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GLYCOPINION MINI-REVIEW

A general approach to the synthesis of *O*- and *N*-linked glycopeptides

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Introduction

In contrast to other proteins, specifically glycosylated proteins are not easily available by gene-technology since they are posttranslational products resulting from the activity of trimming glycosyl hydrolases and transferases [1–7]. Therefore, organic synthesis provides a valuable alternative for the preparation of model glycopeptides. Direct condensation of oligosaccharides with peptides in a convergent fashion which may at first sight seem advantageous has, however, been hampered by the inherent difficulty of reacting specifically and partially protected oligopeptides with suitable activated and protected glycosyl donors [8–12]. The current status has been reviewed recently [6, 13–15]. Therefore other approaches have been studied, particularly the sequential routes for the preparation of glycopeptides and artifical conjugation techniques for glycosylation of proteins. We have found that sequential solid phase peptide synthesis based on Fmoc strategy in combination with the use of suitable activated and glycosylated building blocks has been the most valuable and versatile alternative to the above mentioned convergent approach. Partial structures of glycoproteins consisting of longer peptides with oligosaccharides attached are very useful for the study of structure-function relationships e.g. conformational preferences in the interactions between sugar and peptide [16–22]. Such glycopeptides may also allow the application of glycosyl transferases for the elongation into more complex glycopeptides and the study of protective functions of oligosaccharides on proteins in terms of biodegradation and structural stability. Furthermore, glycopeptides can mimic complex oligosaccharide structures and thus provide a much simpler access to important signal molecules as will be discussed below. This new concept of glycopeptide synthesis and their application as complex oligosaccharide mimics [23] may well be the most important future use of glycopeptides as an alternative to the preparation of naturally occurring fragments thought to be biologically significant [2].

Methodology

Glycopeptides can be prepared by the following conceptually different methods:

(a) Direct glycosylation of suitably protected peptide acceptors to give *O*-linked glycopeptides.

(b) Acylation of glycosyl amines with peptides containing a suitably activated aspartic or glutamic acid to give *N*-linked glycopeptides [9, 10].

(c) Either chemically- or enzymatically promoted segment coupling in a solution of peptides and glycosyl amino acids or small glycopeptide fragments [24].

(d) Chemical conjugation of oligosaccharides or glycosides with peptides (or proteins) through lysine by reductive amination or via activated aspartic or glutamic acid side chains most often leading to non-natural type of glycopeptides or glycoproteins.

(e) Enzyme mediated coupling of suitably activated sugar derivatives and peptides [25] or glycopeptides on either solid phase [26] or in solution [27, 28].

(f) Solid phase glycopeptide synthesis using glycosylated building blocks in standard or multiple synthesis protocols.

The first mentioned methodology (a) has gained some attention and several reports have been published during the last couple of years describing moderate success in the preparation of glycopeptides [8, 11, 29, 30]. However, for more complex of oligosaccharides and also for longer (>3–5 amino acids) peptides with multiple potential *O*- or *N*-linked attachment sites, this methodology will not in the

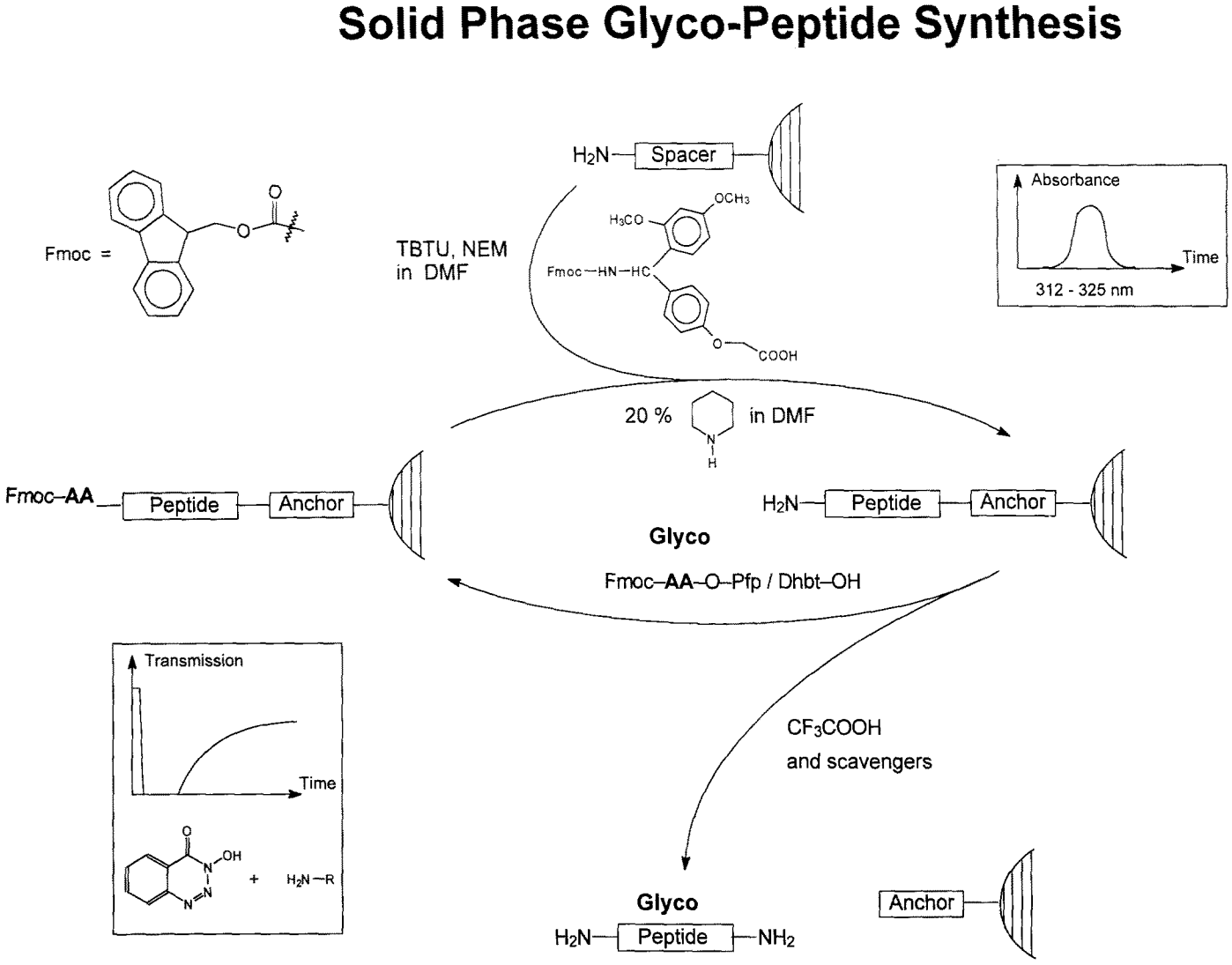


Figure 1. Schematic presentation of the principles used for the solid phase approach to glycopeptide synthesis with monitoring of both the coupling (colour development and disappearance using Dhbt-OH) and deprotection (by UV-absorbance of eluent from Fmoc cleavage).

present author's opinion be an attractive alternative for the construction of glycopeptides.

The second alternative (b) for the *N*-linked glycopeptides has recently been successfully accomplished in several reports. But the same problem as mentioned above for more complex glycopeptides with multiple attachment sites will render this approach unattractive as a general methodology for the preparation of glycopeptides.

The chemically mediated fragment coupling in solution of suitably activated glycosyl amino acid derivatives and peptide fragments or amino acids has until a few years ago been the most successful methodology for the chemical synthesis of glycopeptides [14, 31–34]. Thus several papers particularly from Kunz [31, 35], Ogawa [36, 37] and Paulsen [33, 38–41] have been published describing the synthesis of smaller (~8 amino acid or less) glycopeptides by solution synthesis. However, this methodology is quite laborious and cumbersome to carry out as a more general approach. Segment coupling promoted by proteolytic enzymes has been reported by Wong *et al.* [24] for the preparation of glycopeptides, and also enzyme catalysed modification of preformed (method a) glycopeptides to form more complex carbohydrates on the glycopeptides has been published recently [27, 28, 42].

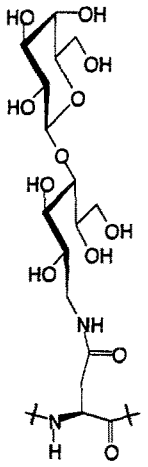
The conjugation of carbohydrate derivatives to proteins or peptides (method d) is beyond the scope of this review but an excellent review by Magnusson *et al.* [43] will appear shortly and present a broad overview of the many different conjugation or polymerization techniques available for the preparation of glycoconjugates.

Successful glycosyl transfer directly to the peptide by use of glycosyl transferases has been reported for *O*-linked GalNAc glycopeptides [25]. However, this methodology is difficult to scale up to larger amounts due to the generally

Table 1. Glycosylation effects on hydrolysis of fluorescence quenched (glyco)peptides by subtilisin Carlsberg [60].

				Scissile bond ↓					
P_5	P_4	P_3	P_2	P_1	P'_1	P'_2	P'_3	P'_4	k_{cat}/K_M ($min^{-1}\,\mu M^{-1}$)
ABz—	Phe—	Gln—	Pro—	Leu—	Asp—	Glu—	Tyr(NO_2)—	Asp—OH	920
ABz—	Asn—	Gln—	Pro—	Leu—	Asp—	Glu—	Tyr(NO_2)—	Asp—OH	2
ABz—	Asn*—	Gln—	Pro—	Leu—	Asp—	Glu—	Tyr(NO_2)—	Asp—OH	0.83
ABz—	Phe—	Asn—	Pro—	Leu—	Asp—	Glu—	Tyr(NO_2)—	Asp—OH	200
ABz—	Phe—	Asn*—	Pro—	Leu—	Asp—	Glu—	Tyr(NO_2)—	Asp—OH	280
ABz—	Phe—	Gln—	Pro—	Asn—	Asp—	Glu—	Tyr(NO_2)—	Asp—OH	18
ABz—	Phe—	Gln—	Pro—	Asn*—	Asp—	Glu—	Tyr(NO_2)—	Asp—OH	0.37
ABz—	Phe—	Gln—	Pro—	Leu—	Asn—	Glu—	Tyr(NO_2)—	Asp—OH	4100
ABz—	Phe—	Gln—	Pro—	Leu—	Asn*—	Glu—	Tyr(NO_2)—	Asp—OH	960
ABz—	Phe—	Gln—	Pro—	Leu—	Asp—	Asn—	Tyr(NO_2)—	Asp—OH	72
ABz—	Phe—	Gln—	Pro—	Leu—	Asp—	Asn*—	Tyr(NO_2)—	Asp—OH	1100

Asn* =



low availability of the transferases at the present time. Furthermore, it suffers from the serious problem of selective reactions at predetermined sites along the peptide chain in cases where more than one serine or threonine unit is present.

Therefore the most versatile and general approach presently available for the preparation of a large variety of glycopeptides with well defined and predetermined structures appears to be the last mentioned methodology (f) using sequential solid phase glycopeptide synthesis. These methods will therefore be described in more detail below.

The preparation of the glycopeptides is based on the well known techniques developed over many years for the assembly of peptides using solid phase methodology. However, due to the acid lability of interglycosidic bonds and the covalent linkage of sugar to the peptide, the Boc strategy is not compatible with glycopeptides synthesis since it requires anhydrous HF or other strong acids in the cleavage from the resin. The milder conditions used in the Fmoc strategy are much more compatible with the nature of the carbohydrate moiety and can therefore be used for the synthesis of glycopeptides. Many different glycosylated amino acid derivatives with more or less permanent protection of the α-amino and the α-carboxyl groups have been reported [31, 44–46]. In many instances the permanent character of these protection groups has, however, prevented the application of these derivatives for the solid phase synthesis of larger glycosylated peptides. Over the last 4 years we have reported [15, 18, 47–60] on the application of the pentafluorophenyl (Pfp) ester of fluorenyl methyloxycarbonyl (Fmoc-)amino acids for the preparation of biologically important glycopeptides. In this simplified version of a building block concept the Pfp ester serves the dual purpose of protecting the carboxylic acid during glycosylation and activating the carboxyl group for the subsequent amide bond formation. Furthermore Pfp-esters are stable under the conditions used both for RP-HPLC and silica-gel chromatography. The method has proved to be general and can be applied for solid phase synthesis of all types of glycopeptides according to their chemical linkage, (1) *N*- [18, 49, 61], (2) aromatic *O*- [56, 57] and (3) aliphatic *O*-linked [50, 51, 53, 54, 58, 60] glycopeptides. As indicated in Fig. 1 the methodology offers monitoring of the coupling step using catalytic

amounts of Dhbt-OH in combination with the Pfp esters [62]. However, UV-monitoring [63] of the Fmoc-cleavage was found also to be important in order to avoid deletion sequences and impure products [64]. We generally use preparative HPLC (if needed at all) for purification and amino acid analysis, sequence analysis, full assignment by NMR spectroscopy and ES-MS for the characterization of the glycopeptides, to assure that the correct compound has been synthesized and that no racemization has taken place. We have not yet observed examples where it has not been possible to accomplish the synthesis of the desired glycopeptides. The examples have been varied from simple mannose-1 → 2-mannose *O*-linked IGF glycopeptides with 18 amino acids [58] to multiple synthesis of over 48 T- and T_N-antigen [52, 65] containing glycopeptides for the study of transferase activity, to the more recent synthesis of linear and cyclic glycopeptide templates as analogues of complex carbohydrates, e.g. the high mannose-type oligosaccharides. The interaction of these templates with the mannose 6-phosphate receptors has been examined with exciting results [66].

Conclusions

Using the above mentioned techniques it has been possible to assess some of the aspects recently put forward about the function of sugars on proteins by Varki [2].

Interestingly it has been observed that:

(1) The most general method for the preparation of well defined glycopeptides today is the use of solid phase glycopeptide synthesis using suitably protected glycopeptide building blocks in combination with Fmoc chemistry and ester protection of the sugar parts.

(2) New protecting groups for the sugar using silyl groups can be extremely useful for the reattachment of larger oligosaccharides to building blocks and subsequent incorporation in well defined glycopeptides [61, 67].

(3) The conformational perturbations of mono-, di- and small oligosaccharides on shorter glycopeptides is marginal. Interactions are observed only between the carbohydrate and the linkage amino acid and its neighbouring units. Larger perturbations have have detected by the current methodology, i.e. primarily NMR spectroscopy, only when several neighbouring units were glycosylated [16, 22].

(4) The protection of peptides by glycosylation towards protease digestion has been demonstrated in several examples in the literature. However, a recent systematic study [61] has shown that this is extremely dependent on the site of glycosylation relative to the scissile bond (data in Table 1) and the results point towards a possible new effect of the glycosylation of proteins, i.e. as specific cleavage signals for enzymes.

(5) The use of glycosylated peptide templates as oligosaccharide mimics has proved to be a very interesting route [66] to be exploited in much more detail, particularly using peptide or glycopeptide library techniques to assay for the biologically active compounds.

Therefore the field of glycopeptide synthesis holds great promise for the next decade and will allow biochemists to assess much more specific and relevant information about the role of sugars on proteins. The challenges for the synthetic chemist are numerous and particularly the use of glycopeptide templates as complex oligosaccharide mimics may open up a completely new and competitive alternative to traditional glycoside synthesis of complex oligosaccharides and eventually for the more general use of glycopeptides or glycopeptide mimetics as potential drugs.

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