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Synthetic glycosylation of peptides using unprotected saccharide β-glycosylamines

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Glycopeptides can be valuable tools in determining the influence of carbohydrate moieties on the intrinsic properties of glycoproteins. However, glycopeptides of sufficient quantity and purity are as yet not readily available from biological sources. The chemical coupling of a β-glycosylamino group of an unprotected carbohydrate with an activated aspartic acid residue of an unprotected peptide is a simple method for synthesizing asparagine-linked glycopeptides. In this report we demonstrate that the use of this method is not restricted to β-glycosylamines of simple monosaccharides or short aspartic acid-containing pentapeptides. This is illustrated by the syntheses of several glycopentapeptides containing *N*,*N*′-diacetylchitobiose, a glutamine-linked glycopentapeptide containing a biantennary complex oligosaccharide, and glycosylated variants of two analogs of a polypeptide hormone, atriopeptin, containing *N*,*N*′-diacetylchitobiose.

Keywords: carbohydrate, attachment, peptide, hormone

Abbreviations: Ac, acetyl; Bzl, benzyl; DMF, dimethylformamide; Fmoc, 9-fluorenylmethoxycarbonyl; Fuc, fucose; Gal, galactose; GlcNAc, *N*-acetylglucosamine; HBTU, *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluroniumhexafluorophosphate; HOBt, 1-hydroxybenzotriazole; Man, mannose; *m*/*z*, mass/charge; NMR, nuclear magnetic resonance; Xyl, xylose; Z, benzyloxycarbonyl; unless otherwise specified, amino acids are abbreviated using their one-letter codes.

Introduction

Secreted and membrane proteins can be subject to a variety of co- or post-translational modifications involving carbohydrate moieties. One of the most common is *N*-glycosylation. This involves the attachment of oligosaccharides to the γ-amide group of asparagine in the consensus tripeptide Asn-X-Thr/Ser, where X is any amino acid other than proline [1]. *N*-linked oligosaccharides are highly branched and structurally complex molecules. A large body of evidence indicates that oligosaccharides can influence not only the physical properties of a glycoprotein [2], but also its bioactivity, biodistribution, immunogenicity, and circulatory life-time [3]. Evidence for the roles of oligosaccharides in biological recognition events is also accumulating [4–7]. Studies utilizing a range of glycopeptides which represent partial structures of glycoproteins will provide insights into how the carbohydrate moiety of a glycoprotein can influence its biological properties. However, glycopeptides of sufficient quantities and purities are not obtained easily from biological sources or recombinant gene technological procedures.

Currently there are two approaches taken to synthesize *N*-linked glycopeptides. The first involves the co-synthetic incorporation of the sugar into the peptide chain as a glycosyl aspartic acid. This method has been successful in producing short *N*-linked glycopeptides having either an *N*-acetylglucosamine or an *N*,*N*′-diacetylchitobiose moiety [8]. The main disadvantage with this method is the exposure of *O*-glycosidic linkages to the acidolytic deprotection conditions commonly used in peptide synthesis [9].

The second and chemically less demanding approach involves the direct coupling of an oligosaccharide β-glycosylamine to an aspartic acid containing peptide. Initial syntheses using this approach resulted in low yields [10, 11]. A more recent study has demonstrated that this approach can be used to obtain *N*-linked glycotri- and pentapeptides in high yields [12] if competing intramolecular succinimide formation [13] is minimized. However, it has not been shown that this method can be applied to larger saccharides or longer peptides, since only the monosaccharide *N*-acetylglucosamine and tri- and pentapeptides were used in the study.

In this report, we demonstrate the usefulness of the post-synthetic route to obtain various *N*-linked glycopeptides by coupling the unprotected β-glycosylamine of

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N,N'-diacetylchitobiose to several pentapeptide amides and two analogs of a polypeptide hormone, atriopeptin. More significantly, we show that an unprotected β-glycosylamine of a complex oligosaccharide can also be coupled to a synthetic pentapeptide.

Materials and methods

Chemicals

Piperidine, chloroacetic anhydride, ammonium hydrogen carbonate, dimethylsulfoxide, 1-hydroxybenzotriazole (HOBt) and *O*-benzotriazol-1-yl-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HBTU) were purchased from Fluka Chemicals Ltd. (Glossop, Derbyshire, UK). Sodium hydrogen carbonate and diethyl ether were purchased from Merck Ltd. (Poole, Dorset, UK). Hydrochloric acid, phenylisothiocyanate and acetic acid were purchased from Pierce and Warriner Ltd. (Chester, UK). Acetonitrile and DMF were purchased from Romil Chemicals Ltd. (Loughborough, Leichestershire, UK). Trifluoroacetic acid and 2,5-dihydroxybenzoic acid were purchased from Aldrich Chemical Company Ltd. (Gillingham, Dorset, UK). All other chemicals used were of AR quality or the highest grade commercially available.

Glycosylamines

N,N'-Diacetylchitobiose was purchased from Sigma Chemical Company Ltd. (Poole, Dorset, UK). G2 biantennary complex sugar [Galβ4GlcNAcβ2Manα6(Galβ4GlcNAc^{V} β2Manα3)Manβ4GlcNAcβ4GlcNAc] purified from transferrin was purchased from Oxford Glycosystems Lrd. (Abingdon, Oxon, UK). Glycosylamines were formed using a modification of the method of Likhosherstov *et al.* [14] as described previously [15]. Dried sugar samples were resuspended in 200–500 µl sterile saturated ammonium hydrogen carbonate. Solid ammonium hydrogen carbonate was added periodically to maintain saturation during an incubation period of 96 h at 30 °C. Samples were then desalted by lyophilization of the reaction mixture. Typically, samples were diluted to 1 ml with sterile distilled water, shell frozen and lyophilized. Sublimation of salt was accelerated by repeated additions of water at 6–8 h intervals. Lyophilized glycosylamine preparations were stored desiccated at −20 °C. Thin layer chromatography and ^{1}H-NMR were used to check the glycosylamine products.

Peptides

Synthetic peptides Fmoc-ADASF-NH_2, Fmoc-ADPTF-NH_2, Fmoc-AEATF-NH_2, and Ac-ADASF-NH_2 were purchased from the Oxford Centre for Molecular Sciences (Oxford, UK). Synthetic peptide Z-MDPT(Bzl)F-NH_2 was purchased from Cambridge Research Biochemicals (Northwich, Cheshire, UK). Atriopeptin analogs were provided by Monsanto Corporate Research (St Louis, Missouri, USA). With the exception of the atriopeptin analogs and Z-MDPT(Bzl)F-NH_2 for all peptides were supplied resin-bound. Cleavage of protected peptides from resin was achieved using a trifluoroacetic acid: water mixture (95: 5 by vol) for 2 h. The peptide solution was dried using a rotary film evaporator, with three flushes of diethyl ether. The residue was dissolved in dimethylformamide (DMF) and the peptides purified by reversed phase HPLC on a Vydac 214TP510 C_4 preparative column (1 cm × 25 cm) as described below. The composition and purity of the isolated peptides were analysed by mass spectrometry and PicoTag.

Synthesis

Glycopeptide synthesis was carried by a method based on that of Anisfeld and Lansbury [12] and as follows. Free peptide (5 µmol) was dissolved in 100 µl DMF, 5 µmol HOBt was dissolved in 50 µl DMF, 15 µmol HBTU was dissolved in 100 µl DMF and 10 µmol *N,N'*-diacetylchitobiose glycosylamine was dissolved in 135 µl dimethylsulfoxide: DMF mixture (1.7:1 by vol). In contrast, 1 µmol G2 oligosaccharide glycosylamine was used to couple to 1 µmol pentapeptide using 1 µmol HOBt and 3 µmol HBTU. For synthesis of glycosylated atriopeptin analogs A and D, 0.5 µmol peptides, 10 µmol *N,N'*-diacetylchitobiose glycosylamine, 0.5 µmol HOBt and 1.5 µmol HBTU were used. These were then mixed together and the mixture stirred gently. The progress of the reaction was followed by reversed phase HPLC of small aliquots using Vydac 214TP54 C_4 (0.46 cm × 25 cm) analytical column. Gradient and monitoring conditions were as described below for the preparative column but with a flow rate of 1 ml min^{-1}.

Purification

Synthetic peptides and glycopeptides were purified by preparative reversed phase HPLC carried out on a Vydac 214Tp510 C4 preparative column using a linear water: acetonitrile gradient over 20 min with a flow rate of 4 ml min^{-1} and monitoring absorbance at 225 nm. Initial gradient conditions were 5% acetonitrile, 0.1% acetic acid in water, final conditions were 100% acetonitrile, 0.1% acetic acid. Fractions containing peptides and glycopeptides were pooled separately. For the separation of nonglycosylated and glycosylated atriopeptin analogs A and D, a sequential application of reversed phase and cation exchange HPLC was required. Following reversed phase HPLC, the unresolved mixture of glycosylated and nonglycosylated atriopeptin analogs A and D was lyophilized and resuspended in 200 µl 50 mM ammonium acetate, pH 5.1, prior to cation exchange HPLC. Cation exchange HPLC was carried out on a PolyCAT A column (0.46 cm × 20 cm) using a combined pH and ionic strength gradient over a 60 min period. Initial conditions were 50 mM ammonium acetate, pH 5.1; final conditions were 250 mM ammonium acetate, pH 7.9. Fractions containing absorbance peaks were pooled and analysed by mass spectrometry and PicoTag.

Analyses

HPLC columns were purchased from Hichrom Ltd. (Reading, Berkshire, UK). HPLC was performed using a Waters HPLC system controlled by Waters Expert Software (version 6.2) running on a DEC 380 computer. The system consisted of two Waters 510 pumps fed through an Erma degasser and a Valco U6 manual injector. Column eluents were monitored with either a Beckman 163 variable wavelength detector or a Waters 490 multi-wavelength detector and collected using a Pharmacia Frac-100 fraction collector.

Amino acid and hexosamine analysis was carried out as described previously [16]. Briefly, peptides were hydrolysed in vapour phase HCl for 6 h at 100 °C for hexosamine analysis or for 24 h at 112 °C for amino acid composition. Derivatization with phenylisothiocyanate was carried out by the Waters PicoTag method according to the manufacturer's protocols. The resultant phenylthiocarbamyl derivatives were separated by reversed phase HPLC on a Hichrom 5 μm Spherisorb ODS2 column (0.46 cm × 25 cm) at 50 °C using modifications of the Waters PicoTag solvent and gradient system (A. C. Willis, personal communication).

Mass spectrometry was carried out using a Finnigan Lasermat laser desorption mass spectrometer fitted with a 337 nm nitrogen laser and controlled by the manufacturer's software running on a Compaq Deskpro 386/20e computer. The laser energy used as just sufficient to ionize the sample. Samples were typically dissolved in water. Sample (0.1 μl) and matrix (0.5 μl 2,5-dihydroxybenzoic acid, 10 mg ml^{-1}, dissolved in acetonitrile: water, 1:4) were applied to standard manufacturer's targets, and allowed to dry thoroughly before being analysed. The ratio of matrix to sample was typically 1000:1.

Results

The chemical reaction scheme for *N*-linked glycopeptide synthesis is illustrated in Fig. 1. The carboxyl side chain of the aspartic acid in the pentapeptide [Z-M-D-P-T(Bzl)-F-NH_2] is first activated with HBTU in the presence of a catalyst HOBt. The unprotected β-glycosylamine is then coupled to the activated aspartic acid to form an asparagine-linked glycopeptide.

The progress of the coupling reaction between peptide and β-glycosylamine was monitored by reversed phase HPLC. The synthetic peptide had a retention time of 25.50 min. An absorbance peak with retention time of 23.80 min appeared after only 2 h of coupling reaction and approximately 60% of the starting peptide with retention time of 25.50 min remained in the mixture. Within 4 h, 85% of the initial peptide had been converted to glycopeptide (Fig. 2a). The putative glycopeptide was characterized by the PicoTag method, which confirmed the amino acid composition of the peptide (approximately equal amounts

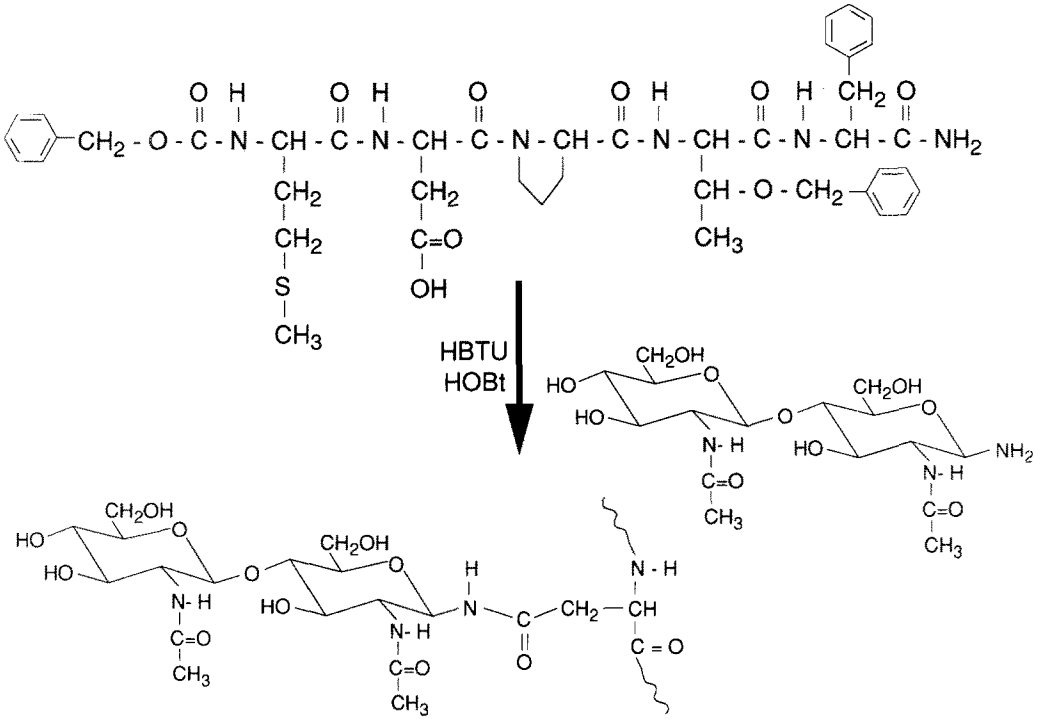

Figure 1. Post-synthetic approach to *N*-linked glycopeptide formation. A schematic diagram illustrating the coupling of an unprotected glycosylamine (*N*,*N*′-diacetylchitobiose) to the carboxyl side chain of an aspartic acid residue in a synthetic pentapeptide amide (Z-M-D-P-T(Bzl)-F-NH_2). Benzyloxycarbonyl (Z), methionine (M), aspartic acid (D), proline (P), threonine (T), benzyl (Bzl), phenylalanine (F), 1-hydroxybenzotriazole (HOBt), and *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluroniumhexafluorophosphate (HBTU).

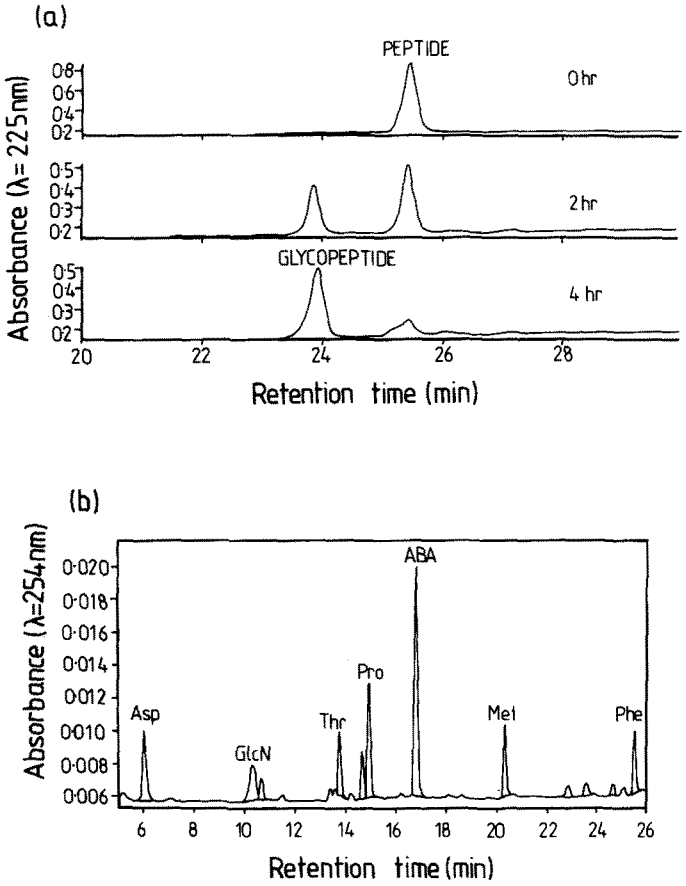

Figure 2. Reversed phase HPLC and PicoTag analysis of *N*-linked glycopeptide. (a) The coupling of *N,N'*-diacetylchitobiose glycosamine to the activated carboxyl side chain of a pentapeptide amide (Z-M-D-P-T(Bzl)-F-NH_2) was monitored at 0, 2, and 4 h by reversed phase HPLC on a C4 analytical column. Fractions containing the putative glycopeptide with retention time of 23.80 min were collected and analysed by PicoTag. (b) The PicoTag analysis of the purified glycopeptide indicated that the amino acids and hexosamine detected in the sample were aspartic acid (Asp), threonine (Thr), proline (Pro), methionine (Met), phenylalanine (Phe), and glucosamine (GlcN). Aminobutyric acid (ABA) was the internal standard used for this analysis. Absorbance was monitored at either 225 nm (a) or 254 nm (b).

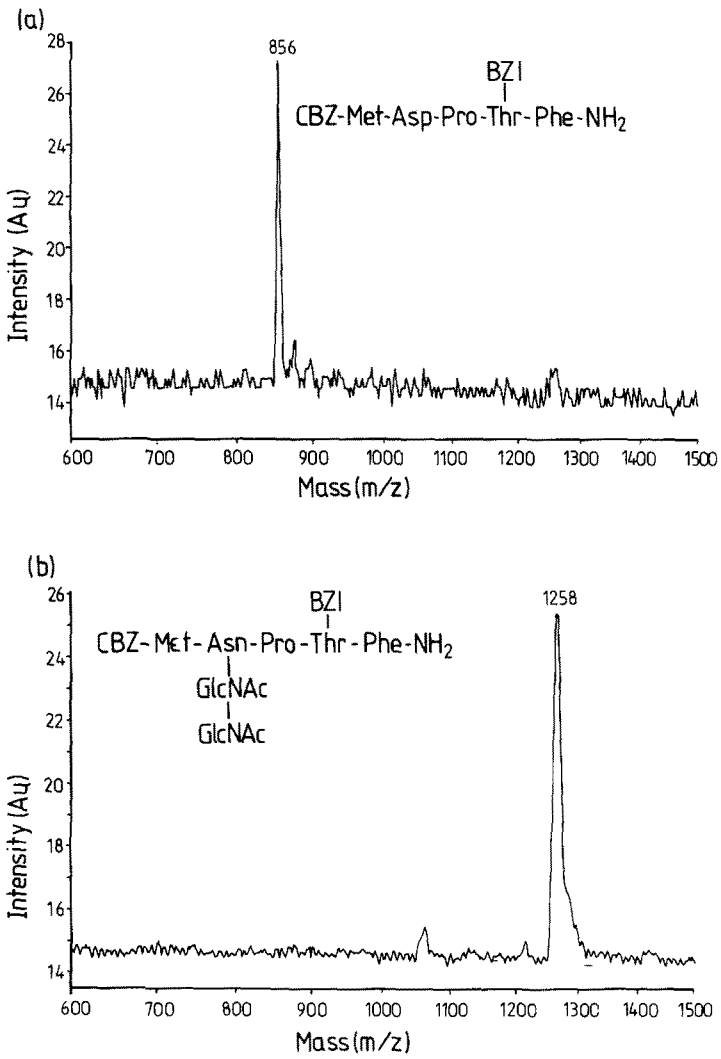

Figure 3. Laser desorption mass spectromety of purified peptide and glycopeptide. The mass of a purified pentapeptide (Z-M-D-P-T(Bzl)-F-NH_2) before and after coupling of the glycosylamine of *N,N'*-diacetylchitobiose was determined by laser desorption mass spectrometry. Both the composition of peptide and glycopeptide and their mass plus sodium adducts are shown in (a) and (b), respectively. Au, arbitrary unit of intensity.

of Asp, Thr, Pro, Met, and Phe were detected) and indicated the presence of glucosamine in the sample (Fig. 2b). Glucosamine is derived from the acid hydrolysis of the acetyl functions and the *O*-glycosidic bond in *N,N'*-diacetylchitobiose. The mole ratio of glucosamine to peptide was 2:1. Further confirmation of the peptide and glycopeptide product was obtained by laser desorption mass spectrometry, which indicated that the sodium adducts of the peptide and glycopeptide had the expected *m/z* values of 856 and 1258 based on their compositions (Fig. 3).

The β-glycosylamine of *N,N'*-diacetylchitobiose was coupled successfully to three other pentapeptides. The compositions and characteristics of these peptides and glycopeptides are summarized in Table 1. Glycopeptides GP1 and GP2 differ from each other in the GP2 contains a *N*-linked sequon whereas GP1 does not. GP3 differs from GP2 in that its amino terminus is blocked by a smaller acetyl functional group than the bulky Fmoc group used in GP2. There were no detectable differences in the rate and yield of the coupling reaction using these peptides and β-glycosylamine of *N,N'*diacetylchitobiose. Glycopeptide syntheses proceeded with similar efficiency (4 h) and yield (85%).

We have taken this post synthetic approach further by demonstrating that this strategy is applicable in generating both natural and unnatural glycopeptides involving larger sugar moieties. As shown in Fig. 4, the β-glycosylamine derivative of a biantennary complex oligosaccharide (G2)

Table 1. Summary of three glycopeptide syntheses listing the compositions, retention times on a C4 reversed phase (RP) HPLC column, and *m/z* values determined for each of the peptides (P1–3) and glycopeptides (GP1–3). The attachment site of β-glycosylamine of *N,N'*-diacetylchitobiose on each of the three peptides to produce asparagine linked glycopeptides is indicated (------). Fmoc, 9-fluorenylmethoxycarbonyl; A, alanine; D, aspartic acid; P, proline; S, serine; T, threonine; and F, phenylalanine.

Peptide	*RP-HPLC retention time (min)*	$[Mass + Na]^+$	
		Calculated	*Measured*
P1 Fmoc-A-D-P-T-F-NH_2	22.85	793	794
GP1 *N,N'*-diacetylchitobiose	21.50	1198	1200
P2 Fmoc-A-D-P-T-F-NH_2	22.65	753	753
GP2 *N,N'*-diacetylchitobiose	21.30	1158	1160
P3 Ac-A-D-P-T-F-NH_2	17.15	573	574
GP3 *N,N'*-diacetylchitobiose	16.35	978	980

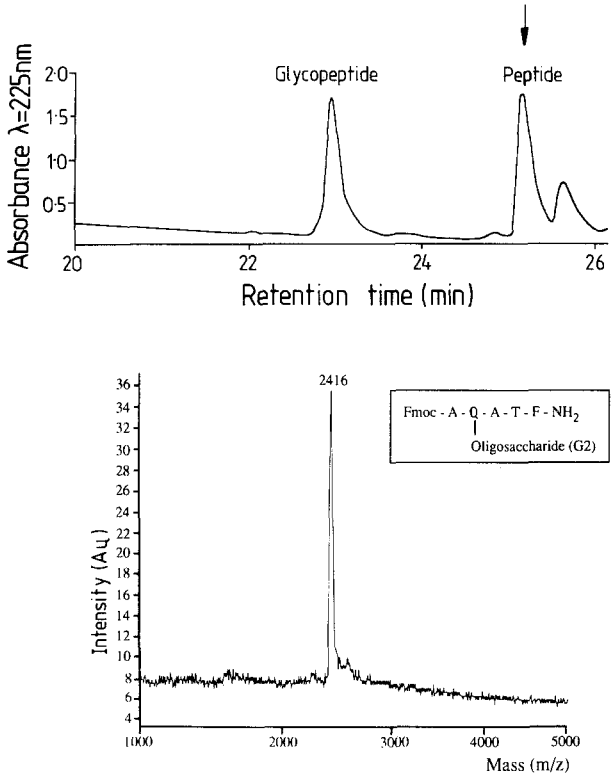

Figure 4. Attachment of oligosaccharide glycosylamine to a synthetic peptide. (a) Reversed phase HPLC profile of the coupling mixture showed two absorbance peaks with retention times of 23.0 and 25.2 min. (b) The putative glycopeptide with retention time of 23.0 min was purified and analysed by laser desorption mass spectrometry. The *m/z* value of 2416 corresponds to the sodium adduct of the putative glycopeptide shown in the inset. G2 oligosaccharide, Galβ1GlcNAcβ2Manα3(Galβ4GlcNAcβ2Manα6)-Manβ4GlcNAcβ4GlcNAc; Fmoc, 9-fluorenylmethyl oxycarbonyl; A, alanine; Q, glutamine; T, threonine; and F, phenylalanine. Peptide used was Fmoc-A-E-A-T-F-NH_2 where E is glutamic acid.

was coupled successfully to a synthetic pentapeptide producing a new peak with retention time of 23 min. Mass spectrometry revealed that this peak has a *m/z* value of 2416, which corresponds to the sodium adduct of the putative glycopeptide. As expected, the mole ratio of glucosamine to peptide was 4:1 since there were four *N*-acetylglucosamine residues in G2 oligosaccharide. The identity of a minor peak which has a longer retention time than the peptide peak (25.3 min) was not investigated. After 2 days, a yield of almost 50% had been achieved. Similar results were obtained using an oligosaccharide purified from horseradish peroxidase: Manα3(Manα6)(Xylβ2)Manβ4GlcNAcβ4(Fucα3)-GlcNAc (data not shown).

Finally, the attachment of *N,N'*-diacetylchitobiose β-glycosylamine to two atriopeptin hormone analogs was investigated. The compositions and sugar attachment sites as well as the laser desorption mass spectra of the coupling mixture are shown in Fig. 5. The *m/z* values are 2543 (peak I) and 2597 (peak III) for the proton adducts of atriopeptin analogs D and A, respectively, and the *m/z* values of 2964 (peak II) and 3002 (peak IV) correspond to the sodium adduct of the glycosylated atriopeptin D and the proton adduct of glycosylated atriopeptin A. The mole ratio of glucosamine to peptide was 2:1 as determined by the PicoTag method. The peptide analogs and their respective glycopeptides were not resolved by reversed phase HPLC on a C4 column. However, they were resolvable by HPLC cation exchange column chromatography. The elution profile (Fig. 6) showed two peaks of similar areas, indicating that the yields of glycopeptides were about 40–50%.

Discussion

The availability of a wide range of synthetic *N*-linked glycopeptides will facilitate investigations into the structural

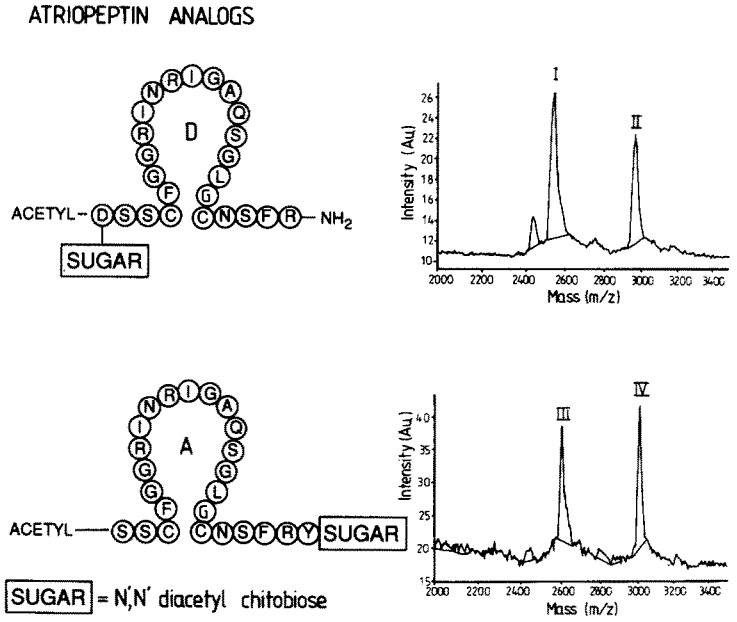

Figure 5. Glycosylated derivatives of atriopeptin analogs. The amino acid compositions and the disulfide linkage of two atriopeptin analogs are shown on the left. *N,N'*-Diacetylchitobiose attachment sites are at the aspartyl side chain and the carboxyl terminus (upper and lower panels, respectively). Laser desorption mass spectra of the synthetically glycosylated peptides and unmodified peptides are shown adjacent to the structures. The measured *m/z* values for the peptides are 2542 and 2595 (peaks I and III in upper and lower panels, respectively). The measured *m/z* values of 2968 and 3002 correspond to the proton adduct of the glycopeptide shown in the upper panel (peak II) and the sodium adduct of the glycopeptide shown in the lower panel (peak IV). The difference in masses between the peptides and their respective glycopeptides is due to the addition of *N,N'*-diacetylchitobiose β-glycosylamine minus a water molecule.

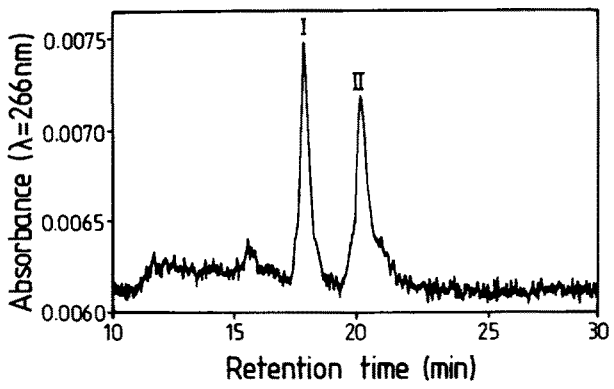

Figure 6. Separation of glycosylated atriopeptin by cation exchange chromatography. Glycosylated and nonglycosylated atriopeptin analog D were first separated from the reaction mixture containing β-glycosylamine of *N,N'*-diacetylchitobiose, and coupling reagents HBTU and HOBt by reversed phase HPLC. The nonglycosylated and glycosylated atriopeptin analog D were then resolved into two distinct peaks (I and II, respectively) by cation exchange HPLC.

and functional roles of carbohydrates in biological systems. In the case of molecules having therapeutic potential, synthetic glycosylation could also be of clinical relevance. However, examples of synthetic glycopeptides achieved in good yields (70–95%) usually consist of short tri- and pentapeptides and/or simple mono- or disaccharides. This is probably due to the limited availability of oligosaccharides from biological sources or the difficulties in glycopeptide synthesis involving oligosaccharides and polypeptides.

The use of unprotected β-glycosylamine in the synthesis of asparagine-linked glycopeptide has been demonstrated previously by the attachment of *N*-acetylglucosamine to several aspartic acid containing tri- and pentapeptides [12]. Since we and others have recently shown that β-glycosylamines of complex oligosaccharides can be obtained in high yields of 70–85% [15, 17], it becomes feasible for us to determine whether the post-synthetic route can be applied to generate more complex glycopeptides.

For our initial studies we employed short pentapeptides and a disaccharide to determine the efficiency of the coupling reaction. The unprotected β-glycosylamine of *N,N'*-diacetylchitobiose was coupled to an aspartic acid containing synthetic pentapeptide to generate an *N*-linked glycopeptide as illustrated in Fig. 1. This sugar was chosen for our studies because it is readily available in large quantities and it is part of the core structure for all *N*-glycosylation. The pentapeptide was chosen to contain a proline residue adjacent to the aspartic acid residue in order to prevent intramolecular succinimide formation [13]. The relatively new coupling reagent HBTU was used since it has been shown to enable faster and more complete couplings than HOBt esters produced by the use of dicyclohexylcarbodiimide [18].

It is clear from our results (Figs 2 and 3) that this simple one-step synthetic method is efficient (4 h) in producing the expected glycopeptide in high yield (85%). The resultant glycopeptide was resolvable from the starting peptide, which facilitated its purification by preparative reversed phase HPLC. An attempt to remove the benzyl blocking group on the threonine residue by trifluoroacetic acid treatment resulted in the partial cleavage of the *O*-glycosidic bond in *N,N'*-diacetylchitobiose (data not shown). This is in contrast to the finding of Otvos *et al.* [8], who prepared *N*-linked glycopeptides containing the same sugar residue using the co-synthetic approach. Acid lability of this *O*-glycosidic bond may be dependent on the conditions of the trifluoroacetic acid treatment. In order to eliminate the problem of having to remove blocking groups without destroying *O*-glycosidic bonds in sugars, unprotected pentapeptides were used for subsequent syntheses.

Similar high efficiency and yield of *N*-linked glycopeptides were also obtained when the unprotected β-glycosylamine of *N,N'*-diacetylchitobiose was used to couple to three pentapeptides with unprotected serine and threonine residues (Table 1). Undesirable side reactions were not detected in these glycopeptide syntheses. This demonstrates

another advantage of using HBTU as the coupling reagent in our glycopeptide syntheses. This reagent generates harmless by-products, which reduces the possible side reactions involving free hydroxyl functions [19]. This property is important if unprotected synthetic peptides and oligosaccharides are to be used to simplify the procedure. The use of unprotected oligosaccharides has an additional advantage in that *O*-acetylated glycosylamines appear to be less nucleophilic [12].

^{1}H-NMR analyses of these glycopeptides indicate that there are signals (δ 3.4–3.9) due to the sugar ring protons (H2–6 and H2′–6′) in the glycopeptides and, more importantly, there is β-stereochemistry at the anomeric position of the asparagine linked *N*-acetylglucosamine residue (δ 5.0) as indicated by a coupling constant ($J_{1,2}$) of 9 Hz (data not shown). Generation of *N*-linked glycoeptides with natural β-stereochemistry has been reported previously using either the co- or post-synthetic routes [8, 12]. Our results demonstrate that the post-synthetic approach could indeed be used for generating large quantities of short *N*-linked glycopeptides, provided that intramolecular succinimide formation is minimized.

Furthermore, the successful coupling of G2 oligosaccharide to a pentapeptide (Fig. 4) in yields of 40–50% suggests that this approach of *N*-linked glycopeptide synthesis can be extended to include much larger saccharides. The lower rate and yield compared with the glycopeptide syntheses involving *N*,*N*′-diacetylchitobiose could be due to a steric hindrance effect of a much larger saccharide and/or the amount of G2 oligosaccharide β-glycosyalmine and peptide used were not optimized. The use of larger amount (>1 μmol) of purified sugar structures such as G2 oligosaccharide for glycopeptide synthesis is possible but as yet not economically feasible. It is also noteworthy that a glutamine linked glycopeptide was produced in this synthesis. This result is significant, since it suggests the possibility of synthetic glycosylation providing a wide range of natural and unnatural short glycopeptides.

The effect of sugar attachment on the activity, biodistribution, and circulatory half-life of normally nonglycosylated bioactive peptides is as yet poorly understood, but could be of major importance if these peptides were to be used as therapeutic agents. For instance, it has been shown previously that the attachment of a galactose residue to enkephalin analogs can increase their analgesic activity by three orders of magnitude [20]. To investigate the possibility of synthetic glycosylation involving bioactive peptides we have attempted to couple β-glycosylamine of *N*,*N*′-diacetylchitobiose to two synthetic peptide analogs of atriopeptin. This polypeptide hormone is produced by the atrial muscle of the heart and exerts its effect on the kidney to regulate blood volume and pressure [21]. The therapeutic potential of this hormone is limited by its short circulatory half-life. The amino acid compositions of these two atriopeptin analogs are ideal for site-specific glycosylation since they have only one free carboxyl group and no lysine residues.

As shown in Figs 5 and 6, synthetic glycosylation of these two atriopeptin analogs was successful. The yield and efficiency were similar to the glycopeptide synthesis involving G2 oligosaccharide β-glycosylamine. This could be due to a steric effect of the polypeptide rather than the sugar in this case. There appear to be no side reactions associated with these two unprotected polypeptides containing arginine, glutamine, and asparagine residues. The addition of a disaccharide to these two polypeptide hormone analogs modified their overall hydrophobicity very little, as suggested by their incomplete separation by reversed phase HPLC (data not shown). However, removal of a negative charge following glycosylation at an activated acidic residue increased their overall net positive charge. This was the basis for the separation of glycosylated and nonglycosylated atriopeptin analogs by cation exchange HPLC (Fig. 6). The confirmation of β-stereochemistry by NMR analysis was not carried out due to insufficient amounts of the glycosylated atriopeptin analogs. It will be of interest to determine the biological activities and circulatory half-life of these glycosylated atriopeptin analogs.

In summary, we have shown that the post-synthetic route enables the generation of natural as well as unnatural glycopeptides by the synthesis of an *N*-linked glycopeptide containing the normally nonglycosylated sequon (*N*-P-T), and the attachment of a sugar to a glutamic acid side chain of a pentapeptide and the carboxyl terminus of an atriopeptin analog. More significantly, our results suggest that it is possible to extend this methodology to couple unprotected oligosaccharide β-glycosylamines to short peptides. This method should be applicable to many peptide sequences and sugar structures, although alternative strategies are required for peptide sequences containing multiple carboxyl functions and those containing unprotected lysine residues.

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