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Recent advances in the synthesis of complex *N*-glycopeptides

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Abstract

This review describes the recent advances in the development of efficient strategies, chemical, enzymatic and chemo-enzymatic, for the synthesis of complex *N*-glycopeptides. A selected number of illustrative examples will also be discussed.

Contents

1. Introduction

The surge of interest in glycoproteins[1] arises from the increasing awareness of their importance in many diverse biochemical processes including cell growth regulation, binding of pathogens to cells,[2] intercellular communication and metastasis.[3] Glycoproteins[4] serve as cell differentiation markers. Among other roles, the saccharide fragment in glycoproteins assists in protein folding and transport,

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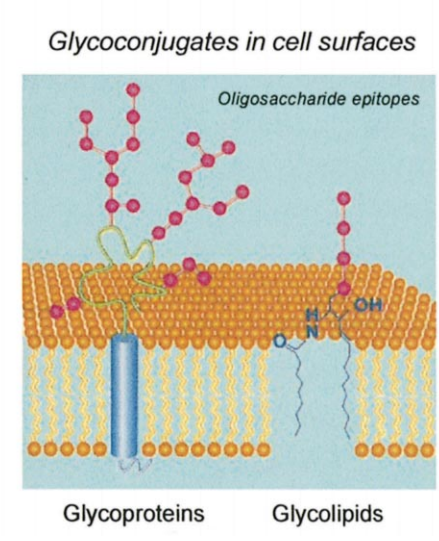
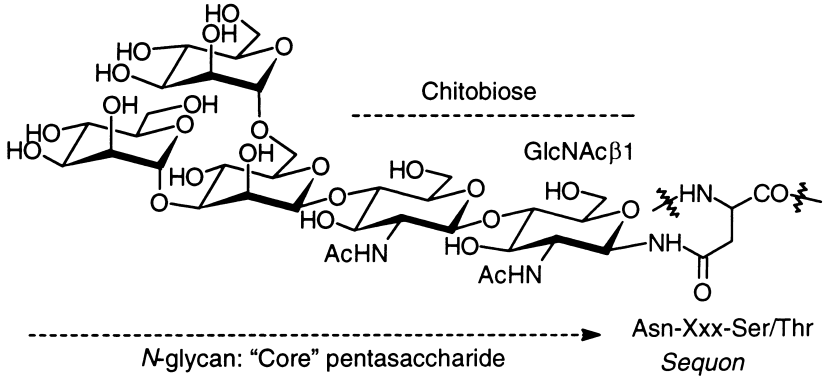
possibly by providing protection against proteolysis. However, they also play a role in the biological action of glycoproteins. Thus, characteristic carbohydrate moieties which are present in cell adhesion molecules, tumour-associated antigens, viral or bacterial invasion targets and blood group determinants are most commonly presented and involved in the binding process of molecular recognition systems in the form of glycoproteins.

Further insight into the important biological roles of the carbohydrate moieties in glycoproteins is gained considering the existence of the elaborate machinery involved in the biosynthesis of glycosylated proteins, and their widespread occurrence, which suggests that these bidomainal structures are assembled by cells in purposeful and regulated ways (Scheme 1).



Scheme 1.

Two main forms of protein glycosidation are naturally found. In a previous review article[5] we dealt with *O*-glycosidation; in the present one we only consider *N*-protein glycosidation which is exclusively effected on asparagine (Asn) residues. Naturally occurring *N*-asparagine glycoproteins and glycopeptides are β-*N*-linked to an *N*-acetyl glucosamine segment of a chitobiose, which is part of a high mannose antennary structure (Scheme 2).

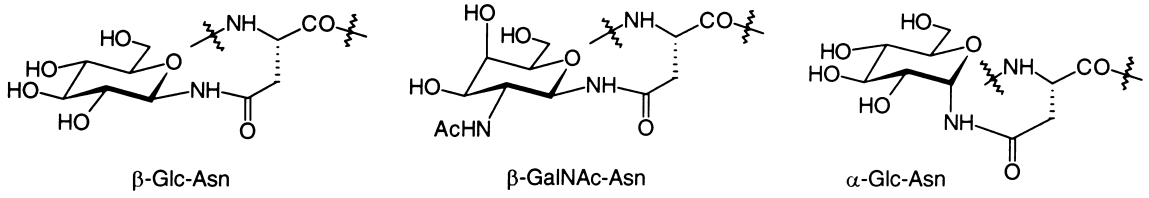


Scheme 2.

This pentasaccharide core carbohydrate is the minimal structure at the attachment point of many *N*-asparagine linked glycoproteins. A common structural feature found in the protein section is that the

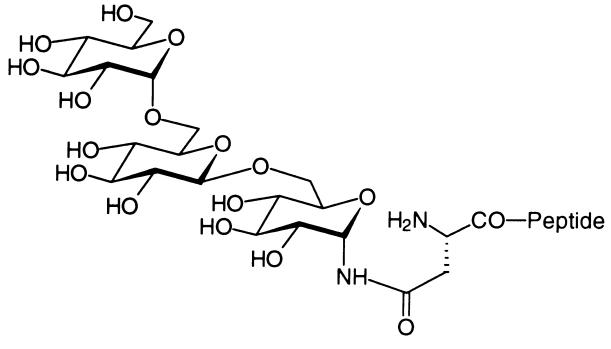
Asn residue is always located in a specific amino acid sequence or sequon of the type Asn-Xxx-Ser/Thr (where Xxx is any amino acid except Pro).

In recent years, novel forms of protein glycosylation have been characterized.[6] It has been found that monosaccharides and oligosaccharides other than the ones constituting the typical core can be attached to the Asn side chains including β-D-glucose[7] and β-*N*-acetylgalactosamine[8] (Scheme 3). The β-GlcNAcAsn linkage unit was found not only in eukariotes but also in the extreme halophilic archaebacteria *Halobacterium halobium* and *H. volcanii*, in the cell surface glycoproteins.[9] Messner and Sleytr[10] have described a trisaccharide of L-rhamnose attached to an Asn residue in GP I of *Bacillus stearothermophilus*. In these cases, too, the Asn-Xxx-Ser/Thr motif is a prerequisite to glycosylation.



Scheme 3.

There are two known exceptions to the motif rule. The first is a glycopeptide called nephritogenoside[11] with a glucose α-linked to the amide side chain in the sequence Asn-Pro-Leu located in the 21 amino acid full sequence (Scheme 4). This *N*-glycopeptide was isolated and purified as a minor component from the glomerular basement membrane of normal rats. This compound is active for the induction of glomerulonephritis in experimental animals.

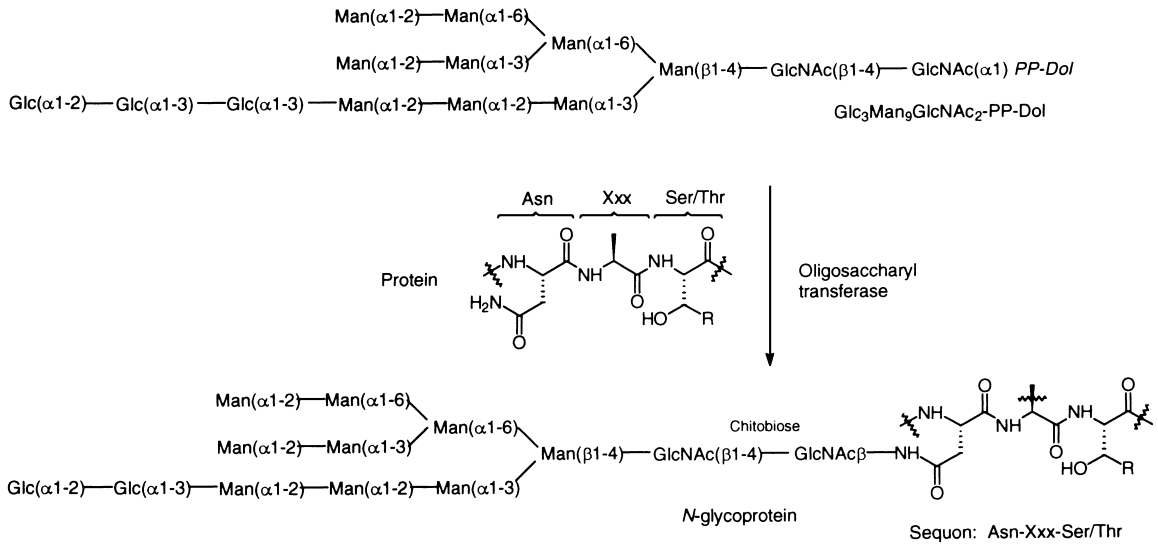


Scheme 4.

The second example[12] is the heavy chain of bovine protein C. This protein contains three carbohydrate structural groups. The first two are attached in typical environments: $Asn_{93}Tyr_{94}Thr_{95}$ and $Asn_{154}Arg_{155}Thr_{156}$. The third site of glycosylation, however, is $Asn_{170}Ala_{171}\mathit{Cys}_{172}$ where a Cys occupies the Ser/Thr position and is involved in a disulfide bridge.

1.1. Biosynthetic pathway: origin of glycoforms

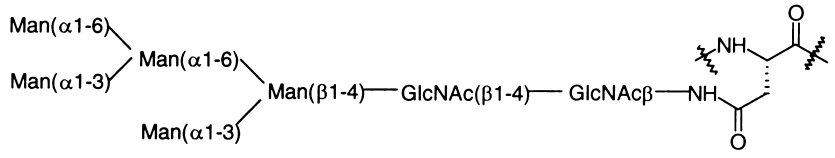
The biosynthesis[13] of all *N*-glycans begins in the rough endoplasmic reticulum with the co-translational transfer of a large oligosaccharide from a common precursor, the dolichol pyrophosphate oligosaccharide to an Asn residue in the polypeptide (en-bloc synthesis, Scheme 5). This lipid-linked oligosaccharide is composed of nine mannoses, three glucoses and two *N*-acetyl glucosamine residues ($Glc_3Man_9GlcNAc_2$).



Scheme 5.

This first event is followed by the removal of three glucose and four mannose residues within the lumen of the endoplasmic reticulum and Golgi apparatus due to the *processing* actions of specific α-glucosidases[14] and α-mannosidases. The product of this processing is the structure $Man_5GlcNAc_2$-R which is the starting point for the synthesis of all complex and hybrid *N*-glycans.

Another key enzyme[15] is GlcNAc transferase I which adds a GlcNAc in β-1,2 linkage to the Man(α1,3)-Man(β1,4)-GlcNAcβ arm of the core (Scheme 6), converting the high mannose-containing fragment to complex and hybrid *N*-glycans. The presence of a β2-linked GlcNAc residue at the non-reducing terminus of this arm is essential for the subsequent actions of several enzymes in the processing pathway.



Scheme 6.

The *N*-glycosylation of proteins is a highly conserved process in eukaryotic evolution. The oligosaccharides $Glc_xMan_9GlcNAc_2$ (x=1–3) are involved in a number of important steps during the biosynthesis and folding of glycoproteins. However, the specific role of *N*-linked glycoproteins is not well understood.[16] Some insight into the mechanisms has been obtained by NMR conformational studies[17] of $Glc_xMan_9GlcNAc_2$.

1.2. Microheterogeneity

The asparagine-bound oligosaccharides found in glycoproteins (*N*-glycans) vary depending on their degree of branching, the variation of terminal structures in the side chains, and the substitution of the core pentasaccharide.[18] A divergent combination of the possible linkages and substitutions leads to thousands of related oligosaccharide structures. This phenomenon is known as microheterogeneity.[19] Therefore,

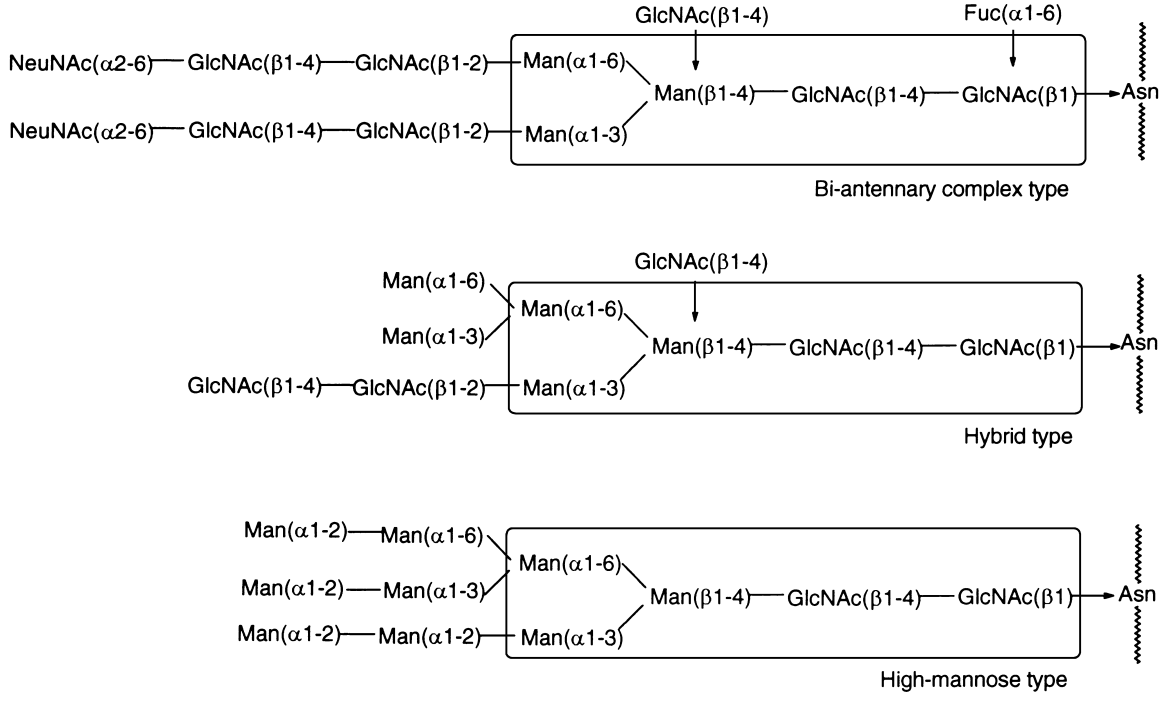
glycoproteins can be viewed as *natural libraries*, or *glycoforms*,[20] whose elements are very similar but show a high degree of diversity in their detailed structures.

Single eukaryotic cell lines often produce many glycoforms of any given protein sequence. For instance, in the case of pharmaceutically important glycoprotein hormone erythropoietin (EPO), a clinically useful red blood cell stimulant for anaemia, when expressed in Chinese hamster ovary cells (CHO) is found to be glycosylated by more than 13 known types of oligosaccharide chains. The efficacy of EPO is heavily dependent on the type and extent of glycosylation.[21] The in vivo biological activity of the glycoform with tetra-antennary *N*-glycans was significantly greater than that of biantennary structures.

Obviously, elucidation of the biological relevance of particular glycoprotein oligosaccharide chains would benefit from isolation of homogeneous entitites. However, glycoprotein heterogeneity renders this process particularly labour intensive. Some relief may be in sight if particular cell lines[22] can be selected to produce more homogeneous glycoproteins for structure–activity relationship studies.

1.3. *Types of* N-*glycans*

As already said, *N*-glycoprotein glycans share the common structure $Man_3GlcNAc_2$-Asn. There is, however, an enormous variety and complexity in the oligosaccharide chains attached to this core. Depending on these additional moieties the structures of *N*-linked oligosaccharides of complex *N*-glycans of glycoproteins fall into four categories[23] (see Scheme 7):



Scheme 7.

(i) High mannose *N*-glycans: which contain only D-mannose (Man) residues attached to the core.
(ii) Complex *N*-glycans: which have 'antennae' or branches attached to the core. These antennae are initiated by the action of four mammalian GlcNAc transferases and may be further elongated by the

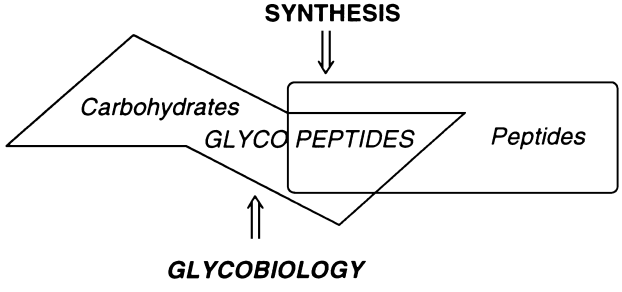
addition of D-Gal, *N*-GlcNAc, L-Fuc, sialic acid and sulfate. The number of antennae in mammals ranges from two (biantennary) to four (tetra-antennary).

(iii) Hybrid *N*-glycans: which have only Man residues on the Man(α1,6) arm of the core and one or two antennae on the Man(α1,3) arm.

(iv) Poly-*N*-acetyllactosamine *N*-glycans: which contain repeating units of Gal(β1,4)-GlcNAc(β1,3) attached to the core.

1.4. Complex N-*glycosylamino acids and* N-*glycopeptides: strategies*

Owing to the complexity of these biomolecules and their microheterogeneity, the understanding of the roles conferred by the carbohydrate moieties upon glycoproteins had to be based on the use of synthetic rather than natural models. Such a need for unequivocally synthesized molecular models of glycoproteins has been fuelling the marriage between both carbohydrate[24] and peptide chemistries with the aim of preparing glycopeptides (Scheme 8). After all this effort, glycopeptides can now be made available with variations in both the peptide and carbohydrate part in higher quantities and superior purities than for their parent glycoproteins, therefore becoming the model compounds of choice for biomedical and structural investigations where glycoproteins are involved. These homogeneous and structurally defined glycopeptides could, therefore, serve as models for the study of how the carbohydrate and polypeptide domains in each of these classes influence one another in terms of conformation and presentation to complementary ligands, receptors, or antibodies.



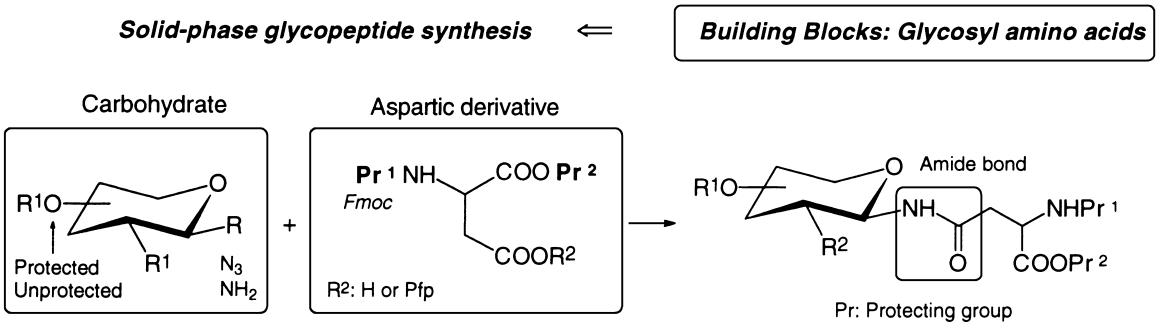
Scheme 8.

Considering the complexity of the problem, advances in the synthesis of glycopeptides[25] have been remarkable. Accomplishments from the laboratories of Paulsen, Kunz, Meldal, T. Ogawa, Schmidt, Unverzagt and Danishefsky, among others, have been of particular significance in fostering this progress.[26] In this regard, one of the most promising directions consists of the elegant interplay of chemically and enzymatically mediated couplings pioneered by Wong.

Currently, there are two basic approaches for synthesizing *N*-linked glycopeptides: (i) the convergent approach; and (ii) the building block stepwise approach.

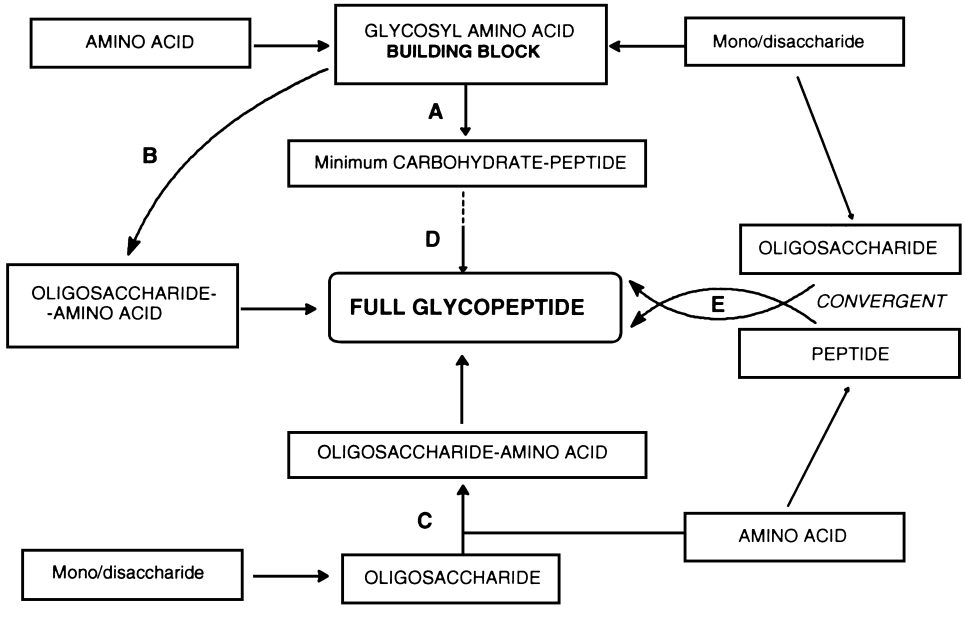
The stepwise approach normally proceeds through a glycosyl asparagine intermediate which usually serves as the building block for a solid-phase construction of a peptide sequence (Scheme 9). This approach requires a protection scheme for N^{α}-amino and the C^{α}-carboxyl functions of the Asn that sometimes reveals side reactions such as aspartimide formation. This protection strategy should take into account that at a certain point the carboxylic acid side-chain of Asn should be free or activated to allow *N*-glycosidic formation.

Versatility is one of the main advantages of this stepwise procedure which allows the synthesis of glycopeptides with variations in the peptide part and gives access to structures containing more than one carbohydrate residue as well.



Scheme 9.

Thus, a suitably modified aspartic amino acid derivative linked to a mono- or disaccharide, undergoes further extension on the carbohydrate moiety (strategy B in Scheme 10) or lengthening of the peptide part (strategy A) and even further elaboration of the saccharide part, notably by enzymatic means once the peptide is completed to give the final glycopeptide (strategy D). According to strategy C, another possibility is to construct the oligosaccharide which is then coupled to a suitably protected derivative of an aspartic derivative, followed by peptide extension of the glycopeptide intermediate.



Scheme 10. Strategies in *N*-glycopeptide synthesis

The convergent approach is represented in strategy E where the required carbohydrate chain and peptide are each built independently, and the amide linkage is created late in the synthesis. These different strategies (Scheme 10) will be illustrated in the following sections.

2. The *N*-glycosidic bond: an amide bond formation

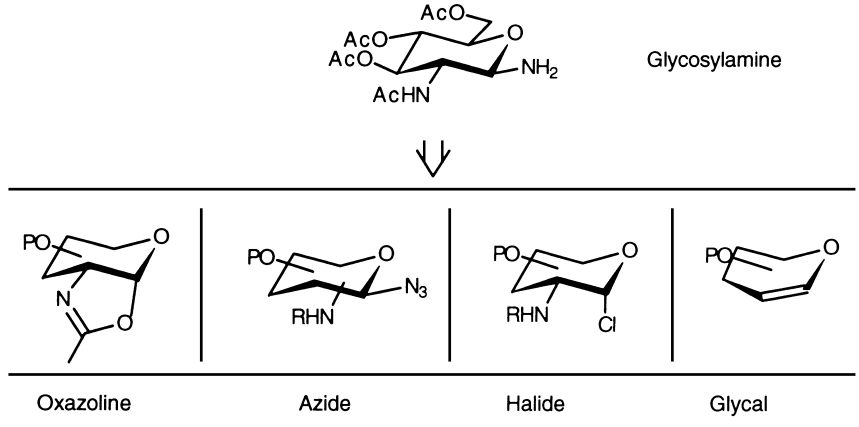
2.1. Glycosyl derivatives

Direct glycosylation of the amide side-chain chain of Asn has not been reported.[27] The routinely adopted approach for the formation of *N*-glycopeptides is via reaction of a glycosylamine or glycosyl precursor and a suitably protected and activated Asp derivative. While this leads to an *N*-glycosidic linkage, the chemistry is an amide bond formation rather than a glycosylation. A requisite for this approach is access to protected or unprotected glycosyl precursors. *N*-glycosides can be prepared from:

– Anomeric glycosylamines (Section 2.1.1)
– Anomeric isothiocyanates (Section 2.1.2)
– Anomeric pentenyl glycosides (Section 2.1.3)
– Anomeric glycosyl azides (Section 2.1.4).

2.1.1. Via glycosylamines

2.1.1.1. Protected glycosylamines. Glycosylamines are accessible from different precursors (Scheme 11). They have been prepared by displacement of glycosyl bromides with ammonia.[28] However, the glycosyl halide is more often converted to the glycosyl azide,[29] either by reaction with silver azide[30] or by using sodium azide under phase transfer catalysis,[31] and then the glycosyl azide is catalytically reduced to yield the corresponding glycosylamine. The azido function itself can be a part of the protecting group strategy. The azide can be introduced via an intermediate oxazoline that is reacted with trimethylsilyl azide in the presence of tin tetrachloride.[32]

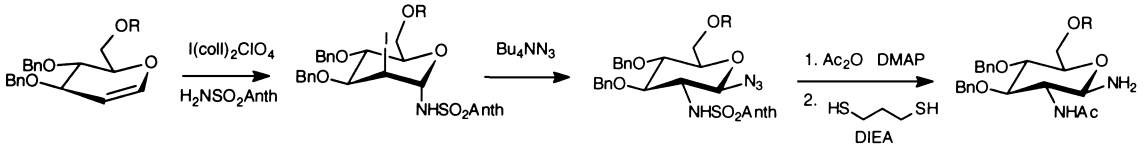


Scheme 11. Common precursors of protected glycosylamines

As already stated, glycosylamines are prepared either by hydrogenolysis of a glycosyl azide catalyzed by Pd/C,[33] Lindlar catalyst,[34] PtO_2, Ni Raney[35] or by using propanedithiol in the presence of triethylamine and methanol.[36] However, under reduction conditions the anomerization of glycosylamine can occur. In order to avoid glycosylamine anomerization, the reduction of glycosyl azides in the presence of symmetric or mixed anhydrides of the β-carboxylic acid of aspartic acid derivatives, has been investigated.

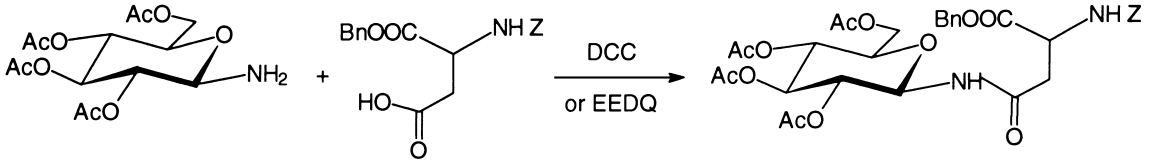
Other methods for glycosylamine preparation include the one developed and extensively used by Danishefsky[37] (Scheme 12). The reaction of a glycal with a combination of an arylsulfonamide and iodonium di-*sym*-collidine perchlorate (IDCP) stereoselectively affords 2-β-iodo-1-α-sulfonamide intermediates which later undergo azidolytic rearrangement. Acetylation of the sulfonamido NH group and

subsequent reduction with propane-1,3-dithiol in the presence of diisopropylethylamine (DIEA) leads to the formation of the corresponding protected glycosylamine.



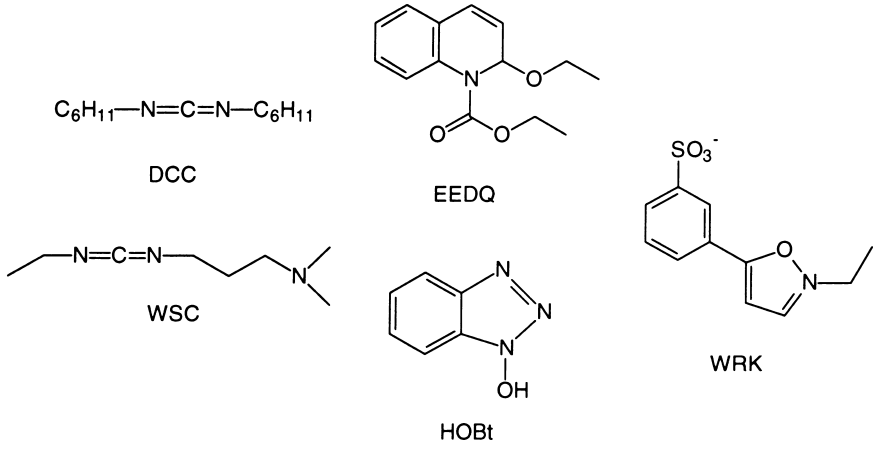
Scheme 12.

The first synthesis of an *N*-glycosylamino acid was described in 1961 by Marks and Neuberger[38] (Scheme 13). They were trying to obtain direct proof for the proposed carbohydrate–protein linkage between a carbohydrate and a protein in egg albumin.[39] Since then, a number of glycoproteins have been found to contain this or other types of carbohydrate linkages.



Scheme 13.

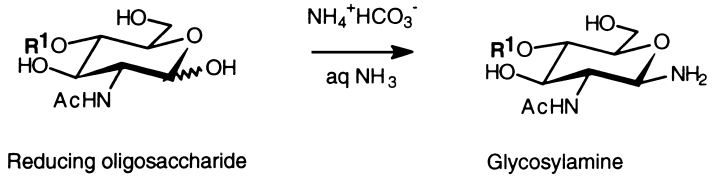
Reagents applicable for peptide bond formation are also useful for *N*-glycosylation (Scheme 14). Usually, the asparagine–glucosamine linkage is formed with the aid of acylating reagents such as dicyclohexyl carbodiimide (DCC)[40] in the presence of additives such as 1-hydroxybenzotriazole or dihydroquinoline derivatives such as the 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ).[41] The related 2-isobutoxy-1-isobutoxycarbonyl-1,2-dihydroquinoline (IIDQ)[42] has been used for the coupling of 1-glycosylamines to protected aspartyl residues in the process of preparing *N*-glycoasparagine building blocks.[43] Subsequently, the amino acid is selectively deprotected and peptide chain extension is carried out either in solution or on solid support.



Scheme 14. Coupling reagents used for amide bond formation

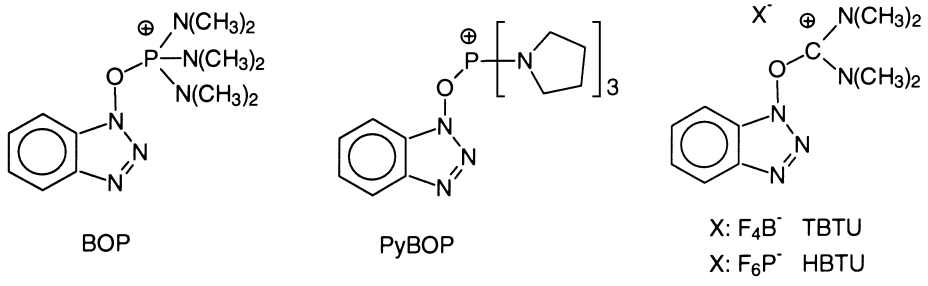
2.1.1.2. Unprotected glycosylamines. Alternatively, unprotected glycosylamines[44] are obtained after extensive treatment of reducing oligosaccharides with saturated ammonium bicarbonate solution[45] (Scheme 15). However, this process is not useful for protected oligosaccharides. This method has been

improved recently by the utilization of equimolar amounts of ammonium bicarbonate in the presence of ammonia.[46]



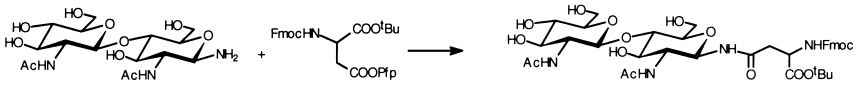
Scheme 15.

Unprotected glycosylamines can be coupled to carboxyl groups of protected amino acids by means of a number of uronium and phosphonium salts in the presence of excess base, commonly DIPEA. For glycopeptide synthesis the most commonly used reagents include benzotriazol-1-yloxytris(dimethylamino) phosphonium hexafluorophosphate (BOP or Castro's reagent), *O*-(1*H*-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) or the essentially similar hexafluorophosphate (HBTU) (Scheme 16). These reagents produce active acylating species, in situ, presumably by generating the corresponding hydroxybenzotriazolyl esters.



Scheme 16. Coupling reagents used for amide bond formation

Crude glycosylamines can be coupled to the pentafluorophenyl (Pfp) activated ester of FmocAspO^{t}Bu in *N*,*N*-dimethylformamide/water mixtures to give *N*-glycosyl compounds containing an unprotected sugar unit (Scheme 17). Neither pentafluorophenyl esters nor the symmetrical anhydrides acylate the unprotected hydroxyl groups of the sugar moieties during peptide synthesis.[47]



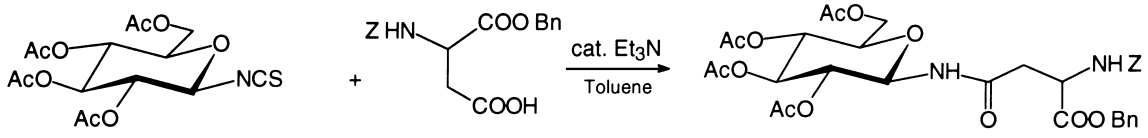
Scheme 17.

After removal of the ^{t}Bu ester with TFA, the unprotected disaccharide (in this example chitobiosyl) building block was used via the pentafluorophenyl ester for the solid phase synthesis of a T cell glycopeptide amide.

2.1.2. Via glycosyl isothiocyanates

An alternative method for *N*-glycosylation employs glycosyl isothiocyanates, obtained from the corresponding glycosyl bromide, thus avoiding the azide step.[48] These anomeric isothiocyanates have been coupled with aspartic acid to give an amide linkage[49] (Scheme 18). An elegant application of this methodology for the synthesis of a β-mannosyl–chitobiosyl–asparagine conjugate was reported by Kunz et al.[50] The isothiocyanide was obtained by reaction of an oxazoline with potassium rhodanide (potassium thiocyanate)/HBF_4 in the presence of 18-crown-6 as catalyst. Reaction of the isothiocyanate with α-*t*-

butyl *N*-allyloxycarbonyl L-asparaginate under strictly anhydrous conditions led to the formation of the β-mannosyl–chitobiosyl–asparagine in 75% yield.

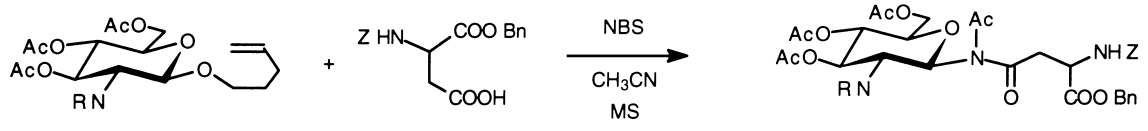


Scheme 18.

The mechanism of the reaction involves nucleophilic attack on the isothiocyanate by the carboxylic acid leading to the formation of a symmetrical anhydride and a glycosyl thiocarbamic acid. Loss of S=C=O gives the glycosylamine, which reacts with the anhydride to form the amide.

2.1.3. Via pentenyl glycosides

The methods described above require a modification of the anomeric centre which is followed by the introduction of a peptidyl moiety. Fraser-Reid et al.[51] developed a new approach that allows for the direct coupling of a peptidyl residue with a glycosyl moiety (Scheme 19). They envisaged that an intermediate anomeric β-nitrilium ion, obtained by reaction of an anomeric oxonium ion and nitrile would, after hydrolysis with water, give an amide.

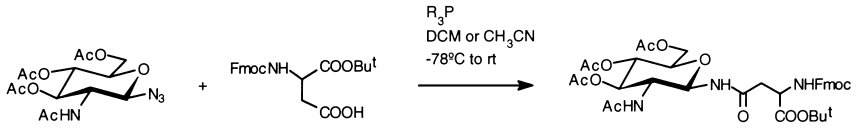


Scheme 19.

Treatment of either anomer of a pentenyl glycoside with *N*-bromosuccinimide (NBS) in acetonitrile is proposed to lead to the formation of an oxonium ion. Contrary to what would be predicted by the reverse anomeric effect, this intermediate is trapped by acetonitrile solvent to give an α-nitrilium ion. A carboxylic acid can then add to the nitrilium ion, giving rise to an imino anhydride, which rearranges to give an imide. The acetyl group can be removed efficiently by treatment with piperidine in DMF to give the *N*-glycosylamino acid. The methodology was employed for the preparation of a protected *N*-linked chitobiose–asparagine conjugate.

2.1.4. Via glycosyl azides

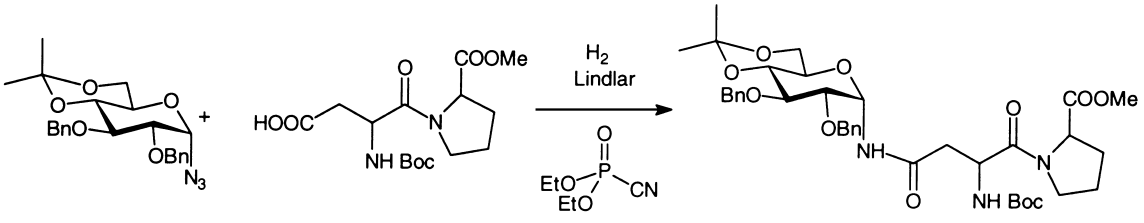
The reaction of a carboxylic acid and an azide in the presence of tertiary phosphine to give an amide linkage is well known.[52] Following this method, Maunier et al.[53] reported the synthesis of a glycosylamide from a glycosyl azide and acyl chloride in the presence of trialkylphosphine. Inazu et al.[54] have developed an efficient method for preparation of N^{α}-protected N^{β}-glycosylated-L-asparagine derivatives by reacting a glycosyl azide and N^{α}-Fmoc L-aspartic acid α-monoester in the presence of triethylphosphine in dichloromethane at room temperature. By this method, a glycosyl asparagine derivative was obtained in satisfactory yield only when triethylphosphine, *N*-acetylglucosaminyl azide and dichloromethane were employed. They recently described an improved procedure for glycosylasparagine and glycosylglutamine derivatives by optimization[55] of the latter method using different trialkylphosphines at low temperature (Scheme 20).



Scheme 20.

2.2. *Special case:* α-N-*glycopeptide synthesis*

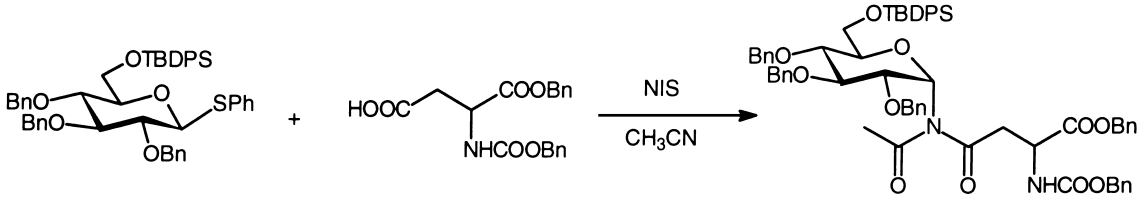
As in the case of *O*-glycopeptides the selective preparation of α-linked *N*-glycopeptides is always a difficult task. One example was reported by Takeda et al.[56] on the synthesis of an α-*N*-linked dipeptide *N*-glucopyranoside derivative (Scheme 21). A benzyl-protected glucosyl azide was employed as the starting material to avoid the later use of basic conditions. Hydrogenation of the azide with Lindlar's catalyst gave an α/β mixture of glucopyranosylamines which were coupled to a dipeptide in the presence of *O*,*O*-diethylcyanophosphonate resulting in the formation of an anomeric mixture of glycopeptides. These two anomers could not be separated by column chromatography. However, resolution was possible on the starting α/β mixture of glycosylamines, which were later individually coupled to render the corresponding glycosyl dipeptides.



Scheme 21.

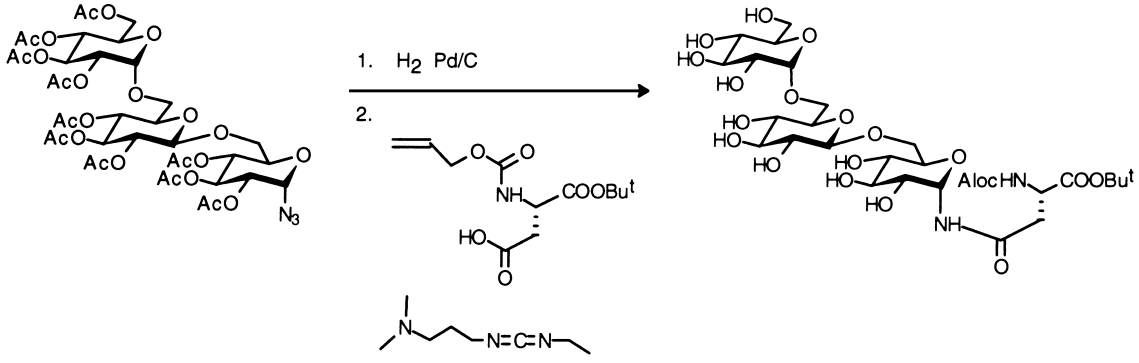
This strategy was employed when preparing an *N*-triglycosyl dipeptide that represented a partial structure of nephritogenoside.

An efficient and stereocontrolled synthesis of a core trisaccharide of nephritogenic glycopeptide was reported by Sasaki et al.[57] (Scheme 22). Conversion of the thioglycoside into the α-imide proceeded via the α-acetonitrilium ion under the influence of *N*-iodosuccinimide (NIS) in acetonitrile at room temperature in a higher yield (85%) than that described for the corresponding pent-4-enyl glycoside.[58]



Scheme 22.

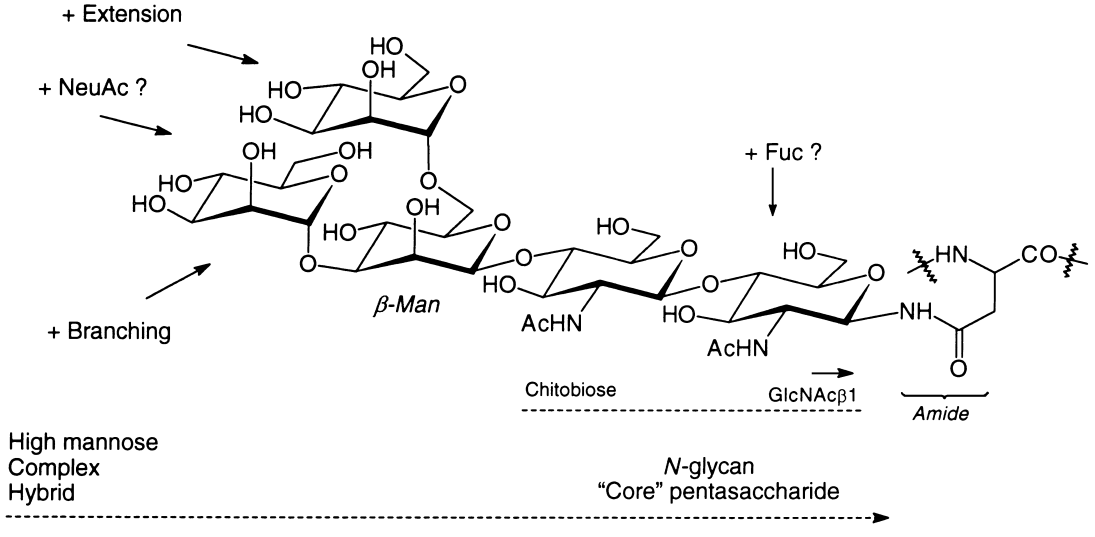
A total synthesis of this bioactive glycopeptide was later reported by Shiba et al.[59] The triglucosyl azide was prepared by coupling hepta-*O*-acetyl-α-D-isomaltose fluoride with a protected glucopyranosyl azide. The azide was reduced by catalytic hydrogenation using Pd/C and the resulting amine coupled to the Asp derivative to give an anomeric mixture of glycosylamino acids that could be separated by column chromatography (Scheme 23).



Scheme 23.

3. Problems in the synthesis of complex *N*-glycopeptides. State of the art

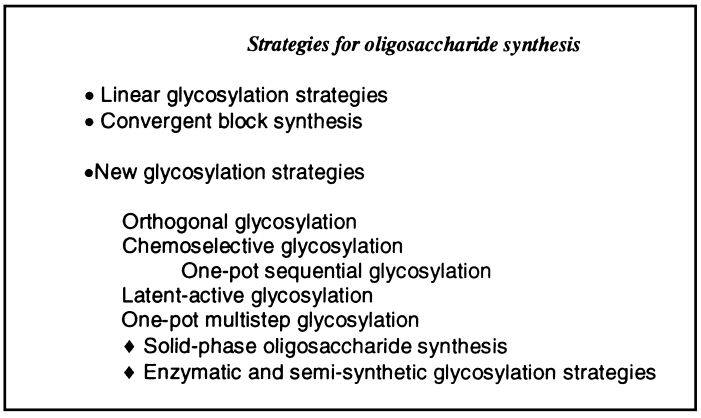
In spite of remarkable progress over the last few decades, the routine synthesis of complex *N*-glycopeptides is a difficult task still presenting many challenges. The main problems are those inherent not only in the synthesis of the complex *N*-glycan precursor, but also those encountered during the coupling between the carbohydrate and the amino acid/peptide moieties and when attempting modifications at the *N*-glycan of the already assembled *N*-glycopeptide (Scheme 24).



Scheme 24.

Oligosaccharide synthesis is well behind other disciplines dealing with the synthesis of other biopolymers such as peptides and oligonucleotides in the sense that there are no general and reliable methods for the formation of glycosidic linkages. Regio- and stereoselectivity problems always have to be faced when coupling any pair of glycosyl donors and acceptors. To date, the only tools available are the choice of protecting groups and reagents and reaction conditions. This means that lengthy optimization operations are needed. However, this does not deter the progress in oligosaccharide synthesis as seen by the increasing number of new glycosyl donors and reaction conditions being reported in the literature[60] along with new strategies for the synthesis of oligosaccharides[61] (Scheme 25).

Only one β-1,2-*cis* mannoside linkage is found in mammalian oligosaccharides but this is located at the *N*-glycan pentasaccharide core of all *N*-glycoproteins. This ubiquitous bond has, however, become



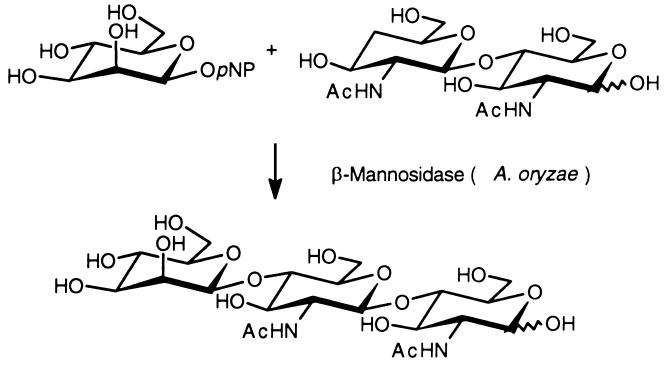
Scheme 25.

one of the most difficult issues in oligosaccharide chemistry.[62] The β-mannoside can be built initially in a precursor building block or obtained in the whole complex *N*-glycan.[63] So far, preparation methods developed for the β-D-mannoside linkages fall into one of the categories outlined in Table 1.

Table 1

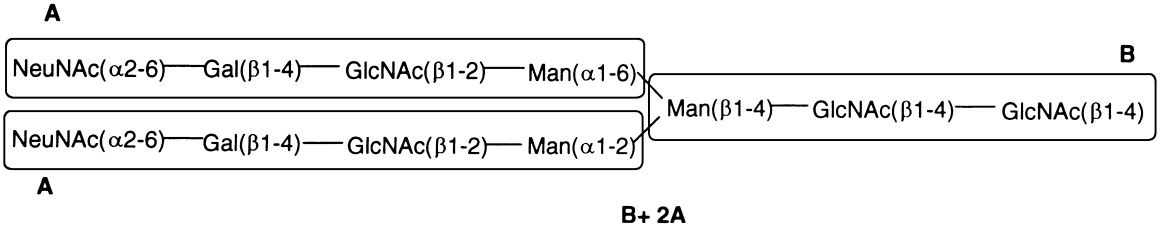
Methods for β-mannoside formation	*Ref.*
Chemical methods	
The insoluble promoter strategy	64
Conversion of β-D-glucoside to β-D-mannoside (oxidation and reduction steps)	65
From 2-oxo-glycosyl halides (insoluble promoter and oxidation-reduction)	66
Epimerization of β-glucosides into β-D-mannoside	67, 68
Intramolecular aglycon delivery	69
Enzymatic methods: β-mannosidase	70

A particularly interesting recent enzymatic method for the synthesis of the core trisaccharide of all *N*-linked glycoproteins uses β-mannosidase from *Aspergillus oryzae* to produce this β-mannoside linkage[71] (Scheme 26). Other groups have also investigated the application of β-mannosyltransferases[72] to the preparative synthesis of *N*-linked core oligosaccharides.



Scheme 26.

Among many other achievements, a milestone[73] in the synthesis of complex *N*-glycans was the synthesis of a sialylated biantennary undesaccharide by Ogawa in 1986 (Scheme 27), since the synthesis of the α-D-sialic acid linkage can compare in difficulty to the β-mannosyl found in the *N*-glycan core. Further methods for modification on the complex *N*-glycan, by extending either the terminal *N*-glycan with sialylation or polylactosaminylation, or by fucosylation of the inner core, are still needed. Sulfation and phosphorylation should also be considered key reactions in the synthesis of certain complex *N*-glycans of relevant biological glycoconjugates.



Scheme 27.

At this point, and after introducing the main problems in *N*-glycopeptides synthesis, we now proceed to discuss the progress in this field.

3.1. Chemical methods

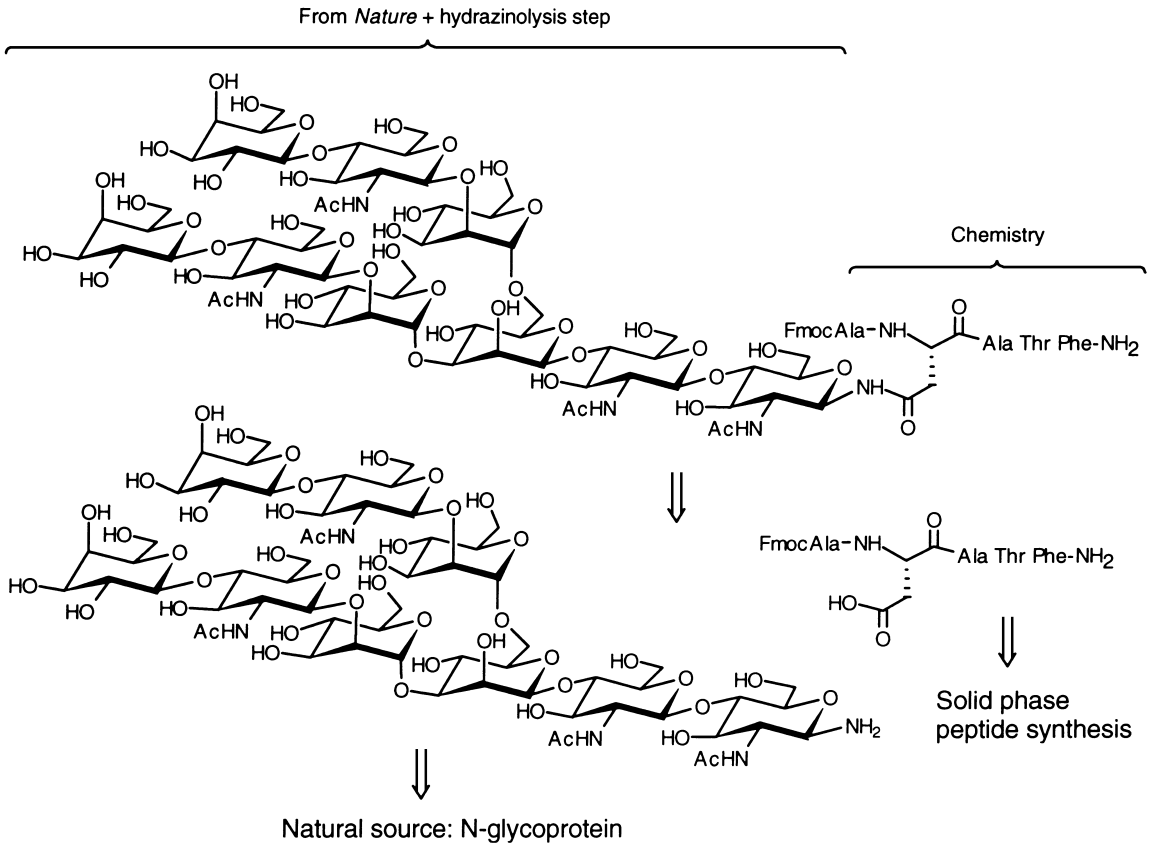
3.1.1. Obtaining complex N*-glycans from nature*

To avoid facing the difficulties of oligosaccharide synthesis, a very wise strategy is to obtain complex *N*-glycans from natural sources such as glycoproteins. A first step for such a goal is releasing glycans from glycoconjugates.

The release of *N*-linked oligosaccharides in small amounts was first accomplished by enzymatic[74] and chemical methods. Enzymatic methods have a high degree of specificity, although the enzyme must be validated for every new application. An important endoglycosidase which has been very useful in the analysis of *N*-linked glycans was identified in *Flavobacterium meningosepticum*. PNGase F is commonly used for deglycosylation but is specific for removing only *N*-glycans. However, the success of enzymatic methods for preparative purposes is unfortunately highly dependent on the substrate specificity and on the availability of the enzyme.

Chemical methods have the advantage of broad specificity towards the substrate and include a large variety of chemical reagents, e.g. hydrazine,[75] β-elimination by mild alkaline treatment or under strongly alkaline conditions,[76] and lithium aluminium borohydride.[77] The reducing agent converts the reducing sugar to a sugar alditol residue. On the other hand, hydrazine as the cleaving reagent offers the possibility of obtaining the released oligosaccharides in their unreduced hemiacetal form and has the added benefits of being non-selective (with respect to the glycan) and amenable to automation. Hydrazinolysis was first used to release only *N*-glycans[78] but reaction conditions have been described for the optimal release of *O*- and *N*-glycans from proteins, and the method can be optimized to permit the selective removal of only *O*-linked residues. Hydrazinolysis of glycoproteins on an analytical scale has become a popular method for the isolation of *N*-linked oligosaccharides.[79]

The concept of obtaining complex *N*-glycans for glycopeptide synthesis was first described[80] in the European Peptide Symposium in 1992. The report described both the synthesis of a long peptide based on chitobiose and a short peptide linked to a complex oligosaccharide using an *N*-glycan obtained from a natural source (Scheme 28).



Scheme 28.

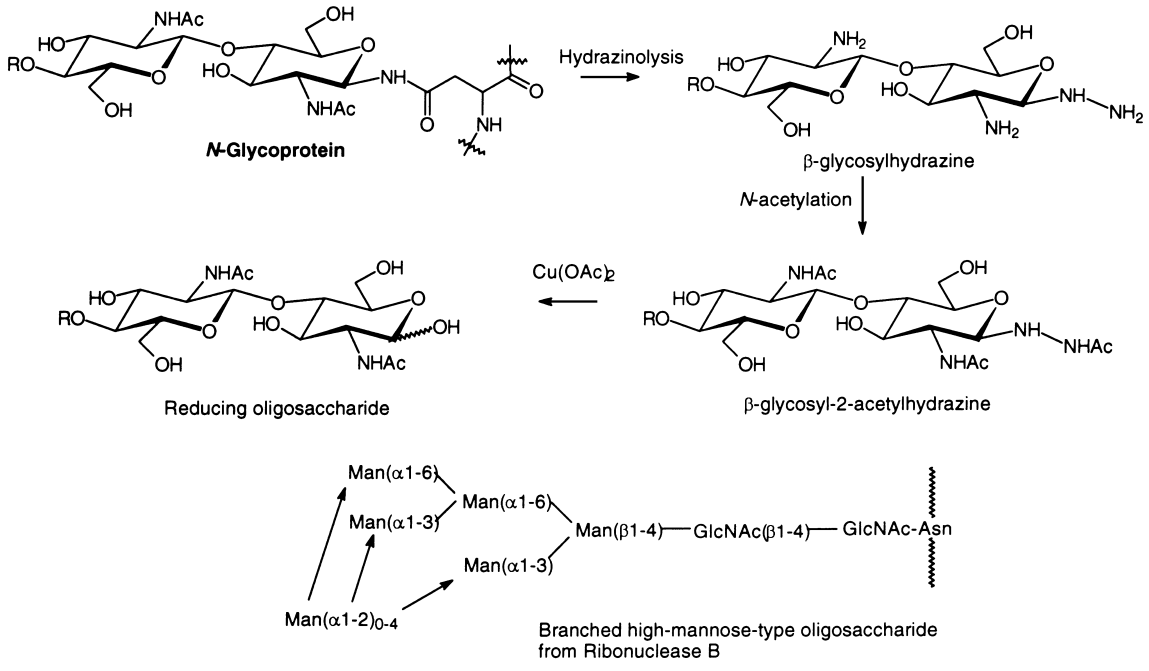
The complex *N*-glycan was isolated from human fibrinogen after hydrazinolysis. Formation of the unprotected complex glycosylamine by the ammonium carbonate method and coupling to an Asp-containing pentapeptide yielded the first complex *N*-glycopeptide from a glycan obtained from natural sources.

In 1993, Lansbury et al.[81] prepared, by a solution-phase convergent approach, a complex glycopeptide derivative of a pentapeptide (Ac-Tyr-Asp-Leu-Thr-Ser-NH_2) with a glycosylamine derivative of the $(Man)_5(GlcNAc)_2$ heptasaccharide which was isolated from the urine of sheep with swainsonine-induced α-mannosidosis, a disease in which the catabolism of mannose-containing glycoproteins is impaired, causing the build-up of mannose-containing oligosaccharides.[82]

In 1997, Meldal et al.[83] described for the first time a method for large-scale hydrazinolysis to release *N*-linked oligosaccharides from *N*-glycoproteins. The aim was to recover these *N*-linked oligosaccharides from natural sources, e.g. from fetuin and ribonuclease B, in high overall yields and purity in their non-reduced form by employing mild hydrazinolysis conditions. Both fetuin and ribonuclease B are commercially available, well-characterized, affordable and offer the complex-type (fetuin) or the high-mannose (ribonuclease B) oligosaccharides.

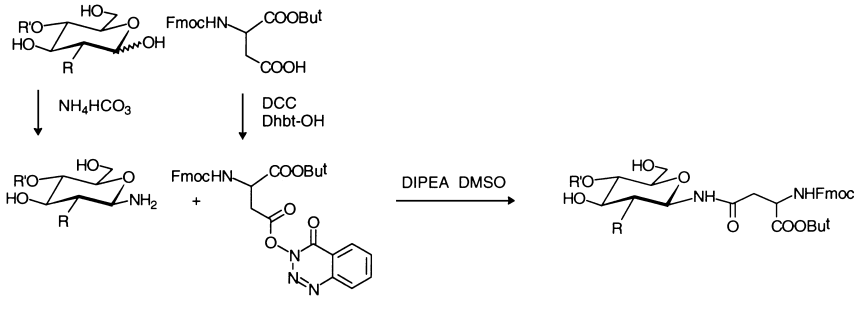
Mild hydrazinolysis of fetuin[84] and ribonuclease B[85] was performed under controlled and optimized conditions (Scheme 29). Thus, after exhaustive dialysis and lyophilization of proteins, heating with anhydrous hydrazine in a sealed tube afforded the corresponding β-glycosyl hydrazides. After *N*-acetylation with acetic anhydride, the crude material was subjected to cellulose column chromatography to separate the *N*-linked oligosaccharides. To cleave the formed hydrazides the crude material was first

treated with copper(II) acetate and then 25 mM sulfuric acid at 80°C to release the neutral desialylated oligosaccharides.



Scheme 29.

Treatment of the reducing sugars with a saturated solution of ammonium hydrogen carbonate in either water or dimethylsulfoxide gave in almost quantitative yields the corresponding glycosylamines. Owing to the unstable nature of these amines, the lyophilized crude products were used directly for coupling to the side-chain-activated aspartic acid derivative [FmocAsp(ODhbt)O-Bu^t] to afford the *N*-glycosylated asparagine derivatives[86] (Scheme 30). Subsequent acetylation of the carbohydrate hydroxyl groups and cleavage of the *tert*-butyl ester by trifluoroacetic acid yielded the *N*-linked, ready to use, building blocks.



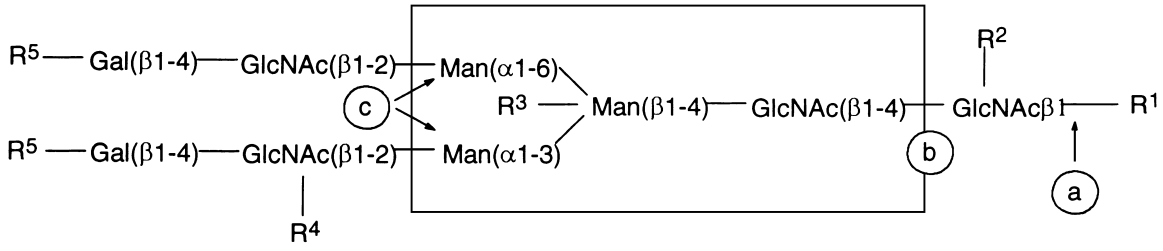
Scheme 30.

Meldal and Paulsen have used these *N*-glycans to synthesize *N*-linked glycosyl asparagine building blocks for multiple-column peptide synthesis[87] (MCPS) of *N*-linked glycopeptides to study putative immunogenic epitopes binding to the major histocompatibility complex (MHC) Class II molecule and T-cell response.

3.1.2. Trichloroacetimidates as powerful glycosyl donors

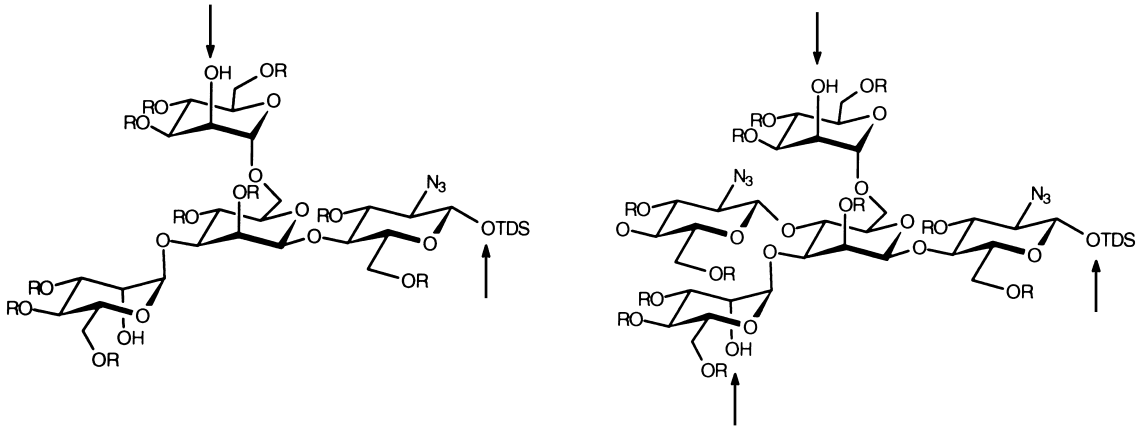
The group of Schmidt has recently described a versatile strategy[88] for the construction of eventually all complex *N*-glycans including bisected oligosaccharides required for *N*-glycopeptide synthesis. It is based on a flexible protecting group pattern and on the use of *O*-glycosyl trichloroacetimidates as glycosyl donors which have been developed over the years for the efficient synthesis of *N*-glycans.[89]

In particular, they describe the synthesis of a biantennary disaccharide (R^1=R^2=R^3=R^4=R^5=H) and the corresponding bisecting decasaccharide where R^3 is a GlcNAc(β1–4) residue. This is the first example of a bisecting type *N*-glycan synthesis (Scheme 31).



Scheme 31.

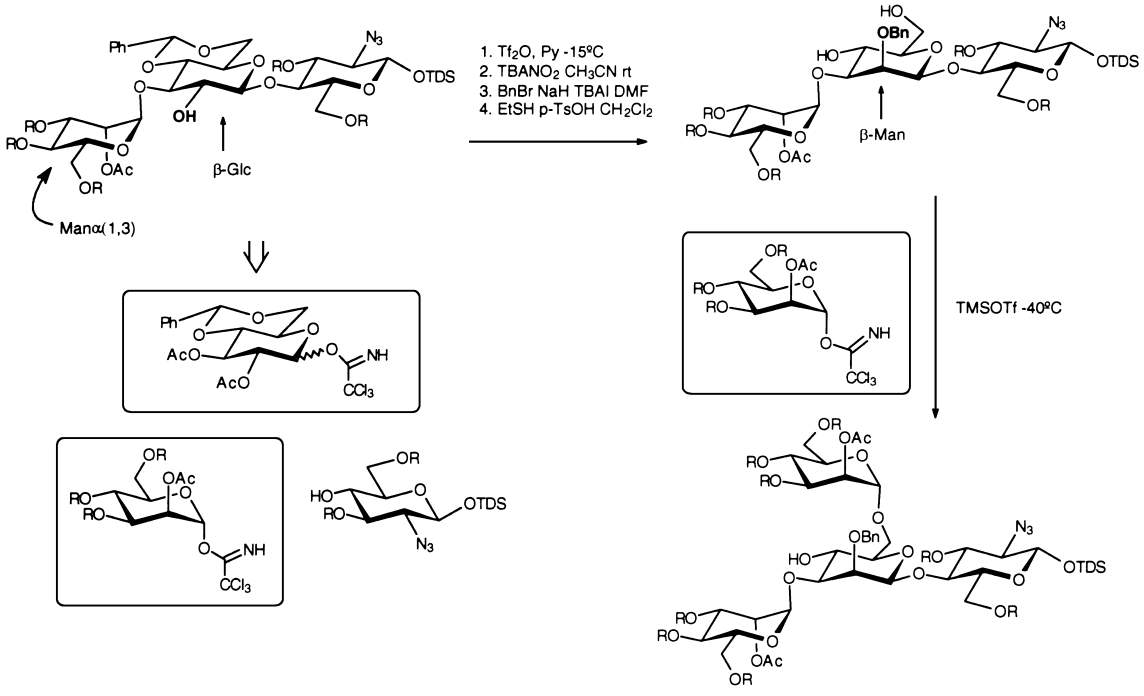
This strategy leads to tetra- and pentasaccharides as basic structures that can be further elaborated (Scheme 32). Thus, by coupling other moieties at R^4 and R^5 and different antennae at the 2-hydroxy groups of the two α-linked mannose residues (disconnection **c** in Scheme 31). Also, either an acetylglucosamine (R^2=H) or a fucosyl-α(1,6)-*N*-acetylglucosamine [R^2=Fuc α(1,6)] residue can be attached in a β(1,4) linkage at the reducing end (disconnection **b** in Scheme 31). When R^1 is Asn these compounds can be directly employed for *N*-glycopeptide synthesis (disconnection **a** in Scheme 31).



Scheme 32.

For the β-mannopyranoside linkage found in the central core, transformation of a β-linked glucopyranosyl to a β-mannopyranosyl residue was envisaged (Scheme 33). However, this epimerization was planned *after* the attachment of an α(1,3)-linked mannosyl residue to a 4,6-benzylidene protected glucose. The benzylidene group manipulation provides complete flexibility to establish further regioselective linkages to the 2-, 4- and/or 6-positions of the mannosyl residue. Epimerization was effected by treatment with Tf_2O in pyridine at −15°C, addition of *tetra*-butylammonium nitrite ($TBANO_2$) and then hydrolysis to yield the 2-*O*-unprotected β-mannoside.

In addition, in an example of the synthesis of bisected structures, the tetrasaccharide was first glycosylated with a known azidoglucose moiety (Scheme 34). After removal of the *O*-acetyl groups, the



Scheme 33.

pentasaccharide was glycosylated with a lactosamine donor in the presence of TMSOTf as the catalyst to afford the nonasaccharide. Other antennae could be prepared at this point by glycosylating with different glycosyl donors. The anomeric OTDS was deprotected with TBAF in THF and then the nonasaccharyl imidate was prepared as the donor with the azide glucosamine acceptor to yield exclusively the β-linkage in the decasaccharide.

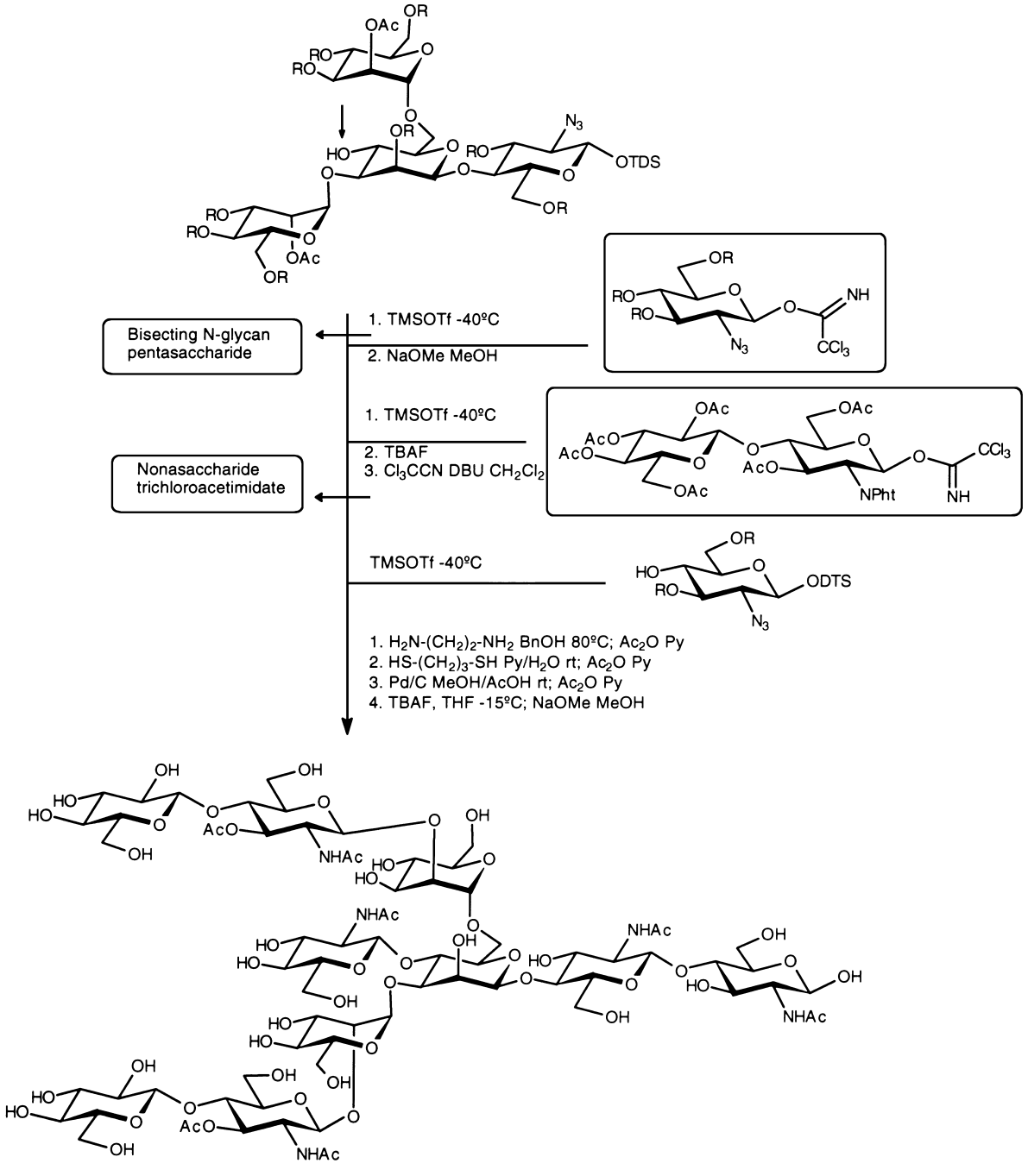
Finally, to obtain the target molecule, the derivative was selectively dephthaloylated (with simultaneous *O*-deacetylation). *N*- and *O*-Acetylation, reduction of the azido groups, and hydrogenolytic debenzylation was followed by complete acetylation. Only the TDS was retained. TBAF treatment and de-*O*-acetylation furnished the target bisected disaccharide.

3.1.3. The convergent approach: in solution and in solid phase

3.1.3.1. Using unprotected glycosylamines. An impressive demonstration of the feasibility of accomplishing a convergent synthesis of the carbohydrate and peptide domains was first provided by Cohen-Anisfeld and Lansbury,[90] who described the reaction conditions for the direct coupling of naturally derived anomeric oligosaccharide unprotected β-glycosylamines and minimally protected aspartic-containing synthetic peptides.

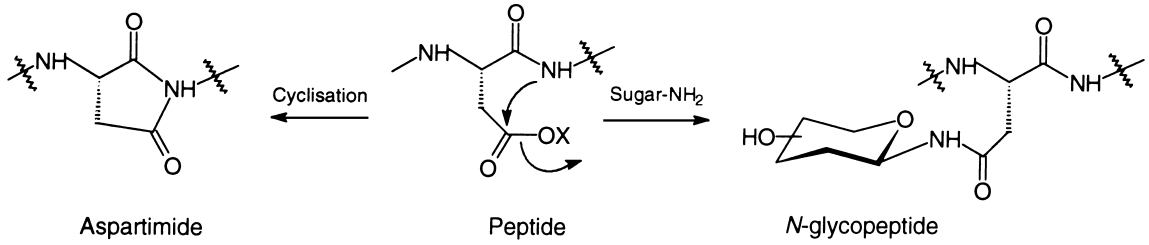
In order to make the convergent approach a viable alternative to the stepwise strategy, three problems had to be solved. Firstly, since glycosylation of a peptide is expected to be slower and more difficult than the glycosylation of an amino acid, a potent coupling reaction is needed in order to carry out high-yielding glycosylation of a peptide.[91] Secondly, when a peptidyl Asp side chain is activated for glycosylation, there is the potential for a competing, easy intramolecular reaction, namely, cyclization to the succinimide,[92] which must be minimized. Thirdly, a protective group scheme must be developed allowing selective deprotection of one Asp residue, while other protecting groups remain intact.

In practice, the coupling of protected glycosylamines to α-esters of aspartic acid proceeds in good



Scheme 34.

yields, while coupling to Asp-containing peptides is interfered with by competing intramolecular succinimide formation (Scheme 35). In order to minimize succinimide formation and achieve the best coupling yields several factors had to be carefully controlled including the activation of the peptide aspartate carboxyl group, the minimization of base in the reaction medium, and the choice of protection for the carbohydrate hydroxyls.

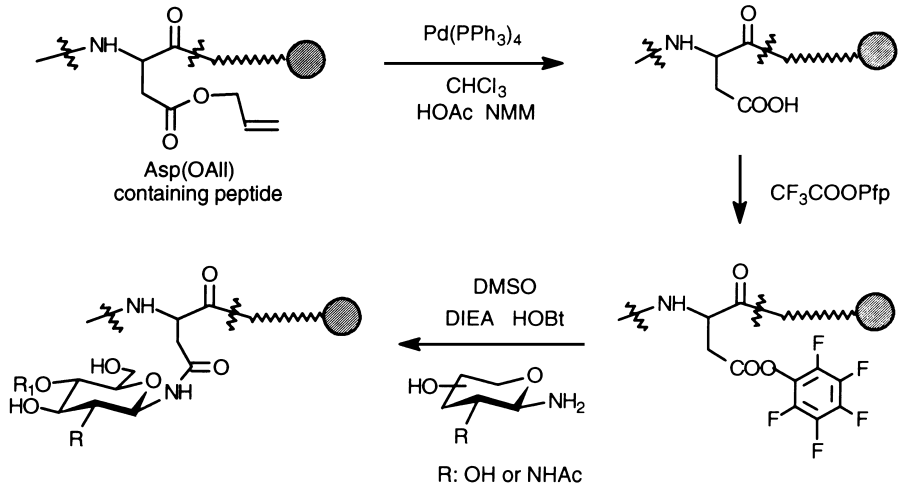


Scheme 35.

Although Lansbury's team also reported a preliminary result on the glycosylation of resin-bound aspartic acid, the extension of this concept to solid-phase protocols has been described by two other groups. In both cases, the peptide is prepared as peptidyl resin following stepwise solid-phase synthesis protocols and the unprotected glycosylamine is later coupled to the peptidyl resin to yield a glycopeptidyl resin.

Albericio's group[93] developed a method based on a three-way orthogonal solid-phase strategy and direct coupling of a single monosaccharide (unprotected 2-acetamido-1-amino-1,2-dideoxy-β-D-glycopyranose) to the carboxyl function of aspartyl-containing peptidyl resins. In this strategy the side-chain aspartyl containing peptidyl resins were initially protected as allyl esters which were selectively removed by Pd(0) catalysis prior to *N*-glycosidic bond formation.

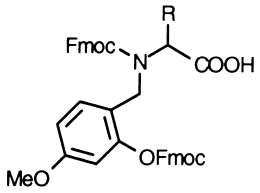
Alternatively, the key step of the procedure developed by Gallop et al.,[94] is the coupling of an unprotected glycosylamine to a pentafluorophenyl carboxylate (OPfp) on the Asp or Glu side chain of a resin-bound peptide[95] (Scheme 36). The activated aspartic acid derivative OPfp ester can be prepared on the resin after selective removal of allyl protecting groups by Pd(0) catalysis, by using a pentafluorophenyl acetate reagent.[96] This procedure allows for the introduction of one or more saccharide moieties at a defined position of a peptide, by efficient amidation of the corresponding glycosylamines without concomitant esterification of the sugar free hydroxyl groups. This method has been also applied to glutamic acid containing peptidyl resins. It was applied to the generation of glycopeptide libraries[97] by combinatorial methods.



Scheme 36.

One main problem with these procedures is finding that the orthogonal allyl side-chain-protecting group for aspartyl residues is prone to aspartimide formation. One approach[98] for preventing unwanted

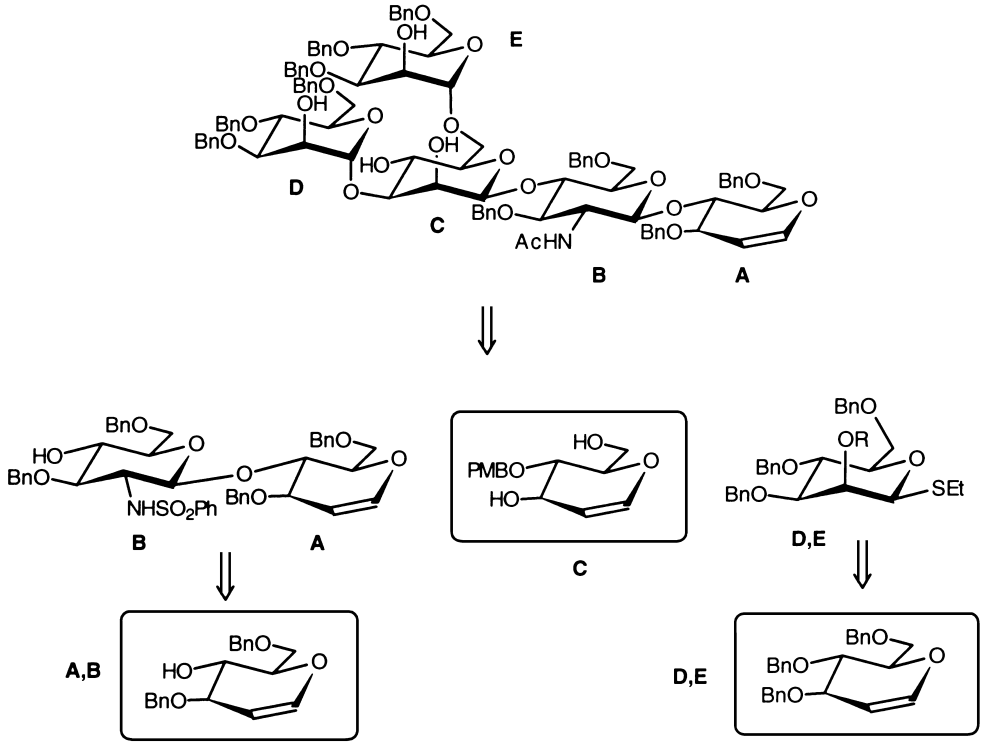
aspartimide formation is to replace the amide nitrogen proton of the aspartyl bound with an acid labile backbone protecting group, 2-hydroxy-4-methoxybenzyl (Hmb, Scheme 37).



Scheme 37.

3.1.3.2. The glycal approach. An important achievement in the preparation of a naturally occurring high-mannose 'core' carbohydrate domain terminating in an anomeric amine by a highly convergent synthesis[99] and the subsequent coupling to a synthetic peptide carrying the Asn-Xxx-Ser/Thr sequon was reported by Danishefsky et al.[100] in 1997.

Following the retrosynthetic scheme (Scheme 38), the pentasaccharide corresponding to the 'core' region of all asparagine-linked glycoproteins, was assembled by means of glycal-derived thioethyl donors[101] and glycal acceptors[102] as key building blocks. In turn, the high mannose pentasaccharide anomeric amine was condensed with various aspartic acid containing tri- and pentapeptides to yield a series of *N*-linked glycopeptides.



Scheme 38.

The key issues in Danishefsky's strategy, as exemplified by this synthesis, are:

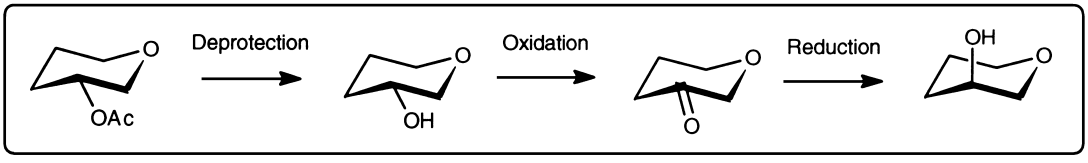
(i) the availability of protected glycals;

(ii) the construction of the β-mannoside linkage;

(iii) the acetamidoamination of the terminal glycal to obtain the corresponding protected complex glycosylamine which is accomplished by iodoanthracenesulfonamidation followed by azidolytic rearrangement; and

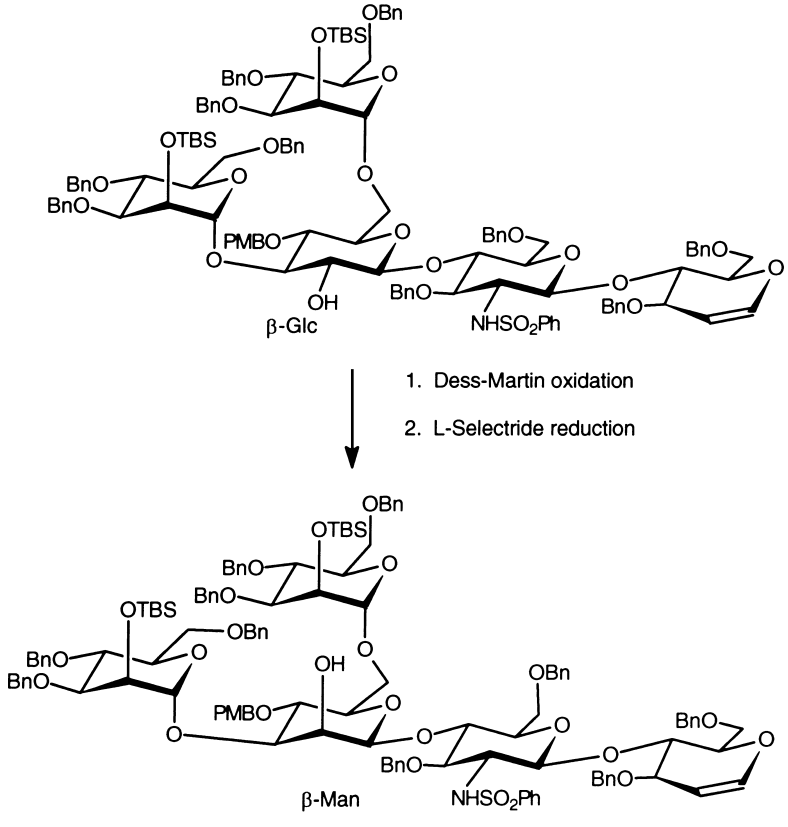
(iv) the efficient reduction of the β-anomeric azide in a highly complex high mannose setting that competes with anomerization of the resultant amine and *trans*-acylation through the neighbouring acetamido linkage.

It is also interesting to note that owing to the wide applicability of the glycal assembly, several protected glycals have become commercially available from Aldrich. Moreover, as far as the β-mannoside linkage is concerned, it was fashioned by inversion (through an oxidation–reduction sequence) of a β-glycoside[103] (Scheme 39). To date, this is the most complex setting in which a β-mannoside has been synthesized by inversion of a β-glycoside.[104]



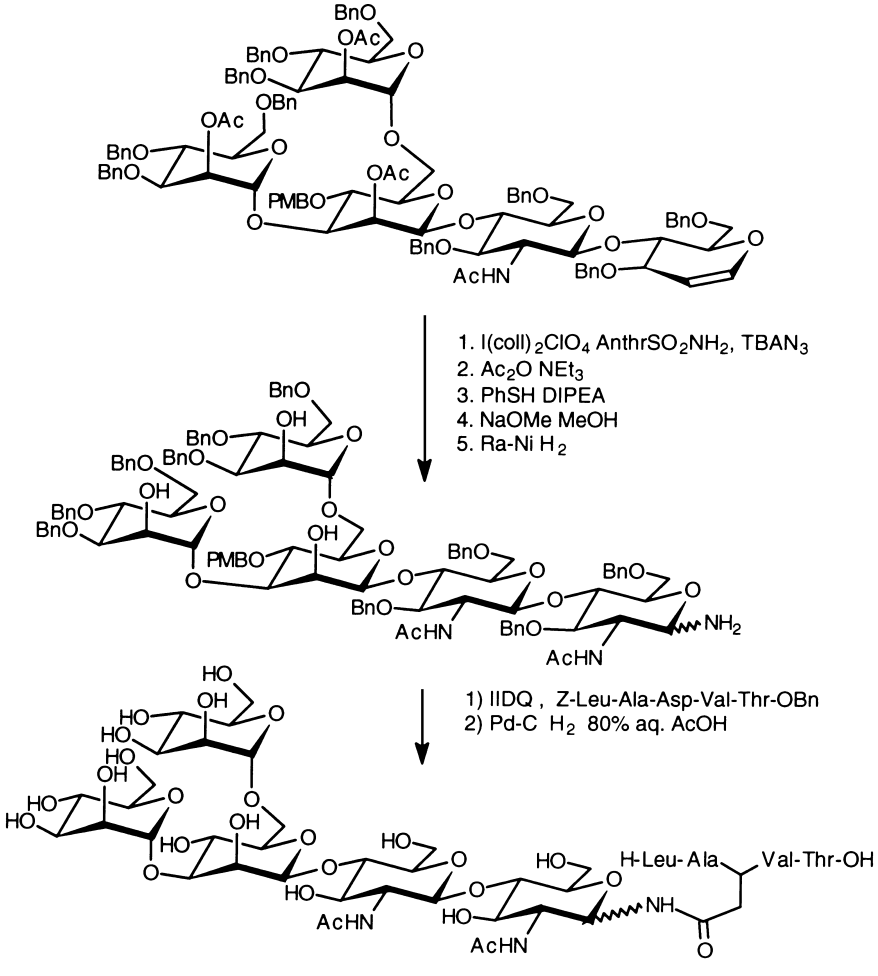
Scheme 39.

One of the crucial synthetic strategies is the reductive deacetylation of the unique ester at C2, followed by Dess–Martin oxidation[105] of the unique equatorial alcohol that afforded the unstable ketone which was further reduced with L-Selectride[106] to give the β-mannoside (Scheme 40).



Scheme 40.

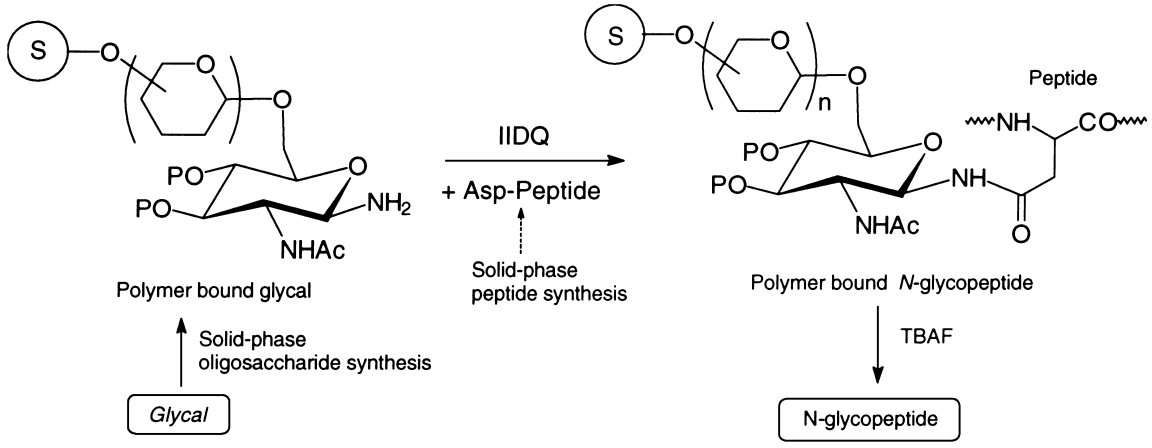
Other synthetic final operations such as the introduction of the amino group and the coupling/deprotection procedures deserve some comments. The TBS groups were deprotected with TBAF and the three free hydroxyl groups were acetylated. Concomitant acetylation of the sulfonamido NH group enabled the reductive removal (thiophenol in the presence of diisopropylethylamine) of the sulfonamide. At this stage after deacetylation of the acetyl groups with a catalytic amount of sodium methoxide only Bn groups were present in the pentasaccharide. Iodoanthracenesulfonamidation[107] of the terminal pentasaccharide glycal was followed by azidolytic rearrangement[108] yielding a protected complex anomeric azide (Scheme 41). The protecting group strategy allows the extension of the pentasaccharide through the terminal mannose units. Unfortunately, reduction of the azidopentasaccharide under Raney-nickel conditions with or without triethylamine yielded a mixture of glycosylamines[109] which were coupled to the peptide in the presence of IIDQ yielding an anomeric mixture of glycopeptides.



Scheme 41.

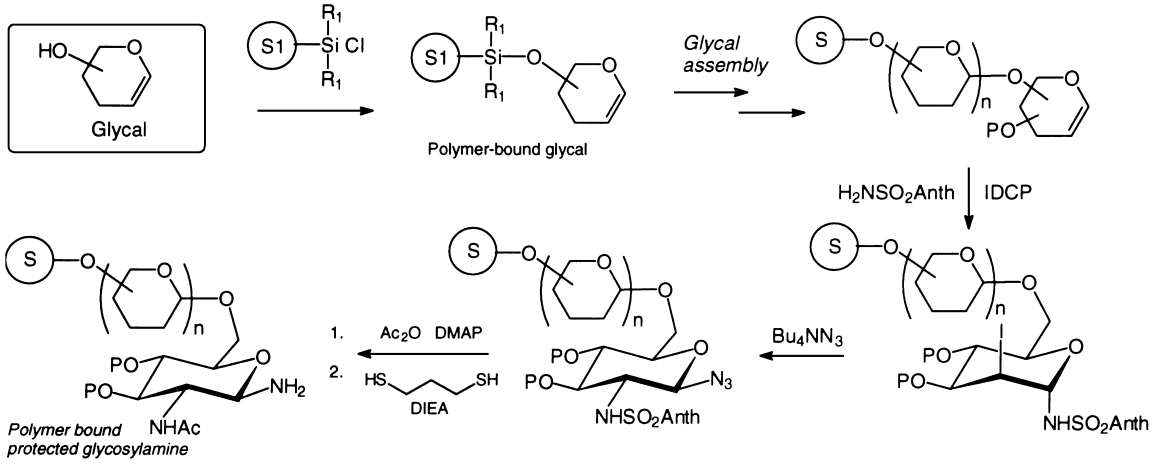
Similar approaches have been developed for the convergent synthesis of *N*-linked glycopeptides on a solid support[110] due to the inherent advantages of solid-phase methodology. Towards this goal the glycal assembly method and other reactions involved in these strategies have been transferred to the solid phase.[111] The striking difference to the previously reported convergent solid-phase approaches is that

the complex *N*-glycan is polymer bound,[112] i.e. the complex *N*-glycan is synthesized using solid-phase synthesis, and then coupled to a preformed peptide (Scheme 42).



Scheme 42.

Glycals are attached to a polystyrene polymer by a silyl ether bond and are activated to function as glycosyl donors with 3,3-dimethyloxirane. Glycosidations are performed by reactions with a solution-based acceptor that is itself a glycal. Thus, the polymer-bound construct terminates in a glycal (Scheme 43). The terminal double bond can be functionalized to provide a C2-*N*-acetyl glucosamine linkage with an amino group in the anomeric position. Iodosulfonamidation of the polymer bound glycal to the *N*-acetyl glucosamine using anthracenesulfonamide is crucial for the success of the solid-phase synthesis. Iodosulfonamidation of glycal with iodonium bis(*sym*-collidine)perchlorate,[113] a powerful iodinating reagent, and anthracenesulfonamide[114] gives the polymer bound diaxial iodosulfonamide in good yield. After treatment with soluble tetra-*n*-butyl ammonium azide and acetylation of the nitrogen of sulfonamide, the sulfonamide could be removed either stepwise with thiophenol or 1,3-propanedithiol and Hunig's base (DIEA). Under these conditions the azide is concomitantly reduced to give polymer bound *N*-acetyl glucosamine. All reactions are performed in THF which is the optimal solvent not only for the efficiency of solid-phase reactions but also for a good swelling of the polystyrene support.

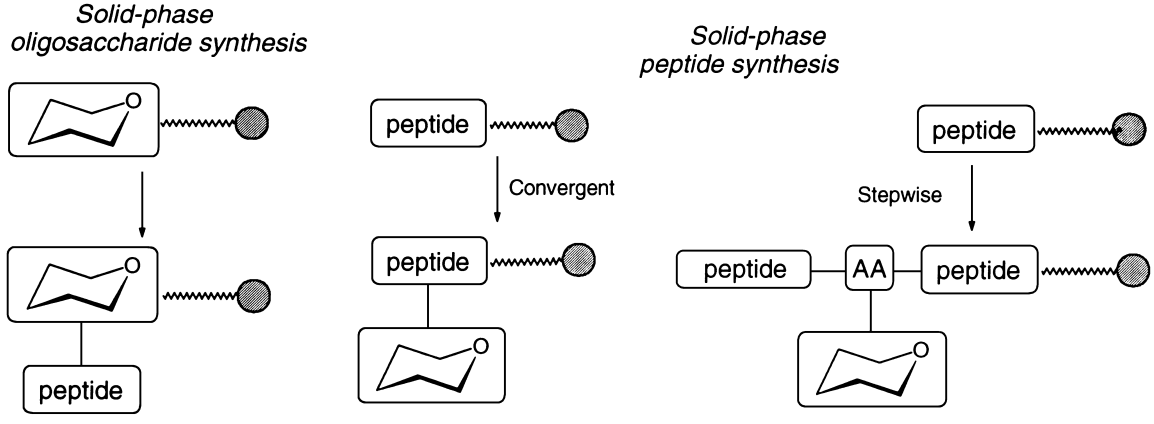


Scheme 43.

The glucosamine can be coupled in a convergent manner with the β-carboxyl group of an aspartyl residue on a preformed peptide. Finally, *N*-glycopeptides are obtained from the polymers by the addition of tetra-*n*-butyl ammonium fluoride.

This scheme allows the inclusion of either unnatural (artificial) sugars or amino acids on the glycopeptide. To date, the problem of interpolating a suitable β-linked mannose, in turn, branched with α-mannose residues, has not yet been implemented in solid-phase approaches. Such experiments will represent a remarkable development.

The polymer supported convergent synthesis of *N*-glycopeptides by Danishefsky is here outlined to exemplify the role and benefits of the development and implementation of new methodologies based on the solid-phase oligosaccharide synthesis (Scheme 44). These strategies would not only allow the rapid and efficient synthesis of target molecules, such as the complex *N*-glycans of glycopeptides, but also the synthesis of polymer bound, fully deprotected saccharides for use directly and repeatedly in relevant biological assays, as invaluable tools in glycobiology.



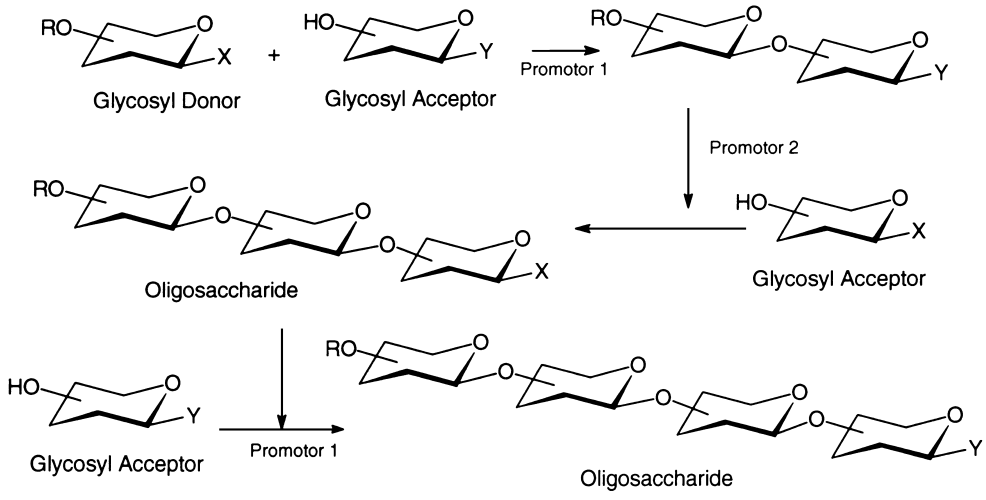
Scheme 44. Comparison on solid-phase approaches to *N*-glycopeptide synthesis

Since the first reported work[115] on the glycosylation of a polymer bound glycosyl acceptor with anomeric bromides there have been an increasing number of papers published on solid-phase oligosaccharide and glycopeptide synthesis which has been recently reviewed by different authors.[116] A number of patents[117] have also appeared over recent years which shows the economic potential of these successful approaches. Two main strategies can be envisaged entailing either attachment of the acceptor or of the donor to the solid support. In the former strategy, an acceptor is bound to the solid support, usually at the anomeric position, and a solution based donor and promoter are added for the coupling step. In the second approach, glycosyl donors are bound to the solid support by a suitable hydroxyl group and then reacted with solution phase acceptors. Recent reviews on polymer bound reactions highlight the different type of polymers and linkers[118] available as well as the different methods for monitoring polymer supported reactions. However, there is still little mention of reactions relevant to oligosaccharide assembly.[119] Although the polymer supported synthesis of oligosaccharides still lags behind that of peptides and oligonucleotides great efforts are being made in this field that have already enabled preparation of the first carbohydrate combinatorial libraries.[120]

3.1.4. β-Mannosylation with a variant of the intramolecular aglycon delivery

A key building block for the synthesis of complex *N*-linked glycopeptides, of the biantennary heptasaccharide–asparagine conjugate[121] type was efficiently prepared by the Ogawa group.[122]

Ogawa et al. designed several monosaccharide units, whose anomeric centres could be activated or transformed under different conditions for the preparation of a key trisaccharide intermediate based on an orthogonal glycosylation strategy. In this orthogonal approach, two anomeric groups (X and Y) are used which both act as anomeric protecting groups as well as leaving groups and a schematic representation of this approach is depicted in Scheme 45.



Scheme 45.

The complex-type biantennary–asparagine conjugate of *N*-linked glycoproteins shown in Scheme 46 was synthesized from five monosaccharide units in seven steps with an overall yield of 18.4%. This is a remarkable example of an efficient synthetic route that could be conducted on a multi-gram scale. An additional advantage is the fact that the anomeric azide heptasaccharide precursor is a suitable intermediate for the chemical synthesis of even more complicated biantennary *N*-oligosaccharides.

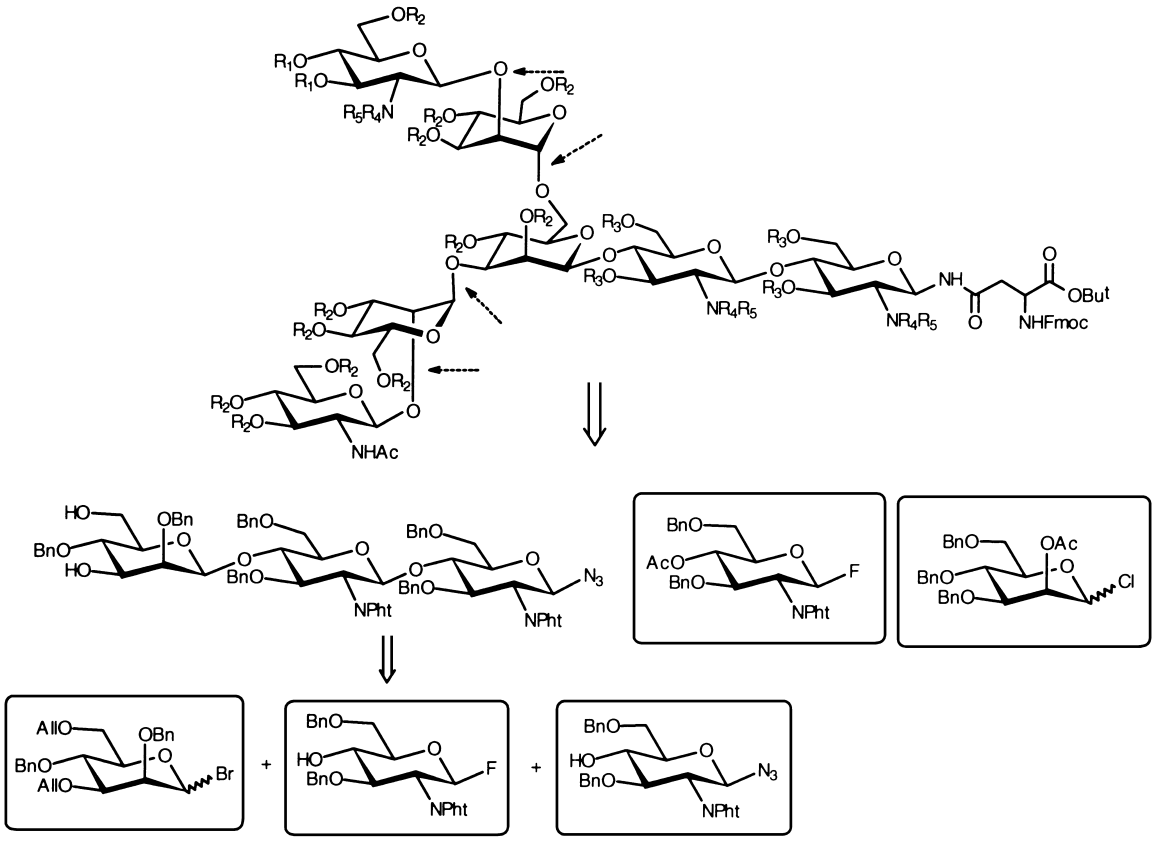
Unverzagt[123] has also prepared a partially benzylated and partially acetylated heptasaccharide precursor of this type by selective glycosylations in 11 steps with an overall yield of under 4% starting from protected mono- and disaccharides.

The protecting group scheme was based on phthaloyl for the 2-amino functions and Bn or allyl for the carbohydrate hydroxyl groups. Interestingly, the allyl group is selectively removed by an iridium-catalyzed process.[124] In this synthesis, the formation of the β-mannose linkage was promoted by silver alumina silicate that afforded the disaccharide in 91% yield as a mixture of the two isomers without influencing the anomeric fluoride (Scheme 47).

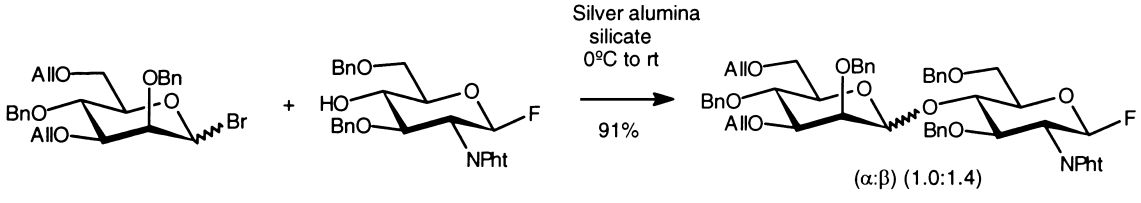
The same procedure was used by Ogawa to prepare a key β-mannoside trisaccharide which is the first example of the introduction of a β-mannosyl residue onto the C-4 hydroxyl group of a GlcNAc carrying a glycosyl residue at C-6 by means of silver silicate promotion. The trisaccharide was obtained in 53% yield as a mixture of α- and β-isomers in a 1.4:1.0 ratio (Scheme 48).[125]

This trisaccharide building block was used by Ogawa et al.[125] for the synthesis of derivatives of a novel Asn-linked core structure with the purpose of unveiling the configuration of a new linkage (Scheme 49). The novel *N*-linked core structure, with an additional GlcNAc, was isolated by Stanley et al.[126] from Chinese hamster ovary (CHO) cell-LEC18. It was suggested that the new GlcNAc residue markedly alters the conformation of related oligosaccharides and thus causes the high resistance of LEC18 cell to both pea lectin (PSA) and *Lens culinaris* agglutinin (LCA).

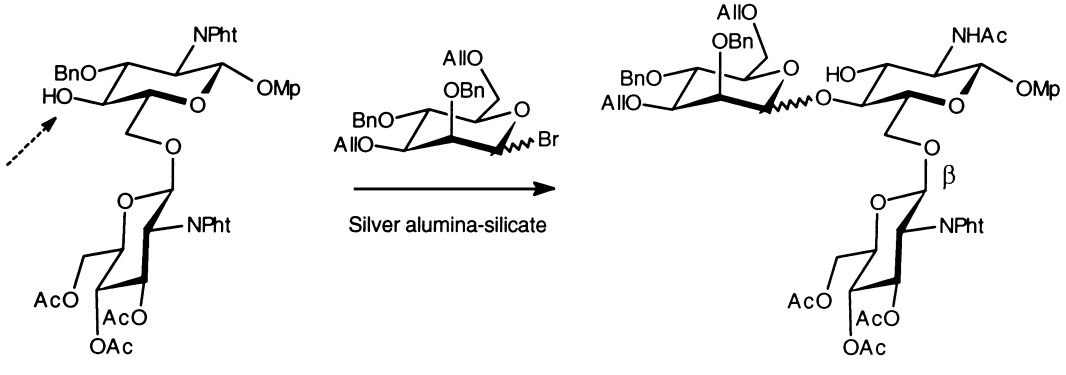
The intramolecular aglycon delivery (IAD) approach which was introduced by Barresi and



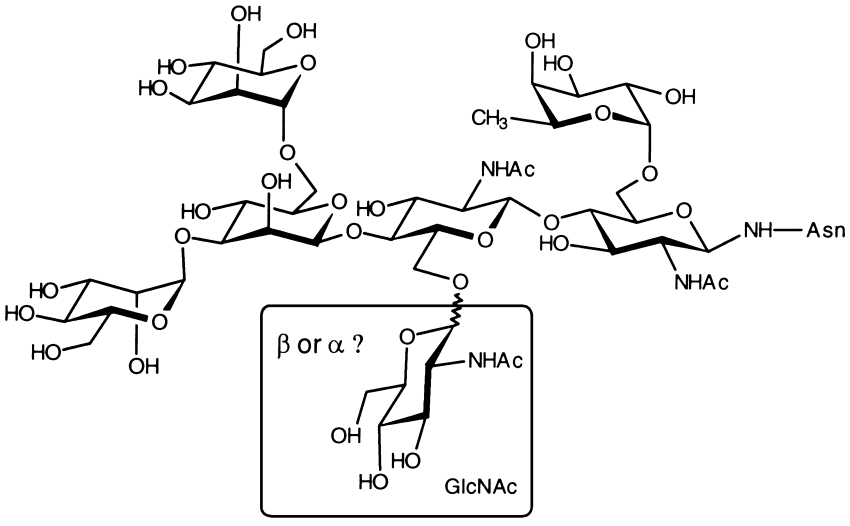
Scheme 46.



Scheme 47.



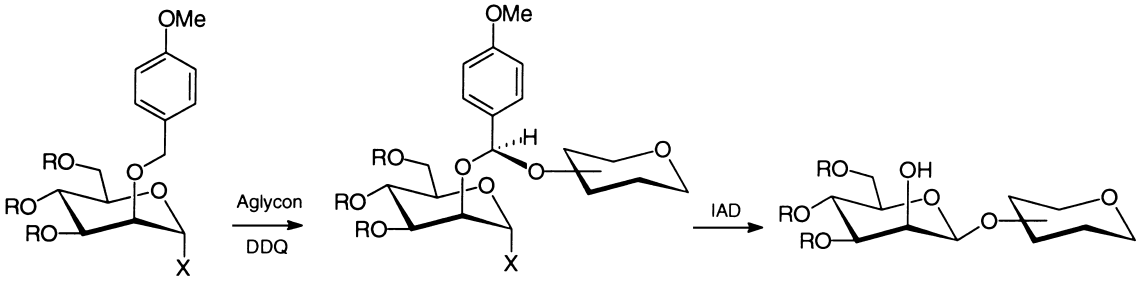
Scheme 48.



Scheme 49.

Hindsgaul[127] and later by Stork et al.[128] is endowed with an evident advantage over the others, because it guarantees the exclusive formation of the correct anomer as a result of the geometrical constraint.

Ogawa et al. has described a new variant of intramolecular aglycon delivery (IAD) by the use of a 2-*O*-*p*-methoxybenzyl protected mannosyl donor[129] delivery (PMB-assisted approach, Scheme 50) to solve one of the main obstacles in the chemical synthesis of Asn-linked glycoprotein oligosaccharides which is the formation of β-mannoglycoside linked to the 4-position of *N*-acetylglucosamine.

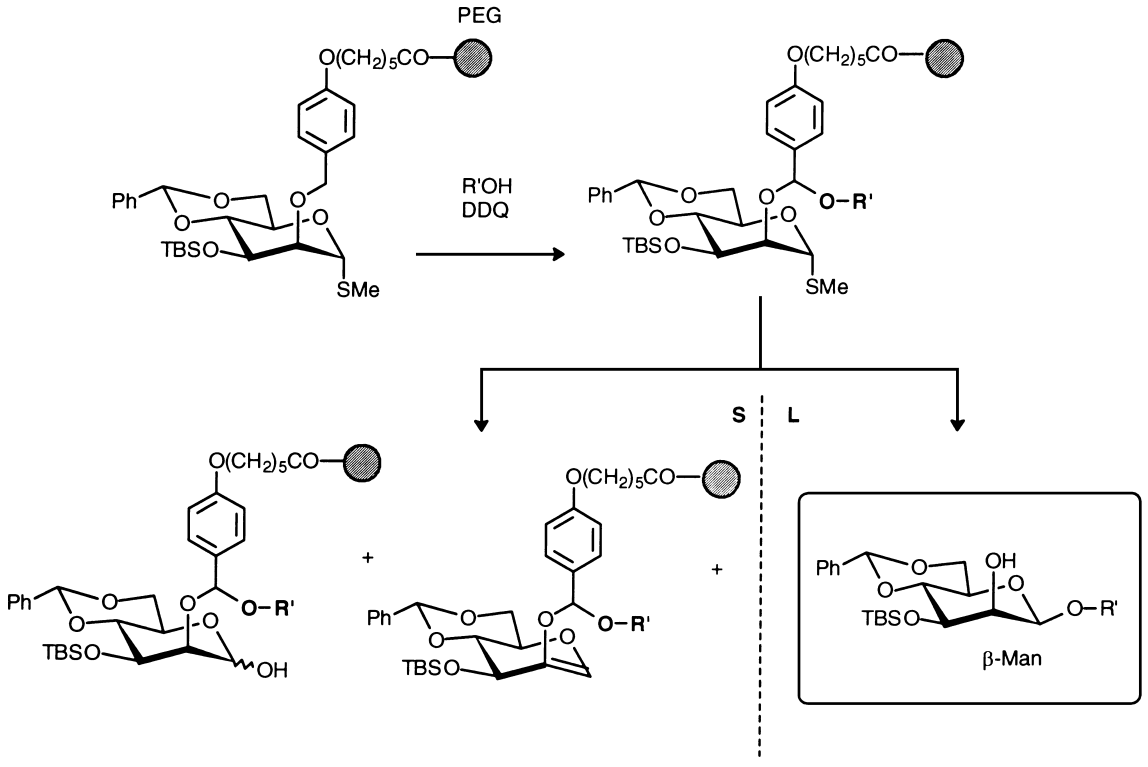


Scheme 50.

Recently, Ogawa et al. have demonstrated that polymer bound protecting groups can stereospecifically direct the introduction of a reagent, as shown in the polymer-supported synthesis of β-mannoglycosides[130] (Scheme 51). It is important to note that only the desired glycosylation products were liberated from the polymeric resin (liquid phase: L). Side products such as the hydrolysis or the elimination product remained on the solid phase (S). Thus, the polymer support serves as a 'gatekeeper'. This example illustrates that protecting groups[131] can be actively used in synthesis and achieve more than temporary blocking of a functional group.

The flexibility of the *p*-methoxybenzylidene-tethered β-mannosylation for stereoselective synthesis of asparagine-linked glycan chains is exemplified in the synthesis of a fucose containing hexasaccharide[132] which constitutes the core structure of biomedically significant glycoproteins[133] (Scheme 52). It is most significant to point out that this is the first example of the exclusive formation of a β-mannoglycoside at the oligosaccharide block-condensation stage.

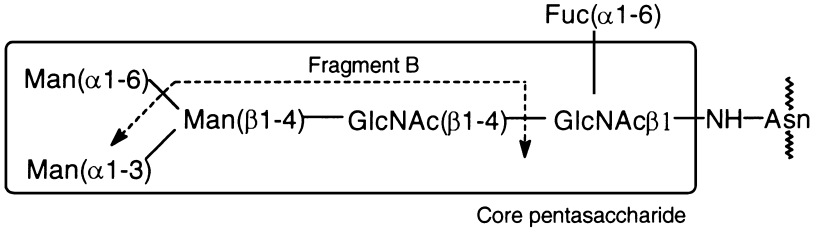
Thus, β-mannosylation was performed with the mannoside disaccharide and the latent GlcNAc



Scheme 51.

component as an acceptor. Initial transformation into the mixed acetal trisaccharide was effected by DDQ (Scheme 53). The crucial IAD steps were conducted by exposure of the acetal to methyl triflate and 2,6-di-*tert*-butyl-4-methylpyridine (DBMP) in 1,2-dichloroethane to afford the β-mannoglycoside product (fragment B) of Asn-linked glycan chains.

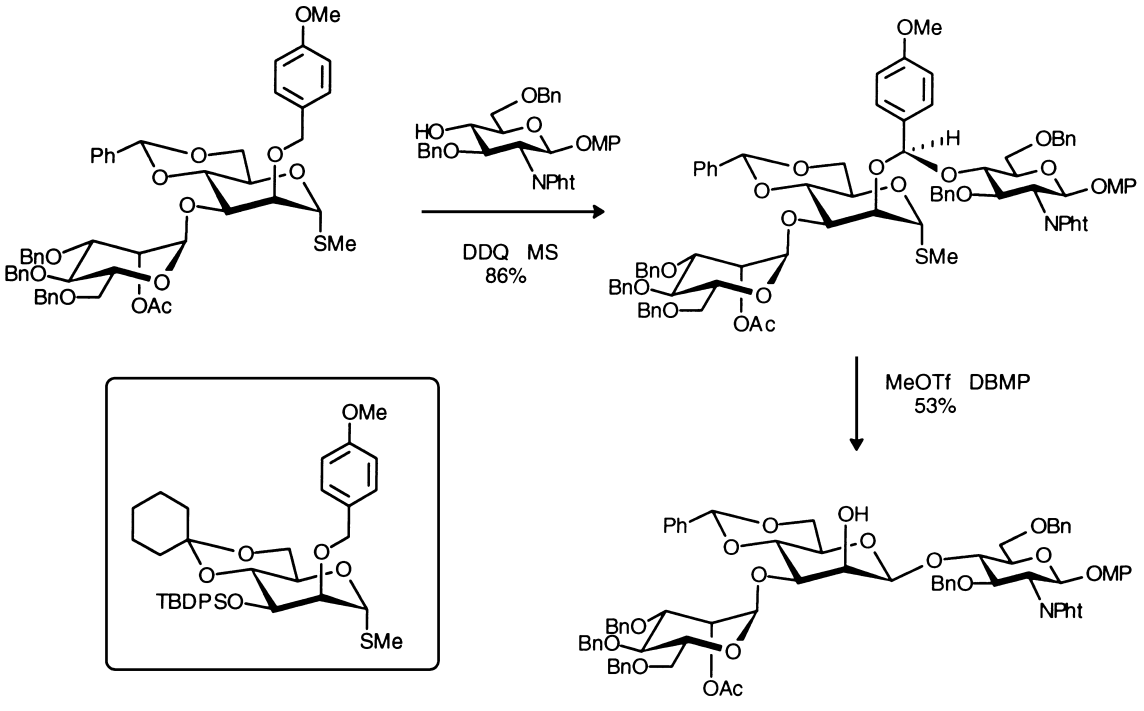
The efficiency of PMB-assisted β-mannosylation has been further improved[134] by changing the protecting group at the 4- and 6-positions from the previously utilized benzylidene to cyclohexylidene or tetraisopropyldisiloxane groups in the protected mannosyl donor. The single stereoisomers are obtained in 75–85% yield.



Scheme 52.

3.2. Chemo-enzymatic and enzymatic approaches to the synthesis of complex N*-glycopeptides*

A milestone in *N*-glycopeptide synthesis has been the preparation of glycopeptides containing complex *N*-glycans. In fact, to date, the synthesis of biantennary sialylated *N*-glycans has only been demonstrated in a few examples. One main problem is found when attempting the assembly of complex oligosacchari-



Scheme 53.

des into glycopeptides by chemical means that requires an increasing demand on protective groups of the peptide and sugar part that normally leads to severe complications. However, the final enzymatic solution has yet to be achieved since, for instance, only a few of the *N*-acetylglucosaminyltransferases responsible for branching have been cloned and overexpressed. In spite of these difficulties novel concepts have been developed for the synthesis of *N*-glycans and their conjugates.

The use of enzymatic methods for glycoside and oligosaccharide syntheses in principle avoids some of the problems posed by chemical synthesis (Scheme 54).

GLYCOZYMES	
	Examples
Glycosyltransferases	Sialyltransferases
	Oligosaccharyltransferases
	N-acetylglucosaminyltransferases
Glycosidases	
Exoglycosidases	β-Glucosidase, β-Mannosidase
Endoglycosidases	Endo-A, Endo-M
Mutant glycosidases	Glucansynthase, Glycosynthase
Glycoamidases	PNGase F

Scheme 54.

Two approaches are dominating enzyme-catalyzed glycoside[135] and oligosaccharide[136] synthesis, glycosyltranferase- and glycosidase-catalyzed glycosidic bond formation. The first uses the normal

biosynthetic machinery that produces the same compounds in living organisms. In the second, enzymes that formally catalyze transfer of an enzyme-bound glycosyl residue to water are induced to do so to a different acceptor.

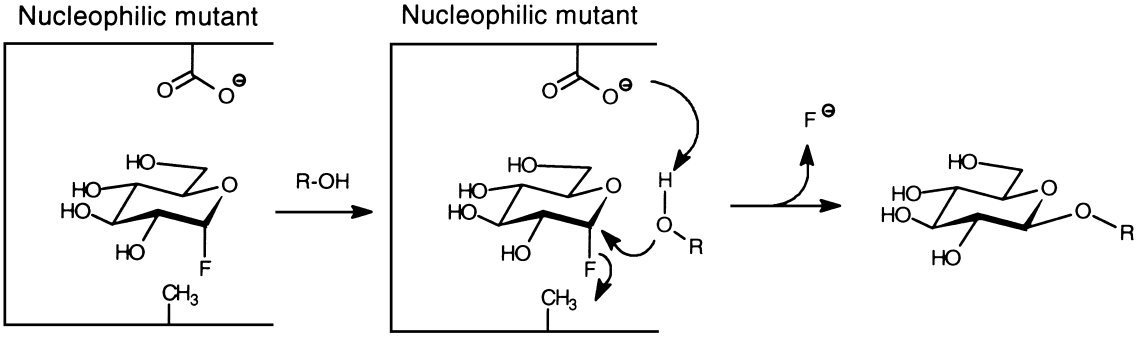
Glycosyltransferases are most often used for the assembly of oligosaccharide chains, but the different carbohydrate combinations which are necessary for the construction of complex oligosaccharide chains requires ready availability of a large number of specific enzymes. To date not only are glycosyltransferases relatively inaccessible but complex glycosyl donors are also required. Glycosyltransferase reactions with cofactor regeneration in situ have made possible the large-scale synthesis of complex carbohydrates.[137] In the same way it is expected that an increasing number of recombinant glycosyltransferases and other carbohydrate-related enzymes should provide enough of all the enzymes necessary for the synthesis.

Glycosidases normally catalyze the hydrolysis of the glycosidic bond; however, under appropriate conditions the enzymes can also catalyze the formation of a glycosidic bond. The potential for retaining glycosidases as tools for the regio- and stereospecific synthesis of glycosides has been realized by many research groups since the early part of this century.[138] The two main approaches to glycosidase-catalyzed synthesis of glycosidic linkages involve direct reversal of hydrolysis (equilibrium-controlled synthesis) and trapping of a glycosyl–enzyme intermediate (kinetically controlled process).

Most glycosidases used for synthetic purposes are exoglycosidases, the glycosyl transfer takes place only to the non-reducing terminal monosaccharide unit of substrates. In addition, several *endo*-type glycosidases[139] have been found to perform either transglycosylation or the reverse reaction. However, in aqueous solution, transglycosylation and glycoside formation by these enzymes are carried out with low yields, therefore necessitating laborious separation of the reaction mixture.

An interesting goal in glycosidase-catalyzed oligosaccharide synthesis studies is to have a library of glycosidases capable of selectively catalyzing the formation of any desired glycosidic bond. As the number of synthetically useful glycosidases grows, the possibility of building up oligosaccharide structures by the successive application of these appropriate glycosidases is getting closer.

A further approach for oligosaccharide synthesis which is currently emerging is based on the rational design of the enzyme's catalytic inner machinery. Two independent groups[140] have recently developed a new concept based on the preparation of mutants of glycosidases (Scheme 55). The mutated glycosidase lacks its catalytic nucleophile and its main advantage is that the reaction products cannot be hydrolyzed.

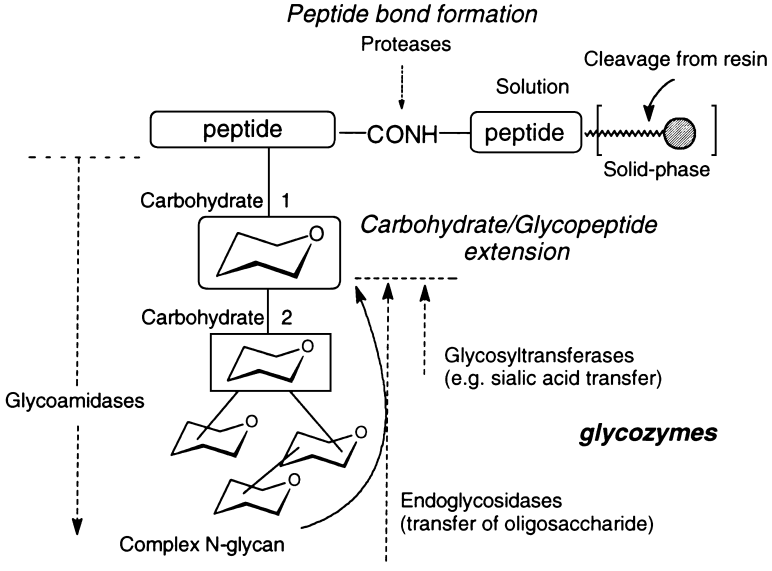


Scheme 55. Glucansynthase a mutant glycosidase

This new approach to oligosaccharide synthesis has the twin advantage of utilizing inexpensive glycosyl donors and enzyme availability, and therefore, has considerable potential as an alternative strategy for oligosaccharide synthesis, particularly on a large scale.

All these enzymatic tools and strategies obviously apply when solving a different part of *N*-

glycopeptide synthesis which is *N*-glycan preparation. Accordingly, in Scheme 56 we have summarized *state of the art* uses of glycozymes and other enzymes such as proteases in the enzymatic synthesis of complex *N*-glycopeptides either in solution phase or solid-phase.



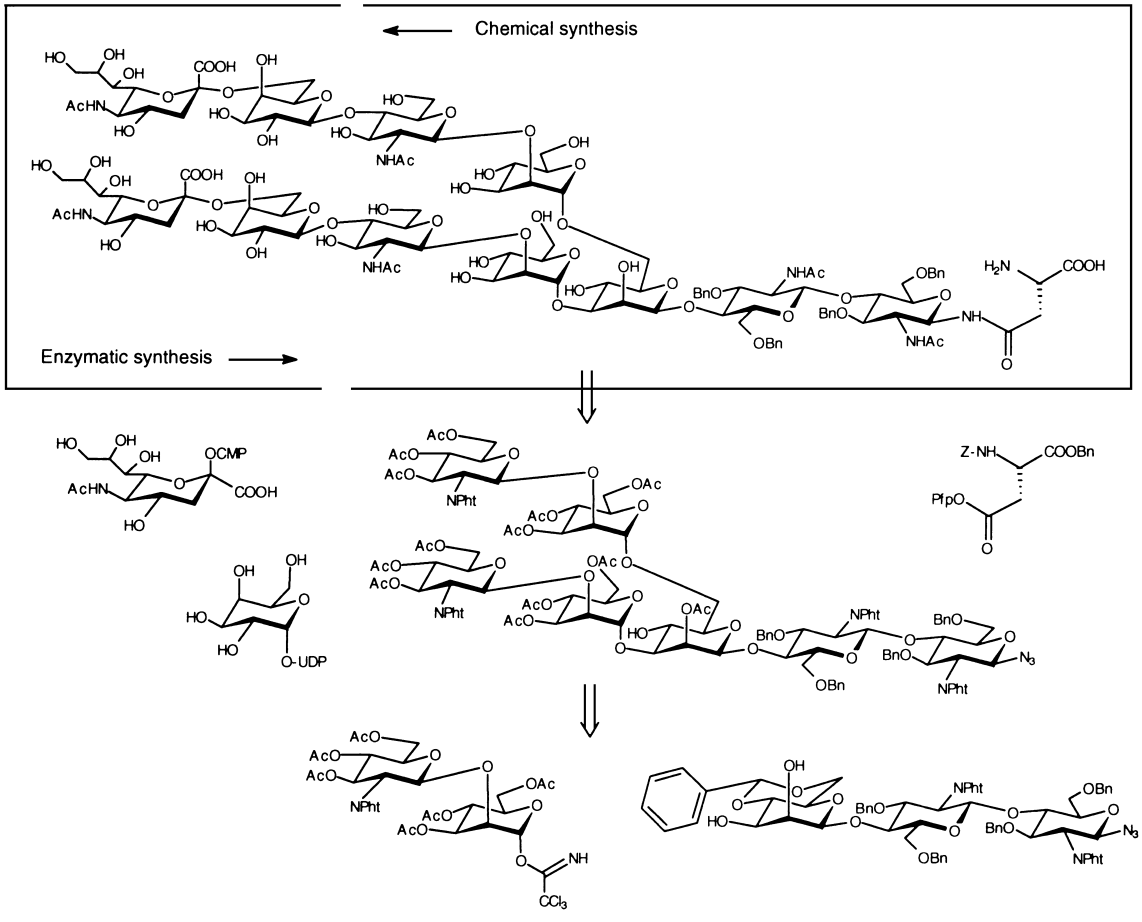
Scheme 56.

Illustrative examples of the enzymatic synthesis of complex *N*-glycopeptides will be given below to show how they have not only been advantageously employed for the extension of the oligosaccharide part in complex *N*-glycan synthesis, but also have led to pure glycoforms of proteins.

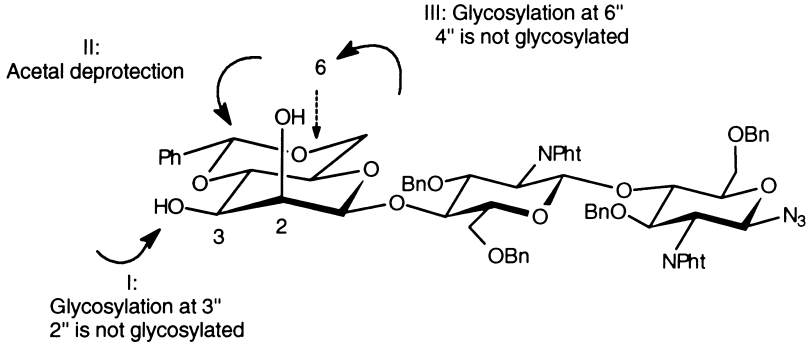
3.2.1. Using glycosyltransferases

An analysis of the difficulties encountered in the chemical synthesis of complex *N*-glycans has revealed three key reactions. The construction of the central β-mannose unit, the introduction of sialic acid and the deprotection of the sialylated *N*-glycan. To circumvent the latter two problems, Unverzagt has come up with a new strategy to obtain complete *N*-glycans by combining chemical synthesis with the ease of enzymatic carbohydrate chain elongation using glycosyltransferases. A highly efficient method to enzymatically introduce galactose and sialic acid in the presence of alkaline phosphatase was developed earlier[141] and used as a basis for this new chemo-enzymatic approach.[142] The main achievement of this methodology is the total synthesis[143] of a glycosylated asparagine carrying a complex type undesaccharide *N*-glycan (sialylated undesaccharide) which is a partial structure of many glycoproteins (Scheme 57).

The key step of the strategy is the double regioselective glycosylation of the benzylidene mannoside at positions 3′′ and 6′′. The core trisaccharide, synthesized according to the clever procedure for β-mannosylation introduced by Kunz and Günther,[144] was glycosylated at the equatorial 3′′-OH function and *not* at the axial 2′′-OH (Scheme 58). The presence of a single benzylidene protecting group in the β-mannoside portion of an appropriately functionalized core-trisaccharide led to the development of a double regioselective glycosylation strategy.[145] A temporary 4,6-*O*-benzylidene acetal on the β-mannoside allows the regio- and stereoselective introduction of the (1–3) and the (1–6) arm of *N*-glycans. Following this approach, not only were diantennary *N*-glycans obtained, but also triantennary and tetra-antennary *N*-glycans could be synthesized chemically.



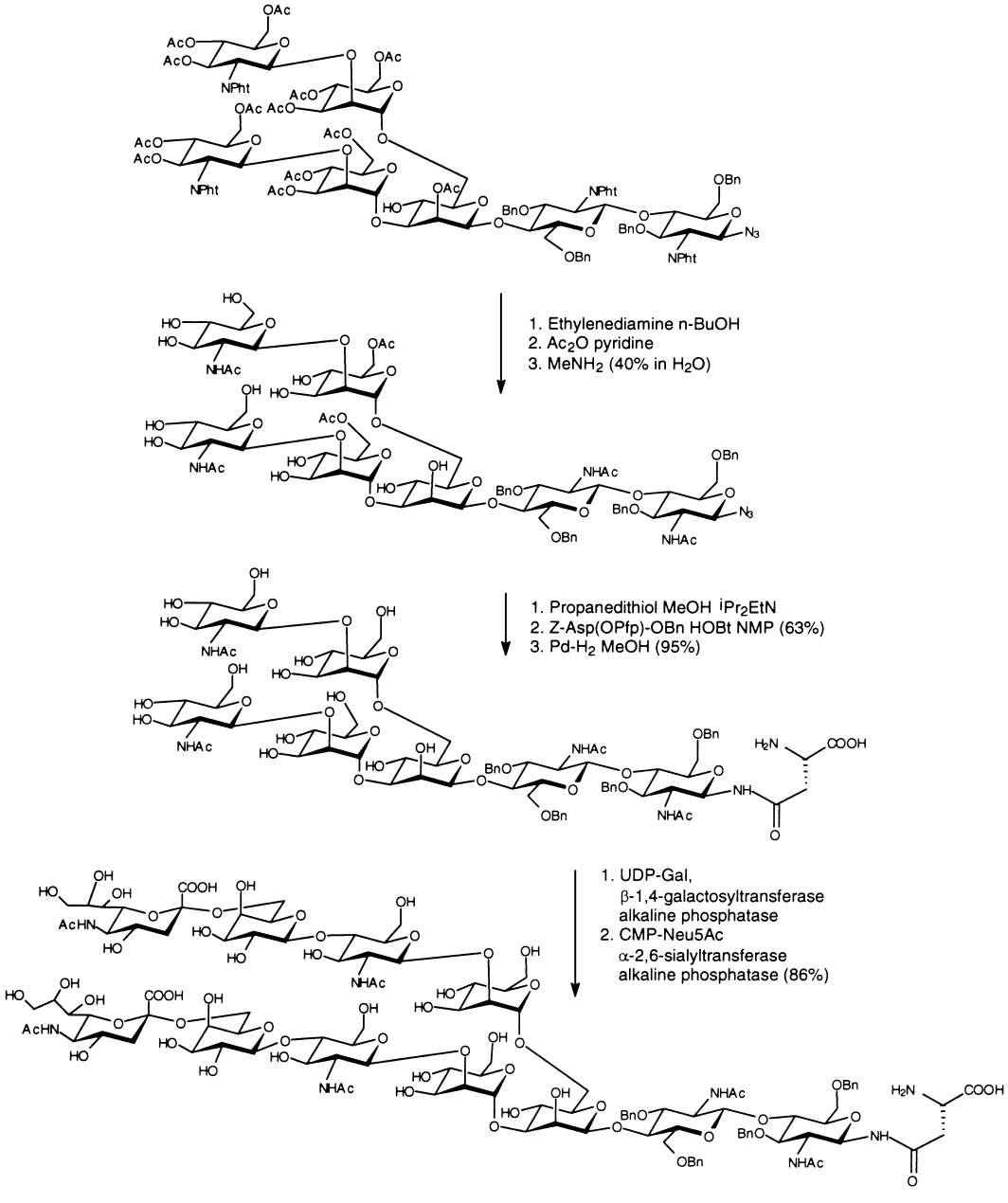
Scheme 57.



Scheme 58.

Elongation with a disaccharide trichloroacetimidate in the presence of boron trifluoride etherate gave the desired pentasaccharide regio- and stereoselectively. Then, after acetylation of the 2′′-OH and removal of the benzylidene acetal, the pentasaccharide carried two OH groups, at 4′′ and 6′′, and reacted with the disaccharide trichloroacetimidate to afford an α-1–6-linked heptasaccharide regio- and stereoselectively. This procedure could be applied to obtain over 5 g of this heptasaccharide. Dephthaloylation by heating with ethylenediamine and *n*-butanol[146] was carried out prior to the coupling to

the aspartic acid derivative (Scheme 59). After *N*-acetylation and subsequent deacetylation of *O*-acetates with aqueous methylamine, the partially protected heptasaccharide was water-soluble bearing four benzyl hydrophobic groups. As starting material a chemically synthesized diantennary heptasaccharide azide was deprotected in a three-step sequence in high yield. The reduction of the anomeric azide was accomplished with propanedithiol in methanol–ethyldiisopropylamine. Coupling of the glycosylamine to an activated aspartic acid (Z-Asp(OPfp)OBn) gave the benzyl protected asparagine conjugate. After removal of the six benzyl functions the resulting heptasaccharide asparagine was elongated enzymatically on the oligosaccharide portion.

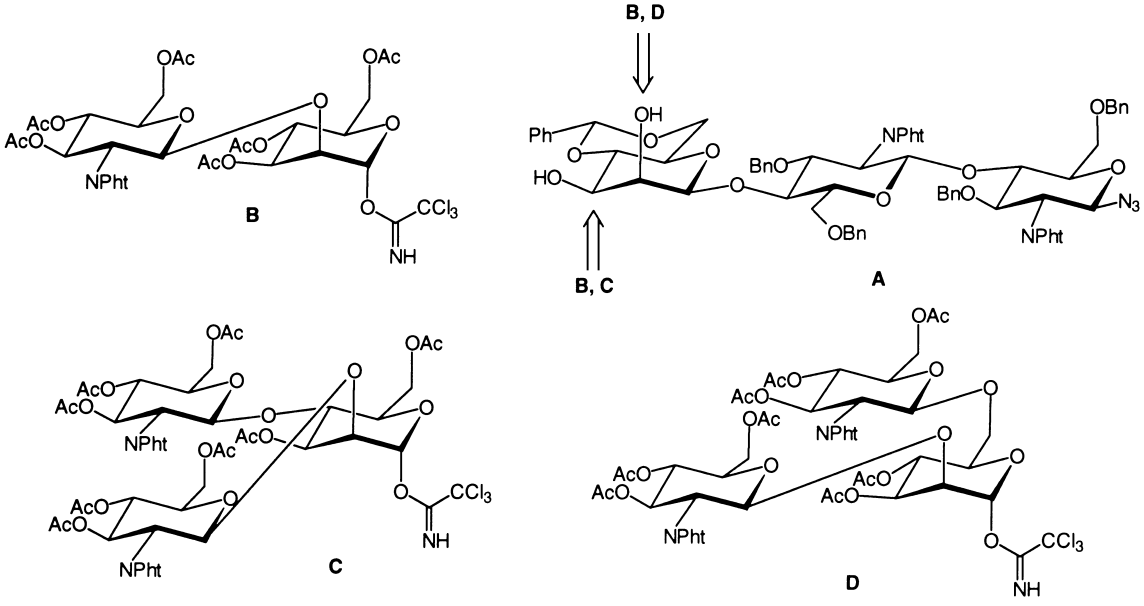


Scheme 59.

The use of β-1,4-galactosyltransferase and the α-2,6 sialyltransferase in the presence of alkaline phosphatase allowed the efficient transfer of four sugar units to the acceptor, resulting in a full length *N*-glycan, a sialylated diantennary undecasaccharide–asparagine conjugate. This complex glycoconjugate, which up to now could only be isolated from natural sources,[147] was successfully synthesized on a 5 mg scale.

Alternatively, after the deprotection of the six benzyl groups, the resulting heptasaccharide–asparagine conjugate could be used in solution-phase peptide synthesis to elongate the *C*- and *N*-peptide termini. The glycopeptide obtained was elongated stepwise by enzymatic transfer yielding a complex biantennary undecasaccharide-containing glycopeptide that represented the amino acids 21–25 sequence of bovine ribonuclease B. This represented the first synthesis of a glycoprotein fragment carrying a full length asparagine-linked oligosaccharide.[148]

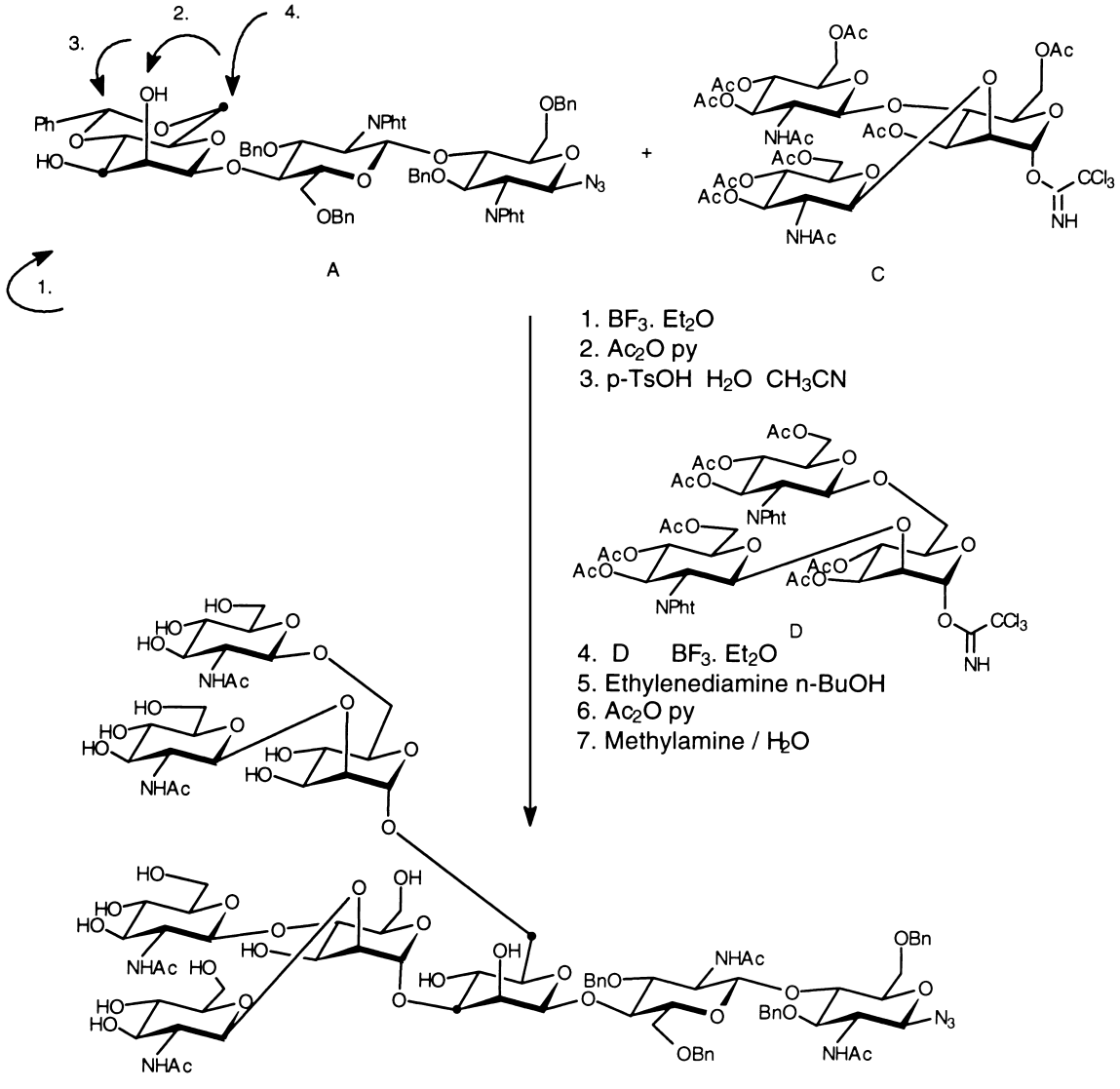
As can be seen from the previous example, the most frequent basic structures of *N*-glycans could be assembled by a single synthetic scheme based on a number of building blocks. It is important to appreciate that together with **A** and **B** the newly synthesized trisaccharide building blocks **C** and **D** form a modular system which allows the multiple use of valuable intermediates to obtain various basic structures of *N*-glycans (Scheme 60). These versatile building blocks **A**–**D** provide an efficient chemical equivalent for the modular structure of these natural oligosaccharides.



Scheme 60.

As an example of the versatility of this modular approach the synthesis of a nonasaccharide is outlined here.[149]

Coupling between the trisaccharide trichloroacetimidate **C** and the linear trisaccharide **A** proceeds with high yield (93%) to form a new α-1,3 linkage (Scheme 61). Acetylation of the free hydroxyl group followed by debenzylidenation with *p*-toluenesulfonic acid gives a hexasaccharide diol (Scheme 61). The sterically most demanding glycosylation of this hexasaccharide acceptor takes place regio- and stereoselectively with building block **D** to give a nonasaccharide azide in 78% yield (Scheme 61). Dephthaloylation with ethylenediamine in *n*-butyl alcohol followed by an acetylation–decetylation sequence yields a minimally protected nonasaccharide azide.



Scheme 61.

The remaining protecting groups in the chitobiosyl part are strategically valuable. They allow the construction of glycoconjugates through an *N*-glycosidic bond at the anomeric centre, and facilitate purification by reverse-phase HPLC. This nonasaccharide was completed with the aid of transferases to a sialylated tetra-antennary heptadecasaccharide.

3.2.2. The use of endoglycosidases endowed with transglycosylation activity

endo-β-*N*-Acetylglucosaminidase (*endo*-β-GlcNAc-ase, EC 3.2.1.96) is an endoglycosidase that hydrolyzes the glycosidic bond in the *N*,*N*′-diacetylchitobiose moiety of *N*-linked sugar chains in glycoproteins and leaves one *N*-acetyl glucosamine (GlcNAc) residue on the protein. The enzymes of this class are widely distributed in microorganisms,[150] plants,[151] animals,[152] and humans.[153] They are also the most studied of the endoglycosidases since they are useful for the structural analysis of glycoproteins and

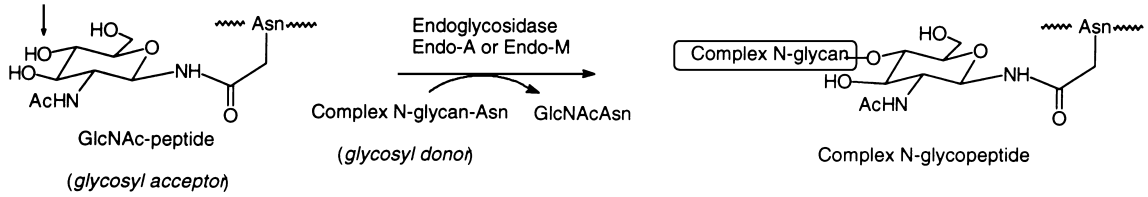
can be of help in isolating both *N*-linked oligosaccharides and partially deglycosylated proteins without degradation.

endo-Type glycosidases have been found to perform either transglycosylations or the reverse reactions. Methods based on the use of such enzymes have the advantage that an oligosaccharide fragment rather than a monosaccharide residue is transferred at once. Large fragments can be transferred using, e.g. *endo*-β-*N*-acetylglucosaminidases such as *endo*-F (*Flavobacterium meningosepticum*),[154] *endo*-A (*Arthrobacter protophormiae*) and *endo*-M (*Mucor hiemalis*).

Other endoglycosidases such as *endo*-α-*N*-acetylgalactosaminidase[155] from *Diplococcus pneumoniae* and ceramide glycanase from leech (*Macrobdella decora*), also have important potential for neoglycoconjugate preparation.[156] However, in aqueous solution, transglycosylation and hydrolysis proceeds leading to mixtures.

The synthesis of *N*-glycopeptides using endoglycosidases can be carried out in two steps:

(i) chemical synthesis of GlcNAc-containing peptides;
(ii) enzymatic glycosyl transfer of the complex oligosaccharide from the donor to the terminal GlcNAc of the glycopeptide (acceptor) in an aqueous medium containing organic solvents (Scheme 62).

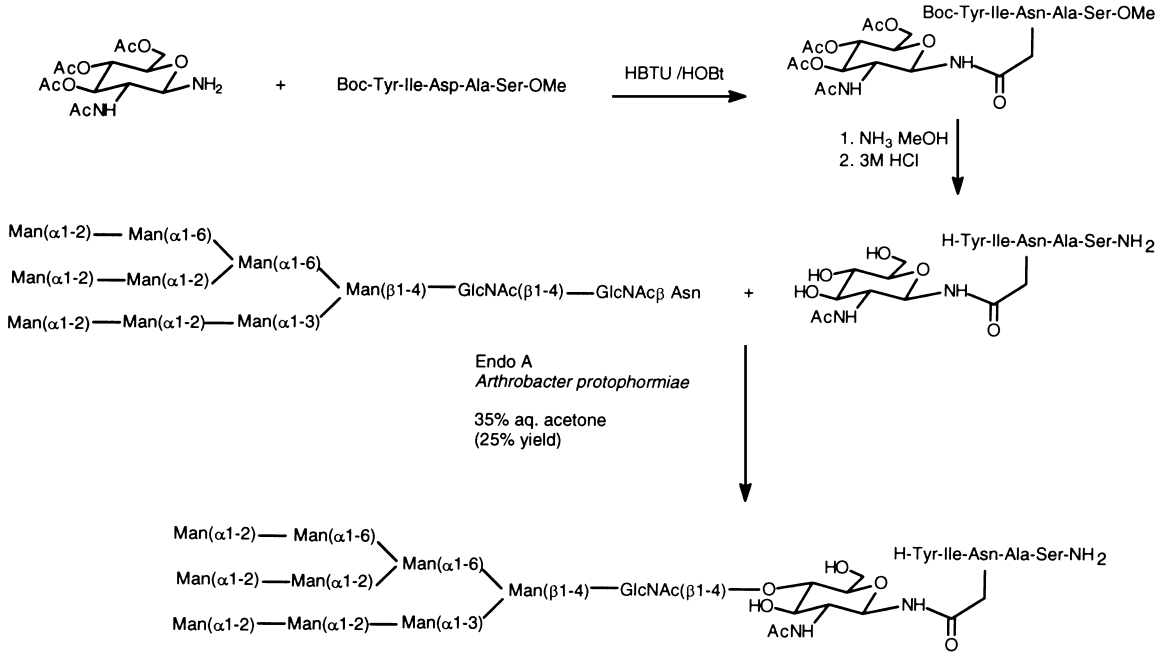


Scheme 62.

3.2.2.1. endo-*A Synthetic applications.* *Arthrobacter protophormiae*, a gram-positive bacterium, produces *endo*-β-GlcNAc-ase (*endo*-A) when the cells are grown in medium containing ovalbumin.[157] The purification and properties of the enzyme have been reported.[158] Although the hydrolytic activity still predominates, *endo*-A has transglycosylation activity, the oligosaccharides being transferred to suitable acceptors such as glucose, mannose, and gentibiose during chitobiose core cleavage by the enzyme.[159] Therefore, the enzyme has been used for neoglycoprotein synthesis by tranglycosylation.[160] As reported by Lee et al.[161] the transglycosylation activity of this enzyme can be enhanced by inclusion of organic solvents in the reaction mixture to near complete suppression of hydrolysis in the media containing organic solvents. The enzyme was stable in media containing up to 30% acetone, 30% dimethylsulfoxide, or 20% *N*,*N*-dimethylformamide at 37°C for at least 30 min. The acceptor (GlcNAc) concentration must be greater than 0.2 M for efficient transglycosylation. A suitable glycosyl donor is a high mannose-type *N*-glycan asparagine derivative ($Man_9GlcNAc_2Asn$) that can be conveniently prepared from soybean agglutinin (isolated from soybean flour) by exhaustive protease digestion and subsequent gel filtration separation.[162]

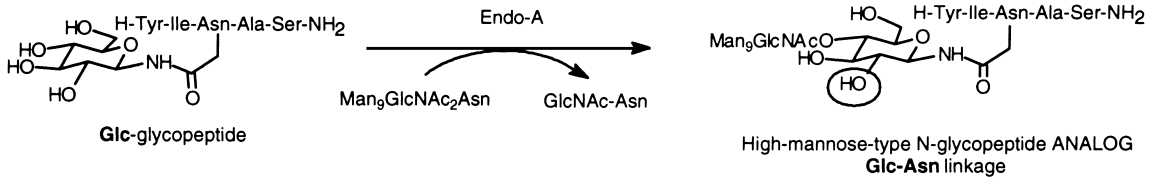
The enzyme has been shown to be very useful for synthesizing not only novel oligosaccharides[163] but also neoglycoproteins and neoglycoconjugates, as exemplified by the work of Lee et al. on the synthesis of complex *N*-glycopeptides[164] to study the mechanism and biological functions of glycoamidases (Scheme 63). Thus, they have described the chemoenzymatic synthesis of not only high mannose-type *N*-glycopeptides but also of *C*-glycopeptides using the *endo*-A transglycosylation reaction.

Using the same approach the synthesis of a high-mannose-type glycopeptide analogue containing a glucose–asparagine linkage instead of the natural *N*-acetylglucosamine–asparagine linkage has been



Scheme 63.

reported[165] (Scheme 64). In both cases, either the GlcNAc or the Glc pentapeptide served as the acceptor in the transglycosylation *endo*-A catalyzed reaction.



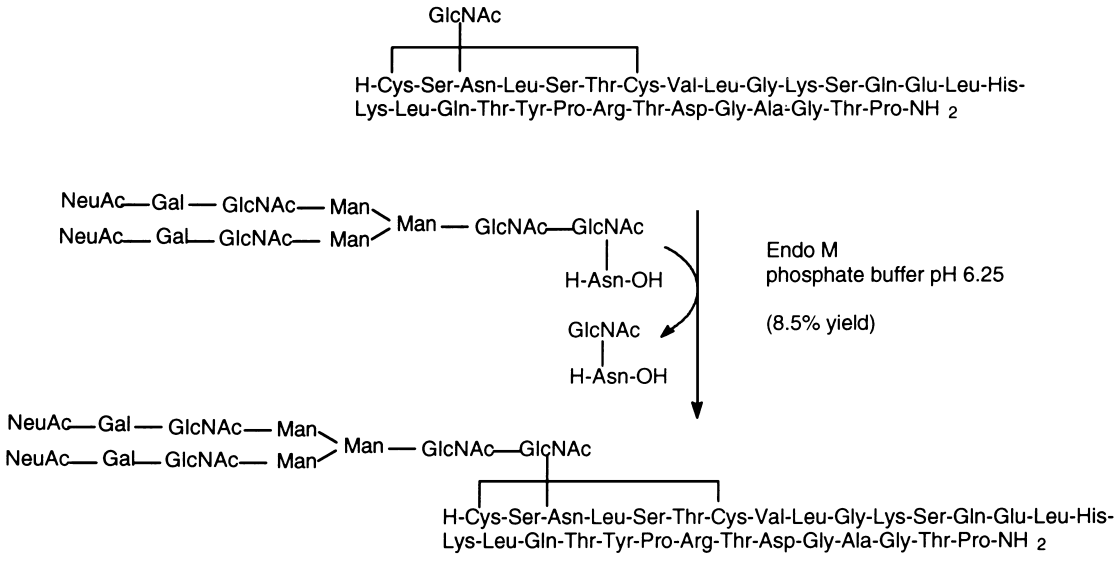
Scheme 64.

3.2.2.2. endo-*M Synthetic applications.* Yamamoto et al.[166] have found a novel *endo*-β-GlcNAc-ase in the culture medium of *Mucor hiemalis* isolated from soil which could also cleave the complex type of oligosaccharide *N*-glycans unlike other *endo*-β-GlcNAc-ases which can act only on high mannose and hybrid type oligosaccharides. This enzyme also shows transglycosylation activity and could transfer the oligosaccharides from glycopeptides to suitable acceptors with a GlcNAc residue during hydrolysis of the glycopeptide.[167] These early observations suggested that the transglycosylation of *endo*-M might be useful for the synthesis of glycopeptides.[168]

Since sialo- or asialotransferrin glycopeptide,[169] obtained from human transferrin have proven to be good substrates for *endo*-M, glycopeptide analogues containing sialo-complex-type and the desialo complex-type oligosaccharides have been prepared by *endo*-M catalyzed transglycosylation. These reactions make use of the transferrin glycopeptide as the glycosyl donor and a GlcNAc-containing synthetic peptidyl substrate as a glycosyl acceptor.

Several synthetic complex glycopeptides have also been recently prepared using *endo*-M enzymatic catalysis. Thus, glycosylated analogues[170] of Peptide T, a partial sequence of the HIV envelope glycoprotein gp120 which has been reported to block infection of human T cells by human immunodeficiency virus,

have been prepared to improve the conformational stability and resistance against protease digestion. A recent paper[171] describes the first report on the artificial addition of *N*-linked oligosaccharides to the bioactive peptide eel calcitonin having *no* natural sugar chains (Scheme 65). Eel calcitonin (eCT) is a calcium regulating hormone that consist of 32 amino acid residues and has a consensus sequence of 'Asn-Leu-Ser' for *N*-glycosylation but no sugar chains. The complex eCT glycopeptide was synthesized to study the influence of the oligosaccharide attached to the Asn residue on the structure and biological activity of the eel calcitonin.



Scheme 65.

3.3. Enzymatic glycoprotein synthesis: pure homogeneous glycoforms

Solid-phase peptide synthesis (SPPS) provides rapid and efficient access to peptides in the range of 3–40 amino acid residues. During the synthesis of larger fragments, uncoupled sequences, side-products and epimers accumulate resulting in dramatically decreased yields and purities of the final products.

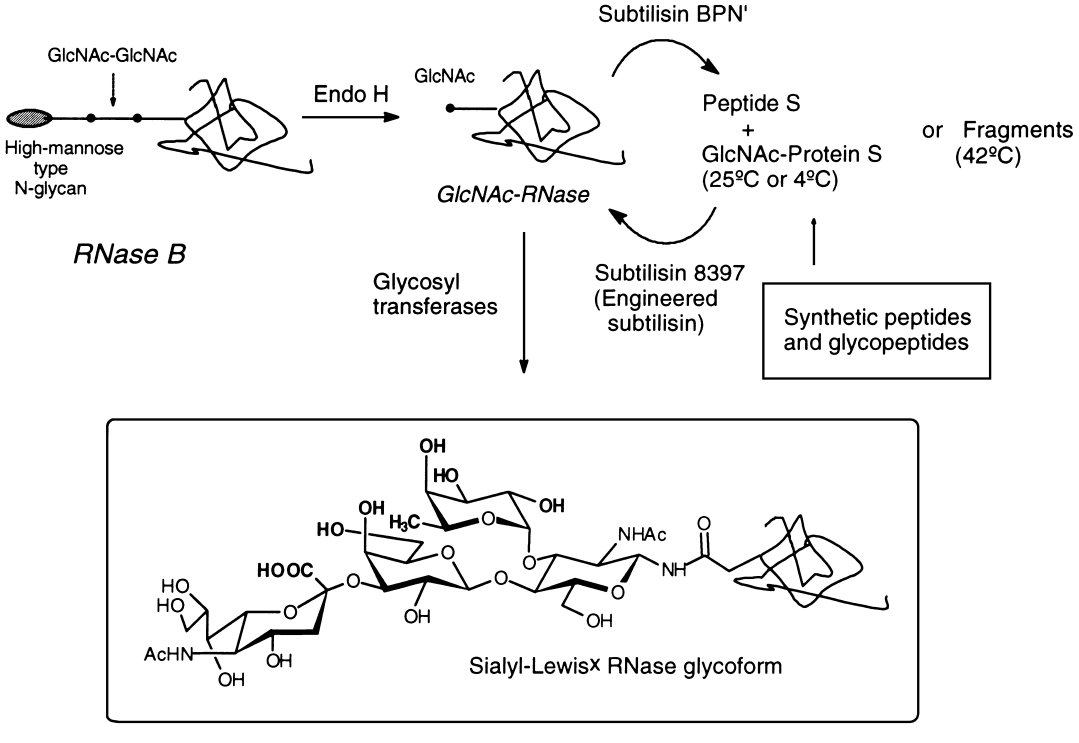
The synthesis of large peptides via condensation of peptide fragments synthesized by solid-phase methods seems to be more feasible. The intermediates can be purified, and isolation of the product, which differs greatly in molecular weight from the individual fragments, is carried out easily. Chemical fragment condensations, however, suffer from poor solubility of the protected peptide fragments and are prone to racemization at the C-terminus of the acyl donor. In contrast, enzyme-catalyzed fragment condensations are free of racemization and use side-chain unprotected substrates, increasing the solubility of the peptide fragments. Most commonly the kinetically controlled approach is exploited using proteases together with peptide esters as acyl donors. Because of its broad substrate specificity, subtilisin and engineered variants thereof have found wide application in peptide and glycopeptide coupling reactions. This development is the result of the availability of several engineered thermostable subtilisins and new mechanistic insights into controlling its preference for peptide bond formation or peptide bond cleavage.

This enzymatic method can be applied to glycopeptide synthesis and is especially valuable for the synthesis of large glycopeptides. In this approach not only proteases but also glycosyltransferases[172] have also been exploited for the solution and solid-phase synthesis of glycopeptides.[173]

In order to study the effects that carbohydrates have on glycoprotein structure and function, it is imperative to be able to synthesize the appropriate natural and non-natural glycoprotein variants in a single form. Progress has been made towards the use of in vivo techniques such as glycosyl engineering[174] and cell line mutations to prepare specific glycoproteins.[175] However, these methods still produce only heterogeneous mixtures[176] of different glycoforms (glycoproteins possessing heterogeneous distributions of carbohydrates) and enzymatic in vitro chemo-enzymatic methods have been pursued to overcome this problem. For example, homogeneous glucocerebrosidase used in the treatment of Gaucher's disease has been prepared via enzymatic removal of the heterogeneous sugar chains,[177] and neoglycoproteins[178] have been prepared via chemical glycosylation at sites other than the normal *N*- and *O*-glycosylation sites.[179]

The enzymatic condensation strategy under kinetic control has been used in protein synthesis[180] in conjugation with glycosyl transferase reactions by Wong et al.[181] as a important approach for preparing pure glycoproteins. Much of the success of this approach has to be credited to the impressive studies of Wells who engineered others for the total synthesis of ribonuclease A and analogues through enzymatic peptide synthesis.[182]

In Wong's approach, glycoproteins possessing heterogeneous distributions of carbohydrates (glycoforms) that have been prepared by recombinant DNA may be remodelled to a homogeneous species via enzymatic synthesis by use of endoglycosidases, proteases and glycosyl transferases (Scheme 66). As shown in the scheme, two strategies are used in the synthesis of glycoproteins. The first uses endoglycosidases to remove heterogeneous carbohydrates of glycoproteins and glycosyltransferases to attach the desired carbohydrates. The second strategy is a new chemo-enzymatic process using synthetic peptides and glycopeptides as substrates for enzymatic ligation catalyzed by proteases followed by further enzymatic glycosylations.



Scheme 66. *Glycoprotein synthesis*. Enzymatic synthesis of ribonuclease glycoforms through carbohydrate remodelling and glycopeptide coupling

To prove this concept ribonuclease B was chosen by Wong as the model system since it contains a single glycosylation site at asparagine 34. Several glycoforms exist in nature but all are of the high mannose type. A variant of RNase possessing a single *N*-acetylglucosamine (GlcNAc) at Asn34 was synthesized by treating RNase B (a mixture of glycoforms) with endoglycosidase H (*endo*-H) to produce the homogeneous product GlcNAc-Rnase. The initial transferase reaction was mediated by β-1,4-galactosyltransferase (GalT) and the oligosaccharide moiety was further enlarged by use of two different glycosyltransferases: (i) the α-1,3-fucosyltransferase (FucT); and (ii) the α-2,3-sialyltransferase (SialT). Alternatively, the GlcNAc-RNase was subjected to limited proteolysis by subtilisin BPN′ under two different sets of conditions. At 4 or 25°C two fragments were produced, GlcNAc-Protein S and peptide S that were religated by addition of 9 vol. of glycerol and subtilisin 8397.

This strategy offers access to previously unavailable unnatural glycopeptide/proteins through manipulation of monoglycosylated species which are available via selective enzymatic cleavage of the carbohydrate moiety. Wong et al.[183] have recently described the solution- and solid-phase synthesis of glycopeptides containing a GlcNAc moiety at different positions and have investigated them as substrates for subtilisin-catalyzed glycopeptide condensation, with the aim of developing enzymatic syntheses of complex glycopeptides and glycoproteins.

4. Conclusions

Molecular recognition of carbohydrates and related structures, such as glycoproteins, in biological systems represents a new frontier of research.

Current synthetic methodologies are nowadays able to give access to most of the known glycans found in glycoproteins. However, most of the synthetic routes are still very cumbersome and low yielding and so we are far from the ‘custom’ synthesis level of other biopolymers such as peptides and oligonucleotides. Accordingly, there is plenty of room for new methodologies in carbohydrate chemistry, in particular for glycosylation methods, which need to be more regio- and stereoselective. Particularly valuable will be those methods that will not require the assistance of protecting group schemes.

If complex glycan targets have become less than a synthetic challenge in molecular glycobiology it is because of powerful alliances between chemical and biological disciplines. In particular, the availability of many natural and engineered glycosidic enzymes throughout molecular cloning and expression has led to decisive advances and will continue fostering the process by providing enzymes such as sulfotransferases,[184] fucosyl transferases,[185] GlcNAc transferases[186] and sialidases.[187]

More important advances will definitely come from approaches that view living cells as complex machines[188] whose architecture and metabolic pathways can be understood and manipulated at the molecular level. It will not be long before, by integrating chemical and genetic approaches, it will be possible to direct the construction of cell surfaces with well-defined oligosaccharide landscapes.

In the meantime, chemistry will continue to play a key role in uncovering the molecular mechanism of carbohydrate recognition, by providing synthetic oligosaccharide materials and by designing and developing novel structures to combat diseases with new therapeutic and diagnostic carbohydrate agents.

5. Abbreviations

AA: amino acid; Ac: acetyl; AgOTf: silver trifluoromethanesulfonate; All: allyl; Anth: anthracene; Aq.: aqueous, Asp: aspartic; Asn: asparagine; BOP: benzotriazol-1-yloxytris(dimethylamino)

phosphonium hexafluorophosphate, Bn: benzyl; CHO: Chinese hamster ovary; GlcNAc: *N*-acetyl glucosamine; DBMP: 2,6-di-*tert*-butyl-4-methylpyridine; DCC: dicyclohexylcarbodiimide; DDQ: 2,3-dichloro-5,6-dicyano-1,4-benzoquinone; Dhbt-OH: 3,4-dihydroxy-3-hydroxy-4-oxo-1,2,3-benzotriazole; DIEA/DIPEA: ethyldiisopropylamine; DMAP: 4-dimethylaminopyridine; Dol: dolichol; EEDQ: 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline; Fmoc: (9-fluorenyl)methoxycarbonyl; Fuc: Fucose; HBTU: *O*-(1*H*-benzotriazol-1-yl); *N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; Hmb: 2-hydroxy-4-methoxybenzyl; HOBt: 1-hydroxybenzotriazol; IAD: intramolecular aglycon delivery; IDCP: iodonium di-*sym*-collidine perchlorate; IIDQ: 2-isobutoxy-1-isobutoxycarbonyl-1,2-dihydroquinoline; Man: mannose; MCPS: multiple column peptide synthesis; MeOTf: methyltrifluoromethanesulfonate; Mp/MP: *p*-methoxyphenyl; NeuAc: *N*-acetylneuraminic acid, sialic acid; NMM: *N*-methylmorpholine; PEG: poly(ethylene) glycol; Pfp: pentafluorophenyl; Pht: phthaloyl; PMB: *p*-methoxybenzyl; *p*-NP: *p*-nitrophenyl; Pr: protecting group; Py: pyridine; PyBOP: benzotriazole-1-yl-oxy-tripyrrolidino-phosphonium hexafluorophosphate; RNase: ribonuclease; TBAF: tetrabutylammonium fluoride; $TBANO_2$: tetrabutylammonium nitrite; TBDPS: *tert*-butyldimethylsilyl; TBS: *tert*-butyldiphenylsilyl; TBTU: *O*-(1*H*-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; TDS: thexyldimethylsilyl; Tf: triflate; Tf_2O: triflic anhydride; TMSOTf: trimethylsilyl trifluoromethanesulfonate; WRK: 'Woodward's reagent K', *N*-ethyl-5-phenylisoxazolium-3′-sulfonate; WSC: 'water soluble carbodiimide' 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide; Z: benzyloxycarbonyl.

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