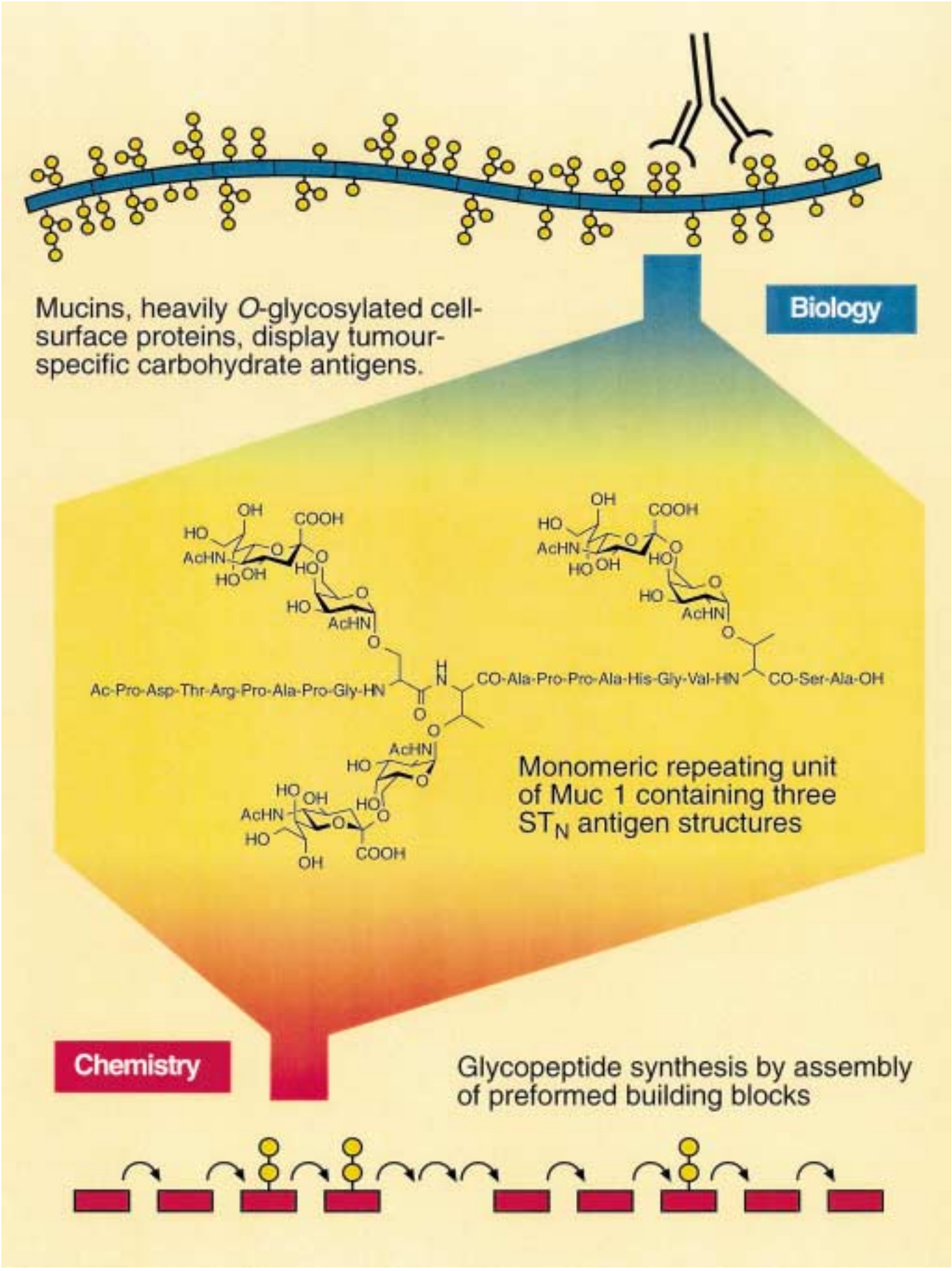

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Glycopeptide Synthesis and the Effects of Glycosylation on Protein Structure and Activity

Oliver Seitz*[a]

Dedicated to Professor Horst Kunz on the occasion of his 60th birthday

Despite the omnipresence of protein glycosylation in nature, little is known about how the attachment of carbohydrates affects peptide and protein activity. One reason is the lack of a straightforward method to access biologically relevant glycopeptides and glycoproteins. The isolation of homogeneous glycopeptides from natural sources is complicated by the heterogeneity of naturally occuring glycoproteins. It is chemical and chemoenzymatic synthesis that is meeting the challenge to solve this availability problem, thus playing a key role for the advancement of glycobiology. The current art of glycopeptide synthesis, albeit far from being routine, has reached a level of maturity that allows for the access to homogeneous and pure material for biological and medicinal research. Even the ambitious goal of the total synthesis of an entire glycoprotein is within reach. It is demonstrated that with the help of synthetic glycopeptides the effects of glycosylation on protein structure and function can be studied in molecular detail. For example, in immunology, synthetic (tumour-specific) glycopeptides can be used as immunogens to elicit a tumour-cell-specific immune response. Again, synthetic glycopeptides are an invaluable tool to determine the fine specificity of the immune response that can be mediated by both carbohydrate-specific B and T cells. Furthermore, selected examples for the use of synthetic glycopeptides as ligands of carbohydrate-binding proteins and as enzyme substrates or inhibitors are presented.

KEYWORDS:

bioorganic chemistry · glycobiology · glycopeptides · glycoproteins · solid-phase synthesis

1. Introduction

The majority of proteins are posttranslationally modified, the most abundant modification being the attachment of carbohydrates to the side chains of asparagine, serine and threonine. The glycans modulate the structure of the proteins and regulate their biological half-life and activity.[1] Glycoproteins mediate biological recognition events as illustrated by their involvement in cell adhesion, cell differentiation and cell growth.[2–4] Parasites have evolved that use the heavily glycosylated membrane-bound proteins protruding into the extracellular space as points of entry.[5] Aberrant glycosylation is associated with various plagues such as autoimmune and infectious diseases and cancer. It comes as no surprise that researchers of chemistry, biochemistry, immunology and medicine are interested in providing specific tools for the diagnosis and therapy of glycoconjugate-related diseases. However, only through a cooperative effort can a rapid progress be achieved. Such an interdisciplinary project requires both information transfer within and between disciplines. It is along these lines that the current state in glycopeptide synthesis will be presented here, as well as selected examples of the studies on the biological role of protein and peptide glycosylation in lectin binding, epitope mapping, T-cell stimulation and immunotherapy. The examples presented throughout this review were chosen for instructive purposes. It is not intended to give a comprehensive overview[6–12] of the entire area of glycopeptide chemistry and glycopeptide biology, which appears to be unachievable anyway. Furthermore, it is important to note that this review is focussed on synthetic glycopeptides containing natural *N*- and *O*-glycosyl linkages, since these most closely resemble the structure of naturally ocurring glycopeptides and glycoproteins and therefore allow for the assessment of the biological role of protein glycosylation.

Glycosylation introduces an enormous structural diversity to proteins. Glycoproteins exist in various glycoforms possibly reflecting the subtle mechanism by which heterogeneous glycosylation regulates biological activity. The isolation of well-defined glycopeptides from natural sources is difficult and even with current techniques virtually impossible. In addition, recombinant proteins, which are synthesised by host cells in a necessary but artificial environment, might display an altered glycosylation pattern. It is chemistry that can solve this availability problem.

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2. The glycosidic linkage

Almost all of the naturally occuring glycosidic linkages can be classified into two principal groups: the *N*-glycosides, which are attached to the amide side chain of asparagine, and the more diverse *O*-glycosides, which are linked to the side chains of serine, threonine and tyrosine.[13, 14] Less common are *O*-glycosidic linkages to hydroxylysine and hydroxyproline. In vivo, *N*-glycosylation can occur only at asparagine residues embedded in the consensus tripeptide sequence Asn-Xxx-Ser/Thr, Xxx being any amino acid but proline.[15] During co-translational *N*-glycosylation, the enzyme oligosaccharyltransferase transfers one common oligosaccharide unit from dolichol phosphate to the protein. Various glycosylhydrolases then trim the oligosaccharide to a core pentasaccharide on which all further glycosylations occur. Other *N*-glycosidically linked monosaccharides such as β-D-glucose[16] and β-D-*N*-acetylgalactosamine[17] have been observed (Figure 1).

In contrast, the biosynthesis of *O*-glycosides is not following a common pathway. The core fragment that is most commonly displayed is 2-acetamido-2-deoxy-D-galactose (GalNAc)[*] attached to serine and threonine (Figure 2). A variety of different tissue-specific glycosyltransferases work upon the α-D-GalNAc bridgehead, leading to a diverse set of so-called mucin-type *O*-glycosides. Mucins are excessively *O*-glycosylated proteins that are expressed on the surface of various epithelial cell types.[18] These proteins constitute one important class of tumour-associated antigens and hold much promise as potential target for tumour therapy (see below).

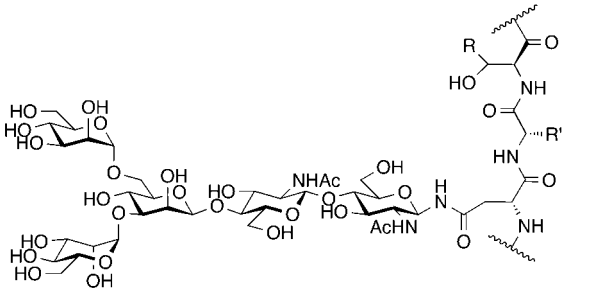

Figure 1. *Pentasaccharide core fragment of oligosaccharides N-glycosidically bound to asparagine in the consensus sequence Asn-Xxx-Thr/Ser.*

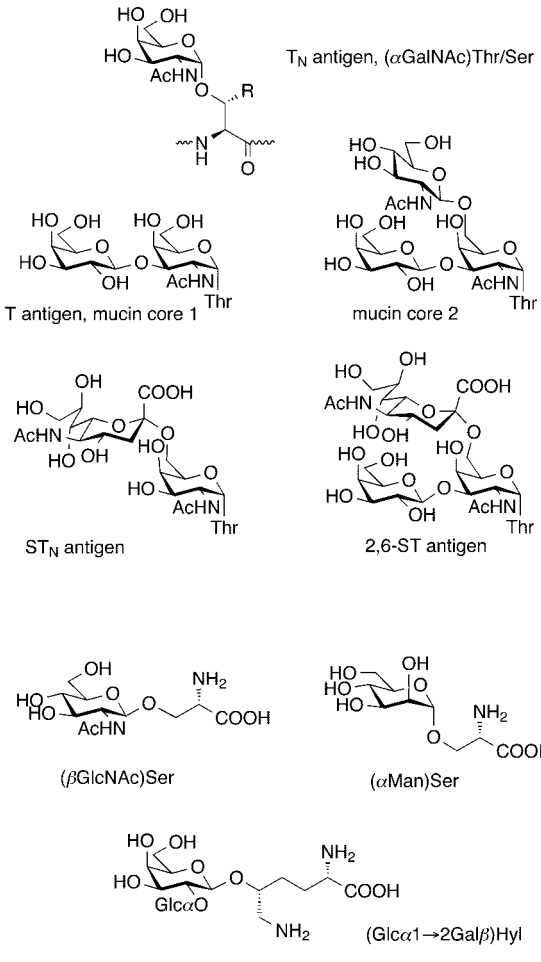

Figure 2. *Selected examples of carbohydrates O-glycosidically linked to serine and threonine.*

Recently, the β-*O*-glycosidic attachment of 2-acetamido-2-deoxy-D-glucose units to serine ((βGlcNAc)Ser) has been discovered (Figure 2).[19] The introduction of the β-*O*-GlcNAc moiety into nuclear pore proteins, transcription factors and cytoskeletal proteins seems to be involved in transcriptional regulation, analogous to the regulation of protein activity by phosphorylation.[20, 21] Many glycoproteins that were isolated from yeast carry α-D-mannose units linked to serine ((αMan)Ser).[22] This includes recombinant human proteins such as the insulin-like

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born in 1966, received his Diploma in chemistry from the University of Mainz in 1992 and obtained his Ph.D. degree there in 1995 under H. Kunz, developing a new linker for solid-phase glycopeptide synthesis. In 1996 he became a postdoctoral fellow working in the laboratories of C.-H. Wong at the Scripps Research Institute in La Jolla, California. He moved to the University of Karlsruhe in 1997 and started work towards his Habilitation under H. Waldmann. In 2000 he moved to Dortmund where he leads a group in the Department of Chemical Biology at the Max Planck Institute of Molecular Physiology and the Institute of Organic Chemistry at the University of Dortmund. His research interests include the synthesis and functionalisation of biopolymers such as peptides, glycopeptides, nucleic acids and analogues for further use as biomolecular tools in biological and medicinal research, and, most recently, the development of new strategies for the functionalisation of peptide nucleic acids.

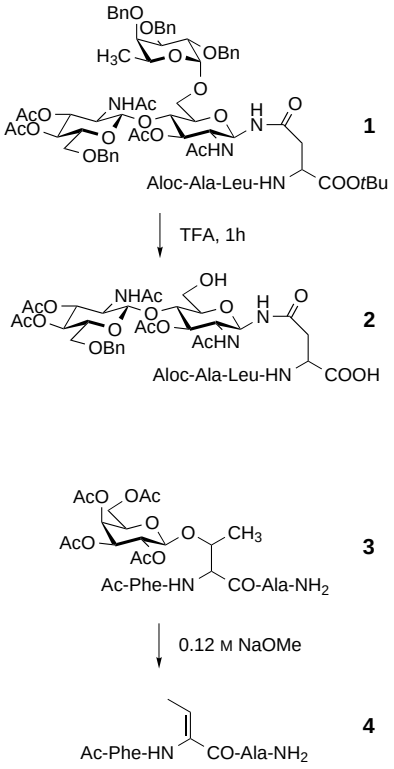
[*] *A list of abbreviations can be found at the end of the article.*

growth factor (IGF) and illustrates that non-human cell lines can produce human proteins with a non-human glycosylation pattern.[23] Structural proteins such as collagens contain hydroxylysine (Hyl) and frequently are found to be *O*-glycosylated by either β-D-galactose or Glcα1 $\rightarrow$2Galβ1 moieties.[24, 25] Interestingly, specific T cells seem to be stimulated upon presentation of β-D-Gal-containing peptide fragments of type II collagen leading to rheumatoid arthritis in a mouse model (see below). Many more types of *O*-glycosidic attachments are known. For more information on type and function of *O*-linked carbohydrates not presented in this review, the reader is referred to literature references [26 – 36].

3. Synthetic considerations

The crucial step of any synthesis of a glycopeptide is the introduction of the carbohydrate part. In principle, there are two strategies. Most commonly, preformed glycosylated amino acid building blocks are employed in the stepwise assembly of the peptide backbone. Alternatively, the carbohydrate can be conjugated to a full-length peptide. The latter will be discussed in the context of peptide assembly in Section 5.

Although the block glycosylation approach promises high convergence, it is hampered by the very nature of glycosyl-bond-forming reactions. For the introduction of *N*-glycosides, the side chain carboxy group of aspartic acid has to be activated, which in most cases almost inevitably leads to the formation of aspartimide by-products (see below). Even more complex is the formation of *O*-glycosidic bonds. Reliable *O*-glycosylation reactions are difficult to achieve stereoselectively when more complex targets are involved. Furthermore, *O*-glycosidic and intersaccharidic linkages turn out to be labile towards acids. Thus, the proper selection of protecting groups, necessary to block the numerous functional groups on both the glycan and the peptide part, is by no means a trivial matter. In a well-documented case, the acidolysis of the glycopeptide *tert*-butyl ester **1** was plagued by the undesired cleavage of the fucosidic linkage (Scheme 1).[37] Fortunately, acetylation of the trisaccharide rendered the fucoside less labile. Global acetyl protection of the glycan hydroxy groups is now a standard technique in solid-phase glycopeptide synthesis. Glycosidic linkages of common carbohydrates such as GalNAc, GlcNAc, Gal, Glc and Man, especially when they are acylated, withstand short treatment with TFA used for removal of side-chain-protecting groups in solid-phase synthesis. Particularly stable glycosides such as *N*- and *O*-linked GlcNAc residues can withstand treatment with HF.[38] However, the stability of *O*-glycosidic bonds depends on several parameters like the amount of scavenger used and, of course, the particular structure of the glycopeptide.[39] The number of possible protecting groups is further limited by the base lability of the *O*-glycosidic linkage. Under basic conditions such as a 0.12 M solution of sodium methylate in methanol, normally used for the removal of carbohydrate *O*-benzoyl groups, abstraction of the serine or threonine α-proton induced the β-elimination of the carbohydrate in **3** (Scheme 1).[40] Morpholine or piperidine are not basic enough to promote β-elimination. Thus, application of the well-established and probably most versatile Fmoc protecting group strategy (Fmoc = 9-fluorenylmethoxycarbonyl) is feasible.[41]



Scheme 1. *Acid lability of the α-fucosidic linkage (**1** $\rightarrow$**2**) and β-elimination of O-linked glycans under basic conditions (**3** $\rightarrow$**4**).*

It is because of the complexity outlined above that the construction of neoglycopeptides through chemoselective ligation reactions is an attractive alternative. Several excellent reviews describe the synthesis as well as the biological application of neoglycoconjugates.[42–45] However, neoglycopeptides contain non-natural glycosyl linkages that could possibly but not necessarily interfere with the biological activity. One possible rescue might be offered by chemoenzymatic methods for the assembly of glycopeptides.[46, 47] Mimicking the natural biosynthesis pathways certainly has great potential to facilitate the access to complex glycans. A requirement, however, is the availability of the corresponding glycosyl- and oligosaccharyl-transferases.

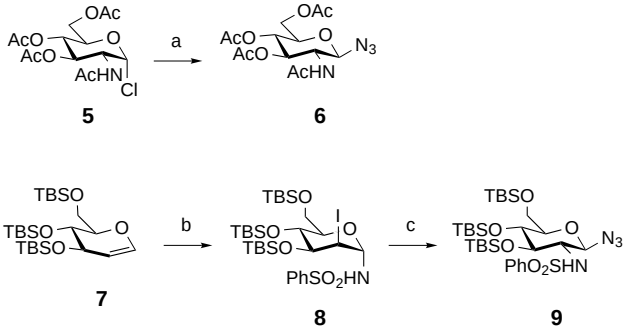
4. Synthesis of preformed glycosyl amino acids

Recently, reviews appeared that were focused on the construction of glycosyl amino acid linkages in solution and on the solid phase.[48, 49] This review does not intend to give a comprehensive overview of all methods of glycopeptide and glycosyl amino acid synthesis. The examples presented in the following Sections were selected with the aim to outline some principles of current synthetic methodology and, thus, serve instructive purposes rather than comprehensiveness.

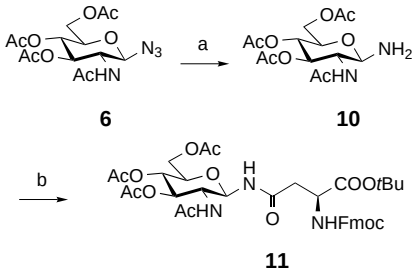
4.1. *N*-Glycosides

4.1.1. β-*GlcNAc-Asn*

With a few exceptions all *N*-glycosides are linked through a β-GlcNAc bridgehead. Consequently, the vast majority of research in the field had been focused on the preparation of this particular linkage. The construction of the *N*-glycosidic bond usually proceeds through amide-bond-forming rather than glycosylation reactions. Therefore, the predominant tactic used is that of synthesizing a peptide coupling between an N- and C-terminally protected aspartic acid and a glycosylamine. Thus, a key step in the synthesis of *N*-glycosides is the preparation of glycosylamines. The most common procedure involves the synthesis of glycosyl azides **6** and the subsequent reduction to the labile amines (Schemes 2 and 3). Useful precursors for glycosyl azides are glycosyl halides such as **5**, which in



Scheme 2. *A typical example of a glycosyl azide synthesis. a) NaN_3, CH_2Cl_2, $NaHCO_3$, H_2O, Bu_4NHSO_4, 98%;[56] b) $I(coll)_2ClO_4$, H_2NSO_2Ph, CH_2Cl_2, $-10\,^{\circ}C$, 60%; c) NaN_3, DMF, 92%.*



Scheme 3. *A typical example of the reduction of a glycosyl azide and the coupling of glycosylamine with Fmoc/tBu-protected aspartic acid. a) H_2, Raney-Ni;[37] b) DCC, HOBt, THF, 59%.[72]*

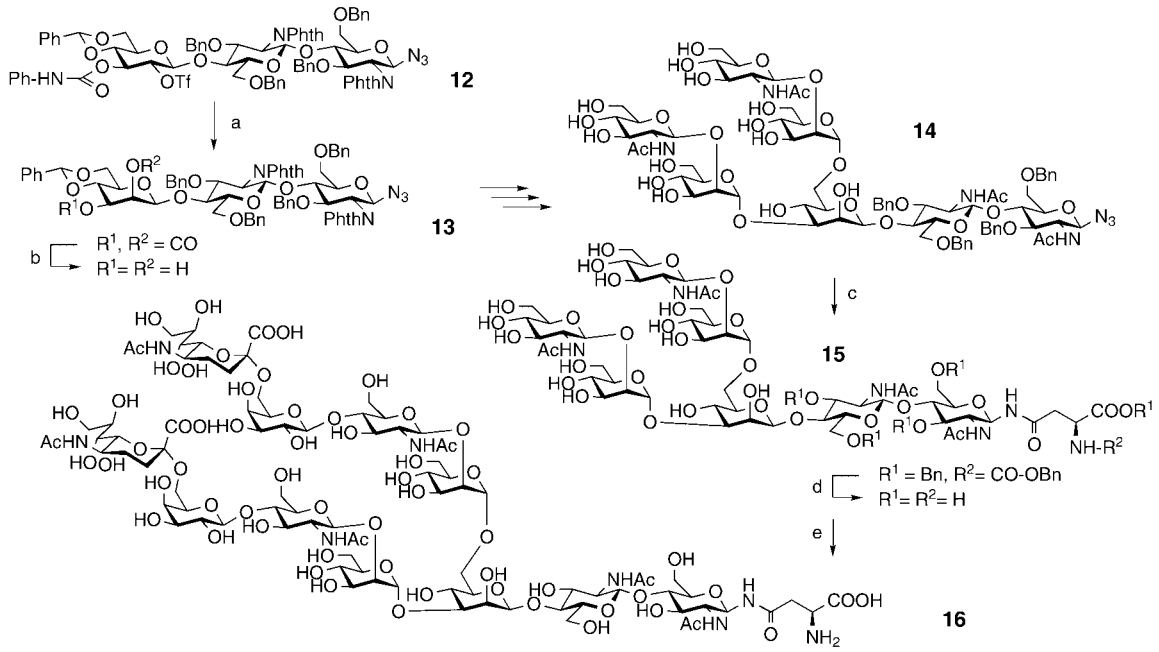
homogeneous systems have to be treated with silver azide, sodium azide or tetramethylguanidinium azide in organic solvents such as toluene, DMF or acetonitrile.[50–54] Most conveniently, phase transfer catalysis provides the glycosyl azides in high yield.[55, 56] An alternative can be the treatment of anomeric acetates or bromides with trimethylsilyl azide.[57–59] McDonald and Danishefsky reported[61] a different route to access glycosyl azides, in which glycals[60] were used. For example, treatment with iodocollidinium perchlorate in the presence of phenylsulfonamide led to iodosulfonamidation of the TBS-protected glycan **7** (Scheme 2).[61] The subsequent reaction of the iodosulfonamide **8** with sodium azide furnished the glycosyl azide **9**. After acetylation of the sulfonamide moiety, the removal of the phenylsulfonyl group or the anthracenylsufonyl group,[62] which is used for the synthesis of more complex oligosaccharides, was achieved by photolysis or thiolysis.

The conversion of a glycosyl azide such as **6** to the corresponding amine has been accomplished by heavy-metal-catalysed hydrogenation,[37, 63–71] Staudinger reaction,[72] transfer hydrogenation[73] and hydride reduction.[74] For the construction of the *N*-glycosidic bond, protected or unprotected glycosylamines were subjected to a suitably activated aspartic acid building block. Scheme 3 shows a typical procedure, in which the *O*-acetylated glycosylamine **10** was acylated to obtain the protected *N*-glycosyl amino acid **11**.[72] Virtually all protecting group combinations appear to be possible.[50, 75–81]

Glycosylamines have been prepared directly from unprotected sugars by using ammonia in methanol or ammonium hydrogen carbonate.[75, 82] This technique has been particularly useful for carbohydrates from natural sources. Meldal and co-workers utilised this approach for the synthesis of protected glycosylamines.[83] An alternative method for the synthesis of the β-*N*-glycosidic bond involves glycosyl isothiocyanates that are readily available through the reaction of glycosyl halides with potassium rhodanide.[84] Günther and Kunz applied this method to the synthesis of β-mannosylchitobiosyl asparagine, a compound that comprises the central trisaccharide core of *N*-glycoproteins.[85]

For the synthesis of more complex *N*-glycans, the introduction of the carbohydrate moiety is commonly performed by using a block condensation of the full-length glycosylamine with the aspartic acid derivative. One example was described by Ogawa and co-workers in their elegant synthesis of the Asn-linked core pentasaccharide.[86] An impressive report on the chemoenzymatic synthesis of the sialylated undecasaccharide – asparagine conjugate **16** was published by Unverzagt (Scheme 4).[73] A crucial intermediate was the trisaccharide building block **13**. According to a protocol from Kunz and Günther,[87, 88] heating the 2-*O*-triflate **12** in DMF and pyridine led to inversion of the configuration at C2 through cyclisation, thus installing the *manno* configuration in **13**. A series of two subsequent glycosylation reactions and protecting-group manipulations yielded the heptasaccharidyl azide **14**.[89] After reduction of the azide, coupling with the Z/Bn-protected aspartic acid proceeded by preferential formation of the β-*N*-glycoside **15**. The completely deblocked heptasaccharide – asparagine conjugate was used as a substrate in the enzymatic double galactosylation with galactosyltransferase, UDP-GalNAc serving as a donor.[90] The regio- and stereoselectivity of glycosyltransferase-catalysed reactions was implemented a second time by performing an ($\alpha 2 \rightarrow 6$)-sialyltransferase-catalysed double sialylation with CMP-*N*-acetylneuraminic acid (CMP-NeuNAc) as a donor substrate.

Recently, an alternative method for the synthesis of complex *N*-linked glycosyl amino acid building blocks was reported.[91] This approach was based on the mild hydrazinolysis procedure for releasing naturally occurring *N*-linked oligosaccharides in an intact, unreduced form from natural glycoproteins and subsequent formation of the corresponding glycosylamines. For



Scheme 4. *a) i DMF, Pyr, 60 °C; ii AcOH, dioxane, H_2O, 0 °C; $BF_3 \cdot Et_2O$, CH_2Cl_2, 85 %; b) NaOMe, MeOH, CH_2Cl_2, 70 %; c) i propanedithiol, NEt_3, MeOH; ii Z-Asp(Pfp)-OBn, HOBt, NMP, 63 %; d) Pd, H_2, MeOH, AcOH, 95 %; e) i UDP-Gal, β-1,4-galactosyltransferase, alkaline phosphatase; ii CMP-NeuNAc, α-2,6-sialyltransferase, alkaline phosphatase, 86 %.*

example, the major glycoprotein of foetal calf serum, bovine fetuin (**17**), was treated with hydrazine to afford the triantennary-complex-type oligosaccharidylhydrazine **18** (Scheme 5). After *N*-acetylation, the acetohydrazide **19** was isolated. Subsequent treatment with copper acetate yielded the oligosaccharide **20**. The conversion to the glycosylamine **21** was accomplished by applying the method of Kochetkov et al.[75] Coupling with Fmoc/*t*Bu-protected aspartic acid Dhbt-active ester, subsequent *O*-acetylation and acidolysis furnished the triantennary building block **22**. It should be noted that the preparative separation can be problematic when proteins are used that occur in various glycoforms.

4.2. *O*-Glycosides

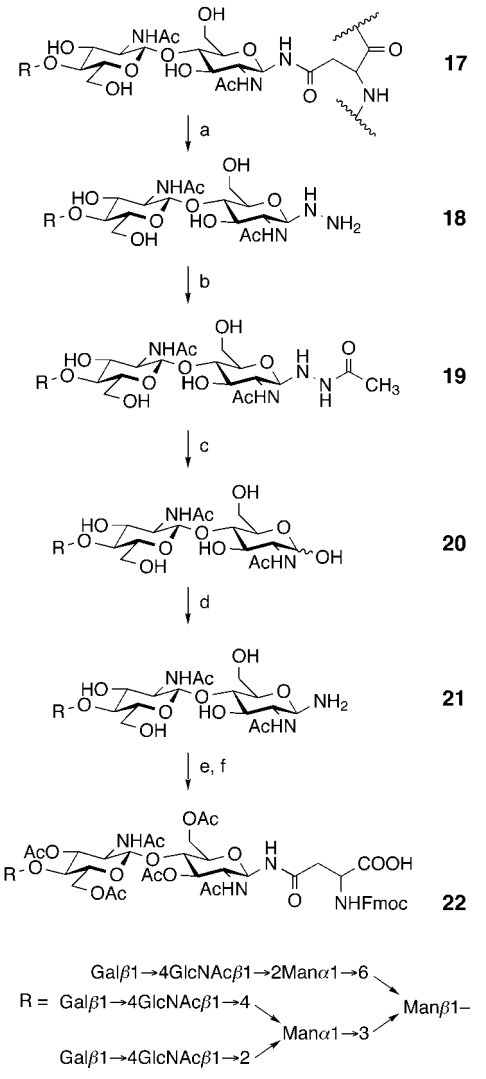
4.2.1. α-GalNAc-Thr/Ser

By far the most abundant carbohydrate that is found in naturally occurring *O*-glycoproteins is the 2-acetamido-2-deoxy-D-galactose (GalNAc) residue, which is α-*O*-glycosidically bound to serine or threonine. Accordingly, a great deal of work was devoted towards establishing suitably protected GalNAc(α1 $\rightarrow$O)Thr/Ser building blocks. Nearly all syntheses utilised the non-participating azido group for protection of the 2-acetamido moiety according to the protocol of Lemieux and Ratcliffe (Scheme 6).[92] Most commonly, the 1-bromo-[93, 94] and 1-chlorosugars[41, 95, 96] such as **23** were employed in Königs – Knorr type glycosylations of Fmoc-protected serine and threonine esters. Highest α-selectivities were obtained when an insoluble promotor such as silver perchlorate was used. For reestablishing the 2-acetamido group, the azido group in **24** was reduced to yield an amine which was subsequently acetylated to give **25**.[95, 96] Alternatively, reductive acetylation can be performed with thioacetic acid.[41, 93, 94] Protecting the carboxy group as pentafluorophenyl ester yielded building blocks that are suitable for direct use in Dhbt-OH-mediated peptide couplings.[97, 98]

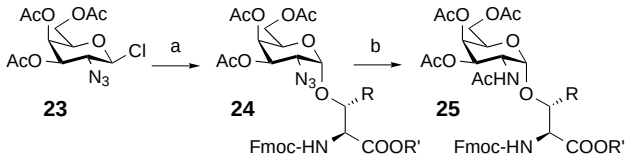
A conceptionally different route towards α-GalNAc-Ser/Thr building blocks was realised in the laboratory of Schmidt.[99] Rather than performing a glycosylation, a Michael-type reaction of 2-nitrogalactal with Boc/*t*Bu-protected serine or threonine was utilised to establish the α-glycosidic bond.

For the synthesis of *O*-glycosyl amino acids of higher complexity, the glycan can be introduced as a block or by using a suitably protected α-GalNAc-Ser/Thr unit as an acceptor, to which further glycosyl donors would be appended. The former approach offers a high degree of convergence for the assembly of glycopeptides as represented in the reaction of disaccharide donor **26** with Fmoc/Pfp-protected serine or threonine **27** (Scheme 7).[100] Depending on the promotor and the temperature, either α-glycoside **28** or β-glycoside **29** was preferentally formed.

Similar synthetic approaches towards the synthesis of T antigen building block **28** and related mucin-type glycosyl amino acids were previously reported.[96, 101–104] Although very popular, bromo- and chlorosugars are not always the optimal glycosyl donors for stereocontrolled glycosylation reactions. In the synthesis of the sialyl-(2 $\rightarrow$6)-T-antigen building blocks **31**, the Königs – Knorr-type glycosylation was compared with the phosphite and trichloroacetimidate methods (Scheme 8).[105] Interestingly, the trichloroacetimidate activation displayed the highest α-selectivity for the glycosylation of serine, whereas the Königs – Knorr reaction was found to be optimal for threonine. The

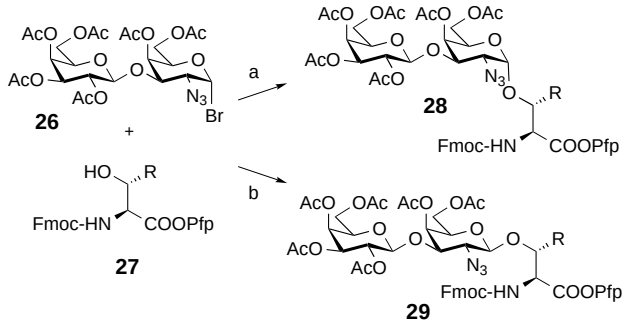


Scheme 5. *a) H_2NNH_2, 85 °C; b) Ac_2O, sat. $NaHCO_3$, 0 °C, Dowex AG-50X12; c) $Cu(OAc)_2$, 0.1 M AcOH, r.t., 30 min, purification; d) sat. NH_4HCO_3, 45 °C, 30 h, 95 %; e) Fmoc-Asp(ODhbt)-OtBu, $EtNiPr_2$, DMSO, r.t., 3 h; f) i Ac_2O, Pyr; ii TFA, r.t., 1 h.*

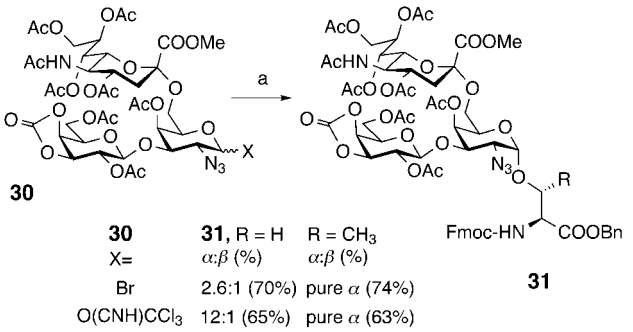


Scheme 6. *a) Fmoc-NH-CH(CHROH)-COOR', $Ag_2CO_3/AgClO_4$ (R' = Bn: R = CH_3, 60 %, pure α-anomer; R = H, 65 %, pure α-anomer;[95] R' = tBu: R = CH_3, 81 %, α:β = 9:1; R = H, 70 %, α:β = 8:1;[96] R' = All: R = CH_3, 50 %, pure α-anomer; R = H, 55 %, pure α-anomer;[41] b) R' = Bn: i $NaBH_4$, $NiCl_2$, ii Ac_2O, Pyr, 85 % (R = CH_3), 86 % (R = H);[95] R' = tBu: i H_2S, Pyr, ii Ac_2O, Pyr, 81 % (R = CH_3), 75 % (R = H);[96] R' = All, CH_3COSH, 82 % (R = CH_3), 81 % (R = H).[41]*

trichloroacetimidate method appears to have a general and broad applicability for the anomeric activation of high molecular weight oligosaccharides. For example, even a bissialyl-T-antigen building block was prepared through trichloroacetimidate activation of a tetrasaccharide.[106]



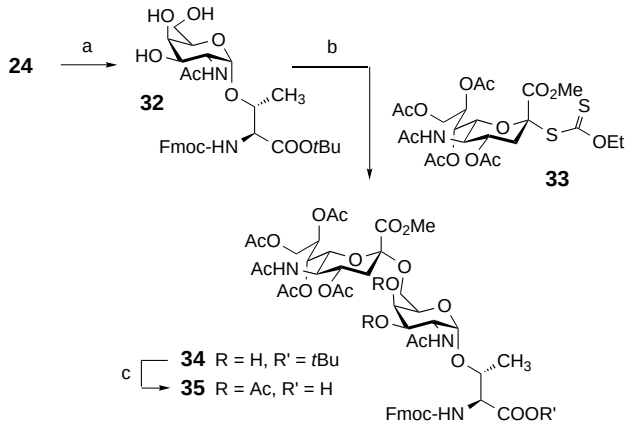
Scheme 7. *a) $AgClO_4$, CH_2Cl_2, $PhCH_3$, −40 °C (R = H, 51 % pure α-anomer; R = CH_3, 68 % pure α-anomer); b) $AgClO_4/Ag_2CO_3$, CH_2Cl_2, $PhCH_3$, 25 °C (R = H, 56 % pure β-anomer; R = CH_3, 69 % pure β-anomer).*



Scheme 8. *a) X = Br: $AgClO_4$, CH_2Cl_2, r.t.; X = O(CNH)CCl_3: $BF_3 \cdot Et_2O$, THF, −30 °C.*

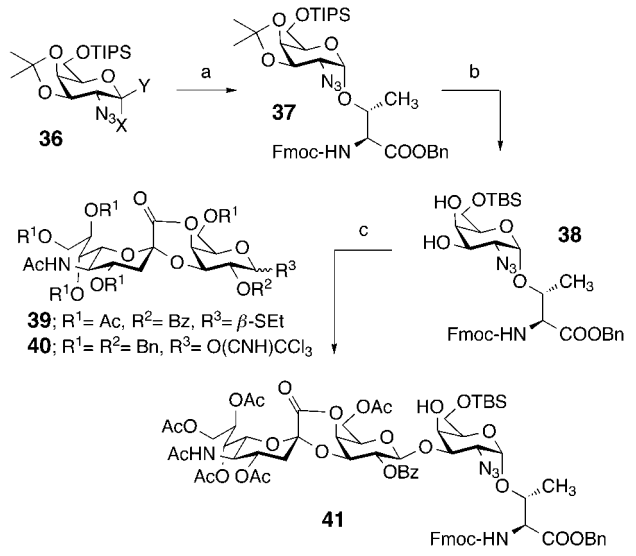
Although remarkable with respect to the size and complexity of the glycosyl donors, the modest yields as well as the poor α-selectivities achieved at this advanced stage render the block glycosylation approach inefficient. One major drawback is that the stereoselective outcome of *O*-glycosylation reactions becomes less predictable as the complexity of the glycosyl donor increases. To this end, the strategy in which a preformed α-GalNAc-Thr/Ser glycoside serves as an acceptor for further elaboration on the carbohydrate part offers an appealing alternative. Liebe and Kunz fashioned this approach, which Danishefsky recently termed the "cassette approach",[110] in the synthesis of a sialyl-T_N antigen.[94] From the α-glycoside **24** (see Scheme 6) all *O*-acetyl groups were removed (Scheme 9). The resulting glycoside **32** was subjected to a regioselective sialylation with the sialyl xanthogenate **33**. Acetylation of the remaining two hydroxy groups of **34** and acidolysis of the *tert*-butyl ester readied the 5-step synthesis of building block **35**, which is suitable for the use in solid-phase synthesis. A similar building block was accessed in the laboratory of Kihlberg in a 10-step synthesis.[107] Mathieux and co-workers used the 4,6-benzylidine-protected form of glycoside **32** as a universal precursor for the synthesis of mucin core 1, core 2, core 4 and core 6 building blocks.[102]

Recently, the group of Danishefsky started an ambitious program towards the synthesis and immunological evaluation of tumour-associated glycopeptides.[108] In the reported syntheses of the building blocks containing the T antigen (core 1), the sialyl-T_N antigen, the sialyl-T antigen, the bissialyl-T antigen and a



Scheme 9. *a) i CH_3COSH, 84%; ii MeOH, NaOMe, pH < 8.5, 77%; b) MeSBr, AgOTf, MeCN, CH_2Cl_2, −62 °C, 32% pure α-anomer; c) i Ac_2O, Pyr, 88%; ii TFA/PhOMe (13:1), quant.*

basic substructure of a Lewis Y blood group determinant, the broad applicability of the "cassette approach" was demonstrated.[109] As a common precursor, the 2-azidogalactosylthreonine derivative **37** was prepared by using either the fluoride or the trichloroacetimidate of the 6-silyl-protected acetonide of 2-azidogalactose **36** (Scheme 10).[110] Removal of the isopropylidene

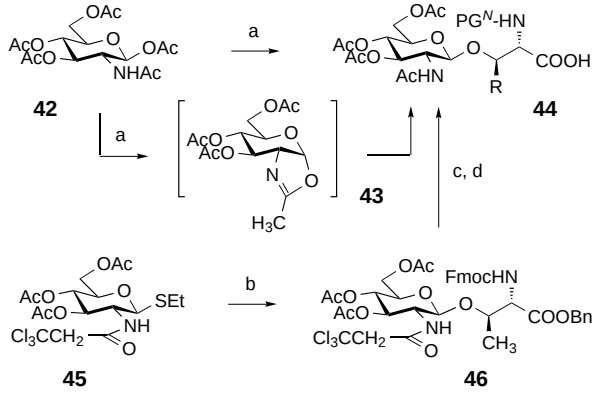


Scheme 10. *a) X = H, Y = O(CNH)CCl_3: 0.5 equiv TMSOTf, THF, R = H: 86% pure α-anomer, R = CH_3: 15% pure α-anomer; X = F, Y = H: $Cp_2ZrCl_2/AgClO_4$, CH_2Cl_2, R = H: 89%, α:β = 2:1, R = CH_3: 87%, α:β = 6:1; b) i I_2, MeOH; ii TBSCl, imidazole, DMF, 85% c)* ***39****, NIS, TfOH, CH_2Cl_2, molecular sieves (MS; 4 Å), 10 min, 62%.*

and the triisopropylsilyl ether groups and subsequent TBS-protection of the primary hydroxy group yielded the 3-acceptor **38**.[111] Activation of the thioethyl glycoside **39** with NIS in the presence of trifluoromethanesulfonic acid furnished trisaccharide **41** in high yield and with excellent β-selectivity. In a similar approach, Nakahara and co-workers previously used the trichloroacetimidate **40**, in which the carboxy group of the sialic acid was also protected as a lactone.[112]

4.2.2. β-GlcNAc-Thr/Ser

Because of the putative regulatory role in the transient modification of proteins, much attention has been drawn to the synthesis of the β-*O*-GlcNAc-Ser/Thr moiety. In the event of the glycosylation reaction, α-1,2-*trans* selectivity has to be achieved, which usually is guaranteed by exploiting neighbouring-group participation. Since 2-acetamido-2-deoxyglucose (GlcNAc) seems to have such a group already in place, early attempts focused on the use of 2-acetamido-2-deoxy-3,4,6-tri-*O*-protected glucosyl halogenides as glycosyl donors.[113] However, the yields were low due to the poor reactivity of the formed oxazoline intermediate **43** (Scheme 11). As the presence of Lewis acid catalysts increased the oxazoline reactivity, $BF_3 \cdot Et_2O$ activation of glycosyl acetates **42** has been demonstrated to



Scheme 11. *a) MS (4 Å), CH_2Cl_2, $BF_3 \cdot Et_2O$, NEt_3, PG^N = Fmoc: R = H, 55%, R = CH_3, 53%;[114] PG^N = Z: R = H: 49%, R = CH_3, 41%;[115] PG^N = PhacOZ: 54%;[116] b) DMTST, CH_2Cl_2, 78%; c) i Zn, AcOH, ii Ac_2O, Pyr, 77%; d) Pd/C (5%), H_2, EtOAc, EtOH, 88%.[115]*

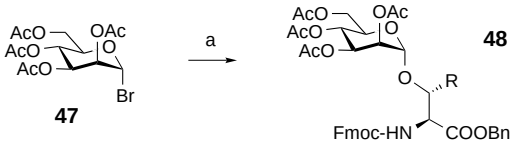
improve the yields of *N*-protected β-*O*-GlcNAc-serine and -threonine derivatives **44**.[114–116] Interestingly, the use of the readily available anomeric acetates requires no protection of the amino acid carboxy group, thus rendering protecting-group manipulations unnecessary.[117] In principle, the reaction of the peracetylated glucosamine **42** with *N*-protected serine or threonine provides the quickest access to the GlcNAc-substituted amino acid building blocks **44**. However, the necessity for laborious purification procedures, particularly after scale-up of the glycosylation reaction, can be a serious limitation.[118]

Replacement of the *N*-acetyl group by strongly electron-withdrawing groups reduces the extent of oxazoline formation, and highly efficient glycosylations were reported when the Aloc-,[119] Troc-[120–122] or Dts-protected[123] donors were employed. A typical example is given in Scheme 11. The DMTST-promoted reaction of the thioglycoside **45** with Fmoc/Bn-protected threonine furnished conjugate **46** in high yield.[115] The subsequent reductive cleavage of the *N*-Troc group was followed by *N*-acetylation and hydrogenation delivering glycosyl amino acid **44** (PG^N = Fmoc, R = CH_3). Palladium-catalysed cleavage of the Aloc group or reductive removal of the Troc or the Dts group can be performed in the presence of acetic anhydride, thus minimising the number of steps and possible side reactions.

This is of particular importance when Fmoc-protected Pfp-active ester is used, since the unprotected amino group easily participates in an intramolecular acylation reaction. Alternatively, the Dts group can be removed on the solid phase with thiols, although the reported yields are lower.[124]

4.2.3. α-Man-Thr/Ser

The synthesis of α-mannosylserine and -threonine is relatively straightforward since both the anomeric effect and the neighbouring-group participation increase the α selectivity. For example, the reaction of the peracetylated mannosyl bromide **47** with Fmoc/Bn-protected serine or threonine in the presence of silver triflate afforded the α-mannosyl amino acids **48** in high yield (Scheme 12).[125] The Königs – Knorr-type coupling of disaccharide donors was also used in the glycosylation of Fmoc/Pfp-protected amino acids.[126, 127] A similar approach was exploited for the synthesis of α-mannosylthreonine derrivatives containing a phosphate moiety at the 6-position.[128] Peracetylated mannose was used as the glycosyl donor in the reaction with C-terminally unprotected Fmoc-threonine.[118]

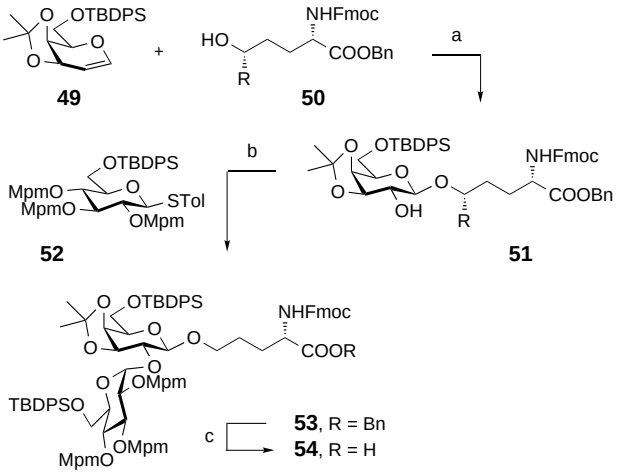


Scheme 12. a) AgOTf, CH_2Cl_2, 71% (R = CH_3), 68% (R = H).

4.2.4. β-Gal-Hyl

The attachment of β-galactose or β-glucosyl-(α1 $\rightarrow$2)-galactose to the hydroxylysine residues of collagen was suggested to be involved in the induction of rheumatoid arthritis.[129] Broddefalk and co-workers reported the synthesis of (Galβ)-Hyl and (Glcα1 $\rightarrow$2Galβ)-hydroxynorvalin building blocks **51** and **54** (R = H), the latter serving as a model compound for the corresponding hydroxylysine (Scheme 13).[130] Both the hydroxylysine **50** (R = BocHNCH$_2$) and the hydroxynorvaline **50** (R = H) were glycosylated by using the glycal **49**. During the synthesis, the glycal **49** was converted to the α-1,2-anhydrosugar that, after treatment with $ZnCl_2$, furnished the β-galactosides **51**. Attempts to attach the α-glucosyl residue to the 2-hydroxy group of the hydroxylysine **51** (R = BocHNCH$_2$) failed. However, the model compound **51** (R = H) proved to be a suitable acceptor for the glycosylation reaction with the methoxybenzylated thioglucoside **52**. Recently, it was demonstrated that glucosylation of **51** is possible when an *N*-Z blocking group is installed instead of the *N*-Boc protecting group.[131]

Due to the vast number of glycosides that were found to be *O*-glycosidically linked to hydroxy-group-containing amino acids, only a few examples were presented here. However, for the type of glycosides that were not considered, some literature syntheses are given in refs. [132 – 142] for the readers' perusal.



Scheme 13. a) i DMDO, acetone, CH_2Cl_2, 0 °C; ii $ZnCl_2$, THF, AW-300, −50 °C → r.t., R = H: 50%, R = CH_2NHBoc: 37%; b) NIS, AgOTf, CH_2Cl_2, MS (4 Å), −45 to −15 °C, 71%; c) H_2, 10% Pd/C, NH_4OAc, EtOAc, 87%.

5. Synthesis of glycopeptides

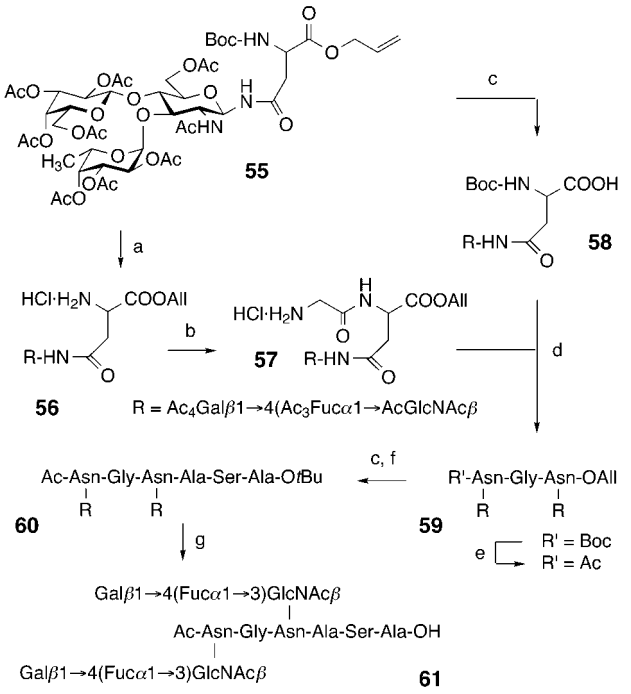
In developing a methodology for glycopeptide synthesis, the additional complexity and lability as conferred by the carbohydrate part (see Section 3) must be considered. Hence, glycopeptide synthesis presents a synthetic challenge, particularly with respect to the protecting-group chemistry, which has to allow for selective removal without harming the acid- and base-labile glycoconjugates (see Scheme 1). Herein, this feature will receive particular attention and the only examples presented are the ones in which complete deblocking was demonstrated. Principally, glycopeptides are synthesised in solution or on the solid phase. Solution and solid-phase approaches will be discussed in independent sections and each approach will be further subdivided into *N*- and *O*-glycopeptide synthesis by using either preformed glycosyl amino acid building blocks or peptide and glycopeptide glycosylation techniques.

5.1. Synthesis of glycopeptides in solution

In the early days of glycopeptide assembly, it was believed that coupling reactions involving rather bulky glycosyl amino acids would be low-yielding. In combination with the lack of assessing a proper reaction control in solid-phase synthesis and the attempt to avoid the use of a large excess of valuable building blocks, most of the early syntheses were performed in solution rather than on the solid phase. In addition, many of the linkers that are compatible with the present solid-phase synthesis techniques of glycopeptides were not available then. However, it is by no means true that solution-phase synthesis would be old-fashioned. Particularly, when larger amounts of target compounds are desired, carrying out reactions in solution allows for the efficient and economical synthesis of small to medium-sized oligomers.

5.1.1. N-Glycopeptides

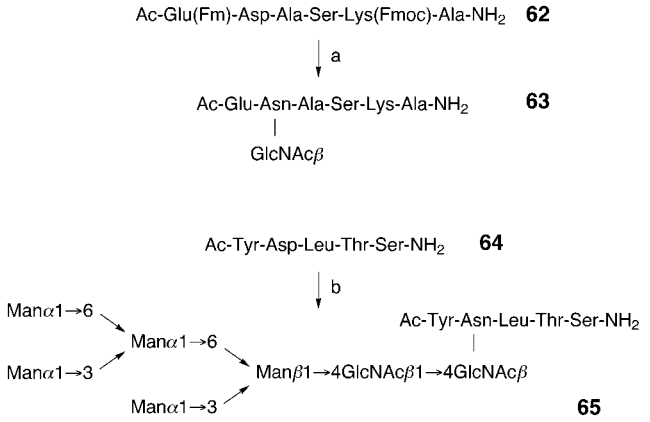
A typical example of the current art of solution-phase assembly has been reported by von dem Bruch and Kunz.[70] In the synthesis of the *N*-glycopeptide cluster **61** that contains two Lewis X residues, the carbohydrate hydroxy functions were equipped with acetyl groups (→**55**), which allowed for easy removal of the Boc group without damaging the labile α-fucoside moiety (Scheme 14). The resulting hydrochloride **56** was subjected to a coupling with *N*-Boc-protected glycine followed by acidolytic Boc removal to obtain the amine **57**. Rh^{I}-catalysed deallylation of **55** afforded **58**. **57** and **58** were coupled



Scheme 14. *a) HCl, Et_2O, 92%; b) i Boc-Gly-OH, IIDQ, CH_2Cl_2; ii HCl, Et_2O, 86%; c) [$(PPh_3)_3RhCl$], EtOH/H_2O (9:1), 70 °C, 93%; d) EDC, DIPEA, HOBt, CH_2Cl_2, 64%; e) i HCl, Et_2O; ii Ac_2O, Pyr, 82%; f) TBTU, HOBt, H-Ala-Ser-Ala-OtBu, MeCN, 73%; g) i HCOOH, 20 °C; ii NaOMe, MeOH, pH 8.5, 87%.*

in the presence of EDC and HOBt to afford the protected glycotripeptide cluster **59**. Removal of the *N*-Boc group, subsequent *N*-acetylation and deallylation yielded an intermediate that was coupled to a tripeptide to furnish the fully protected conjugate **60**. Successive treatment with formic acid and with highly diluted sodium methylate in methanol removed the *tert*-butyl ester and the *O*-acetyl groups, respectively.

A convergent method for the synthesis of *N*-glycopeptides was reported by Cohen-Anisfeld and Lansbury.[82] As a key reaction, the acylation of *O*-unprotected glycosylamines with aspartic-acid-containing peptides was optimised. Appropriately protected peptides such as **62** and **64** (Scheme 15) were synthesised on the solid phase by employing a double-protection scheme according to the Boc strategy. Scheme 15 shows the introduction of the glycosylamines, which were synthesised by using the procedure of Kochetkov et al.[75] For

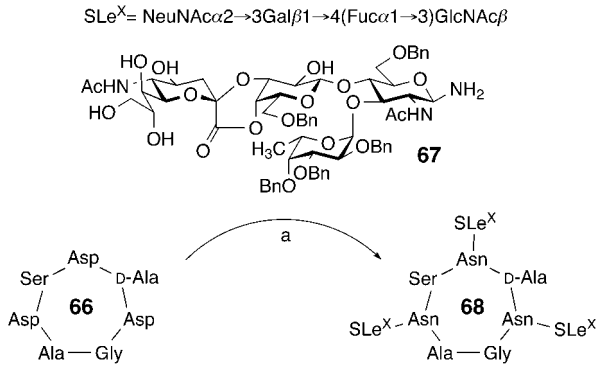


Scheme 15. *a) i DMSO, HOBt, HBTU, 2-acetamido-2-deoxyglucosylamine; ii piperidine, 33% (37% aspartimide); b) DMF, DMSO, HOBt, HBTU, complex glucosylamine, 55%.*

example, the reaction of peptide **62** with 2-acetamido-2-deoxyglucosylamine in the presence of HBTU, HOBt and DIPEA and subsequent treatment with piperidine afforded the *N*-glycoconjugate **63**. This technique allowed for the coupling of small amounts of glycosylamines, demonstrated best by the synthesis of glycopeptide **65** that contains a heptasaccharide of which only 35 μmol were available. Although the reactions were optimised, the formation of aspartimides (see Scheme 21, p. 227) could not be prevented.

For the synthesis of the trivalent sialyl-Lewis X (SLe^X) conjugate **68**, Sprengard and co-workers scaffolded the tetrasaccharide amine **67** to the cycloheptapeptide **66** (Scheme 16).[143] The formation of three *N*-glycosidic bonds was accomplished by activation with HATU, HOAt and DIPEA. Due to the use of the conformationally constrained cyclopeptide **66**, aspartimide formation was not observed. Global deprotection was achieved by hydrogenolytic debenzylation, which was followed by methylate-induced saponification.

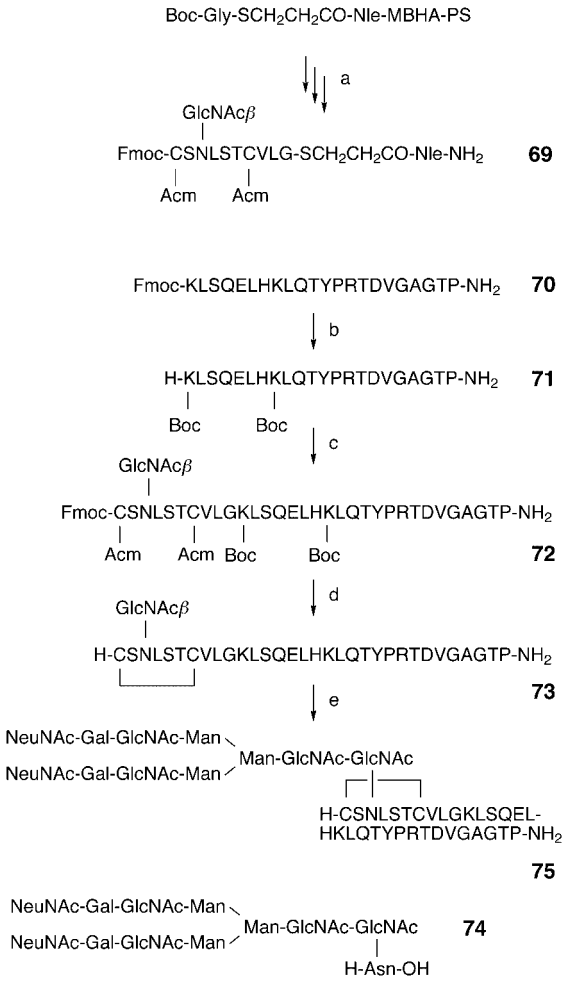
An elegant approach towards the enzymatic synthesis of single-glycoform proteins has been developed by Wong and co-workers.[144] By the use of glycosidases, they removed the natural *N*-linked glycan structure from ribonuclease B, leaving only the β-*N*-GlcNAc residue. This protein was subjected to a cascade of enzymatic glycosyl transfer reactions developed for the syn-



Scheme 16. *a) i HATU, HOAt, DIPEA, DMF, 48%; ii H_2, Pd/C, MeOH/dioxane/AcOH (5:1:1), 88%; iii NaOH in H_2O/MeOH, pH 10.4, 96%.*

thesis of the SLe^X tetrasaccharide (see also Scheme 26).[90] In their report, the successive treatment with galactosyl, sialyl and fucosyl transferase in combination with the corresponding sugar donors established a well-defined ribonuclease B containing only *N*-linked SLe^X saccharides. A similar strategy was applied in the synthesis of a glycodelin A glycopeptide by using enzyme-catalysed trimming of a diantennary glycopeptide obtained from asialofibrinogen.[145]

Mizuno and co-workers[146] developed an enzyme-catalysed transglycosylation reaction[147] to transfer an entire oligosaccharide onto a GlcNAc-containing peptide. The preparation of eel calcitonin analogues carrying natural *N*-linked oligosaccharides started with the solid-phase synthesis of the *N*-GlcNAc-peptide thioester **69** by using the Boc strategy (Scheme 17). The desired product was obtained in a modest overall yield of 12%, possibly due to losses occurring during the HF cleavage and inefficient coupling of and to the (GlcNAc)Asn building block, which was employed without protection of the carbohydrate hydroxy groups. The Fmoc-protected C-terminal peptide segment **70** was prepared by standard solid-phase peptide synthesis. Prior to its use as nucleophile in the thioester fragment condensation,[148] Boc groups were introduced to block the side-chain amino groups followed by removal of the Fmoc group. Thioester **69** and the partially protected peptide segment **71** were added to silver nitrate, Dhbt-OH and DIPEA in DMSO to furnish the partially protected 31-mer **72** in 55% yield. Subsequent removal of the Boc and the Fmoc groups yielded a conjugate that was subjected to the silver-nitrate-induced Acm cleavage to install the disulfide bridge in **73**. Then **73** was incubated with the glycosylasparagine **74** containing a disialo-complex-type oligosaccharide from human transferrin in the presence of *endo*-β-*N*-acetylglucosaminidase from *Mucor hiemalis*. After 6 h the desired product **75** was formed in 9% yield. It has to be considered that in order to transfer an oligosaccharide to a peptide, the preformed glycosyl amino acid has to be available. However, the principal value of this strategy is that it allows for the addition of *N*-linked oligosaccharides to bioactive peptides or proteins that might be difficult to synthesise chemically.

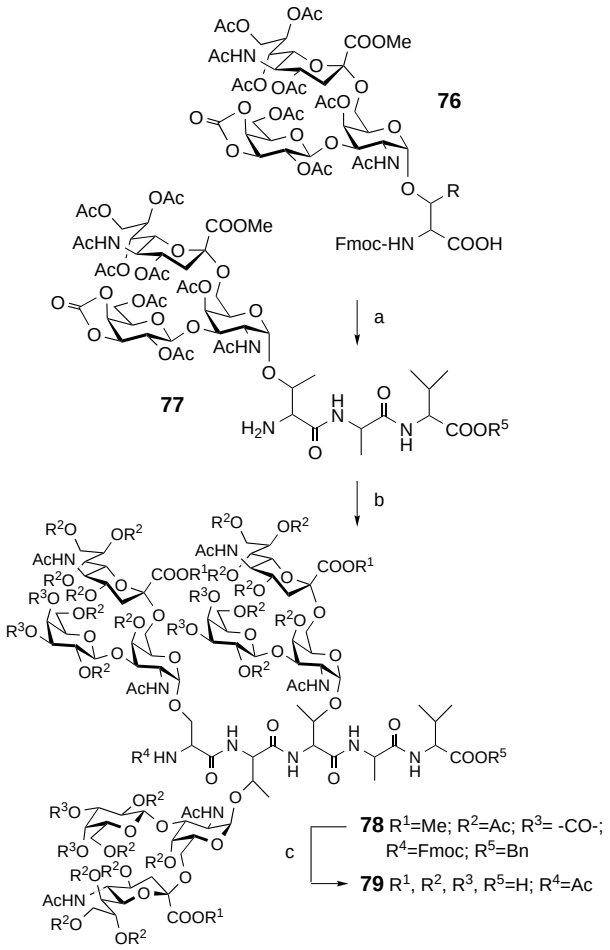


Scheme 17. *a) Peptide synthesiser model 430A (Applied Biosystems) protocol, last three amino acids coupled manually, cleavage: HF/PhOMe (9:1), 0 °C, 12%; b) i Boc-OSu, DIPEA, DMSO; ii 5% piperidine in DMSO, 19% (based on the amino group on the starting resin); c)* ***69****, $AgNO_3$, DIPEA, Dhbt-OH, DMSO, 55%; d) i 5% 1,4-butanedithiol, TFA; ii 5% piperidine, DMSO, 83%; iii $AgNO_3$, H_2O, DIPEA, DMSO; iv, 1 N HCl, DMSO, 67%; e) phosphate buffer (pH 6.25),* endo-β-*GlcNAc-ase, 9%.*

5.1.2. O-Glycopeptides

As part of their studies on carbohydrate-based vaccine constructs that mimic the cell surface of tumour antigens, the group of Danishefsky has synthesised various *O*-glycopeptides containing mucin-type carbohydrate structures. The assembly of the CD43-related glycopeptide **79** carrying sialyl-TF epitopes was commenced by coupling building block **76** to Ala-Val-benzyl ester with the help of IIDQ (Scheme 18).[109] Upon treatment with potassium fluoride in DMF and [18]crown-6, the Fmoc group was removed. Iterative peptide coupling steps between the N terminus and the protected glycosyl amino acid **76** gave the desired pentapeptide **78**. Global deprotection was accomplished by successive steps of Fmoc removal, *N*-acetylation, hydrogenolytic debenzylation and saponification. A similar reaction sequence was applied to the synthesis of a nonapeptide clustering three sialyl-TF saccharides[105] and a tripeptide displaying three TF antigens.[111] Recently, the Danishefsky group demonstrated the power of their aproach by preparing a glycopeptide that contained three *O*-linked hexasaccharides with Lewis Y structure.[149] For immunological studies this impressive glycopeptide was conjugated to the immunostimulating Pam_3Cys moiety, thereby circumventing the need of conjugation to a carrier protein such as KLH (keyhole limpet hemocyanine).

It was already mentioned that enzymes can be used for the assembly of *N*-glycopeptides. The same holds true for the synthesis of *O*-glycopeptides. For example, Schultz and Kunz employed $\beta 1 \rightarrow 4$-galactosyltransferase to catalyse the regioselective galactosylation of short synthetic *O*-glycopeptides.[120] More recently, a set of three glycosyltransferases was employed to construct an *O*-linked SLe^X peptide in solution and on the solid phase (see Scheme 26).[115] Omitting any preformed glycosylation products, enzymes can allow for the de novo glycosylation. Microsomal membranes of the colorectal cancer cell line LS180 provided the source for the enzyme polypeptide: GalNAc transferase that catalyses the unspecific *O*-glycosylation



Scheme 18. *a) i H-Ala-Val-OBn, IIDQ, CH_2Cl_2, 85%; ii KF, DMF, [18]crown-6, 95%; b) i* ***76****, IIDQ, CH_2Cl_2, 87%; ii KF, DMF, [18]crown-6; iii* ***76****, IIDQ, CH_2Cl_2, 90%; c) i KF, DMF, [18]crown-6; ii Ac_2O, CH_2Cl_2; iii H_2, Pd/C, MeOH/H_2O; iiii NaOH, MeOH, H_2O, pH 10 – 10.5, 80%.*

of threonine residues of a synthetic peptide corresponding to the human MUC2 tandem-repeat domain.[150]

5.2. Synthesis of glycopeptides on the solid phase

The synthesis of glycopeptides in solution has been successfully applied, but the repetitive isolation of the intermediates renders this approach cumbersome. Solid-phase synthesis, however, offers the opportunity to automate the repetitive process.[151–154] In addition, the use of a large excess of the building blocks can drive peptide couplings to completion, which sometimes is difficult to achieve in solution-based approaches. As a result, glycopeptides often can be synthesised in higher yields on the solid phase than in solution. But what might be even more important is the high speed of automated solid-phase synthesis and the possibility to readily implement parallel or combinatorial synthesis formats.[155, 156] Ultimately, once a high level of maturity is reached and glycosyl amino acid building blocks are commercially available, non-chemists could access biologically relevant glycopeptides, analogously to standard peptide and oligonucleotide synthesis.

Due to the many types of *N*- and *O*-linked saccharides, the literature on solid-phase synthesis of glycopeptides is vast. Rather than presenting examples for the solid-phase synthesis of any class of glycopeptide, an overview about the general tactics and strategies will be given in the following sections.

5.2.1. Linkers and resins

A decisive step in the design of solid-phase glycopeptide synthesis is the choice of the anchor group, which connects the polymeric support and the growing glycopeptide. The linkers that were originally designed to suite the Boc/Z strategy such as the PAM[157] or the MBHA linker[158] require cleavage conditions (HF or TFMSA) that are too harsh to accomodate the acid-labile glycosidic linkage. Thus, most commonly more acid-labile linkers such as the Wang,[159] the HMPA,[160] the Rink[161] or the PAL linker[162] are used (Figure 3). The much milder cleavage conditions leave the glycosidic bonds unaffected, when employed in combination with the Fmoc/*t*Bu strategy.[163] The introduction of

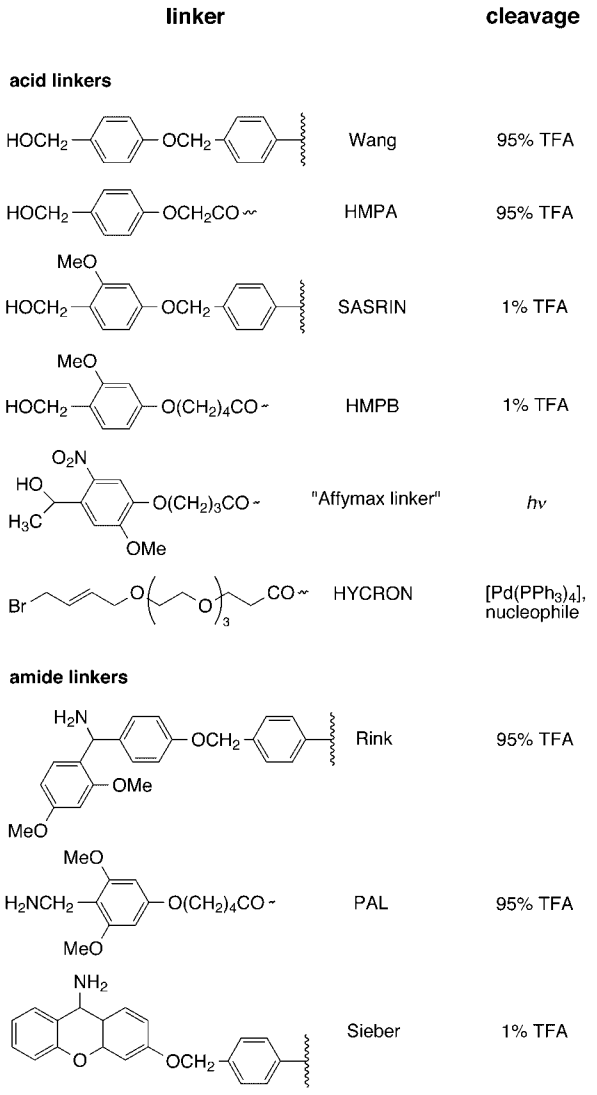


Figure 3. *Useful linkers for the solid-phase synthesis of peptides and glycopeptides.*

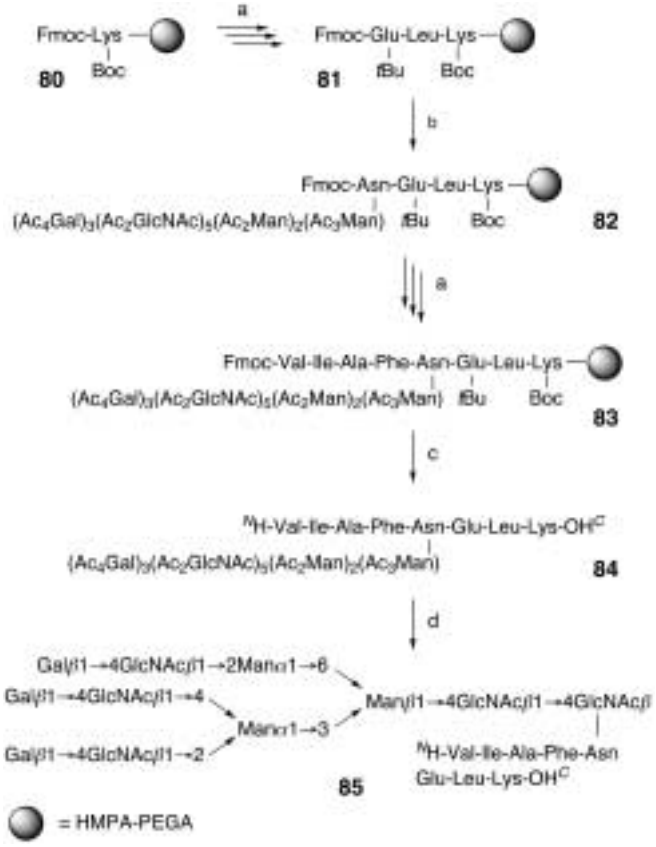
additional alkoxy substituents renders the linker system even more susceptible to acidolytic cleavage. Accordingly, the mild acid treatment of Fmoc/*t*Bu-protected glycopeptides linked through SASRIN,[164] HMPB[165] or Sieber anchors[166] releases fully protected fragments suitable for further manipulations. UV-labile linkers also allow for the liberation of fully protected glycopeptides. However, the early generation of photolabile linkers were cleavable in low yields only or were base-sensitive and, thus, not fully compatible with Fmoc chemistry.[167] A significant advance was the introduction of the nitroveratryl-based Affymax linker, which is stable towards reagents used in Fmoc synthesis.[168] Linkers of the allyl type[169] such as the HYCRON linker[170] are cleavable under almost neutral conditions and are orthogonally stable to the commonly used acid- and base-labile protecting groups.

The choice of the solid support itself is of particular importance for the success of solid-phase synthesis. However, there is no particular resin for solid-phase glycopeptide synthesis. The central criterion is the swelling capacity. Highly swellable supports allow for a high rate of mass transfer, which is of special significance when bulky reagents such as enzymes have to penetrate the polymeric support. Most frequently, polystyrene-based polymers crosslinked with divinyl benzene[171] were used including grafted polyethylene – polystyrene block copolymers with enhanced swelling capacity such as TentaGel.[172] For continous flow procedures, more hydrophilic resins such as kieselguhr-supported poly(dimethylacryl amide) might be preferable.[173] PEGA, a copolymer of polyethylene glycol and polyacryl amide, has been reported to have a high swelling capacity even in aqueous solvents, similar to TentaGel.[174] Recently, two novel types of PEG-based supports have been introduced, POEPOP[175] and SPOCC.[176] Both appear to display excellent swelling characteristics.

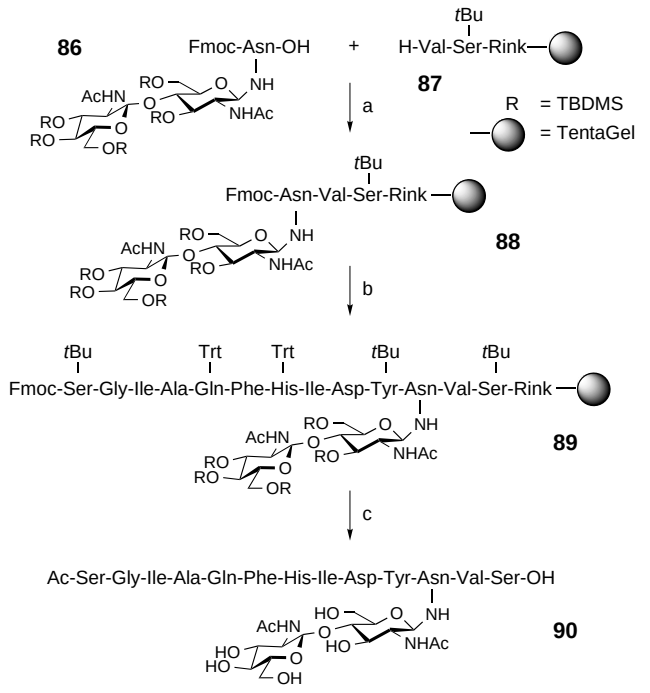
5.2.2. N-*Glycopeptides*

The most commonly used strategy employs the coupling of preformed glycosyl amino acids. An instructive example of current *N*-glycopeptide synthesis is shown in Scheme 19. Starting from the supported lysine **80**, which was linked through the acid-labile HMPA linker, Meinjohanns and co-workers first synthesised resin-bound tripeptide **81** by iterative Fmoc removals and amino acid couplings.[91] Only 1.1 equivalents of the glycosylasparagine building block **22** (Scheme 5) were coupled. The resulting glycotetrapeptide resin **82** was extended to resin **83**. Then, the *O*-acetylated glycopeptide **84** was released from the resin by treatment with TFA/water. Finally, the sodium-methylate-promoted *O*-deacetylation afforded the fully deprotected glycopeptide **85** in 35% overall yield based on the initial loading of the resin.

Kihlberg and co-workers reported problems during the final *O*-deacetylation of an *N*-linked chitobiosyl glycopeptide fragment of protein S.[177] Hence, they fashioned the use of the asparagine building block **86**, in which the *N*-linked chitobiose disaccharide was masked with acid-labile TBDMS protecting groups (Scheme 20). The solid-phase synthesis was performed on the TentaGel resin functionalised with the Rink linker. After



Scheme 19. *a) i 20% piperidine, DMF; ii 3 equiv Fmoc-aa-Pfp, Dhbt-OH, DMF; b) i 20% piperidine, DMF; ii 1.1 equiv* ***22****, TBTU, DIPEA, DMF; c) TFA/H_2O (95:5); d) NaOMe, MeOH, pH 9, 35% overall yield.*



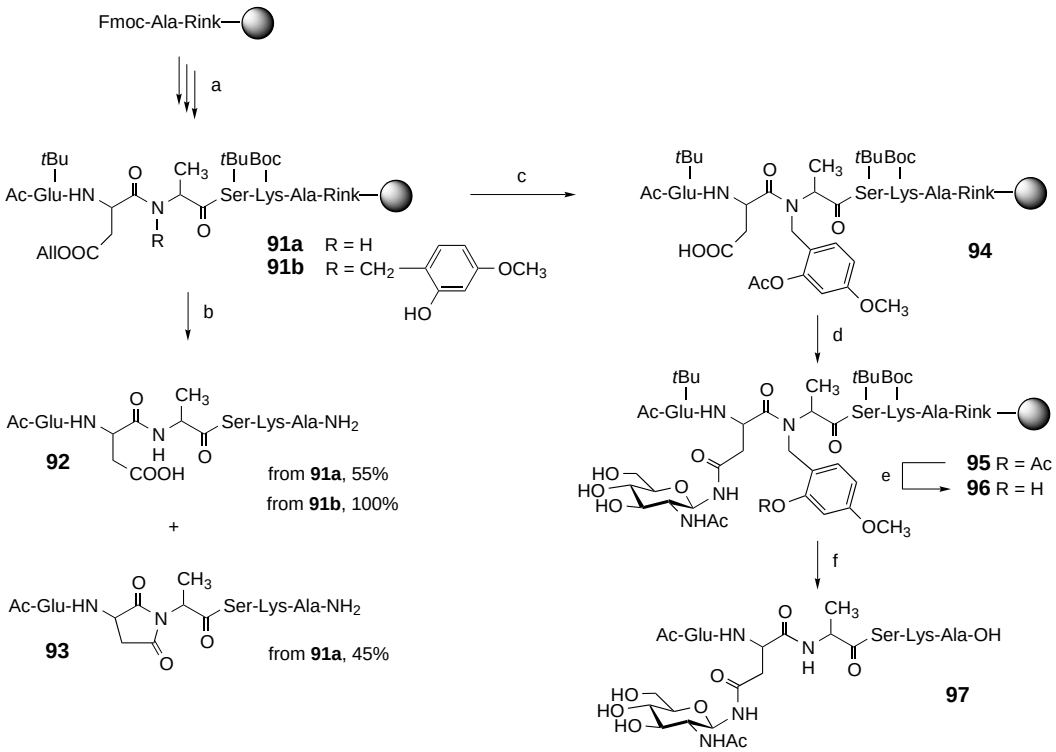
Scheme 20. *a) i 2 equiv Fmoc-(TBS$_5$-chitobiosyl)Asn-OH, DIC, HOAt, DMF; ii Ac_2O, Pyr; b) i 20% piperidine, DMF; ii 4 equiv Fmoc-aa-OH, DIC, HOBt, DMF; iii Ac_2O, Pyr; c) i 20% piperidine, DMF; ii Ac_2O, Pyr; iii TFA/H_2O/ethanedithiol/PhSMe (35:2:2:1); iv TFA/H_2O (3:1), 28%.*

the solid-phase assembly of **89**, treatment with TFA/water/thioanisole/ethanedithiol released glycopeptide **90** from the solid support. Concomitantly, the amino acid side chain protecting groups and almost all TBDMS groups were removed. A trace of monosilylated target compound was deprotected by dissolving it in TFA/water (3:1) to give the target glycopeptide in 28% overall yield.

In the synthesis of a CD52 glycopeptide containing the *N*-linked core pentasaccharide (Figure 1), Guo and co-workers favoured *O*-benzyl protection of the carbohydrate hydroxy groups.[86, 178] Although *O*-benzylation renders the glycosidic bonds more acid-labile, this type of protection in some cases might be preferable since the higher reactivity in glycosylation reactions facilitates the synthesis of the glycosylasparagine building blocks. For complete *O*-debenzylation, however, hydrogenolysis was carried out for four days using 20% $Pd(OH)_2$ on charcoal in aqueous ethanol.

Despite the excellent results obtained with preformed glycosyl amino acids, the synthesis of the corresponding building blocks is time-consuming and cumbersome. Methods that utilise the coupling of glycosylamines to aspartic-acid-containing peptides are potentially faster and offer a higher degree of convergence. Albericio's group incorporated the *N*-Fmoc-protected aspartic acid β-allyl ester in solid-phase peptide synthesis. On the full-length resin-bound peptide, the aspartyl side chain carboxy group was selectively deallylated and subsequently coupled to the glycosylamine.[179] However, this method was plagued by a severe side reaction, the formation of aspartimides, which upon hydrolysis gave rise to peptides linked through the α- and β-carboxy groups. The aspartimide formation occurs during both the initial synthesis of the Asp(OAll)-containing fully protected peptide and, after selective deprotection, during the glycosylamine coupling. Vetter et al. bypassed these problems by attaching glycosylamines to the γ-carboxy group of glutamic-acid-containing peptides, thereby forming neoglycopeptides.[180] Offer and co-workers presented a strategy by which the formation of aspartimides in the on-resin solid-phase synthesis of natural *N*-glycopeptides could be completely prevented.[181] The key feature was the application of the *N*-(2-acetoxy-4-methoxybenzyl) (AcHmb) group as a protecting group for the backbone amide function. The hexapeptide **92** was chosen as a sensitive model compound to examine the susceptibility of aspartyl β-All-protection towards aspartimide formation (Scheme 21). HPLC analysis of the crude mixture obtained after cleavage from resin **91a** revealed that the use of the Fmoc-Asp(OAll) building block resulted in almost 50% formation of aspartimide **93**. The aspartimide formation was completely prevented when the Hmb-mediated backbone protection was employed.

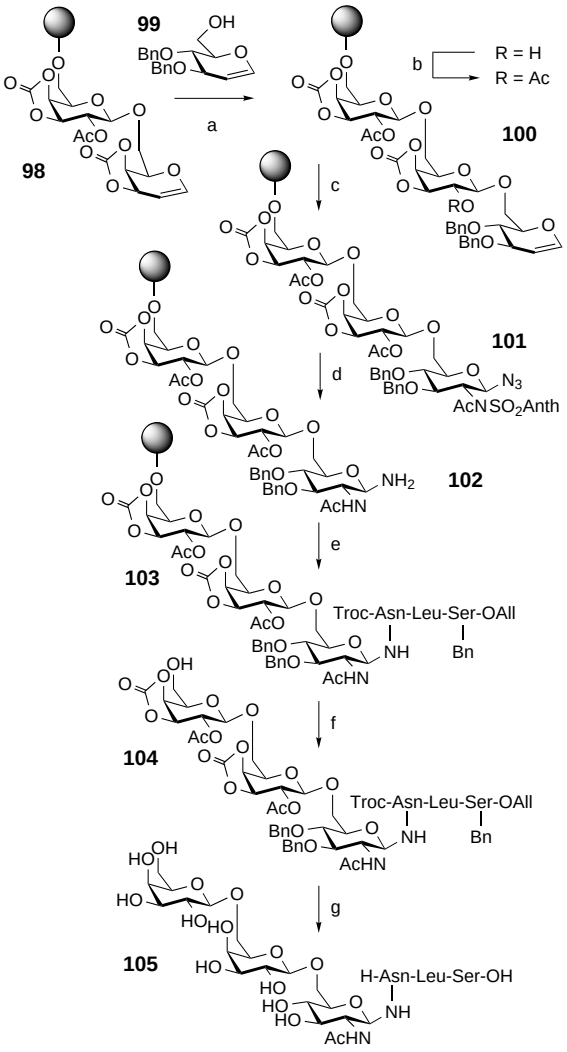
The strategy for the on-resin coupling of glycosylamines was commenced with the selective removal of the β-OAll group from the Hmb-protected peptide resin **91b**. The Hmb group was *O*-acetylated to prevent potential side reactions in the subsequent coupling of **94** with *O*-unprotected glucosamine. As the *O*-acetylation stabilises the Hmb group, *O*-deacetylation is a necessary step to reestablish its lability towards TFA. Brief treatment of **95** with hydrazine-hydrate ensured *O*-deacetylation. The final TFA-promoted cleavage of **96** removed all



Scheme 21. *a) Standard Fmoc solid-phase peptide synthesis; b) i $[Pd(PPh_3)_4]$, NMM, AcOH, DMF, $CHCl_3$; ii TFA/H_2O/Et_3SiH (90:5:5); c) i Ac_2O, DIPEA; ii $[Pd(PPh_3)_4]$, NMM, AcOH, DMF, $CHCl_3$; d) glucosylamine, BOP, HOBt, DIPEA, DMSO, DMF; e) N_2H_4, DMF; f) TFA/H_2O/ethanedithiol/Et_3SiH (91:3:3:3); 30%.*

protecting groups including the Hmb group and liberated the GlcNAc-substituted peptide **97**. The subsequent HPLC analysis showed that the Hmb backbone protection conferred complete stability towards aspartimide formation. However, it has to be noted that this strategy suffers from two drawbacks. It cannot be guaranteed that the glycosylamine coupling will lead to the exclusive formation of β-*N*-glycosides, and removal of the α-anomers might be difficult on the level of the full-length product. Another limitation might be set by the order of the deprotection steps. Labile glycosidic linkages such as the α-fucosidic bond (Scheme 1) survive TFA treatment only in their *O*-acetylated form. The Hmb group, however, has to be *O*-deacetylated prior to acidolysis, resulting in deacetylation and concomitant enhancement of the acid lability of problematic carbohydrates like α-fucosides and sialosides.

A conceptionally different route towards the convergent solid-phase synthesis of the *N*-linked glycopeptide **105** was introduced by Danishefsky's group (Scheme 22).[182] Rather than linking the glycopeptide through its peptide part, the saccharide was anchored to the solid support. The known disaccharide glycal **98** was linked to the polystyrene resin by using a silyl tether. Epoxidation with dimethyldioxirane and subsequent glycosylation with 3,4-di-*O*-benzyl glucal **99** afforded the trisaccharide polymer **100**. After acetylation and iodosulfonamidation (see Scheme 2), treatment with tetrabutylammonium azide induced the sulfonamide rearrangement, and the subsequent *N*-acetylation furnished the β-disposed azide **101**. The cleavage of the sulfonamide and subsequent azide reduction gave the polymer-bound glycosylamine **102**, which was subjected to the coupling reaction with *N*-Troc-protected peptide allyl ester. Treatment of the trisaccharide – tripeptide conjugate **103** with HF · pyridine complex liberated glycopeptide **104** in 35 % overall yield. For global deprotection, the allyl, the Troc, the benzyl and the acetyl protecting groups were removed by Pd^0-catalysed allyl transfer, Zn-mediated reductive cleavage, hydrogenolysis and KCN-induced methanolysis, respectively. Alternatively, before cleavage from the resin the peptide could also be extended either from the N terminus or from the C terminus. Despite of the elegance of this approach, the possible formation of aspartimides could set limitations to its general applicability.

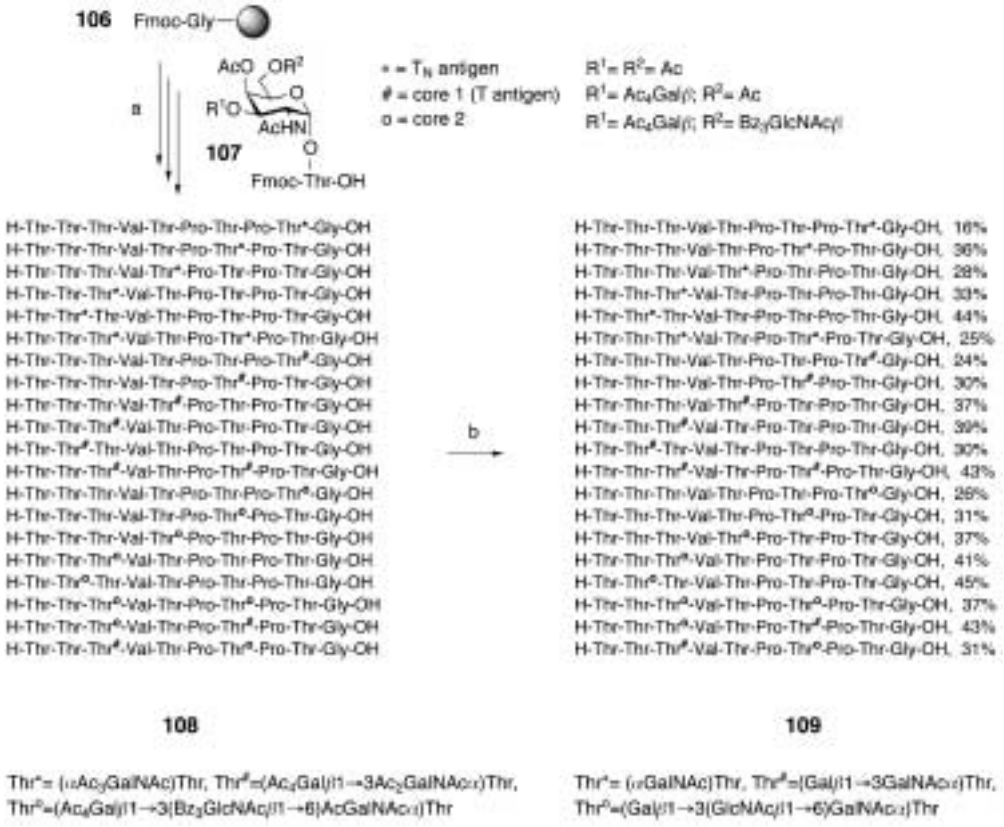


Scheme 22. *a) i DMDO, CH_2Cl_2; ii $ZnCl_2$, THF; b) Ac_2O, coll, THF, DMAP; c) i $I(coll)_2ClO_4$, THF, -10 to 0 °C, Anth-SO_2NH_2; ii Bu_4NN_3, THF; iii Ac_2O, DMAP, THF; d) propanedithiol, DIPEA, DMF; e) Troc-Asn-Leu-Ser(OBn)-OAll, IIDQ, CH_2Cl_2; f) HF · Pyr, PhOMe, CH_2Cl_2, -10 °C, 35 %; g) i $[Pd(PPh_3)_4]$, dimethylbarbituric acid, THF; ii Zn, AcOH, MeOH; ii $Pd(OAc)_2$, H_2, AcOH, MeOH; iv KCN, MeOH, 65 %.*

The problematic aspartimide formation can be prevented when a supported glycopeptide is synthesised that has the *N*-linked GlcNAc residue already in place for further extension of the carbohydrate part. Schuster et al. reported an approach by which glycosyltransferases were utilised to extend the glycan of an immobilised *N*-GlcNAc peptide.[183] Enzymatic on-resin glycosylations generally suffer from the intolerance of the biocatalyst towards many of the commonly used supports. Silica or controlled-pore glass, non-swellable solid supports with high porosity and surface area, enable biocatalysis. An alternative approach utilises resins that swell in both aqueous and organic solvents to a high degree, thus allowing the biocatalyst to penetrate the polymeric network. This strategy was favoured by Hindsgaul, Meldal, Thiem and co-workers who demonstrated that the galactosyltransferase-catalysed synthesis of LacNAc-disaccharides was feasible on polyacrylamide or PEGA.[184, 185]

5.2.3. O-Glycopeptides

The abundance of α-*O*-linked GalNAc moieties in *O*-glycopeptides stimulated many research groups to devise solid-phase synthesis schemes. The groups of Bock, Meldal and Paulsen largely contributed to the field as they recognised the potential of solid-phase synthesis and realised parallel and combinatorial synthesis formats. In the synthesis of MUC 2 and MUC 3 peptides containing oligosaccharides with T_N antigen, core 1 (T antigen), core 2, core 3, core 4 and core 6 structure, preformed *O*-acyl-protected glycosyl amino acid building blocks were employed in a multiple-column synthesis of 45 differently glycosylated decamers.[102] For example, the synthesis of the T_N-antigen-, core-1- (T antigen) and core-2-containing MUC 2 decamers **109** was performed in parallel on a manual 20-column peptide synthesiser (Scheme 23). As solid support the Fmoc-Gly-modified Wang resin **106** was chosen. TBTU/HOBt activation was

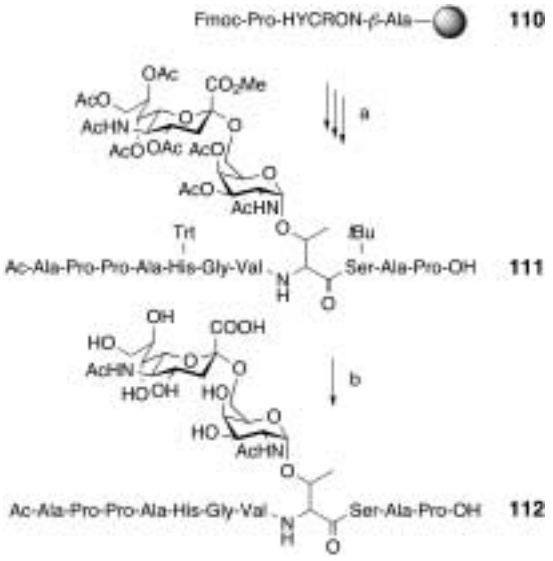


Scheme 23. *a) i 20% piperidine, DMF; ii 3 equiv Fmoc-aa-Pfp, Dhbt-OH, DMF (or 1.5 equiv glycosyl amino acid, TBTU, DIPEA, DMF); cleavage: TFA/H_2O (95:5); b) NaOMe, MeOH.*

performed for the core 1 and the core 2 building blocks **107** (1.5 equiv) and Pfp esters with addition of Dhbt-OH for the unglycosylated Fmoc-protected amino acids. After the TFA cleavage, the glycopeptides **108** were treated with sodium methylate in methanol to remove the *O*-acetyl and *O*-benzoyl protecting groups.

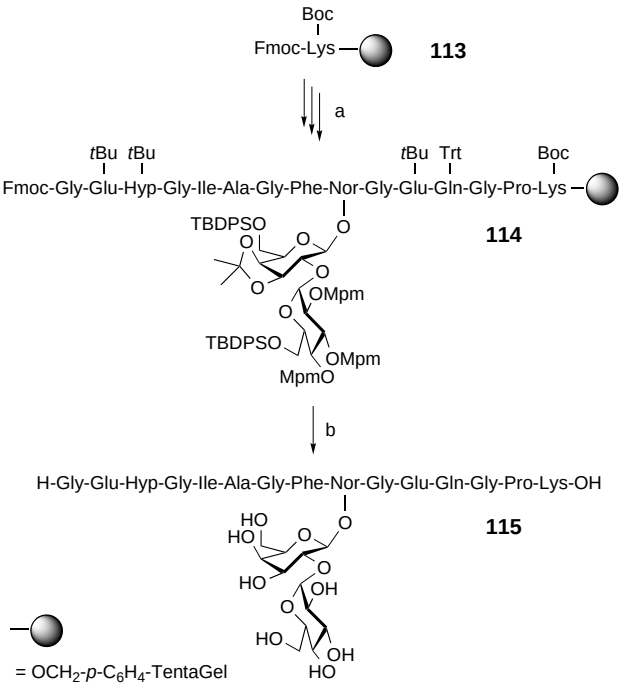
The conditions required for the removal of *O*-benzoyl protecting groups can give rise to β-elimination and epimerisation (Scheme 1). The use of peracetylated building blocks allows for the application of milder conditions. The groups of Kihlberg[107] and Kunz[94] independently reported the incorporation of *O*-acetyl-protected *O*-sialyl-T_N-threonine building blocks. Liebe and Kunz employed the HYCRON linker in their synthesis of a sialyl-T_N-containing MUC 1 undecamer. The HYCRON linker was already reported to enable the highly efficient synthesis of mucine-type *O*-glycopeptides.[186] In a reported case an overall yield of 95% was achieved. The Fmoc-proline-derivatised HYCRON polystyrene resin **110** was extended by using TBTU/HOBt activation and the less basic DMF/morpholine for the removal of the Fmoc groups rather than DMF/piperidine (Scheme 24). The release of the protected glycopeptide **111** was accomplished by applying the Pd^0-catalysed allyl transfer to the nucleophile morpholine. Subsequent treatment with TFA and aqueous sodium hydroxide removed the protecting groups to give the desired sialyl-T_N glycopeptide **112** in 32% total yield. In Kihlbergs synthesis of a 16-mer with HIV gp120 sequence the coupling of the glycosyl amino acid was performed by using only one equivalent. This coupling did not reach completion. TFA cleavage and subsequent saponification liberated the glycopeptide and the terminated peptide in 14 and 12% overall yields, respectively.

Although acetylation is by far the most popular and usually the most versatile means for protecting the carbohydrate hydroxy groups and stabilising the glycosidic bonds, there are cases in which side reactions were reported. On the condition that the coupling of the *O*-acetylated glycosyl amino acid to the resin-bound peptide is unusually slow, an O→*N* acetyl shift might irreversibly block the nucleophile.[107] The basic conditions needed for the *O*-deacetylation can cause cysteine-induced degradations[41] and, if hydrazine is used, hydrazide formation of Asn residues,[137] which might proceed through the initial formation of an aspartimide. Of course, β-elimination as well as epimerisation can be problematic if the *O*-deacetylation conditions are not carefully adjusted.



Scheme 24. *a) i 50% morpholine, DMF; ii Fmoc-aa-OH, TBTU, HOBt, NMM, DMF; iii repetitive cycles of i – ii; iv [$Pd(PPh_3)_4$], morpholine, DMF/DMSO (1:1), 42%; b) i TFA, PhOMe, EtSMe; ii NaOH, MeOH, 76%.*

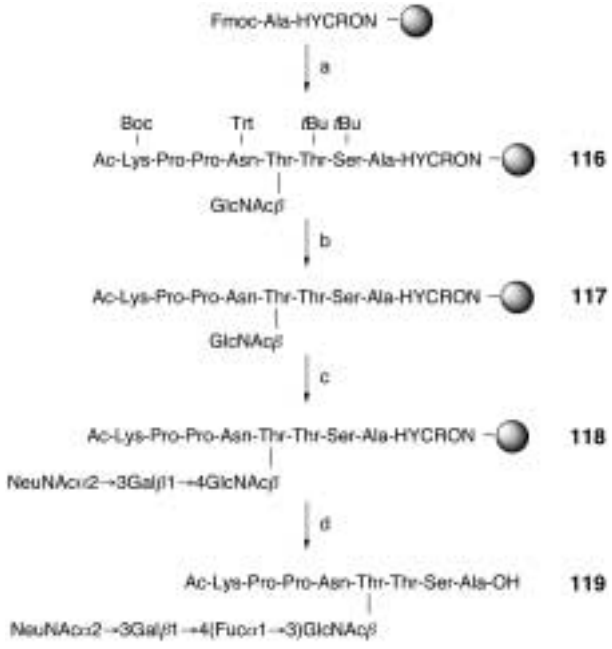
Nakahara and co-workers favoured *O*-benzyl protection for the synthesis of the B chain of the α2HS glycoprotein.[187] *O*-Benzyl groups, however, are sometimes difficult to remove in the presence of cysteine and methionine residues. In addition, as *O*-benzylation is used in carbohydrate chemistry to prepare armed/activated glycosyl donors and acceptors, it inevitably enhances the acid lability of glycosidic bonds. One rescue is the use of acid-labile carbohydrate protecting groups. Christiansen-Brams and co-workers proposed *O*-trimethylsilyl protection, which unfortunately is too labile to be of general utility.[188] The group of Kihlberg fashioned the use of hindered silyl-type protecting groups such as TBDMS and TBDPS ethers as well as protection through 4-methoxybenzyl ethers.[189] For the solid-phase synthesis of glycopeptides derived from type II collagen, the disaccharide–5-hydroxynorvaline conjugate **54** (Scheme 13) was incorporated into an Fmoc-based protocol (Scheme 25). After the TFA cleavage of glycopeptide resin **114**, the desired *O*-disaccharide–peptide conjugate **115** was obtained in 45% overall yield. One advantage of this protecting-group pattern is that the carbohydrate as well as the peptide side chains are liberated during the TFA-induced cleavage from the resin.



Scheme 25. *a) i 20% piperidine, DMF; ii Fmoc-aa-OH, DIC, HOBt, DMF (glycosyl amino acid, DIC, HOAt, DMF); b) TFA/H_2O/PhSMe/ethanedithiol (87.5:5:5:2.5).*

On the quest towards strategies that minimise the number of steps for the building-block preparation, Bock, Meldal, Paulsen and co-workers synthesised glycopeptides containing *O*-linked 2-azido-2-deoxygalactosyl residues.[190] The conversion of the 2-azido into a 2-acetamido group was performed on the resin-bound glycopeptide rather than on the building-block level. Unfortunately, in all syntheses a by-product was formed in 10–15% yield. The reduction of the azido group with thioacetic acid, which had to be carried out for 2–8 days, led to the formation of thioacetamides, which in some cases were difficult to remove. A similar strategy was used for the solid-phase synthesis of glycopeptides containing β-*O*-linked *N*-acetylglucosamine.[124, 191]

The limiting step for the synthesis of glycopeptides containing complex carbohydrates is the synthesis of the glycosyl amino acids. A convergent route, which would utilise resin-bound glycopeptides as acceptors for on-resin glycosylation reactions, would omit the need to synthesise complex glycosyl amino acid building blocks in solution. Glycosyltransferases are versatile tools for glycosylations of complex acceptors as they have shown to provide for selective glycosylation reactions in the absence of protecting groups.[46] The application of glycosyltransferases in solid-phase synthesis, however, requires a linkage enabling the removal of protecting groups without detaching the supported substrates. In addition, the liberation of the target molecule from the support should proceed under conditions that would not affect the acid- and base-labile glycoconjugate. The HYCRON linker, which enables both the application of the Boc and the Fmoc strategy, satisfies these demanding properties as demonstrated by the chemoenzymatic solid-phase synthesis of glycopeptides containing the SLe^X tetrasaccharide β-glycosidically linked to threonine residues.[115] To allow for the use of both aqueous and organic solvents, controlled-pore glass was used as a solid support. The synthesis was performed following the usual Fmoc protocol. The carbohydrate was incorporated in the form of the *O*-unprotected amino acid glycoside. For the removal of all side chain protecting groups, the supported glycopeptide **116** was treated with TFA (Scheme 26). The unprotected glycopeptide **117** remained on the solid support

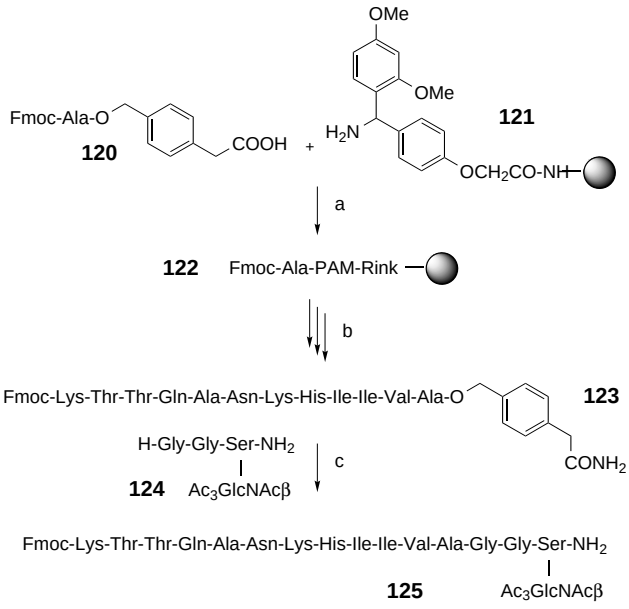


Scheme 26. *a) i 50% morpholine, DMF; ii Fmoc-aa-OH, HBTU, HOBt, NMM, DMF; iii* N*-acetylation: AcOH, HBTU, NMM, HOBt, DMF; b) TFA/H_2O/ethanedithiol (40:1:1); c) i UDP-Gal, GalTase, 50 mM HEPES (pH 7), 5 mM $MnCl_2$, 37°C; ii CMP-NeuNAc, α-2,3-sialyltransferase, 0.1 M HEPES (pH 7), 5 mM $MnCl_2$, alkaline phosphatase, 37°C; d) i [$Pd(PPh_3)_4$], morpholine, DMF, DMSO, 9%; ii FucTase, GDP-Fuc, 0.1 M HEPES (pH 7), 37°C, 59%.*

and was subjected to enzymatic galactosyl- and sialyltransfer reactions as pioneered by Schuster and Wong et al.[183] The glycoconjugate **118** was released under mild conditions, taking advantage of the Pd^0-catalysed cleavage of the allylic linkage. An enzymatic fucosylation was performed to complete the synthesis of the SLe^X peptide **119**.

The group of Paulsen developed a chemical approach of the aforementioned strategy. A selectively masked glycopeptide was subjected to on-resin glycosylation reactions by using different resins such as Polyhipe, Macrosorb and TentaGel.[192] Interestingly, only the Polyhipe-supported acceptors gave rise to glycosylation products. An even higher degree of convergence can be achieved when a direct glycosylation of peptidic hydroxy groups is possible. Such a strategy eliminates the bottleneck of glycosyl amino acid synthesis and facilitates the access to highly diverse *O*-glycopeptides with variable peptide and carbohydrate structure. Until recently, most efforts were plagued by low yields in the *O*-glycosylation of resin-bound peptide acceptors.[186, 193, 194] A recent study shows that this might be due to an effect of the resin.[195] An inert support, the POEPOP resin, was prepared by anionic polymerisation of mono- and bisepoxy-poly(ethylene glycol), thus avoiding the presence of amide bonds that could possibly act as scavengers.[175] A supported pentapeptide was subjected to glycosylation reactions by employing various trichloroacetimidate-activated donors. The yields of the glycopeptides obtained after TFA cleavage were reported to be in the range of 41 – 78% relative to the yield determined for the synthesis of the acceptor peptide. This approach is certainly of high attractiveness for combinatorial syntheses since it allows to access a diverse universe of *O*-glycopeptides without synthesising any preformed building blocks. However, as the authors emphasised the importance of avoiding the presence of amide groups in the polymeric support, it has to be noted that this is indeed the very nature of peptides. It is not unreasonable to speculate that lower glycosylation yields would be expected if longer resin-bound peptides were employed as glycosyl acceptors.

A convergent strategy with respect to the peptide part is offered by the on-resin condensation of peptide fragments. Fragment condensations can be performed on both the N and the C termini when the glycopeptide is connected through the carbohydrate or the peptide side chain to the solid support. Similar to the strategy of Danishefsky et al. (Scheme 27),[182] Nakahara and co-workers linked the primary 6-hydroxy group of an Fmoc/All-protected Tantigen – threonine conjugate to a silyl tether.[196] After removal of the C-terminal allyl group, segment couplings were performed. Lampe, Weitz-Schmidt and Wong employed an acetal-type linkage to attach a fucosyl-threonine through the 3- and 4-hydroxy groups of fucose.[197] Further elaboration on both the C- and the N-terminal end was demonstrated as well as the mild acid cleavage to release fucopeptides that serve as SLe^X mimetics. Chemical fragment condensations often suffer from the poor solubility of the protected glycopeptide fragments[198] and are prone to racemisation at the C terminus of the acyl donor. In contrast, enzyme-catalysed fragment condensations are free of racemisation and use substrates that have unprotected side chains, thereby

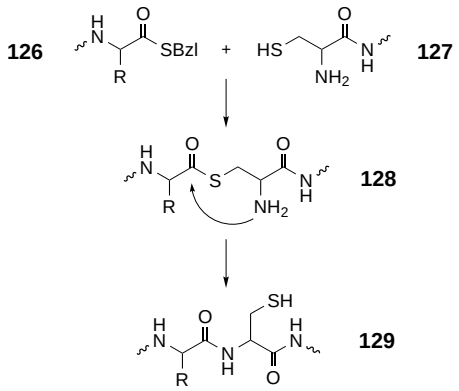


Scheme 27. *a) HBTU, HOBt, NMM, DMF; b) standard Fmoc solid-phase peptide synthesis, cleavage: TFA/Et_3SiH/H_2O (95:2.5:2.5), 89%; c) subtilisin 8397 K256Y, 50 mM triethanolamine/DMF (1:9), 84%.*

increasing the solubility of the peptide fragments. Witte, Seitz and Wong reported on the solid-phase synthesis of peptide and glycopeptide esters for subsequent use in enzyme-catalysed fragment condensations.[199] For the synthesis of a partial sequence of the C-terminal region of ribonuclease B, conjugate **120**, consisting of *N*-Fmoc-protected alanine and the acid- and base-stable PAM linker, was coupled to the Rink resin **121** (Scheme 27). The elongation of **122** followed standard Fmoc protocols. Standard TFA cleavage conditions removed all acid-labile side-chain-protecting groups and also detached the *N*-Fmoc-protected peptide PAM ester **123** from the solid support. Peptide esters of this type with unprotected side chains can serve as acyl donors in enzyme-catalysed peptide couplings. Accordingly, the segment condensation of **123** with the N-terminally unprotected glycotripeptide **124** was achieved with the protease subtilisin, affording the glycopentadecapeptide **125** in 84% yield.

A chemical approach towards a condensation of unprotected peptide fragments was developed in Kent's laboratories.[200] This technique, which is known as "native chemical ligation",[201] utilises the selective coupling between a C-terminal peptide thioester **126** and an N-terminal cysteine **127** (Scheme 28). The highly nucleophilic thiol group of the N-terminal cysteine **127** attacks the activated thioester **126**. The thioester intermediate **128** is subject to a spontaneous intramolecular $S \rightarrow N$ acyl transfer by which the final ligation product **129** is formed.

Recently, the group of Bertozzi made use of native chemical ligation for the total chemical synthesis of a 82-residue glycoprotein.[202] Crucial for the native chemical ligation is the access to the peptide thioesters **126**. The commonly used standard Fmoc/*t*Bu strategy, however, is not applicable for the solid-phase synthesis of thioesters, since thiosters would not

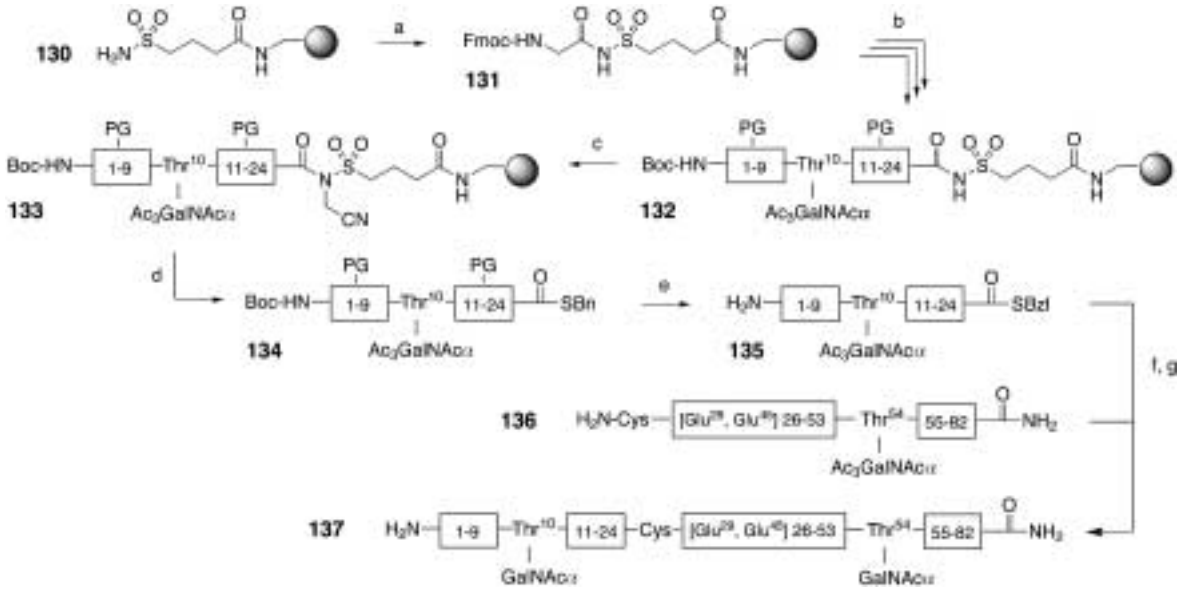


Scheme 28. *The concept of "native chemical ligation".*

withstand the conditions needed for the removal of the Fmoc groups. This problem was solved by making use of Ellman's modification of Kenner's sulfonamide linker,[203] which allows for a post-assembly formation of the glycopeptide thioester. In the report, the supported N-terminal 24-mer fragment **132**, which contained one *O*-linked GalNAc moiety, was synthesised on the sulfonamide-modified resin **130** according to the Fmoc/*t*Bu strategy (Scheme 29). The cleavage of the acid- and base-stable sulfonamide **132** was commenced by iodoacetonitrile alkylation. The tertiary sulfonamide **133** was then cleaved by thiolysis with a large excess of benzenethiol to yield the protected glycopeptide thioester **134**. Subsequent removal of the acid-sensitive protecting groups finished the synthesis of the acyl donor **135**. The C-terminal glycopeptide fragment **136** was synthesised by a standard peptide synthesis protocol. For the native chemical ligation, the two fragments **135** and **136** were coupled in the presence of thiophenol which is known to accelerate the ligation reaction. Finally, *O*-deacetylation afforded the fully deprotected 82-mer glycoprotein **137**. It seems reasonable to assume that this technique could provide the basis for the synthesis of a variety of biologically interesting glycoproteins having a degree of complexity that was almost impossible to implement by previous tactics.

6. Biological and structural studies with synthetic glycopeptides

The recent improvements in the synthetic methodology and technology make it possible to gain access to homogeneous glycopeptides complex enough to study the structural and biological influences of protein glycosylation.[10, 204, 205] One of the main influences that glycosylation exerts is the stabilisation of protein conformations, which is of central importance for the immunogenicity of glycopeptides. Thus, in the following sections selected examples will be presented in which synthetic glycopeptides were employed to unravel the role of carbohydrate attachment in the stabilisation of peptide conformations, in epitope mapping and in T-cell induction. Because of the particular role in cell–cell recognition and cell adhesion, the interaction of glycoproteins with carbohydrate-binding proteins (CBPs) will be discussed. However, this field has been reviewed extensively and the discussion will thus be very brief.[206–208] Glycopeptides are subject to enzymatic transformations like any other protein. Accordingly, a short section serves to illustrate how synthetic glycopeptides were used to examine the effect of glycosylation on the substrate properties of a given peptide. The knowledge acquired in the above-mentioned studies can be advantageously applied in the development of glycopeptide-based vaccines—promising candidates for the immunotherapy of cancer, which will be discussed in the last section.



Scheme 29. *a) Fmoc-Gly, PyBOP, DIPEA, DMF; b) standard Fmoc solid-phase peptide synthesis, N-terminal amino acid coupled as* N*-Boc-protected building block; c) ICH_2CN, DIPEA, NMP; d) BnSH, THF; e) TFA/PhOH/H_2O/PhSMe/EDT (82.5:5:5:5:2.5), 4 h, 21% overall yield (based on resin capacity); f) 6 M Gn·HCl, 100 mM NaH_2PO_4, pH 7.5, 4% PhSH, 55%; g) 5% equiv H_2NNH_2, DTT, 53%.*

6.1. The structure and conformation of glycopeptides

Mass-spectrometrical analysis is one of the most sensitive methods for obtaining peptide sequence information. Homogeneous glycopeptides as accessed by chemical or chemoenzymatic synthesis can be used as model compounds for the calibration of sequencing data. Goletz and co-workers reported a MALDI-MS-based sequencing strategy that allows for the localisation of peptide *O*-glycosylation sites.[209] A synthetic glyconanapeptide was analysed to demonstrate the feasibility of the new strategy, which was then applied for the characterisation of MUC 1 glycopeptides.

In the early days, the conformation of glycopeptides has been analysed mainly by using CD spectroscopy, which provides information about secondary structure such as helical and β-sheet content. The recent progress in NMR spectroscopy in combination with computational methods allows for a more detailed insight into the local structure and dynamic processes.[210] In principle, two types of glycosylation patterns can be distinguished. One is represented by the clustered mode of glycosylation typical for the *O*-linked mucin-type glycopeptides and the other by the singular attachment of a carbohydrate at a specific site. For studying the latter, O'Connor and Imperiali have utilised a synthetic hemagglutinin nonapeptide, which carried a chitobiose disaccharide *N*-linked to a natural glycosylation site.[211] The glycosylation site of peptide **138** adopted a turn structure, the Asx turn,[212] which appears to be important for natural *N*-glycosylation (Figure 4). The analysis of the ROESY NMR spectra and the amide proton variable temperature (VT) coefficients of peptide **138** and the chitobiosyl peptide **139** revealed that *N*-glycosylation induced a major alteration of the peptide backbone conformation. The intense NOE between the amide protons of asparagine and glycine and the very low temperature coefficient of the glycine amide signal suggested the presence of a β-turn structure. One hundred nine NOE-derived distance restraints were included in a simulated-annealing procedure. These calculated structures were consistent with a type I β-turn. Although no NOEs were observed between the peptide backbone and the carbohydrate, the very nature of the latter proved to be of central importance for the induction of the β-turn conformation. Attachment of GlcGlc, GlcGlcNAc, GlcNAcGlc and GlcNAc revealed that the only sugars that maintained the β turn were those that contained the 2-acetamido group at the proximal sugar unit.[213]

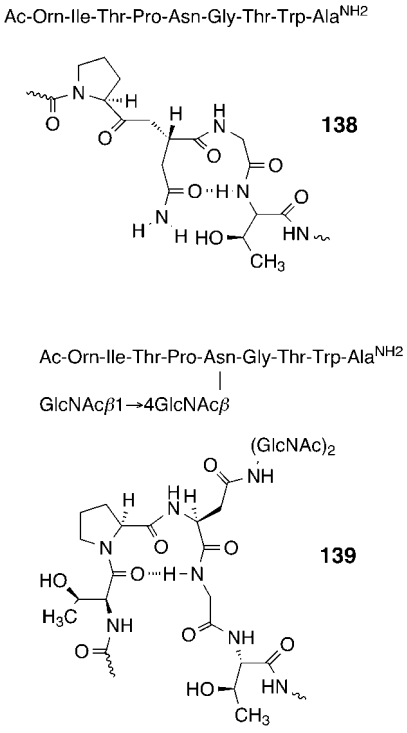
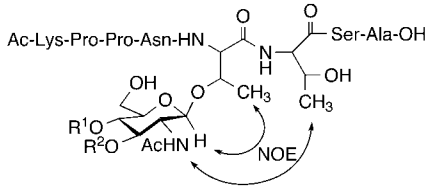


Figure 4. *Peptide **138** adopts an Asx-turn structure. Glycosylation induces a type I β-turn structure of glycopeptide **139**.*[211, 212]

NOEs between the methyl group protons of the 2-acetamido moiety and backbone amide protons were observed in a conformational study of the glycopeptide Ala-Leu-(Glcβ1 →6Glcβ1 →6GlcNAcβ)Asn-Leu-Thr.[214] The analysis of the NH to Hα couplings along with the effect of temperature on chemical shift of the amide resonances indicated the presence of a conformational equilibrium between an ordered structure and a random coil conformation. The unglycosylated peptide failed to show any secondary structure, which suggested that the glycan exerted a conformational bias to the peptide backbone. Fifty-two NOE-derived distance constraints were implemented in simulated-annealing calculations to emulate a structural family whose members all displayed similar conformations. Similar results were obtained in a study of the repeating unit of RNA polymerase II.[215] *O*-Glycosidic attachment of a single GlcNAc residue to a threonine was reported to induce a turn-like structure of glycopeptide Ac-Ser-Tyr-Ser-Pro-(6GlcNAcβ)Thr-Ser-Pro-Ser-Tyr-Ser-NH_2. Using computation guided by NMR-derived distance constraints, it was concluded that the carbohydrate lies over the plane of the turn. Importantly, two independent calculations, Monte Carlo and molecular dynamics calculations, provided the same low-energy structure. As a consequence of these calculations Ser 3 and Ser 6 came into close proximity. Since these residues were identified as *O*-phosphorylation sites, a model was proposed in which phosphate-mediated charge repulsion and glycosylation-induced turn formation would act as a mutually exclusive conformational switch.

A detailed analysis of the conformational interplay between an octapeptide fragment of the mucin domain of MAdCAM-1 and an *O*-linked carbohydrate was performed in the Wong group.[216] NMR and computational modelling were applied to study the *O*-linked SLe^X peptide **119** and its synthetic intermediates, the *O*-GlcNAc, *O*-LacNAc and the *O*-sialyl-LacNAc peptides (Figure 5). Whereas the *O*-linked SLe^X tetrasaccharide moiety of **119** and a free SLe^X tetrasaccharide were found to adopt the same conformation, the conformation of the peptide backbone was significantly altered upon glycosylation. Although the amide – amide ROESY pattern varied with the nature of the appended glycan, the molecular dynamics calculations indicated that all carbohydrates stabilised a turn-like structure near the glycosylation site. Interestingly, the ROESY peaks between the 2-acetamido group and the methyl group of Thr 6 disappeared after introduction of the fucosyl residue along with a significant



119, R^1 = NeuNAcα2→3Galβ; R^2 = Fucα
140, R^1 = NeuNAcα2→3Galβ; R^2 = H
141, R^1 = Galβ; R^2 = H
142, R^1 = R^2 = H

Figure 5. *NMR and computer modelling suggested a conformational interplay between the carbohydrate and the peptide backbone. A balance between hydrophobic carbohydrate – peptide interactions and hydrophilic carbohydrate – carbohydrate interactions affected the NOEs between carbohydrate and peptide protons. Thus, the induced conformational differences depend on the extent of glycosylation.*[216]

decrease of the ROESY peaks between GlcNAc-H1 and Thr 5-Hγ. As a likely explanation, it was suggested that glycosylation of the *O*-GlcNAc peptide **142** induced a sequence-specific reorientation of the carbohydrate moiety. Since this affected the conformation of the 2-acetamido group, the reorientation also influenced the conformation of the peptide backbone.

Although the presented examples indicate that glycosylation can induce turn-like structures, it has to be emphasised that strong influences of a single monosaccharide residue are rare.[217, 218] In contrast, the extended conformation of the rod-like mucin peptides seems to be stabilised by the multiple attachment of *O*-linked carbohydrates.[219] This stiffening effect was demonstrated by using the hexaglycosylated glycophorin A decapeptide fragment **143** as a model compound (Figure 6).[220]

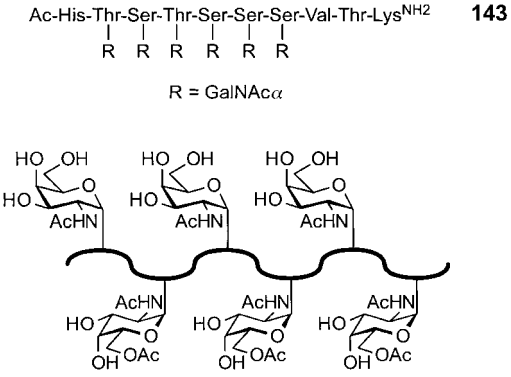
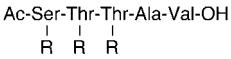


Figure 6. *The multiple attachment of a monosaccharide residue to a mucin forced the peptide backbone to adopt a "wave-type" structure.*[220]

Each GalNAc residue exhibited one NOE contact between the 2-acetamido proton and the amide proton of the amino acid to which the carbohydrate was attached. The absence of NH_i – NH_{i-1} mid-range and long-range contacts was in accordance with an extended conformation. A constrained molecular dynamics simulation provided a structural motif in which the backbone adopted a "wave-type" conformation. The carbohydrate attachment sites were suggested to alternate between the tops and the bottoms of the "wave". These investigations are remarkable as the stabilisation of the native-like extended conformation was already obtained by glycosylation with the GalNAc monosaccharide.

Recently, the solution conformation of mucin peptides containing α-*O*-linked T_N-, T- and 2,6-sialyl-T-antigen clusters along with a non-natural β-*O*-linked T-antigen cluster were studied by a combined NMR and computer modelling approach (Figure 7).[221]



R = GalNAcα, Galβ1→3GalNAcα, Galβ1→3(NeuNAcα2→6)GalNAcα, Galβ1→3GalNAcβ

Figure 7. *Mucin glycopeptides that were studied by a combined NMR and computer modelling approach. The peptide backbone of the glycoconjugates adopted an extended conformation on the condition that the glycans are linked through* α-O-*glycosidic bonds.*[221]

The sequential attachment of the α-*O*-linked GalNAc moieties induced a conformationally highly stable structure. Remarkably, the NOE pattern of the three α-linked saccharides was virtually identical. This indicated that also in this case attachment of the GalNAc monosaccharide was sufficient for stabilisation of the extended conformation, which is typical for the structure of mucin glycoproteins. Interestingly, the corresponding β-*O*-linked saccharide failed to induce the extended backbone formation, pointing towards the specific role of the α-*O*-GalNAc linkage.

6.2. Carbohydrate-binding proteins

Carbohydrate-binding proteins (CBPs) are involved in biological recognition events such as intercellular cell adhesion, clearance of aged proteins and protein sorting. According to their binding mechanism, mammalian CBPs are classified in C-type, S-type and P-type lectins. Often, the binding of a single saccharide is relatively weak, leading to dissociation constants in the micromolar range. However, multivalent presentation of the carbohydrate ligands increases the affinity for reasons that are the subject of intense studies.[206]

C-type lectins are calcium-dependent, the most prominent being the E-, P- and L-selectins. The interaction between selectins and their glycoprotein and glycolipid ligands is mediated through the SLe^X tetrasaccharide and sulfated derivatives thereof. These interactions establish the adhesion between leukocytes and endothelial tissue during the early phase of an inflammatory response (E- and P-selectins) or to the lymph nodes (L-selectins). This highly studied field was reviewed in detail in refs. [207, 208]

Recently, Suzuki and co-workers reported on a C-type lectin that is expressed on the cell surface of human macrophages.[222] It preferentially binds to mucin peptides carrying the carcinoma-associated T_N antigen and was proposed to be involved in the immunorecognition of malignant cells. In order to study the co-operative effects of polyvalent carbohydrate ligands, Iida and co-workers prepared the C-type human macrophage lectin (HML) in

recombinant form (rHML) and the multiply glycosylated mucins **144** – **146** (Figure 8).[223] The kinetics of the binding between rHML and the GalNAc ligands were examined by surface plasmon resonance (BIAcore apparatus). Relative to **144**, the

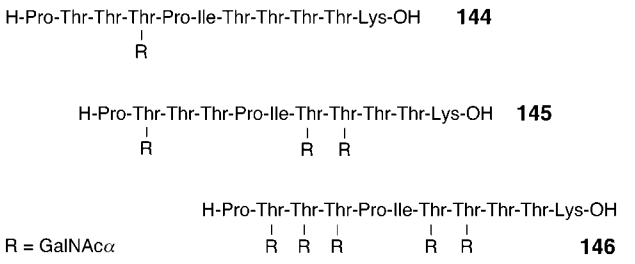


Figure 8. *Glycosylated mucin peptides that were used in binding studies with recombinant C-type human macrophage lectin (rHML). After normalisation to the number of carbohydrate residues, rHML binds with increased affinity to peptides that display multiple GalNAc moieties.*[223]

binding of **145** and **146** increased by a factor of 3.5 and 7.6, respectively, when normalised to the number of GalNAc residues. Dense immobilisation of the glycopeptide ligands led to high-affinity association. The K_D values that were obtained by fluorescence polarisation equilibrium binding studies with the labelled ligands in a solution assay were consistent with the findings from the surface plasmon resonance assay. The carbohydrate-density-dependent increase of the affinity was not observed when functional fragments spanning the HML carbohydrate recognition domain (CRD) were used. Along with chemical cross-linking studies these results indicated that rHML and not the recombinant CRD forms trimers in aqueous solution.

Many CBPs bind only to a few carbohydrate residues at the non-reducing end of a complex glycan. In a rapidly progressing field, peptides are used to mimic the scaffolding and even elements of the recognised carbohydrate structure itself.[197, 224] An illustrative example was presented by St. Hilaire and colleagues.[225] A 300 000-member glycopeptide library containing the three different glycosyl amino acids **147** – **149** was prepared and screened for binding to a C-type lectin from *Lathyrus odoratus* (Figure 9). The most active glycopeptides were detected by incubating the PEGA-resin-bound library with a fluorescently labelled lectin in a solid-phase binding assay. Brightly fluorescing beads were selected and submitted to mass spectrometrical analysis. As a small portion of the amino groups was capped with carboxylic acid tags during each cycle of elongation, ladder sequencing enabled the rapid identification of high-affinity binders. The most active compounds contained mannose only. None of them contained the mannosyl disaccharide, indicating that the lectin binding site is too narrow to accommodate disaccharides.

6.3. Antibody epitope mapping

Monoclonal antibodies directed against tumour-associated glycopeptide antigens are used in tumour diagnostics, both in vitro and in vivo. Often, the first generation of tumour-epitope-directed antibodies is obtained by immunising mice with human

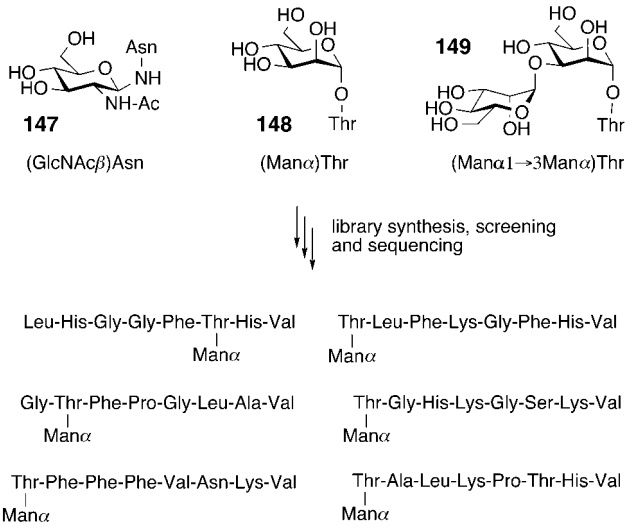


Figure 9. *Building blocks for the construction of a glycopeptide library screened for binding to the C-type lectin from* Lathyrus odoratus. *The most active compounds contained mannose and not the mannosyl disaccharide.*[225]

cancer cells. The first-generation antibodies allow for the isolation of the immunogens by immunoprecipitation or affinity chromatography. The purified immunogens can be employed for a further round of immunisation. Although monoclonal carbohydrate-directed antibodies might display a highly specific binding event, the direct elucidation of the recognised structure is complicated by the multivalent nature and the heterogeneity of the non-immunogenic part of the glycoconjugates. In order to study the binding characteristics of these diagnostically relevant carbohydrate-directed antibodies, synthetic glycopeptides are an invaluable tool.

Mucins, heavily *O*-glycosylated proteins expressed on epithelial cells, have been the subject of intense research efforts. The mucin structure is dominated by a variable number of tandem-repeat sequences. In the MUC 1 mucin these span a 20 amino acid sequence, which is highly immunogenic. Upon carcinogenesis certain glycosyltransferases are expressed in lower concentrations leading to aberrant glycosylation of the mucins.[226] Among the most important tumour-associated antigens are the T_N and the sialyl-T_N antigens, which are found in human colon cancer, ovarian cancer and breast cancer.[227–231] The T antigen was demonstrated to be even tumour-specific in breast tissue.[232] A plethora of monoclonal antibodies directed against tumour-relevant mucin structures exist. For example, the group of Singhal and Toyokoni studied the binding of the monoclonal antibodies SM3 and HMPV to the synthetic MUC 1 repeat sequence and its pentaglycosylated variant in an enzyme-linked immunosorbant assay (ELISA).[233] Surprisingly, both antibodies bound equally well to both peptides. Furthermore, binding of SM3 and HMPV to breast cancer mucin purified from patient pleural effusion was inhibited by both the unglycosylated and the penta-T_N conjugate. Mucins from normal tissues were not recognised. Thus, it was suggested that the attachment of five GalNAc residues is not sufficient to mask the peptide epitopes of the mucin core, which in normal tissue is heavily glycosylated.

The studies of Spencer and co-workers revealed that although antibodies might recognise a peptide epitope, increasing glycosylation can confer a significant enhancement of the binding affinity to the peptides.[234] Fluorescence binding quenching studies with the monoclonal antibody C595, which recognises a Pro-Asp-Thr-Arg (PDTR) epitope, showed that the introduction of three GalNAc residues at threonines 9 and 21 and at serine 20 increased the MUC 1 peptide – antibody association constant (**150** versus **151**, Figure 10). Interestingly, this increase appeared to coincide with a population increase of the PPII helix conformation as indicated by CD spectroscopy in cryogenic mixtures. Hence, it was proposed that the PPII helix is stabilised by MUC 1 glycosylation with GalNAc residues and thus the antibody's binding affinity.

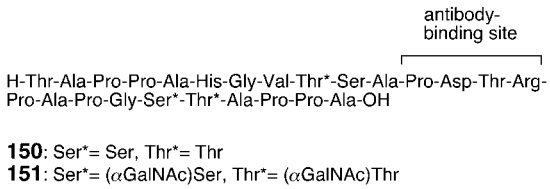


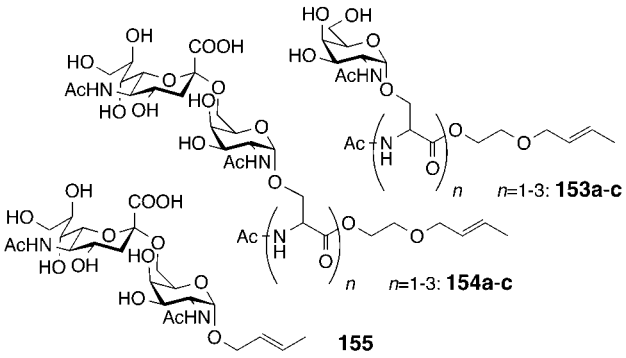
Figure 10. *Attachment of three GalNAc residues to a 25-mer mucin peptide is sufficient to increase both the population of peptides adopting a polyproline II (PPII) helix conformation and the binding of the monoclonal antibody C595.[234]*

A similar result was obtained by Karsten and co-workers who examined the binding of 28 Asp-Thr-Arg (DTR)-specific anti-MUC 1 antibodies to 12 synthetic MUC 1 20- and 21-mers containing T- and T_N antigens at varying positions.[235] The DTR motif is a preferred target for the majority of the peptide-specific anti-MUC 1 antibodies. Albeit previous studies reasoned that this motif represents an effective target for B and T cells only if it is unglycosylated, the binding and inhibition analyses performed by Karsten and co-workers revealed that glycosylation within this motif enhanced the binding. When compared to the unglycosylated peptide, 12 of the 28 antibodies tested showed enhanced binding to three of the seven glycopeptides **152** (Figure 11). The structural feature in common was that

Figure 11. *Selected glycopeptides used for evaluating the fine specificity of monoclonal antibodies. Glycopeptides **152 b, d** and **f** displayed enhanced binding to 12 of 28 tested antibodies indicating that contrary to previous suggestions, glycosylation of the immunodominant DTR motif (indicated in bold) might confer an increased antigenicity of monomeric MUC 1 repeats.[235]*

152 b, d and **f** were glycosylated at the DTR motif with the T and the T_N antigens. Only three antibodies were inhibited upon glycosylation. The binding and inhibition experiments with the T- and the T_N-substituted peptides **152 b, d** and **f** demonstrated that peptide antigenicity is already exerted on the monosaccharide level (see Section 6.1).

The fine specificities of carbohydrate-specific antibodies obtained by immunising mice with immunogens derived from human cancer cell lines was compared with that of antibodies which were generated by using synthetic clustered sialyl-T_N and T_N glycopeptides.[236] To the former group belong the antibodies B72.3 and CC49, which were in clinical use for radioimaging adenocarcinomas. Binding and inhibition experiments with the synthetic peptide conjugates shown in Figure 12 revealed that the monoclonal antibodies B72.3 and CC49 strongly reacted to ST_N serine clusters (**154 b**, **154 c**, **158**). While partial inhibition of binding of B72.3 to ovine submaxillary mucin was observed with the ST_N monomer **154 a**, CC49, a second-generation antibody



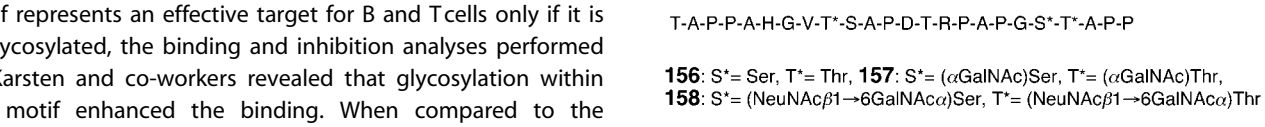


Figure 12. *Glycopeptides **153** – **158** were used for the determination of the fine specificity of anti-sialyl-T_N and anti-T_N monoclonal antibodies. Two clinically used antibodies, which were identified to recognise sialyl-T_N epitopes such as **154** and **158**, were demonstrated to be cross-reactive with T_N-containing glycopeptides **153** and **157**. The monoclonal antibody B195.3, however, showed a true specificity for sialyl-T_N epitopes **154**, **155** and **158**, although without the involvement of the core O-glycosidic bond.[236]*

that was generated following immunisation with B72.3-affinity-purified immunogen, showed no such binding. Hapten recognition included the serine glycosidic bond and both antibodies cross-reacted with T_N-serine clusters **153 b** and **c**. Another set of monoclonal antibodies, B195.3, B230.9 and B239.6, was obtained by immunising mice with KLH conjugates of ST_N **154 a**, T_N trimer **154 c** and ST_N trimer **154 c**, respectively. Only B195.3 showed a high specificity to the ST_N disaccharide including the crotylglycoside **155**. This demonstrated that serine was not involved in the recognition. B239.6 reacted with the ST_N-serine clusters **154 b**, **c** and **158** with strong cross-reactivity to the T_N-serine clusters **153 b**, **c** and **157**. The authors suggested that immunohistological data based on the binding of anti-ST_N antibodies in assessing

the degree of ST_N expression have to be interpreted with caution since many antibodies in clinical use might be cross-reactive to T_N clusters.

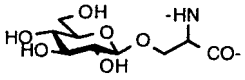
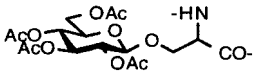
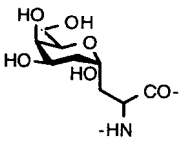
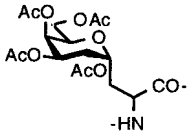
6.4. Glycopeptides as enzyme substrates or inhibitors

It is well recognised that the attachment of carbohydrates serves to maintain the structural integrity of glycoproteins.[3, 237] Both *N*- and *O*-glycosides stabilise the conformation of the protein backbone and protect the glycoprotein from proteolytic attack. Accordingly, the introduction of glycan residues can confer a slower clearance of putative peptide therapeutics in vivo.[238] In a recent example, Mehta et al. investigated the effect of glycosylation on the enzymatic hydrolysis of peptides.[239] The proteolytic enzyme studied was savinase, a subtilisin-type enzyme. A panel of 8-mer peptide substrates was synthesised in which each amino acid but a single proline was replaced with an unglycosylated and a lactose-containing asparagine residue. Generally, introduction of the lactose residue rendered the glycopeptide less vulnerable towards proteolytic degradation. This stabilising effect was particularly pronounced at the site N-terminal to the cleavage site (P1 site).

In addition to the protection against proteolytic degradation, glycosylation can also enhance the delivery of a peptide drug. Polt et al. have shown that glucosylated enkephalins were transported through the endothelial barrier by a glucose transporter and retained their biological activity.[240] Likewise, the intestinal absorption of peptide drugs was improved by glycosylation.[241] Recently, Negri et al. evaluated the antinociceptive activity of glycosylated analogues of dermorphin and deltorphin (Table 1).[242] The synthetic analogues differed in the carbohydrate moiety that was attached through either an *O*- or a *C*-glycosidic bond. All glycosides were linked to the C terminus since a related study demonstrated the favourable pharmacological activity of this modification.[243] The introduction of glycosides and acetylated glycosides conferred a significant delay of the degradation as determined by the biological activity that remained after incubation with brain and liver homogenates. A competition assay, in which the binding site of the μ-receptor was occupied by a radioactively labelled ligand revealed that substitution of the C-terminal serine in **159** with alanine (→**160**) did not affect the binding of dermorphin. Introduction of β-*O*-linked glucose (→**161**) as well as α-*C*-linked galactose (→**163**) resulted in a slight reduction of the receptor affinity. The affinity dropped significantly when the carbohydrates were acetylated (→**162**). The in vitro activities were following the same trend. However, in in vivo studies, in which all analogues were evaluated for their antinociceptive activity after systemic administration, a different behaviour was observed. This analgesia hot-plate test showed that the *O*- and *C*-glycosylated analogues **161** and **163**, respectively, were more analgesic than the unmodified dermorphin (**159**). As a possible explanation the authors suggested that the delayed clearance might result in higher in vivo concentrations. Alternatively, the carbohydrate residue might confer a transporter-mediated transfer through the blood–brain barrier. It should be noted that the attachment of carbohydrates can increase cellular uptake of a variety of medicinally interesting compounds such as toxophores,[244–246] oligodeoxynucleotides,[247] proteins[248] and polymers.[249]

In order to assess the role of protein glycosylation, the biological properties of a glycoprotein can be studied before and after enzymatic or chemical deglycosylation. *N*-Deglycosylation mainly is carried out by using *N*-glycanases, which catalyse the

Table 1. *Opioid receptor affinity and analgesic potency of glycosylated analogues **161**–**164** of dermorphin (H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-X-NH_2; **159**).*

Compound	X	μ-Opioid receptor affinity,[a] K_i [nM]	In vitro activity[b] IC_{50} [nM]	Analgesia hot-plate test;[c] AD_{50} [μmol kg^{-1}]
159	Ser	1.1 ± 0.2	1.5 ± 0.5	2.4
160	Ala	1.2 ± 0.9	1.6 ± 0.2	n.d.
161		2.4 ± 0.3	3.5 ± 0.4	1.4
162		7.9 ± 0.9	8.2 ± 0.7	4.8
163		2.5 ± 0.4	4.0 ± 0.5	1.1
164		11.2 ± 2.1	6.0 ± 0.5	6.6

[a] Competition assay using 0.5 nM [^{3}H][D-Ala^2, MePhe^4, Glyol^5]enkephalin (DAGO) as μ-opioid receptor ligand. [b] Tissue preparations of the myenteric plexus-longitudinal muscle obtained from small intestine of male guinea pigs were used for the evaluation of agonists **159**–**164** with respect to their inhibition of the electrically evoked twitch. [c] AD_{50} = median antinociceptive dose, which was assessed by placing mice on a 55 °C heated surface and recording the mean time to licking of the back paws or an escape jump. n.d. = not determined.

hydrolysis of the *N*-glycosidic bond. Fan and Lee reported a detailed study on the substrate structure requirements of the two commercially available *N*-glycanases, peptide:*N*-glycosidases A and F (PNGase A and F).[250] A panel of amino acid and peptide conjugates containing *N*-linked GlcNAc, cellobiosyl, lactosyl and chitobiosyl residues was employed to determine the hydrolytic activities of PNGases A and F on these synthetic substrates (Figure 13). Neither PNGase A nor F was able to hydrolyse lactose- or cellobiose-containing peptides (**165** and **166**, respectively). The chitobiosyl (**168**, **170**) and the GlcNAc tripeptides (**167**, **169**) were hydrolysed by both glycoamidases indicating that the 2-acetamido group of GlcNAc or chitobiose is mandatory. Single glycosyl amino acids such as **171** were not accepted as substrate neither by PNGase A nor by PNGase F. The minimum length of the peptide backbone required for *N*-deglycosylation by PNGase F appeared to be that of a tripeptide whereas PNGase A also accepted dipeptides (compare **168** and **170** with **171** – **173**). PNGase A seemed to prefer substrates that contained the *N*-glycosylation site at the N terminus to those that were C-terminally glycosylated. Interestingly, a threonine at the C + 2 position (consensus sequence for *N*-glycosylation) of the glycosylated asparagine improved PNGase F activity (compare **167** with **169** and **168** with **172**).

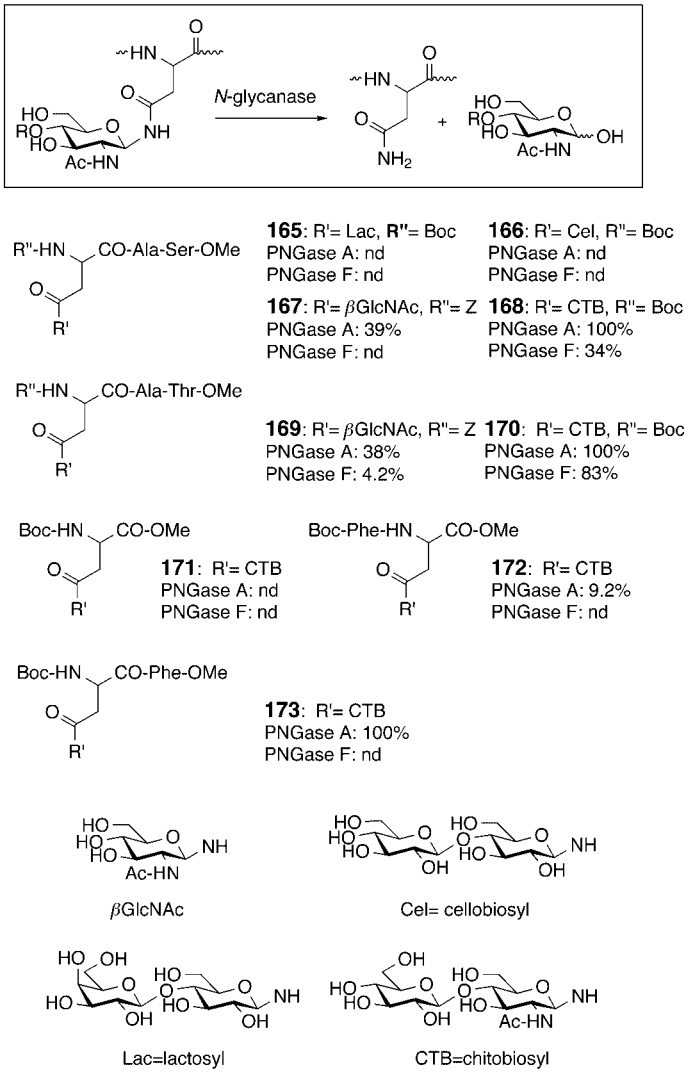


Figure 13. *Selected examples of synthetic glycopeptides that were used for the evaluation of the substrate structure requirements of PNGases A and F. The activities were assessed by analysing the hydrolysis mixture for the percentage of cleavage after 18 h incubation at 37 °C and pH 5.0 for PNGase A and pH 8.0 for PNGase F. nd=not detectable.*[250]

6.5. Glycopeptides in binding to MHC molecules and T-cell recognition

In the event of the specific immune response towards foreign substances, the immune system follows two principal pathways. One is termed humoral immunity, in which antibodies specifically bind to the challenging antigens. The other, cellular immunity, is mediated by cells rather than molecules and employs cytolytic T lymphocytes for recognition and lysis of antigen-charged cells. Both the cellular and the humoral immune response depend on the assistance of T-helper cells. The latter can also be stimulated by a few thymus-independent antigens, which usually are of high molecular weight such as lipopolysaccharide (LPS), a component of bacterial cell walls. However, the induction of high-affinity antibodies is possible only with the aid of T-helper cells. In brief, T cells recognise antigens on the surface of accessory cells. Every cell constantly converts endogeneous proteins to small-size peptides by means of its proteasome. The fragments are transported into the endoplasmic reticulum (ER), in which the 10 – 20-mer peptides bind to MHC class I molecules. After transfer through the Golgi network, the peptide – MHC I complex is located on the cell surface and presented to $CD8^+$ cytolytic T cells provided that certain residues anchor the peptide to the MHC-binding cleft. If the T-cell receptor (TCR) recognises non-self peptides in the MHC I peptide-binding groove, a cytolytic response towards the antigen-presenting cell (APC) can be triggered. Specialised APCs such as macrophages internalise proteins by endocytosis. After passage through an acidic compartment these proteins are degraded and the resulting peptides are bound to MHC class II molecules. Recognition of MHC II – peptide complexes by T-helper cells then is able to induce the production of cytokines, which are necessary for B-cell activation.

It was known that carbohydrates do not bind to MHC molecules. Whether glycopeptide fragments would bind to MHC molecules was not known until the early 1990s. An excellent review presented the data that had been collected between 1992 and 1997.[154] Briefly, binding studies revealed that synthetic glycopeptides can bind well to MHC I and MHC II molecules when the glycosylation site was at a non-anchoring amino acid. Immunisation studies and T-cell proliferation assays confirmed that glycopeptides are immunogenic and able to induce a carbohydrate-specific T-cell response. More recently, Jensen and co-workers immunised mice with the synthetic glycopeptide **174b** and raised MHC-class-II-E^k-restricted T-cell hybridomas that proliferated and secreted interleukin 2 (IL-2) upon activation with **174b** (Figure 14).[251] The fine specificity of 22 T-cell hybridomas was investigated by determining the growth of an IL-2-dependant cell line. The synthetic glycopeptides that were used for activation of the T-cell hybridomas

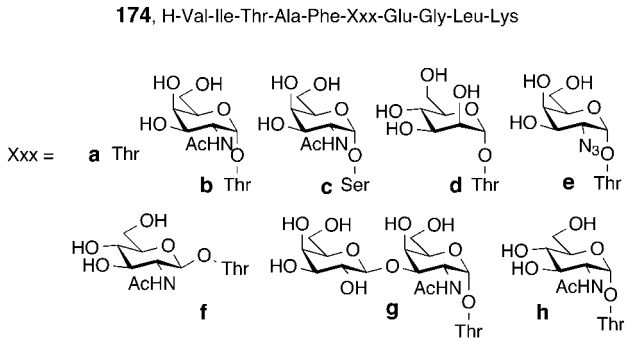
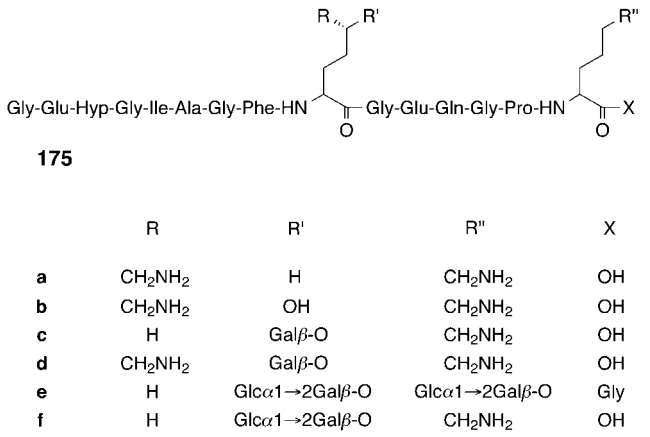


Figure 14. Selected examples of synthetic peptides and glycopeptides that were used for the evaluation of the carbohydrate specificity of MHC-class-II-restricted T-cell hybridomas raised against the O-glycosylated self peptide ***174 b****.*[251]



	R	R'	R''	X
a	CH_2NH_2	H	CH_2NH_2	OH
b	CH_2NH_2	OH	CH_2NH_2	OH
c	H	Galβ-O	CH_2NH_2	OH
d	CH_2NH_2	Galβ-O	CH_2NH_2	OH
e	H	Glcα1→2Galβ-O	Glcα1→2Galβ-O	Gly
f	H	Glcα1→2Galβ-O	CH_2NH_2	OH

Figure 15. Synthetic peptides and glycopeptides used to demonstrate that the majority of T cells obtained after immunisation with native type-II collagen specifically recognise glycopeptide ***175 d*** *with no cross-reactivity to the unglycosylated peptides* ***175 a*** *and* ***b****.*[130]

differed in the glycan moiety, which was attached to a known E^k-binding peptide derived from haemoglobin. Remarkably, 19 of the hybridomas responded only to glycopeptide **174 b**. The three other clones responded to the unglycosylated peptide **174 a** displaying a total lack of cross-reactivity between glycopeptide and unglycosylated peptide. Most of the hybridomas were equally activatable by the GalNAc-serine- and GalNAc-threonine-containing peptides **174 c** and **b**, respectively. Seventeen of nineteen hybridomas responsive to glycopeptide **174 b** were able to distinguish between the αGalNAc-containing glycopeptides **174 b** and **c** and the αGlcNAc-containing glycopeptide **174 h**, indicating that the glycan is the entity recognised by the T-cell receptor. However, αGalNAc peptides in which amino acids pointing to the T-cell receptor were replaced by alanine were unable to activate the hybridomas. It was thus concluded that the T-cell hybridomas specifically recognised both the glycan and the solvent-exposed parts of the glycan-carrying peptide.

T cells were shown to recognise a glycopeptide derived from type-II collagen.[130] The IL-2-producing hybridomas obtained after immunisation of mice with type-II collagen were incubated with peptides **175 a** and **b** together with glycopeptides **175 c** – **f** (Figure 15). The synthetic peptide **175 a** activated only a few collagen-reactive T-cell hybridomas although a peptide spanning the same sequence was demonstrated to be even immunodominant when obtained through proteolytic cleavage of native collagen.[252] It was reasoned that post-translational modification of the lysine residues such as hydroxylation and glycosylation would be responsible. However, even less hybridomas responded to peptide **175 b** containing two hydroxylysines instead of lysine. The response of 76% of the T-cell hybridomas was associated with carbohydrates since they all recognised type-II collagen but not peptides **175 a** and **b**. Interestingly, the majority of the hybridomas was stimulated upon incubation with glycopeptide **175 d**, in which the central hydroxylysine carried the β-galactosyl moiety. The same hybridomas proved unreactive towards glycopeptides **175 e** and **f**, which contained the Glcα1→2Gal disaccharide β-linked to hydroxynorvaline, but a few were cross-reactive with glycopeptide **175 c** containing an β-galactosylated hydroxynorvaline at an identical position. These investigations presented the first example that immunisation with a natural glycoprotein can elicit carbohydrate-specific T cells.

A recent study addressed the question whether presentation of glycosylated peptides by MHC class I molecules occurs in vivo.[253] MHC class I molecules present peptides obtained through processing of endogeneous proteins, which in the cytosol often carry the GlcNAc monosaccharide β-linked to serine. Therefore, an assay was developed which was able to detect GlcNAc residues attached to MHC-I-bound peptides. MHC – peptide complexes were purified from normal human spleen and treated with acetic acid for elution of the bound peptides. The isolated MHC-derived peptides were incubated with ^{3}H-labelled UDP-galactose and GlcNAcβ1→4-galactosyltransferase to transfer the radioactively labelled galactose moiety to any of the peptides that eventually contained the GlcNAc modification. Roughly 0.1% of the peptides presented by class I MHC molecules were found to carry the radioactive label, which after chemical deglycosylation was identified to be part of the Galβ1→4GlcNAc disaccharide. A requirement for the presentation of peptides by MHC class I molecules in vivo is that the peptides are transported by TAP (transporter associated with antigen presentation; for a recent review see ref. [254]) into the endoplasmic reticulum. Indeed, synthetic glycopeptides carrying the *O*-β-GlcNAc substitution were demonstrated to be substrates for TAP-mediated transport across the ER membrane.

The molecular basis of the recognition of MHC class I – glycopeptide complexes by the T-cell receptor has been suggested based on crystal structures of glycopeptides **177** and **178** with H-2D^b MHC (Figure 16).[255] Both glycopeptides were used to raise H-2D^b-restricted, carbohydrate-specific cytotoxic T lymphocyte (CTL) clones. Interestingly, all clones that were directed against **178** showed a strong cross-reactivity to **177**.[256] In contrast, the CTL clones obtained through immunisation with **177** were highly specific for **177**. The crystal structures revealed that in both structures the *O*-GlcNAc group of the MHC-I-associated glycopeptides **177** and **178** were solvent-exposed and therefore accessible for recognition by the T-cell receptor. However, the

176, H-Phe-Ala-Pro-Gly-Asn-Tyr-Pro-Ala-Leu-OH

177, H-Phe-Ala-Pro-(GlcNAcβ)Ser-Asn-Tyr-Pro-Ala-Leu-OH

178, H-Phe-Ala-Pro-Gly-(GlcNAcβ)Ser-Tyr-Pro-Ala-Leu-OH

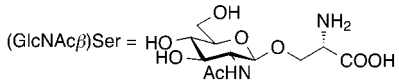


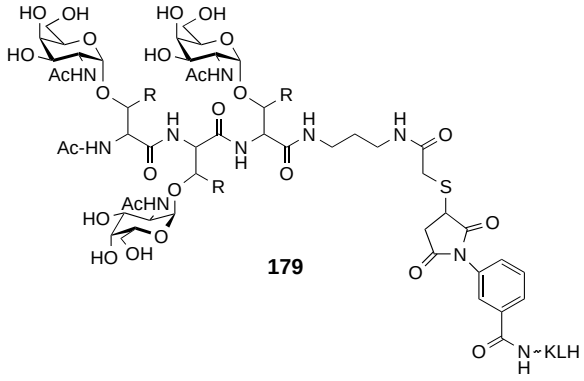
Figure 16. *The crystal structures of the H-2D^b MHC class I molecule in a complex with peptide **176**, glycopeptide **177** or glycopeptide **178** suggested a molecular basis for the non-reciprocal pattern of cross-reactivity of cytotoxic T cells that were obtained through immunisation with **177** and **178**.[255]*

backbone conformations of the two glycopeptides bound by the H-2D^b groove were strikingly different. MHC-bound **177** adopted a conformation that was almost identical to the wild-type peptide **176**. In glycopeptide **178**, the glycan part occupied an anchor position. As a result, the peptide portion surrounding this residue was rotated by 180° to expose the glycan moiety to the solvent. This reorganisation of the bound conformation was accompanied by different recognition patterns of the T-cell receptors. The highly cross-reactive CTL clone displayed a TCR with a short CDR3 loop to allow access to glycan **178**, which accommodates a large volume due to the omission of one anchor residue. This led to the selection of a highly promiscuous TCR, which also recognised **177**. The TCR directed against MHC-bound **177** contained a longer CDR3 loop, which was suggested to be able to make additional contacts to the peptide residues.

6.6. Glycopeptides for the immunotherapy of cancer

During carcinogenesis the majority of cells experience a dramatic transformation of the glycosylation machinery. As a result, many proteins that are expressed on the surface of the cancer cells display an altered glycosylation pattern.[257] A few types of oligosaccharides are tumour-associated and for some tissue even tumour-specific structures. These include T_N, sialyl-T_N and T antigens and Lewis-X and Lewis-A structures, whose expression is increased in cancer cells. Furthermore, increased β1,6-GlcNAc-branching of *N*-linked glycans and a general increase in sialylation are commonly observed. A large body of data obtained in clinical and experimental settings revealed that the increased expression level of certain saccharides is correlated with a poor prognosis.[257] These altered glycan structures can thus be regarded as a means to distinguish the tumour cell from a normal cell. The immune system, which evolved to eradicate non-self structures, perpetually eliminates most tumour cells. However, the carbohydrates attached to surface proteins might be expressed at levels too low to induce a powerful immune response. Vaccination with synthetic tumour antigens with the help of immunostimulatory adjuvants could target the immune system to the cancer cells. Usually, synthetic antigens are of low molecular weight and hence poorly immunogenic. Conjugation to immunogenic carrier proteins such as KLH or BSA, however, was shown to elicit an immune response that was directed against the synthetic carbohydrate or glycopeptide hapten as well as the tumour cells.[258] Until now, the immune responses to synthetic carbohydrate-derived vaccines were largely restricted to the stimulation of antibody production. The high antibody titers obtained through vaccination can prevent metastasis by the eradication of circulating tumour cells. In addition, the antibody response could mediate tumour destruction by targeting the cytolytic complement system to the cancer cells.

The group of Danishefsky synthesised trimeric T_N peptides as partial structures of mucin-related antigens (**179** in Figure 17).[111]

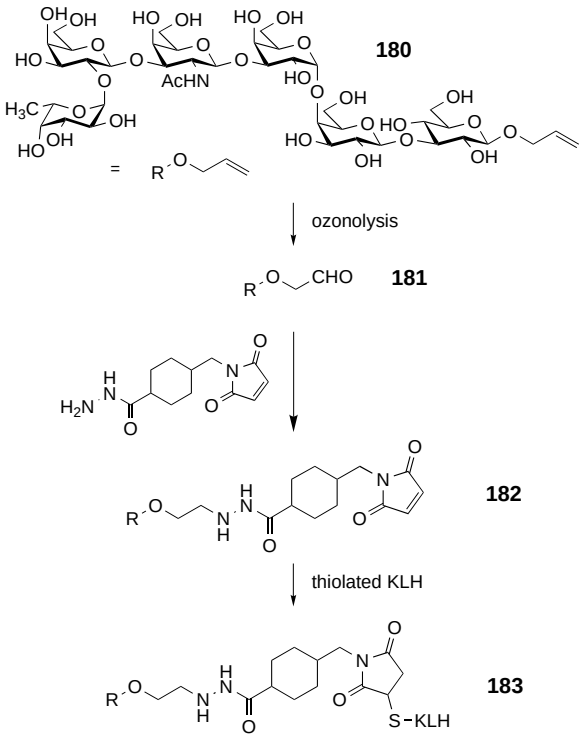


179

Figure 17. *The mucin-derived glycopeptide – KLH conjugate contains three clustered T_N antigens and was used in clinical trials as a putative synthetic vaccine against prostate cancer.[111]*

Immunisation studies revealed that conjugation to KLH induced high IgM and moderate IgG titers in mice. The sera from mice were able to attack T_N-positive LS-C colon cancer cells as evaluated by flow cytometry assays (detection of surface-bound antibodies) and complement-mediated cytotoxicity assays (^{51}Cr release upon complement-mediated lysis of target cells). However, despite repeated booster immunisations, the immune response was dominated by high IgM titers and there was no evidence for a IgM to IgG class switch. Along with the lack of secondary response this indicates a T-cell-independent antibody response, which is characteristic for many carbohydrate antigens.

Danishefsky's group also synthesised the most complex vaccine to date that was evaluated in phase-I clinical trials.[259] The globo H allyl glycoside **180** was converted to the aldehyde **181** by ozonolysis and linked to the amino group of the bifunctional cross-linker **182** (Scheme 30). The maleimide group served to attach the oligosaccharide to thiolated KLH. The resulting globo H – KLH conjugate **183** was used for the immunisation study including 18 patients with relapsed prostate cancer. A comparison of pre- and post-treatment sera revealed that 10 of 18 patients showed a two- to ninefold increase in IgM reactivity against globo-H-expressing MCF-7 cells. Only in two patients an increased IgG reactivity was noted. The sera of nine patients showed an increase in complement-mediated lysis of the globo-H-positive human breast cancer cell line MCF-7. As a part of this study, the PSA-status (PSA = prostate-specific antigen), which correlates with the progression of prostate cancer, was monitored. All patients showed rises of the PSA levels at the beginning of the trial. Two of five minimal-disease patients showed modest PSA decreases. It was suggested that for an



Scheme 30. *The fully synthetic globo H hexasaccharide conjugate **183** was used in phase-I clinical vaccination studies of prostate cancer patients.[259]*

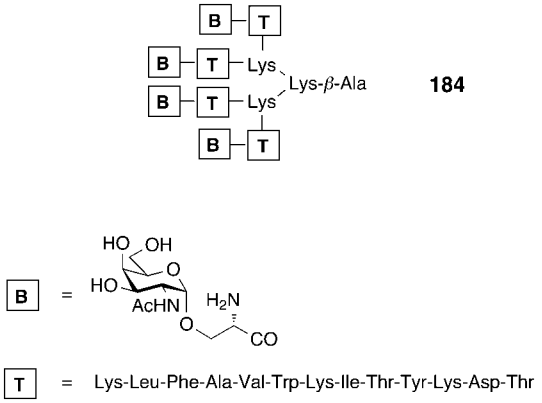


Figure 18. *The fully synthetic immunogen **184** that contains a multiply antigenic T_N glycopeptide as B-cell epitope (B) and a well-known T-cell epitope (T) was shown to elicit a T-cell-dependent immune response that increased the survival of tumour-bearing mice.[262]*

effective immunotherapy an increased vaccine potency is required.

MacLean and co-workers investigated a sialyl-T_N–KLH conjugate as cancer vaccine in clinical trials including ovarian, colorectal and breast cancer patients.[260] It was found that high levels of antibodies against the mucin-associated sialyl-T_N epitope correlated with the survival of metastatic adenocarcinoma patients. In a following study, it was shown that pretreatment with cyclophosphamide enhanced the effect of the sialyl-T_N–KLH cancer vaccine.[261] Cyclophosphamide is believed to predominantly inhibit supressor-T cells induced by low levels of the tumour antigens. Patients who received cyclophosphamide before the vaccination developed higher anti-sialyl-T_N titers than untreated patients. The sera also reacted against naturally occurring sialyl-T_N epitopes from ovine submaxillary mucin. The phase-II clinical study demonstrated that cyclophosphamide-treated patients showed superior antibody responses. An inverse correlation between tumour growth and anti-sialyl-T_N titers was noted. It seemed that survival of patients, who had received cyclophosphamide before vaccination, was increased.

Recently, the first successful attempt to induce a T-cell-dependent immune response against carbohydrate epitopes was reported.[262] The synthetic immunogen **184** was designed as a multiply antigenic T_N glycopeptide containing a well-known T-cell epitope from the type-I poliovirus (Figure 18). Remarkably, an IL-2-secreting T-cell hybridoma cell line specific for the unglycosylated poliovirus peptide (T, Figure 18) showed a strong cross-reactivity to **184**. T-cell stimulation was achieved with a 10000-fold lower dose compared to a construct in which the T_N antigen was omitted. Clearly, presentation of **184** by MHC was enhanced through either a favourable intracellular processing or an increased endocytosis by the antigen-presenting cells. The sera obtained from immunised mice were able to recognise the native T_N antigen on human Jurkat T-lymphoma and LS180 adenocarcinoma cell lines. It was remarkable that the humoral response was dominated by IgG antibodies supporting the notion that a T-cell-dependent response was induced.

7. Summary and outlook

The examples that were selected for this review demonstrate that the recent improvements in the synthetic methodology have enabled the synthesis of glycopeptides that exhibit an ever increasing complexity of both the peptide and the carbohydrate part. Our increased understanding of glycosylation reactions facilitates the preparation of glycosyl amino acid building blocks for subsequent use in the assembly of the peptide backbone. Particularly solid-phase synthesis allows for the rapid and—if desired—combinatorial access to various glycopeptides. It appears that through the combined use of chemical and enzymatic methods and the application of orthogonal ligation strategies such as native chemical ligation even the ambitious goal of the total synthesis of glycoproteins comes within reach. A promising approach certainly is the utilisation of intein-mediated protein-splicing—a splicing event that allows for a general biocatalysis of a peptide fragment condensation. To date, however, there is no general method for the synthesis of any glycopeptide or glycoprotein. A crucial step is the introduction of the carbohydrate part, and as long as there are only a few glycosyltransferases available, chemical synthesis is meeting the challenge to provide access to homogeneous glycopeptides of a complexity that allows for biological and medicinal investigations.

From the biological studies a picture emerges in which glycosylation is reflecting a subtle mechanism by which both the structure and the activity of a protein can be regulated. For example, a single glycan residue can induce a turn-like structure

whereas the attachment of multiple glycan residues can force the peptide backbone to accommodate an extended conformation. Carbohydrates can decrease the in vivo clearance of a peptide. However, an increase is possible when the glycosylation targets peptides to specialised cells that express carbohydrate-binding proteins on their surface. It also became apparent that the conjugation with carbohydrates can mask as well as create peptide epitopes, which was shown for both B and T cells. Carbohydrates can be highly immunogenic when conjugated to a suitable peptide T-cell epitope, and the carbohydrate-specific antibodies thus generated are versatile tools for tumour diagnostics. In addition, the immunisation with natural and synthetic glycopeptides can induce T cells that specifically recognise the glycan of an MHC-bound glycopeptide. This is of utmost importance for the design of effective glycopeptide immunogens, as one hope for the future is that an immune response against tumour-specific glycopeptide antigens can be directed against tumour cells, thus providing a means to eradicate circulating metastases.

Abbreviations

aa	amino acid
Ac	acetyl
Acm	acetamidomethyl
All	allyl
Aloc	allyloxycarbonyl
Anth	anthranyl
Bn	benzyl
Boc	*tert*-butyloxycarbonyl
BOP	1-benzotriazolyloxytris(dimethylamino)phosphonium hexafluorophosphate
BSA	bovine serum albumin
CDR	complementarity-determining region
CMP	cytidine monophosphate
coll	collidine
Cp	cyclopentadienyl
CTL	cytotoxic T lymphocyte
DCC	*N*,*N*′-dicyclohexylcarbodiimide
Dhbt-OH	3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine
DIC	diisopropylcarbodiimide
DIPEA	*N*-ethyl-*N*,*N*-diisopropylamine
DMDO	dimethyldioxirane
DMF	*N*,*N*-dimethylformamide
DMSO	dimethyl sulfoxide
DMTST	dimethylmethylthiosulfonyl triflate
Dts	*N*-dithiasuccinyl
DTT	1,4-dithiothreitol
EDC	1-(3-dimethylaminopropyl)-3-ethylcarbodiimide
Fm	fluorenylmethyl
Fmoc	fluoren-9-ylmethyloxycarbonyl
Fuc	fucose
FucTase	α-1,3-fucosyltransferase
Gal	galactose
GalNAc	2-acetamido-2-deoxygalactose
GalTase	β-1 $\rightarrow$4-galactosyltransferase
Glc	glucose
GlcNAc	2-acetamido-2-deoxyglucose
Gn·HCl	guanidine hydrochloride
HATU	*O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate
HBTU	*O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate
HEPES	2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid
HOAt	1-hydroxy-7-azabenzotriazole
HOBt	1-hydroxy-1*H*-benzotriazole
Hyl	hydroxylysine
Hyp	4-hydroxyproline
$I(coll)_2$	iodocollidinium
Ig	immunoglobulin
IIDQ	1-isobutyloxycarbonyl-2-isobutyloxy-1,2-dihydroquinoline
IL	interleukin
KLH	keyhole limpet hemocyanine
MAdCAM	mucosal adressin cell-adhesion molecule
MALDI	matrix-assisted laser desorption/ionisation
Man	mannose
MBHA	4-methylbenzhydrylamine
MHC	major histocompatibility complex
Mpm	methoxyphenylmethyl
MUC	mucin
NeuNAc	*N*-acetylneuraminic acid
NIS	*N*-iodosuccinimide
Nle	norleucine
NMM	*N*-methylmorpholine
NMP	*N*-methylpyrrolidone
NOE	nuclear Overhauser effect
Nor	norvaline
Orn	ornithine
PAM	phenylacetamido
Pam_3Cys	tripalmitoyl-*S*-glycerylcysteinylserine
PEGA	polyethylene glycol – acrylamide copolymer
Pfp	pentafluorophenyl
PG	protecting group
Phac	phenylacetyl
Phth	phthaloyl
PPII helix	polyproline II helix
PyBOP	benzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphat
Pyr	pyridine
PS	polystyrene
ROESY	rotating-frame Overhauser spectroscopy
Su	succinimidyl
TBDMS	*tert*-butyldimethylsilyl
TBDPS	*tert*-butyldiphenylsilyl
TBS	tributylsilyl
TBTU	*O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate
*t*Bu	*tert*-butyl
Tf	trifluoromethanesulfonyl
TFA	trifluoroacetic acid
TFMSA	trifluoromethanesulfonic acid
THF	tetrahydrofuran
TIPS	triisopropylsilyl
TMS	trimethylsilyl
Tol	tolyl
Troc	trichlorethyloxycarbonyl
Trt	trityl = triphenylmethyl
UDP	uridine diphosphate
Z	benzyloxycarbonyl

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