Solid-phase synthesis of a fucosylated glycopeptide of human factor IX with a fucose-α-(1→O)-serine linkage

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The chemical synthesis of a glycopeptide with L-fucose directly linked to the hydroxy group of L-serine is reported. Two building blocks containing a protected and an unprotected fucose residue α-glycosidically linked to N^{α}-Fmoc-Ser-OH were prepared and used in the synthesis of a glycopeptide fragment of the EGF domain of Human Factor IX 55–65. It was demonstrated that both building blocks were completely compatible with the standard Fmoc-based solid-phase peptide synthesis protocol and furthermore that OH protection of the carbohydrate is necessary only during the final acid treatment for cleavage of the glycopeptide from the resin.

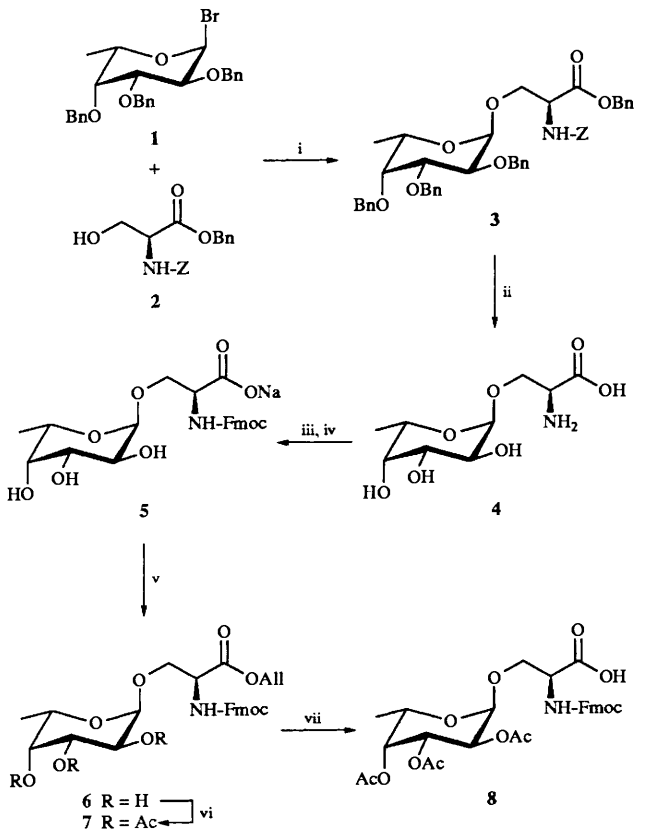
Introduction

The majority of proteins found in nature are glycosylated and the structural diversity of the oligosaccharides and the variety of linkages between reducing sugar and protein is enormous.[1] The common structure of N-linked glycoproteins consist of a pentasaccharide core with an *N*-acetylglucosamine–asparagine linkage. In O-glycoproteins the connection of *N*-acetylgalactosamine to serine and threonine, characteristic for mucin glycoproteins, occurs most frequently, but other carbohydrates, *e.g.* *N*-acetylglucosamine or mannose, have also been found attached to hydroxy amino acids. A novel type of O-glycosylation has been reported recently by several authors investigating the N-terminal Epidermal Growth Factor (EGF) domains of various coagulation and fibrinolytic proteins.[2–7] L-Fucose has been found α-glycosidically linked to serine or threonine residues within the peptide motif Cys-X-X-Gly-Gly-Thr/Ser-Cys. More recently, Harris *et al.* have identified the tetrasaccharide NeuAcα(2→6)Galβ(1→4)GlcNAcβ(1→3)-Fucα1→*O* linked to serine 61 of Human Factor IX.[7] The biological function of this unusual glycosylation is unknown and therefore a chemical synthesis of fucosylated peptides would be interesting for biological and structural investigations. Furthermore, L-fucose is known to be an important residue of branched oligosaccharides in glycoconjugates that bind to selectins, and synthetic fucosylated glycopeptides might be potential mimetics of selectin-binding oligosaccharides.[8]

Results and discussion

In this paper the chemical synthesis of a fucosylated peptide is demonstrated. The EGF domain of Human Factor IX 55–65, in which Ser-61 has been found α-fucosylated,[7] was selected as the target sequence. The assembly of glycopeptides is most efficiently done by the preparation of a glycosylated amino acid which is suitably protected for incorporation into the peptide chain by solid-phase peptide synthesis.[9,10] The synthesis of such a building block is performed by reaction of a serine derivative protected at the amino and carboxylic group and an L-fucose derivative which is activated at the anomeric position and protected at the other hydroxy groups. It is well established that α-fucosides can be synthesized in high stereoselectivity with benzyl-protected fucose donors by application of the *in situ* anomerisation procedure.[11,12] However, benzyl protection is not always compatible with peptide chemistry due to the conditions required for deprotection and the possible presence of sulfur-containing or aromatic amino acids. This means that the benzyl protecting groups of the fucose must be removed after glycosylation and that the carbohydrate part must be alternatively protected, *e.g.* with acetates or benzoates. Considering the possibility of a β-elimination under basic conditions, acetates are preferred because they are removable under mild basic conditions. The choice of benzyl protection of the fucose for the glycoside synthesis also has an influence on the selection of protecting groups of the serine acceptor. The fluoren-9-ylmethoxycarbonyl (Fmoc) group is the standard N^{α}-amino protecting group that has been used for glycopeptide synthesis. However, the Fmoc group is not completely stable under the hydrogenolytic conditions used to remove benzyl ethers. Therefore the use of the benzyloxycarbonyl (Cbz)-group as N^{α}-amino and the benzyl ester as C^{α}-carboxylic acid protecting groups was investigated.

Glycosylation of N^{α}-Cbz-serine benzyl ester **2** with 2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl bromide **1**[13] was performed in the presence of Et_4NBr in CH_2Cl_2–dimethylformamide (DMF) to afford the α-linked glycosyl amino acid **3** in 85% yield after chromatography on silica gel (Scheme 1). The presence of the α-glycosidic linkage was confirmed by the small coupling constant $J_{1,2}$ = 4 Hz in the ^1H NMR spectrum. Hydrogenolysis with a palladium/carbon catalyst gave the fully deprotected derivative **4** in quantitative yield. Introduction of the N^{α}-Fmoc group was performed using fluoren-9-ylmethyl succinimidyl carbonate (Fmoc-OSu) in aq. acetone. The sodium salt **5** was converted into the allyl ester **6** by reaction with allyl bromide in 51% yield. Acetylation of the carbohydrate OH groups with acetic anhydride–pyridine gave compound **7** (87% yield). The allyl ester was removed by a palladium-catalysed allyl transfer to morpholine to afford the building block **8** in 90% yield. The direct conversion of compound **5** into compound **8** by acetylation was not feasible due to the formation of a mixed anhydride which led to extensive decomposition during the reaction. The glycosyl amino acid **8** is a suitable building block for solid-phase glycopeptide synthesis. Activation of the carboxylic acid can be performed with *in situ* coupling reagents, *e.g.* *O*-(1*H*-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU).[14] The penalty for having acetate protection on the fucose was a considerable loss of material due to low yield in the allylation step. Therefore we investigated whether the glycosyl amino acid **5**, with unprotected hydroxy groups, could be used for solid-phase synthesis. It has been shown that free aliphatic hydroxy functions are not esterified by



Scheme 1 *Reagents:* i, Et_4NBr, CH_2Cl_2–DMF; ii, Pd/C, HCl, EtOH; iii, Fmoc-OSu, $NaHCO_3$, aq. acetone; iv, NaOH; v, AllBr, DMSO; vi, Ac_2O, pyridine; vii, $(Ph_3P)_4Pd$, morpholine, THF

TBTU activation[14] and therefore it should be feasible to couple building block **5** without any significant esterification of the carbohydrate OH groups taking place.

The two building blocks N^{α}-Fmoc-Ser(α-L-FucAc$_3$)-OH **8** and N^{α}-Fmoc-Ser(α-L-Fuc)-OH **5** were both used in the solid-phase synthesis of undecapeptide **10** (synthesis A with building block **8**, synthesis B with building block **5**). Glycopeptide **10** represents a partial structure of the EGF domain of Human Factor IX with the glycosylation site at Ser-61. Both cysteine residues, Cys-62 and Cys-56, were protected at the side chain by acetamidomethyl (Acm) groups. The syntheses were performed using a custom-made continuous-flow peptide synthesizer with monitoring of coupling reactions by the Dhbt-colour reaction.[15,16] The suitably protected, non-glycosylated N^{α}-Fmoc amino acids were activated as Pfp-esters with addition of 0.1 mol equiv. of 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (Dhbt-OH) as the acylation catalyst and indicator for the spectrophotometric monitoring of the coupling reactions. In each of the two glycopeptide syntheses, the glycosylated amino acids **5** and **8** were coupled by activation with TBTU/*N*-ethylmorpholine (NEM). Two mole equivalents of the glycosylated amino acids were used. A poly(ethylene glycol) poly(dimethylacrylamide) copolymer (PEGA),[17] derivatised with a trifluoroacetic acid (TFA)-labile Rink-Amide-Linker,[18] was used as the solid support. It has been demonstrated by Kunz *et al.* that the O-glycosidic bond of ether-type protected fucose residues show an enhanced acid lability.[19] In a previous study we have investigated the acid stability of a non-protected fucose residue linked to a small model peptide and found that the fucose will be partially cleaved upon a 2 h treatment with 95% aq. TFA.[8] For this reason the carbohydrate OH groups in synthesis B were acetylated with acetic anhydride–pyridine directly after the coupling of building block **5**. The actual acylation times for peptide elongation were 2 h for each amino or glycosyl amino acid in both syntheses, although the solid phase monitoring indicated quantitative reactions in most cases after 15–20 min. N^{α}-Fmoc deprotections were effected in 10 min by treatment with 20% piperidine/DMF.

The protected glycopeptide **9** from synthesis A was cleaved from the resin with 95% aq. TFA in 2 h and the product was purified by preparative RP-HPLC. Cleavage of the protected glycopeptide **9** from synthesis B was carried out in the same manner. Analytical HPLC of the crude product showed a major peak and a series of other peaks having shorter retention times. On a preparative scale, the fraction of the main peak was collected and the compound was analysed by electron spray mass spectrometry (ES-MS) and NMR spectroscopy, confirming the correct structure of compound **9**. The ES-MS analysis of the combined other fractions indicated the presence of compounds derived from compound **9** but lacking one or two acetates. This was further confirmed by complete deacetylation to afford compound **10**. Thus only partial acetylation is required to confer stability to fucosylated glycopeptides towards TFA. The presence of partially acetylated compounds is probably due to incomplete acetylation of the fucose hydroxy groups on the solid phase by the acetic anhydride–pyridine mixture, indicating that in contrast to acetylation in solution an extended reaction time is required for complete solid-phase acetylation of hydroxy groups.

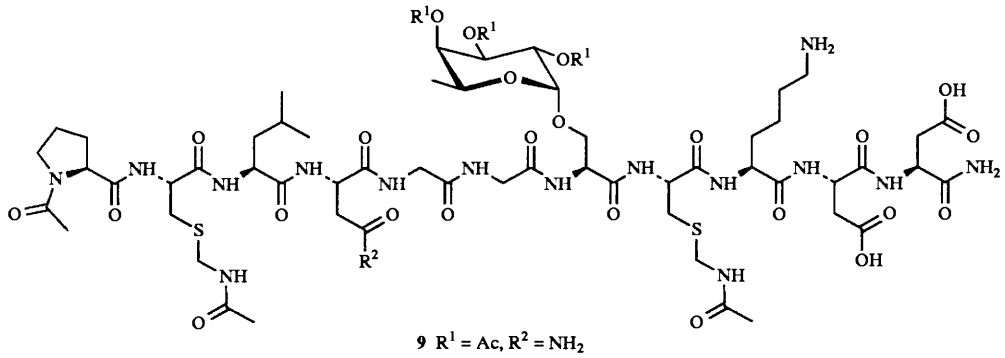
The deacetylation of the carbohydrate was performed with hydrazine hydrate in aq. methanol at a pH of 9–9.5 within 4–6 h. The excess of hydrazine hydrate was quenched with acetone after completion of the reaction. According to analytical HPLC on a standard gradient the product was more than 95% pure; however, upon applying an extended gradient a shoulder became visible. The two products were separated by preparative RP-HPLC and analysed by ES-MS and NMR spectroscopy. The analytical data of the main product (65% yield) were in agreement with the expected structure **10**. By using 2D NMR experiments a complete assignment of all ^1H and ^{13}C resonances was achieved (Tables 1 and 2). The by-product had a mass of M + 15, and the C–H-correlated spectra of compounds **10** and **11** were virtually identical except for the shift of the Asn-58 α-C which was shifted 2 ppm upfield. From these data it was concluded that the carboxamide of Asn-58 had been converted into the corresponding carbohydrazide **11**. No intermediate product resulting from reaction with methanol could be observed. This result is consistent with the absence of methyl esters during reaction of compounds containing carboxamides with dil. sodium methoxide. This side-reaction had been observed previously[8] in cases of C-terminal amides but it has not previously been observed for the side-chain of Asn residues. Deacetylation of fucose glycopeptides with stronger bases, *e.g.* sodium methoxide, caused cleavage of the sugar by β-elimination.[8]

In conclusion, a new and convenient method for incorporating α-L-fucosyl-serine residues into peptides by solid-phase synthesis has been described. The shortest and in terms of overall yield most efficient route employed a fucosyl-serine building block with an unprotected fucose residue. In order to confer sufficient stability during the TFA cleavage from the resin the carbohydrate was acetylated on the solid phase. The use of hydrazine hydrate was successful for the deacetylation of the carbohydrate: however, conversion of an Asn carboxamide into a carbohydrazide was observed as a side-reaction.

Experimental

General procedures

TLC was performed on Merck Silica Gel 60 F_{254} (E. Merck, Darmstadt) with detection by quenching of fluorescence and/or



9 R^1 = Ac, R^2 = NH_2
10 R^1 = H, R^2 = NH_2
11 R^1 = H, R^2 = $NHNH_2$

Table 1 ^{1}H NMR chemical shifts for compound **10**[a]

Amino acid	α-NH	α-H	β-H	γ-H	δ-H	ε-H	Others
Asp-65	8.17	4.76	2.92, 2.84				7.10, 7.41 ($CONH_2$)
Asp-64	8.27	4.75	2.99, 2.88				
Lys-63	8.10	4.34	1.87, 1.78	1.44	1.72	3.06	7.46 (NH-ε)
Cys-62	8.26	4.64	3.10, 2.96				
Ser-61	8.58	4.66	4.18, 3.68				
Gly-60	8.17	4.09, 4.01					
Gly-59	8.23	4.01					
Asn-58	8.21	4.76	2.92, 2.84				*b*
Leu-57	8.10	4.40	1.70	1.62	0.92, 0.85		
Cys-56	8.34	4.64	3.10, 2.93				
Pro-55		4.43	2.28, 1.95	2.00	3.70, 3.61		1.97 ($COCH_3$)
Acm	8.35	4.35					2.03 ($COCH_3$)
Acm	8.37	4.43, 4.32					2.03 ($COCH_3$)
Fucose	1-H	2-H	3-H	4-H	5-H	6-H	
	4.89	3.85	3.92	3.80	3.93	1.21	

[a] Measured in 50% CD_3CO_2D–water, internal reference acetic acid at 2.03 ppm. [b] The $CONH_2$ was not resolved because of signal overlap.

Table 2 ^{13}C NMR chemical shifts for compound **10**[a]

Amino acid	C-α	C-β	C-γ	C-δ	C-ε	Others
Asp-65	50.34[b]	36.73[b]				
Asp-64	50.75[b]	35.82[b]				
Lys-63	54.22	30.79	22.43	26.82	40.15	
Cys-62	54.45	31.90[c]				
Ser-61	54.12	66.87				
Gly-60	43.26					
Gly-59	43.54					
Asn-58	51.06	35.56[b]				
Leu-57	53.10	39.95	24.69	22.72, 20.92		
Cys-56	53.85	31.80[c]				
Pro-55	60.84	30.25	24.76	49.03		21.81 ($COCH_3$)
Acm	41.43					22.44 ($COCH_3$)
Acm	41.20					22.44 ($COCH_3$)
Fucose	C-1	C-2	C-3	C-4	C-5	C-6
	98.73	68.79	70.40	72.49	67.24	15.80

[a] Measured in 50% CD_3CO_2D–water, internal reference acetic acid at 19.997 ppm. [b,c] Assignments can be interchanged.

by charring with sulfuric acid. All organic solvents were of p.a. quality from Labscan (Ireland), DMF for peptide synthesis was of DNA grade. Column chromatography was performed on Silica Gel 60 (E. Merck 40–60 μm, Darmstadt). Concentrations were performed under reduced pressure at temperatures below 40 °C. *p*-[(α-Fluoren-9-ylmethoxycarbonylamino)-2,4-dimethoxybenzyl]phenoxyacetic acid (Rink-Linker) and suitably protected N^{α}-Fmoc amino acids were purchased from NovaBiochem (Switzerland), and TBTU and Dhbt-OH from Fluka (Switzerland). NMR spectra were recorded on a Bruker AM 300, a Bruker WM 360 or a Bruker AM 500 MHz spectrometer at 300 K and were referenced to internal $SiMe_4$

(δ_H, δ_C 0.00) for solutions in $CDCl_3$ and CD_3OD. The ^1H and ^{13}C resonances were assigned by ^1H, ^{13}C, ^1H–^1H double quantum filtered phase-sensitive homonuclear chemical-shift correlation spectroscopy (COSY), nuclear Overhauser effect spectroscopy (NOESY) and heteronuclear ^{13}C–^1H-correlated experiments. Optical rotations were measured with a Perkin-Elmer 241 polarimeter at 22 ± 2 °C, and $[\alpha]_D$-values are given in units of 10^{-1} deg cm^2 g^{-1}. FAB mass spectra were recorded using a Kratos AEIMS9 instrument on samples suspended in a matrix mixture of dithioerythritol and dithiothreitol (5:1) with xenon as the bombarding gas. ES-MS spectra were recorded in the positive mode on a VG Quattro Mass Spectrometer, with 50% aq. acetonitrile as the liquid phase. HPLC was performed on a waters system using Deltapak reversed-phase C_{18} columns (analytical: 8 × 10, 1 cm^3 min^{-1}; preparative: 19 × 300, 10 cm^3 min^{-1}). Solvent system A: 0.1% TFA and B: 0.1% TFA in 90% acetonitrile–10% water. Detection was at 215 nm.

N^{α}-Benzyloxycarbonyl-3-*O*-(2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl)-L-serine benzyl ester 3

N^{α}-Benzyloxycarbonyl-L-serine benzyl ester **2** (dried under vacuum overnight with phosphorus pentaoxide) (5.04 g, 15.3 mmol) and tetraethylammonium bromide (3.47 g, 16.83 mmol) were dissolved in dichloromethane (30 cm^3)–DMF (2 cm^3) containing crushed 4 Å molecular sieves (15 g). The solution was stirred under argon for 30 min. To this slurry was added a solution of freshly prepared 2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl bromide **1** (38.3 mmol) in dichloromethane (20 cm^3) and the reaction was allowed to proceed for 15 h under argon. Methanol (10 cm^3) was added and stirring was continued for 30 min. The reaction mixture was filtered, diluted with dichloromethane, and washed successively with water and brine. After the mixture had been dried and the solvent removed, the residue was chromatographed on silica gel [hexane–ethyl acetate (3:1)] to give *compound* **3** (9.49 g, 85%) as an oil which solidified upon storage, $[\alpha]_D$ −57.7 (*c* 0.91, $CHCl_3$); R_f 0.30 [hexane–ethyl acetate (3:1)] (Found: C, 72.3; H, 6.45; N, 1.9. $C_{44}H_{47}NO_9$ requires C, 72.01; H, 6.46; N, 1.91%); δ_H(360 MHz; $CDCl_3$) 7.10–7.15 (25 H, m, ArH), 6.09 (1 H, d, $J_{NH,CH}$ 9.5, NH), 5.23 (1 H, d, *J* 11.5, PhCH_2), 5.13 (2 H, s, PhCH_2), 5.05, 4.92, 4.76 and 4.77 (each 1 H, d, *J* 11.5, PhCH_2), 4.52–4.69 (5 H, m, 3-PhCH_2, Ser α-H, 1-H), 4.16 (1 H, dd, $J_{\alpha H,\beta H}$ 2.5 and J_{gem} 10, Ser β-H), 3.94 (1 H, dd, $J_{1,2}$ 4 and $J_{2,3}$ 10, 2-H), 3.60 (1 H, dd, $J_{3,4}$ 2.5, 3-H), 3.48 (1 H, d, J_{vic} 3.5, Ser β-H′), 3.29–3.40 (2 H, m, 4- and 5-H) and 0.91 (3 H, d, $J_{5,6}$ 6.5, 6-H); δ_C(300 MHz; $CDCl_3$) 170.10 (CO ester), 156.30 (CO carbamate), 138.88, 138.53, 136.35 and 135.51 (Ph quat.), 128.62, 128.57, 128.50, 128.38, 128.19, 128.14, 127.83, 127.64 and 127.54 (Ph methine) 98.87 (C-1), 78.87 (C-4), 77.36 (C-3), 76.50 (C-2), 74.74, 73.31 and 73.23 (PhCH_2), 69.03 (Ser C-β), 67.15 and 67.07 (PhCH_2), 66.68 (C-5), 54.51 (Ser C-α) and 16.48 (C-6).

3-*O*-(α-L-Fucopyranosyl)-L-serine 4

The protected glycosyl amino acid **3** (6.5 g, 8.86 mmol) was dissolved in ethanol (75 cm^3)–water (20 cm^3), 2 mol dm^{-3} HCl (4.5 cm^3) and 10% palladium on carbon (2.5 g) were added and the mixture was stirred under a flow of hydrogen for 24 h. After completion of the reaction, the catalyst was filtered off and the solvent was evaporated off to give title compound **4** in quantitative yield (2.22 g) as a solid [Found: (FAB-MS) *m/z*, 252.0 (M + H), 59.3%; 289.9 (M + K), 10.4%. $C_9H_{17}NO_7$ requires *M*, 251.24]: δ_H(360 MHz; D_2O) 4.90 (1 H, d, $J_{1,2}$ 1.5, 1-H), 4.20–4.29 (2 H, m, Ser β- and α-H), 3.93 (1 H, q, $J_{5,6}$ 6.5, 5-H), 3.75–3.85 (4 H, m, Ser β-H′, 2-, 3- and 4-H) and 1.19 (3 H, d, $J_{5,6}$ 6.5, 6-H); δ_C(300 MHz; $CDCl_3$) 171.39 (CO), 98.83 (C-1), 72.48 (C-4), 70.28 (C-3), 68.77 (C-2), 68.01 (C-5), 65.71 (Ser C-β), 54.37 (Ser C-α) and 16.01 (C-6).

Sodium N^{α}-(fluoren-9-ylmethoxycarbonyl)-3-*O*-(α-L-fucopyranosyl)-L-serinate 5

Compound **4** (2.22 g, 8.86 mmol) was dissolved in a mixture of acetone (100 cm^3) and water (50 cm^3). Sodium hydrogen carbonate (745 mg, 8.86 mmol) and Fmoc-OSu (6.0 g, 17.8 mmol) were added and the solution was stirred overnight. The reaction mixture was extracted with ethyl acetate, the pH of the aqueous layer was brought to 9 (pH-meter) by the addition of aq. sodium hydroxide, and the aqueous layer was lyophilised to give compound **5**. R_f 0.21 [dichloromethane–methanol (2:1)], which was not further purified; $[\alpha]_D$ −49.9 (*c* 1, MeOH) [Found: (ES-MS) *m/z*, 496.17 (M + Na), 100%. $C_{24}H_{27}NO_9$ requires *M*, 473.17]; δ_H(360 MHz; CD_3OD) 7.15–7.70 (8 H, m, ArH), 4.64 (1 H, d, $J_{1,2}$ 3.5, 1-H), 4.21–4.33 (3 H, m, 2 Fmoc CH_2, Ser α-H), 4.06–4.15 (2 H, m, Ser β-H, Fmoc CH), 3.80 (1 H, q, $J_{5,6}$ 6.5, 5-H), 3.65 (1 H, dd, $J_{2,3}$ 9.5, 2-H), 3.56 (1 H, dd, $J_{3,4}$ 2.5, 3-H), 3.50 (1 H, d, $J_{3,4}$ 2.5, 4-H), 3.46 (1 H, dd, J_{gem} 9.5 and $J_{\alpha H,\beta H'}$ 1, Ser β-H′) and 1.08 (3 H, d, $J_{5,6}$ 6.5, 6-H_3).

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-3-*O*-(α-L-fucopyranosyl)-L-serine allyl ester 6

Compound **5** was dissolved in dimethyl sulfoxide (100 cm^3) and allyl bromide (25 cm^3) was added. After being stirred for 4 h, the reaction mixture was diluted with ethyl acetate and washed successively with water and brine. The organic layer was evaporated to dryness and the residue was chromatographed [dichloromethane–methanol (9:1)] to give title compound **6** (2.32 g, 51% from **4**) as a yellow foam, R_f 0.51 [dichloromethane–methanol (9:1)] [Found: (FAB-MS) *m/z*, 514.1 (M + H), 1.1%; 536.1 (M + Na), 1.1%; 179 (dibenzofulvene + H fragment), 100%. $C_{27}H_{31}NO_9$ requires *M*, 513.24]; δ_H(360 MHz; CD_3OD) 7.28–7.81 (8 H, m, ArH), 5.93 (1 H, dddd, $J_{b,c}$ 17 and $J_{a,c}$ 10.5, $J_{c,d}$ 5.5 and $J_{c,e}$ 5.5, allyl H^c), 5.37 (1 H, dddd, $J_{a,b}$ 1.5, $J_{a,d}$ 1.5 and $J_{a,e}$ 1.5, allyl H^a), 5.27 (1 H, dddd, $J_{b,d}$ 1.5 and $J_{b,e}$ 1.5, allyl H^b), 4.80 (1 H, d, $J_{1,2}$ 3.5, 1-H), 4.62–4.71 (2 H, m, H^d and H^e allyl), 4.59 (1 H, t, $J_{H\alpha,H\beta}$ 2.5, Ser α-H), 4.47 and 4.38 (each 1 H, dd, J_{gem} 10 and J_{vic} 7, Fmoc CH_2), 4.22–4.32 (2 H, m, 2- and 3-H), 3.72–3.82 (2 H, m, Fmoc CH, 5-H), 3.61–3.68 (2 H, m, 4-H, Ser β-H), 3.58 (1 H, dd, J_{gem} 9 and $J_{H\alpha,H\beta}$ 2.5, Ser β-H′) and 1.23 (3 H, d, $J_{5,6}$ 6.5, 6-H).

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-3-*O*-(2,3,4-tri-*O*-acetyl-α-L-fucopyranosyl)-L-serine allyl ester 7

To a solution of compound **6** (3.68 g, 7.18 mmol) in dichloromethane (75 cm^3) were added pyridine (7 cm^3, 86.15 mmol) and acetic anhydride (8.15 cm^3, 86.15 mmol). After being stirred overnight, the reaction mixture was diluted with dichloromethane and then was washed successively with water, aq. sodium hydrogen carbonate and brine. The organic layer was evaporated to dryness and the residue was chromatographed [ethyl acetate–hexane (2:1)] to give *title compound* **7** (3.97 g, 87%) as a foam, $[\alpha]_D$ −77.0 (*c* 1.65, $CHCl_3$); R_f 0.60 [hexane–ethyl acetate (1:1)] (Found: C, 62.0; H, 5.75; N, 2.2. $C_{33}H_{37}NO_{12}$ requires C, 61.97; H, 5.83; N, 2.19%); δ_H(360 MHz; $CDCl_3$) 7.28–7.80 (8 H, m, ArH), 5.96 (1 H, dddd, $J_{b,c}$ 17 and $J_{a,c}$ 10.5, $J_{c,d}$ 5.5 and $J_{c,e}$ 5.5, allyl H^c), 5.59 (1 H, d, $J_{\alpha,NH}$ 8.5, Ser NH), 5.38 (1 H, dddd, $J_{a,b}$ 1.5, $J_{a,d}$ 1.5 and $J_{a,e}$ 1.5, allyl H^a), 5.28 (1 H, dddd, $J_{b,d}$ 1.5 and $J_{b,e}$ 1.5, allyl H^b), 5.18–5.28 (2 H, m, 3- and 4-H), 5.13 (1 H, d, $J_{1,2}$ 3.5, 1-H), 5.03–5.11 (1 H, m, 2-H), 4.63–4.79 (2 H, m, H^d and H^e allyl), 4.60 (1 H, ddd, $J_{\alpha H,\beta H}$ 2.5 and $J_{\alpha H,\beta H'}$ 3.0, Ser α-H), 4.49 and 4.39 (each 1 H, dd, J_{vic} 7 and J_{gem} 10.5, Fmoc CH_2), 4.23 (1 H, t, J_{vic} 7, Fmoc CH), 4.20 (1 H, dd, J_{gem} 10.5, Ser β-H), 4.03 (1 H, q, $J_{5,6}$ 6.5, H-5), 3.68 (1 H, dd, Ser β-H′), 2.17, 2.02 and 1.98 (each 3 H, 3 s, 3 × Ac) and 1.10 (3 H, d, $J_{5,6}$ 6.5, 6-H); δ_C(300 MHz; $CDCl_3$) 170.54, 170.38, 169.96 and 169.41 (CO ester), 155.87 (CO carbamate),

143.83, 143.66 and 141.33 (Ph quat), 131.37 (CH_2=$CHCH_2O$), 127.77, 127.11, 125.03 and 120.03 (Ph methine), 119.47 (CH_2=$CHCH_2O$), 96.84 (C-1), 70.89 (C-4), 68.51 (CH_2=$CHCH_2O$), 68.02 (C-3), 67.95 (C-2), 67.19 (Fmoc CH_2), 66.52 (Ser C-β), 64.83 (C-5), 54.31 (Ser C-α), 47.16 (Fmoc CH), 20.69 and 20.62 ($COCH_3$) and 15.83 (C-6).

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-3-*O*-(2,3,4-tri-*O*-acetyl-α-L-fucopyranosyl)-L-serine 8

Compound **7** (3.70 g, 5.79 mmol) was dissolved in tetrahydrofuran (75 cm^3) and then morpholine (1 cm^3, 11.58 mmol) and tetrakis(triphenylphosphine)palladium (670 mg, 0.579 mmol) were added. After being stirred for 5 min, the reaction mixture was diluted with dichloromethane and then was washed successively with 0.5 mol dm^{-3} hydrochloric acid, water and brine. The organic layer was evaporated to dryness and the residue was chromatographed [dichloromethane–methanol (9:1)] to give title compound **8** (3.11 g, 90%) as a foam, R_f 0.43 [dichloromethane–methanol (9:1)] [Found: (FAB-MS) *m/z*, 622.16 (M + Na), 3.47%; 638.17 (M + K), 100%. $C_{30}H_{33}NO_{12}$ requires *M*, 599.20]; δ_H(360 MHz; CD_3OD) 7.16–7.73 (8 H, m, ArH), 5.06–5.23 (2 H, m, 3- and 4-H), 4.88–4.98 (2 H, m, 1- and 2-H), 4.35 and 4.28 (each 1 H, dd, J_{gem} 10 and J_{vic} 6.5, Fmoc CH_2), 4.02–4.18 (3 H, m, 5-H, Fmoc CH and Ser α-H), 3.91 (1 H, dd, $J_{\alpha H,\beta H}$ 5.5 and J_{gem} 9.5, Ser β-H), 3.68 (1 H, dd, $J_{\alpha H,\beta H'}$ 3.0, Ser β-H'), 2.04 (3 H, s, Ac), 1.86 (6 H, 2 s, 2 × Ac) and 0.95 (3 H, d, $J_{5,6}$ 6.5, 6-H); δ_C(300 MHz; CD_3COD) 172.29, 171.98 and 171.64 (CO ester), 158.34 (CO carbamate), 145.33, 145.23 and 142.57 (Ph quat), 128.76, 128.22, 126.11 and 120.93 (Ph methine), 97.33 (C-1), 72.51 (C-4), 69.55 (C-3), 69.38 (C-2), 67.89 (Fmoc CH_2, Ser C-β), 65.81 (C-5), 56.85 (Ser C-α), 48.35 (Fmoc CH), 20.71, 20.63 and 20.45 ($COCH_3$) and 16.14 (C-6).

N^{α}-Ac-Pro-Cys(Acm)-Leu-Asn-Gly-Gly-Ser(Ac_3-α-L-Fuc)-Cys(Acm)-Lys-Asp-Asp-NH_2 9

Synthesis A applying N^{α}-Fmoc-Ser(Ac_3-α-L-Fuc)-OH 8 as building block. Synthesis of compound **9** was performed as described under synthesis B by using a PEGA resin (280 mg, subst. 0.23 mmol g^{-1}). The glycosylamino acid **8** (75.6 mg, 2 mol equiv.) was incorporated into the peptide chain by activation with TBTU (1.95 mol equiv.) and NEM (3 mol equiv.) for 2 h. The glycopeptide was cleaved from the resin and purified as described below to yield glycopeptide **9** (60 mg, 64%).

Synthesis B applying N^{α}-Fmoc-Ser(α-L-Fuc)-OH 5 as building block. The glycopeptide was synthesized in a custom-made continuous-flow peptide synthesizer with on-line monitoring of the acylation reactions by a solid-phase spectrophotometer and application of the Dhbt-colour reaction. The PEGA resin (280 mg, subst. 0.35 mmol g^{-1}) was derivatised with norleucine and the Rink-amide linker. The non-glycosylated N^{α}-Fmoc-amino acids were coupled by using 3 mol equiv. of the corresponding pentafluorophenyl esters plus addition of 1 mol equiv. of Dhbt-OH and with the following side-chain-protecting groups: Bu^t for Asp, Bu^tOCO (Boc) for Lys and Acm for Cys. The glycosylated amino acid N^{α}-Fmoc-Ser(α-L-Fuc)-OH (2 mol equiv.) was coupled by activation with TBTU (1.9 mol equiv.) and NEM (3 mol equiv.). The coupling times were 2 h for all amino acids. N^{α}-Fmoc deprotections were effected in 10 min with 20% piperidine–DMF. After coupling of the glycosylamino acid **9**, the resin was washed with DMF and treated with acetic anhydride–pyridine (1:2) for 2 h. The protected glycopeptide **9** was cleaved from the solid support with 95% aq. TFA (6 cm^3). The resin was washed 5 times with 95% TFA (3 cm^3), and the combined TFA fractions were evaporated under reduced pressure. The crude product was analysed by analytical RP-HPLC [buffer A–buffer B (100:0–50:50) (30 min)] and ES-MS indicated that only partial acetylation of the fucose had been achieved. The correct compound **9** was isolated by preparative RP-HPLC [buffer A–buffer B (100:0–50:50) (60 min)] in 49 mg (32%) yield. A second fraction contained a mixture of partially acetylated compounds [ES-MS: Found: *m/z* 1521.92 (M − Ac + H) and 1479.9 (M − 2Ac + H)] was isolated in 69 mg (45%) yield. Compound **9** [Found: (ES-MS) *m/z*, 1563.8 (M + H). $C_{62}H_{98}N_{16}O_{27}S_2$ requires *M*, 1562.64]; $[\alpha]_D$ −61.3 (*c* 1.0, water).

N^{α}-Ac-Pro-Cys(Acm)-Leu-Asn-Gly-Gly-Ser(α-L-Fuc)-Cys(Acm)-Lys-Asp-Asp-NH_2 10

Compound **9** (45 mg, 28.8 μmol) was dissolved in methanol–water (1:1; 8 cm^3), hydrazine hydrate (450 mm^3) was added and the solution was stirred at room temperature. The pH of the solution was 9–9.5 as determined by pH-paper. After 1 h, additional hydrazine hydrate (100 mm^3) was added. The deacetylation was followed by analytical RP-HPLC [buffer A–buffer B (100:0–50:50) (30 min)] and was finished after 4 h. Acetone (10 cm^3) was added and the solution was concentrated. Analytical HPLC of the crude product using an extended gradient [buffer A–buffer B (100:0–60:40) (60 min)] indicated the presence of two compounds, which were separated by preparative HPLC [buffer A–buffer B (100:0–60:40) (120 min)]. Fraction 1 (8 mg, 19.3%) was identified as by-product **11** [Found: (ES-MS) *m/z*, 1453.36. $C_{56}H_{93}N_{17}O_{24}S_2$ requires *M*, 1451.6]. The correct glycopeptide **10** was isolated in 27 mg (65%) yield (Found: (ES-MS) *m/z* 1437.96. $C_{56}H_{92}N_{16}O_{24}S_2$ requires *M*, 1436.59]; NMR data are presented in Tables 1 and 2; $[\alpha]_D$ −65.8 (*c* 1, water).

Acknowledgements

This work was supported by the Alexander von Humboldt Foundation through a Feodor Lynen Fellowship (S. P.), by a NATO-grant AS.5-2-05(CRG.920734)1408 92/JARC-501 (M. M. and O. H.) and the Alberta Heritage Foundation for Medical Research (T. L. L.).

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Paper 5/02709H
Received 28*th April* 1995
Accepted 13*th July* 1995