Synthesis of Glycopeptides Containing Carbohydrate and Peptide Recognition Motifs

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I. Introduction

The majority of natural proteins contain (oligo)-saccharide side chains and therefore are called glycoproteins. The saccharide residues are covalently linked to the protein backbone either *N*- (via asparagine) or *O*-glycosidically (via serine, threonine, tyrosine, or hydroxylysine).[1,2] It has become evident that glycoproteins play an important role in biological processes: besides the influence saccharide residues have on the conformational and physicochemical properties of the proteins, important biological recognition processes depend on the interplay between peptidic and saccharidic elements.[2−7]

Eucaryotic cells synthesize glycoproteins by modifying ribosomal proteins enzymatically. Without this cellular machinery in hand and being unable to isolate sufficient quantities of homogeneous material, the synthesis of glycosylated peptides of exactly specified structure remains a challenge of chemical synthesis. Recent efforts and achievements shall be described throughout this article, demonstrating how organic chemists are able to turn the task of constructing glycopeptides of enormous complexity into a story of success.

The synthesis of glycopeptides is demanding because of the polyfunctionality of the target molecules. A simple calculation reveals up to 10 different functionalities that can be encountered during the synthesis of a glycopeptide. Moreover, the complexity of the problem is heavily increased by the simultaneous occurrence of some of the functional groups in a single molecule. Even a simple monosaccharide as glucopyranose contains five hydroxy groups, three of them having almost identical reactivity.

Throughout this section the major achievements in glycopeptide chemistry reported during the last five years will be discussed. Earlier work already has been reviewed.[8−13] Furthermore, no particular attention will be paid to the synthesis of neoglycopeptides[14] (e.g., dendrimers[15,16]) or -proteins or to the preparation of *C*-glycopeptides.[17,18]

First, the construction of the *O*-glycosylated undecapeptide **1** shall be analyzed as an example to introduce typical methodologies that have been de-

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Tanja Reipen was born in Cologne (Germany) in 1973. She studied at the University of Cologne, where she graduated with a diploma in chemistry in 1998. She is currently working on her doctoral thesis in the group of Professor Horst Kunz at the Johannes Gutenberg–Universität Mainz. The major topic of her work is the synthesis of cadherin-derived glycopeptides.

Dr. Michael Schultz was born in Hadamar (Germany) in 1963. He studied chemistry in Mainz and finished his doctoral thesis in 1994 under the supervision of Professor Horst Kunz working on chemoenzymatic glycopeptide synthesis. Since 1992 he has been a senior research fellow with Professor Kunz. His major research interests are in the field of glycopeptide chemistry and selectin-ligand synthesis.

Horst Kunz, born in Frankenhausen (Saxony) in 1940, studied chemistry at the Humboldt-Universität Berlin and at the Universität Mainz. He completed his Ph.D. degree with Professor L. Horner on the synthesis of cyclic orgonophosphorus compounds in 1969. His Habilitation dealt with ester analogues of acetylcholine and their application in protecting-group chemistry. He was appointed Associate Professor for Organic Chemistry in 1979 and Full Professor of Bioorganic Chemistry in 1988 at the Universität Mainz. His research centers on stereoselective synthesis, the chemistry of peptides and carbohydrates, solid-phase synthesis, and combinatorial chemistry. He received the Max Bergmann medal in 1992 and the Emil Fischer medal in 2000. In 1998, he was elected as a member of the Saxony Academy of Sciences at Leipzig.

veloped to overcome the above-mentioned key issue of polyfunctionality.

A. Synthesis of a Glycoundecapeptide—Major Problems and Solutions

The target sequence **1** (Chart 1) represents a fragment from the tandem repeat of MUC1, which is a heavily glycosylated protein found in mucus and on epithelial cells.[19−23] It is rich in serine, threonine, and proline, and its saccharide side chains are altered due to incomplete glycosylation in the case of cancer.[24−26] An important example for one of those tumor-associated carbohydrate antigens is the sialyl−Tn antigen (αNeu-2,6-αGalNAc-1-*O*-Ser/Thr). Synthetic fragments such as **1** are being tested as vaccines against cancer.[27]

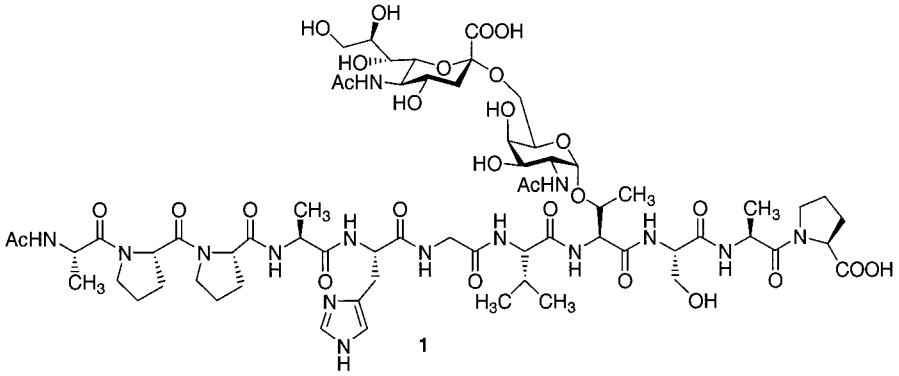
The synthesis of the depicted molecule was carried out following a common strategy: A glycosylated amino acid building block[1] with suitable protecting groups on both carbohydrate and amino acid functionalities was formed; this building block was introduced into a solid-phase peptide synthesis using adapted polymer support, linker, coupling methods, and protecting groups; the fully protected fragment was detached from the solid support; and the protecting groups were removed without affecting the sensitive parts of the glycopeptide.

1. Synthesis of the Sialyl–Tn Building Block

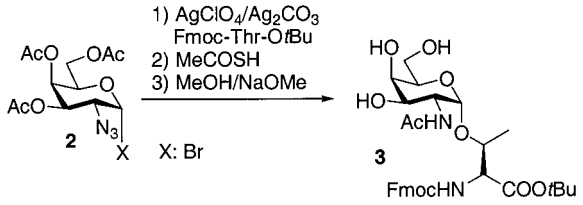
The synthetic strategy[28,29] towards the preparation of the disaccharide threonine unit starts with the formation of the αGalNAc−Thr building block (Scheme 1). Azidonitration of galactal,[30,31] conversion into the azido galactosyl bromide **2**, and glycosylation of a threonine derivative using the Koenigs−Knorr methodology gave a fully protected intermediate.

Apart from this "classical" approach, other combinations of leaving groups and activation principles for the formation of the desired acetal often are applied. Trichloroacetimidates ($X = OC(CCl_3)NH$)[32,33] are formed in a base-catalyzed reaction from the 1-*O*

Chart 1



Scheme 1



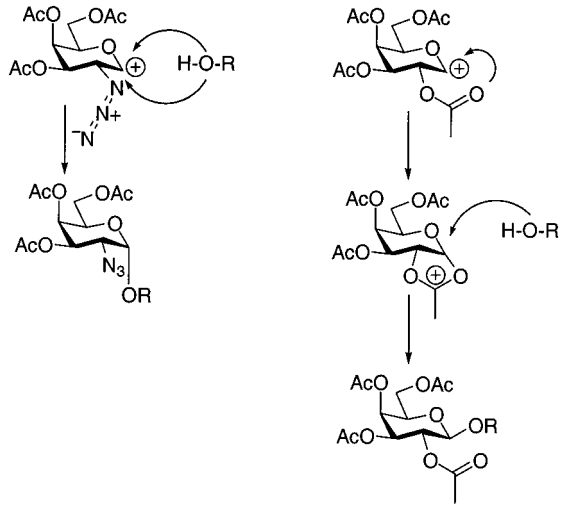
unprotected saccharides and can efficiently be activated by catalytic amounts of a Lewis acid (TMSOTf, BF_3-OEt_2). Thioalkyl or -aryl donors[34] (X = SEt, SMe, SPh) usually are prepared from the 1-*O*-acetyl derivatives and are activated by soft electrophiles (MeOTf, DMTST {$(CH_3SS(CH_3)_2)OTf$}[35]). These donors have found broad application. 1,2-Oxiranes of saccharides prepared from glycal precursors have recently been used for the synthesis of demanding target molecules.[36] Oxiranes can be activated with Lewis acids ($ZnCl_2$). In the case of 2-acetamido sugars, however, oxiranes are not applicable in a straightforward manner.

Apart from the efficiency of the glycosylation step, the stereoselectivity of these reactions is decisive: typically, GalNAc−Ser/Thr derivatives have to be obtained in the α-(*cis*)-configuration. In the example shown above, α-glycoside formation is achieved by use of the 2-azido group which does not show neighboring group participation during the activation of the anomeric position (Scheme 2).

Other neighboring groups that favor the formation of α-glycosides are *O*-benzyl-, *O*-methoxybenzyl-, or thioethers. If *β*-(1,2-*trans*)-anomers are required, acyl groups have to be used in the neighboring position.[31]

In the next stage, a suitable sialic acid residue has to be stereoselectively coupled to the GalNAc−Thr moiety. To this end, the azido group was reduced and acetylated by use of thioacetic acid. Other reagents such as H_2S or $HS(CH_2)_2SH$ followed by acetic acid anhydride also are useful. Next, the *O*-acetyl groups were removed by treatment with NaOMe in MeOH below pH 8.5. The selectivity of this reaction might be surprising since Fmoc is usually cleaved by weak bases such as morpholine. However, unlike morpholine, the catalytic amount of methanolate present during the Zémplen transesterification does not efficiently trap the fulvene formed. Due to this orthogonal stability of Fmoc and *O*-acetyl groups, subsequent steps to introduce the Fmoc group at a later stage, prior to the solid-phase synthesis, can be avoided.

Scheme 2



The development of efficient methods for the formation of α2,3- or α2,6-sialosides has been the focus of saccharide chemistry for several years.[37] Usually, yields and stereoselectivity of the sialylation reactions are low due to the destabilization of the glycosidic cation by the neighboring carboxy group and because of the lacking neighboring group at C-3. Recently, anomeric xanthates,[38,39] thioalkyl and -aryl donors,[40] and sialosyl phosphites[41,42] were shown to be valuable precursors for the formation of α-sialosides.

In contrast to other strategies reported,[43,44] the sialylation of the primary hydroxy function in **3** was carried out in the presence of the two unprotected secondary hydroxy groups (Scheme 3).[28] In fact, the glycosylation with sialyl xanthate **4** activated by methyl sulfenyl triflate proceeded regioselectively with satisfying stereoselectivity. After HPLC purification, the desired sialyl−Tn antigen conjugate **5** was obtained in a yield of 32%.

Prior to the use of the disaccharide threonine conjugate in a solid-phase synthesis, the C-terminal *tert*-butyl ester has to be cleaved using TFA and a scavenger (to trap cationic species). Earlier investigations have shown that this treatment can only be performed safely if the acid-sensitive intersaccharidic linkages are shielded by acylic protecting groups.[45] With ether-type hydroxy protections, particularly sensitive saccharides such as fucosides are affected.

Scheme 3

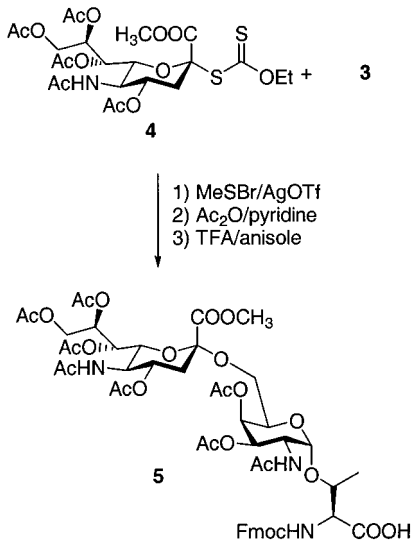
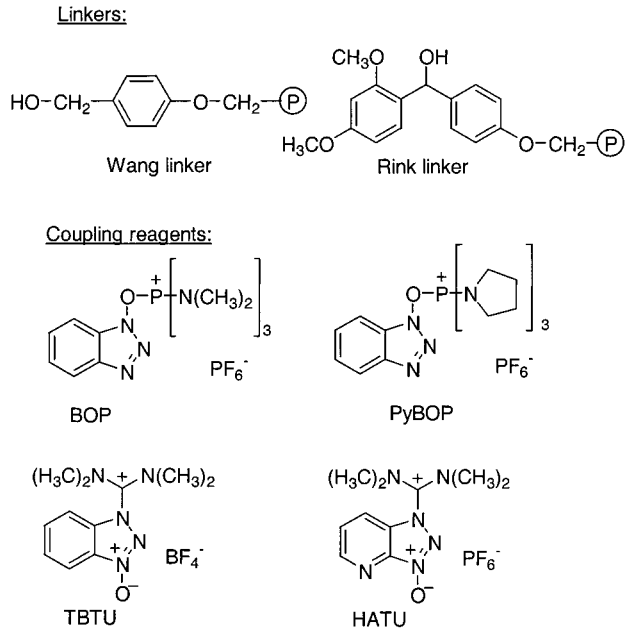


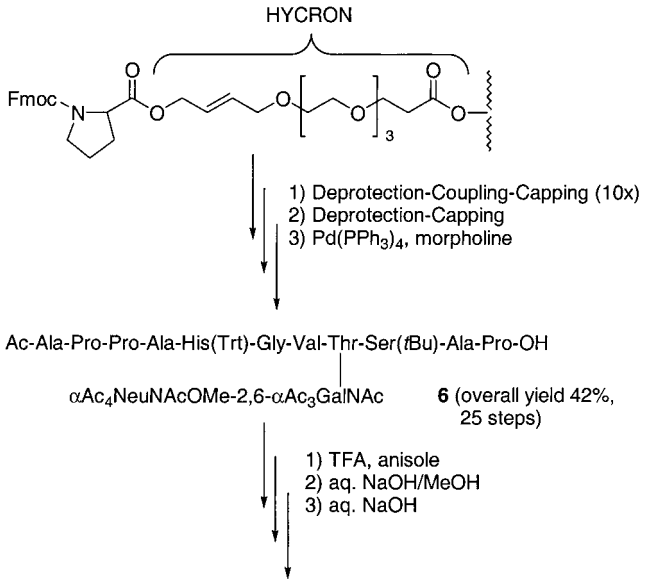
Chart 2



2. Solid-Phase Synthesis of Undecaglycopeptide 1

Solid-phase peptide synthesis (SPPS), originally developed by Merrifield and co-workers,[46] has become a powerful tool for the rapid and efficient assembly of peptides, oligopeptides, and even small proteins.[47] The methodology used for modern SPPS can also be applied for the construction of glycopeptides (Chart 2). Suitable polymer supports either hydrophobic (aminomethyl polystyrene) or hydrophilic (Tentagel, PEGA) are available. Linkers attached to the polymer, usually acid-labile (Wang,[48] RINK[49]) or even super acid-sensitive (SASRIN,[50] cleavable with 1% TFA), and a great variety of other linkers have been developed and are commercially available. Coupling reagents based on benzotriazole (TBTU,[51] HATU,[52,53] BOP,[54] PyBOP) forming intermediate active esters with the protected amino acids effect quantitative and rapid coupling reactions (Chart 2). According to the Fmoc strategy, repetitive quantitative and mild removal of the *N*-terminal protecting group is attainable using piperidine or morpholine.[10,55] Different combinations and variations of the tools mentioned have successfully been used for the synthesis of a variety of glycopeptides as will be discussed below.

Scheme 4



In the model synthesis discussed here, an aminomethyl polystyrene resin modified with a proline-loaded allylic HYCRON anchor was used (Scheme 4).[56,57] HYCRON consists of a hydrophilic triethylene glycol spacer etherified with dihydroxybut-2-ene and hydroxypropionic acid. The allyl-type ester formed with the starting amino acid can be cleaved under almost neutral conditions via Pd(0)-catalyzed allyl transfer onto a suitable nucleophile (e.g., *N*-methylaniline, morpholine, *N*-methylmorpholine) which irreversibly traps the allylic moiety.[58]

Extension of the peptide chain was performed using TBTU[51] and a 4-fold excess of the protected amino acids. Fmoc removal was carried out with morpholine, and unreacted amino groups were capped with acetic anhydride after each coupling step. To minimize diketopiperazine formation, often observed with proline or glycine being one of the two C-terminal amino acids, the second amino acid (Ala) was introduced as the Boc-protected derivative. The ammonium salt resulting from the TFA-promoted cleavage of the Boc group does not undergo undesired cyclization. It is noteworthy that the feasibility of this strategy is an advantageous outcome of the allylic anchoring principle stable to both bases and acids.

It is desirable to monitor the assembly of the peptide backbone analytically. Here, the UV absorption of the fulvene−morpholine adduct resulting from the Fmoc deprotection step was recorded. Alternatively, the conductivity of the piperidine−fulvene conjugate can be measured. DIC/HOBt couplings can be monitored continuously by the use of bromophenol blue.[59] Recently, solid-phase NMR methods were used to analyze the formation of the peptide chain on the resin.[60]

Removal of the glycopeptide from the polymer support and cleavage of the amino acid side chain

protecting groups are usually achieved with TFA in one step. If, however, protected fragments are required, these conditions often are not applicable. Using the HYCRON methodology, selectively carboxy-deblocked fragments such as **6** can be obtained under the neutral conditions applied.

For a complete deprotection of the glycopeptide (→ **1**), the side chain protecting groups were removed with TFA and the *O*-acetyl groups were cleaved with sodium methoxide in methanol. The removal of the rather stable *N*-acetyl neuraminic acid methyl ester required optimized conditions using aqueous sodium hydroxide at pH 11−11.5. This final step must be carried out very carefully because already at pH 12 a number of side reactions occur, including β-elimination of the carbohydrate or epimerization within the peptide.[29]

This introductory example was meant to list the major problems encountered and some of the solutions to these problems. Of course, many other specific questions will be discussed throughout the following systematic report. To this aim, glycopeptides containing *O*-linked mono- and disaccharides (section II), *O*-glycopeptides with complex side chains (section III), and glycopeptides with *N*-linked saccharide side chains (section IV) will be shown.

II. O-Glycopeptides Carrying Mono- and Disaccharides

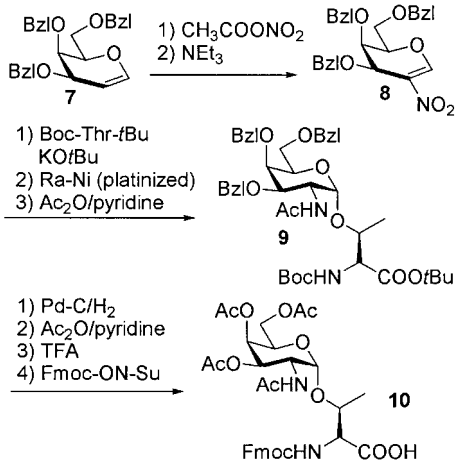
The occurrence of aberrant carbohydrate side chains in natural glycoproteins often parallels pathological phenomena. In particular, the Thomsen−Friedenreich antigen (T antigen, βGal-1,3-αGalNAc-1-*O*-Ser/Thr) and its derivatives (Tn, sialyl−Tn) have been described as tumor-associated antigens.[24−26,61] Consequently, the synthesis of glycopeptides carrying these side chains has been thoroughly investigated.

A. *O*-Glycopeptides Carrying αGalNAc

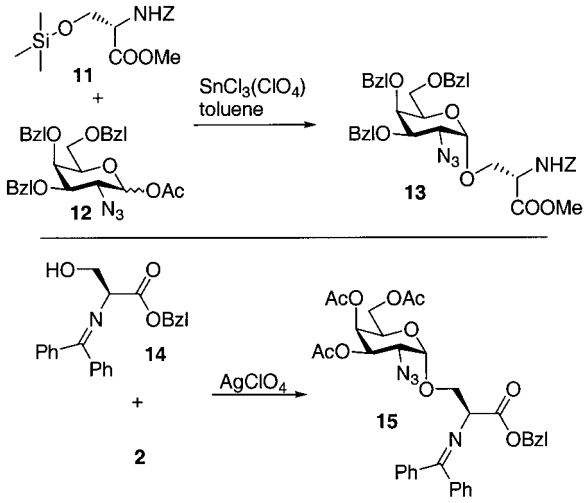
The preparation of αGalNAc−Ser/Thr building blocks for the synthesis of glycopeptides belongs to the standard repertoire of glycopeptide chemistry (Scheme 1).[10] Thus, only recent results covering either interesting methodological developments or target structures of particular complexity and biological relevance will be discussed here.

One of the drawbacks on the standard route to αGalNAc-1-*O*-Ser/Thr building blocks is the azidonitration reaction[30] needed for the introduction of the 2-azido substituent. The procedure requires ceric ammonium nitrate and gives only moderate yields of an unstable compound after lengthy chromatographic purification. An improved procedure avoiding chromatography of synthetic intermediates has been published.[62] To circumvent the azidonitration reaction, the use of 2-nitroglycal **8** was envisaged (Scheme 5).[63] This Michael acceptor was prepared from the corresponding tri-*O*-benzylgalactal **7** via the addition of acetyl nitrate and subsequent elimination of acetic acid. Well-known Boc- and Fmoc-protected amino acid derivatives were reacted with 2-nitrogalactal **8** and KOtBu/THF to give predominantly α-glycosides (α/β 8:1) in high yield. The nitro group of the resulting

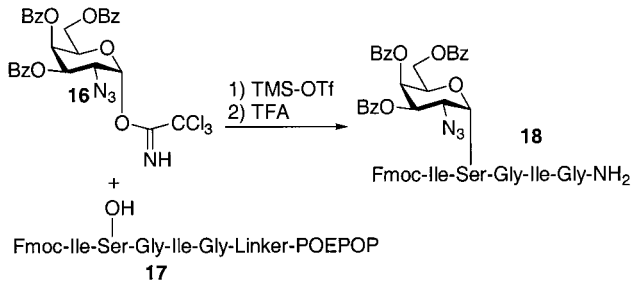
Scheme 5



Scheme 6



Scheme 7

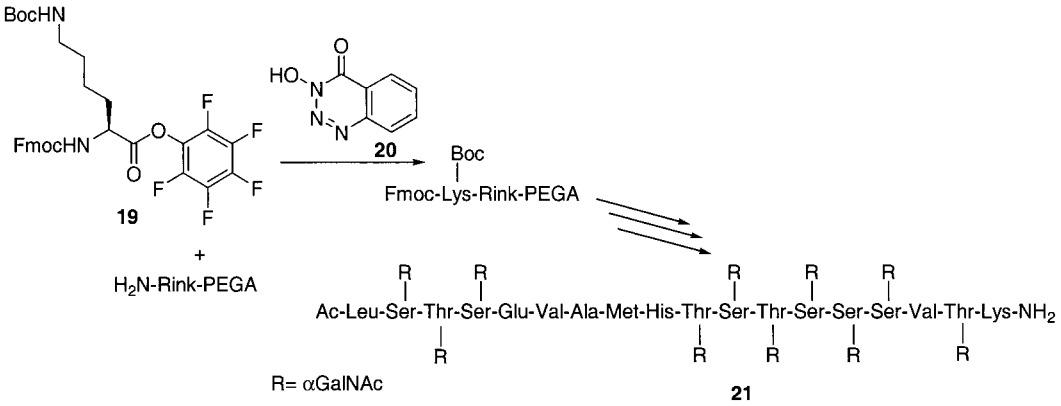


Boc derivatives (**9**) was selectively hydrogenated and acetylated before the *O*-benzyl ethers were exchanged for *O*-acetyl groups. After the introduction of the Fmoc instead of the Boc group, the desired GalNAc−Ser/Thr derivative (**10**) was obtained. Despite these circumstantial manipulations, the reaction sequence is considered an interesting innovation.

Other investigators concentrated on glycosyl acceptors with improved nucleophilicity combined with suitable promotors.

Thus, trimethylsilylated serine and threonine derivatives (**11**) were reacted with 1-*O*-acetyl-2-azido galactose **12** and $SnCl_3(ClO_4)$ to give αGalNAc−Ser/Thr derivatives (**13**) almost quantitatively in α/β-ratios of >9:1 (Scheme 6).[64] However, no results with

Scheme 8



Fmoc-protected serine or threonine TMS-ethers were reported.

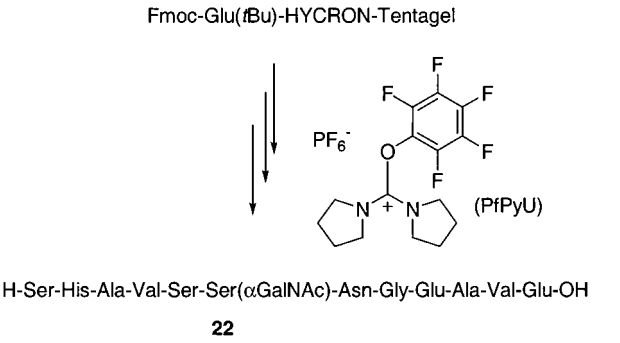
Polt et al. utilized Schiff bases derived from serine or threonine.[65,66] The authors propose higher reactivity of the hydroxy groups in **14** due to a favorable hydrogen-bonding pattern. In fact, **14** was efficiently glycosylated with the common 2-azido galactosyl bromide **2** promoted by silver perchlorate. Four additional steps were required to transform **15** into a suitable building block.

An effective methodology for the solid-phase glycosylation of peptides with carbohydrate donors was recently introduced.[67] Key improvement was the development of a suitable polymer support based on a polyoxyethylene−polyoxypropylene (POEPOP) copolymer devoid of disturbing amide linkages. With this polymer support combined with the acid-labile Rink linker, pentapeptide **17** was synthesized (Scheme 7). Solid-phase glycosylation of the serine side chain with excess of tri-*O*-benzoylated 2-azido galactosyl trichloroacetimidate **16** and cleavage from the resin (TFA) gave the protected glycopentapeptide **18** in a yield of 78%. Since other glycosyl donors (e.g., fucose, galactose, glucose, mannose) could be reacted as well, this methodology holds some potential for the construction of glycopeptide libraries.

A biologically interesting target molecule carrying GalNAc residues is glycophorin A, a transmembrane glycoprotein found in erythrocytes. To investigate the influence of the GalNAc side chains on the protein conformation, an octadecapeptide carrying nine αGalNAc units was synthesized (Scheme 8).[68] Throughout the solid-phase synthesis, coupling reactions were carried out using preformed Fmoc glyco−amino acid pentafluorophenyl (Pfp) esters **19** in the presence of triazine derivative **20** (Dhbt-OH).[69] With the help of **20**, peptide couplings were followed directly by detecting the color-alteration Dhbt−O^- → Dhbt−OH at λ = 440 nm. After completion of the glycopeptide chain elongation, treatment of the resin-linked intermediate with carefully purified thioacetic acid, hydrazine in methanol,[70] and TFA gave target glycooctadecapeptide **21** in a yield of 51%.

With the synthesis of a 82 amino acid GalNAc-containing glycopeptide from the family of diptericins (antibacterial glycopeptides found in insects), it was shown that very large structures can be prepared using a modern SPPS methodology.[71] The starting amino acid conjugated via the Rink-amide linker to

Scheme 9



a polystyrene-based resin was elongated with Fmoc-protected amino acids and with glycosylated building block **10** (Scheme 5). From a variety of fragments prepared, the largest (82 amino acids, two GalNAc residues) could be obtained in a yield of 3.5%.

Particulary difficult glycopeptide sequences still motivate the search for alternative coupling methods, e.g., reagents of improved efficiency. This was the case for the dodecapeptide fragment **22** (Scheme 9) from the loop sequence of the homophilic recognition region of E-cadherin.[72] Cadherins are decisively involved in cell adhesion and morphogenesis. Because TBTU as a coupling reagent gave only low yields of the target molecule, the pentafluorophenol-derived uronium salt (PfPyU) was developed.[73,74] With PfPyU as the coupling reagent in the solid-phase synthesis, the GalNAc-containing dodecapeptide **22** was synthesized on HYCRON−Tentagel resin (see section I) in an overall yield of 55%.

B. Glycopeptides Carrying the T Antigen (βGal-1,3-αGalNAc)

Chain-extending syntheses of glycopeptides carrying the T antigen side chain have been described about 18 years ago.[75−77] Until today, the strategy once developed for the preparation of T antigen building block **26** is still applied (Scheme 10). [78] It involves the synthesis of the disaccharide unit from 2-azido galactose acceptor **23** and peracylated galactose donor **24**. The resulting disaccharide is converted into donor **25** and linked to serine/threonine derivatives.

A series of mucin-type glycopeptides carrying up to three T antigen side chains was synthesized on solid phase using an acid-labile linker (PAL) conju-

Scheme 10

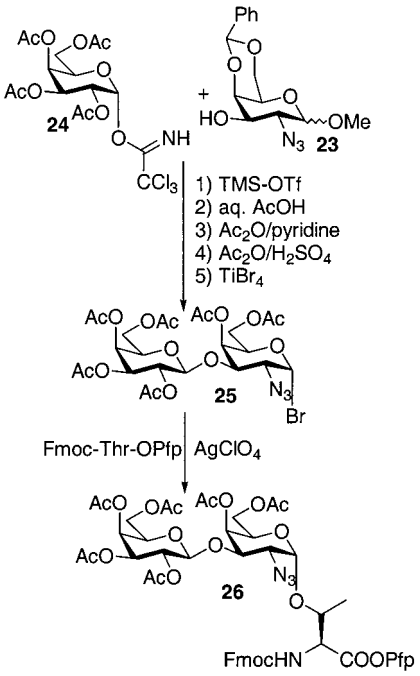
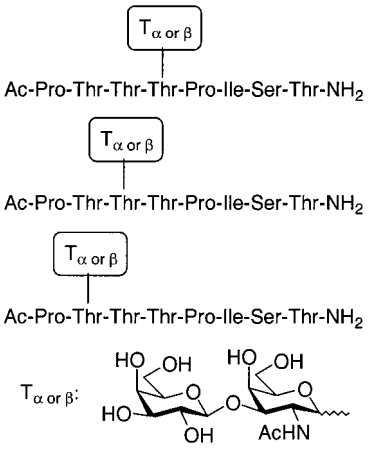


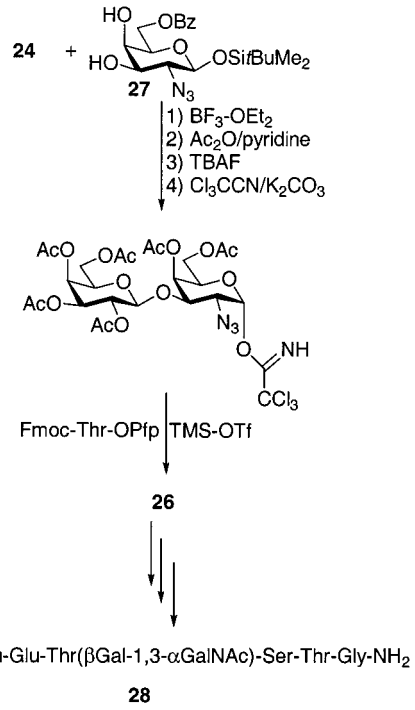
Chart 3



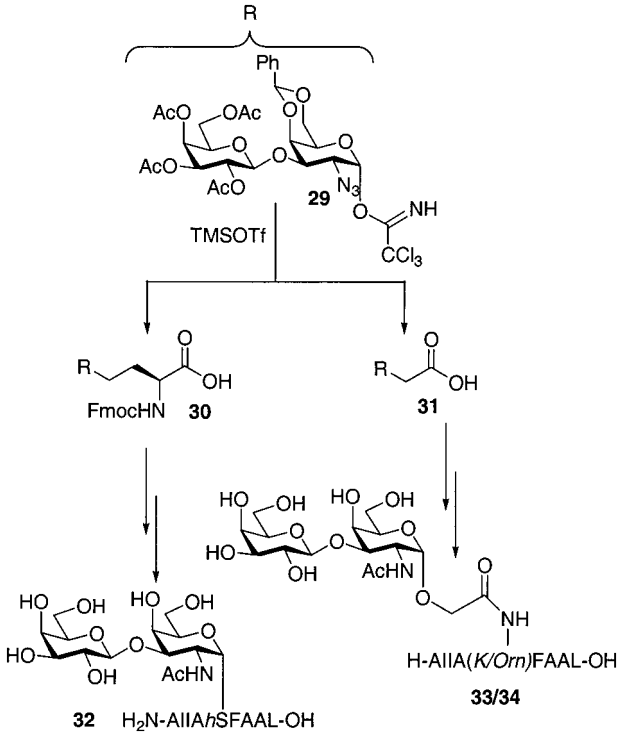
gated to a polyacrylamide resin.[78] As described above, Pfp esters in combination with Dhbt−OH (**20**) allowed the progress of the solid-phase couplings to be followed. After *N*-terminal acetylation, treatment with thioacetic acid and TFA the deprotection was completed with sodium methoxide in methanol. Interestingly, a mixture of anomers of building block **26** could be used in the same fashion and the resulting mixtures could be separated after deacetylation by HPLC providing 18 different glycopeptides carrying either α- or *β*-linked T antigen side chains (Chart 3, selected structures shown).

A similar approach was used to synthesize a fragment of the leukocyte surface glycoprotein *sialophorin* (Scheme 11).[79] Synthesis of the disaccharide−serine/threonine building block was accomplished via the trichloroacetimidate methodology employing 1-*O*-TBDMS protected acceptor **27**. The resulting Pfp-ester **26** was used in a solid-phase glycopeptide synthesis which was followed colorimetrically with the help of Dhbt−OH **20** and the dye violet acid 17. Thioacetic acid treatment, TFA-promoted cleavage

Scheme 11



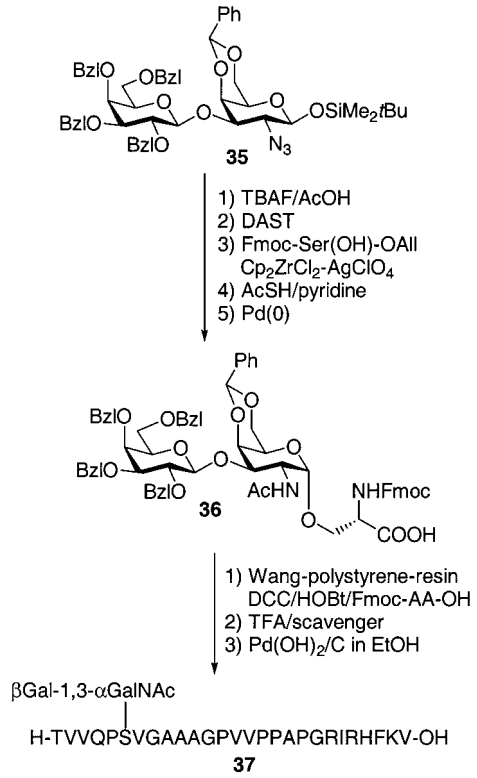
Scheme 12



from the PAL resin, and deacetylation afforded target glycopeptide **28**.

A crucial question in glycoprotein biology is how the glycan side chain influences the protein backbone. In this context, one important parameter is the distance between carbohydrate and biologically active peptide sequence. For a systematic investigation, the T antigen was attached to the side chains of homoserine, lysine, and ornithine via two different strategies (Scheme 12).[80] In the case of homoserine, a building-block approach was followed employing

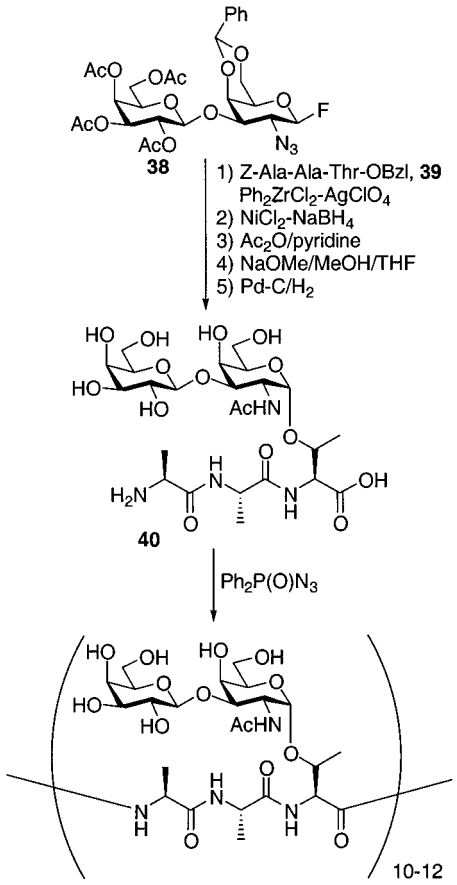
Scheme 13



residue **30** in the solid-phase synthesis of a peptide with known affinity for a MHC class I molecule.[81] Here, the acid-labile HMPA linker was used. On the other hand, the T antigen linked to a glycolic acid derivative (**31**) was introduced via a convergent approach. The Boc groups of the lysine and ornithine side chains of the preformed resin-bound peptide were cleaved, and the liberated amino groups were acylated with glycosylated compound **31**. In this case, a base-labile linker (HMBA) was used to facilitate Boc removal from the lysine or ornithine side chains prior to the attachment of the T antigen derivative. In both cases, the 2-amino function was generated using dithiothreitol before acetylation and cleavage from the resin was accomplished either with TFA or 0.1 M NaOH. As a result, T antigen-carrying peptides (**32**−**34**) with distances of three, six, and seven atoms between glycan and peptide scaffold were obtained and can be compared with the compound derived from serine directly glycosylated with the T antigen.

The "natural" T antigen serine building block was used for the synthesis of a 27 amino acid glycopeptide fragment from human α2HS glycoprotein.[82] However, in contrast to most other approaches, a benzyl ether protected carbohydrate was used for the synthesis of building block **36** and this protecting group pattern was maintained throughout the solid-phase synthesis (Scheme 13). Starting from disaccharide silyl ether **35**, the anomeric protection was removed and the α/β-glycosylfluoride was prepared. Zirconium/silver salt-mediated glycosylation of Fmoc-protected serine allyl ester, treatment with thioacetic acid, and cleavage of the allyl ester (Pd(0)) gave the benzyl- and benzylidene-protected T antigen derivative **36**. However, after solid-phase synthesis on a Wang polystyrene resin using DCC/HOBt as coupling reagents, a mixture of mono-, di-, tri-, and tetrabenzylated products resulted from the TFA-promoted cleavage. Pooling of those benzyl-protected compounds and hydrogenation finally afforded the target heptacosaglycopeptide **37** in a yield of 26%.

Scheme 14



It is well-known that the occurrence of clustered glycan side chains can significantly increase the biological activity of a given glycoprotein.[83] This observation motivated the construction of a sequential glycopeptide polymer carrying 10−12 T antigen units.[84] To this end, tripeptide **39** was glycosylated using disaccharide fluoride **38** and a zirconium/silver salt promotor (Scheme 14). Reduction of the azide function, acetylation, and removal of all protecting groups gave glycotripeptide **40**, which could be subjected to polycondensation using diphenylphosphoryl azide (DPPA). The resulting oligomer (M_w 6000−7300 u) represents a typical fragment from an antifreeze glycoprotein found in polar and deep-sea fish.[85]

An alternative to the standard solid-phase peptide chemistry was developed by employing hydroxy functions of either serine/threonine or saccharide moieties as links to the polymer via a silyl linker.[86,87] Into chloro(α,α-dimethylbenzyl)dimethylsilane a *p*-nitro group was introduced. The resulting *p*-nitro(α,α-dimethylbenzyl)dimethylsilyl chloride **(42)** was etherified with the 6-hydroxy group of Gal-GalNAc derivative **41** upon treatment with sodium iodide and *N*-methylmorpholine in DMF (Scheme 15). Subsequent reduction of the *p*-nitro group, condensation of the resulting aniline derivative with succinic anhydride, and coupling to glycine-loaded Wang resin

Scheme 15

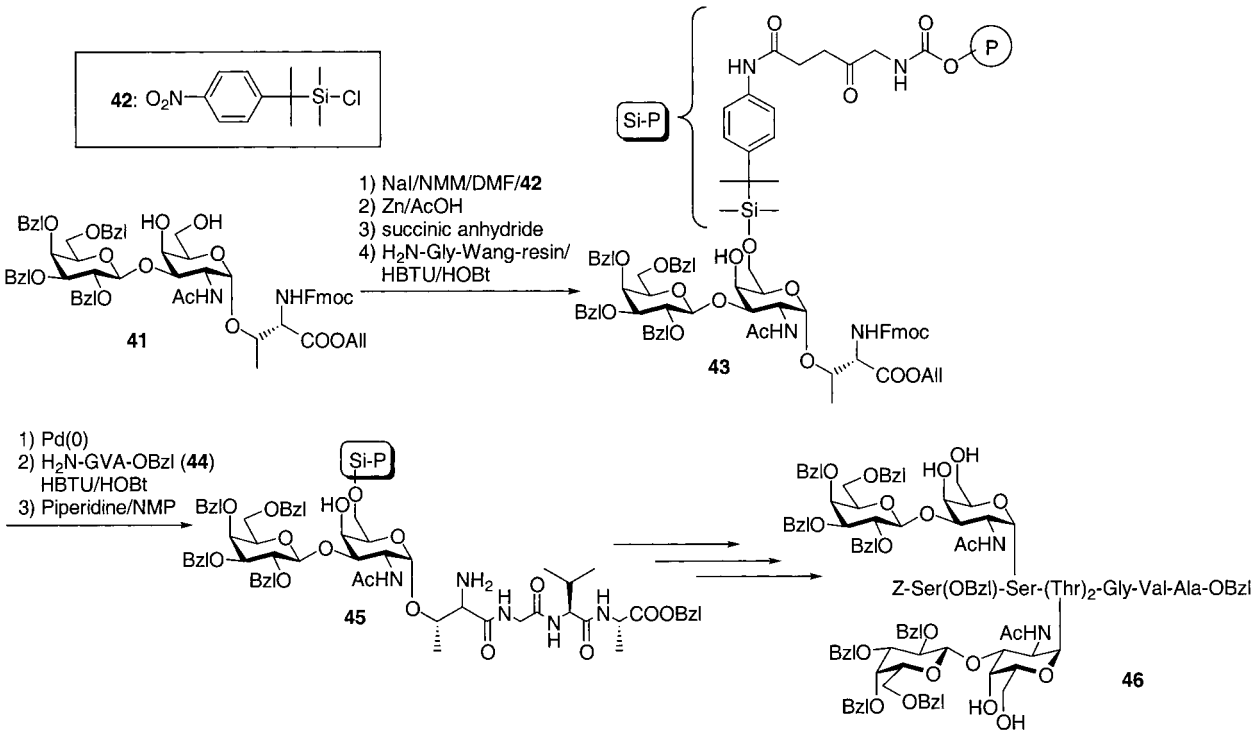
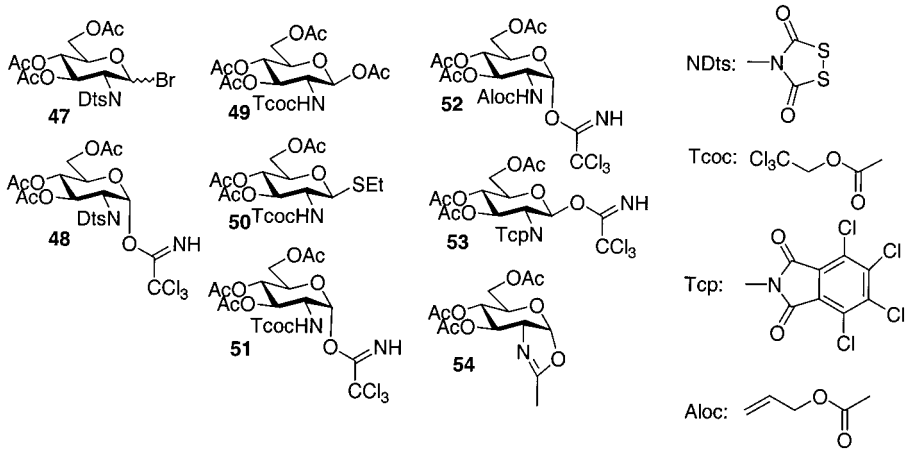


Chart 4

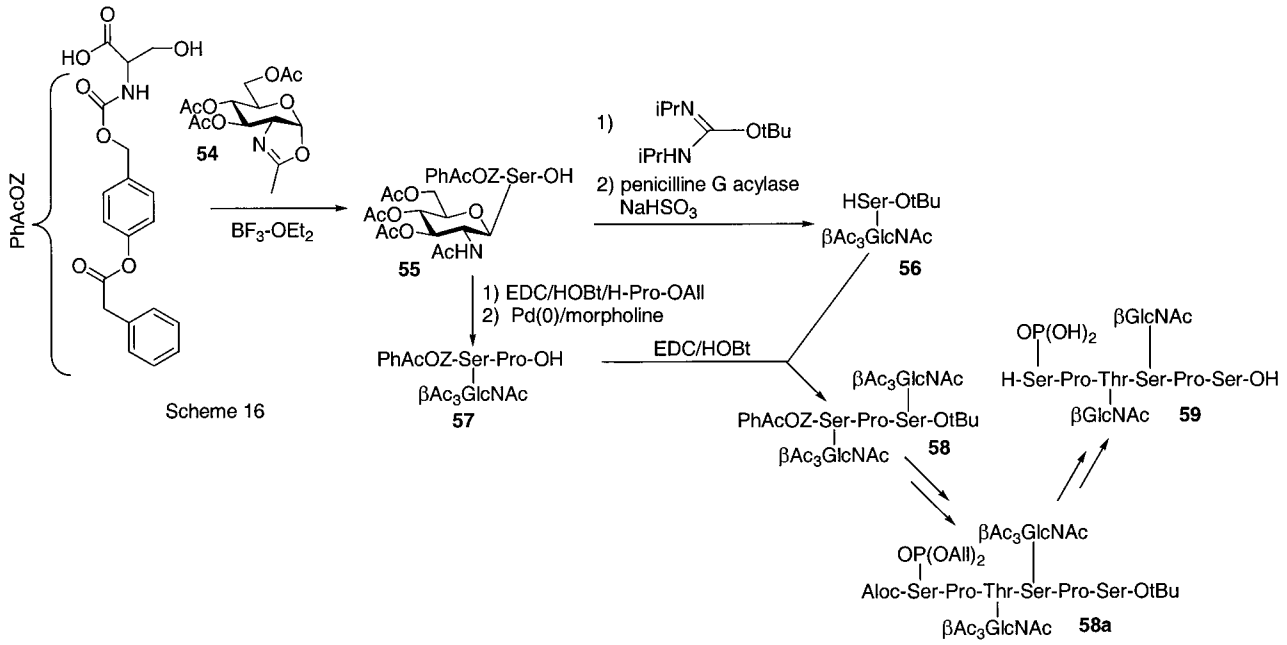


gave compound **43** ready for extension of the peptide chain in both the *C*- and *N*-terminal directions. First, the allyl ester was cleaved and the carboxy group condensed with tripeptide benzyl ester **44**. Removal of the Fmoc group (**45**) then facilitated the stepwise attachment of two Gal-GalNAc−Ser/Thr residues and a Z-protected serine unit. Final treatment with tetrabutylammonium fluoride (TBAF) in THF furnished glycoheptapeptide **46** in 55%. Deprotection of this molecule has not been reported.

C. *O*-Glycopeptides Carrying β-GlcNAc

In contrast to most other glycan side chains found in nature, *O*-2-acetamidoglucopyranose always occurs as unmodified monosaccharide side chain linked to serine or threonine. This is particularly remarkable since the *O*-GlcNAc-containing peptides were first detected by the transfer of ^{14}C-labeled galactose in an enzyme-catalyzed reaction using a common transferase.[88] *O*-GlcNAc residues occur ubiquitiously but predominantly in nucleoplasmic and cytoplasmic cell compartments.[89] The biological function of this post-translational modification is not yet fully understood. There is, however, evidence that a dynamic equilibrium between *O*-GlcNAc glycosylation and phosphorylation of serine or threonine residues influences the nucleocytosolic transport of glycoproteins, e.g., RNA II polymerase.[89] Other important biological aspects of *O*-GlcNAc glycosylation (e.g., Alzheimer's disease) have also been discussed. *O*-GlcNAc-containing peptides were the first synthetic glycopeptides ever prepared.[90] Despite this tradition, building blocks of general applicability were not described until the beginning of the 1990s. Since that time, temporary 2-amino protections were employed in order to facilitate a mild and efficient glycosylation of serine and threonine derivatives.[91,92] A selection of suitable GlcNAc donors are depicted in Chart 4.

Scheme 16



Scheme 16

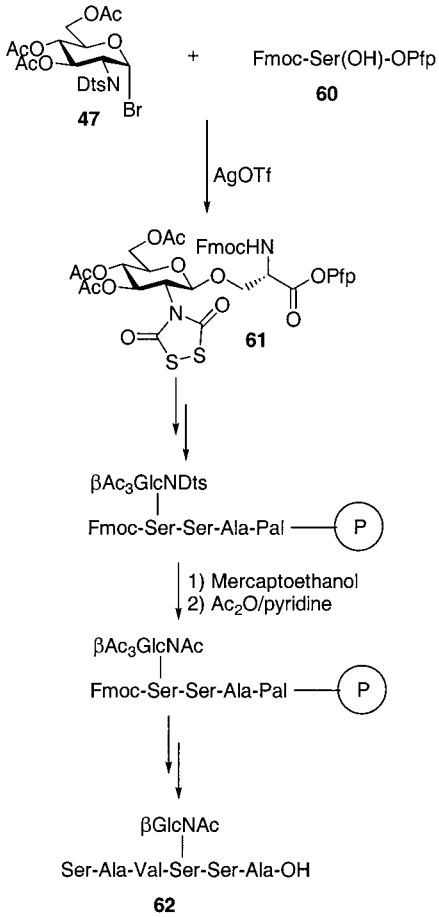
An elegant new method for the synthesis of *O*-glycosides containing the naturally occurring 2-acetamido group has recently been published. Starting from glycals, this one-pot reaction directly furnished the corresponding C2-acetamidoglycosides. However, this procedure has not yet been used for the synthesis of glycopeptides.[93]

A building block with a free carboxy group was obtained from oxazoline **54**.[94] The resulting *p*-(phenylacetoxy)benzyloxycarbonyl (PhAcOZ) protected GlcNAc-serine derivative **55** (Scheme 16) was employed for the solution-phase synthesis of a bisglycosylated and phosphorylated fragment of RNA polymerase II.[95] First **55** had to be modified: the C-terminal carboxylic acid was converted into the *tert*-butyl ester, and the urethane was cleaved enzymatically using penicilline G acylase (→ **56**). Dipeptide GlcNAc fragment **57** was obtained after condensation of **55** with proline allyl ester and cleavage of the C-terminal protection. Coupling of both GlcNAc compounds (**58**), *N*-terminal deprotection, introduction of a phosphorylated serine residue, and deprotection gave target **59**.

A solid-phase synthesis of a GlcNAc-containing hexapeptide started with the *N*-dithiasuccinoyl-protected donor **47**.[96] Glycosylation of Fmoc-serine pentafluorophenyl ester **60** gave *β*-configurated compound **61** (Scheme 17). SPPS on polyethylene−polystyrene resin with acid-labile anchoring (PAL) was accomplished using diisopropylcarbodiimide-(DIC)/HOBt as coupling reagent. After incorporation of **61**, the *N*-Dts group had to be exchanged due to its sensitivity toward piperidine or even morpholine. Treatment with mercaptoethanol/DIPEA and acetic anhydride gave the *N*-acetylated derivative. The fully assembled peptide was detached and deprotected with TFA and sodium methoxide (→ **62**).

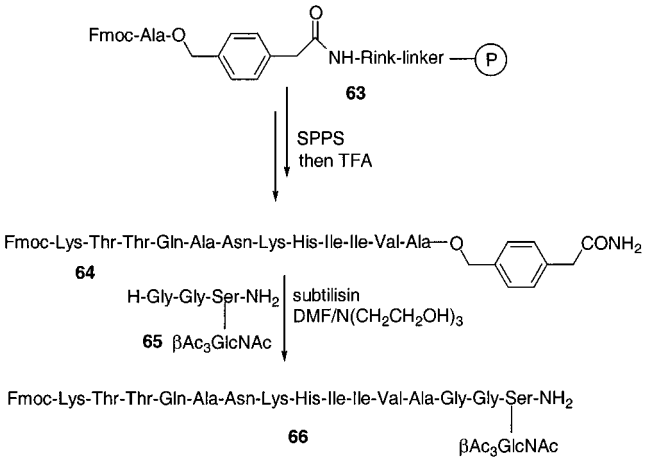
An interesting chemoenzymatic approach for the formation of larger fragments of *O*-GlcNAc-containing peptides was proposed.[97] The strategy applied is

Scheme 17

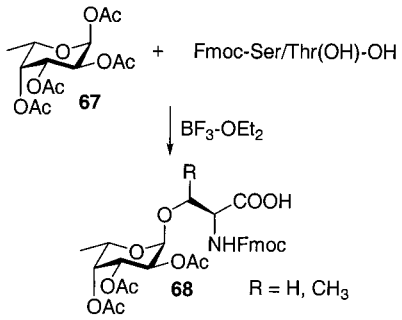


based on the subtilisin-catalyzed aminolysis of peptide benzyl-type esters by peptides bearing a free amino group (Scheme 18). The required peptide PAM (*p*-(hydroxymethyl)phenylacetamide) ester **64** was obtained by introducing the starting amino acid via a PAM linker to the Rink−amide resin (**63**). After the assembly of the peptide, TFA-mediated detach-

Scheme 18



Scheme 19



ment furnished dodecapeptide PAM amide **64**. This served as a substrate for the subtilisin-catalyzed reaction with GlcNAc-containing tripeptide amide **65**. To decrease the competing hydrolysis reaction, the ratio of DMF/buffer in the solvent was increased to 9:1.

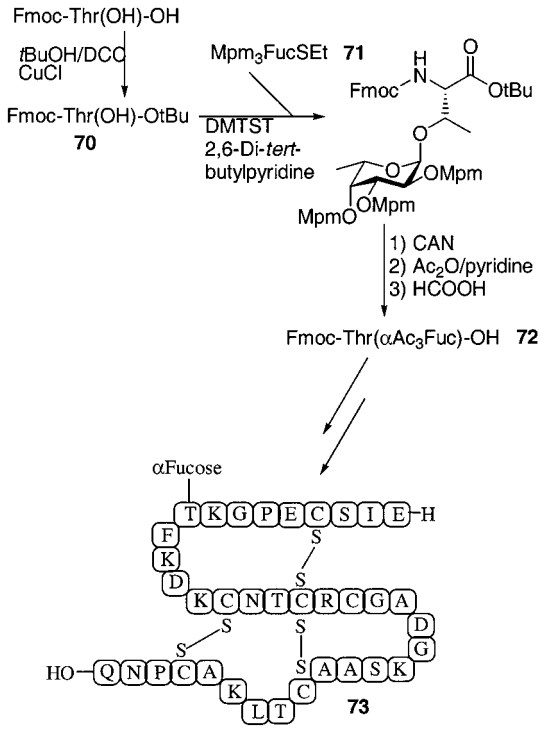
D. *O*-Fucosylated Glycopeptides

Although the occurrence of *O*-fucosylated proteins has rarely been described, this structure is related to natural products of particular biological relevance. L-Fucose α-linked to serine or threonine has been found in human factor IX, in an insect neuropeptide, and in epidermal growth factor (EGF) domains of various coagulation and fibrinolytic proteins.[98] This background motivated the synthesis of fucose-carrying glycopeptides in order to investigate the biological function of the *O*-fucosidic residue.

Rapid access to the required fucosylated serine or threonine building blocks is provided by treating a mixture of peracetylated fucose **67** and Fmoc-Ser/Thr-OH with borontrifluoride etherate (Scheme 19).[99] In the course of this transformation, the β-fucoside formed at first rearranges to the thermodynamically more stable α-fucoside **68**, which can be obtained in a yield of 35% (R = CH_3) and 44% (R = H), respectively. However, the resulting mixtures have to be purified by HPLC. This opposes the scaling up of this one-step procedure.

Pure α-fucosides are obtained by the in-situ anomerization reaction using benzyl ether-type protected fucosyl donors. Because the *O*-glycosidic linkage of *O*-benzyl-protected fucose is distinctly sensitive toward acidic conditions (e.g., TFA), an exchange of

Scheme 20



O-benzyl for *O*-acetyl protection has to be carried out. Two similar protocols providing the required αAc$_3$-Fuc-Ser/Thr building blocks were reported almost simultaneously. In one approach, Fmoc-protected threonine *tert*-butyl ester **70** (Scheme 20) was glycosylated with *O*-methoxybenzylated thiofucoside **71**.[100] In the resulting α-fucosylated threonine, the hydroxy groups were deprotected with ceric ammonium nitrate and acetylated prior to the acidolysis of the *tert*-butyl ester. Building block **72** was then incorporated into the SPPS of a 36 amino acid neuropeptide. Wang resin served as the solid support and BOP[54] (see section I) as coupling reagent. In a methyldiethylamine/oxygen-mediated reaction, three sulfide bridges were formed whereby the *O*-acetyl groups were simultaneously removed (→ **73**).

In a similar approach, tri-*O*-benzylated fucosyl bromide (Scheme 21) was used to glycosylate Z-protected serine benzyl ester (→ **74**).[101] All protections could then be removed by hydrogenolysis, and Fmoc was introduced at the *N*-terminus. Formation of the allyl ester, acetylation, and allyl ester removal gave the target building block **75**. Direct acetylation of the carboxy intermediate was not feasible.

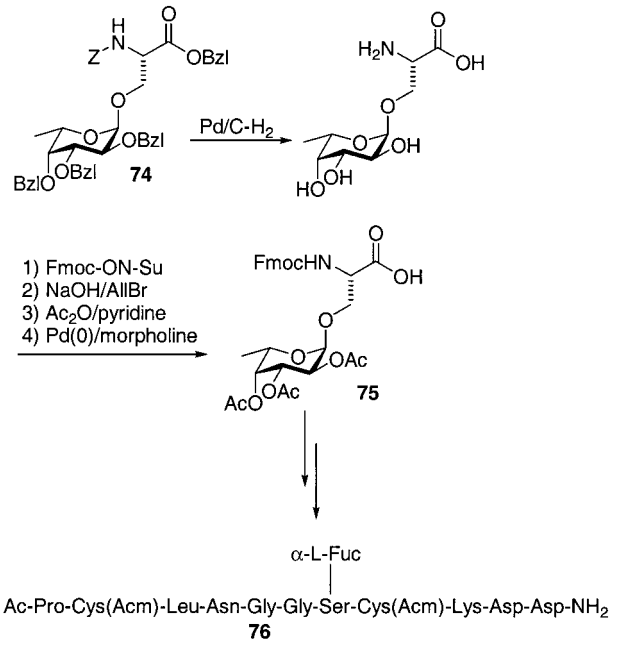
With the help of the Pfp/Dhbt-OH methodology, the fucosylated undecapeptide **76** from human factor IX was obtained. Deprotection of the target molecule was not described.

Interestingly, it was observed that partial acetylation of the fucose moiety was sufficient to prevent the TFA-promoted cleavage of the fucosidic bond[45] during the detachment/deprotection procedure.

E. *O*-Glycopeptides Carrying the Sialyl−Tn Antigen (αNeuNAc-2,6-αGalNAc)

The biological relevance of the sialyl−Tn antigen and the first successful synthetic approach toward

Scheme 21



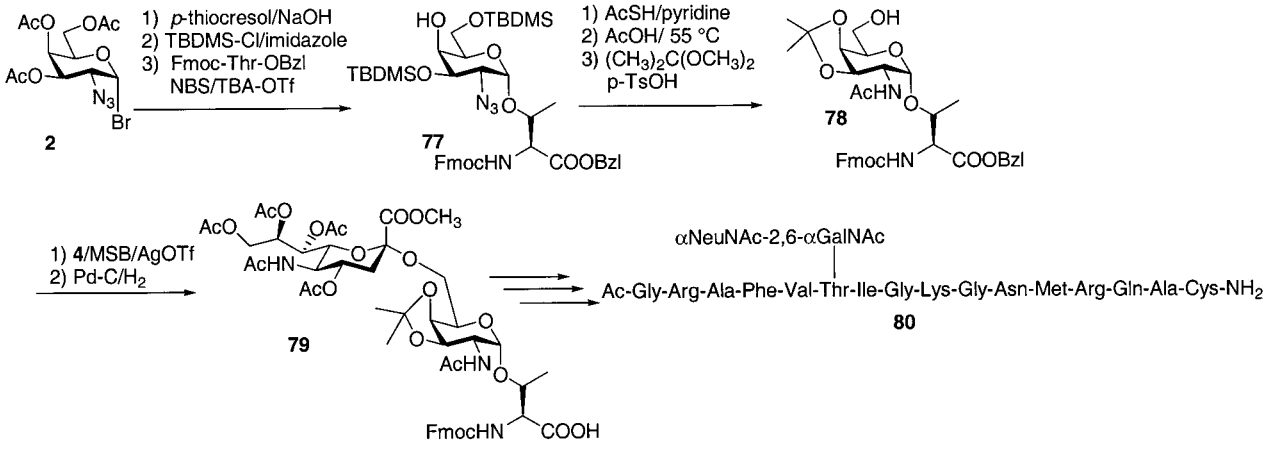
STn-containing glycopeptides are described in the Introduction.[102]

A different strategy toward the sialyl−Tn building block for solid-phase synthesis was reported at the same time.[43,103] Herein, sterically demanding silyl ether protection of the galactose moiety was applied. First, 2-azidogalactosyl bromide **2** was converted into the deacetylated 1-(*p*-cresylthio)glycoside by treatment with *p*-thiocresol and sodium hydroxide (Scheme 22). Subsequently, reaction with *tert*-butyldimethylsilyl chloride (TBDMSCl) and imidazole gave the 3,6-disilylated donor derivative. Glycosylation of Fmoc-protected threonine benzyl ester was achieved via the 1-bromo galactosyl intermediate using NBS and tetrabutylammonium triflate (TBA-OTf, → **77**). The following reduction of the azide turned out to be difficult. Only a mixture of thioacetic acid in pyridine gave the 2-*N*-acetamide in satisfying yields. Removal of the TBDMS groups and 3,4-isopropylidenation furnished suitable acceptor **78** that was sialylated at the 6-hydroxy group employing sialyl xanthate **4**. Hydrogenolysis gave target building block **79**, which was incorporated into the SPPS of a glycopeptide fragment from HIV gp 120. Polystyrene/PEG polymer, an acid-labile linker, and DIC/HOBt as coupling reagents were used. The STn derivative was coupled via its azabenzotriazolyl ester (DIC/HOAt). After completion of the solid-phase synthesis, the glycopeptide was detached from the resin with TFA. Finally, the *O*-acetyl groups and the methyl ester were cleaved by careful treatment with sodium methoxide and aqueous sodium hydroxide, respectively (→ **80**). Due to the C-terminal cysteine residue, about 1% of the disulfide derivative was formed.

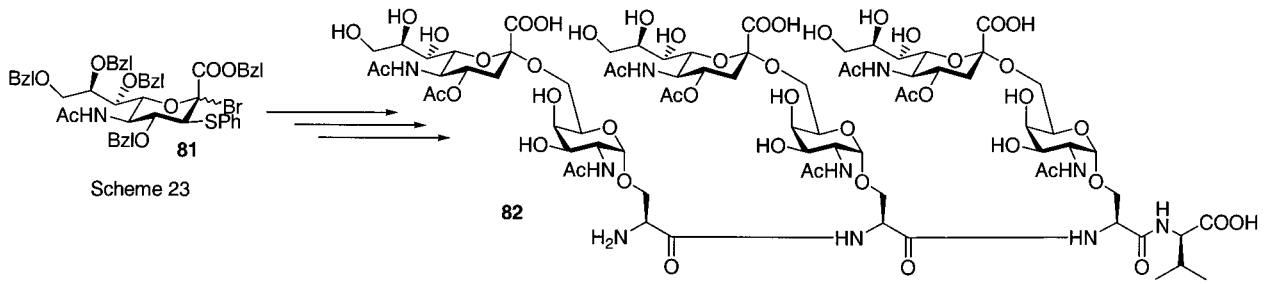
It should be mentioned that the solution-phase synthesis of a tetrapeptide bearing three sialyl−Tn epitopes has been described earlier.[44,104] In this context, sialic acid donor **81**, carrying a 3-thiophenyl substituent, was used to ensure the stereoselective formation of the required building block precursor (Scheme 23).

Almost the same tris-sialyl−Tn derivative has been mentioned only recently;[105] however, no details about the synthetic strategy were given.

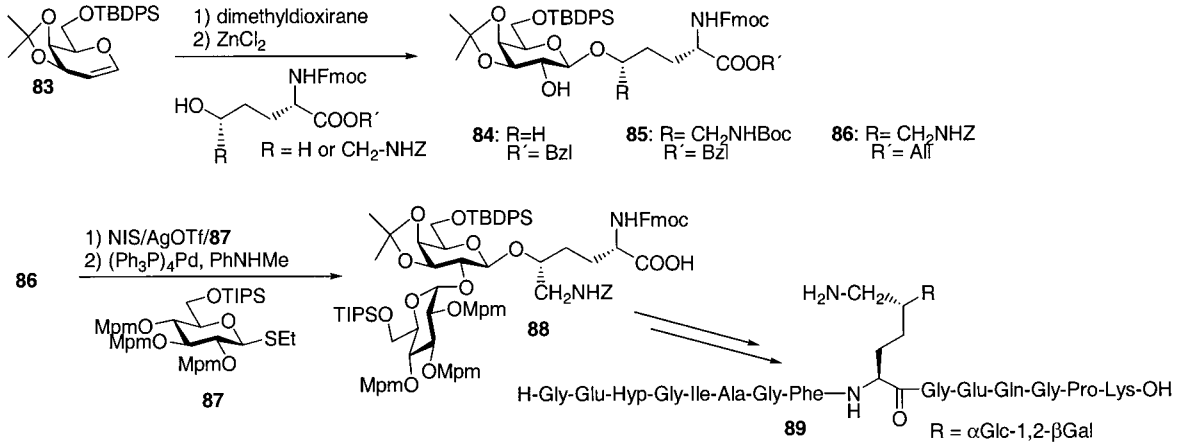
Scheme 22



Scheme 23



Scheme 24



F. Collagen Type II Derived Glycopeptides Carrying β-Gal and αGlc-1,2-βGal Side Chains

For studies concerning the T-helper-cell-induced immune response by type II collagen, hydroxylysine- or hydroxynorvaline-containing peptides with β-galactose or αGlc-1,2-βGal side chains were synthesized (Scheme 24).[106,107]

The β-galactosylated hydroxylysine/norvaline building blocks were prepared from the oxirane of glycal **83** in a $ZnCl_2$-mediated glycosylation. It should be noted that hydroxylysine is a precious compound that has to be converted into a suitable acceptor in a four-step reaction sequence. In the case of the galactosylated hydroxynorvaline unit (**84**), the applied strategy allowed direct α1,2-glucosylation with thioaryl donor **87**. Interestingly, conditions for the subsequent hydrogenolysis of the benzyl ester were found that neither affected the *O*Mpm ethers nor the Fmoc group. Various attempts to couple the glucosyl residue to the galactosylated hydroxylysine derivative **85** failed as well because of the lability of the Boc group under the acidic conditions required for the glycosylation. Recently, utilization of the more stable Z-group instead of the Boc group furnished diglycosylated hydroxylysine **88** in 47% yield.[108]

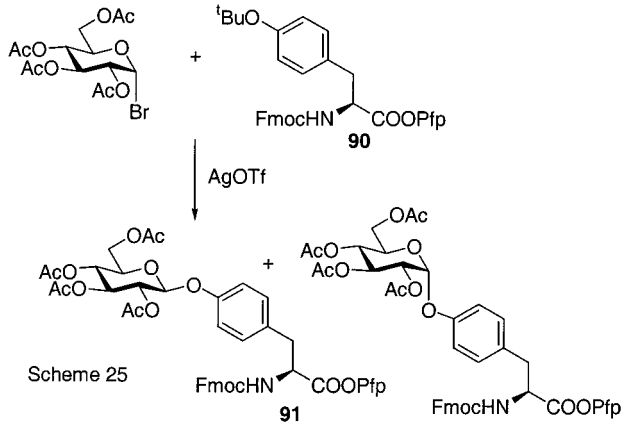
Using building block **88**, Tentagel−Wang resin, and DIC/HOBt or DIC/HOAt as coupling reagents, collagen II-derived glycopeptides were successfully synthesized. Detachment from the resin and deprotection (→ **89**) was achieved in one step as the Z-group could be cleaved simultaneously with the other acid-labile protecting groups by treatment with TFA and thioanisole as nucleophile.[109]

Subsequent biological investigations revealed that a glycopeptide from type II collagen having a centrally located β-Gal-Hyl structure was recognized by the majority of the autoimmune T-cells obtained in a mouse model for rheumatoid arthritis.[107]

G. Glycopeptides Containing Tyrosine *O*-Glycosides

There are only few reports about the chemical synthesis of tyrosine-*O*-glycosides in the literature. On the one hand, tyrosine residues carrying saccharide side chains are seldom found in nature;[110−113] on the other hand, chemical glycosylation of tyrosine often results in moderate yields or requires harsh reaction conditions due to the low nucleophilicity of phenolic hydroxy groups.

Scheme 25



Substantial progress was achieved by the use of tyrosine *tert*-butyl ether **90** (Scheme 25) as glycosyl acceptor, which was reacted with glycosyl halides under Koenigs−Knorr conditions. The resulting *O*-pentafluorophenyl-protected building block **91** could be used directly for the solid-phase synthesis of glycopeptides.[114]

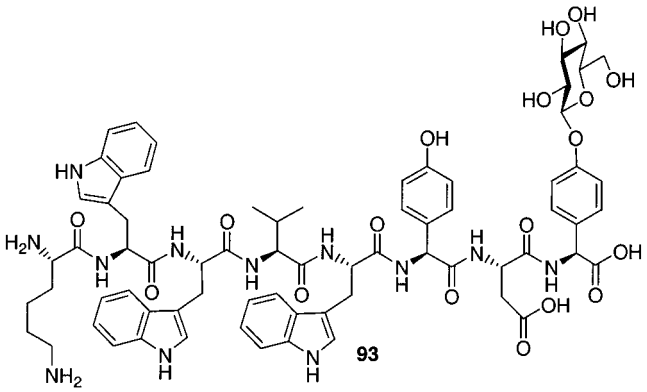
This strategy was demonstrated in the synthesis of a NK_2 tachykinin receptor antagonist.[115] The glycosylated model peptide **92** was obtained by a solid-phase protocol based on Fmoc methodology (Chart 5).

In a similar approach, Fmoc-protected tyrosine trichlorophenyl esters were glycosylated with peracetylated glucose in a BF_3-promoted reaction.[116] The resulting active esters were applied in the solid-phase synthesis of glycopeptides related to dermorphine, a morphinomimetic heptapeptide.

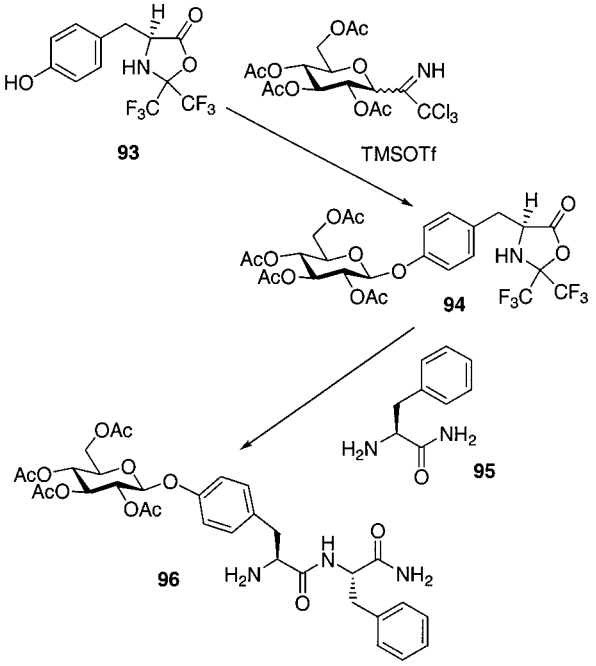
Recently, a new strategy for the synthesis of glycosylated tyrosine building blocks was published.[117] This strategy circumvents multistep procedures by employing a cyclic protection for both amino and carboxy function. For this purpose, the amino acid was reacted with an excess of hexafluoroacetone to yield the 2,2-bis(trifluoromethyl)-1,3-oxazolidine-5-one **93** (Scheme 26).

Glycosylation of **93** was favorably performed with glycosyl trichloroacetimidates. The resulting oxazo-

Chart 5



Scheme 26



lidinone (e.g., **94)** and the amino acid amide **95** were converted into glycosylated dipeptide **96**, which can be further elongated at the free amino group.

III. O-Glycopeptides with Complex Carbohydrate Structures

Despite the development of methodologies in glycopeptide synthesis, only a few syntheses of *O*-glycopeptides carrying a complex carbohydrate side chain were described. A summary of the different approaches that have taken place during the past few years will be given below.

A. *O*-Glycopeptides Containing Core Structures

A characteristic feature of mucin-type core carbohydrates is the linking region of the protein backbone with the saccharide side chain. The first saccharide is always 2-acetamido-2-deoxy-D-galactose (T_N antigen), which is α-glycosidically linked to the hydroxy group of threonine or serine building the inner part of several characteristic saccharide core chains. So far, eight different core structures have been identified: Core 1, βGal-1,3-αGalNAc-1-Thr(Ser) (T antigen); Core 2, βGal-1,3-[βGlcNAc-1,6-]αGalNAc-1-Thr-(Ser); Core 3, βGlcNAc-1,3-αGalNAc-1-Thr(Ser); Core 4, βGlcNAc-1,3-[βGlcNAc-1,6-]αGalNAc-1-Thr(Ser); Core 5, αGalNAc-1,3-αGalNAc-1-Thr(Ser); Core 6, βGlcNAc-1,6-αGalNAc-1-Thr(Ser); Core 7, αGalNAc-1,6-αGalNAc-1-Thr(Ser); and Core 8, αGal-1,3-αGalNAc-1-Thr(Ser). To these core structures other saccharide units of varying structure and length can be attached consisting of GalNAc, GlcNAc, Gal, Fuc, and NeuNAc.

The carbohydrate chains found on cancer-associated mucins are incomplete, resulting in prematurely terminated abnormal structures that are potential targets for diagnosis and immunotherapy of cancers.

Solid-phase methods using Fmoc-protected glycosylated amino acid building blocks are a flexible and reliable way to prepare series of glycopeptides. According to this approach,[118] "active esters" like Fmoc-AA-OPfp were glycosylated and used as building blocks in multiple-column peptide synthesis.[119,120] The synthesis of eight different *O*-glycopeptides of the mouse haemoglobin-derived decapeptide Hb (67−76) VITAFNEGLK and their photoaffinity labeled analogues was described. Varied building blocks such as β-D-GlcNAc-*O*-Ser/Thr, α-D-GalNAc-*O*-Ser/Thr, core 1, core 2, core 3, and core 4 structures were introduced instead of the central Asn-72. In addition, the thiolytic reduction of azido and *N*-dithiasuccinyl (*N*-Dts) groups on the solid support was presented.

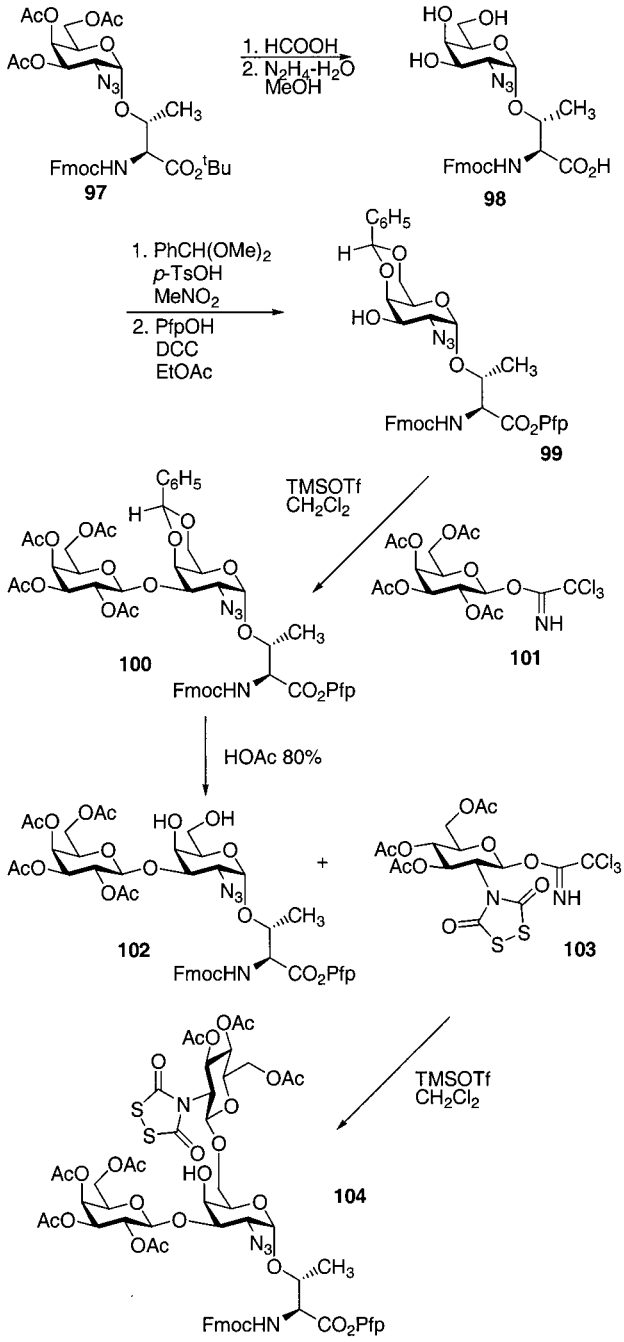
The glycopeptide synthesis was performed using either acetamido building blocks or *N*-Dts- and azido-containing building blocks.[121,122] The synthesis of core 2 building block **104** based on the *N*-Dts methodology[121] is described as an example of these syntheses (Scheme 27). Amino acid derivative **97** was transformed into the 4,6-*O*-benzylidene derivative Fmoc-Thr(α-D-GalN_3)-OPfp **99** by a four-step synthesis sequence.

The disaccharide **100** was obtained by glycosylation of **99** with 2 equiv of the peracetylated galactose imidate **101** (89%). After removal of the benzylidene group with aq. acetic acid, the *N*-Dts-protected imidate **103** was condensed with diol **102** using trimethylsilyl triflate (TMSOTf) as promotor. The regioselectively β-(1,6)-linked trisaccharide **104** was obtained in 67% yield and used as building block for solid-phase synthesis.

Macrosorb resin, equipped with the acid-labile HMPA linker, served as solid support. The use of the Pfp esters in combination with Dhbt-OH allowed monitoring of the peptide bond formation. Fmoc groups were cleaved by treatment with 20% piperidine in DMF. The base-sensitive *N*-Dts group was removed before resuming the peptide synthesis. The azido and the *N*-Dts functionality of compound **105** were reduced on solid phase (Scheme 28) by thiolysis with dithiothreitol and DIPEA in dichloromethane. The less reactive propane-1,3-dithiol in the presence of DIPEA selectively reduced the *N*-Dts group without affecting the azido group.

After subsequent *N*-acetylation with acetic anhydride in DMF, peptide synthesis was carried out, the glycopeptide deprotected, released from the solid-

Scheme 27

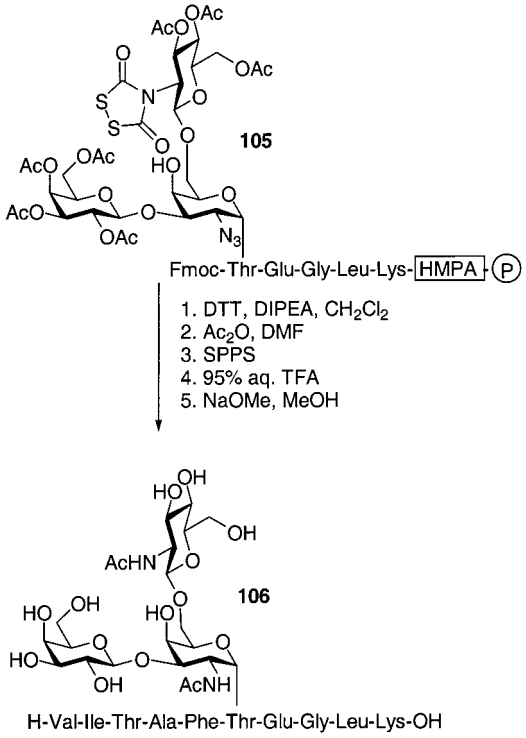


phase using 95% aq. TFA, and finally deacetylated with sodium methoxide in methanol. The decapeptide **106** containing the core 2 structure as tumor-associated antigen was obtained in a yield of 45%.

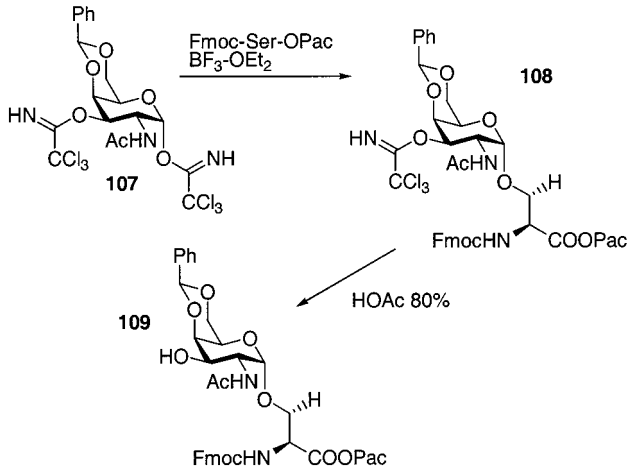
In a subsequent report[123] decapeptide sequences from repeating units of MUC1 and MUC2 were synthesized in which the threonine residues were glycosylated with core 1, core 2, core 3, core 4, or core 6 structures.

In another report the synthesis of core 5 and its sialylated analogue as glycopeptide building blocks was carried out successfully.[124] Particularly interesting herein was the use of 1,3-di-trichloroacetimidates **107** as glycosyl donors (Scheme 29). The trichloroacetimidate group on position 3 of *N*-acetylgalactosamine **107** served as acid-labile protecting group.

Scheme 28



Scheme 29



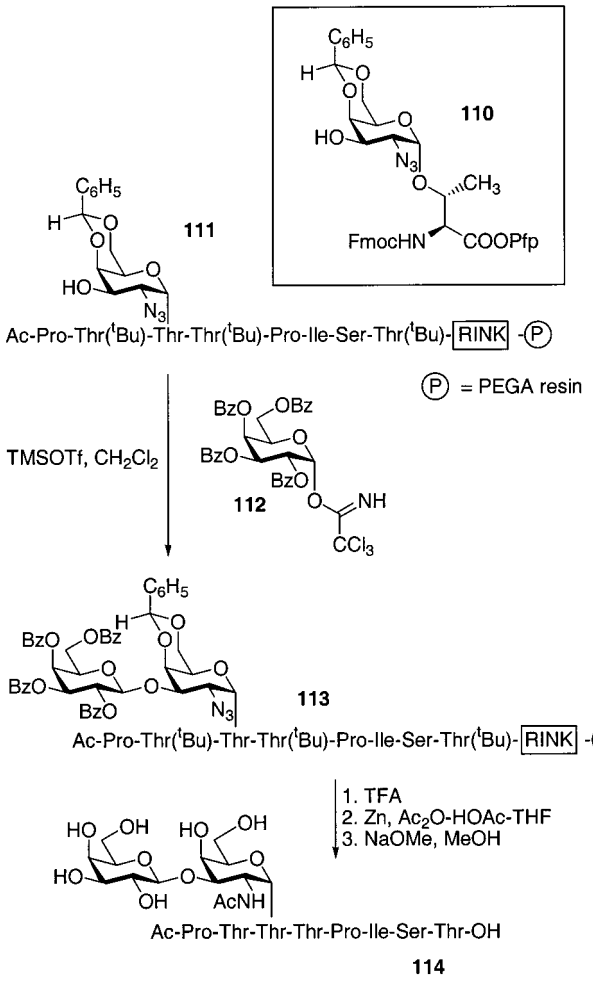
B. *O*-Glycopeptides Containing Saccharides Derived from Core Structures

Glycopeptides containing the tumor-associated F1-α structure (βGal-1,4-βGlcNAc-1,6-αGalNAc-1-Thr-(Ser)), a trisaccharide generated from core 6 structure by abnormal glycosylation, have been synthesized recently.[125,126]

An unusual strategy for the introduction of this carbohydrate moiety is discussed below.[127] A partial sequence from MUC 2 carrying 2-azido-2-deoxy-α-D-galactose as carbohydrate side chain was prepared (Scheme 30). This residue with unsubstituted 3-OH or 6-OH groups served as glycosyl acceptor on solid phase for the extension of the saccharide side chain.

Building block **110** with deblocked 3-OH group was used for the solid-phase glycopeptide synthesis providing compound **111**. The synthesis of the glycoconjugate was carried out using PEGA resin[128] deriva-

Scheme 30

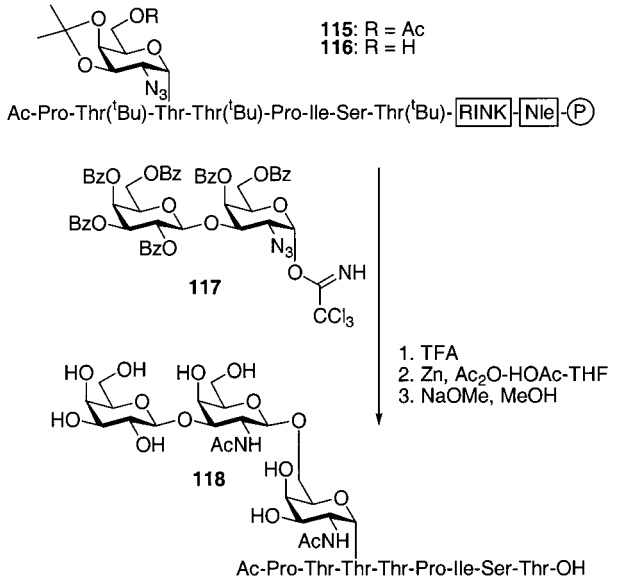


tized with acid-labile Rink linker[49] as polymer support. Coupling was performed with Fmoc-amino acid pentafluorophenyl (Pfp) or 3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl (Dhbt) esters. Using trichloroacetimidate **112** as glycosyl donor, the glycosylation took place in dichloromethane with TMSOTf as the promotor. High yields were achieved if donor **112** was used in excess (8 mol equiv). The reaction proceeded stereoselectively with neighboring-group participation and afforded only the β-glycosylated product (**113**). After cleavage from the resin with TFA, the protected glycopeptide was obtained in 67% yield.

The same peptide sequence was prepared by using other resins such as Polyhipe, Macrosorb, and Tenta Gel as solid support. The compounds immobilized by Macrosorb and Tenta Gel could not be glycosylated. On Polyhipe resin the formation of an anomeric mixture (α:β = 1:2.3) was observed. This result shows that the choice of the resin can exhibit a significant influence on the stereoselectivity of glycosylation on solid phase. Finally, the azido group of the carbohydrate moiety was reduced with activated zinc in an acetic anhydride−acetic acid−THF mixture.[121] The resulting compound was debenzoylated with catalytic amounts of sodium methoxide in methanol to give **114**.

To glycosylate the 6-OH group of GalNAc, an amino acid building block with suitable protecting group pattern was used in solid-phase peptide synthesis to

Scheme 31



provide compound **115** (Scheme 31). The acetyl group of the saccharide residue was selectively removed by mild alkaline hydrolysis, yielding glycosyl acceptor **116**. Glycosylation was performed with disaccharide **117** as glycosyl donor under the conditions described above. After cleavage from the resin, the protected peptide was obtained in a yield of 53%.[127] Deprotection was performed under standard conditions and provided trisaccharide octapeptide **118**.

C. *O*-Glycopeptides with αMan-Ser/Thr Linkages

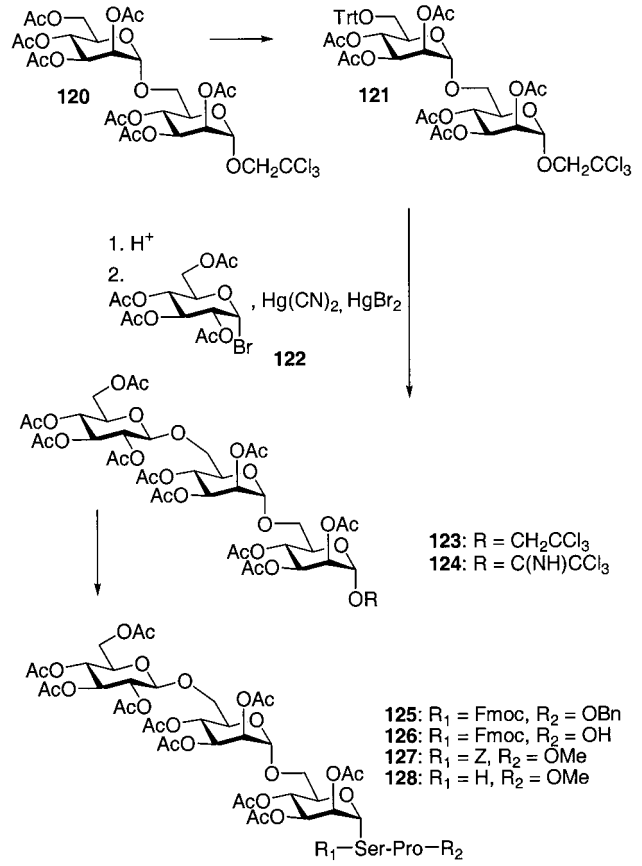
The synthesis of the hexaglycosyl tetrapeptide **119**[129] as a model compound for a phytoalexin elicitor-active glycoprotein[130] proceeded via fragment condensation in solution (Scheme 33). The target structure contains a partial structure in which the reducing mannosyl residue of a trisaccharide is *O*-glycosidically attached to serine.[131]

Mannosylbromide was coupled to the acceptor 2,2,2-trichloroethyl-2,3,4-tri-*O*-acetyl-α-D-mannopyranoside in the presence of $Hg(CN)_2$ and $HgBr_2$ in dichloromethane (Scheme 32). The disaccharide **120** was deacetylated, tritylated at 6′-OH, and reacetylated to give compound **121**. After detritylation, the disaccharide was glycosylated with readily available glucosyl bromide **122** in the presence of $Hg(CN)_2$ and $HgBr_2$. The trichloroethyl group was eliminated using zinc−copper in acetate buffer, and the resulting hydroxyl group was transformed into trichloroacetimidate **124**.

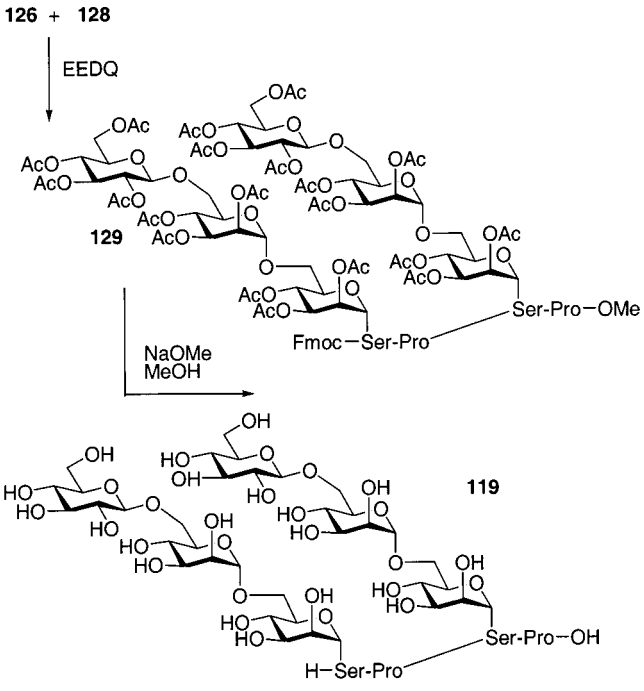
The BF_3−OEt_2-mediated reaction of trisaccharide trichloroacetimidate **124** with Fmoc- and benzyl-protected dipeptide Fmoc-Ser-Pro-Bn gave trisaccharide derivative **125**. A second building block was obtained by coupling of **124** with Z- and methyl-protected dipeptide Z-Ser-Pro-OMe. After deprotection, condensation between the corresponding dipeptides (**126** and **128**) took place in the presence of EEDQ in dichloromethane (Scheme 33). Removal of the protecting groups with NaOMe in MeOH afforded the desired tetrapeptide **119**.

Recently, the synthesis of a tetrasaccharide building block containing an α-*O*-mannosyl serine/threo-

Scheme 32

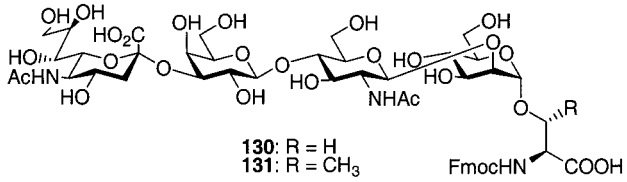


Scheme 33

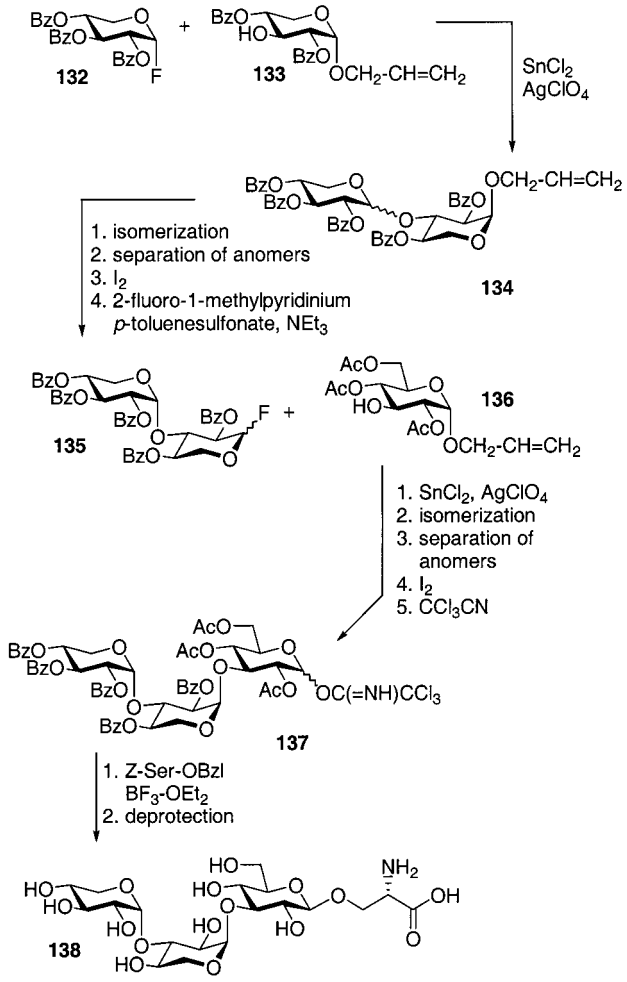


nine linkage was reported (Chart 6).[132] The saccharide moiety αNeuNAc-2,3-βGal-1,4-βGlcNAc-1,2-αMan-Ser/Thr (**130**/**131**) is the major constituent of the *O*-linked carbohydrates of α/β-dystroglycan complex and contributes to laminin binding.[133] An incorporation of this compound into a peptide sequence has not yet been reported.

Chart 6



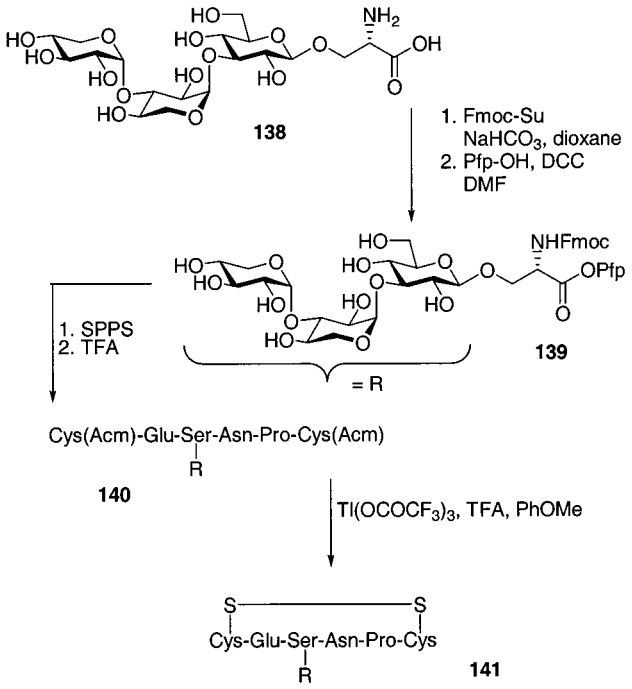
Scheme 34



D. *O*-Glycopeptides with βGlc-Ser Linkage

Blood clotting factor IX is a plasma glycoprotein which is involved in the blood coagulation cascade.[134,135] The glycopeptide sequence containing the trisaccharide α-D-Xyl-1,3-α-D-Xyl-1,3-β-D-Glc and corresponding to residues 51−56 was synthesized using the Fmoc/Dhbt method.[136] The synthesis of the unprotected carbohydrate building block had been reported earlier (Scheme 34).[137] It started with the xylosyl fluoride **132**. The glycosylation of α-allyl xyloside **133** with **132** using tin(II) chloride and silver perchlorate as glycosylating agents led to an anomeric mixture of the disaccharide **134** (α:β = 1:0.6). Isomerization of the allyl group using an iridium complex[138] gave 1-*O*-propenyl glycosides. Subsequently, the separation of the anomers was possible. The 1-propenyl group was cleaved with iodine. Conversion into fluoride **135** was carried out with 2-fluoro-1-methylpyridinium *p*-toluenesulfonate and trieth-

Scheme 35



ylamine (α:β = 3.2:1). The allyl glycoside **136** was then glycosylated with **135** to give the α-linked trisaccharide preferentially (α:β = 3.7:1).

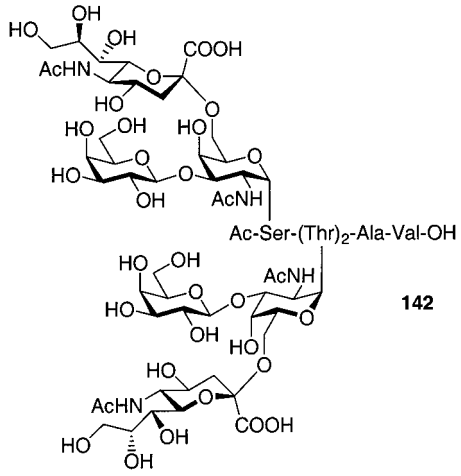
The allyl group was isomerized to facilitate the separation of the xyloside anomers. The removal of the 1-*O*-propenyl group was followed by reaction with trichloroacetonitrile in a yield of 94% (→**137**). After glycosylation of Z-Ser-OBzl with trichloroacetimidate **137**, final removal of all the protecting groups afforded the desired *O*-glycosyl serine **138**.

Since glycoconjugate **138** was available only in small quantities, the hydroxy groups of the carbohydrate residue were left unprotected in order to limit the number of synthetic steps. The serine residue was protected with the Fmoc group, and its carboxy function was transformed into the Pfp ester (Scheme 35). In the solid-phase peptide synthesis, only 0.37 equiv of the valuable trisaccharide amino acid building block **139** was used to afford an efficient incorporation of the glycosylated amino acid. The unreacted amino functions were not capped during or after the synthesis to avoid acetylation of the sugar hydroxy groups. The cysteine residues were protected with the acetamidomethyl (Acm) group to protect them from oxidation.

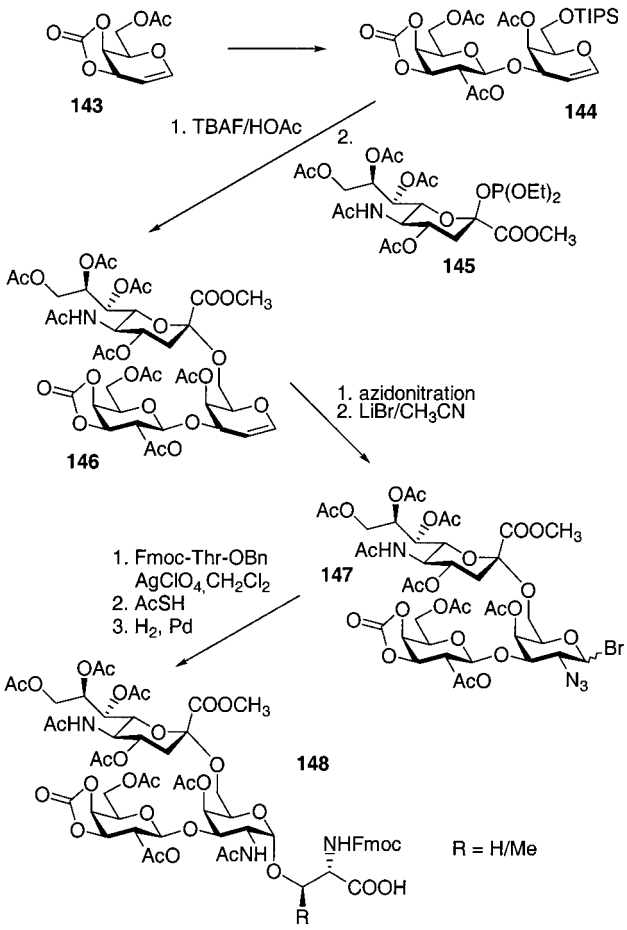
Treatment with 95% TFA resulted in cleavage of the glycopeptide **140** from the resin with simultaneous removal of the *tert*-butyl and Acm protection. The glycopeptide **140** was purified by means of gel filtration chromatography and repeated reversed-phase HPLC and isolated in a yield of 24% (based on the amount of building block **139** consumed).

The cyclic peptide **141** was finally obtained by an intramolecular oxidation procedure[139] with thallium-(III) trifluoroacetate and TFA in 75% yield.

Chart 7



Scheme 36



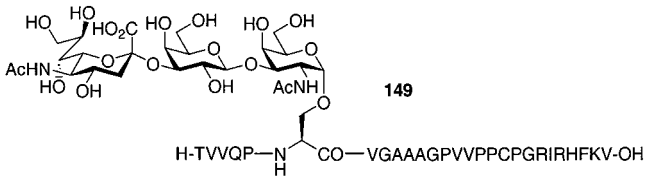
E. *O*-Glycopeptides Containing Sialyl-T Structures

1. O-Glycopeptides Containing the 2,6-Sialyl-T Antigen

The synthesis of a CD43-derived glycopeptide[140] with clustered 2,6-sialyl-T antigen epitopes (**142**) was recently described (Chart 7).[141,142]

The construction of the glycosylated amino acid building block corresponding to the 2,6-STF antigen was carried out using the glycal approach.[143] The complete glycan was assembled before it was coupled to a serine or threonine residue (Scheme 36). The 6-*O*-TIPS galactal was reacted with compound **143**

Chart 8



to provide disaccharide **144**. After removal of the silyl group, sialylation was performed with the sialyl diethyl phosphite[41,144] **145** and TMSOTf as the promoter in 84% yield (α:β = 4:1). Trisaccharide **146** was azidonitrated[30] and converted into a variety of glycosyl donors. The reaction with threonine acceptors proceeded with complete stereoselectivity. Highest yields were achieved with glycosyl bromide **147**. The reaction of **147** with protected serine led to mixtures of anomers but still provided the desired α-*O*-linked anomer as the major product.

The azido groups of the serine and threonine building blocks were reduced with thioacetic acid[145] and subsequently debenzylated (**148**).

The *O*-glycosyl amino acids were used to prepare a clustered portion of the CD43 glycopeptide **142**. The glycopeptide backbone was assembled by the use of IIDQ[146] as coupling reagent to furnish the protected pentapeptide. Deprotection yielded the desired glycopeptide **142** containing three STF antigen residues.

2. O-Glycopeptides Containing the 2,3-Sialyl-T Antigen

The first total synthesis of the native B-chain of α2HS glycoprotein[147,148] carrying the (2→3) Sialyl-T antigen (**149**) was recently reported (Chart 8).

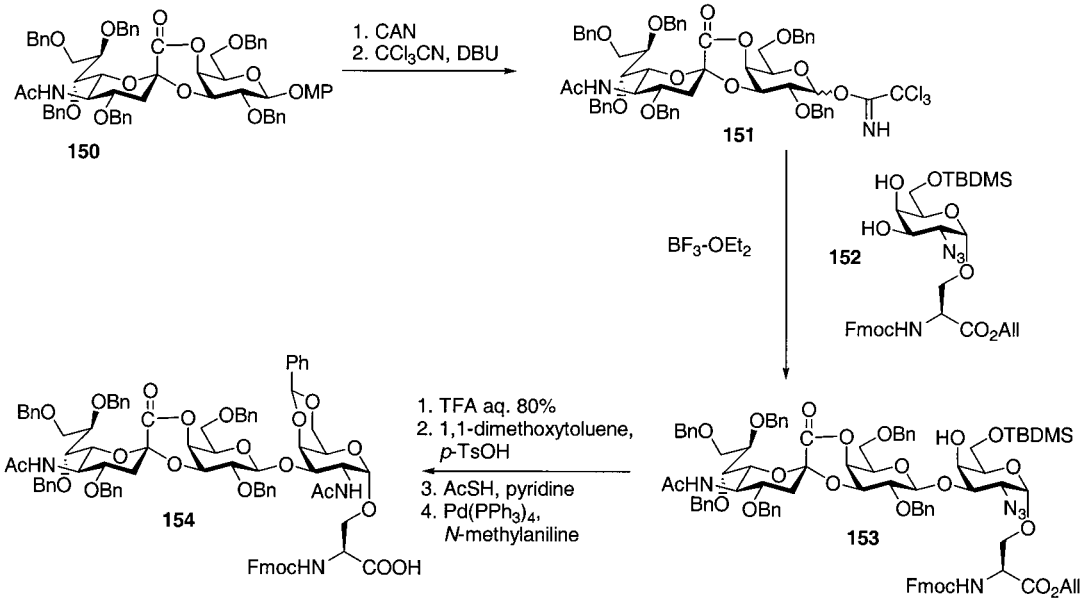
Human plasma globulin α2HS is involved in a variety of significant biological events, such as bone mineralization, endocytosis, and opsonization.[149] The synthesis was carried out using Fmoc/benzyl protecting-group strategy.[77] The glycosylated building block was synthesized as follows (Scheme 37). The preformed disaccharide **150** carrying the lactone structure[150] as internal protection was treated with ceric ammonium nitrate to afford the hemiacetal, which was converted into trichloroacetimidate **151** (α:β = 3:1). Glycosylation of the Fmoc-protected glycosyl serine allyl ester **152** with **151** in the presence of BF_3-OEt_2 yielded the sialyl-T antigen serine conjugate **153**. The compound was desilylated with 80% aq. TFA and subsequently benzylidenated. Subsequent treatment with thioacetic acid-pyridine[145,151] and Pd(0)-catalyzed cleavage[152,153] of the allyl ester afforded the desired building block **154**.

The stepwise synthesis of the 27 amino acid-containing glycopeptide was performed on HMP resin (4-hydroxymethylphenoxymethyl-polystyrene-copolymere−1% divinylbenzene). DCC/HOBt in *N*-methylpyrrolidinone served as activating agents. The glycopeptide was detached from the resin using a mixture of 95% aq. TFA−phenol−thioanisole−1,2-ethanedithiol. The cleavage was accompanied by partial debenzylation. Complete deprotection was achieved with TMSOTf−thioanisole in TFA to yield the heptacosapeptide **149** (Chart 8). The usual hydrogenolytic debenzylation procedure failed because of simultaneous desulfurization of the cysteine residues.

Recently, a related strategy for the design of glycosylated building blocks was reported.[142] Another group described a block condensation approach yielding sialyl-(2→3)-T antigen.[154]

An alternative access to sialyl-T glycopeptides is based on the cyclodextrin-assisted enzymatic glycan chain extension of a protected glycosyl building block.[155] Herein, chemical and enzymatic methods were combined to obtain a sialyl-T-containing building block efficiently. The solubility of Fmoc-Thr-(αGalNAc)-O^{t}Bu[29,102] **155** building block in water is low and had to be enhanced by the use of cyclodextrins. Application of a cascade of β-galactosidase and α2,3-sialyl transferase-catalyzed glycosylatious of **155** allowed the one-pot assembly of building block **157** (Scheme 38).

Scheme 37



Scheme 38

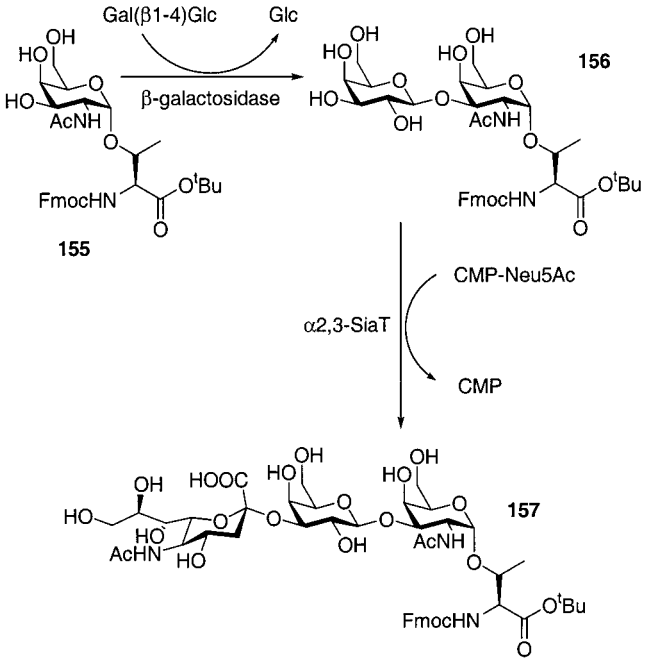
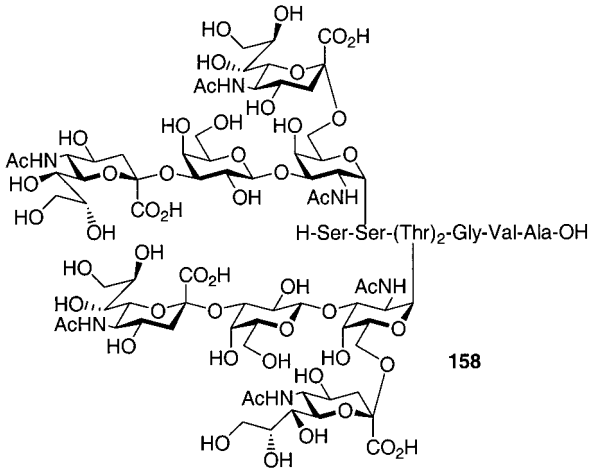


Chart 9



3. O-Glycopeptides Containing Disialylated Saccharide Chains

Glycophorin A is a major transmembrane sialoglycoprotein found in human erythrocytes. The extracellular region is highly glycosylated with tetrasaccharides containing two sialic acids.[44,156] The polymorphic *N*-terminal pentapeptide sequences correspond to the MN blood group epitopes.[157,158] The synthesis of the *N*-terminal glycoheptapeptide **158** (Chart 9) containing three complex carbohydrate side chains was carried out successfully in solution.[150] The tetrasaccharide-linked amino acid building blocks were designed for Fmoc strategy and synthesized via stereocontrolled glycosylations.

EEDQ or IIDQ served as coupling agents to elongate the peptide chain in CH_2Cl_2 as solvent, while IIDQ was favored for condensation of the amino acids carrying the carbohydrate residues. Fmoc groups were cleaved by treatment with morpholine. It should be mentioned that the reaction between the two glycosylated amino acids afforded 81% coupling yield, despite the sterical hindrance of both reaction partners. After deprotection, the target structure **158** was purified by gel permeation chromatography.

Chart 10

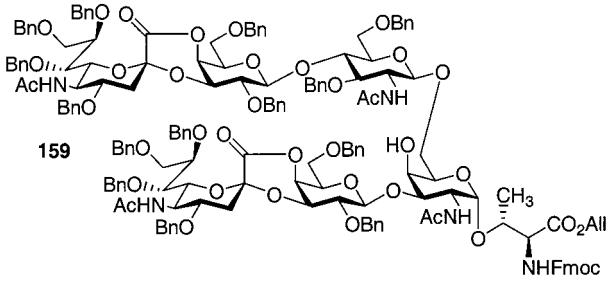
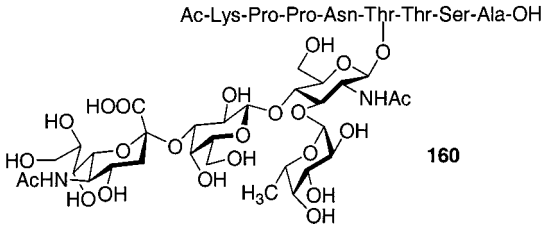


Chart 11



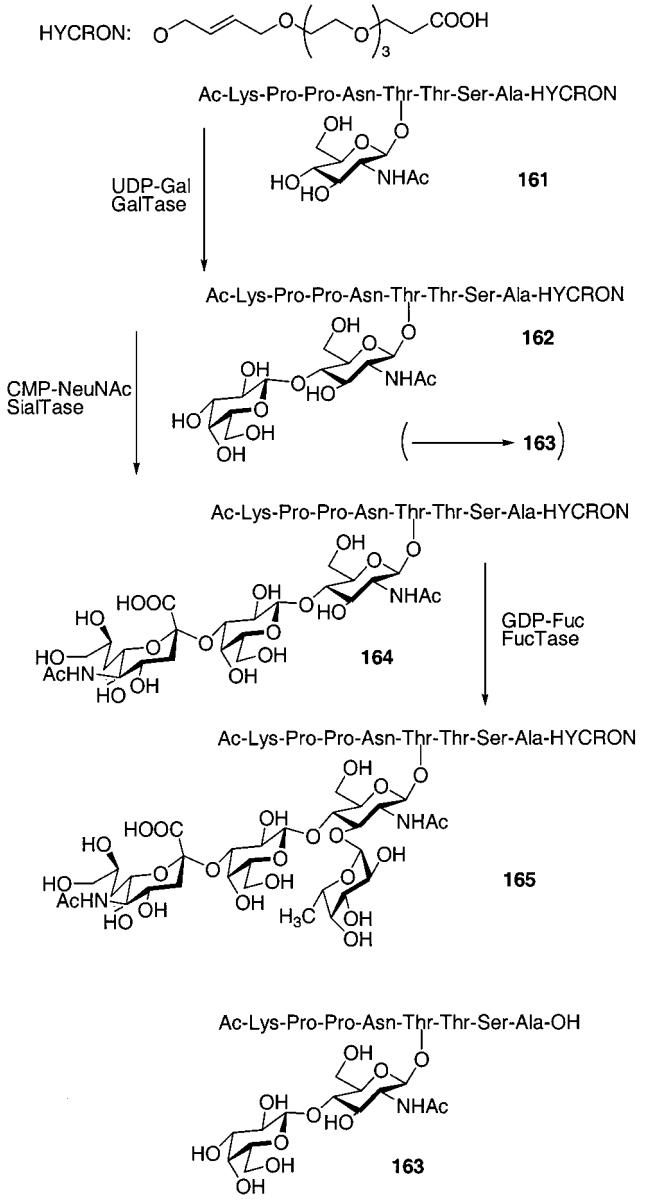
A core class II disialylated hexasaccharide **159** as building block for glycopeptide synthesis using Fmoc strategy was accomplished recently (Chart 10).[159] It is the major oligosaccharide of the cell surface glycoprotein leukosialin[160] and is associated with immunological disorders such as leukemia[161,162] and AIDS.[163,164] This complex structure has not yet been incorporated into a glycopeptide sequence.

F. *O*-Glycopeptides Containing Sialyl-Lewis-X Structures

Recently, a chemoenzymatic strategy for the synthesis of *O*-linked sialyl−Lewis-X (sLe^x) tetrasaccharide was published.[165] A variety of investigations concerning enzymatic reactions in solution and on solid phase have been described, of which—on account of their extent—only a few will be considered here in detail. Glycopeptide **160** (Chart 11) was chosen as target for the enzymatic approach containing a threonine residue carrying the sLe^x tetrasaccharide. This glycoconjugate is part of the mucin domain of mucosal addressing cell adhesion molecule-1 (MAdCAM-1), which is a ligand of P- and L-selectin. The peptide backbone was assembled employing the established techniques of solid-phase peptide synthesis via the Fmoc/^{t}Bu strategy. *O*-Protected and *O*-unprotected GlcNAc residues were incorporated by coupling the preformed glycosyl amino acids Fmoc-Thr(βAc$_3$GlcNAc)-OH and Fmoc-Thr(βGlcNAc)-OH, respectively.

The peptide backbone was assembled on PEGA resin,[128] but CPG[166] was employed as the solid support prior to the enzymatic reactions, since the PEGA resin shrank after contact with the aqueous enzyme solution. All coupling reactions were performed in DMF using HBTU and NMM. The acid- and base-stable HYCRON linker[56] was cleaved in a Pd(0)-catalyzed reaction, providing a mild and efficient method to liberate acid- and base-sensitive compounds.

Scheme 39



HYCRON conjugate **161** was used as substrate for the glycosyltransferases in solution to examine the substrate specifity of the enzymes (Scheme 39). Substrate **161** was efficiently glycosylated to give the *O*-LacNAc octapeptide **162** in 87% yield. Unexpectedly, at longer reaction times, cleavage of the allylic ester was observed (→ **163**).

After careful investigations, the preparative sialylation was performed at high concentrations of substrate CMP−NeuNAc and Triton X-100, known to randomize peptide conformations. The *O*-sialyl-LacNAc octapeptide **164** was isolated in almost quantitative yield.

The enzymatic fucosylation proceeded at pH 6.0 without concomitant ester hydrolysis observed in the sialylation step. However, it was not possible to drive the reaction to completion. After 5 days the *O*-sLe^x octapeptide conjugate **165** was isolated in 55% yield.

Subsequently, the attempts from solution synthesis were transferred to solid phase. Supported glycopeptide **166** (Chart 12) was submitted to the galactosyltransferase reaction after protecting-group removal.

Chart 12

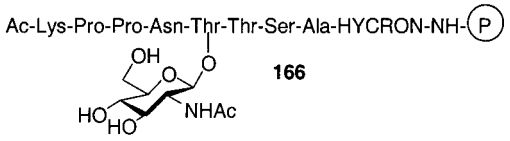
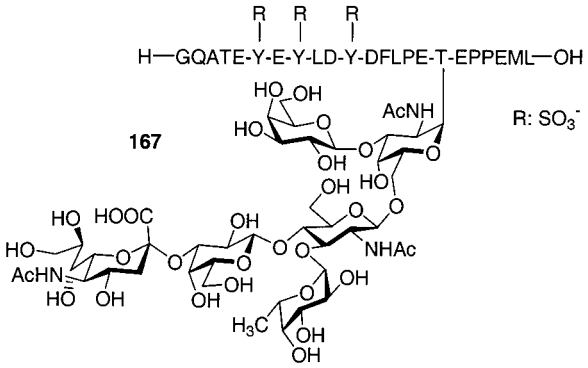


Chart 13



The complete on-resin assembly suffered from incomplete glycosylation reactions. In addition, undesired release of the glycopeptide from the ester linkage was observed during the enzymatic reactions, so that both *O*-LacNAc and *O*-sialyl-LacNAc octapeptide were found in the supernatant.

Just recently, an enzymatic synthesis of a novel glycopeptide GSP-6 **167** (Chart 13) was published.[167] This complex glycoconjugate containing three sulfated tyrosine (TyrSO_3) residues and a monosialylated, core 2-based *O*-glycan with a sialyl-Lewis^x motif at a specific threonine residue represents a partial structure of the *N*-terminus of P-selectin glycoprotein ligand-1 (PSGL-1), predicted to be important for P-selectin binding.[168−170] PSGL-1 is responsible for adhesion of leucocytes to the vascular endothelium during inflammatory diseases.

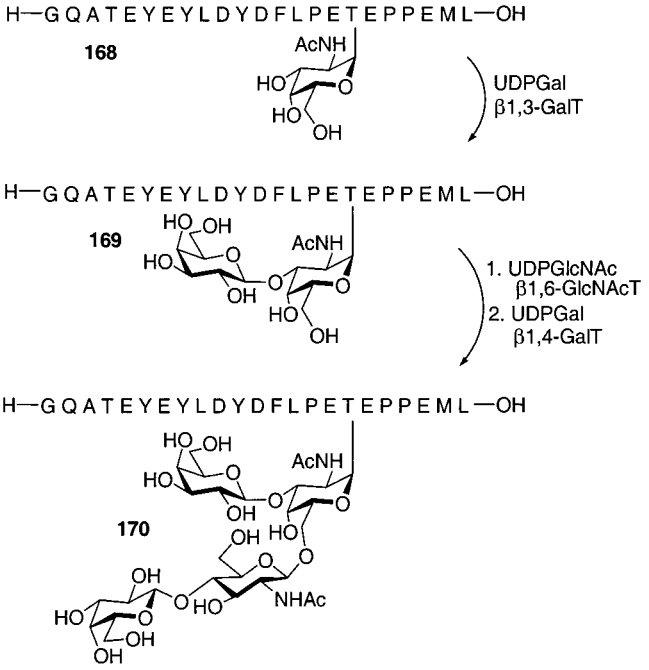
GSP-6 binds tightly to immobilized P-selectin, whereas the glycopeptide lacking either sulfate or sLe^x shows distinctly reduced affinity.

In the described synthesis[167] the peptide was assembled by standard solid-phase peptide synthesis. During the solid-phase synthesis, tri-*O*-acetylated GalNAc was incorporated into the peptide using Fmoc-Thr(α-Ac$_3$GalNAc)-OH derivative. The crude peptide was deacetylated with sodium methoxide in methanol and purified by reversed phase HPLC to yield **168**.

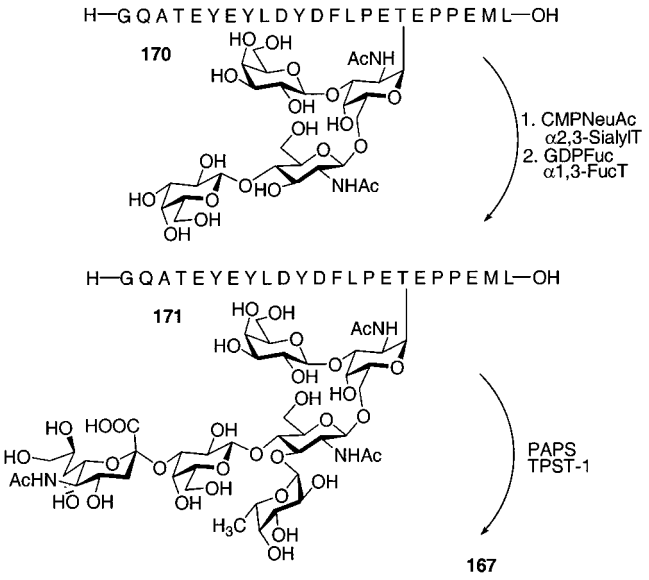
Galactosylation of **168** was carried out using UDP−Gal and purified core 1 β1,3-GalT, resulting in a yield of more than 95% (**169**) (Scheme 40).

The core 2 structure was obtained by core 2 β1,6-GlcNAcT and UDP-GlcNAc quantitatively and was further converted into the tetrasaccharide **170** by using β1,4-GalT and UDP−Gal. Glycoconjugate **170** was purified on a Sephadex G-50 column and sialylated by α2,3-sialylT and CMP−NeuAc. The reaction mixture was directly treated with α1,3-FucT and GDP−Fuc. A purified sample was analyzed by HPLC and showed complete conversion into hexasaccharide **171** (Scheme 41).

Scheme 40



Scheme 41



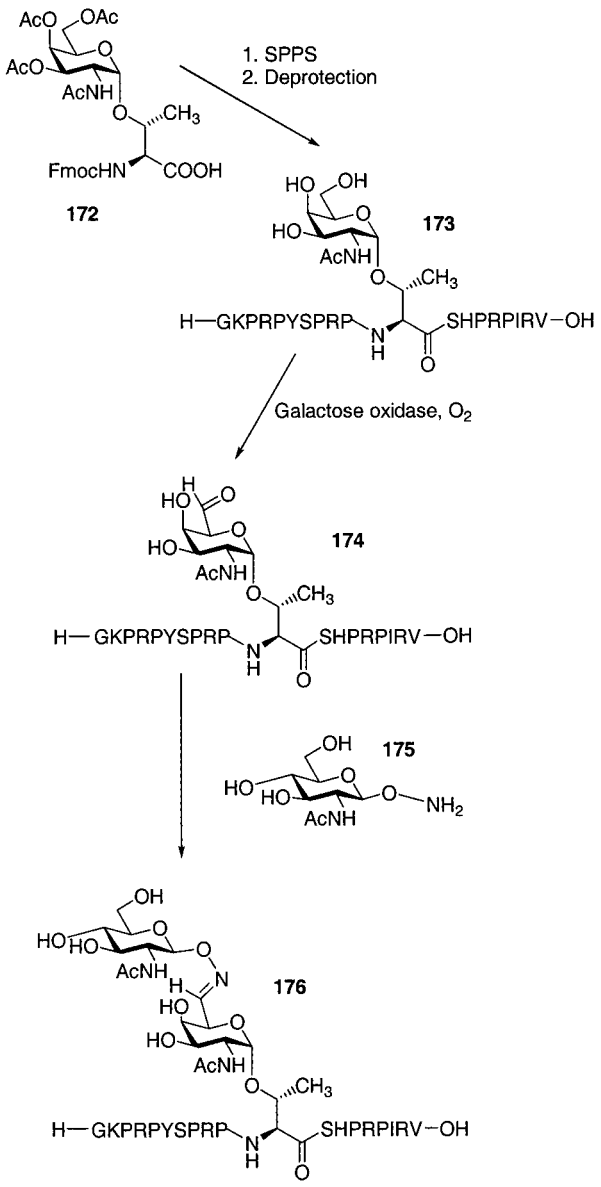
Compound **171** was sulfated using PAPS and recombinant human tyrosyl-protein sulfo-transferase-1 (TPST-1) within 35 h. After extraction of protein and detergent with chloroform/methanol (2:1), the reaction mixture was desalted.

HPLC analysis confirmed that conversion from **171** to GSP-6 (**167**) succeeded in a yield of more than 95%.

G. *O*-Glycopeptides with Unnatural Intersaccharidic Linkages

Recently, a different strategy for the chemoselective synthesis of *O*-linked glycopeptides with unnatural sugar−sugar linkages was published.[171] The synthetic pathway used the principle of chemoselective ligation[172] and afforded an oxime-linked analogue of the β-(1,6) glycosidic linkage which is frequently observed in naturally occurring *O*-linked glycans (Scheme 42).[173]

Scheme 42

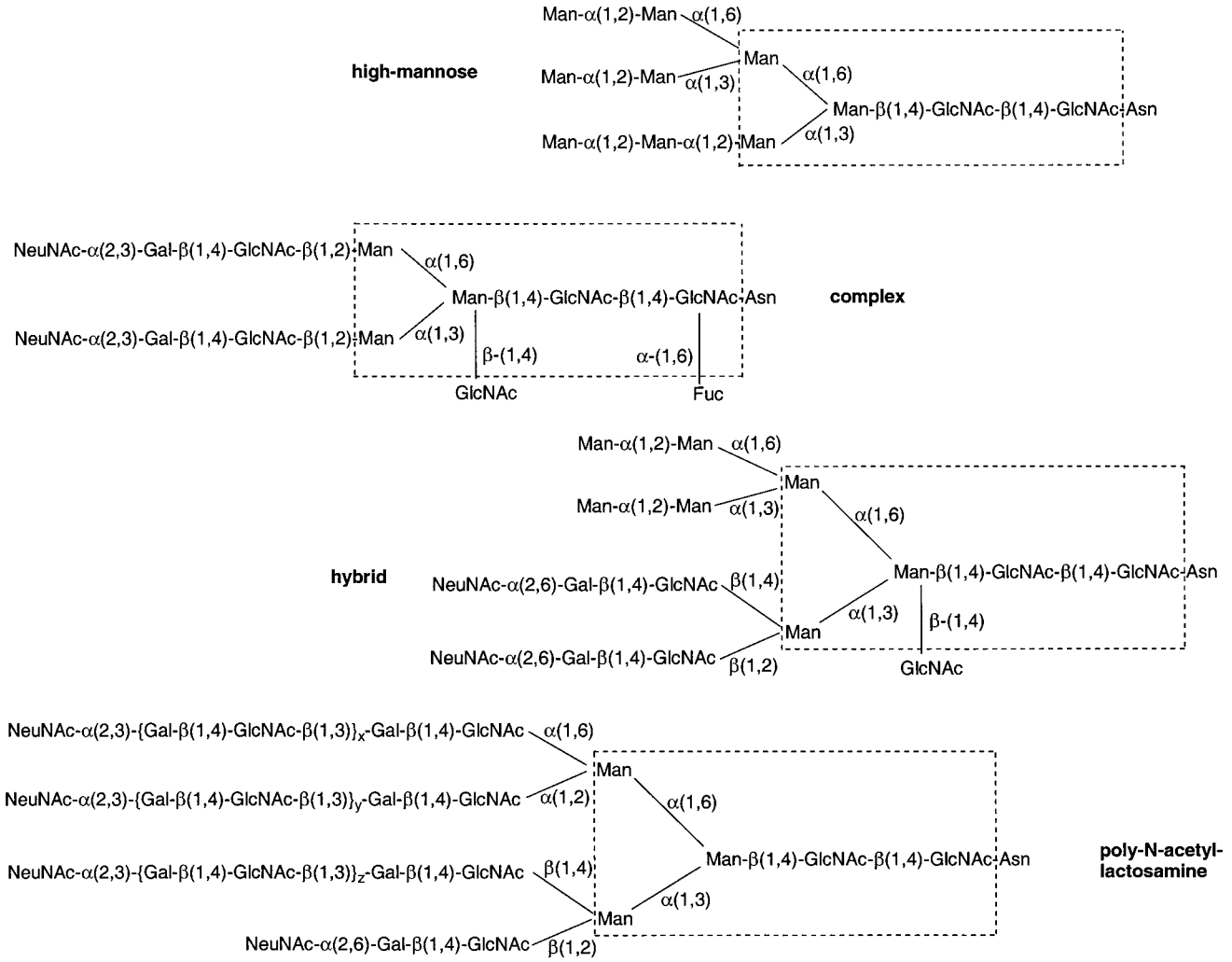


First, building block **172** [Fmoc-Thr(α-D-Ac$_3$GalNAc)]-OH was incorporated into a glycopeptide by standard solid-phase peptide synthesis (Scheme 42). The synthesis yielded the insect-derived, antibacterial 19-amino acid glycopeptide drosocin, the biological activity of which is influenced by glycosylation.[174,175] After deprotection, the GalNAc residue of **173** was oxidized with galactose oxidase to give the corresponding C-6 aldehyde **174**. The aldehyde was coupled with the hydroxylamino derivative of galactose (**175**)[176] to give the oxime **176** in a high yield. This glycan **176** mimics the naturally occurring core 6 structure and showed a 4-fold increase in the blocking of bacterial growth compared to the unglycosylated drosocin.

IV. Glycopeptides Carrying N-Linked Saccharides

The biosynthesis of natural *N*-glycopeptides occurs co- and posttranslationally in the presence of numerous enzymes, e.g., glycosyltransferases, glycosidases, epimerases, etc.[177] As a result, a single protein species

Chart 14



can exist in hundreds of glycoforms that differ in saccharide composition. Despite this microhetereogeneity, *N*-glycoproteins can be categorized into four major classes.[178] All categories share a common pentasaccharidic core structure built up from a branched trimannosyl unit which is linked to the reducing *N*,*N*′-diacetylchitobiose (Chart 14). On the basis of the structure and location of the glycan residues bound to the trimannosyl core, high-mannose, complex, hybrid, and poly-*N*-acetyl-lactosamine-type side-chains can be differentiated.

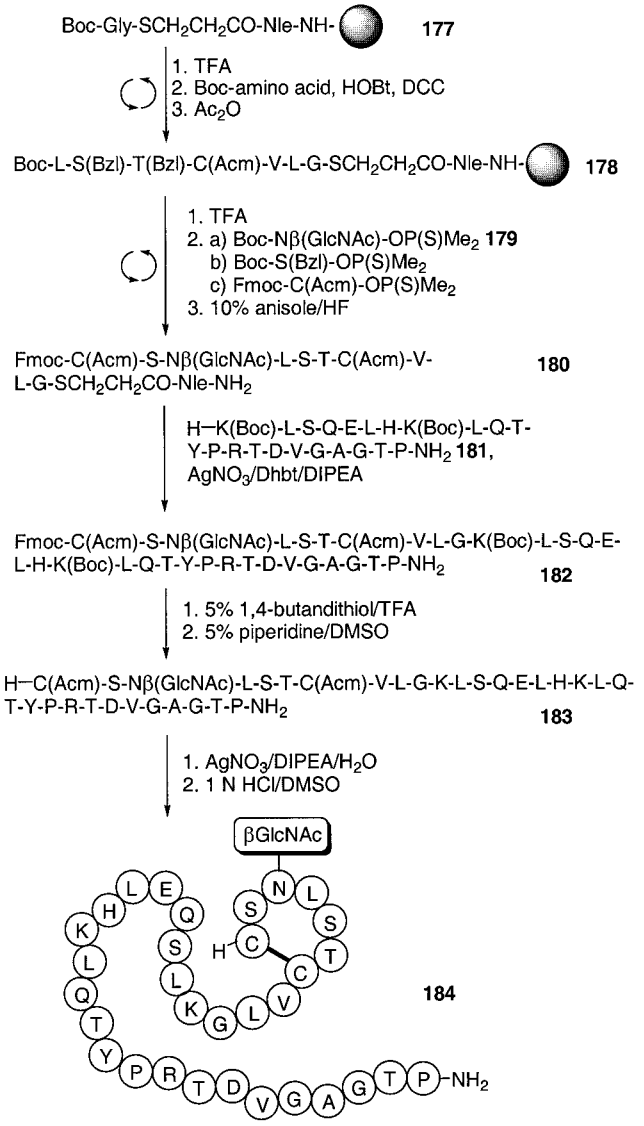
Apart from the chitobiosyl core, high-mannose-type *N*-glycans contain almost exclusively α-mannosyl residues linked to the two mannosyl branches at the nonreducing end of the core structure. Complex-type glycans contain no mannose except for the innermost trimannosyl core, which is extended by *N*-acetylglucosamine or galactose. Hybride-type glycans contain fragments of the high-mannose and of the complex type. The poly-*N*-acetyllactosamine glycans contain repeating units of {(*β*Gal-1,4-*β*GlcNAc)$_x$-1,3-} attached to the pentasaccharidic core. Their reducing ends are often substituted with sialic acid.

The saccharide chains in natural *N*-glycoproteins are always linked to asparagine. There are no confirmed reports on glycosylation of glutamine. The sequence Asn-Xaa-Ser/Thr, where Xaa can be any amino acid except proline, serves as a consensus sequence for *N*-glycosylation, being, however, just a necessary but nonsufficient condition.[179,180]

For the chemical synthesis of *N*-glycopeptides, the generation of an anomeric glycosylamine is a crucial step. It can either be performed by treatment of unprotected saccharides with a saturated solution of ammonium hydrogencarbonate according to the method of Kochetkov[181,182] or by reduction of anomeric glycosyl azides, which are favorably obtained by reaction of 1-*O*-acetyl saccharides with TMS−N_3 or from glycosylhalides by nucleophilic substitution with N_3^-.[183]

The incorporation of the glycosylamine in a glycopeptide can be achieved by two strategies: the use of building blocks and the convergent approach. The building block approach is characterized by the generation of a suitably protected glycosyl amino acid, which is used as synthon for glycopeptide synthesis. In particular, for the synthesis of glycopeptides containing complex saccharides, the convergent approach is more favorable because it requires less of the precious glycosylamine. This strategy begins with the synthesis of a protected peptide which is subsequently deprotected at the aspartic acid residue. Afterwards, condensation with the glycosylamine furnishes the desired *N*-glycopeptide.

Scheme 43



Another variant of the convergent approach consists of the synthesis of a glycopeptide containing a simple glycosyl amino acid building block such as *N*-acetylglucosaminyl aspartic acid. After completion of the peptide chain, the saccharide is extended by chemical or enzymatic methods.

A. Glycopeptides Containing *N*-Linked Monosaccharides

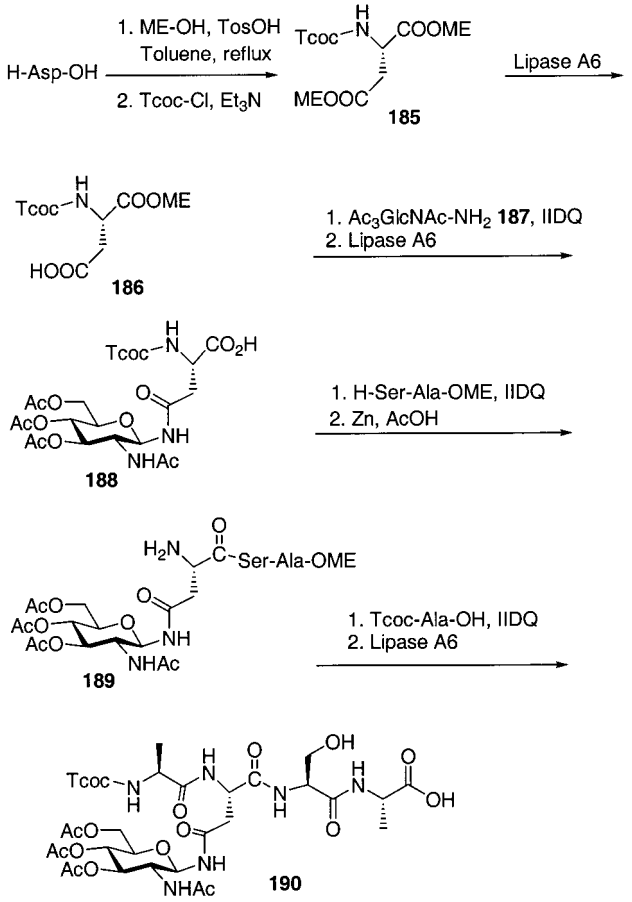
Eel calcitonin is a 32 amino acid peptide hormone that decreases the concentration of calcium in the blood. A convergent synthesis of a glycosylated analogue of eel calcitonin has been described.[184] Starting from thioester resin **177**,[185] the *N*-terminal decapeptide fragment of calcitonin **180** was prepared by applying the Boc strategy and DCC/HOBt-mediated coupling of the first seven amino acids (Scheme 43). After this sequence a change from DCC/HOBt activation to the use of amino acids activated as dimethylphosphinothioic mixed anhydrides[186] had to be made, because the intended coupling of the glucosylated asparagine building block **179**, carrying no hydroxyl protection, was not compatible with the application of carbodiimides. Treatment of the MBHA resin (methyl benzhydrylamine resin) with anhydrous HF containing 10% anisole cleaved the glycopeptide from the polymer and simultaneously removed the benzyl ester side chain protecting groups. The *N*-terminal glycopeptide thioester **180** was finally isolated in a yield of 12%.

The C-terminal peptide segment **181** was prepared by standard solid-phase Boc chemistry as described above. Only the terminal lysine was introduced as Fmoc amino acid. Deprotection and detachment from the MBHA resin was readily achieved by reaction with HF/anisole/1,4-butanedithiol. For the following thioester segment condensation[187] with the *N*-terminal fragment **180**, the lysine side chains were protected by reaction with Boc-OSu/DIPEA. The *N*-terminal Fmoc group was removed, and the partially protected peptide segment **181** was added to a mixture of glycopeptide thioester **180**, silver nitrate, 3-hydroxy-3,4-dihydro-4-oxo-1,2,3-benzotriazine (Dhbt-OH), and DIPEA in DMSO. Glycopeptide **182** was obtained in 78% yield after purification. Careful choice of the coupling conditions secured that the Acm protecting groups of the cysteine side chains remained unaffected.[185] Removal of the side chain protection groups and of the amino terminal Fmoc group furnished linear eel calcitonin precursor **183**. The intramolecular disulfide bond between Cys^1 and Cys^7 was formed after removal of the Acm groups by treatment with 1 N HCl/DMSO at room temperature. The yield of glycosylated eel calcitonin analogue **184** was 9% based on the initial loading of the starting resin.

Because of the mild reaction conditions employed, enzymes can be highly useful for various protecting group manipulations in glycopeptide synthesis. The 2-methoxyethyl (ME) ester as carboxyl protection group, cleavable by a number of lipases, was applied in the solution-phase synthesis of tetrapeptide **190**,[188] carrying an asparagine-linked 2-acetamido glucosyl side chain. Starting from aspartic acid, the carboxyl groups were protected as methoxyethyl (ME) esters by azeotropic esterification in the presence of *p*-toluenesulfonic acid (Scheme 44). Treatment of the *N*-protected aspartic acid di(2-methoxymethyl) ester **185** with lipase A6 from *aspergillus niger* regioselectively cleaved the ME ester from the side chain carboxyl group (→ **186**). IIDQ promoted coupling with GlcNAc derivative **187**,[189] and subsequent incubation with lipase N furnished C-terminally deprotected building block **188**. Condensation with seryl-alanine ME ester and removal of the Tcoc group with zinc/acetic acid[190] yielded glycosylated tripeptide **189**. The amino-deblocked compound was coupled to Tcoc-alanine, furnishing the desired *N*-glycotetrapeptide **190** after enzymatic removal of the C-terminal ME ester.

A strategy for the construction of combinatorial libraries of *N*-glycopeptides was developed by Vetter et al.[191] The key step of the methodology used was the condensation of an unprotected glycosylamine with the pentafluorophenylester of the carboxyl side chain of a resin-bound peptide. This activated ester was generated from Fmoc- and *tert*-butyl-protected

Scheme 44

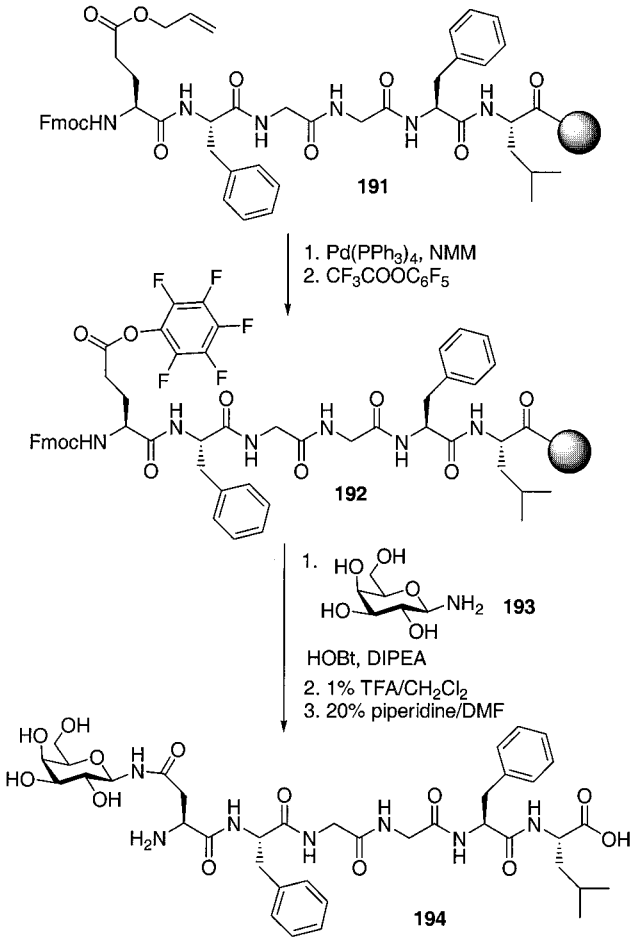


polymer-bound peptides **191** with temporary allylester side chain protection for the glutamic or aspartic acid residue (Scheme 45). Palladium(0)-mediated cleavage of the allylester[192] and subsequent conversion of the liberated carboxyl group to the pentafluorophenylester **192** (Scheme 45) enabled the coupling reaction between the peptide and glycosylamine **193**. Residual protecting groups were removed, and the glycopeptide was detached from the solid support (→**194**). This synthetic procedure was applied to the Leu-enkephalin sequence (Asp-)Tyr-Gly-Gly-Phe-Leu, because it has been shown that glycosylation of a related peptide drastically increased its affinity to opiate receptors.[193] By repeating the described reactions with 5 different amines and 18 saccharides, a library of 23 glycopeptides, including sulfated saccharides and uronic acids, was obtained.

B. Glycopeptides Containing Chitobiose (βGlcNAc-1,4-GlcNAc)

Although the carbohydrate parts of *N*-linked glycoproteins are usually highly complex branched oligosaccharides, most of them share the common $Man_3(GlcNAc)_2$ core linked by an amide bond to the side chain of asparagine. Since it is known that the chitobiose at the reducing end of these saccharides exerts the greatest influence on the conformation of *N*-glycosylated peptides,[194] this commercially available disaccharide has been chosen for the chemical synthesis of various glycopeptides as an approximation of the natural carbohydrate structures.

Scheme 45



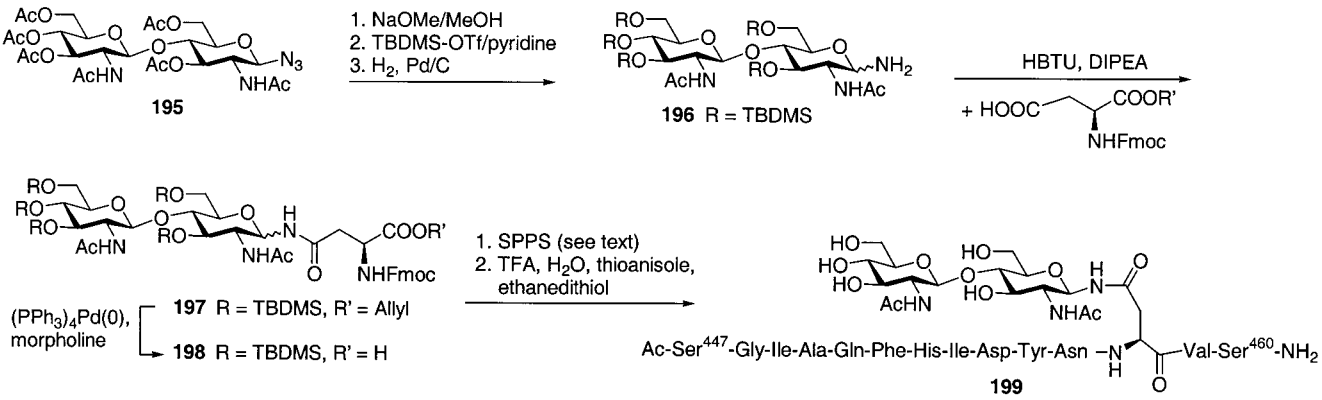
For example, the effect of glycosylation on the binding properties of protein S to C4b binding protein (C4BP),[195] one of the proteins of the complement system, was investigated.[196] Protein S can act as an anticoagulant and is a vitamin K-dependent cofactor in the regulatory system of blood clotting. The concentration of free protein S in the blood is drastically decreased by forming a strong complex with C4b binding protein. The resulting increase of the propensity of the blood to coagulate is of high medicinal and pharmacological interest.

For the binding of protein S to C4BP, the fragment from $Ser^{447}-Ser^{460}$ containing a potential *N*-glycosylation site at Asn^{458} is of particular importance. Chemical synthesis of the glycosylated and of the unglycosylated peptides was therefore a helpful tool for the investigation of the effect of glycosylation of protein S 447−460 on binding to C4BP.

For the synthesis of glycopeptide **199**, a suitably protected N^4-chitobiosyl asparagine was synthesized which afterwards was incorporated into the solid-phase synthesis of the glycopeptide: Proceeding from peracetylated chitobiosyl azide **195**,[197] the hydroxy groups were deprotected and converted into TBDMS ethers with TBDMS−OTf/pyridine, followed by catalytic hydrogenation of the azide to furnish the anomeric chitobiosylamine **196** (Scheme 46).

In contrast to the analogous peracetylated disaccharide, this persilylated compound showed good solubility in organic solvents. HBTU-promoted cou-

Scheme 46



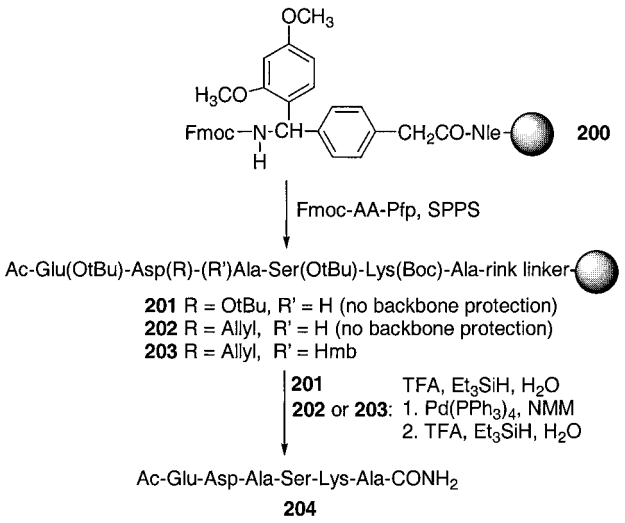
pling of the glycosylamine to Fmoc-Asp(OH)-OAll resulted in an anomeric mixture of glycosylasparagines **197** (20% and 38% yield, respectively) which was separated by chromatography. The unsatisfactory stereochemical outcome of the reaction can be explained by a distortion of the carbohydrate ring from the normal $^4C_1$ chair conformation caused by the bulky TBDMS groups.[198] The allyl protection of the β-configurated building block was selectively cleaved by palladium(0)-catalyzed transfer to morpholine,[152] resulting in the C-terminally unprotected building block **198**, which was directly used in the solid-phase synthesis of the peptidic backbone of the desired protein S fragment **199**.

Peptide synthesis was carried out on a tentagel resin functionalized with the acid-labile Rink amide linker.[49] Coupling reactions were achieved by activating the amino acids as benzotriazoyl or, in the case of the glycosylated aspartic acid building block **198**, as azabenzotriazoyl ester. After completion of the synthesis, acidolytic treatment of the resin with TFA released the glycopeptide from the resin and cleaved the silyl ether protecting groups from the sugar moiety without affecting the glycosidic bonds. Purification by reversed phase HPLC yielded the desired chitobiosyl-protein S fragment **199** in 28% yield.[196]

The affinity of the glycopeptide as well as of the analogously synthesized unglycosylated peptide to C4BP was determined in a competitive assay in which the ability of the peptides to inhibit binding of protein S to C4BP was quantified. A 4-fold lower concentration of the glycopeptide as compared to the unglycosylated peptide was sufficient to give the same inhibitory effect, giving strong evidence that natural protein S is also glycosylated at Asn^{458}.

Another chitobiosyl peptide was synthesized in the course of investigations about undesired aspartimide formation during convergent glycopeptide synthesis.[199] The convergent approach is characterized by a two-step procedure: The fully protected peptide chain was synthesized and, in the second step, selectively deblocked at the Asp residue(s) to be glycosylated. If common Fmoc methodology was applied, the side chains of the aspartic acid residues are usually protected as *tert*-butyl or allyl esters. However, it was shown that both protection groups cannot prevent the formation of aspartimides under the basic conditions required for Fmoc removal.[200] Additional aspartimide formation occurs when the

Scheme 47

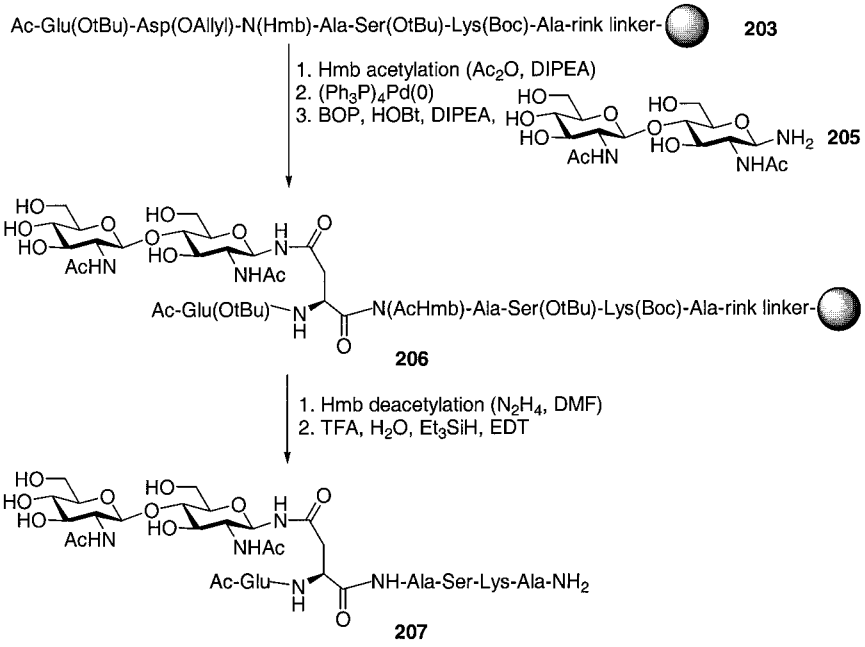


aspartyl residues are activated for condensation with glycosylamines. Unfortunately, the intermediate active ester of aspartic acid can also attack the amide nitrogen between aspartic acid and its C-terminal neighbor, leading to aspartimide formation. This side reaction can be avoided by introducing mono-*N*-alkyl amino acid derivatives (see Scheme 47).

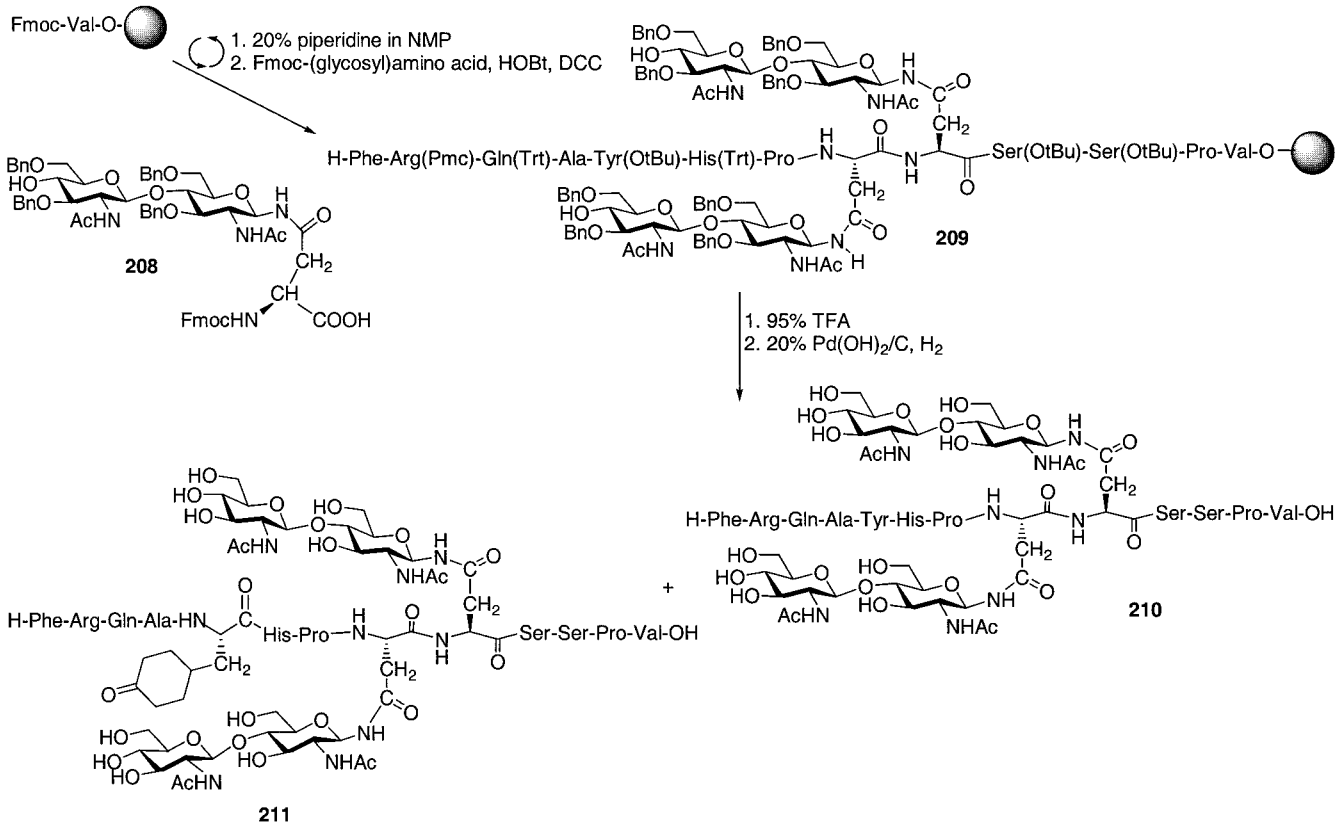
Thus, the *N*-(2-hydroxy-4-methoxybenzyl) group (Hmb) was used to prevent aspartimide formation. As a model sequence to examine the benefits of this approach, hexapeptide Ac-Glu-Asp-Ala-Ser-Lys-Ala-$CONH_2$ **204**, which is known to be particularly susceptible to the formation of aspartimides, was chosen.[201,202] In initial studies, the hexapeptide was synthesized three times: once with classical *tert*-butyl ester protection for the aspartic acid side chain (→ **201**), then with allyl ester side chain protection (→ **202**), and again with allylester side chain protection but additional backbone protection of the alanine preceding the aspartic acid (→ **203**) (Scheme 47).[199]

Coupling reactions were performed using pentafluorophenyl esters on Kieselgur-supported poly(dimethylacrylamide), employing the acid-labile Rink amide linker **200**. After completion of the peptide synthesis, the *N*-termini were acetylated and, in the case of the allyl ester protected peptides, the aspartic acid side chains liberated by palladium(0)- catalyzed transfer of the allyl group to *N*-methylmorpholine.[153] HPLC and MALDI-TOF analysis of the products

Scheme 48



Scheme 49



obtained after cleavage from the resin and removal of protecting groups revealed that the peptide with *tert*-butyl-protected Asp side chain was generated in high purity. In contrast, its allyl ester-protected analogue without backbone protection could only be obtained as a mixture with the corresponding aspartimide in a ratio of about 1:1. The hexapeptide synthesized with allyl ester-protected Asp side chains and additional Hmb backbone protection showed no contamination with aspartimide, featuring aspartyl amide bond backbone protection as a highly efficient method to prevent aspartimide formation.

After this basic strategy was established, the on-resin coupling of chitobiosylamine **205** to the backbone-protected hexapeptide was investigated next (Scheme 48). To prevent potential side reactions, the Hmb hydroxy function was protected by acetylation with acetic anhydride/DIPEA.

Treatment of the peptide resin with palladium(0) reagent in the presence of *N*-methylmorpholine liber-

ated the side chain β-carboxylate of the aspartyl residue, which was then glycosylated with *N*,*N*′-diacetyl-chitobiosylamine **205** through activation with BOP/HOBt/DIPEA (→**206**).[199] *N*,*N*′-diacetyl-chitobiosylamine was obtained according to the procedure of Kochetkov.[181] Only 1.1 equiv of the glycosylamine proved to be sufficient to drive the coupling reaction to completion. In previously described synthesis[203,204] several quantities of the often precious glycosylamines had to be used instead of an auxiliary base (e.g., DIPEA) in order to minimize aspartimide formation.

To obtain the target glycopeptide **207** the backbone-protecting AcHmb group was deacetylated with hydrazine in DMF to reestablish its sensivity to TFA. Acidolytic cleavage of the glycopeptide from the resin and simultaneous removal of the Hmb backbone protecting groups as well as of the side chain protecting groups gave the desired glycopeptide **207** in an overall yield of 30% after purification by preparative HPLC.[199]

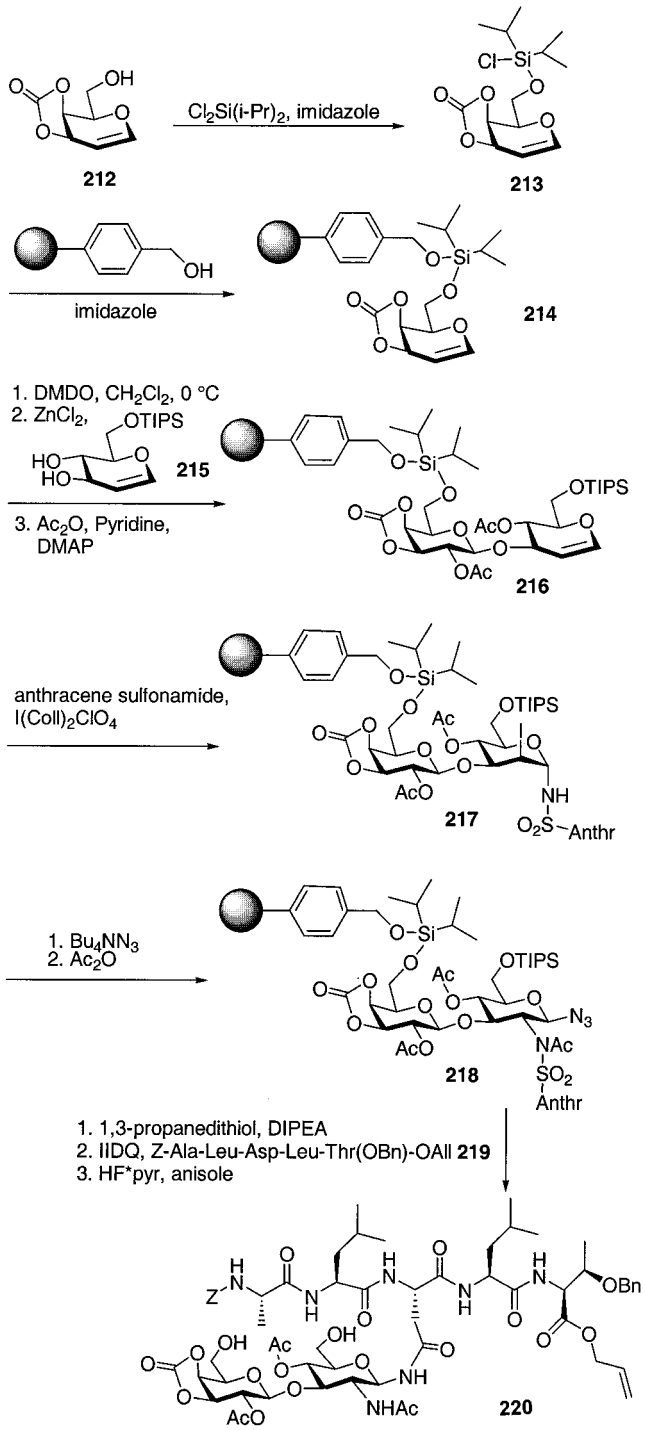
As an example of a glycopeptide with two vicinal *N*-glycosylation sites a peptide fragment of the interleukin 8 (IL8) receptor was synthesized.[205] IL8 is a pro-inflammatory polypeptide chemokine. Its biological activity can be efficiently inhibited by peptide fragments of the extracellular regions of IL8 receptors, characterizing these peptides as potential therapeutics.

Key building block for the synthesis of the desired bis-glycosylated IL8 receptor fragment **210** was benzylated chitobiosyl asparagine conjugate **208** (Scheme 49),[205] the origin of which was not outlined. Peptide synthesis was performed on valine-preloaded hydroxymethyl polystyrene (HMP) resin using DCC−HOBt as the coupling reagent. Coupling of the two chitobiosyl-asparagine building blocks was done manually. Both coupling steps were reported to proceed in high yields giving the resin-linked bis-glycosylated hexapeptide **209** in over 90% yield. After completion of the synthesis, release of the glycopeptide from the resin and concomitant deprotection of the amino acid side chains was achieved by treatment with a mixture of TFA and different scavengers. The crude product was subjected to reductive debenzylation. The unprotected glycopeptide **210** could be obtained in only 19% yield (related on **209**) because of an astonishing reduction of the tyrosine side chain to cyclohexanone (→ **211**).

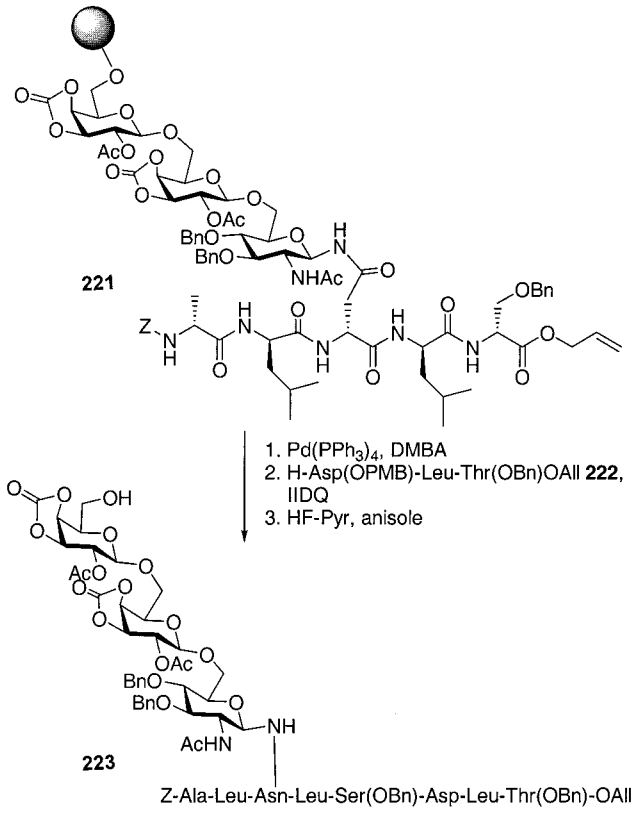
An improved method for the chemical synthesis of oligosaccharides and glycopeptides on solid phase is based on common hydroxymethyl-modified Merrifield or Wang resin to which the nonreducing end of a glycal is linked via a diisopropyl silyl ether (Scheme 50).[206]

Starting from 3,4-galactal carbonate **212**, silyl ether **213** was obtained by reaction with dichlorodiisopropylsilane/imidazole. Taking advantage of the enhanced reactivity of the dichlorosilane compared to its monohalogenated derivative, formation of bis-glycal diisopropylsilyl ether could be prevented. Intermediate **213** was not isolated but directly coupled to the hydroxybenzyl resin (→ **214**). Unreacted hydroxy groups of the polymer were capped by treatment with diisopropyldichlorosilane/imidazole. Conversion of the glycal into the epoxide using dimethyldioxirane and $ZnCl_2$-promoted glycosylation of 6-*O*-TIPS-glucal **215** provided resin-linked disaccharide **216**, which was acetylated at the 4-hydroxy function. Treatment with iodonium bis-*sym*-collidine perchlorate and anthracenesulfonamide[207] furnished iodosulfonamide **217**, which was transformed into the 2-anthracenesulfonamidoglyosyl azide **218** by reaction with tetra-*n*-butylammonium azide[208] and subsequent acetylation of the sulfonamido group. The anthracenesulfonamide offered the advantage that the nitrogen−sulfur linkage was cleavable by a variety of mild methods, e.g., by 1,3-propanedithiol

Scheme 50



Scheme 51



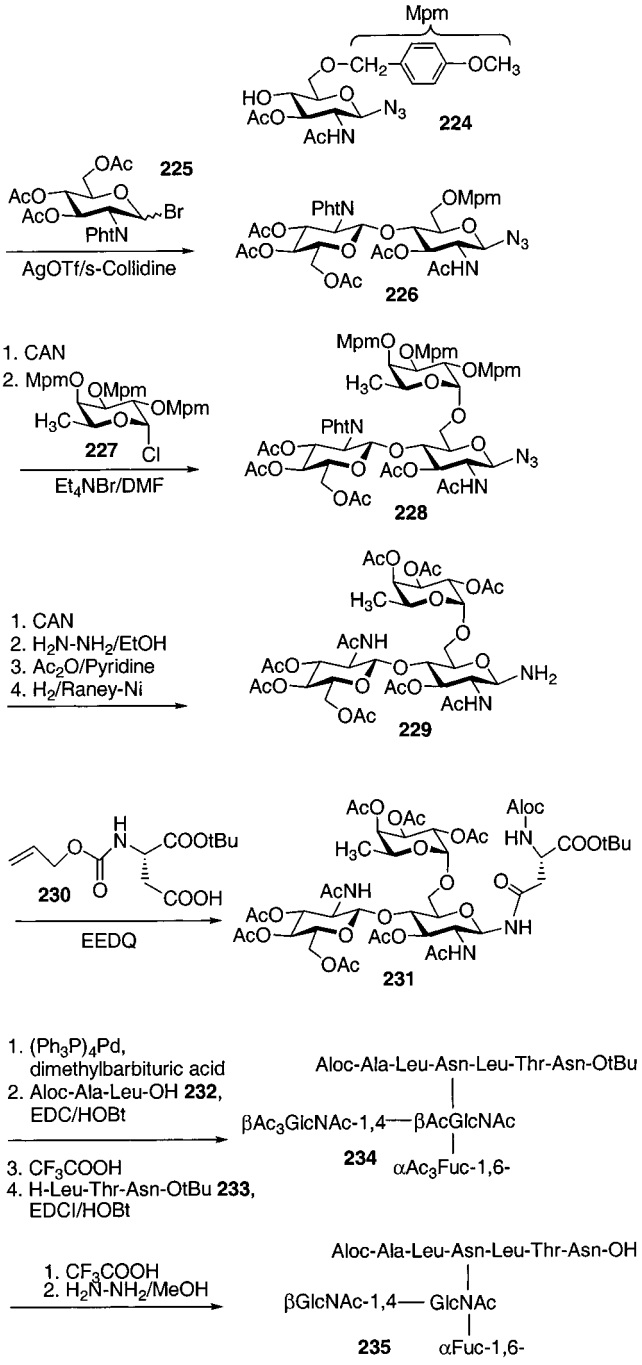
which simultaneously reduced the anomeric azide to the lactosylamine. Coupling of the disaccharide amine to pentapeptide **219** with IIDQ yielded the resin-bound glycopeptide, which was readily disengaged from the solid support with HF/pyridine and anisole at 0 °C. Prolonged treatment with fluoride at room temperature removed the 6-*O*-TIPS group from the disaccharide, furnishing glycopeptide **220** in 44% overall yield.

It is noteworthy that the binding of a glycopeptide to the solid support *via* one of the hydroxyl groups of the saccharidic moiety (see also section II.B) allows further on-resin chain elongation of the peptide in both the *C*- and *N*-terminal directions. However, an appropriate orthogonal protecting-group strategy is prerequisite for this procedure, as demonstrated by the reaction of the glycopentapeptide **221** with tripeptide **222**, furnishing glycooctapeptide **223** in 18% overall yield (Scheme 51).[209,210]

C. Glycopeptides Containing Trisaccharides

There are only a few reports concerning the synthesis of naturally occurring glycoprotein fragments carrying fucose-containing saccharides. The synthesis of trisaccharide hexapeptide **235**, representing a partial structure of the envelope glycoprotein of a murine leukemia virus,[211] has been reported.[45] Starting from 4-hydroxyglucosyl azide **224**,[183] protected chitobiosyl azide **226** was obtained by glycosylation with 3,4,6-triacetyl-2-deoxy-2-phthalimido glucosyl-bromide **225**[212] (Scheme 52). Cleavage of the *p*-methoxybenzyl ether with $Ce(NH_4)_2(NO_3)_6$[213] and subsequent reaction with 2,3,4-tri(*p*-methoxybenzyl)-

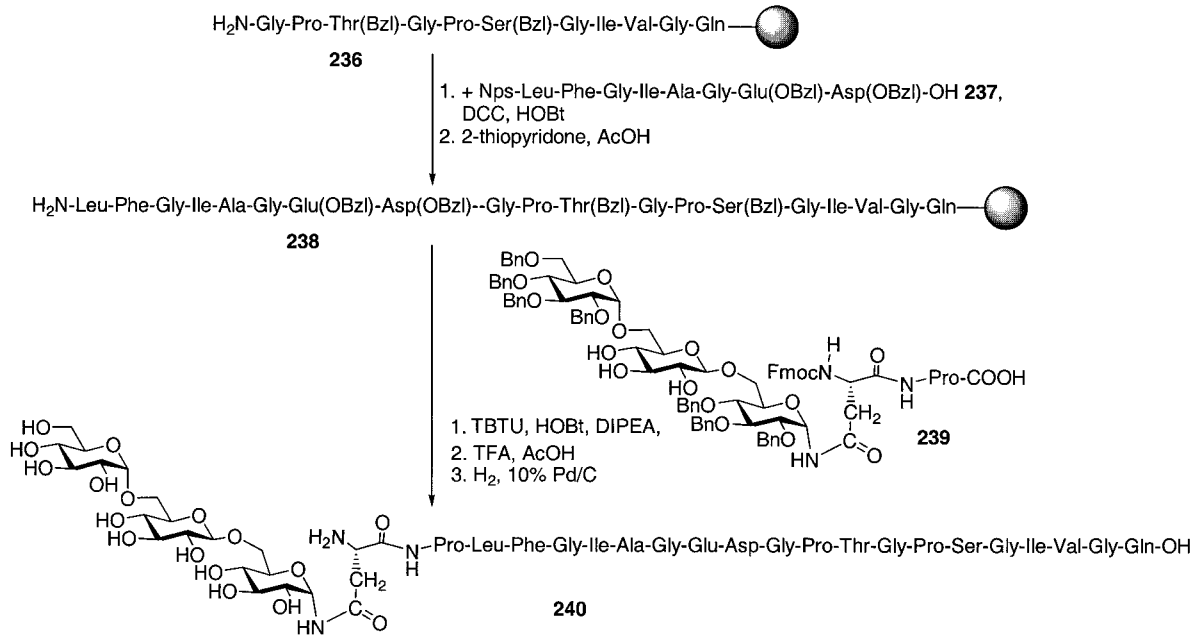
Scheme 52



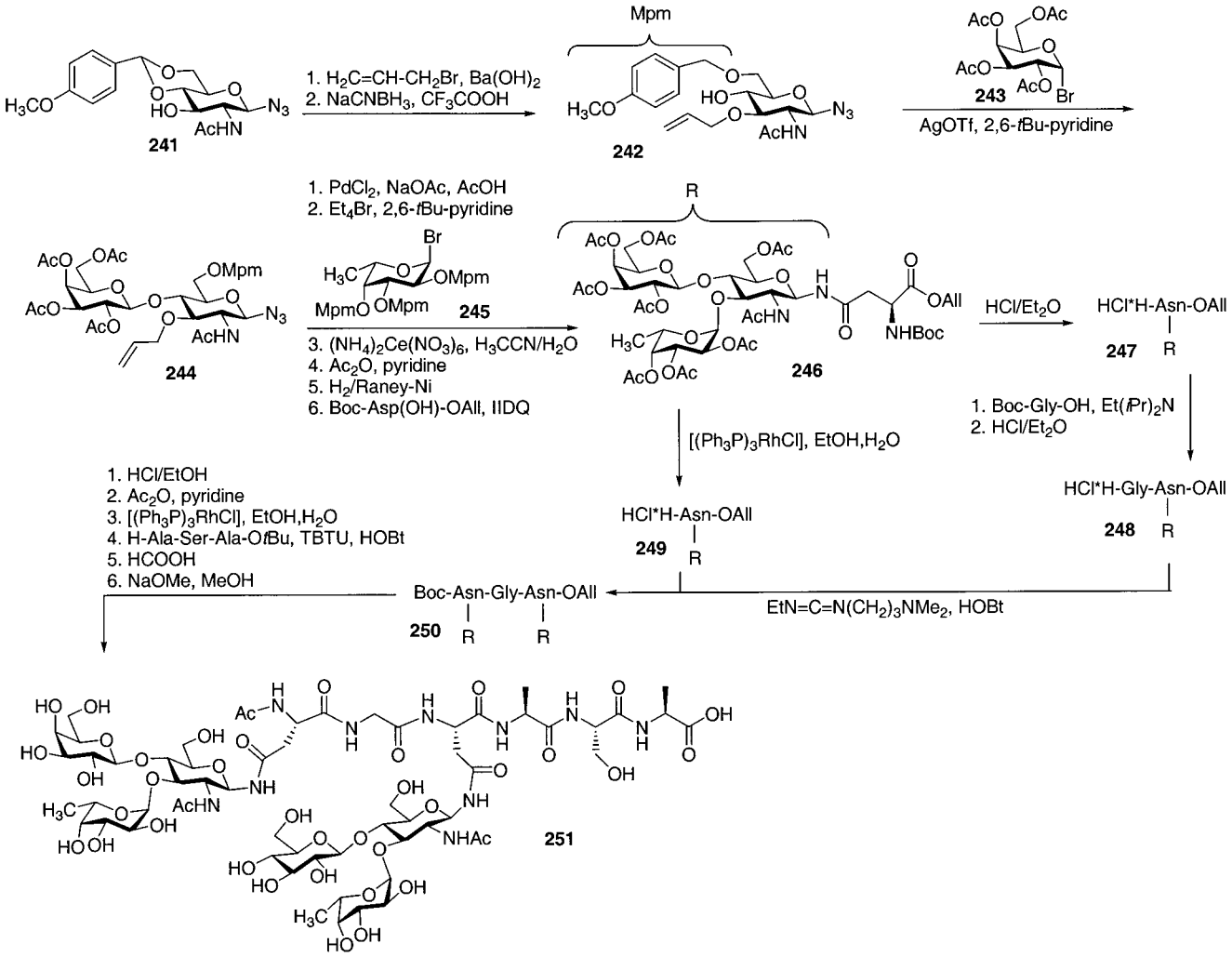
fucosyl chloride **227**[214] stereoselectively furnished α-fucosyl glycosyl azide **228**. Trisaccharide **228** could not be used directly for the synthesis of glycopeptides because of the acid-sensitivity of the fucosidic linkage. Therefore, an exchange of the ether-type (Mpm) protection for acetyl protection was carried out by oxidative cleavage of the Mpm ethers using cerium ammonium nitrate (CAN), cleavage of the phthalimido group, and peracetylation. From the resulting glycosyl azide, the anomeric *β*-amine **229** was obtained by Raney-nickel-catalyzed hydrogenolysis.

The fucosyl chitobiosylamine **229** was condensed with 1-*tert*-butyl *N*-allyloxycarbonyl aspartate **230** to give the fully protected trisaccharide asparagine conjugate **231**. After removal of the Aloc group,[215] coupling with dipeptide **232**, and extension of the

Scheme 53



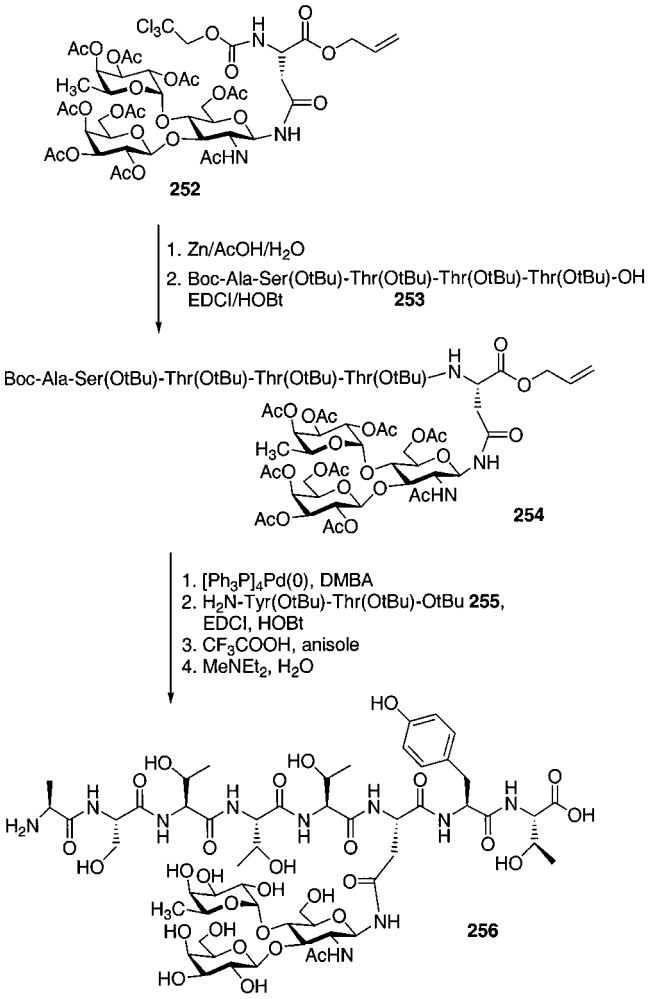
Scheme 54



deprotected C-terminus with tripeptide **233**, protected hexapeptide **234** was obtained. Treatment of **234** with trifluoroacetic acid and, subsequently, hydrazine in methanol gave the glycohexapeptide **235**.

A strategy to avoid aspartimide formation in the course of the synthesis of a glycopeptide containing the highly susceptible Asp-Gly sequence has been demonstrated in a report about the synthesis of

Scheme 55

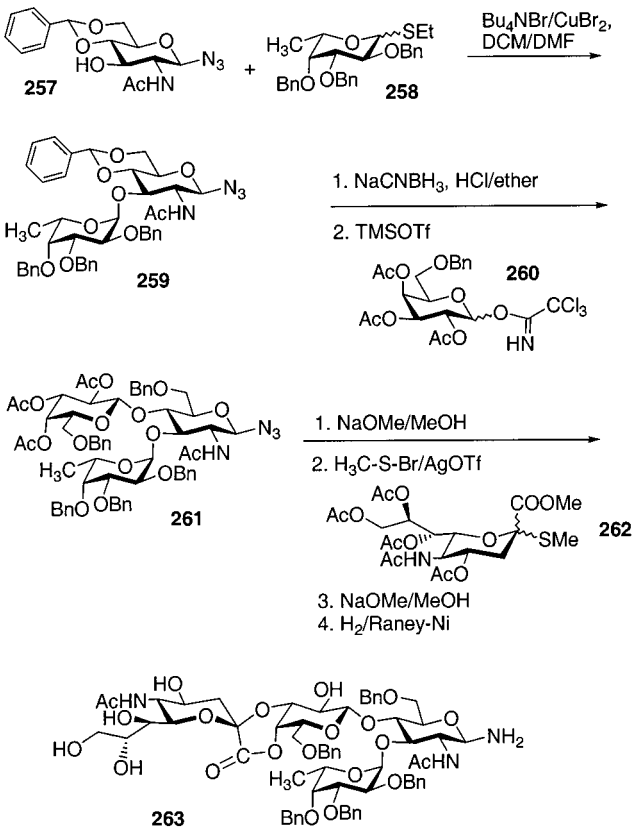


nephritogenoside.[216] Nephritogenoside is a 21 amino acid glycopeptide having a trisaccharide directly linked to the *N*-terminal aspartyl residue via an unusual α-*N*-glycosidic bond.[217] It is a strong inductor of chronic progressive glomerulonephritis (renal inflammation). However, isolation and purification of this compound is very difficult because only 5−6 mg of nephritogenoside could be purified from the perfused renal cortices of 1200 rats.[218]

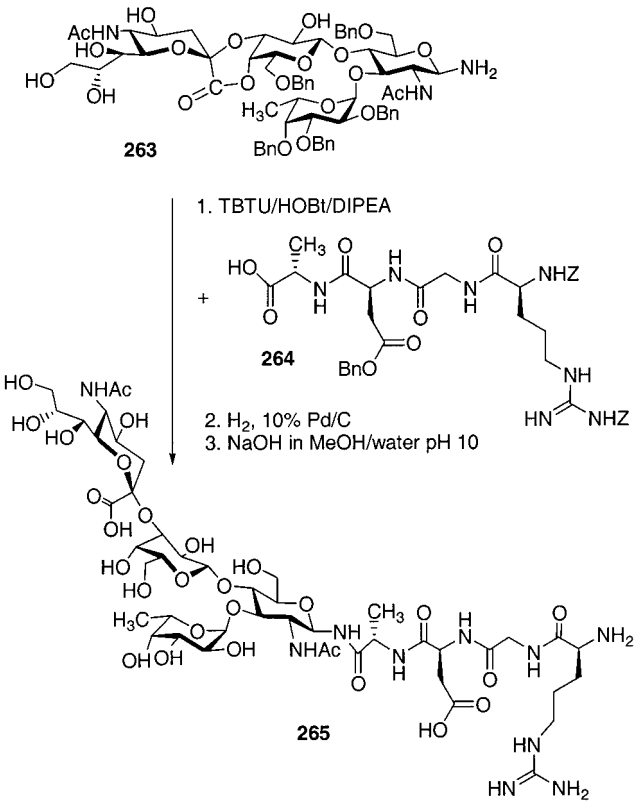
For its chemical synthesis, the peptidic bond between a benzyl-protected aspartic acid and glycine was formed by fragment condensation of two smaller (glyco)peptides, **236** and **237** (Scheme 53).

The C-terminal fragment **236** was readily obtained on a 2-chlorotrityl resin[219] using TBTU as coupling reagent. The synthesis of the amino terminal fragment **237** started with Fmoc-Asp(OBn)-OH. As for the synthesis of the C-terminal fragment, Fmoc strategy was applied except for the *N*-terminal leucine for which *o*-nitrophenyl sulfenyl (Nps) protection was chosen. Acidolytic cleavage of the assembled *N*-terminal fragment from the resin and coupling to C-terminal peptide resin **236** yielded the Nps-protected nonadecapeptide peptide resin. Deprotection of the Nps group was achieved quantitatively with 2-thiopyridone/AcOH (→**238**).[220] Due to the acidic conditions, no aspartimide formation of the sensitive Asp(OBn)-Gly sequence was observed. Peptide resin **238** was subsequently coupled to glycodipeptide **239**[216] using HBTU/HOBt/DIPEA as condensation reagents. Cleavage from the resin and hydrogenolysis gave free nephritogenoside glycopeptide **240** in good yield.

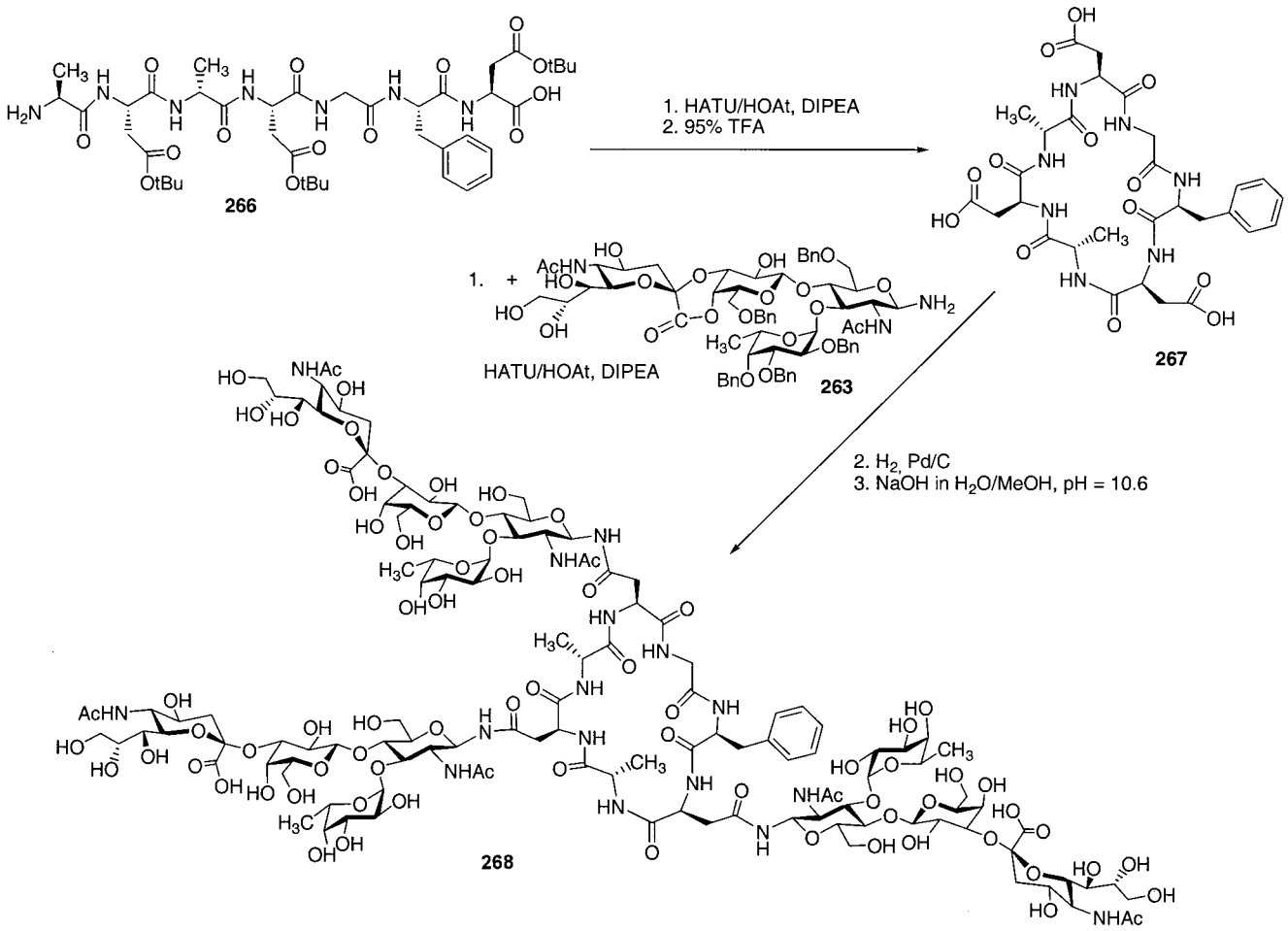
Scheme 56



Scheme 57



Scheme 58



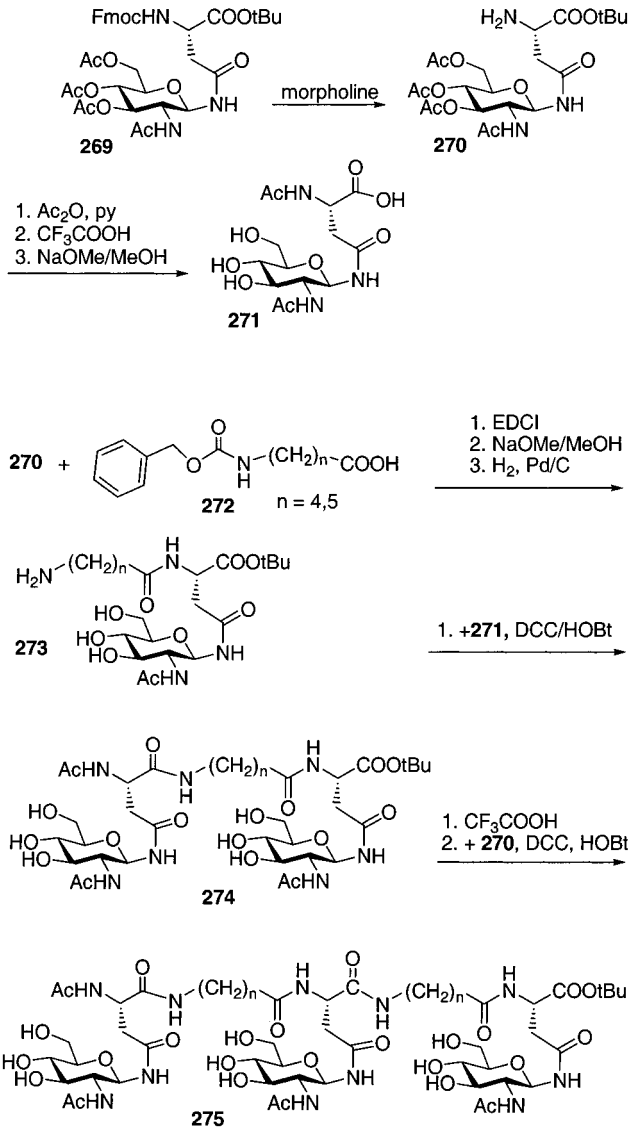
Glycoconjugates containing the Lewis^x trisaccharide are of particular pharmacological importance since they have been described as stage-specific embryonic and tumor-associated antigens. The construction of hexapeptide **251** (Scheme 54), containing two Lewis^x saccharides, was motivated by the aforementioned cluster effect.[83] For the syntheses of the saccharide moiety, *p*-methoxy benzylidenated glucosyl azide **241**[183] was protected as allyl ether in the 3-position prior to the regioselective opening of the benzylidene acetal (→ **242**). Glycosylation of **242** with peracetylated galactosylbromide **243**[221] furnished disaccharide **244**. The 3-*O*-allyl ether was cleaved with palladium chloride/sodium acetate.[222] After fucosylation using Mpm_3FucBr **245** as donor, the Mpm groups of the protected Lewis^x derivative were exchanged for *O*-acetyl groups, the anomeric azide was reduced to the 1-amino group, which was condensed with Boc-Asp(OH)-OAll (→ **246**). Removal of the Boc protecting group was achieved with HCl in diethyl ether without affecting the fucosidic linkage, because of the stabilizing effect of the *O*-acetyl groups (→ **247**).[223] On the other hand, the allyl ester was cleaved by a Rh(I)-catalyzed reaction, furnishing glycosyl amino acid **249**. Both building blocks were introduced in a solution-phase synthesis of glycohexapeptide **251**, as outlined in Scheme 54. After complete deprotection, the bivalent Lewis^x conjugate **251** was coupled to carrier proteins such as bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH) by carboxy activation with water-soluble carbodiimide/*N*-hydroxysuccinimide, thus providing structurally defined neoglycoconjugates for immunological purposes.[224]

Peptide T is a partial sequence of the envelope glycoprotein gp 120 of HIV-1. The threonine-rich octapeptide can block infection of human T cells by the HIV virus.[225] A glycosylated peptide T sequence carrying the Lewis^a antigen has been synthesized in solution (Scheme 55).[214] The synthetic strategy was based on protected Lewis^a−asparagine conjugate **252**,[226] which was selectively deprotected at the *C*- and the *N*-terminus. Cleavage of the *C*-terminal Tcoc group with zinc in aqueous acetic acid and EDCI/HOBt-promoted condensation with pentapeptide **253** yielded glycohexapeptide **254**. After cleavage of the C-terminal allyl ester, **254** was extended to a glycoheptapeptide by reaction with tyrosyl-threonine ester **255**. Cleavage of the protecting groups with trifluoroacetic acid and, subsequently, methyl diethylamine furnished the deprotected peptide T−Lewis^a conjugate **256** in a yield of 44%.

D. Glycopeptides Containing Sialyl Lewis^x

Invasion of leucocytes constitutes a crucial process in the cause of chronic and acute inflammatory diseases. The diapedeses of leucocytes through the vascular endothelium into the site of inflamed tissue is a key step in the inflammatory response. This

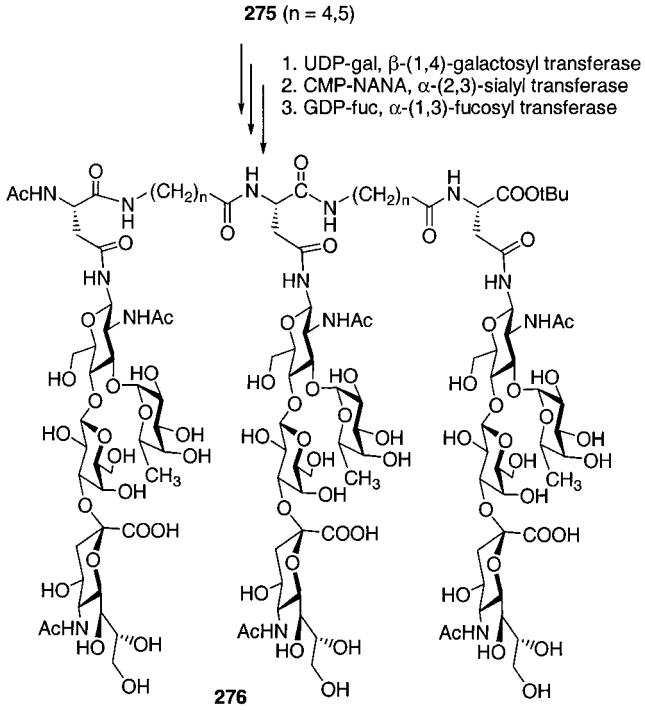
Scheme 59



multistage process begins with the so-called "rolling" of leucocytes,[227] which is caused by interaction of glycoprotein receptors, the selectins (E-,P-), expressed on the surface of the activated endothelial cells, with carbohydrate ligands exposed by the leucocytes.[228] The tetrasaccharide sialyl Lewis^x and sulfated derivatives of Lewis^x have been identified as ligands recognized by the selectins.[229] Not only inflammatory processes, but also the recirculation of lymphocytes and metastasis of cancer cells are correlated with the interaction of sialyl Lewis^x and the selectins. For the understanding of these interactions and the treatment of corresponding diseases, glycopeptides containing the sialyl Lewis^x epitope are of particular interest.

Although in natural glycoproteins sLe^x-saccharides are usually *O*-glycosidically linked to the peptide backbone, a number of *N*-glycopeptides have been synthesized because of the higher stability of these compounds toward enzymatic degradation. As an example, the synthesis of a RGD-sialyl Lewis^x glycoconjugate **265** containing the integrin binding tripeptide RGD in combination with sLe^x as selectin ligand has been performed.[230] As a synthon for the

Scheme 60



synthesis of **265**, a suitably protected sialyl Lewis^x building block was prepared.

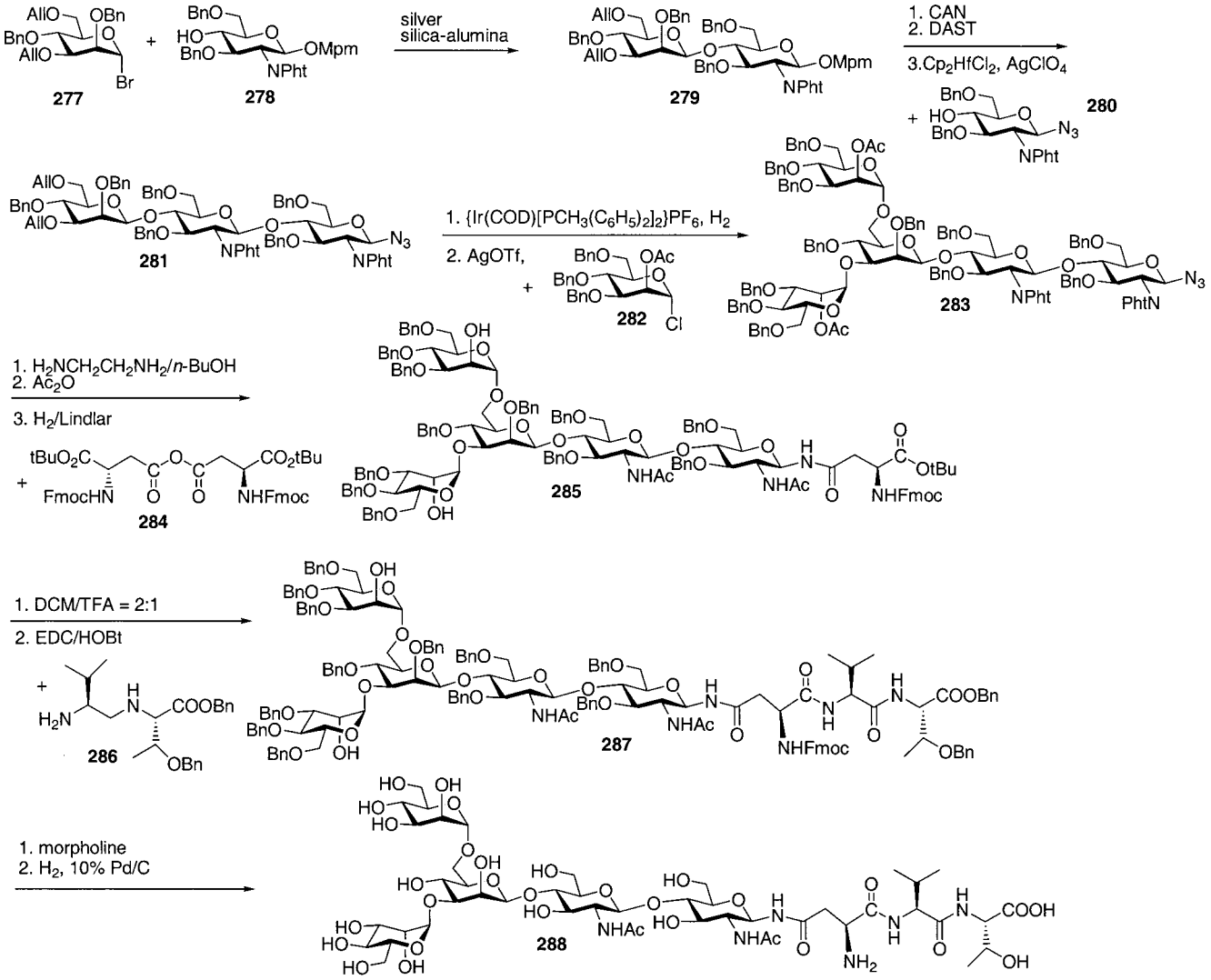
Glycosylation of 4,6-*O*-benzylidene-protected 2-*N*-acetyl glucosamine azide **257**[183,223] with perbenzylated fucoside **258**[231] stereoselectively yielded α-glycoside **259** (92%) (Scheme 56).

Regioselective opening of the benzylidene acetal with $NaCNBH_3/HCl$[232] and subsequent galactosylation with galactosyltrichloroacetimidate **260**[233] furnished protected Lewis^x derivative **261**. After *O*-deacetylation, the trisaccharide was regioselectively sialylated with the methyl thioglycoside of neuraminic acid methyl ester **262**.[40] Chromatographic separation of the product from unreacted Le^x turned out to be difficult. Therefore, the crude reaction mixture was deacetylated with sodium methanolate in methanol, giving the sLe^x-lactone, which could readily be purified. Reduction of the anomeric azide with Raney-nickel furnished sLe^x-amine **263**.

As the second building block, RGDA tetrapeptide **264** was synthesized using Fmoc solid-phase strategy on an acid labile Sasrin resin.[50] The C-terminal alanine served as a spacer between the RGD sequence and the tetrasaccharide. Fragment condensation of glycosylamine **263** and RGDA peptide **264** with TBTU/HOBt/DIPEA gave the protected *N*-glycosyl RGDA conjugate in a yield of 65% (Scheme 57). Hydrogenolytic cleavage of the Z and benzyl groups followed by alkaline saponification of the intramolecular lactone with sodium hydroxide in aqueous methanol furnished the desired glycopeptide **265**.[230]

In a cell-adhesion assay the competitive binding of **265** and tumor cells of cell line HL 60 to a recombinant human P-selectin IgG fusion protein was measured. The synthetic compound showed an IC_{50} of 26 μM and thus proved to be a very efficient ligand to P-selectin. Interestingly, this sLe^x-RGD peptide conjugate showed practically no affinity to E-selectin.

Scheme 61



A cyclic sLe^x-containing glycopeptide, prepared by a combined strategy utilizing solid-phase peptide synthesis and fragment condensation in solution, displayed an opposite behavior (Scheme 58).[234]

First, linear heptapeptide **266** containing D-alanine was synthesized on the solid phase according to the Fmoc strategy on an acid-labile SASRIN resin. Cleavage of the heptapeptide from the solid support with 1% TFA in CH_2Cl_2 was achieved without affecting the *tert*-butyl ester side chain protecting groups of the aspartic acid residues. The linear heptapeptide **266** was cyclized with HATU/HOAt/DIPEA,[52] applying high dilution conditions to prevent intermolecular dimerization. Cleavage of the side chain *tert*-butyl ester furnished cycloheptapeptide **267**, which was condensed with sialyl Lewis^x-amine **263** (see above). After hydrogenolytic removal of the benzyl ethers, the acetyl groups as well as the intramolecular lactone were cleaved by treatment with NaOH in water/methanol at pH 10.6.

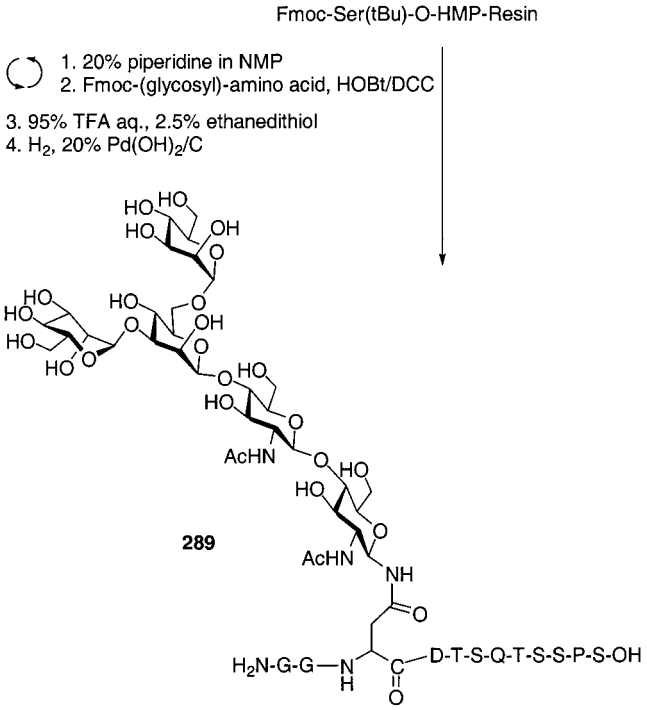
Glycoconjugate **268** inhibited the adhesion of HL 60 cells to E-selectin with an IC_{50} of 0.35−0.6 mM but showed no affinity to P-selectin.

A chemoenzymatic approach was chosen for the syntheses of trivalent sLe^x-containing glycoconjugates.[235] Starting from known *N*-acetylglucosamine asparagine building block **269**,[236] glycosyl amino acid **270** was obtained by removal of the Fmoc group with morpholine (Scheme 59). *N*-Acetylation and cleavage of the remaining protecting groups yielded compound **271**, which was used in a later stage of the synthesis.

EDCI-promoted condensation of Z-ω-amino acids **272**[237] with glycosyl amino acid ester **270** resulted in the formation of the corresponding glycoconjugates, which were deacetylated and deprotected at the amino function (→**273**). Coupling of **273** with the C-terminally deprotected glycosyl amino acid **271** furnished bis-glycosylpeptides **274** in high yield. Cleavage of the *tert*-butyl ester of **274** and condensation with building block **270** gave the trimeric structures **275**. These substrates were now converted into the sialylated glycopeptides **276** by glycosyltransferase-catalyzed reactions with the corresponding glycosyl nucleotides as indicated in Scheme 60. Glycosylation reactions were carried out in the presence of alkaline phosphatase which destroyed the inhibiting nucleoside phosphates formed from the sugar nucleotides.[238]

Compared with monomeric sLe^x bound to *N*-acetyl aspartic acid *tert*-butylester (IC_{50} = 0.6 mM), trimeric

Scheme 62



glycopeptide **276** with $n = 4$ showed a 4-fold higher affinity toward E-selectin (IC_{50} = 0.14 mM). Its homologous compound with $n = 5$ only exhibited an IC_{50} of 0.39 mM, suggesting that the affinity of clustered selectin ligands is dependent on the distance between the saccharide ligands.

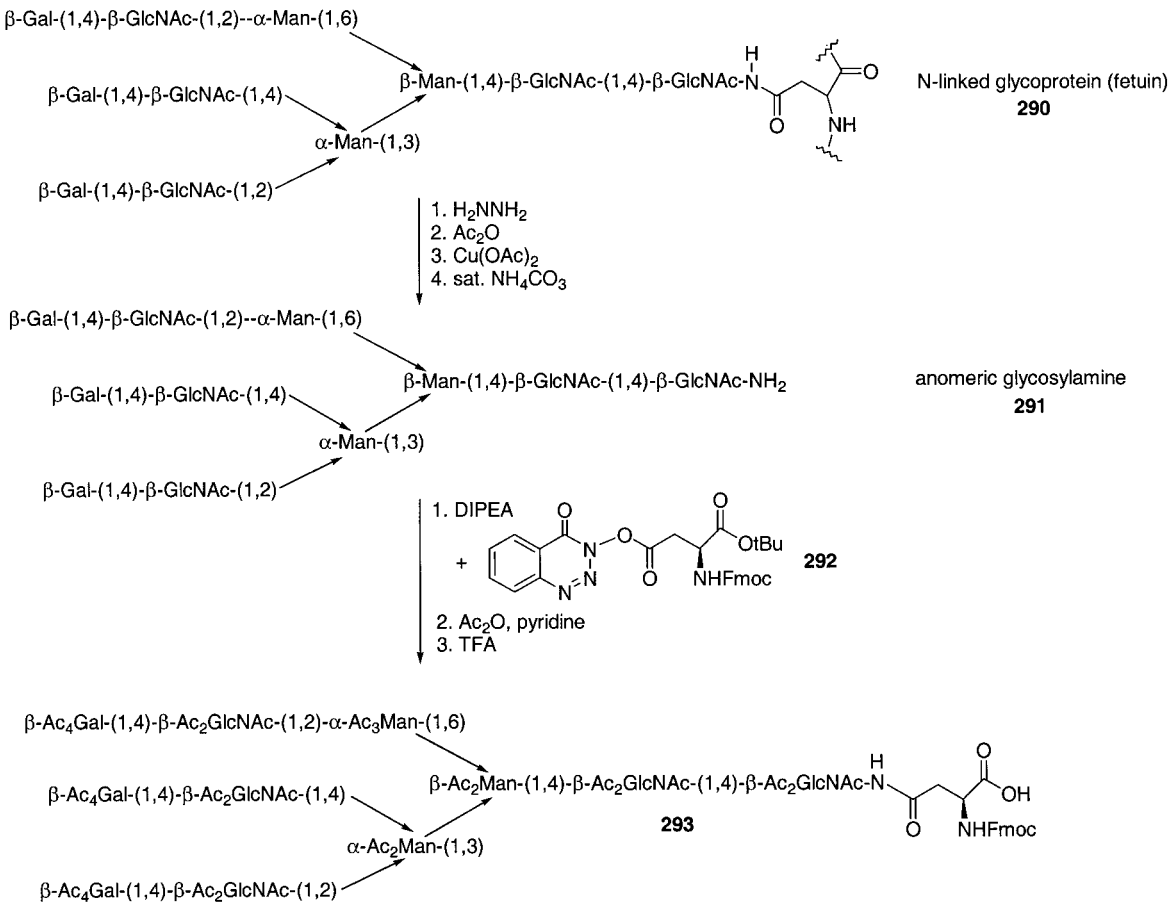
A solely enzymatic synthesis of a sialyl Lewis^x containing glycopeptide has been performed using ribonuclease B as starting material.[239] Ribonuclease B is a 124 amino acid glycoprotein that contains a single glycosylation site at asparagine 34. The natural enzyme consists of several glycoforms, which are all of the high-mannose type. By treatment of RNase B with endoglycosidase H, these natural saccharide substituents were specifically cleaved between chitobiose GlcNAc units to yield homogeneous GlcNAc−RNase. The glycosyl side chain of the truncated protein was elongated in the carbohydrate portion to give the sLe^x-containing glycoprotein by applying the same enzymatic techniques as described above.

E. Complex *N*-Glycopeptides

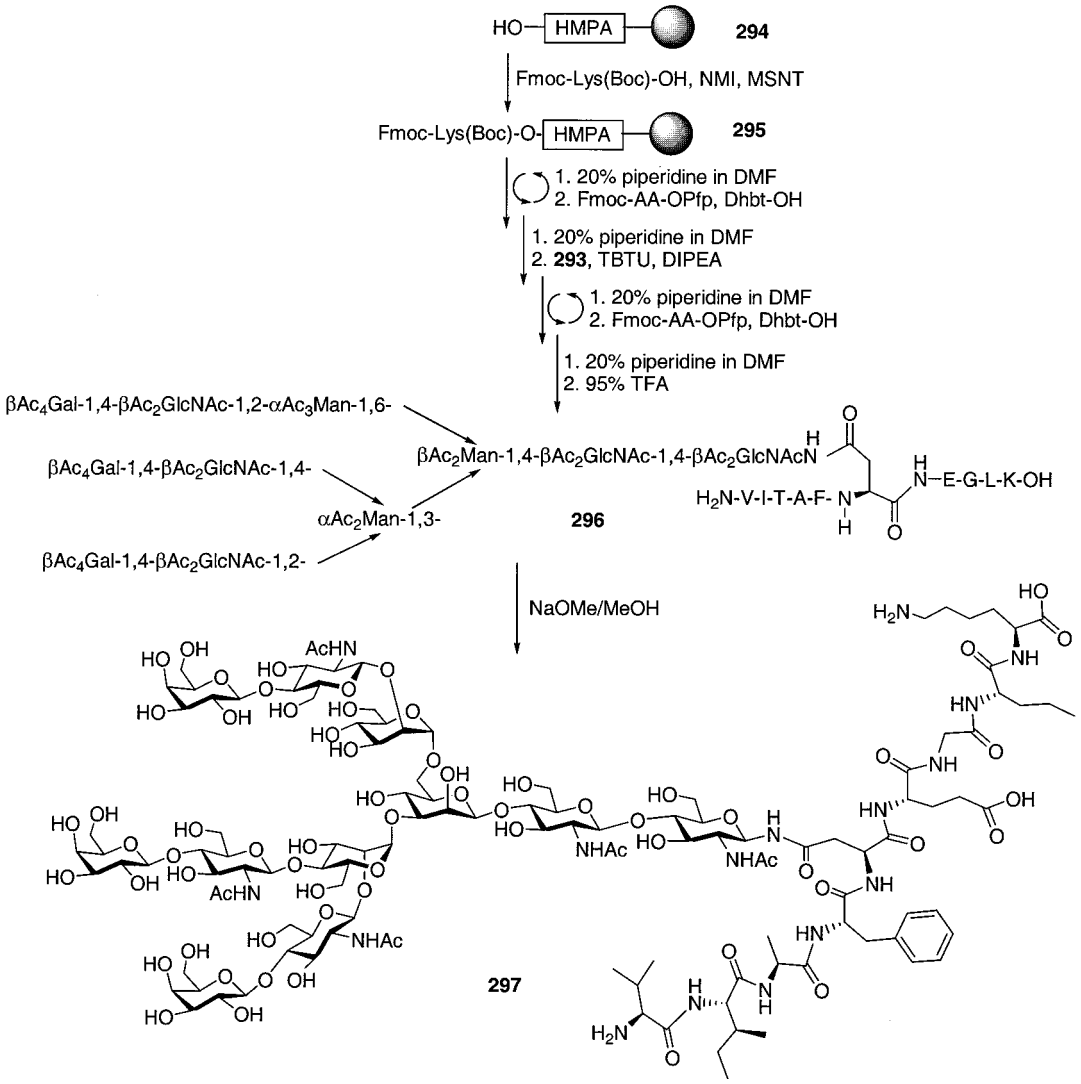
A solution-phase synthesis of *N*-glycotripeptide **288**, carrying the core pentasaccharide of asparagine-linked glycans, has been reported by T. Ogawa et al.[240] The sequence Asn-Val-Thr corresponds to amino acids 78−80 of the α-chain of human chorionic gonadotropin[241] (a glycopeptide hormon), carrying an *N*-linked oligosaccharide attached to Asn.[78] The synthesis of **288** starts with the assembly of glycosylasparagine building block **285** (Scheme 61):

Disaccharide **279** was obtained by glycosylation of *N*-phthaloylated glucosamine derivative **278** with mannosyl bromide **277**,[242] using silver silica−alumina as promotor.[243] Although the combination of an insoluble promotor and a mannosyl donor with ether-type protection in the 2-position favors the formation

Scheme 63



Scheme 64



of β-mannosides, the disaccharide was produced as a mixture of anomers from which **279** had to be separated by chromatography. The *p*-methoxyphenyl group was converted into the anomeric fluoride, which after activation by a hafnocene complex[244] reacted with glucosyl azide **280** to give **281**. Deallylation of **281** was achieved by an iridium-catalyzed process,[138] and the resultant diol was glycosylated with mannosyl chloride **282** to give branched α-mannoside **283**. The phthalimido group was exchanged for acetyl after cleavage with ethylenediamine/*n*-BuOH.[245] Simultaneously, the 2-*O*-acetyl groups of the mannoses also were removed. Hydrogenolysis of the anomeric azide in the presence of aspartic acid anhydride **284** gave the desired building block **285** (three steps). Extension of the peptide was performed by cleaving the *tert*-butyl ester and coupling with dipeptide **286**. Final deprotection of glycoconjugate **287** was achieved in two steps: cleavage of the Fmoc group with morpholine and hydrogenolysis of the benzyl ethers afforded target structure **288** in 58% yield.

In addition to the described solution-phase synthesis, building block **285** has also been used in a solid-phase synthesis of the glycopeptide antigen CD 52.[205,246] CD 52 consists of a dodecapeptide that contains a single *N*-glycosidic linkage. Monoclonal antibodies against CD52 have been used in vitro and in vivo to avoid recjection between transplant and host, e.g., rejection of bone marrow transplants.[247,248]

For the synthesis of target structure **289** (Scheme 62), solid-phase synthesis was performed on a hydroxymethylpolystyrene (HMP) resin.

Coupling was achieved with DCC/HOBt in NMP. Detachment from the resin and cleavage of the *tert*-butyl ether side chain protecting groups was carried out with 95% aqueous TFA containing 2.5% ethanedithiol as scavenger. Mass spectrometric analysis revealed considerable amounts of partially debenzylated glycopeptide. Therefore, the mixture was directly subjected to hydrogenolysis with 20% $Pd(OH)_2$ on charcoal. Purification by gel permeation chromatography followed by HPLC furnished the expected CD 52 antigen **289** in 74% yield.

A triantennary complex type oligosaccharide released from fetuin, the major glycoprotein of fetal calf serum, has been employed in the synthesis of glycodecapeptide **297** (Scheme 64).[249] Fetuin is a 341-amino acid protein with six carbohydrate moieties per molecule, three *O*-glycosidically linked to Ser or Thr and three *N*-glycosidically bound to Asn.[250] Whereas the *O*-glycosides are only small, the majority of the

Asn-linked oligosaccharides (about 80%) are triantennary complex-type structures.

The nonimmunogenic sequence VITAFNEGLK in **297** is derived from mouse hemoglobin. Its *N*-linked glycoform serves as T-cell epitope and has been selected to allow investigation of the activation of T-cells by glycopeptides bound to MHC-class II molecules.

The preparation of **297** started with the release of the saccharides from fetuin by hydrazinolysis (Scheme 63).[251] *N*-Acetylation of the obtained glycosylhydrazides prior to cellulose column chromatography separated the large *N*-linked saccharides from smaller *O*-linked oligosaccharides and peptidic impurities. To obtain the anomeric glycosylamine **291**, the hydrazino group was cleaved with $Cu(OAc)_2$. Heating in 0.25 mM sulfuric acid at 80 °C cleaved residual sialic acids from the oligosaccharide. Treatment of the asialo saccharide with saturated aq. NH_4HCO_3 gave glycosylamine **291** according to the method of Kochetkov.[181] The amine **291** was condensed with Fmoc-Asp(ODhbt)-OtBu **292** and the product purified by preparative HPLC. Acetylation of the free hydroxy groups of the sugar moieties and deprotection of the carboxyl group with neat TFA yielded building block **293** on a 20-mg scale ready for use in solid-phase peptide synthesis.

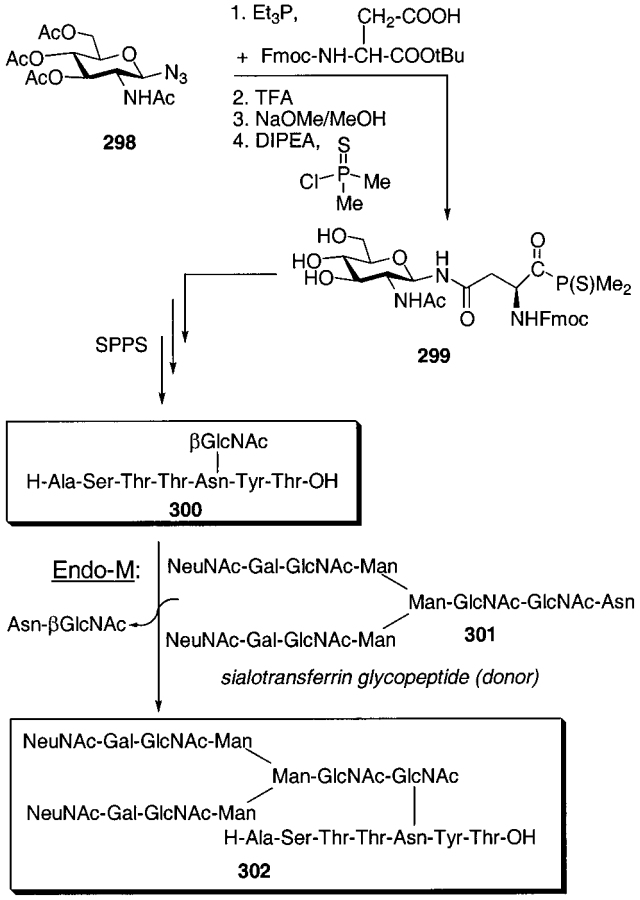
Reactions were performed on PEGA resin equipped with the acid-labile HMPA-linker **294**. The unglycosylated Fmoc amino acids were coupled as Pfp-esters in the presence of Dhbt-OH, whereas glycosylated building block **293** was employed in the presence of TBTU/DIPEA (Scheme 64). After addition of the *N*-terminal amino acid, the Fmoc group was removed. Cleavage of the glycopeptide from the resin and simultaneous amino acid-side chain deprotection was performed by treatment with aqueous 95% TFA. The crude glycopeptide **296** was precipitated by trituration with ether and subsequently deacetylated to give target compound **297**.

A highly glycosylated derivative of peptide T has been obtained by endoglycosidase M-catalyzed transglycosylation of a sialo-biantennary complex-type oligosaccharide from human transferrin.[252] Endo M (EC 3.2.1.96) is a unique endoglycosidase that hydrolyzes *N*,*N*′-diacetylchitobiosyl linkages in oligosaccharides bound to asparaginyl residues of various glycopeptides. In addition to the hydrolytic activity, the enzyme can act as transglycosidase which transfers oligosaccharides to suitable acceptors containing GlcNAc residues.

The first step of the chemoenzymatic synthesis of glycosylated peptide T **302** consisted of the solid-phase synthesis of *N*-acetylglucosamine carrying peptide T **300** (Scheme 65).

The *N*-acetyl glucosamine building block **299** was obtained in a one-pot reaction of 2-acetamido-3,4,6-tri-*O*-acetyl-2-desoxyglucopyranosyl azide **298** with Fmoc-aspartic acid *tert*-butyl ester in the presence of triethylphosphine.[236] Coupling reactions were performed on a polystyrene resin using dimethylthionophosphinic mixed anhydrides without protection of the carbohydrate hydroxyl groups. Cleavage of the glycopeptide from the resin was carried out with a

Scheme 65

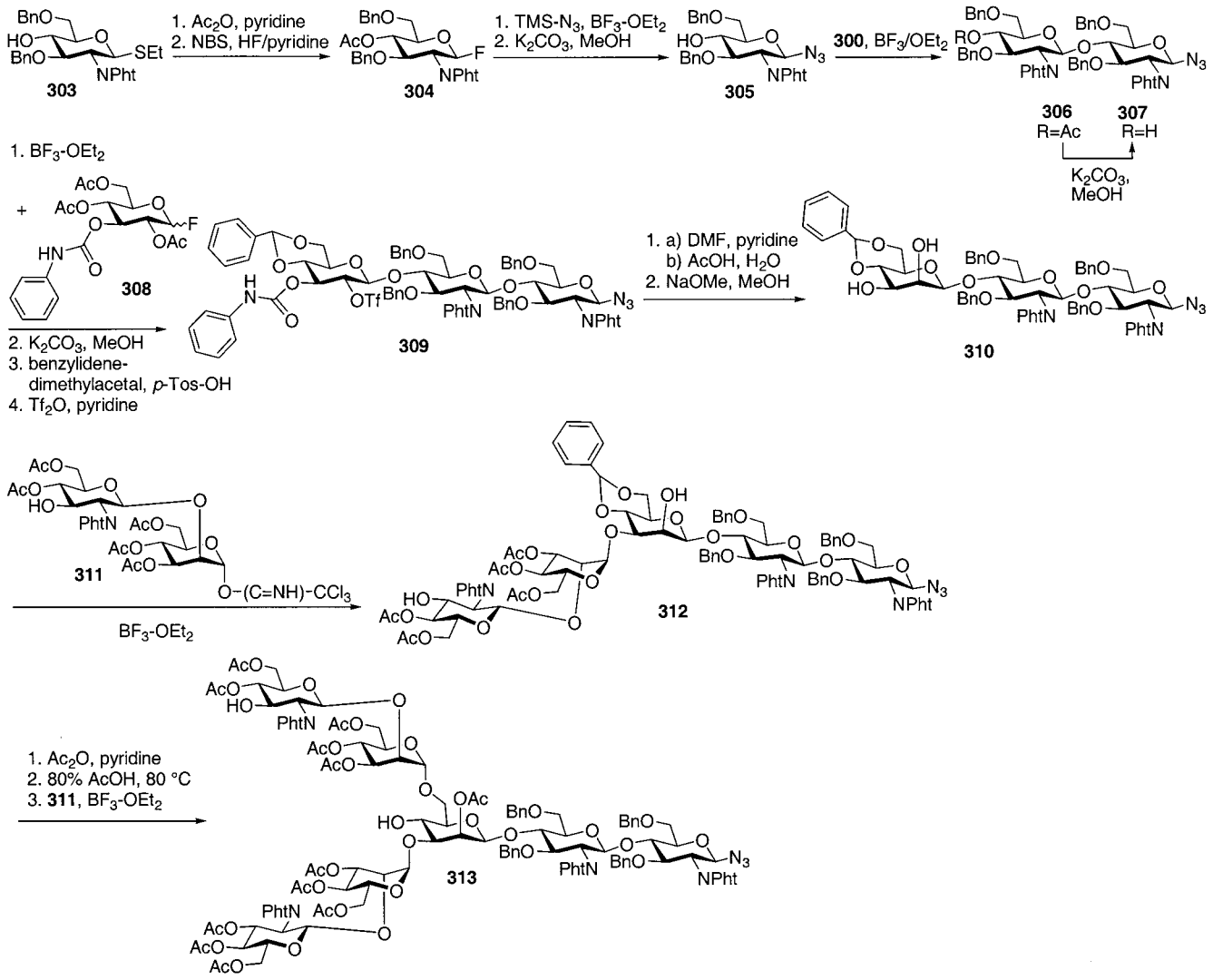


mixture of TFA and different scavengers, which also removed the amino acid protecting groups. For the transglycosylation reaction, sialotransferrin glycopeptide was prepared by exhaustive Pronase digestion of human transferrin and purification of the fragments by Sephadex G-25 gel filtration. Incubation of the sialotransferrin glycosyl asparagine **301** and the *N*-acetylglucosaminyl peptide T **300** with the endo-*β*-*N*-acetylglucosaminidase from *Mucor hiemalis*[253] gave peptide T glycosylated with an intact sialo biantennary complex-type oligosaccharide **302** in 9% yield after purification. The stability of natural, unglycosylated peptide T and the newly synthesized glycosylated derivative **302** were investigated by incubating both compounds with Pronase. After 30 min, almost all peptide T was degraded but about 80% of the glycosylated peptide T remained unaffected.

Further examples for the application of endoglycosidase M in glycopeptide synthesis as the transfer of oligosaccharides from human transferrin glycopeptides to synthetic human chorionic gonadotropin peptide[254] and to eel calcitonin[255] containing an *N*-acetylglucosamine moiety have been reported.

A total synthesis of an undecasaccharide-glycopentapeptide fragment of bovine ribonuclease B was accomplished by solution-phase glycopeptide chemistry combined with enzymatic techniques.[256] Target structure **319** (Scheme 67) was obtained via solution-phase peptide synthesis using heptasaccharide asparagine building block **315**. Subsequent enzymatic protecting-group manipulations and enzymatic ex-

Scheme 66



tension of the diantennary saccharide chain finally furnished undecasaccharide-glycopeptide **318**.

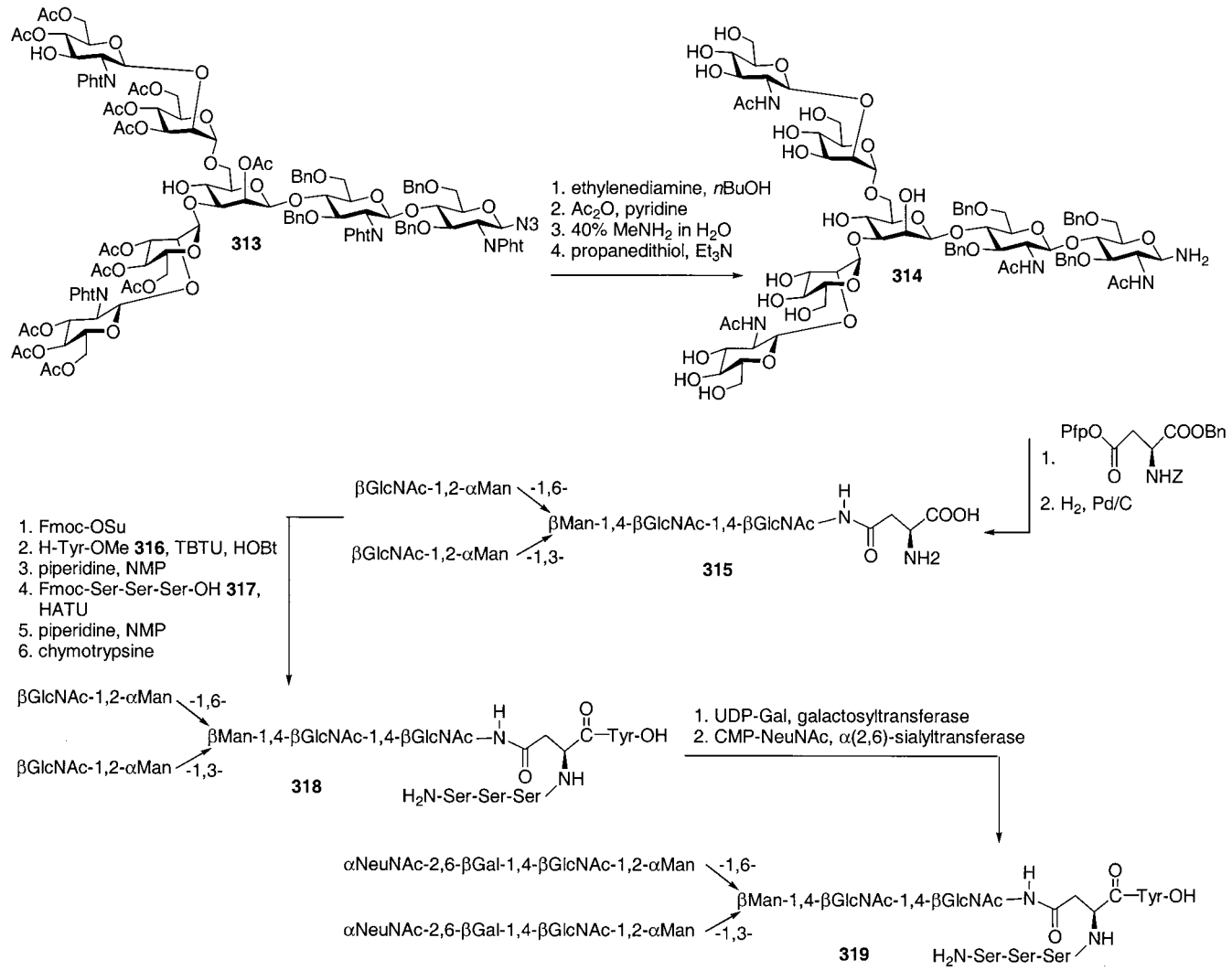
In the synthesis of building block **313**, fluoride **304** played a crucial role, because it served as both glycosyl donor and starting material for the synthesis of acceptor **305** (Scheme 66). It was synthesized from thioglycoside **303**[257] by acetylation of the 4-OH and fluorination of the anomeric position. Replacement of the fluoride by an azido group and deprotection of the 4-*O*-acetyl function furnished acceptor **305**, which was glycosylated with **304** to yield chitobiosyl azide **306**.[258] Cleavage of the acetyl group furnished disaccharide acceptor **307**. β-Mannosylation of **307** was achieved by reaction with 3-phenylcarbamoylglucoside **308** and subsequent intramolecular inversion of the configuration of the 2″-position (Scheme 66).[259] To this aim, the *O*-acetyl groups were removed, the resulting triol was benzylidenated, and the remaining 2″-OH group was converted into the triflate **309**. Treatment with DMF/pyridine at 60 °C inverted β-glucosaccharide **309** to the corresponding β-manno-configurated imidocarbonate, which was hydrolyzed via a carbonate intermediate (→ **310**). Stereo- and regioselective glycosylation of the equatorial 3-hydroxy function with trichloroacetimidate **311** yielded pentasaccharide **312**, which was acetylated in the 2″-position. Acidolytic cleavage of the benzylidene acetal and a second regioselective glycosylation with donor **311** under high dilution conditions furnished heptasaccharide **313**.

The acetyl groups and the phthalimido groups in **313** were cleaved. After *N*-acetylation, the anomeric azide was reduced to glycosylamine **314** with propanedithiol (Scheme 67). Acylation with Z-Asp(OPfp)-OBn and subsequent hydrolygenosis of the Z and benzyl protecting groups accomplished building block **315**.

Solution-phase peptide synthesis was performed in both *C*- and *N*-terminal direction. First, glycosyl amino acid **315** was converted into the Fmoc derivative and *C*-terminally extended by condensation with tyrosine methylester **316**. Selective liberation of the amino group and coupling with Fmoc-protected tripeptide **317** gave heptasaccharide carrying pentapeptide **319**. Despite the numerous hydroxy functions present in the heptasaccharide moiety, acylation occurred chemoselectively at the amino group.

Because of the lability of the remaining protecting groups under the conditions of the following enzymatic elongation of the saccharide chain, both the Fmoc group and the methyl ester were removed. Alkaline cleavage of methyl esters often is accompa-

Scheme 67



nied by epimerization.[260] Therefore, mild enzymatic hydrolysis with chymotrypsin was the method of choice.[261]

Extension of the two branches of the biantennary *N*-glycoside was performed in a stepwise manner. First galactose was transferred to the terminal *N*-acetyl glucosamine residue by reaction of UDP-galactose with galactosyltransferase. The intermediate nonasaccharide was elongated by a regio- and stereoselective sialylation with α(2,6)-sialyltransferase yielding glycopeptide **319** in 91%. In both enzymatic steps the reaction mixture contained alkaline phosphatase to hydrolyze the liberated nucleosides[238] and thus shift the equilibrium toward the formation of the desired glycoside.

Compound **319** is the first example for a total synthesis of a glycopeptide carrying a full-length asparagine-linked oligosaccharide. On the basis of the described methods, the synthesis of whole glycoproteins seems to be within reach.

V. Outlook

Though the given article is far from being complete, this compilation illustrates the efficiency of the present synthetic toolbox that facilitates the preparation of glycopeptides of high complexity. This includes glycopeptides which have particularly critical and sensitive structures, e.g., fucose- and sialic acid-containing saccharides. Again, it must be emphasized that the target molecules depicted are of biological importance. However, this biological background could only be mentioned in passing.

Future efforts in glycoconjugate chemistry will particularly focus on the improved assembly of complex saccharide side chains either in solution or on solid phase employing chemical or chemoenzymatic methodologies. Whereas a purely enzymatic approach can enable elegant access to natural target molecules, unnatural glycopeptides and derivatives of pharmacological interest, as a rule, will require the application of chemical methodologies. If remaining problems such as the efficient construction of glycoconjugates containing β-mannosyl, sialyl, and/or fucosyl residues, particularly on solid-phase, can be solved, a new stage of complexity of synthetic glycopeptides might be attained. Moreover, new findings in the area of biologically important glycoproteins will constantly initiate the development of improved synthetic methods. On the other hand, biological investigations will be increasingly supported by the availability of defined synthetic glycopeptides as model compounds.

VI. Abbreviations

A	alanine
AA	amino acid
Ac	acetyl
Acm	acetamidomethyl
All	allyl
Bn	benzyl
Boc	*tert*-butyloxycarbonyl
BOP	benzotriazole-1-yl-oxy-tris(dimethylamino)phosphoniumhexafluorophosphate
^{t}Bu	*tert*-butyl
Bz	benzoyl
Bzl	benzyl
C	cysteine
CAN	ceric ammonium nitrate
CMP	cytidine monophosphate
Coll	*sym*-collidine
CPG	controlled pore glass
D	aspartic acid
DAST	diethylamino sulfurtrifluoride
DBU	1,8-diazobicyclo[5.4.0]undec-7-ene
DCC	*N*,*N*′-dicyclohexylcarbodiimide
DCM	dichloromethane
Dhbt	3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl
DIC	diisopropylcarbodiimide
DIPEA	diisopropylethylamine
DMAP	(dimethylamino)pyridine
DMBA	dimethylbarbituric acid
DMDO	dimethyldioxirane
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DMTST	dimethylthiomethylsulfonium triflate
DPPA	diphenylphosphoryl azide
Dts	dithiasuccinoyl
DTT	dithiothreitol
E	glutamic acid
EDCI	1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide
EEDQ	2-ethoxy-1-ethoxycarbonyl-1,2-dihydrochinoline
F	phenylalanine
Fmoc	9-fluorenylmethoxycarbonyl
Fuc	fucose
G	glycine
Gal	galactose
GalNAc	2-acetamido-2-deoxygalactose
GDP	guanosine diphosphate
GlcNAc	2-acetamido-2-deoxyglucose
H	histidine
HATU	2-(1*H*-9-azobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HBTU	2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
Hmb	2-hydroxy-4-methoxybenzyl
HMBA	4-hydroxymethylbenzoic acid
HMPA	hydroxymethyl-phenoxyacetic acid
HOAt	1-hydroxy-7-azabenzotriazole
HOBt	1-hydroxybenzotriazole
HPLC	high-performance liquid chromatography
HYCRON	(*E*)-17-hydroxy-4,7,10,13-tetraoxa-15-heptadecanoyl
I	isoleucine
IIDQ	1-isobutyloxycarbonyl-2-isobutyloxy-1,2-dihydrochinoline
K	lysine
L	leucine
Lac	lactose
Le^x	Lewis x
M	methionine
Man	mannose
MBHA	methylbenzhydrylamine
Me	methyl
ME	methoxyethyl
Mp	methoxyphenyl
Mpm	*p*-methoxyphenylmethyl
MSNT	1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole
N	asparagine
NeuNAc	*N*-acetyl neuraminic acid
NMI	*N*-methylimidazole
NMM	*N*-methylmorpholine
NMP	*N*-methylpyrrolidone
Nps	2-nitrophenylsulfenyl
Orn	ornithine
P	proline
Pac	phenacyl
PAL	5-(4′-aminomethyl-3′,5′-dimethoxyphenoxy)valeric acid
PAM	4-(hydroxymethyl)phenylacetamidomethyl
PAPS	adenosine 3′-phosphate 5′-phosphosulfate
PDT	propane-1,3-dithiol
PEGA	poly(ethylene glycol)−dimethylacrylamide copolymer
Pfp	pentafluorphenyl
Ph	phenyl
PhAcOZ	*p*-(phenylacetoxy)benzyloxycarbonyl
POEPOP	polyoxyethylene−polyoxypropylene copolymer
PyBOP	benzotriazole-1-yl-oxy-trispyrrolidinophosphonium hexafluorophosphate
Q	glutamine
R	arginine
S	serine
SASRIN	super acid-sensitive resin
SLe^x	sialyl-Lewis-X
SPPS	solid-phase peptide synthesis
Su	succinimidyl
T	threonine
TBAF	tetrabutylammonium fluoride
TBDMS	*tert*-butyldimethylsilyl
TBDPS	*tert*-butyldiphenylsilyl
TBTU	2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate
Tcoc	*N*-trichloroethoxycarbonyl
Teoc	2-(trimethylsilyl)ethoxycarbonyl
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TIPS	triisopropylsilyl
TMS	trimethylsilyl
TMSOTf	trimethylsilyl triflate
TPST-1	tyrosyl-protein sulfotransferase-1
Trt	trityl
Tos	*p*-toluenesulfonyl
UDP	uridine diphosphate
V	valine
Xyl	xylose
Y	tyrosine
Z	benzyloxycarbonyl

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