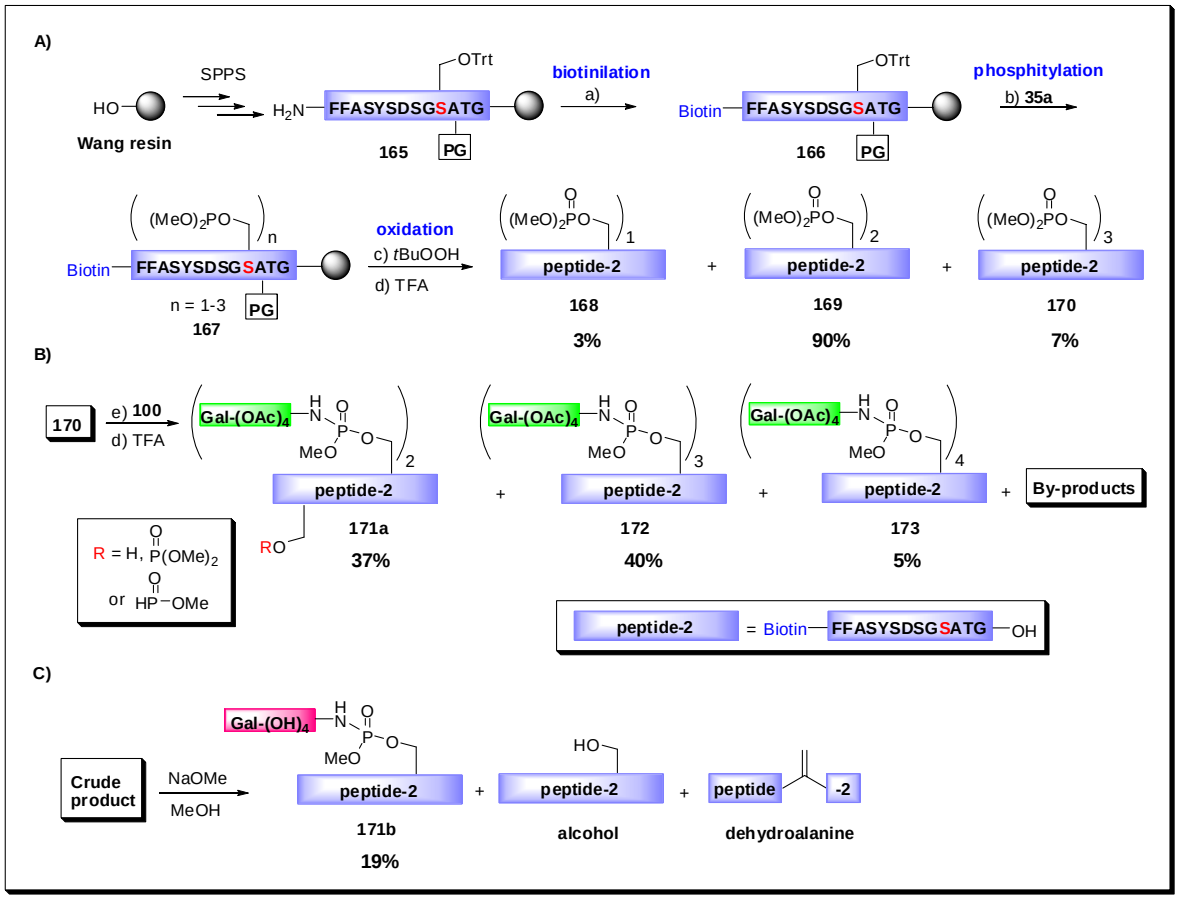
Like **158**, the Staudinger phosphite reaction of **167** with **101** did not proceed at all, whereas with **100** it furnished protected mono- and divalent glycopeptides **171a** and **172**, respectively, in comparable conversions (Scheme 32C).

Next, the crude glycopeptides were treated with NaOMe in methanol for an hour to remove the acetyl groups of the galactose moiety. De-*O*-acetylation provided only the unprotected monovalent glycopeptide **171b** in moderate conversion rate of 19%, whereas the unprotected divalent glycopeptide was not obtained at all.

Hydrazine hydrate in methanol was also used as an alternative de-*O*-acetylating reagent but it afforded only unprotected monovalent glycopeptide in a comparable conversion rate as well.

After this undesired systematic trend we intended to explore whether the biotin immobilization to peptide is involved in this unexpected behavior. Consequently, peptide **174** was synthesized in analogy to the previous protocols. The sequence of peptide **174** is similar to that of **121** which did not encounter any problems in the course of the Staudinger phosphite glycosylation (Scheme 33A).

Peptide **174** involves only one potential site for glycosylation, therefore our expectation was to achieve monovalent glycopeptide at the end. **174** was converted into phosphite-peptide **175** which was then reacted with GlcNAc azide **41** in order to compare it with a previous experiment (See scheme 24A).

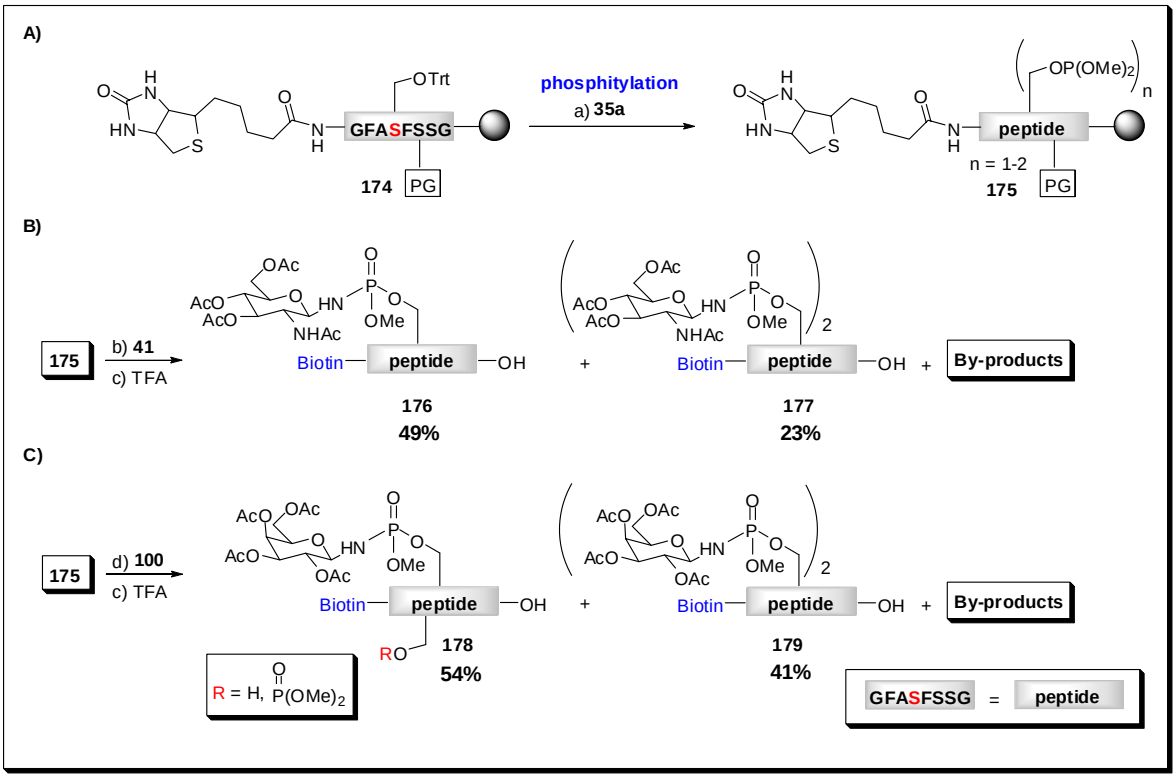


Scheme 32. A) Biotinylation and phosphitylation of immobilized peptide to **167**; B) Staudinger-phosphite reaction; C) De-*O*-acetylation of the crude product of SPR in B. Reagents and conditions: a) Biotin (5 equiv), HBTU (5 equiv), HOBt (5 equiv), DIPEA (5 equiv), dry DMF. 5 h; b) i) 0.5% TFA in CH_2Cl_2 ii) **35a** $(MeO)_2PN(iPr)_2$ (20 equiv.), 1*H*-tetrazole (50 equiv), 25 °C, 1.5h, dry DMF; c) *t*BuOOH, 30 min, 25 °C, DMF; d) 95% TFA, 3h; e) i) **100** (7 equiv), dry DMSO, 40 °C, 45 h; ii) H_2O (110 equiv), 15 h. Conversions are determined by LC-MS analysis. Serine residue marked in red shows the expected glycosylation site.

In contrast to the synthesis of **126**, the current reaction afforded both the mono- and divalent glycopeptides **176** and **177** with a preference for the monovalent glycopeptide formation (Scheme 33B).

The reaction of **175** with azido galactose **100** provided also a mixture of the mono- and divalent glycopeptides **178** and **179** (Scheme 33C).

Considering the synthesis of **126**, the current results indicated that the biotin immobilization to peptide encountered a problem of undesirable partial deprotection for some side chains within the peptide which subsequently provided more potential glycosylation sites than required.



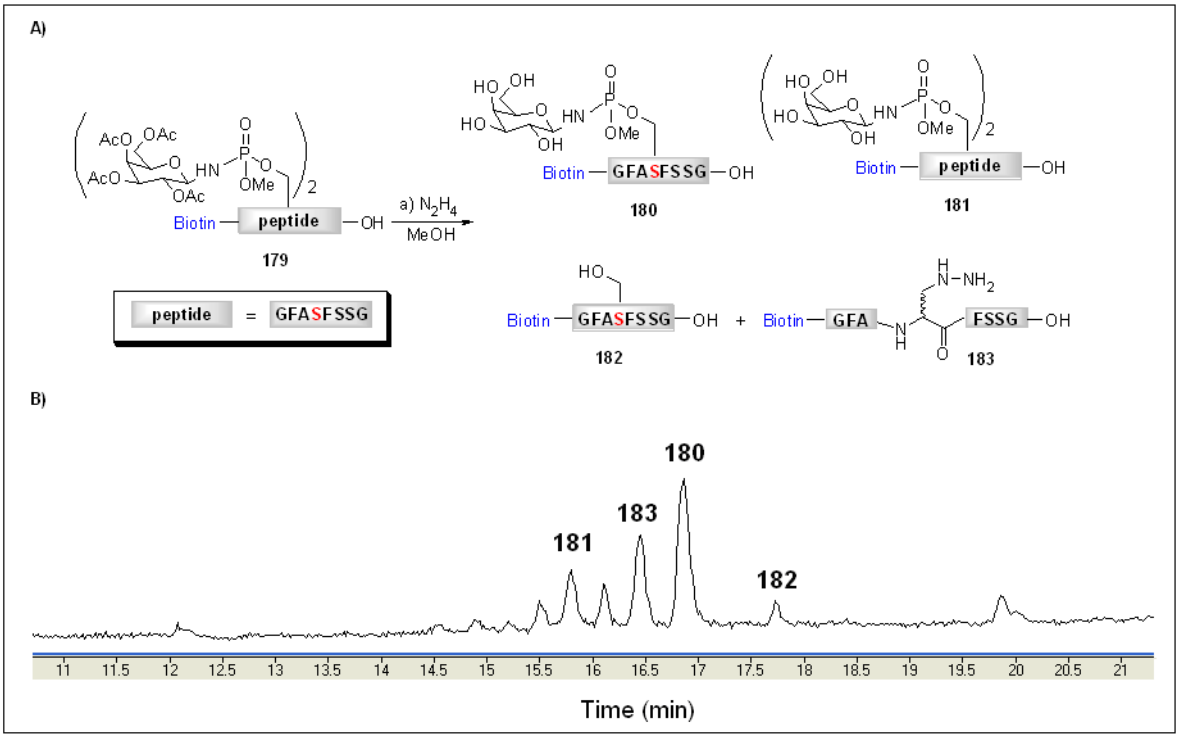
Scheme 33. A) Phosphitylation of immobilized peptide to **175**; B) Staudinger-phosphite reaction with **41** and C) with **100**. Reagents and conditions: a) i) 0.5% TFA in CH_2Cl_2 ii) **35a** $(MeO)_2PN(iPr)_2$ (20 equiv.), 1*H*-tetrazole (50 equiv), 25 °C, 1.5h, dry DMF; b) i) **41** (7 equiv), dry CH_2Cl_2, 40 °C, 45 h; ii) H_2O (110 equiv), 15 h; c) 95% TFA, 3h; d) i) **100** (7 equiv), dry DMSO, 40 °C, 45 h; ii) H_2O (110 equiv), 15 h. Conversions are determined by LC-MS analysis. Serine residue marked in red shows the expected glycosylation site.

Further investigations including peptide fragmentation experiments (MS/MS) for pure **179** were carried out to explore the position of the second glycosylation site. The MS/MS spectrum showed that no glycosylation occurred on the biotin moiety indicating that the second glycosylation occurred within the peptide chain. That means it occurred on one of the serine residues closer to the C-terminus as they are the only potential sites for further glycosylation. From the MS/MS spectrum we could not figure out where the exact site for the second glycosylation is; since the predominant peak was for the glyco-dehydroalanine-peptide.

However, regardless the site of the second glycosylation, **179** was treated with hydrazine hydrate in methanol to deprotect the galactose moiety.

After one hour the unprotected divalent glycopeptide **181** as well as the unprotected monovalent glycopeptide **180** were observed. In addition alcohol **182** and hydrazine-peptide **183** were formed (Scheme 34).

During the revision process of this manuscript, glycopeptides **180** and **181** were sent for Lectin binding study and they are still being investigated.

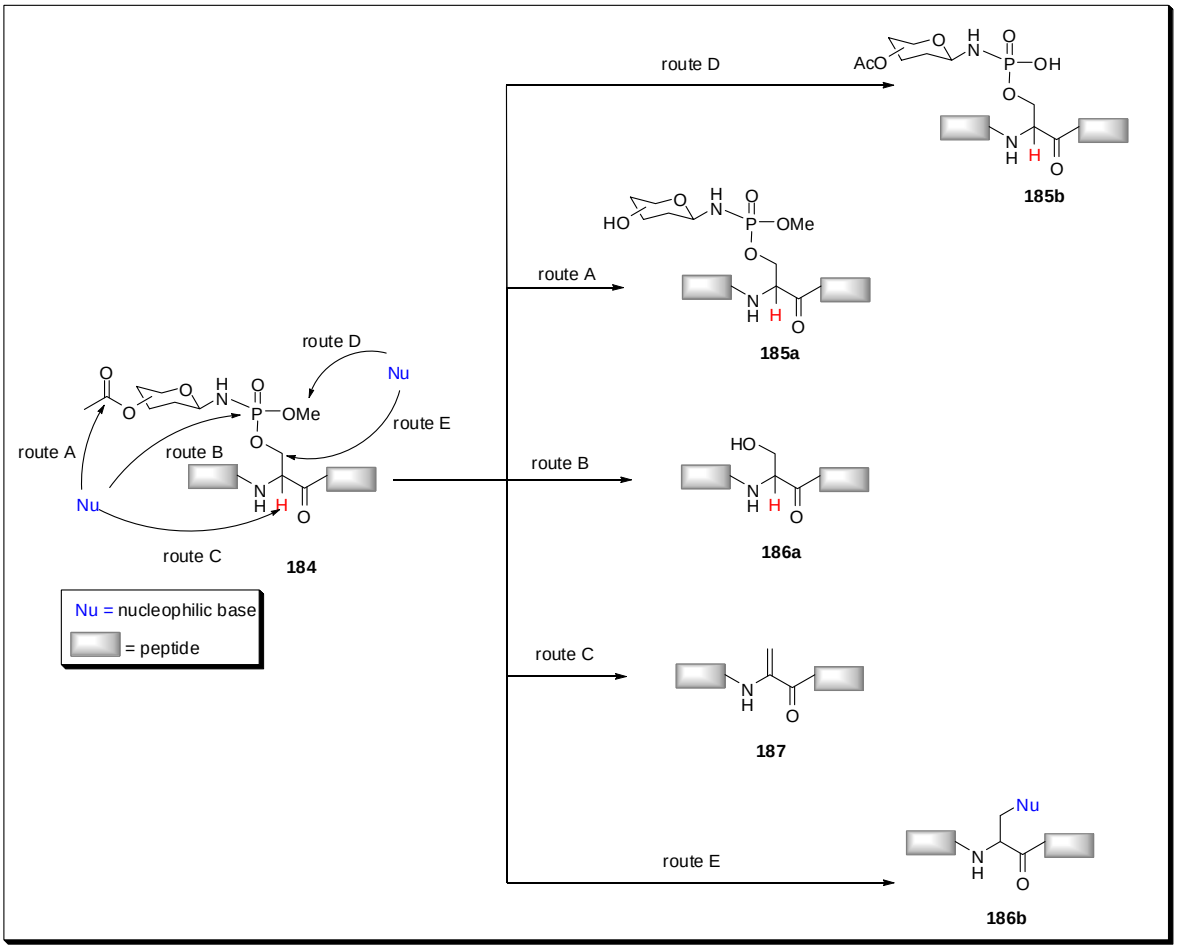


Scheme 34. A) De-*O*-acetylation of glycopeptide **179**; B) LC-MS trace for the De-*O*-acetylation reaction. Reagents and conditions: a) $NH_2NH_2.H_2O$, MeOH, 20 °C, pH = 11, 1 h.

From this and other previous experiments for de-*O*-acetylation of galactose moiety of phosphoramidate-linked glycopeptides **184**, it seems that the nucleophilic base involved in the de-*O*-acetylation can undergo three possible pathways as shown in scheme 35.

The first pathway involves the nucleophilic attack on the carbonyl group of the acetyl group furnishing the desired unprotected glycopeptide **185a** (route A), whereas the second pathway involves the attack on the phosphorous atom to release peptide **186a** (route B). The last pathway proceeds via the abstraction of the acidic α-hydrogen to produce the corresponding dehydroalanine-peptide **187** which can undergo Michael reaction addition in the presence of hydrazine yielding subsequently the hydrazine-peptide adduct (route C).

There are also other two possible pathways, routes D and E, which provide **185b** and **186b**, respectively (Scheme 35).



Scheme 35. Possible pathways during de-*O*-acetylation of glycopeptide containing galactose moiety.

In summary, glycosyl azides and phosphite-peptides required for the Staudinger phosphite reaction were synthesized successfully and further employed to access phosphoramidate-linked glycopeptides as novel glycopeptide mimetics.

The key steps in the Staudinger phosphite reaction were a global phosphitylation of an unprotected Ser residue to a dimethylphosphite-containing peptide, which was followed by glycosylation with a glycosyl azide in high yields and under high retention of the anomeric linkage. Subsequent TFA-mediated cleavage furnishes the phosphoramidate-linked glycoconjugates.

These artificial glycopeptides showed excellent stability under acidic and physiological conditions and their carbohydrate moiety could be deprotected by hydrazine hydrate in methanol in moderate overall yield.

The Staudinger phosphite reaction showed also good chemoselectivity when model phosphite-peptides were reacted with unprotected GlcNAc azide **41**, whereas the reaction with unprotected galactose azide **101** did not proceed at all.

Biotinylated mono- and divalent glycopeptides required for Lectin binding studies could be accessed by Staudinger phosphite reaction but the synthesis encountered a problem of partial deprotection of some amino acid side chains.

The Staudinger phosphite reaction included some limitations such as the formation of dehydroalanine-peptides as well as the unselective hydrolysis which resulted in releasing the carbohydrate moiety, thus no glycopeptide formation.

Chapter 5

Prospects

5 Prospects

The prospective work in this research will be devoted to cover three possible extensions of the described results: first, overcoming the limitation encountered in the synthesis of biotinylated phosphoramidate-linked glycopeptides, second, the glycosylation of hyperbranched polyglycerols (HPGs) by means of the Staudinger phosphite reaction, and finally the solid phase synthesis of GPI-anchored protein mimetics.

5.1 Synthesis of biotinylated mono-, di- and trivalent glycopeptides

As concluded from our previous work on biotinylated glycopeptides, the introduction of the biotin moiety prior to phosphitylation and glycosylation steps resulted in undesirable glycosylation trends. Therefore, we propose to couple the biotin at the final stage after the glycosylation and before the TFA-mediated cleavage.

In order to apply this strategy, the N-terminus should be orthogonally protected to the side chain protecting groups, so that the N-terminus can be deprotected and further biotinylated after the glycosylation step.

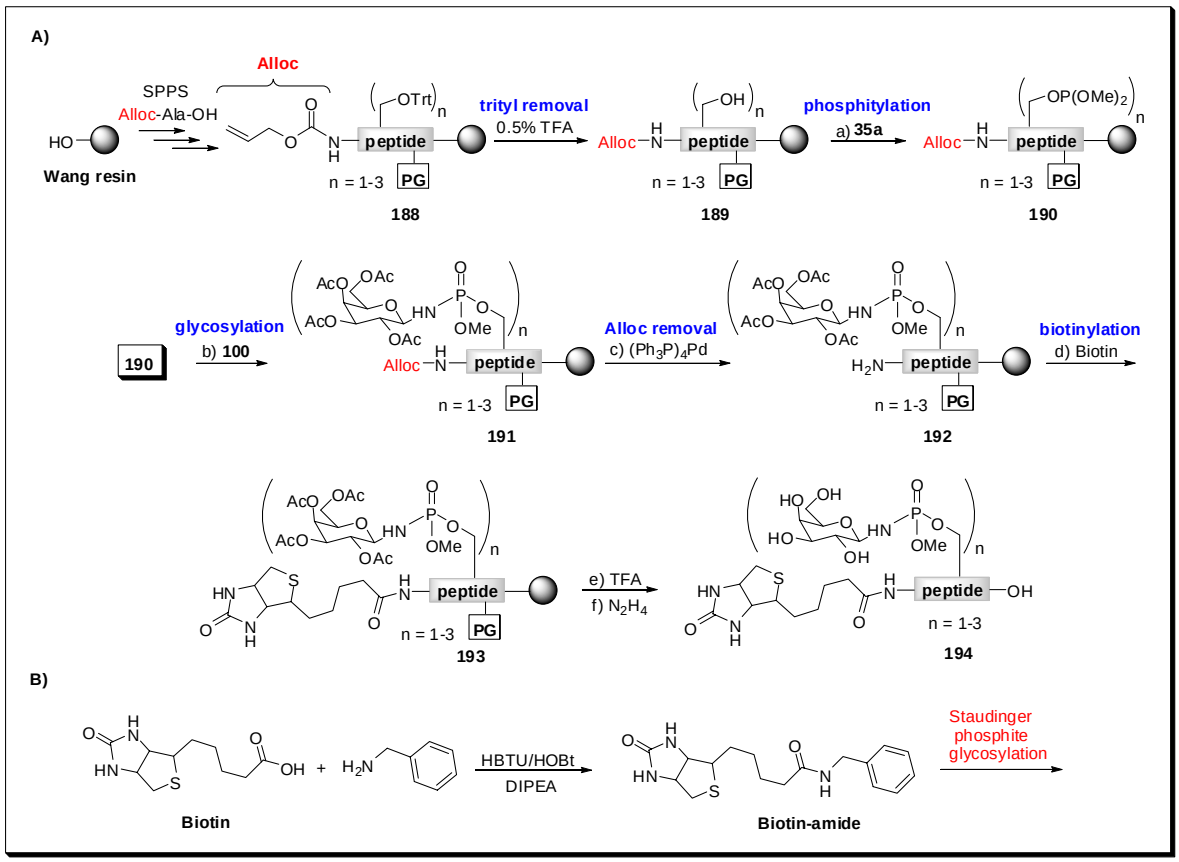
The allyloxycarbonyl (Alloc) protecting group is often used to protect amino group when an orthogonal deprotection scheme is required.[132]

Alloc can be easily removed by Pd(0) catalyzed allyl transfer under mild conditions. The reaction of tetrakis(triphenylphosphine)palladium(0) along with a 37:2:1 mixture of chloroform, acetic acid, and N-Methylmorpholine (NMM) for 2 hours are the most useful conditions to remove Alloc selectively in the presence of standard Fmoc- and *t*Bu-based protecting groups.[133,134]

Peptide **188** with an Alloc-protected N-terminus can be obtained by standard SPPS incorporating Alloc-Ala-OH[135] as a final amino acid residue in the peptide synthesis cycle. Once **188** has been prepared, the trityl groups can be removed with 0.5% TFA to provide free hydroxy groups in **189** which can be phosphitylated and subsequently glycosylated with β-Gal—N_3 **100** to provide the glycopeptide **191**. At this stage the Alloc protecting group should be removed and the nascent free N-terminus should be biotinylated. Subsequent peptide cleavage from the resin with 95% TFA followed by de-*O*-acetylation of the carbohydrate moiety should afford biotinylated glycopeptide **194** (Scheme 36A).

[132] N. Thieriet, J. Alsina, E. Giralt, F. Guibe, F. Albericio, *Tetrahedron Lett.* **1997**, *38*, 7275-7278.
[133] A. Kates, N. A. Sole, C. R. Johnson, D. Hudson, G. Barany, F. Albericio, *Tetrahedron Lett.* **1993**, *34*, 1549-1552.
[134] MERCK, *Novabiochem Peptide Synthesis* 2008/2009, protocol for Alloc protecting group removal.
[135] M. Stawikowski, P. Cudic *Tetrahedron Lett.* **2006**, *47*, 8587-8590.

Apart from that, we also intend to confirm that the biotin immobilization before the glycosylation step influences the Staudinger phosphite reaction. To do so, biotin will be activated according to the previous activation conditions (HBTU/HOBt/DIPEA). Then it will be reacted with benzyl amine to provide biotin-amide which will be subsequently treated with the Staudinger phosphite reaction conditions in the presence of β-Gal—N_3 **100** (Scheme 36B).



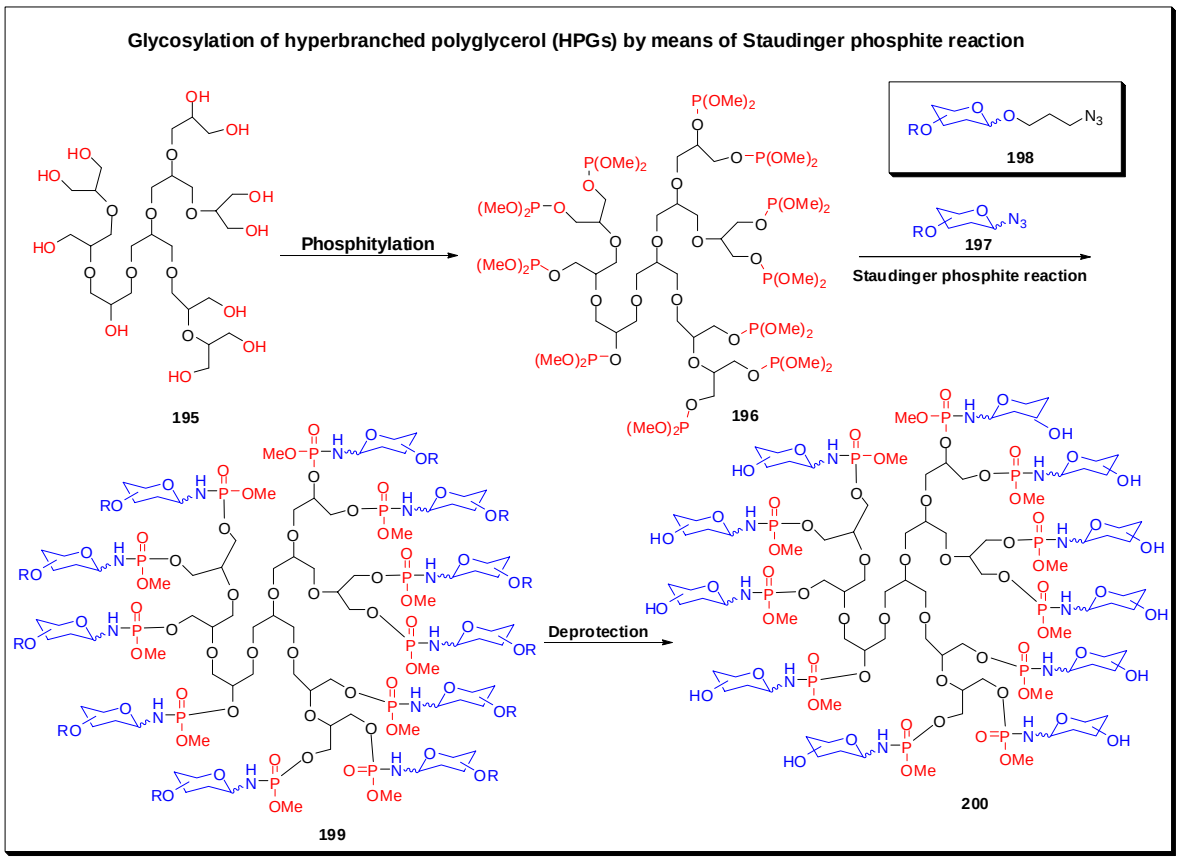
Scheme 36. A) Proposed strategy to access biotinylated glycopeptides **194**; B) Influence of biotin immobilization on SPR. Reagents and conditions: a) **35a** $(MeO)_2PN(iPr)_2$ (20-60 equiv.), 1*H*-tetrazole (50-150 equiv), 25 °C, 1.5h, dry DMF; b) i) **100** (7-21 equiv), dry DMSO, 40 °C, 45 h; ii) H_2O (110-330 equiv), 15 h; c) $(Ph_3P)_4Pd$ in $CHCl_3$: AcOH : NMM (37:2:1); d) Biotin (5 equiv), DIC, (5 equiv), DMAP (cat.), DMF e) 95% TFA, 3h; f) $NH_2NH_2.H_2O$, MeOH, 20 °C, pH = 11, 1 h. SPPS = Solid-phase peptide synthesis, NMM = *N*-methylmorpholine.

5.2 Glycosylation of HPGs by means of Staudinger phosphite reaction

Inspired by the synthesis of multivalent glycoarchitectures[136] and their unique binding to Selectin, we intend to utilize the Staudinger phosphite reaction to access glycosylated hyperbranched glycerol (HPGs).

To achieve this aim, hyperbranched polymer **195** can be phosphitylated and subsequently reacted with protected glycosyl azides **197** or **198** to produce after hydrolysis With water a protected glycosylated dendritic polymer **199** which can be later deprotected affording **200** (Scheme 37).

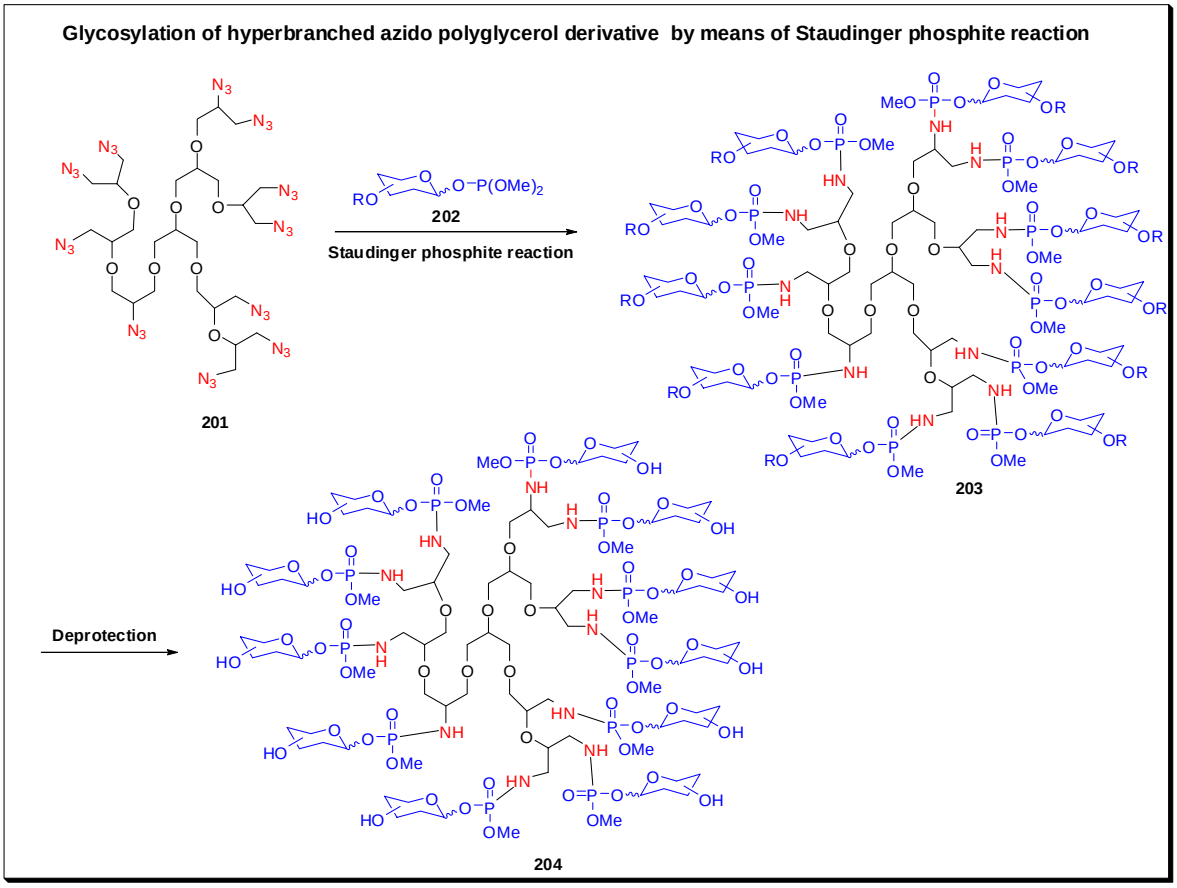
In principle, the hyperbranched polymer **195** can be reacted with unprotected glycosyl azides, avoiding subsequently the deprotection step.



Scheme 37. Proposed strategy to access glycosylated dendritic polyglycerol **200** starting from phosphite-hyperbranched polymer and glycosyl azides.

An alternative strategy to access glycosylated hyperbranched glycerol is to start from azido-dendritic polymer **201** and glycosyl phosphite **202** to yield **203** from the Staudinger phosphite reaction**,** which after deprotection will provide **204** (Scheme 38).

[136] I. Papp, J. Dernedde, S. Enders, R. Haag, *Chem. Commun.* **2008**, 5851–5853.



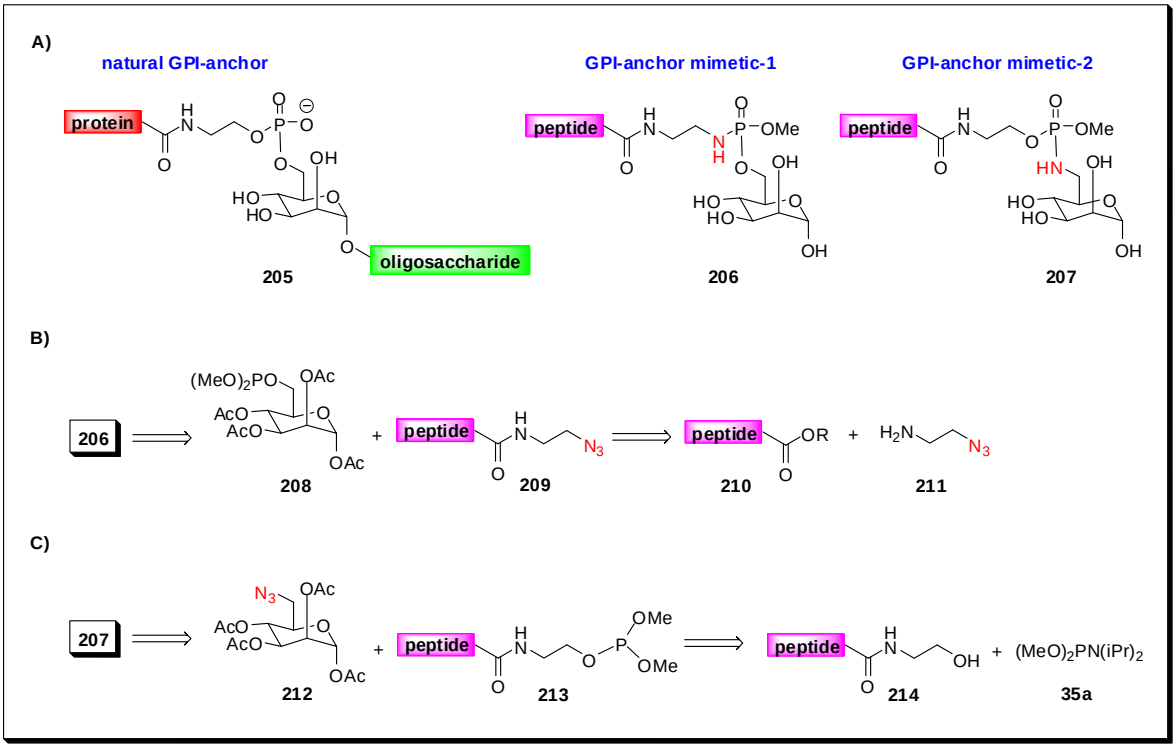
Scheme 38. Proposed strategy to access glycosylated dendritic polymer **204** starting from azido- dendritic polymer and glycosyl phosphites.

5.3 Synthesis of GPI-anchored protein mimetics

The last aim of this research will be the synthesis of GPI-anchored protein mimetics. Our strategy is based on replacing one oxygen atom of the phosphate diester linkage with an NH-moiety (Scheme 39A).

In order to achieve the first mimetic **206**, the oxygen closer to the C-terminus should be replaced. This can be achieved when the Staudinger phosphite reaction starts from glycosyl phosphite **208** and azidopeptide **209** (Scheme 39B).

Scheme 39C shows the alternative synthetic pathway which proceeds via the reaction between glycosyl azide **212** and phosphite-peptide **213**.



Scheme 39. A) Comparison between the naturally occurring GPI-anchored protein **205** and its mimetics **206** and **207**; B) and C) Proposed retro-synthesis to access **206** and **207**.

Chapter 6

Experimental Part

6 Experimental Part

6.1 General information, materials and analytics

Reagents: All reagents, amino acids, and solvents were purchased from commercial suppliers and used without further purification. Dry solvents were purchased from ACROS ORGANICS. Tetrazole solution in acetonitrile was purchased from Sigma Aldrich, acetonitrile was removed under reduced pressure at room temperature and the solid tetrazole was dissolved immediately in dry DMF. ATTENTION! Caution must be considered since solid tetrazole may explode under heating or high pressure.

Thin layer chromatography (TLC) was carried out on Merck silica gel 60, Fluorescenceindikator F_{254}. Detection was possible by UV (wavelength 254 nm or 366 nm) or by a staining agent: 20% H_2SO_4 in Ethanol or by cerium/molybdenium solution [phosphomolybdic acid (10 g), $Ce(SO_4)_2.H_2O$ (4 g), H_2O (375 mL), conc. H_2SO_4 (24 mL)].

Column Chromatography was conducted on Merck silica gel 60 (0.040–0.063 mm, 230–400 mesh ASTM). Specific eluents are given in the synthetic procedures.

Analytical HPLC and HRMS spectra were recorded on an Agilent 6210 TOF LC/MS system, Agilent Technologies, Santa Clara, CA, USA using Agilent Eclipse XDB-C_{18} column (5 µm, 4.6*150 mm) with a flow rate of 0.5 mL/min. Spray voltage and drying gas flow rate were set to 4 kV and 25 psi, respectively.

Preparative HPLC: purification for peptides by semi-preparative HPLC was preformed on a JASCO LC-2000 Plus system using a C_{18} column (5 µm, 25*250 mm with a flow rate of 16 mL/min). Specific gradients are given in the synthetic procedures.

Peptide Synthesis: Peptides were synthesized on an ABI 433A peptide synthesizer using standard amide coupling conditions HBTU/HOBt (Fast-moc protocol) utilizing Wang resin, Rink AM resin or Sieber amide resin which were purchased from Novabiochem.

NMR spectroscopy: ^{1}H, ^{13}C and ^{31}P NMR spectra were recorded by BRUKER AC 250 and BRUKER AM 270 SY instruments (250 MHz, 100 MHz, and 121 MHz). Deuterated solvents, such as $CDCl_3$, CD_3CN and d_6-DMSO were utilized. Chemical shifts δ are given

in ppm relative to TMS as an internal standard or relative to the resonance of the solvent (^{1}H NMR: Chloroform: δ = 7.26 ppm, DMSO: δ = 2.50 ppm; ^{13}C NMR: Chloroform: δ = 77.00 ppm, DMSO: δ = 39.70 ppm).[137]

6.2 General procedures

General procedure I for the preparation of side-chain activated (phosphino)thioester peptides 66a, 66b, 72 and 73 and side-chain activated phosphinophenol-ester peptides 95 and 96 (GP I)

Precursor peptides were synthesized on Rink Amide resin, cleaved from the resin with 99% TFA for 3 hours and precipitated in cold ether. 3 equiv DIC and catalytic amount of DMAP were added to the peptide dissolved in dry CH_2Cl_2 (3 to 5 mL/0.033 mmol) and the reaction mixture was stirred for 10 minutes. Then 4 equivalents of borane protected diphenyl-phosphinomethanethiol **52**, 4 equivalents of phenylmethanethiol **65** or 1.1 equiv of *O*-diphenyl-phosphinophenol **54** were added to the activated peptide and the reaction mixture was stirred for 5 to 12 h.

General procedure II for the preparation of side-chain activated phosphinophenol-ester peptides 85, 92 and 94 (GP II)

Precursor peptides were synthesized on an acid-sensitive Sieber Amide resin with an orthogonally protected Asp(O-2-PhiPr) at the potential glycosylation site and cleaved from the resin with 0.5 % TFA and 2.5 % TIS in CH_2Cl_2 for 15 min. The resin was filtered off and washed with CH_2Cl_2. The filtrate and the washing solution were combined and the solvent was removed under high vacuum. 3 equiv DIC and catalytic amount of DMAP were added to the partially protected peptide dissolved in dry CH_2Cl_2 (3 to 5 mL/0.033 mmol) and the reaction mixture was stirred for 10 minutes. Then 1.1 equiv of diphenyl-phosphinophenol **54** were added to the activated peptide and the reaction mixture was stirred for 5 to 12 h. The solvent was removed under high vacuum and the residue was treated with 99% TFA (1 mL/0.033 mmol) for 3 hours and the peptide was precipitated in cold ether.

[137] H. E. Gottlieb, V. Kotlyar, A. Nudelman, *J. Org. Chem.* **1997,** *62,* 7512-7515.

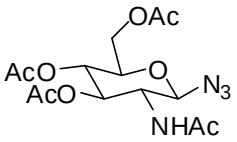
General procedure for the global peptide phosphitylation on a solid support (GP III).
The Ser-residue at the later phosphitylation site was incorporated into the peptide with a trityl side-chain protecting group, which was removed on the resin immediately before the phosphitylation reaction with 0.5 % TFA in dry CH_2Cl_2. The peptidyl resin (0.01 mmol) was placed in a manual peptide synthesis vessel and dried overnight under reduced pressure at 40 °C. A solution of tetrazole (0.5 mmol) and dimethyl-, dibenzyl-, or di-*t*-butyl-*N*,*N*-diisopropylphosphoramidite (0.2 mmol) in dry DMF (0.6 mL) was added to the peptidyl resin and agitated gently for 1.5 hours. The reagents were removed by filtration and the peptidyl resin was washed with dry DMF (3 x 3 mL) and finally with either dry DMSO (3 x 3 mL) or CH_2Cl_2 (3 x 3 mL). Specific dry solvents are given in the synthetic procedures.

General procedure for the oxidation of phosphitylated peptides on the solid support (GP IV).
The phosphitylated peptide was prepared according to GP III with dry DMF washings at the end. Then, dry DMF was added to cover the peptidyl resin and 6 M *t*-butyl hydroperoxide in octane (1 mL) was added and the reaction vessel was gently agitated for 30 minutes. *t*-butyl hydroperoxide was removed by filtration and the peptidyl resin was washed with DMF and CH_2Cl_2 and dried. Finally, the phosphorylated peptide was cleaved from the resin with TFA/TIS/water (95:2.5:2.5) for 3 hours and precipitated in cold ether.

General procedure for solid phase peptide Staudinger-phosphite glycosylation (GP V).
Immobilized phosphitylated peptides were prepared by using dimethyl-*N*,*N*-diisopropylphosphoramidite as a phosphitylating agent unless otherwise specified. Then, glycosyl azide **41**, **42**, **99**, **100** or **101** (0.07 mmol) was dissolved in 1 mL dry or wet solvent (DMF, DMSO or CH_2Cl_2) and added to the peptide resin. The reaction vessel was gently agitated at either 25 °C, 35 °C or 40 °C. After 45h the reaction mixture was quenched by the addition of water (20 μL, 1.1 mmol) and again kept for 15 hours at 25 °C. The reagents were removed by filtration; the resin was washed with DMF (3 x 3 mL) and CH_2Cl_2 (3 x 3 mL) and dried. Finally, the glycopeptide was cleaved from the resin using TFA/TIS/water (95:2.5:2.5 v/v) for 3 hours, precipitated in cold ether and further analyzed or purified by HPLC.

6.3 Synthesis of glycosyl azides

Synthesis of 2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl Azide (acetyl β-GlcNAc-N_3) (41):



41

A suspension A suspension of N-Acetyl-D-glucosamine (2.21g, 10.0 mmol) in acetic anhydride (4.82 mL, 51.0 mmol) was placed in an ice bath with continuous stirring. HBr in acetic acid (33%, 2.45 mL, 10.0 mmol) was added in one portion to the cold suspension of the reaction mixture and was allowed to stir at room temperature until a clear solution was obtained. The reaction mixture was then cooled to 0 °C, additional HBr in acetic acid (33%, 4.9 mL, 20.0 mmol) was added slowly, and stirring was continued for 2-3 hours at room temperature. Solvents were removed under reduced pressure and co-evaporated with dry toluene.

Sodium azide (1.30 g, 20.0 mmol), tetrabutylammonium hydrogen sulphate (TBAHS) (510 mg, 1.5 mmol) and aqueous Na_2CO_3 (1M, 70 mL) were added successively to a solution of the crude reaction product in dry CH_2Cl_2 (50 mL) and the two-phase reaction mixture was stirred vigorously for another 6 hours. The reaction mixture was diluted with CH_2Cl_2 (50 mL). The organic layer was separated and washed with brine, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was chromatographed on a silica gel column with ethylacetate-hexane-acetone (47:2:1). The glycosyl azide **41** was obtained by crystallization from ethylacetate-petroleum ether in 40% yield (1.49 g, 4.0 mmol).

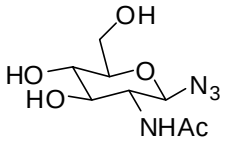
^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.82 (d, 1H), 5.28 (dd, 1H), 5.12 (dd, 1H), 4.78 (d, 1H, CHN_3), 4.28 (dd, 1H), 4.16 (dd, 1H), 3.93 (ddd, 1H), 3.8 (ddd, 1H), 2.1, 2.04, and 1.97 (3s, 3H, 6H, 3H, 3 OAc and NAc).

IR (KBr) ν = 2120 cm^{-1} (N_3).

Further analytical analysis is in accordance with reported results.[138]

[138] E. Meinjohanns, M. Meldal, H. Paulsen, K. Bock, J. *Chem. Soc. Perkin Trans.1* **1995**, 405-415.

Synthesis of 2-Acetamido-2-deoxy-β-D-glucopyranosyl Azide (β-GlcNAc—N_3) (42):



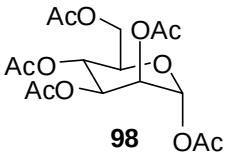
42

Acetyl β-GlcNAc—N_3 **41** (1.0 g, 2.69 mmol) was treated with 0.2M sodium methoxide in methanol (2.5 mL). TLC (ethylacetate:methanol, 9:1) showed after 10 min formation of a new product (R_f 0.0) with complete consumption of the starting material (R_f 0.7). The reaction mixture was neutralized with ion exchange resin (Dowex-H^+), filtered and concentrated *in vacuo* to give 42 in 95% yield (1.2 g, 2.56 mmol) as a white crystalline solid.

^{1}H NMR (400 MHz, MeOD): δ = 4.51 (1H, d, $J_{1,2}$ = 9.4 Hz, H-1), 3.92 (1H, dd, $J_{5,6'}$ = 1.5 Hz, $J_{6,6'}$ = 11.9 Hz, H-6'), 3.66-3.74 (2H, m, H-2, H-6), 3.45 (1H, dd, *J* = 8.1 Hz, *J* = 10.1 Hz, H-4), 3.30-3.39 (2H, m, H-3, H-5), 2.00 (3H, s, CH_3).

Further analytical analysis is in accordance with reported results.[139]

Synthesis of 1,2,3,4,6,-penta-*O*-acetyl-α-D-mannose (98)



98

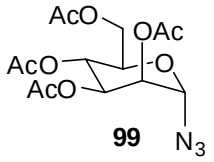
Acetic anhydride (27 mL, 0.288 mol) was mixed with D-mannose (4.98 g, 0.028 mol) and the mixture was stirred for 5 minutes at 0 °C. Sulphuric acid (3 drops) was added and the mixture was stirred for 10 minutes at 0 °C and then allowed to warm to ambient temperature and stirred for a further an hour. Then ice-water (100 mL) was added under stirring and the organic layer was extracted with ethylacetate (100 mL). The combined

[139] S. You Hong, G. Tobias, B. Ballesteros, F. El Oualid, J. C. Errey, K. J. Doores, A. I. Kirkland, P. D. Nellist, M. L. H. Green, B. G. Davis, *J. Am. Chem. Soc.* **2007**, *129*, 10966-10967.

organic extracts were washed with water (3 x 100 mL) and finally with saturated aqueous sodium bicarbonate solution (2 x 100 mL) and dried over $MgSO_4$ and the solvent was removed under reduced pressure. Obtained (9.84 g, 0.025 mol) of D-mannose pentaacetate **98** (89%).

Analytical analysis is in accordance with reported results.[105]

Synthesis of α-Azido-D-mannose tetraacetate (acetyl α-Man—N_3) (99):



Tin (IV) chloride (0.91 mL, 7.75 mmol) was added under argon to a solution of **98** (5.00 g, 12.8 mmol) and trimethylsilyl azide (3.55 mL, 25.6 mmol) in dry dichloromethane (105 mL). The mixture was stirred for 15 hours at room temperature and the solvent was evaporated under reduced pressure. The crude product was purified on a silica gel column with ethylacetate-hexane (1:1) affording **99** as a white solid in 83% yield (4.00g, 10.7 mmol).

^{1}H NMR (400 MHz, CD_3CN): δ = 5.46 (d, *J* = 1.85 Hz, 1H, CHN_3), 5.22 (m, 1H), 5.08 (m, 1H), 4.25 (m, 1H), 4.17 (m, 1H), 4.14 (m, 1H), 2.09, 2.02, 1.99, 1.92 (4s, 12H, 4 OAc).

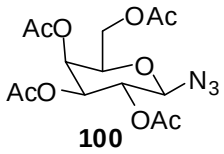
^{13}C NMR (100 MHz, CD_3CN): δ = 171.26, 170.87, 170.66, 170.62, 88.16, 71.51, 69.70, 69.35, 66.00, 62.77, 20.87 (1C, CH_3), 20.83 (1C, CH_3), 20.78 (1C, CH_3), 20.77 (1C, CH_3).

IR (KBr) ν = 2121 cm^{-1} (N_3).

HRMS (ESI-TOF): m/z = 391.146 $[M+NH_4]^+$ (calcd.: m/z = 391.148), 396.104 $[M+Na]^+$ (calcd.: m/z = 396.101)

Further analytical analysis is in accordance with reported results.[103,104]

Synthesis of 2,3,4,6-*O*-acetyl-β-D-galactopyranose azide (acetyl α-Gal—N_3) (100)



100

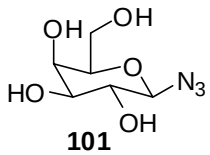
D-galactose (1.876 g, 10.0 mmol) was dissolved in acetic anhydride (5 mL, 51.0 mmol) and cooled with ice bath. 33% HBr/AcOH (3 mL) was then added and the reaction mixture was stirred at room temperature for 90 minutes under argon. The temperature was brought to 0 and 33% HBr/AcOH (5.8 mL) was added. The reaction mixture was then stirred at room temperature for approximately 2.5 hours. Subsequently solvents and reagents were removed under reduced pressure with dry toluene as an auxiliary solvent. The crude bromo galactose (brown syrup) was used without further purification. It was dissolved in dry CH_2Cl_2 (50 mL), sodium azide (1.381 g, 20.0 mmol) and (TBAHS) (524 mg, 1.5 mmol) were added consecutively. Subsequently aqueous K_2CO_3 (1 M, 50 mL) was overlaid and the two phase reaction mixture was stirred vigorously at room temperature for 16 h. Then the reaction mixture was diluted and extracted with CH_2Cl_2. The organic layer was then dried over magnesium sulphate, filtered and evaporated to give crude **100**. The product was then purified by column chromatography (cyclohexane/ethylacetate gradient from 66:34 to 50:50). Recrystallization from cyclohexane/diethylether gave **100** in yield of 39 % (1.44 g, 3.9 mmol)

^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.42 (dd, J = 3.4, 1.1 Hz, 1H), 5.16 (dd, J = 10.4, 8.7 Hz, 1H), 5.03 (dd, J = 10.4, 3.4 Hz, 1H), 4.59 (d, J = 8.7 Hz, 1H), 4.20 - 4.12 (m, 2H), 4.01 (td, J = 6.6, 1.1 Hz, 1H), 2.16 (s, 3H), 2.08 (s, 3H), 2.06 (s, 3H), 1.98 (s, 3H).

^{13}C NMR (100 MHz, $CDCl_3$): δ = 170.51, 170.25, 170.12, 169.50 (4C, 4x CO_2CH_3), 88.43 (1C, CHN3), 72.99 (1C, CH), 70.86 (1C, CH), 68.18 (1C, CH), 66.97 (1C, CH), 61.35 (1C, CH2), 20.89, 20.79, 20.77, 20.65 (4C, 4x CH_3).

HRMS (ESI-TOF): m/z = 396.1029 $[M+Na]^+$ (calcd.: m/z = 396.1014).

Synthesis of β-D-galactopyranose azide (α-Gal—N_3) (101)



101

A suspension of **100** (753 mg, 2 mmol) in dry methanol (6 mL) was stirred for 10 minutes at room temperature. To this mixture a solution of sodium methoxide (30% in MeOH, 0.3 mL) was added and stirred at room temperature until the de-*O*-acetylation reaction was completed (monitored by TLC). Subsequently the reaction mixture was neutralized with an (DOWEX) and filtered. Evaporation of the filtrate and subsequent recrystallization from acetonitrile and diethyl ether yielded 371 mg of **101** (90%).

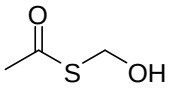
^{1}H NMR (400 MHz, D_2O): δ = 4.63 (d, *J* = 8.7 Hz, 1H), 3.93 (d, *J* = 3.4 Hz, 1H), 3.79-3.71 (m, 3H), 3.65 (dd, *J* = 9.8, 3.4 Hz, 1H), 3.47 (dd, *J* = 9.8, 8.7 Hz, 1H).

Further analytical analysis is in accordance with reported results.[140]

[140] Z. Gyrgydeàk, L. Szilàgyi, *Liebigs Ann. Chem.* **1987**, 235 - 241.

6.4 Synthetic procedures for compounds and peptides involved in TSL

Synthesis of S-hydroxymethyl ethanethioate (47)

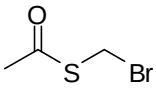


47

Thiolacetic acid **46** (12.5 g, 0.165 mol) and paraformaldehyde (5.00 g, 0.165 mol) were heated to about 100 ^{o}C on an oil bath for 3 h. The colourless reaction mixture turned orange indicating the completion of the reaction. The crude product was then distilled under reduced pressure to give **47** in 80% (14.01 g, 0.132 mmol) as a pale yellow oil.

^{1}H NMR (250 MHz, $CDCl_3$): δ = 5.00 (s, 2H, HOC$\underline{H}_2$S), 4.30 (s, 1H, $\underline{H}$OCH$_2$S), 2.33 (s, 3H, CH_3C(O)S).

Synthesis of S-bromomethyl ethanethioate (48)

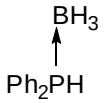


48

Phosphorus tribromide (8.37 g, 0.03 mol) was slowly added to cooled S-hydroxymethyl ethanethioate **47** (10.15 g, 0.095 mol) with stirring under argon atmosphere. The temperature was kept between 5 and 8 ^{o}C. After the complete addition of the PBr_3, the reaction mixture was stirred for additional 30 min at 5-8 ^{o}C and then the temperature was allowed to elevate to room temperature. The reaction mixture was subsequently poured over an ice-water mixture and stirred for another hour. The organic layer was extracted with Et_2O (3 x 100 mL), and the extracts were combined and washed with water (2 x 100 mL), dried over anhydrous $MgSO_4$, and finally concentrated under reduced pressure to give the crude bromomethyl acetyl sulfide **48**. Distillation of the crude product yielded 8.790 g (55%) of **48** as a colourless oil.

^{1}H NMR (250 MHz, $CDCl_3$): δ = 4.69 ppm (s, 2H, BrC$\underline{H}_2$S), 2.38 (s, 3H, C$\underline{H}_3$C(O)S).

Synthesis of borane-diphenylphosphine complex (50)

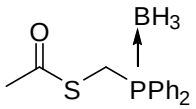


50

A solution of chlorodiphenylphosphine **49** (5.00 g, 0.02 mol) in 9 mL dry THF was cooled to 0 ^{o}C under argon atmosphere. 30 mL of borane-THF complex (1M) was added to the solution followed by the portion-wise addition of $LiAlH_4$ (1.05 g, 0.03 mol). The reaction mixture was stirred at 0 ^{o}C for 2 hours and then was carefully poured onto a mixture of 9 mL conc. HCl and 100 g ice along with stirring. The product was extracted with toluene and the combined extracts were dried over $MgSO_4$ and concentrated under reduced pressure. The residual oil was passed through a short column of silicagel, eluting with toluene to give 3.04 g (67%) **50** as a crystalline solid.

^{1}H NMR (250 MHz, $CDCl_3$): δ = 7.15-7.95 (m, 10 H, aromatic H), 6.30 (dq, $J_{H\text{-}P}$ = 379.00 Hz, $J_{H\text{-}H}$ = 6.38 Hz, 1H), 0.40-1.70 (broad q, 3H, $B\underline{H}_3$).
^{31}P NMR (121 MHz, $CDCl_3$): δ = 6.29 (dq, $J_{H\text{-}P}$ = 379.03 Hz, $J_{H\text{-}H}$ = 7 Hz), 1.09 (dd, $J_{P\text{-}B}$ = 7.23 Hz; 16.55 Hz).

Synthesis of borane-diphenylphosphino methanethiol acetate complex (51a)



51a

Borane-diphenylphosphine complex **50** (3 g, 15 mmol) was dissolved in dry DMF under Ar (g) and cooled to 0 °C. NaH (0.3958 g, 16.5 mmol) was added slowly, and the mixture was stirred at 0 °C until bubbling ceased. Alkylating agent bromomethanethiol acetate **48** (2.5356 g, 15 mmol) was then added and the mixture was allowed to warm to room temperature and stirred for 12 h. Solvent was removed under reduced pressure, and the residue was purified by flash chromatography (silica gel, 10% V/V EtOAc in hexane) providing the product as colorless oil in 34% (1.4562 g).

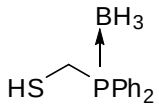
^{1}H NMR (250 MHz, $CDCl_3$): δ = 7.44-7.69 (m, 10H, aromatic H), 3.72 (d, *J* = 7.30 Hz, 2H, $SC\underline{H}_2P$), 2.26 (s, 3H, $C\underline{H}_3C(O)S$), 0.45-1.22 (broad m, 3H, $B\underline{H}_3$).

^{13}C NMR (62.5 MHz, $CDCl_3$): δ = 131.5 (d, $J_{c\text{-}p}$ = 2.30 Hz), 132.3, 192.9 ppm (d, $J_{c\text{-}p}$ = 9.20 Hz), 127.2 (d, $J_{c\text{-}p}$ = 55.00 Hz), 128.7 (d, $J_{c\text{-}p}$ = 11.00 Hz),24.0, 3.0 (d, $J_{c\text{-}p}$ = 36.00 Hz).

^{31}P NMR (121 MHz, $CDCl_3$): δ = 16.88 (d, *J* = 64.90 Hz).

Further analytical analysis is in accordance with reported results.[88]

Synthesis of borane-diphenylphosphinomethanethiol complex (52)



52

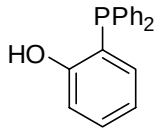
Borane-diphenylphosphino methanethiol acetate complex **51a** (1 g, 3.47 mmol) was dissolved in dry methanol (16 mL), followed by the addition of sodium methoxide (30% in methanol) (0.8 mL). After stirring under Ar(g) for 10 min, the solution was neutralized by 1M hydrochloric acid, and was extracted with ethyl acetate. The combined organic layers were washed by brine, dried over sodium sulfate, filtered, and concentrated under reduced pressure. The product was obtained as colorless oil in 95 % yield (822 mg).

^{1}H NMR (250 MHz, $CDCl_3$): δ =7.67-7.74 (m, 6H, aromatic H), 7.46-7.51 (m, 4H, aromatic H), 3.20 (dd, *J* = 6.38 Hz; 8.20 Hz, 2H, $SC\underline{H}_2P$), 1.89 (ddd, *J* = 8.20 Hz; 15.48 Hz, 1H, $S\underline{H}$), 0.30-1.30 (broad m, 3H, $B\underline{H}_3$).

^{13}C NMR (62.5 MHz, $CDCl_3$): δ = 19.3 (d, $J_{c\text{-}p}$ = 33.00 Hz); 127.2, 128.6, 128.8, 131.5, 132.3, 132.4 (d, $J_{c\text{-}p}$ = 55.00 Hz).

^{31}P NMR (121 MHz, $CDCl_3$): δ = 19.62 (d, $J_{P\text{-}SC\underline{H}2P}$ = 84 Hz); 1.03 ppm (d, $J_{P\text{-}B\underline{H}3}$ = 15.52 Hz).

Synthesis of *O*-diphenylphosphinophenol (54)



54

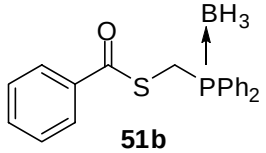
KOAc (2.076 g, 20.76 mmol) was added to a solution of 2-iodophenol (3.7887 g, 17.3 mmol) in 20 mL dry dimethylacetamide (DMA) under argon atmosphere. Cat. amount of $Pd(OAc)_2$ dissolved in dry DMA was then added and the reaction mixture was degassed. Then, diphenylphosphine (3.229 g, 17.3 mmol) was added to the reaction mixture and the temperature was elevated to 130 ^{o}C and stirred for 16 hours. The reaction mixture was then poured onto 70 mL water and the organic layer was extracted with CH_2Cl_2, and washed with brine. The solvent was removed under reduced pressure and the rest was recrystallized from hot methanol. In addition to the desired product, the crystals contained the oxidized product. The product was further purified by column chromatography with only CH_2Cl_2 as an eluent to provide the pure product in 65% yield (3.1293 g, 11.25 mmol).

^{31}P NMR (121 MHz, $CDCl_3$): δ = -26.5 ppm

HRMS (ESI-TOF): m/z = 279.095 $[M+H]^+$ (calcd.: m/z = 279,093)

Further analytical analysis is in accordance with reported results.[141]

Synthesis of Diphenylphosphino(borane)methanethiol benzoate (51b).



51b

DIC (0.975 mmol) and catalytic amount of DMAP were added to benzoic acid (40 mg, 0.325 mmol) dissolved in dry CH_2Cl_2 and the reaction mixture was stirred for 10 minutes. Then (0.65 mmol) of borane protected diphenyl-phosphinomethanethiol **52** was added to the activated benzoic acid and the reaction mixture was stirred for further 4 h and the solvent was then removed under high vacuum. The rest was purified by silica gel chromatography with ethylacetate-hexane (20:80) in 80% yield (92 mg, 0.26 mmol).

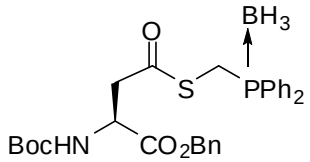
[141] A. Bianchi, A. Bernardi, *J. Org. Chem.* **2006**, *71*, 4565-4577.

^{1}H NMR (250 MHz, $CDCl_3$): δ = 7.40-7.76 (m, 15H), 3.920 (d, 2H), 0.86-1.26 (br, 3H)

^{31}P NMR (121 MHz, $CDCl_3$): δ = 19.70 (m)

HRMS (oxidized **51b**) (ESI-TOF): m/z = 353.0761 [M+H]+ (calcd.: m/z = 353.0760), 375.0582 [M+Na]+ (calcd.: m/z = 375.0580).

Boc-N-β-(diphenylphosphino(borane))methanethiol-L-asparagine benzyl ester (58)



58

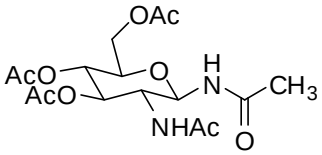
DIC (0.3 mmol) and catalytic amount of DMAP were added to Boc-Asp-OBz **57** (33 mg, 0.1 mmol) dissolved in dry CH_2Cl_2 and the reaction mixture was stirred for 10 minutes. Then (0.2 mmol) of borane protected diphenyl-phosphinomethanethiol **52** was added to the activated aspartic acid and the reaction mixture was stirred for further 4 h and the solvent was removed under high vacuum. The rest was purified by semi-preparative HPLC (C18-column, constant flow: 3 min at 7 % CH_3CN, gradient: 7 % to 95 % CH_3CN over 35 min) in 75% yield (42 mg, 0.075 mmol).

^{1}H NMR (250 MHz, $CDCl_3$): δ = 7.73-7.29 (m, 15H), 5.25-5.13 (m, 2H), 4.55-4.49 (m, 1H), 3.69 (qd, *J* = 14.4, 7.4 Hz, 2H), 3.04 (qd, *J* = 16.6, 5.2 Hz, 2H), 1.44 (s, 3H), 1.54-0.87 (m, 3H).

^{13}C NMR (101 MHz, $CDCl_3$): δ = 194.6, 170.2, 155.0, 35.0, 132.4 (d, J = 15.9 Hz), 132.4 (d, *J* = 2.3 Hz), 131.8, 128.9 (d, *J* = 3.1 Hz), 128.8 (d, *J* = 2.9 Hz), 128.5, 128.3, 128.0, 127.8, 127.6, 127.0 (d, *J* = 13.5 Hz), 80.2, 67.5, 50.4, 44.9, 28.2, 23.6 (d, *J* = 34.2 Hz).

^{31}P NMR (121 MHz, $CDCl_3$): δ = 22.72 ppm

Synthesis of N-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-b-D-glucopyranosyl)acetamide (56a)



56a

Route 1 (acidic deprotection conditions): 20 mg (0.069 mmol) phosphinothioester **51a** was treated with 1 mL 99% TFA for 1 h and the volatiles were removed under high vacuum. The residual phosphonium salt was dissolved without further purification in 1 mL dry DMF and the solution was degassed by three cycles of vacuum and argon. DIPEA (12 equiv) as well as 1 equiv of glycosyl azide **41** were added to this solution and the resulting reaction mixture was stirred for 18 h at 40 ^{o}C before the reaction mixture was concentrated under high vacuum. The residue was chromatographed on a silica gel column with chloroform–methanol (19:1). Pure acetylated GlcNAc (**56a**) was obtained in 62% yield (16.7 mg, 0.043 mmol).

Route 2 (basic deprotection conditions): 20 mg (0.069 mmol) phosphinothioester **51a** was dissolved in 1 mL dry DMF and the solution was degassed by three cycles of vacuum and argon. 3 equiv of DABCO as well as 1 equiv of glycosyl azide **41** were added. The temperature was elevated to 40 ^{o}C and the reaction mixture was stirred for 18 h. The residue was chromatographed on a silica gel column with chloroform–methanol (19:1). Acetylated GlcNAc (**56a**) was obtained in 60% yield (14 mg, 0.031 mmol).

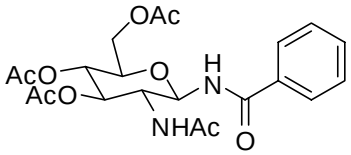
^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.09 (d, 1H), 6.07 (d, 1H), 5.23 (t, 1H), 5.10–5.25 (m, 2H), 4.39 (dd, 1H) 4.16–4.25 (m, 2H), 3.85 (m, 1H), 2.19, 2.17, 2.14, 2.08, 2.06 (5s, 15H, Ac).

HRMS (ESI-ToF): m/z = 389.159 $[M+H]^+$ (calcd: m/z = 389.156), 411.137 $[M+Na]^+$ (calcd: m/z = 411.137).

Further analytical analysis is in accordance with reported results.[142,143]

[142] Z. Gyrgydeak, Z. Hadady, N. Felfldi, A. Krakomperger, V. Nagy, M. Toth, A. Brunyanszki, T. Docsa, P. Gergely, L. Somsak, *Bioorg. Med. Chem.* **2004**, *12*, 4861-4870.
[143] R. S. Talan, A. K. Sanki, S. J. Sucheck, *Carbohydr. Res.* **2009**, *344*, 2048-2050.

Synthesis of N-(2-Acetamido-2,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl) benzamide (56b)



56b

Route 1 (acidic deprotection conditions): 20 mg (0.057 mmol) phosphinothioester **51b** was treated with 1mL 99% TFA for 1 hour and the volatiles were removed under high vacuum. The residual phosphonium salt was dissolved without further purification in 1 mL dry DMF and the solution was degassed by 3 cycles of vacuum and argon. 12 equivalents of DIPEA as well as 3 equivalents of GlcNAc azide **41** were added to this solution and the resulting reaction mixture was stirred for 18 hours at 40 ^{o}C and the reaction mixture was then concentrated under high vacuum. The residue was chromatographed on a silica gel column with ethylacetate-methanol (40:1). Pure benzoylated GlcNAc (**56b**) was obtained in 60% yield (15.3 mg, 0.034 mmol).

Route 2 (basic deprotection conditions): 20 mg (0.057 mmol) phosphinothioester **51b** was dissolved in 1 mL dry DMF and the solution was degassed by 3 cycles of vacuum and argon. 3 equivalents of DABCO as well as 3 equivalents of GlcNAc azide **41** were added to this solution and the temperature was elevated to 40 ºC. The reaction mixture was stirred for 18 hours. Pure **56b** was obtained in 55% yield (14 mg, 0.031 mmol) after column chromatography with ethylacetate-methanol (40:1).

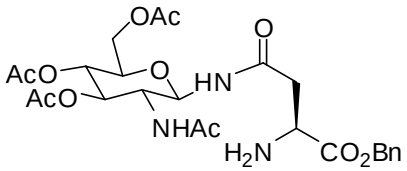
Route 3 (by phosphinophenol activation): DIC (0.975 mmol) and catalytic amount of DMAP were added to benzoic acid (40 mg, 0.325 mmol) dissolved in dry DCM and the reaction mixture was stirred for 10 minutes. Then (0.36 mmol) of *O*-diphenylphosphino-phenol **54** was added to the activated benzoic acid and the reaction mixture was stirred for further 4 h. The solvent was removed under high vacuum. The resulting active ester **81** was dissolved without intermediate purification in 3 mL dry DMF and 3 equivalents of glycosyl azide **41** were added. The reaction mixture was stirred for 18 hour at 40 ºC. The solvent was removed under reduced pressure and the residue was purified by flash chromatography as above yielding 56b in 52% overall yield.

^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.84 (d, 1H), 7.81 (d, 2H), 7.41-7.52 (m, 3H), 6.14 (d, 1H), 5.28 (dd, 1H, J = 9.6, 8.4 Hz, H-1) 5.20 (t, 1H), 5.13 (t, 1H), 4.24-4.40 (m, 2H), 4.12 (d, 1H) 3.85 (d, 1H), 2.1, 2.08, 2.06, 1.92 (4s, 12H, OAc)

HRMS (ESI-TOF): m/z = 451.1727 $[M+H]^+$ (calcd.: m/z = 451.1711), 473.1532 $[M+Na]^+$ (calcd.: m/z = 375.1531).

Further analytical analysis is in accordance with reported results.[142]

Synthesis of glycosylasparagine-benzyl ester (61)



61

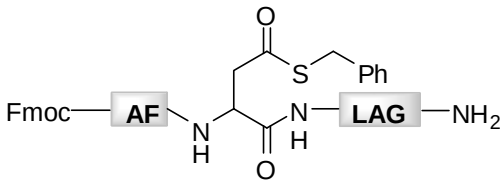
Phosphinothioester **58** (0.033 mmol) was treated with 1mL 99% TFA for 1 hour and the volatiles were removed under reduced pressure. The residual phosphonium salt **59** was dissolved without further purification in 1 mL dry DMF and the solution was degassed by 3 cycles of vacuum and argon. 12 equivalents of DIPEA as well as 3 equivalents of glycosyl azide **41** were added to this solution and the resulting reaction mixture was stirred for 18 hours at 40 ºC. Glycosylated aspartic **61** was purified in 45% yield (8.2 mg, 0.0148 mmol) by HPLC (C18-column, constant flow: 3 min at 7 % CH_3CN (with 0.1 % TFA), gradient: 7 % to 95 % CH_3CN (with 0.1 % TFA) over 35 min).

HRMS (ESI-TOF): m/z = 574.2055 $[M+Na]^+$ (calcd.: m/z = 574.2010), 1125.4086 $[2M+Na]^+$ (calcd.: m/z = 1125.412).

Further analytical analysis is in accordance with reported results.[144,145]

[144] H. Kunz, H. Kauth, *Liebigs Ann. Chem.* **1983**, 337-359.
[145] H. Kunz, H. Kauth, *Angew. Chem.* **1981**, *93*, 918-919.

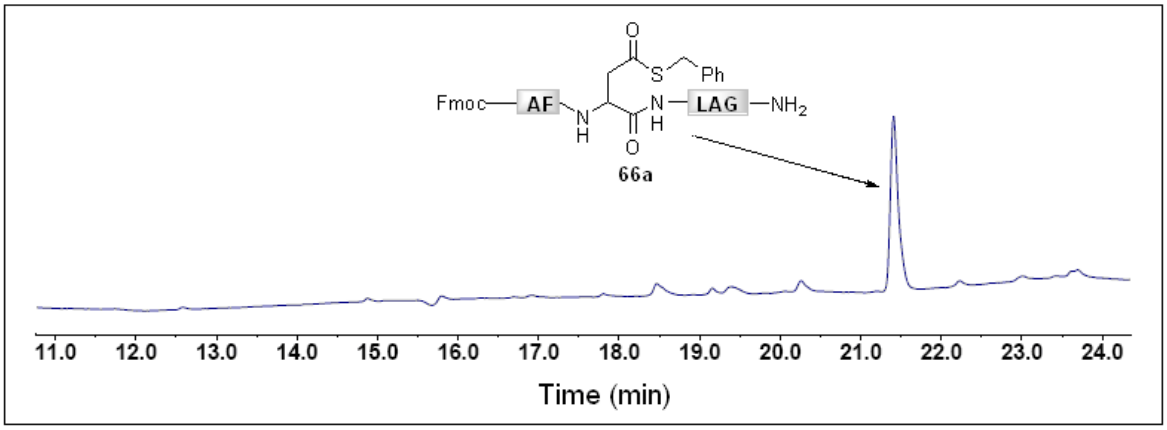
Synthesis of side-chain activated thioester peptide (66a)



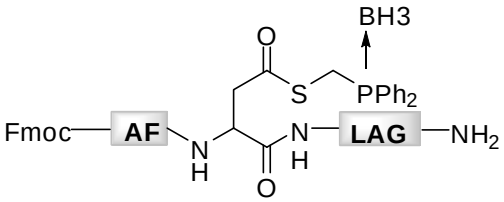
66a

Thioester peptide **66a** was obtained according to GP I using phenylmethanethiol **65** as a reagent for the synthesis of thioester peptide.

Analytical HPLC & HRMS (ESI-TOF): m/z = 920.4039 $[M+H]^+$ (calcd.: m/z = 920.4011)
Peptide **66a** eluted at 21.46 min (constant flow: 5 min at 5 % CH_3CN (with 1.0 % AcOH), gradient: 5 % to 100 % CH_3CN (with 1.0 % AcOH) over 26 min).



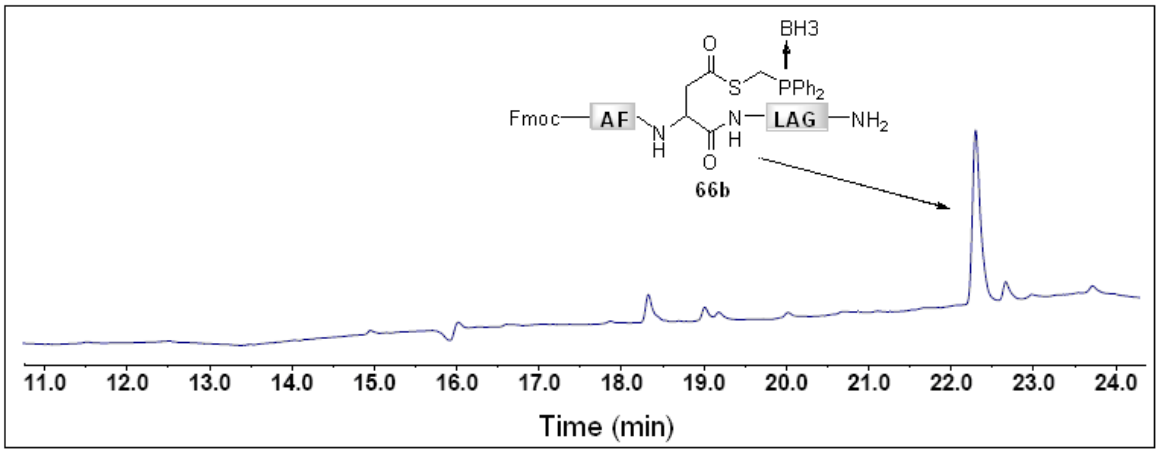
Synthesis of side-chain activated phosphinothioester peptide (66b)



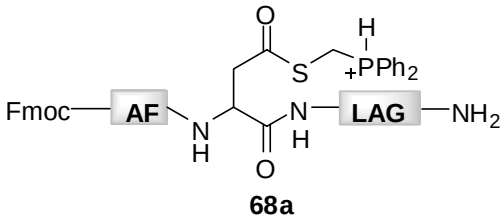
66b

Thioester peptide **66a** was obtained according to GP I using borane-diphenylphosphinothiol complex **52** as a reagent for the synthesis of thioester peptide.

Analytical HPLC & HRMS (ESI-TOF): m/z = 1042.449 $[M+H]^+$ (calcd.: m/z = 1042.447)
Peptide **66b** eluted at 22.30 min (constant flow: 5 min at 5 % CH_3CN (with 1.0 % AcOH), gradient: 5 % to 100 % CH_3CN (with 1.0 % AcOH) over 26 min).



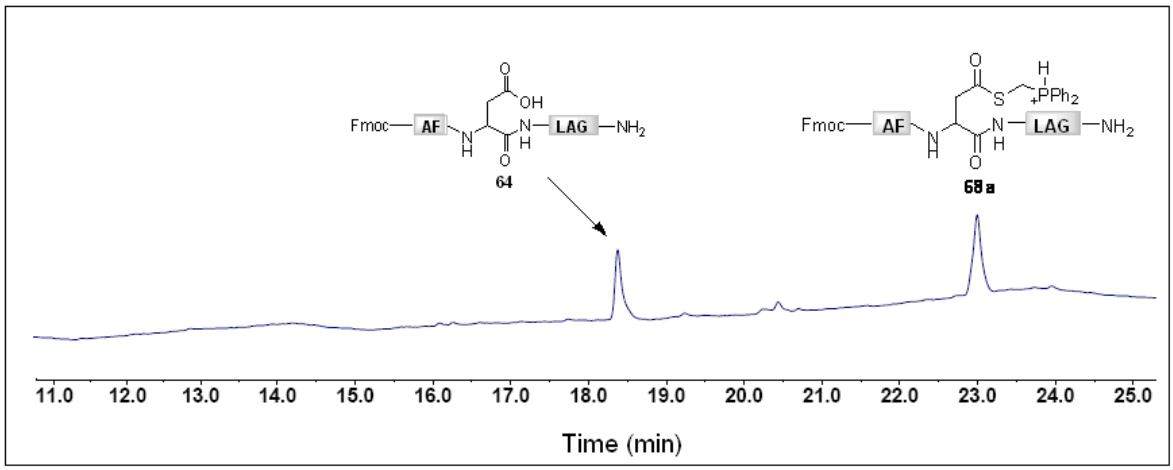
Synthesis of side-chain activated phosphonium salt (68a)



68a

66b was treated with 1 mL 99% TFA for 1 h and the volatiles were removed under high vacuum. The residual phosphonium salt was used in the next synthetic steps without further purification due to its propensity to oxidation during preparative HPLC.
HPLC and HRMS analysis revealed a conversion to **68a** of 65%.

Analytical HPLC & HRMS (ESI-TOF): m/z = 1028.4148 $[M+H]^+$ (calcd.: m/z = 1028.413)
Peptide **68a** eluted at 23.00 min (constant flow: 5 min at 5 % CH_3CN (with 1.0 % AcOH), gradient: 5 % to 100 % CH_3CN (with 1.0 % AcOH) over 26 min).



Synthesis of acetylated peptide (76) by TSL

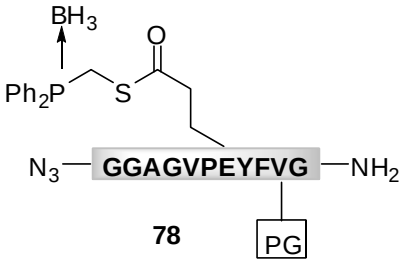
29 mg (0.1 mmol) phosphinothioester **51a** was dissolved was treated with 1 mL 99% TFA for 1 h and the volatiles were removed under high vacuum. The residue was dissolved 1 mL dry DMF and the solution was degassed by three cycles of vacuum and argon. Azido peptide 75 (0.01 mmol) first and then DIPEA (0.12 mmol) were added to the residual reaction mixture and the reaction was monitored by LC-MS.

HRMS analysis revealed that azido peptide **75** was fully converted to peptide **76** after 40 hours at 40 ^{o}C.

Analytical HPLC & HRMS (ESI-TOF): m/z = 767.3723 $[M+H]^+$ (calcd.: m/z = 767.3733)

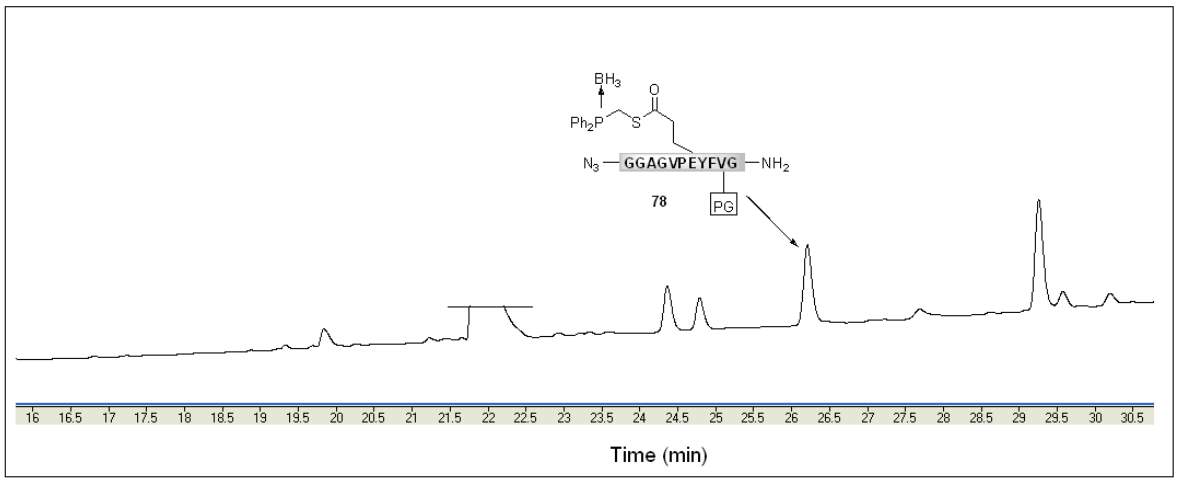
Peptide **76** eluted at 15.84 min (constant flow: 5 min at 5 % CH_3CN (with 1.0 % AcOH), gradient: 5 % to 100 % CH_3CN (with 1.0 % AcOH) over 26 min).

Synthesis of bifunctional azido-phosphinothioester peptide (78)

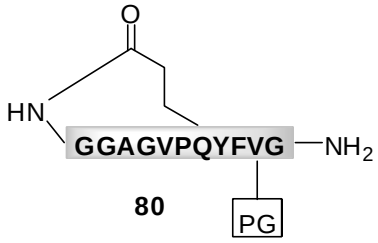


Precursor peptides was synthesized on an acid-sensitive Sieber Amide resin with an orthogonally protected Asp(O-2-PhiPr) at glutamic acid residue. It was cleaved from the resin with 0.5 % TFA and 2.5 % TIS in CH_2Cl_2 for 15 min. The resin was filtered off and washed with CH_2Cl_2. The filtrate and the washing solution were combined and the solvent was removed under high vacuum. 3 equiv DIC and catalytic amount of DMAP were added to the partially protected peptide dissolved in dry CH_2Cl_2 (3 mL/0.033 mmol) and the reaction mixture was stirred for 10 minutes. Then 4 equivalents of borane-diphenyl-phosphinomethanethiol **52** were added to the activated peptide and the reaction mixture was stirred for 12 h. The solvent was removed under high vacuum and the residue was used in other synthetic steps without further purification.

Analytical HPLC & HRMS (ESI-TOF): m/z = 1361.644 $[M+H]^+$ (calcd.: m/z = 1361.643)
Peptide **78** eluted at 26.22 min (constant flow: 5 min at 5 % CH_3CN (with 1.0 % AcOH), gradient: 5 % to 100 % CH_3CN (with 1.0 % AcOH) over 35 min).



Synthesis of cyclized peptide (80) by the base-induced traceless Staudinger ligation



80

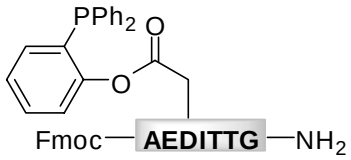
Azido-phosphinothioester peptide **78** (0.033 mmol) was dissolved in 2.5 mL dry DMF and the solution was degassed by 3 cycles of vacuum and argon. 3 equivalents of DABCO were added to this solution and the temperature was elevated to 40 ºC. The reaction mixture was then stirred for 48 hours.

HRMS analysis revealed that cyclized peptide **80** was formed in a very low conversion (< 7%, not isolated).

Analytical HPLC & HRMS (ESI-TOF): m/z = 1089.574 $[M+H]^+$ (calcd.: m/z = 1089.573)

Peptide **80** eluted at 19.10 min (constant flow: 5 min at 5 % CH_3CN (with 1.0 % AcOH), gradient: 5 % to 100 % CH_3CN (with 1.0 % AcOH) over 30 min).

Synthesis of side-chain activated phosphinophenolester peptide (85)



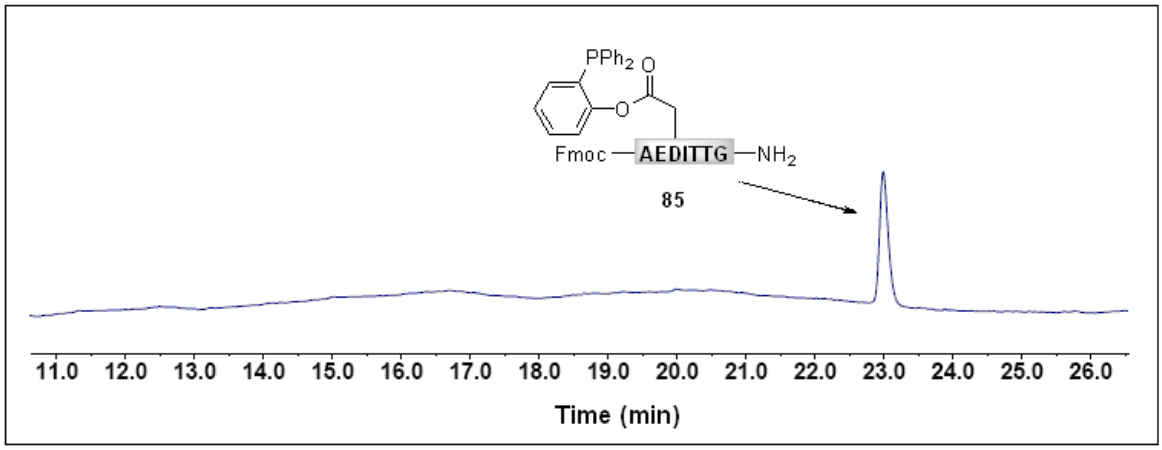
85

Phosphinophenolester peptide **85** was obtained according to GP II using diphenyl-phosphinophenol **54** as a reagent in the synthesis.

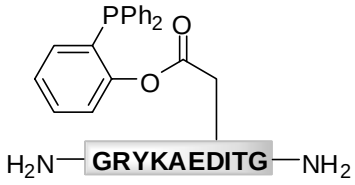
Phosphinophenolester peptide **85** was isolated by semi-preparative HPLC in 60% yield (7.1 mg, 0.006 mmol, C_{18}-column, constant flow: 5 min at 5 % CH_3CN (with 0.1 % TFA), gradient: 5 % to 100 % CH_3CN (with 0.1 % TFA) over 47 min).

Analytical HPLC & HRMS (ESI-TOF): m/z = 1187.483 $[M+H]^+$ (calcd.: m/z = 1187.485)

Peptide **85** eluted at 22.87 min (constant flow: 5 min at 5 % CH_3CN (with 1.0 % AcOH), gradient: 5 % to 100 % CH_3CN (with 1.0 % AcOH) over 30 min).



Synthesis of side-chain activated phosphinophenolester peptide (92)

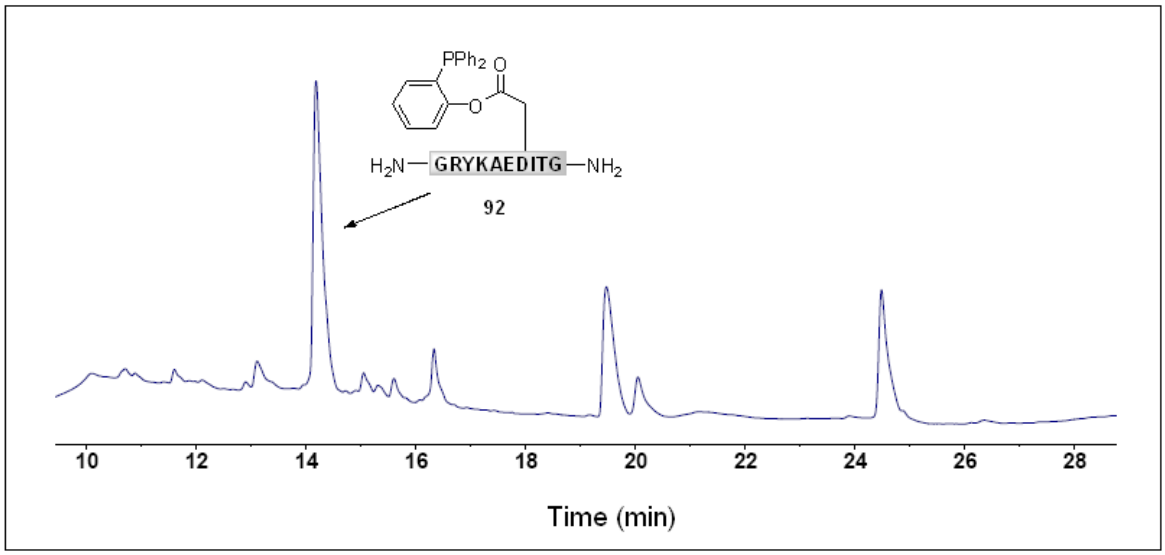


92

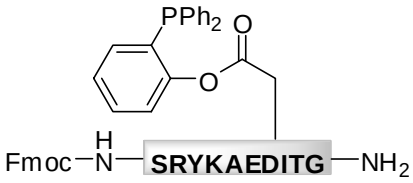
Phosphinophenolester peptide **92** was obtained according to GP II using diphenyl-phosphinophenol **54** as a reagent in the synthesis.

Analytical HPLC & HRMS (ESI-TOF): m/z = 684.8286 $[M+2H]^{++}$ (calcd.: m/z = 684.8287), 1368.649 $[M+H]^{+}$ (calcd.: m/z = 1368.650)

Peptide **92** eluted at 14.15 min (constant flow: 5 min at 5 % CH_3CN (with 1.0 % AcOH), gradient: 5 % to 100 % CH_3CN (with 1.0 % AcOH) over 30 min).



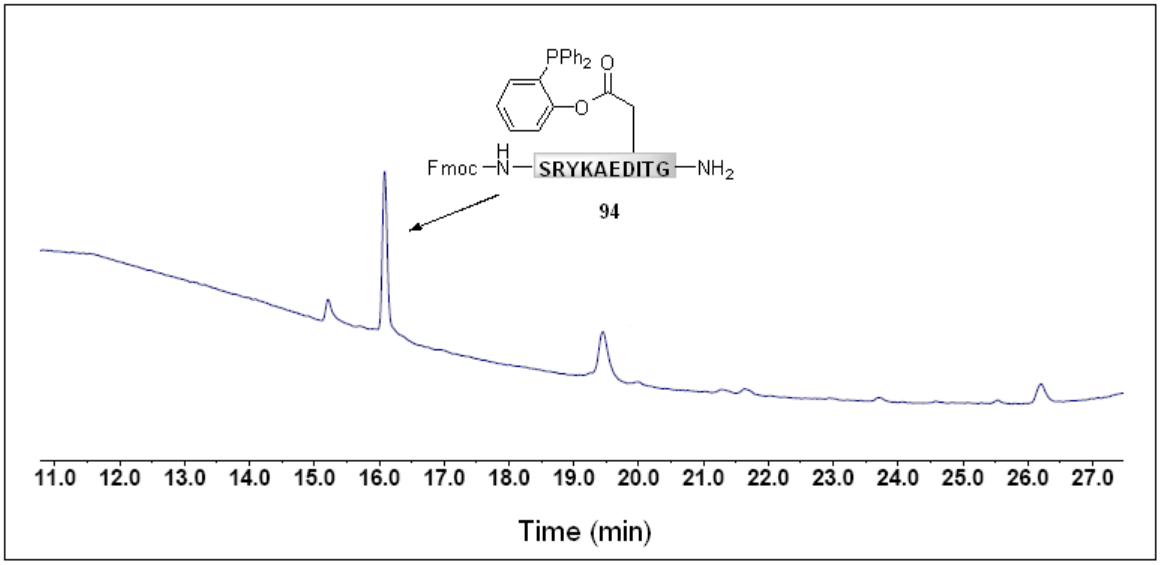
Synthesis of side-chain activated phosphinophenolester peptide (94)



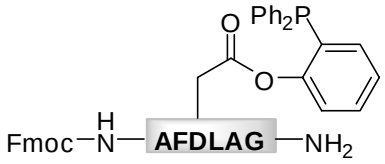
94

Phosphinophenolester peptide **94** was obtained according to GP II using diphenyl-phosphinophenol **54** as a reagent in the synthesis.

Analytical HPLC & HRMS (ESI-TOF): m/z = 810.8695 $[M+2H]^{++}$ (calcd.: m/z = 810.8680), 1620.7287 $[M+H]^{+}$ (calcd.: m/z = 1620.7309).
Peptide **94** eluted at 14.15 min (constant flow: 5 min at 5 % CH_3CN (with 1.0 % AcOH), gradient: 5 % to 100 % CH_3CN (with 1.0 % AcOH) over 30 min).



Synthesis of side-chain activated phosphinophenolester peptide (95)



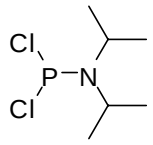
95

Phosphinophenolester peptide **95** was obtained according to GP I using diphenylphosphino-phenol **54** as a reagent in the synthesis.

Analytical HPLC & HRMS (ESI-TOF): m/z = 1074.455 $[M+H]^+$ (calcd.: m/z = 1074.453)
Peptide **95** eluted at 26.24 min (constant flow: 5 min at 5 % CH_3CN (with 1.0 % AcOH), gradient: 5 % to 100 % CH_3CN (with 1.0 % AcOH) over 30 min).

6.5 Synthetic procedures for compounds and peptides involved in SPR

Synthesis of dichloro-*N*,*N*-diisopropylaminophosphine (103)



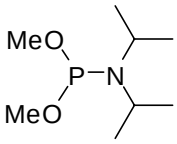
103

Under atmosphere of argon a solution of 21 mL (15.18 g, 150 mmol) diisopropylamine in 60 mL dry THF was added dropwise into a vigorously stirred solution of 6.5 mL (8.93 g, 65 mmol) PCl_3 in 60 mL dry THF at -78 °C. The white suspension was stirred at r.t. for 18 h. The hydrochloride salt was filtered off and washed with 30 mL dry THF, under atmosphere of argon. The filtrate was concentrated to colorless oil with the rotary evaporator and was then purified by fractional distillation under vacuum (Kp = 79 °C, 4.3 mbar) using a vigreux column with avoiding any contact with air. The desired product was obtained as colourless liquid which solidifies at 4°C in 47% (6.11 g, 30.25mmol).

^{1}H-NMR (400MHz, $CDCl_3$): δ = 3.91 (sept, *J*= 6.38Hz, 2H, NC<u>H</u>$(CH_3)_2$), 1.27ppm (d, *J*= 6.84, 12H, CH(C<u>H</u>$_3)_2$)

^{31}P-NMR (400MHz, $CDCl_3$): δ = 170.09 ppm

Synthesis of *N*,*N*-Diisopropylaminodimethoxyphosphine (35a)



35a

Under atmosphere of argon a solution of 2.45 mL (1.94 g, 60.5 mmol) methanol and 18.44 mL (13.68 g, 105.88 mmol) diisopropylethylamine in 31 mL dry diethyl ether was cooled down to 0°C. Afterwards 5.58 mL (6.11 g, 30.25 mmol) dichloro-*N*,*N*-diisopropyl-aminophosphin **103** was added dropwise to the cold stirring solution. The reaction mixture was stirred at r.t. for 19 h. Then, the hydrochloride salt was filtered off and washed with 30 mL dry diethyl ether, under atmosphere of argon. The filtrate was concentrated to colorless oil under reduced pressure and was then purified by fractional distillation under vacuum

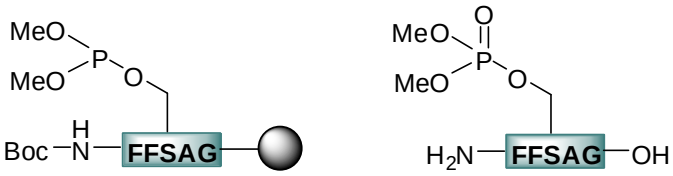
(Kp = 42.5 °C, 4.5 mbar) using a vigreux column with avoiding any contact with air. The desired product was obtained as colorless liquid in 55% (3.21 g, 16.61 mmol). **35a** should be stored at -18 °C under atmosphere of argon.

^{1}H-NMR (400MHz, $CDCl_3$): δ = 3.62-3.52 (m, 2H, NC<u>H</u>$(CH_3)_2$), 3.40 (d, *J*= 13.18Hz, 6H, CH_3O), 1.17 (d, *J*= 6.77Hz, 12H, CH(C<u>H</u>$_3)_2$)

^{31}P-NMR (400MHz, $CDCl_3$): δ = 150.24ppm

<u>I. Global peptide phosphitylation</u>

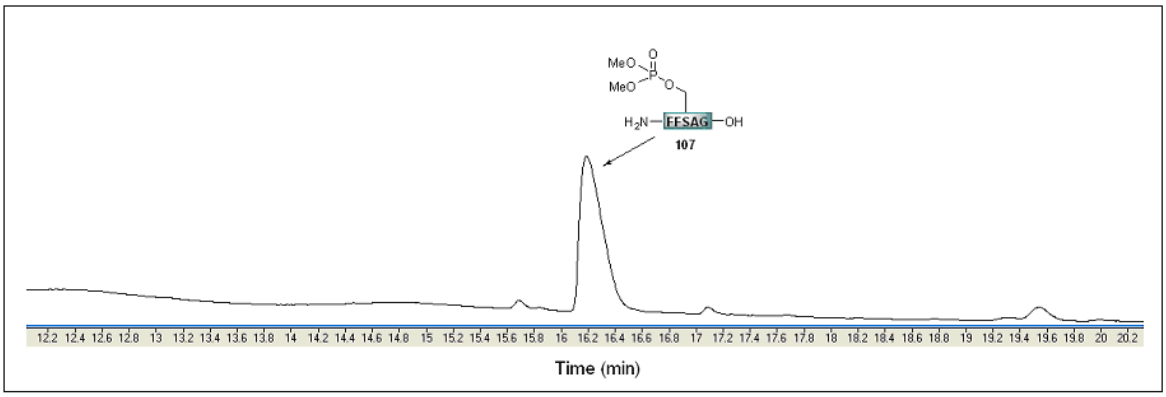
Synthesis of the phosphitylated peptide 106a and determination of the conversion by oxidation to the phosphopeptide triester (107):



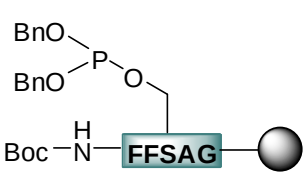
106a **107**

Phosphitylated peptide **106a** was obtained according to GP III using dimethyl-*N*,*N*-diisopropylphosphoramidite **35a** as phosphitylation reagent. Afterwards the conversion was determined by oxidation to the phosphate triester **107** by GP IV. Final HPLC and HRMS analysis revealed a conversion to phosphopeptide **107** of 96%.

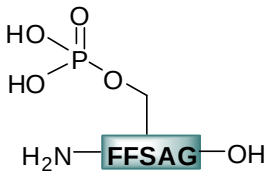
Analytical HPLC & HRMS (ESI-TOF): m/z = 636.2496 $[M+H]^+$ (calcd.: m/z = 636.2429)
Peptide **107** eluted at 16.2 min (constant flow: 5 min at 5 % CH_3CN (with 1.0 % AcOH), gradient: 5 % to 100 % CH_3CN (with 1.0 % AcOH) over 30 min).



Synthesis of the phosphitylated peptide 106b and determination of the conversion by oxidation to the phosphopeptide monoester (108):



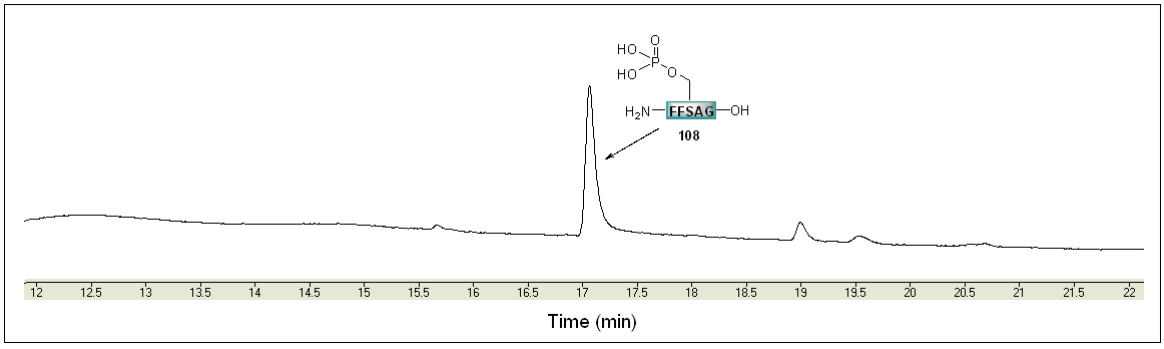
106b



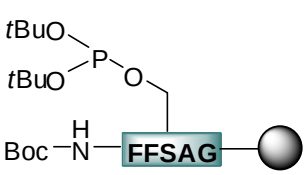
108

Phosphitylated peptide **106b** was obtained according to GP III using dibenzyl-*N*,*N*-diisopropylphosphoramidite as phosphitylation reagent. Afterwards the conversion was determined by oxidation to the phosphate monoester **108** by GP IV. Final HPLC and HRMS analysis revealed a conversion to phosphopeptide **108** of 89%.

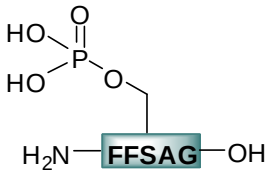
Analytical HPLC & HRMS (ESI-TOF): m/z = 608.2118 $[M+H]^+$ (calcd.: m/z = 608.2116)
Peptide **108** eluted at 17.1 min (constant flow: 5 min at 5 % CH_3CN (with 1.0 % AcOH), gradient: 5 % to 100 % CH_3CN (with 1.0 % AcOH) over 30 min).



Synthesis of the phosphitylated peptide 106c and determination of the conversion by oxidation to the phosphopeptide monoester (108):

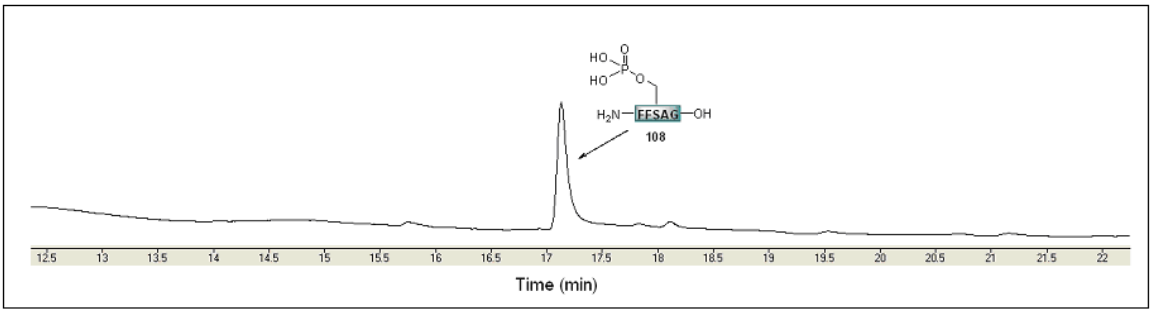


106c



108

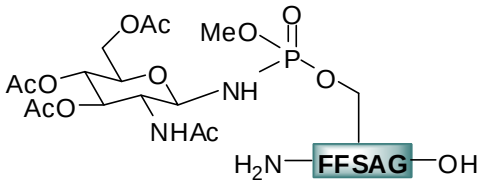
Phosphitylated peptide **106c** was obtained according to GP III using di-*t*-butyl-*N*,*N*-diisopropylphosphoramidite as phosphitylation reagent. Afterwards the conversion was determined by oxidation to the phosphate monoester **108** by GP IV. Final HPLC and HRMS analysis revealed a conversion to phosphopeptide **108** of 94%.



Analytical HPLC & HRMS (ESI-TOF): m/z = 608.2120 $[M+H]^+$ (calcd.: m/z = 608.2116)
Peptide **108** eluted at 17.1 min (constant flow: 5 min at 5 % CH_3CN (with 1.0 % AcOH), gradient: 5 % to 100 % CH_3CN (with 1.0 % AcOH) over 30 min).

II. Staudinger-phosphite reaction

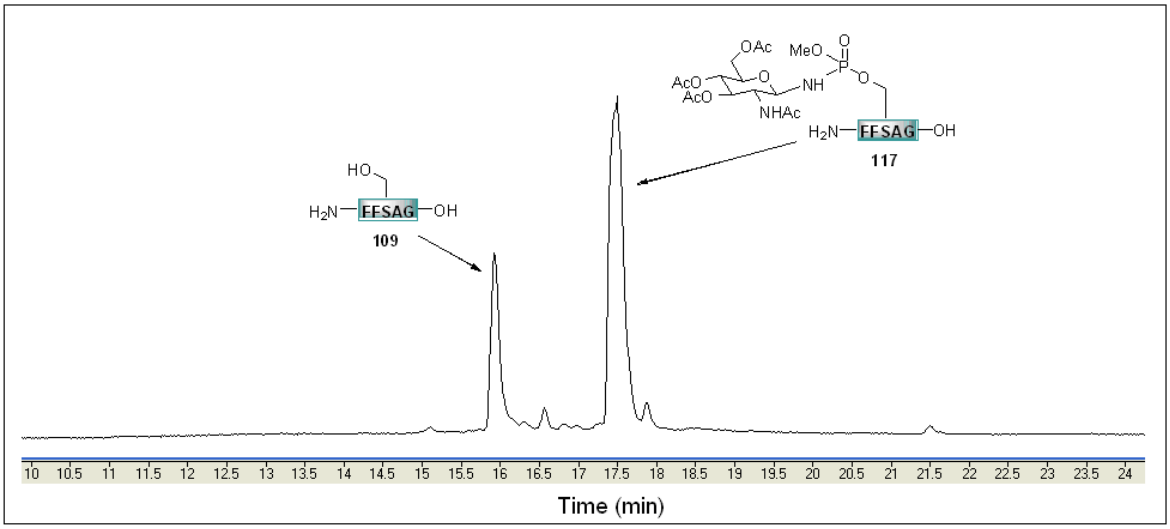
Staudinger-phosphite reaction of 106a to 117.



117

Glycopeptide **117** was obtained according to GP V in which β-GlcNAc—N_3 **41** (0.07 mmol) was dissolved in 1 mL dry CH_2Cl_2 at room temperature. HPLC and HRMS analysis revealed a conversion to glycopeptide **117** of 69%.

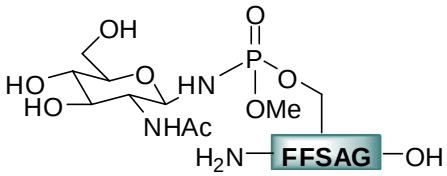
Analytical HPLC & HRMS (ESI-TOF): m/z = 950.3621 $[M+H]^+$ (calcd.: m/z = 950.3543) Glycopeptide **117** eluted at 17.5 min (constant flow: 5 min at 5 % CH_3CN (with 1.0 % AcOH), gradient: 5 % to 100 % CH_3CN (with 1.0 % AcOH) over 30 min).



Basic stability of 117

0.5 mg glycopeptide **117** was treated with 3 mL 2.5 mM Na_2CO_3 in methanol (pH = 9, wet paper) for 21 hours and was monitored by LC-MS. After 90 minutes 16% of **117** underwent β-elimination to the dehydroalanine, which increased to 76% after 13 h and 87% after 21 h. At pH values higher than 10 the corresponding de-*O*-acetylated glycopeptide of **117** formed in addition to the dehydroalanine.

Staudinger-phosphite reaction of 106a to 137.

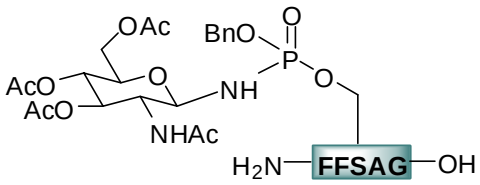


137

Glycopeptide **137** was obtained according to GP V in which β-GlcNAc—N_3 **42** (0.07 mmol) was dissolved in 1 mL wet DMSO at 35 °C. HPLC and HRMS analysis revealed a conversion to glycopeptide **137** of less than 33%.

Analytical HPLC & HRMS (ESI-TOF): m/z = 824.335 $[M+H]^+$ (calcd.: m/z = 824.323)
Glycopeptide **137** eluted at 16.88 min (constant flow: 5 min at 5 % CH_3CN (with 1.0 % AcOH), gradient: 5 % to 100 % CH_3CN (with 1.0 % AcOH) over 30 min).

Staudinger-phosphite reaction of 106b to 118a.

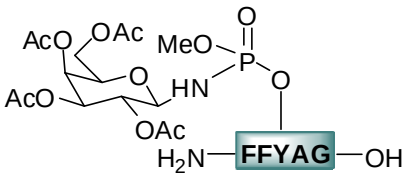


118a

Glycopeptide **118a** was obtained according to GP V in which β-GlcNAc—N_3 **41** (0.07 mmol) was dissolved in 1 mL dry CH_2Cl_2 at room temperature. HPLC and HRMS analysis revealed the monoester **108** as the major reaction product, whereas the conversion to glycopeptide **118a** was less than 25%.[146]

Analytical HPLC & HRMS (ESI-TOF): m/z = 1026.3892 $[M+H]^+$ (calcd.: m/z = 1026.3856)
Glycopeptide **118a** eluted at 19.1 min (constant flow: 5 min at 5 % CH_3CN (with 1.0 % AcOH), gradient: 5 % to 100 % CH_3CN (with 1.0 % AcOH) over 30 min).

Staudinger-phosphite reaction of 141 to 146.

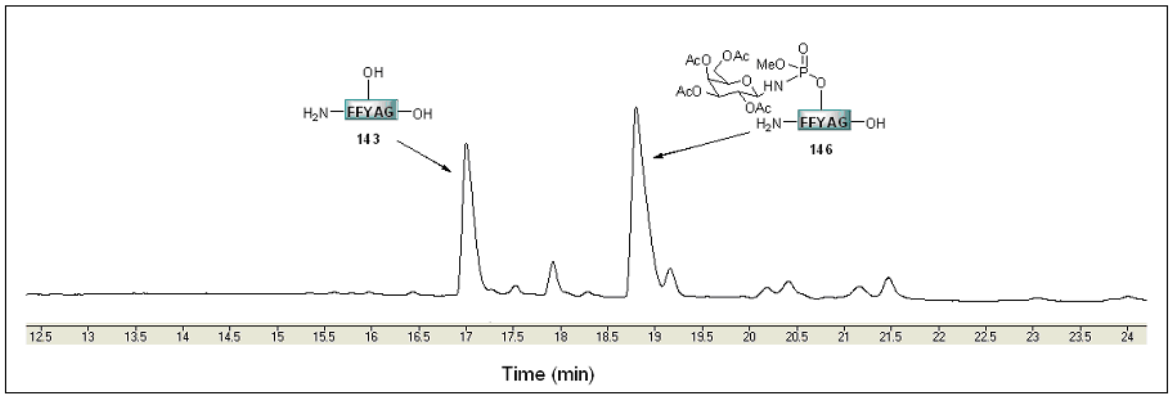


146

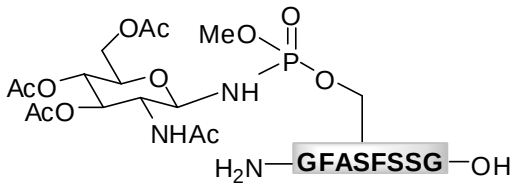
Glycopeptide **146** was obtained according to GP V in which β-Gal—N_3 **100** (0.07 mmol) was dissolved in 1 mL wet DMSO at 35 °C. HPLC and HRMS analysis revealed a conversion to glycopeptide **146** of 58%.

[146] Glycopeptide **118a** contains an additional chromophore in comparison to the byproduct **108**. Since the monoester **108** was the major reaction product and the conversion to glycopeptide **118a** was significantly lower in comparison to glycopeptide **117**, further analysis and optimization were only performed for dimethyl-phosphite containing peptides.

Analytical HPLC & HRMS (ESI-TOF): m/z = 1025.353 $[M-H]^-$ (calcd.: m/z = 1025.353)
Glycopeptide **146** eluted at 18.92 min (constant flow: 5 min at 5 % CH_3CN (with 1.0 % AcOH), gradient: 5 % to 100 % CH_3CN (with 1.0 % AcOH) over 30 min).



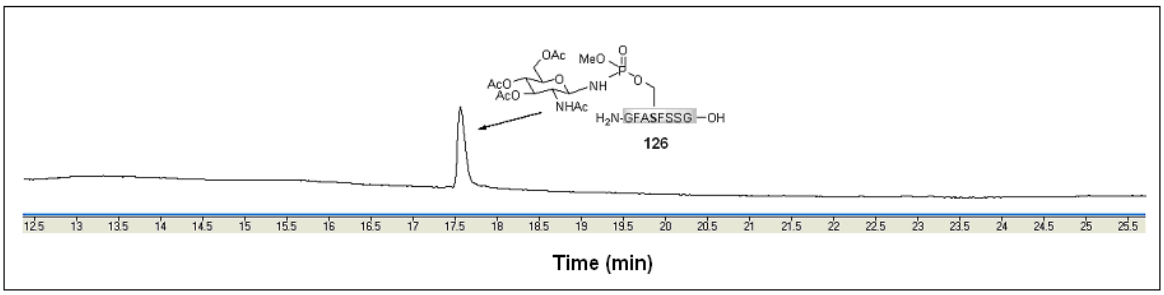
Synthesis of glycopeptide (126) by the Staudinger-phosphite reaction of 121 with 41:



126

Glycopeptide **126** was obtained according to GP V in which β-GlcNAc—N_3 **41** (0.07 mmol) was dissolved in 1 mL dry CH_2Cl_2 at 40 °C and added to the immobilized peptide **121**. Glycopeptide **126** was isolated by semi-preparative HPLC in 49% yield (5.8 mg, 0.0049 mmol, C_{18}-column, constant flow: 5 min at 5 % CH_3CN (with 0.1 % TFA), gradient: 5 % to 100 % CH_3CN (with 0.1 % TFA) over 47 min), in which **126** eluted at 21.0 min.

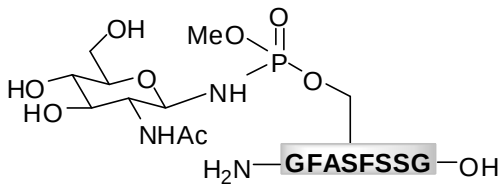
Analytical HPLC & HRMS (ESI-TOF): m/z = 1181.4438 $[M+H]^+$ (calcd.: m/z = 1181.4398)
Pure peptide **126** eluted at 17.55 min (constant flow: 5 min at 5 % CH_3CN (with 1.0 % AcOH), gradient: 5 % to 100 % CH_3CN (with 1.0 % AcOH) over 30 min).



Stability of 126 under aqueous physiological conditions

1 mg glycopeptide **126** was dissolved in 0.5 mL 0.2 M phosphate buffered saline (pH = 7.4). After 24 hours at 37 °C the solution was analyzed by LC-MS, in which no signs of decomposition were observed.

Synthesis of glycopeptide (128) by two routes



128

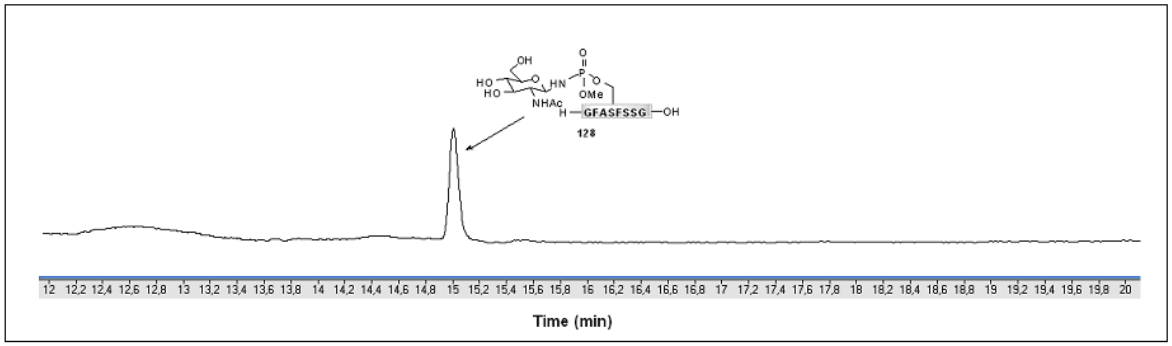
Route 1 (by de-O-acetylation of glycopeptide 126 with hydrazine): 0.5 mL hydrazine hydrate solution (64% in H_2O) was added to **126** (5.0 mg, 0.00423 mmol) dissolved in 2.5 mL methanol. The solution was stirred at 20 °C for 1 hour, then cooled down to 0 °C and stirred for another 5 minutes. 2.5 mL acetone was added before the solution was concentrated under reduced pressure. The residue was dissolved in 0.7 mL TFA/TIS/water (95:2.5:2.5 v/v), precipitated from cold ethyl ether and further purified by semi-preparative HPLC to deliver the unprotected glycopeptide **128** in 67% yield (3 mg, 0.0028 mmol, C_{18}-column, constant flow: 5 min at 5 % CH_3CN (with 0.1 % TFA), gradient: 5 % to 100 % CH_3CN (with 0.1 % TFA) over 47 min), which eluted at 18.6 min. In addition to the glycopeptide the dehydroalanine Michael-adduct of hydrazine was observed.

Route 2 (by the Staudinger-phosphite reaction of 121 with 42):

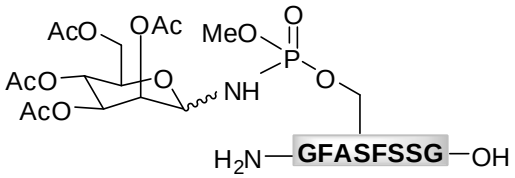
Glycopeptide **128** was obtained according to GP V in which β-GlcNAc—N_3 **42** (0.07 mmol) was dissolved in 1 mL wet DMSO at 35 °C. HPLC and HRMS analysis revealed a conversion to glycopeptide **128** of 34% (not isolated).

Analytical HPLC & HRMS (ESI-TOF): m/z = 1055.4069 $[M+H]^+$ (calcd.: m/z = 1055.4081)

Pure **128** eluted at 15.0 min (constant flow: 5 min at 5 % CH_3CN (with 1.0 % AcOH), gradient: 5 % to 100 % CH_3CN (with 1.0 % AcOH) over 30 min).



Synthesis of glycopeptide (127) by the Staudinger-phosphite reaction of 121 with 99

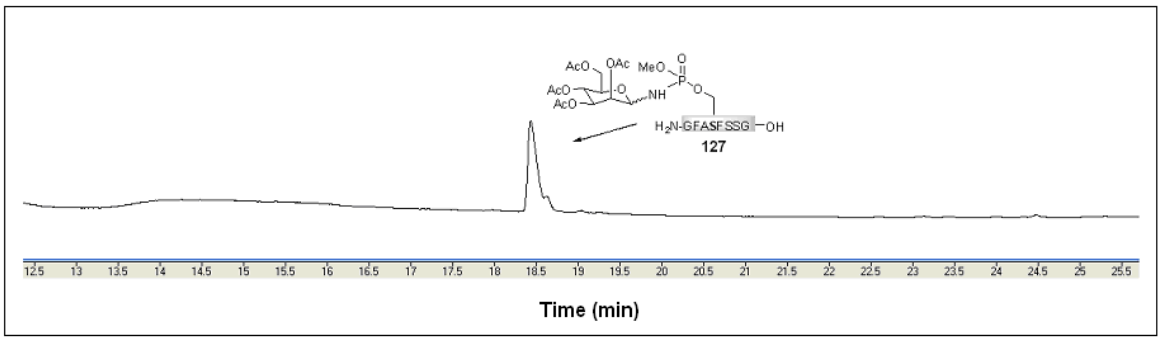


127

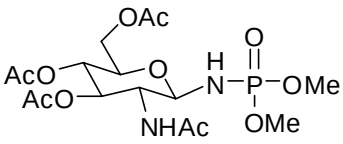
Glycopeptide **127** was obtained according to GP V in which α-Man—N_3 **99** (0.07 mmol) was dissolved in 1 mL dry DMSO at 40 °C and added to the immobilized peptide **121**. Glycopeptide **127** was isolated by semi-preparative HPLC in 64% yield (7.6 mg, 0.0064 mmol, C_{18}-column, constant flow: 5 min at 5 % CH_3CN (with 0.1 % TFA), gradient: 5 % to 100 % CH_3CN (with 0.1 % TFA) over 47 min), in which **127** eluted at 22.3 min.

Analytical HPLC & HRMS (ESI-TOF): m/z = 1182.4128 $[M+H]^+$ (calcd.: m/z = 1182.423)

Pure peptide **127** eluted at 18.44 min (constant flow: 5 min at 5 % CH_3CN (with 1.0 % AcOH), gradient: 5 % to 100 % CH_3CN (with 1.0 % AcOH) over 30 min).



Synthesis of β-5-acetamido-2-(acetoxymethyl)-6-(dimethoxyphosphorylamino)-tetrahydro-2*H*-pyran-3,4-diyl diacetate (β-GlcNAc-phosphoramidate) (132):



132

Trimethyl phosphite (16.7 mg, 16 µL, 0.135 mmol) was added to a solution of β-GlcNAc—N_3 **41** (10 mg, 0.027 mmol) dissolved in 1 mL CH_2Cl_2. The reaction mixture was stirred for 6 hours at room temperature and the solvent was then removed under reduced pressure followed by recrystallization of the resultant syrup from a mixture of hexane and ethyl acetate (1:4) providing a white solid in 88% yield (10.8 mg, 0.024 mmol).

^{1}H-NMR (400 MHz, CD_3CN): δ = 6.81 (d, *J*= 8.97 Hz, 1H), 5.14 (t, *J*= 10.07 Hz, 1H), 4.89 (t, *J*= 9.43 Hz, 1H), 4.54-4.63 (m, 1H, H-1), 4.50 (t, *J*= 10.89 Hz, 1H), 4.15 (dd, *J*= 12.26, 5.67 Hz, 1H), 4.02 (dd, *J*= 12.26, 2.38 Hz, 1H), 3.85 (q, 1H, *J*= 9.94 Hz), 3.75 (o, *J*= 2.14 Hz, 1H), 3.61 (d, *J*= 4.03 Hz, 3H, OCH_3), 3.58 (d, *J*= 3.85Hz, 3H, OCH_3), 1.97, 1.95, 1.93 and 1.82 (4s, 12H, 3 OAc and 1 NAc)

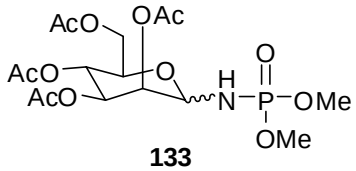
^{13}C NMR (100 MHz, CD_3CN) δ = 171.85, 171.25, 171.19, 170.59, 84.12, 73.67, 73.64, 73.56, 69.79, 63.19, 53.72 (1C, OCH_3), 53.56 (1C, OCH_3), 23.00 (1C, CH_3), 20.87 (1C, CH_3), 20.83 (2C, CH_3).

^{31}P-NMR (121 MHz, CD_3CN): δ = 9.29ppm

HRMS (ESI-TOF): m/z = 455.1422 $[M+H]^+$ (calcd.: m/z = 455.1425), 477.1230 $[M+Na]^+$ (calcd.: m/z = 477.1245).

Further analytical analysis is in accordance with reported results.[125]

Synthesis of 2-(acetoxymethyl)-6-(dimethoxyphosphorylamino)tetrahydro-2*H*-pyran-3,4,5-triyl triacetate (α- or β-Man-phosphoramidate) (133):



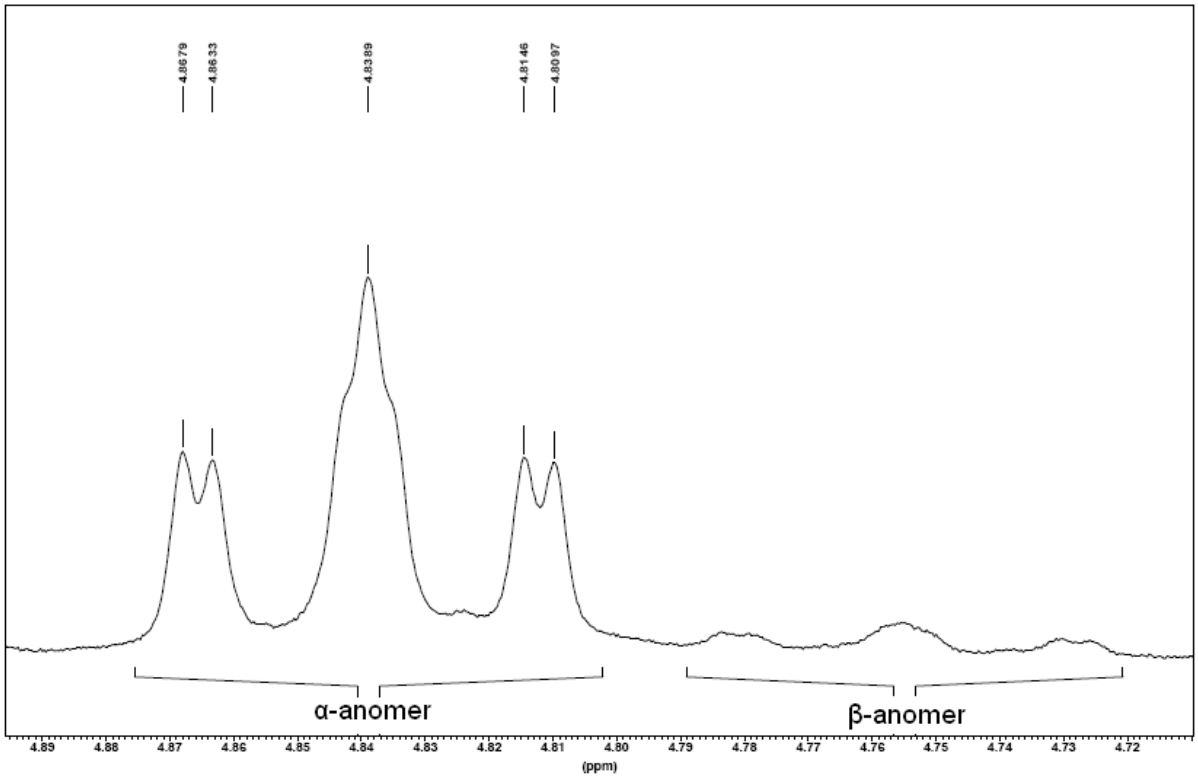
133

Trimethyl phosphite (89 mg, 86 µL, 0.72 mmol) was added to a solution of α-Man—N_3 **99** (55 mg, 0.147 mmol) dissolved in 0.5 mL *d*-DMSO. The reaction mixture was stirred for 15 hours at 40 °C. NMR-analysis revealed a full conversion to **133** with the ratio of 10:1 (α : β).

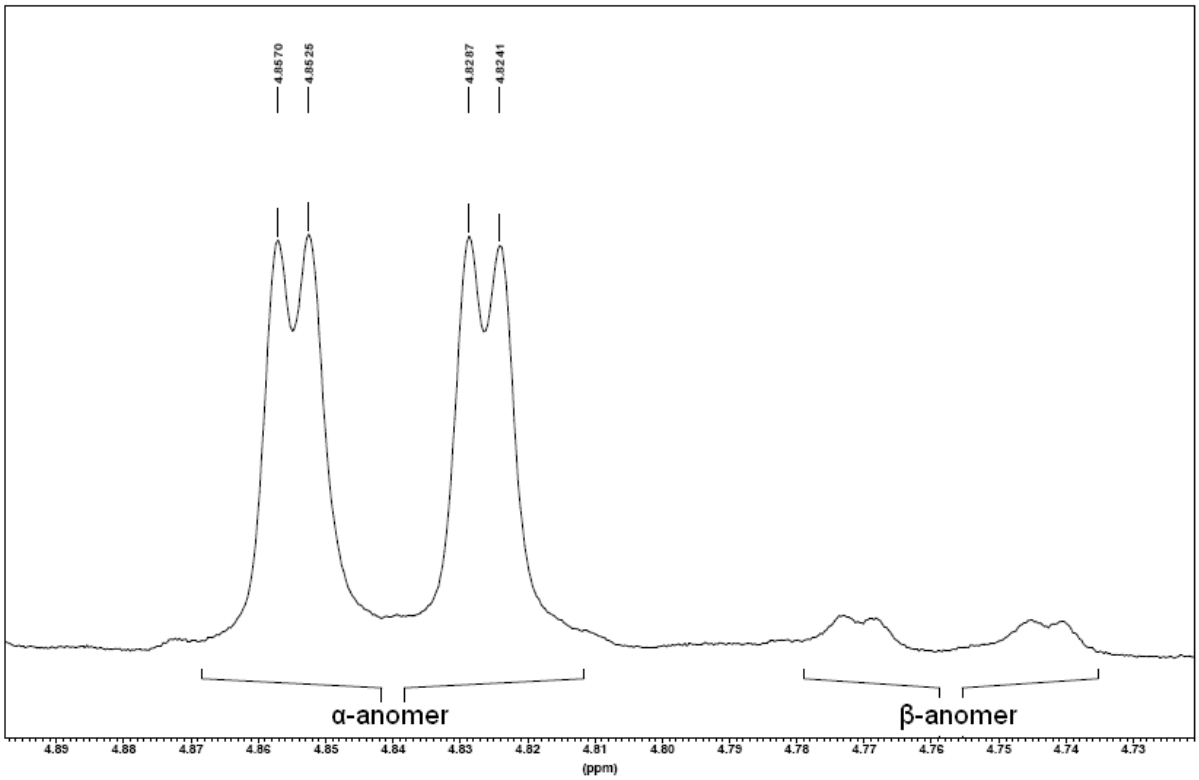
The α-anomeric linkage was confirmed by the appearance of a distorted ddd at 4.84 ppm which on $^1H\{^{31}P\}$ collapsed to dd with $^3J_{H1H2}$ 1.89 Hz and $^3J_{H1NH}$ 11.24 Hz. On D_2O exchange the dd collapsed to d with $^3J_{H1H2}$ 1.89 Hz, revealing trans diequatorial of H1 and H2 with dihedral angle of 60°. [147]

[147] J. Clayden, N. Greeves, S. Warren, P. Wothers, *Organic Chemistry*, Oxford University press, New York, **2001**, pp. 823-848.

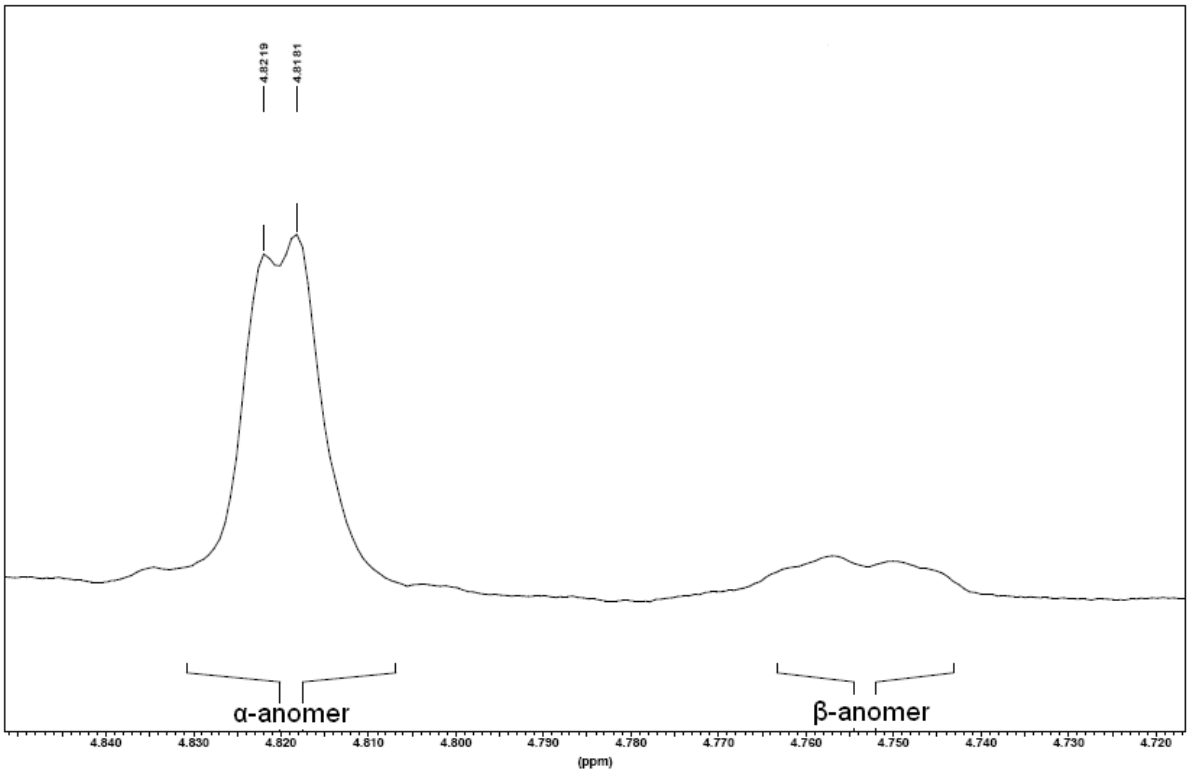
^{1}H-NMR of anomeric protons before ^{1}H{^{31}P} and D_2O exchange.



^{1}H{^{31}P}-NMR of anomeric protons before D_2O exchange.



$^{1}H\{^{31}P\}$-NMR of anomeric protons after D_2O exchange.



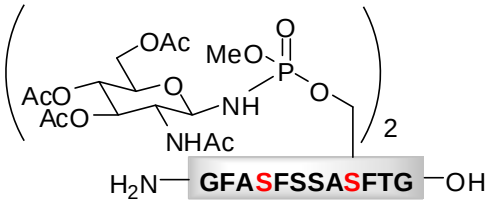
^{1}H-NMR (400 MHz, d_6-DMSO): δ = 6.97 (dd, *J*= 11.24, 2.09 Hz, 1H, NH), 5.60 (dd, *J*= 9.74, 3.59 Hz, 1H), 5.10 (distorted dd, 1H), 5.05 (t, *J*= 9.62 Hz, 1H), 4.84 (distorted ddd, α-anomer H-1), 4.72-4.78 (m, 1H, β-anomer H-1), 4.02-4.18 (m, 3H), 3.65 (d, *J*= 4.87 Hz, 3H, OCH_3), 3.62 (d, *J*= 4.86 Hz, 3H, OCH_3), 2.14, 2.07, 2.02, 1.98 (4s, 12H, 4 OAc).

^{13}C NMR (100 MHz, d_6-DMSO): δ = 170.98, 170.74, 170.59, 170.55, 80.94, 71.58, 71.46, 69.05, 68.96, 67.08, 63.32, 53.59 (1C, OCH_3), 53.44 (1C, OCH_3), 21.60 (1C, CH_3), 21.47 (1C, CH_3), 21.42 (1C, CH_3), 21.38 (1C, CH_3).

$^{31}P\{^{1}H\}$-NMR (121 MHz, d_6-DMSO): δ = 9.86, 9.89.

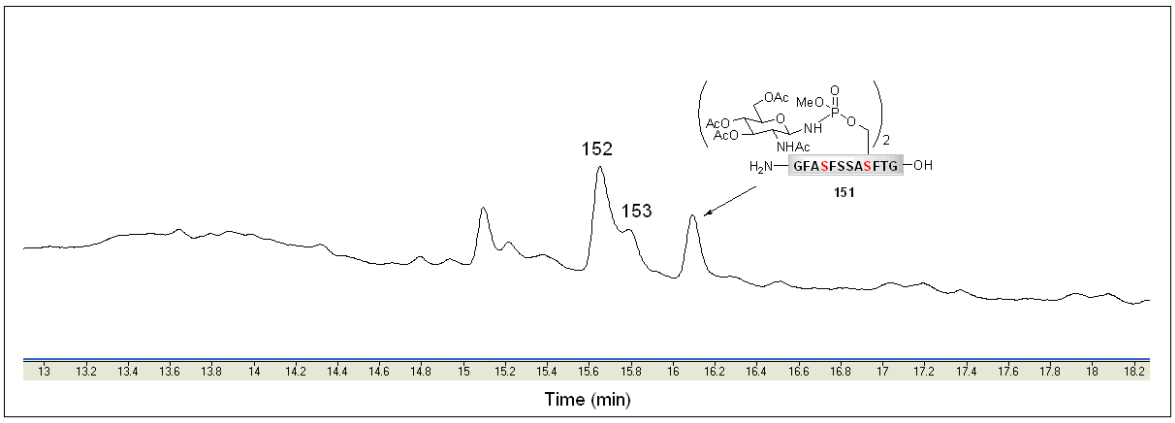
HRMS (ESI-TOF): m/z = 456.1280 $[M+H]^+$ (calcd.: m/z = 456.1265), 478.1098 $[M+Na]^+$ (calcd.: m/z = 478.1085).

Synthesis of divalent glycopeptide (151)



151

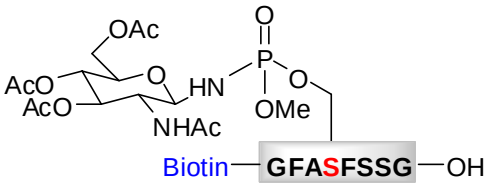
Divalent glycopeptide **151** was obtained according to GP V in which β-GlcNAc—N_3 **41** (0.14 mmol) was dissolved in 2 mL dry CH_2Cl_2 at 40 °C and added to the immobilized peptide **148**. HPLC and HRMS analysis revealed a conversion to glycopeptide **151** of 20%.



Analytical HPLC & HRMS (ESI-TOF): m/z = 1005.370 $[M+2H]^{++}$ (calcd.: m/z = 1005.371), 2009.734 $[M+H]^{+}$ (calcd.: m/z = 2009.734).

Glycopeptide **151** eluted at 16.10 min (constant flow: 5 min at 5 % CH_3CN (with 1.0 % AcOH), gradient: 5 % to 100 % CH_3CN (with 1.0 % AcOH) over 30 min).

Synthesis of biotinylated glycopeptide (176)

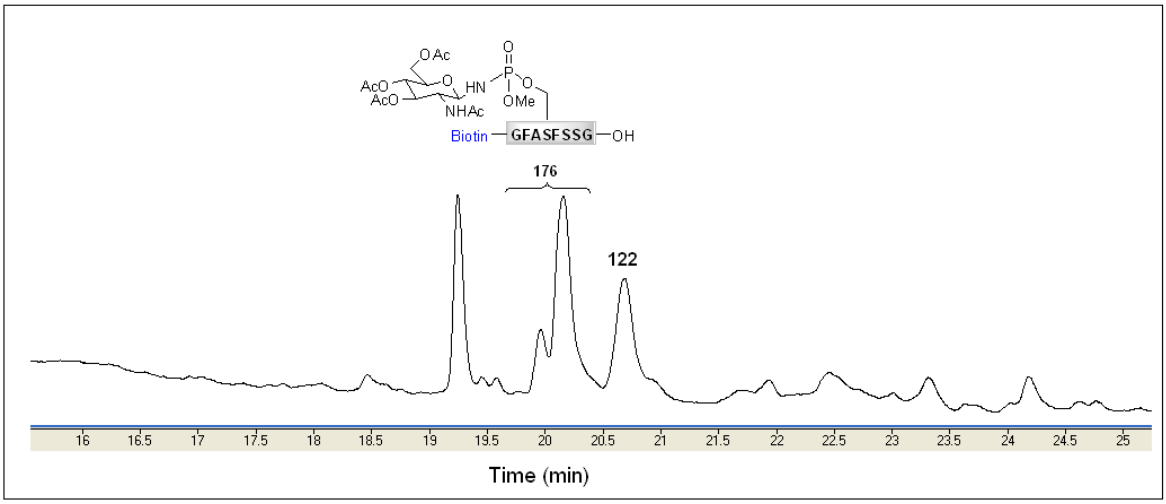


176

Biotinylated glycopeptide **176** was obtained according to GP V in which β-GlcNAc—N_3 **41** (0.07 mmol) was dissolved in 1 mL dry CH_2Cl_2 at 40 °C and added to the immobilized peptide **175**. HPLC and HRMS analysis revealed a conversion to glycopeptide **176** of 49% (not isolated).

Analytical HPLC & HRMS (ESI-TOF): m/z = 1405.518 $[M-H]^-$ (calcd.: m/z = 1405.503)

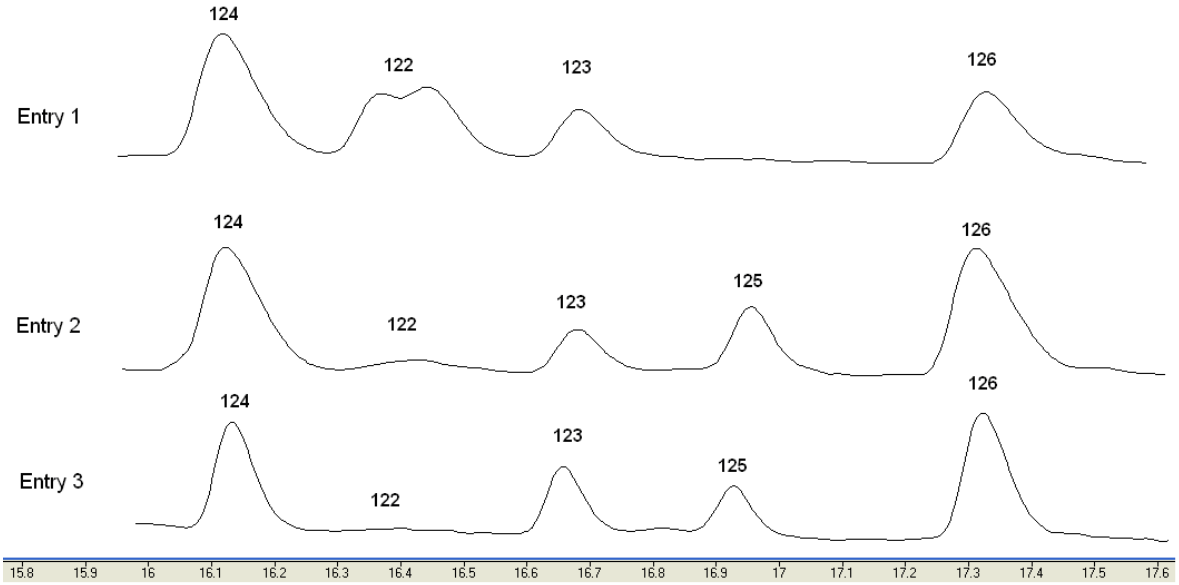
Glycopeptide **176** eluted at 19.9 min and 20.18 min (two peaks) (constant flow: 5 min at 5 % CH_3CN (with 1.0 % AcOH), gradient: 5 % to 100 % CH_3CN (with 1.0 % AcOH) over 30 min).



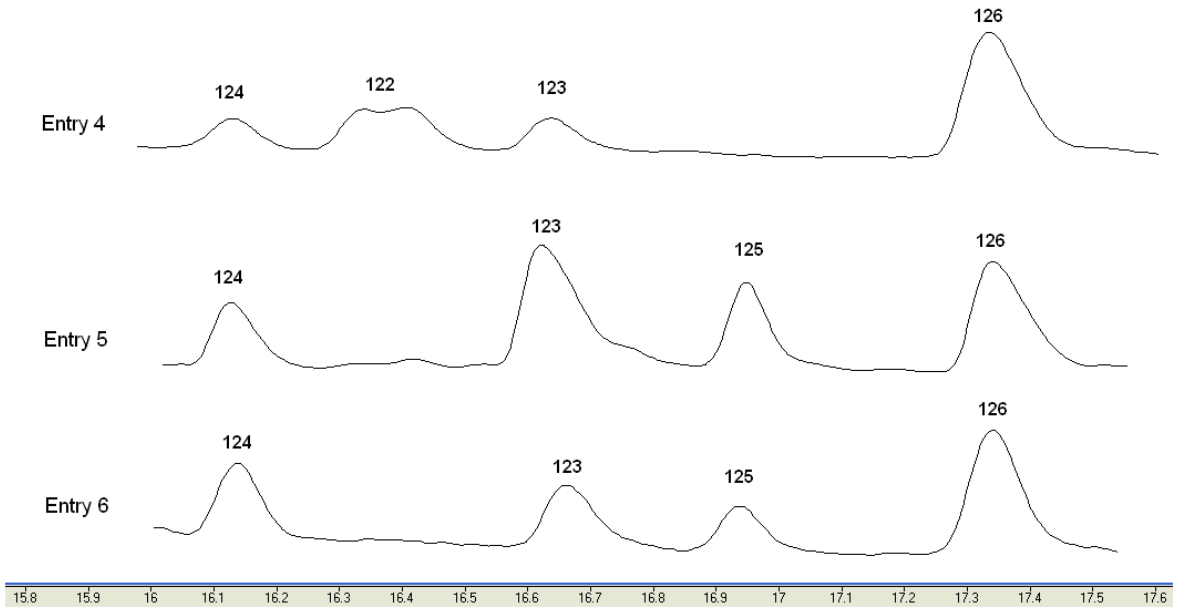
Appendix

I. HPLC-chromatograms at 258 nm for the Staudinger-phosphite reaction of peptide 121 with glycosyl azides 41 and 99 (Table 2, entries 1-15)

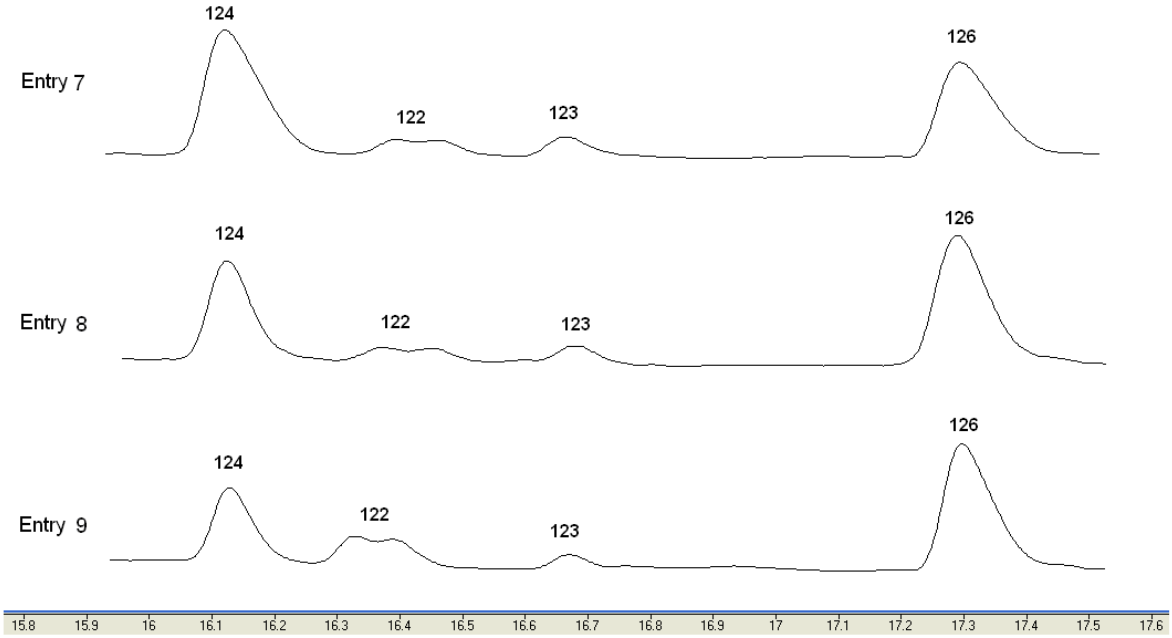
Staudinger phosphite reaction of peptide **121** with β-GlcNAc—N_3 **41** in DMF (table 2, entries 1-3)



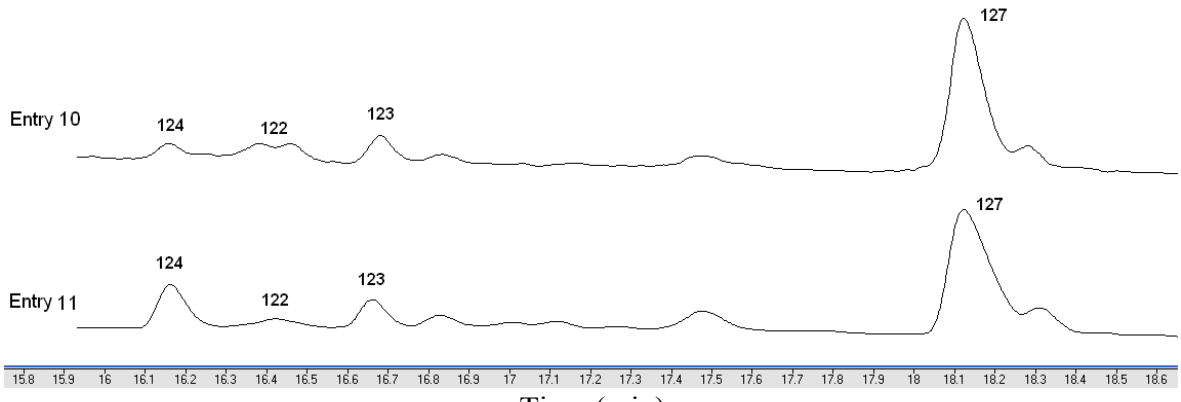
Staudinger phosphite reaction of peptide **121** with β-GlcNAc—N_3 **41** in DMSO (table 2, entries 4-6)



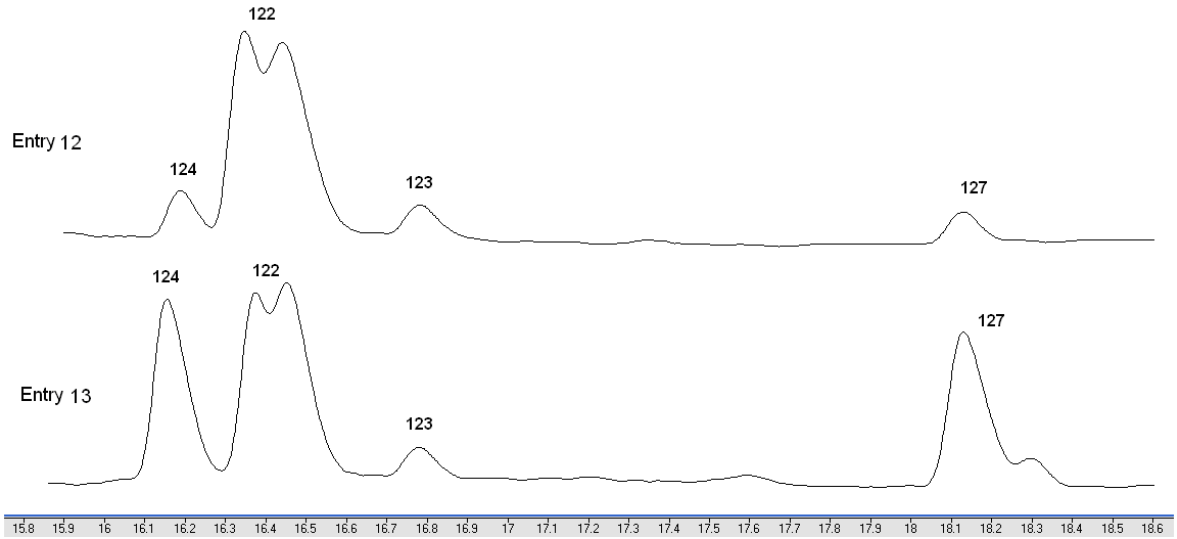
Staudinger phosphite reaction of peptide **121** with β-GlcNAc—N_3 **41** in CH_2Cl_2 (table 2, entries 7-9)



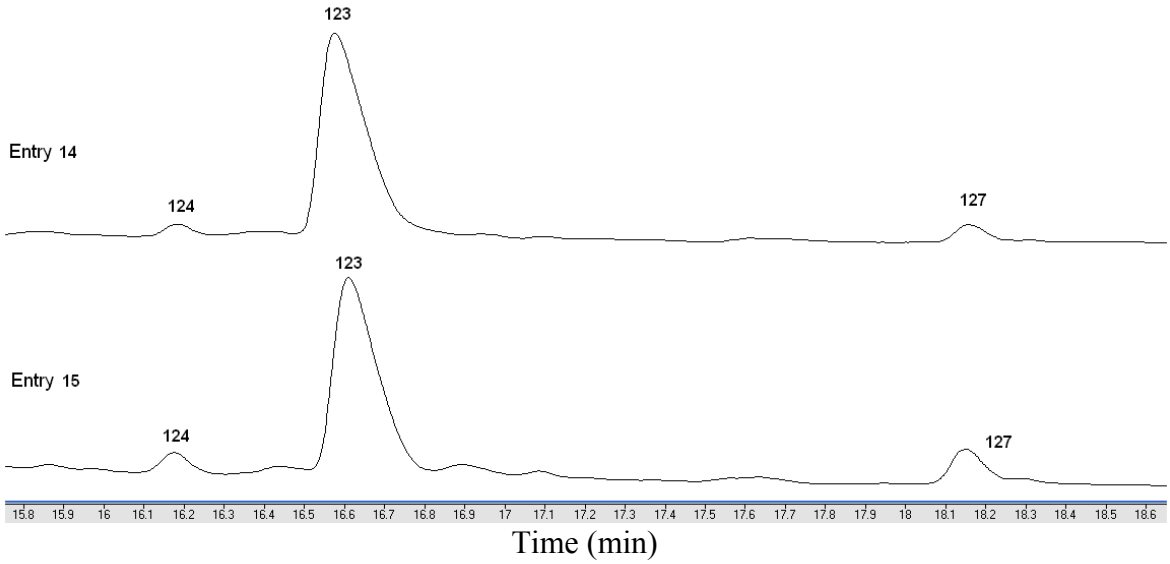
Staudinger phosphite reaction of peptide **121** with α-Man—N_3 **99** in DMSO (table 2, entries 10-11)



Staudinger phosphite reaction of peptide **121** with α-Man—N_3 **99** in CH_2Cl_2 (table 2, entries 12-13)

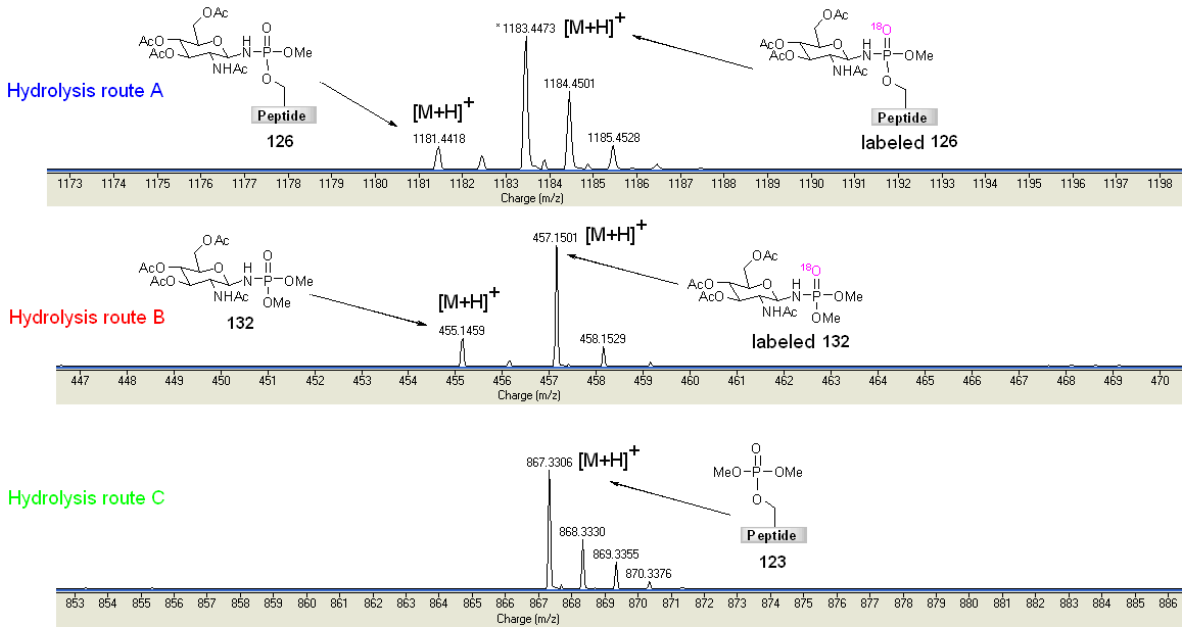


Staudinger phosphite glycosylation of peptide **13** with α-Man-N_3 **5b** in DMF (table 3, entries 14-15)



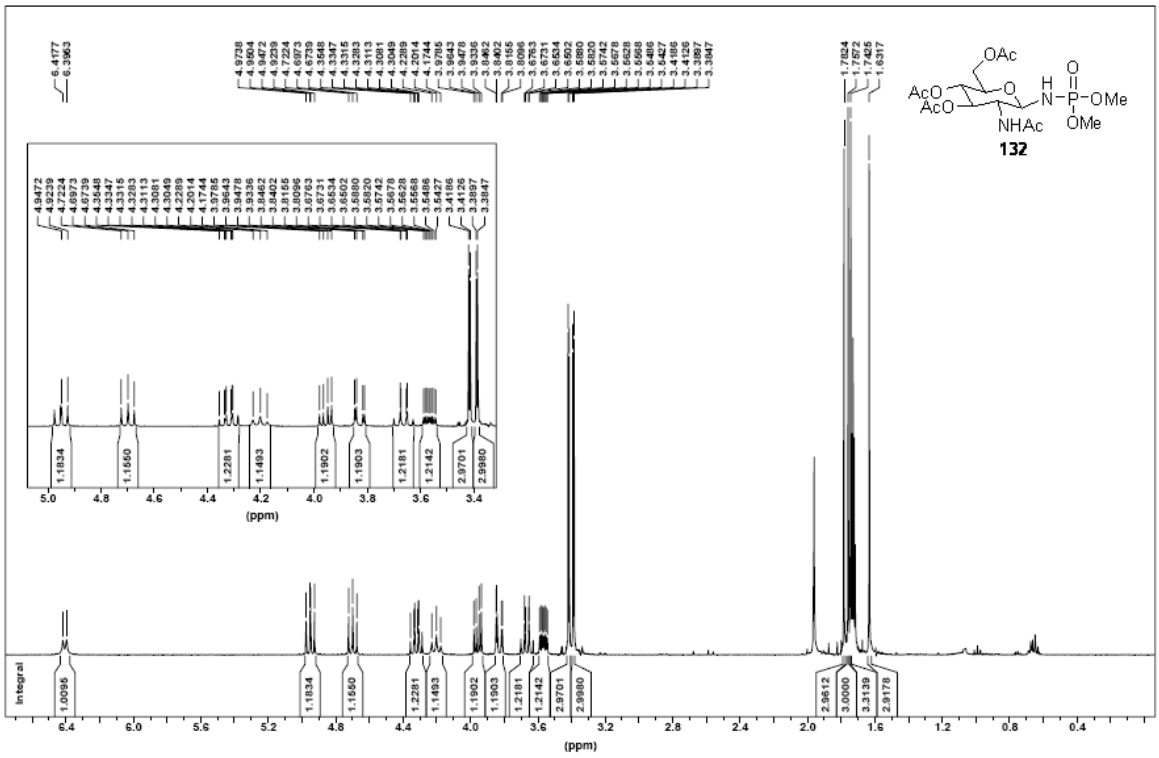
II. Additional Schemes

Scheme A1: MS spectra of obtained peptides labeled **126** and **123** and byproduct labeled **132**.

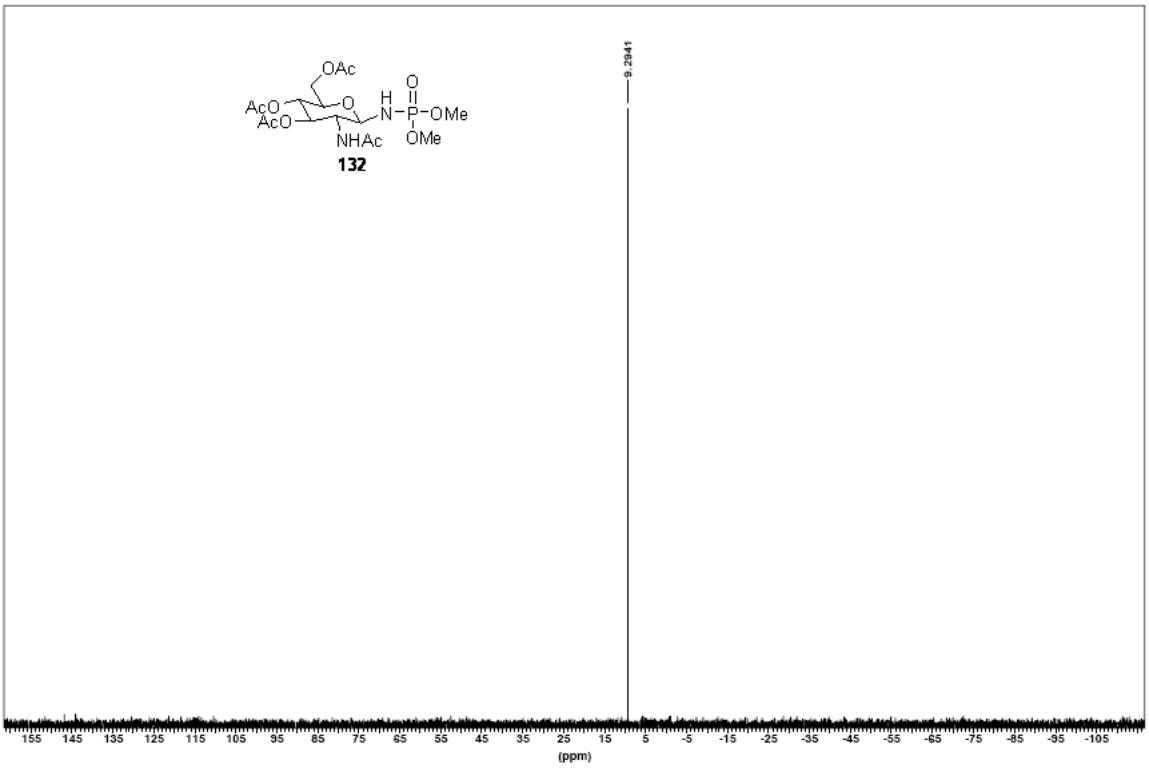


III. Some NMR spectra

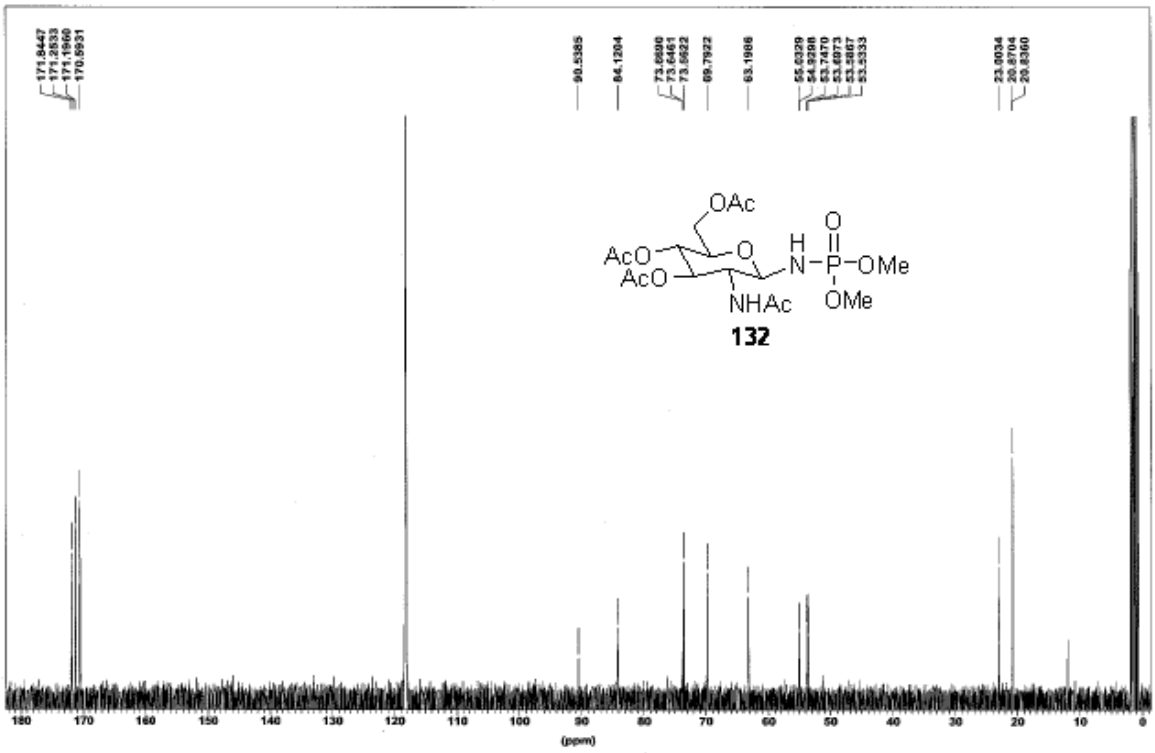
^{1}H NMR (in acetonitrile)



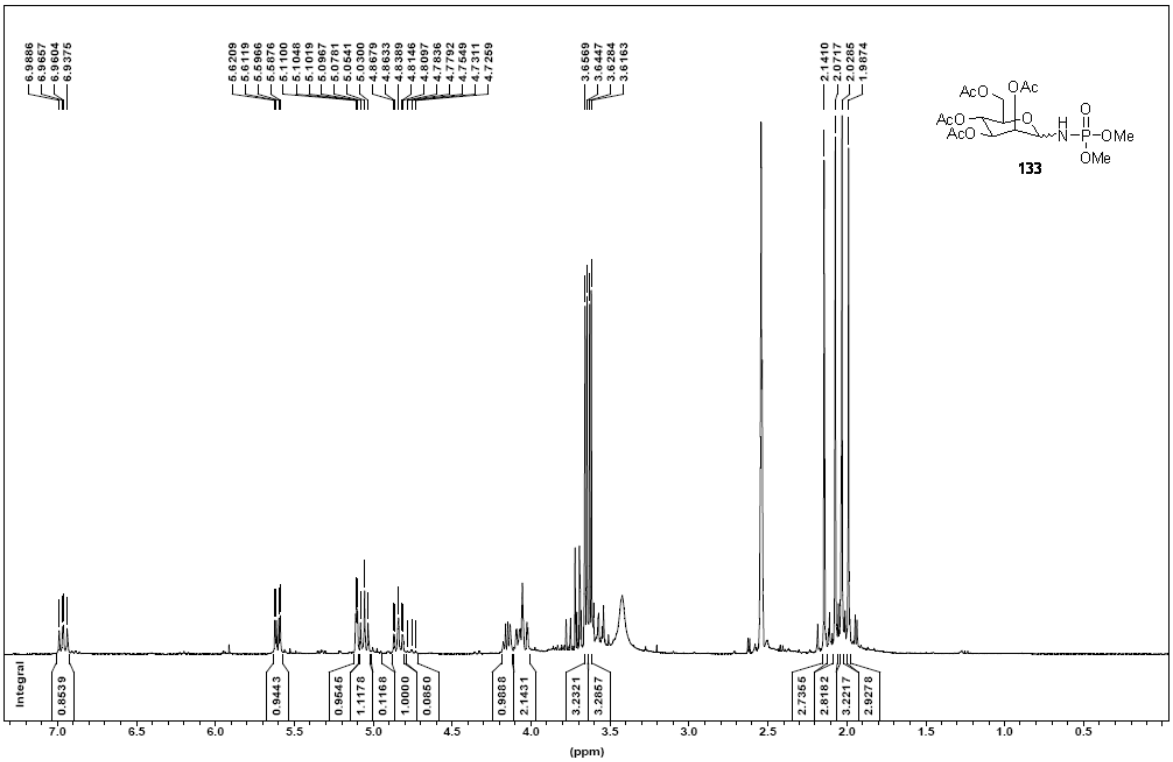
^{31}P NMR (in acetonitrile)



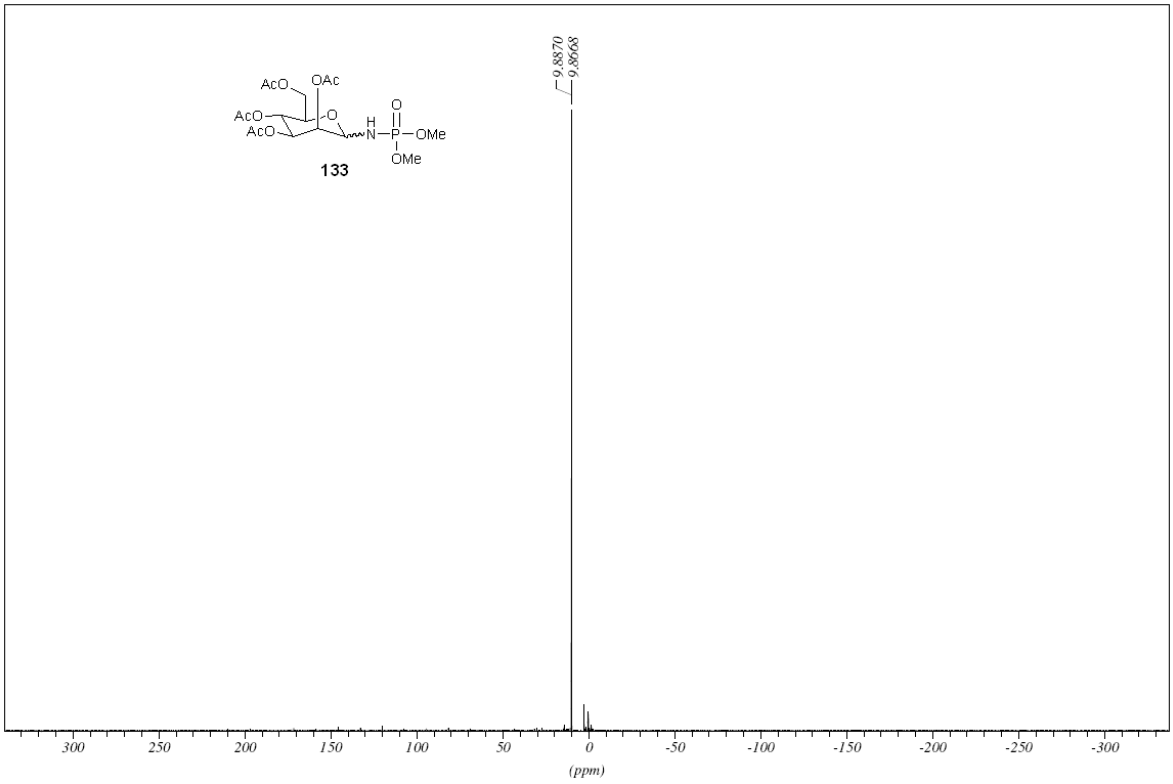
^{13}C NMR (in acetonitrile)



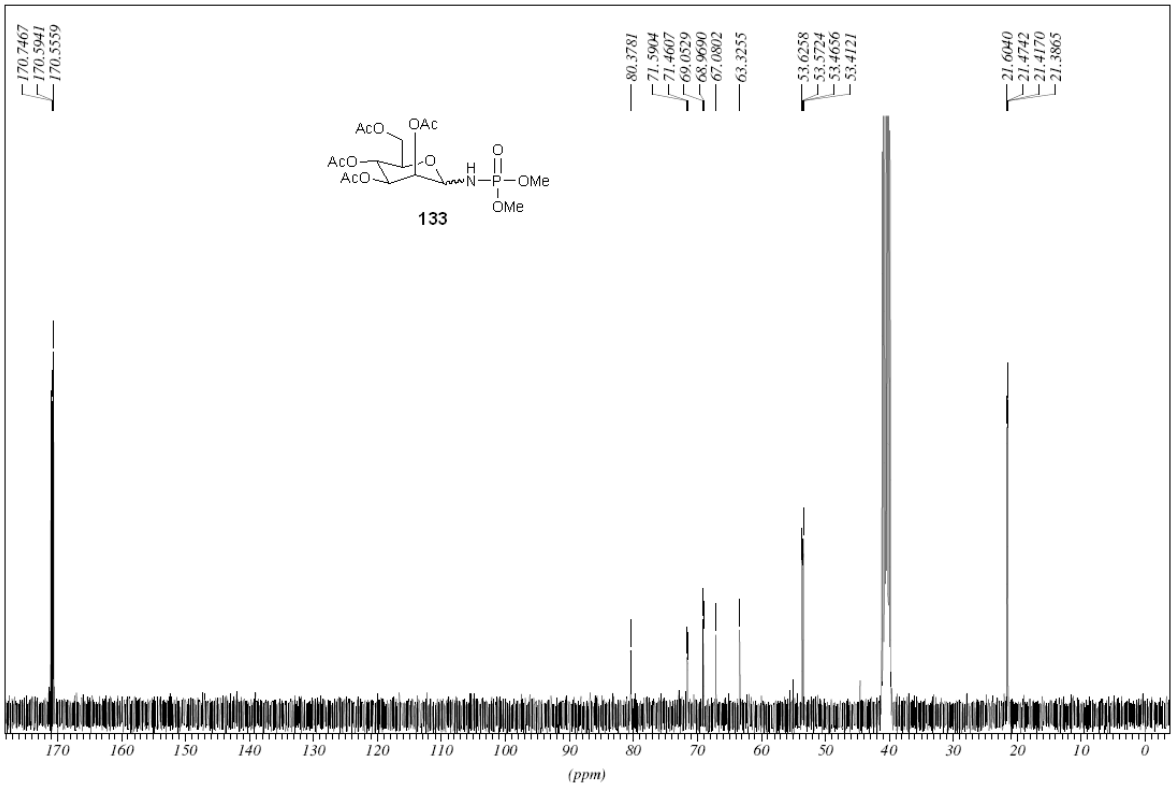
^{1}H NMR (in DMSO)



^{31}P NMR (in DMSO)



^{13}C NMR (in DMSO)



Curriculum Vitae (CV)

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