Small-scale Solid-phase *O*-Glycopeptide Synthesis of Linear and Cyclized Hexapeptides from Blood-clotting Factor IX containing *O*-(α-D-Xyl-1→3-α-D-Xyl-1→3-β-D-Glc)-L-Ser

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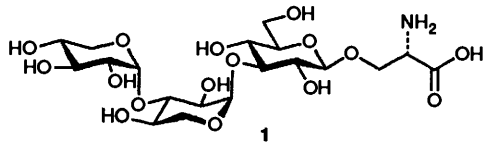
Glycopeptide sequences corresponding to residues 51–56 of the EGF-like domains of human and bovine blood-clotting factor IX have been synthesized using the Fmoc/Dhbt strategy. Building blocks consisting of Fmoc-Ser(*R*)-OPfp, where *R* = β-D-Glc or α-D-Xyl-(1-3)-α-D-Xyl-(1-3)-β-D-Glc, have been synthesized. The building blocks were prepared by treatment of the unprotected glycosylated serine compounds with *N*-(fluoren-9-ylmethoxycarbonyl)succinimide in 1,4-dioxane, followed by treatment with pentafluorophenol and dicyclohexylcarbodiimide in tetrahydrofuran. The glycosylated building blocks were then used in a solid-phase peptide synthesis to give the corresponding glycopeptides. Cyclic glycopeptides were prepared from the acetamidomethyl-protected linear glycopeptides by treatment with thallium(III) trifluoroacetate in trifluoroacetic acid. The cyclic glycopeptides were fully characterized by NMR spectroscopy and mass spectrometry.

Many *O*-glycosylated proteins carry very complex oligosaccharides which are available in only minute amounts from the natural protein and are often heterogeneous when isolated. These oligosaccharides linked to amino acids may be obtained by synthesis, which, depending on its complexity, is often tedious and time consuming, leading to small quantities of glycosyl amino acid to be further manipulated for the incorporation into a target glycopeptide with a well defined composition. We have recently described a method in which oligosaccharides are treated with N^{α}-fluoren-9-ylmethoxycarbonyl (Fmoc) and 2,3,4,5,6-pentafluorophenyl (Pfp) ester protected hydroxyamino acids to yield larger amounts of fully protected building blocks for solid-phase synthesis.[1,2] Techniques for the conversion and incorporation of very small amounts of complex synthetic or isolated glycosyl amino acid into glycopeptides by solid-phase peptide synthesis has not yet been reported.

Blood-clotting factors VII and IX are plasma glycoproteins (zymogens of serine proteases) which are involved in the blood coagulation cascade.[3,4] These proteins are synthesized in the liver and are glycosylated prior to secretion. Recently it has been shown that the first epidermal growth factor (EGF)-like domains of human and bovine blood-clotting factors VII and IX, as well as human and bovine Protein Z[5] and bovine thrombospondin,[6] contain unique *O*-linked sugar chains consisting of glucose and xylose. The above proteins all contain a conserved sequence of amino acids consisting of a Cys-X-Ser-X-Pro-Cys sequence. The sequence for the factor IX blood-clotting proteins is Cys-Glu-Ser-Asn-Pro-Cys, corresponding to residues 51–56. In these proteins the serine residue bears either a disaccharide (α-D-Xyl-1→3-β-D-Glc) or a trisaccharide (α-D-Xyl-1→3-α-D-Xyl-1→3-β-D-Glc) chain.[7] The biological function of these sugar chains remains unknown. The chemical synthesis of the disaccharide[8] and trisaccharide[9] chains conjugated to serine have been carried out.

In order to investigate the biological significance of the oligosaccharide portions of these proteins a sample of the trisaccharide conjugated to serine, compound **1**, was incorporated into a peptide sequence to give a glycopeptide corresponding to residues 51–56 of the human and bovine blood-clotting factor IX.

This glycopeptide synthesis required development of methodology for the protection and incorporation of the completely unprotected *O*-glycosyl amino acid **1** into a solid-phase peptide synthesis. Such methodology will be valuable in the future when it may be expected that such *O*-linked building blocks for solid-phase glycopeptide syntheses become available from over-expression of glycoproteins or from other natural sources.



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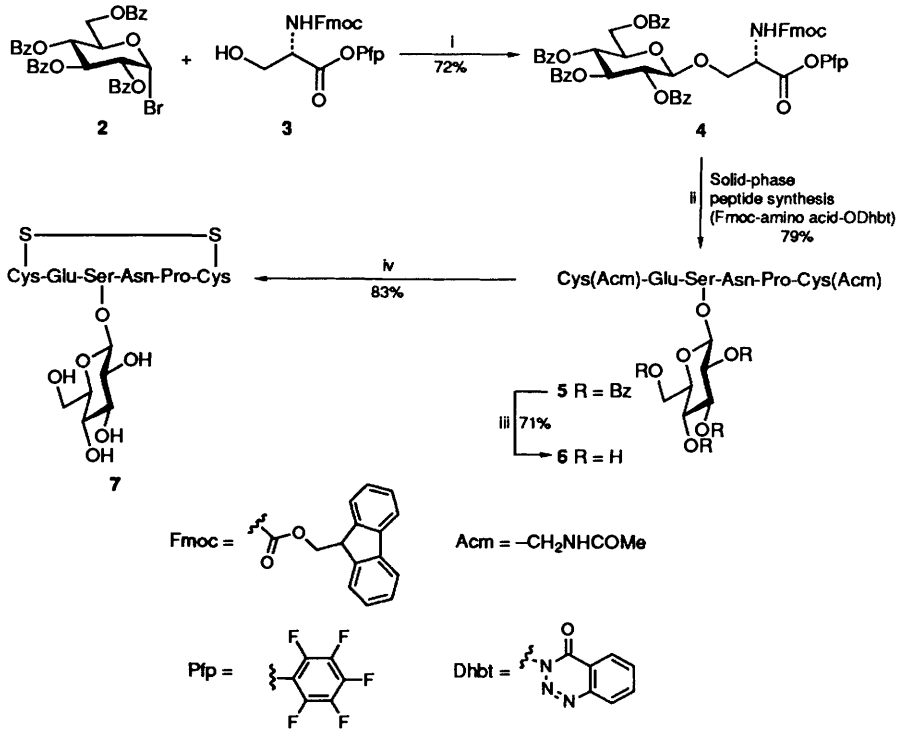
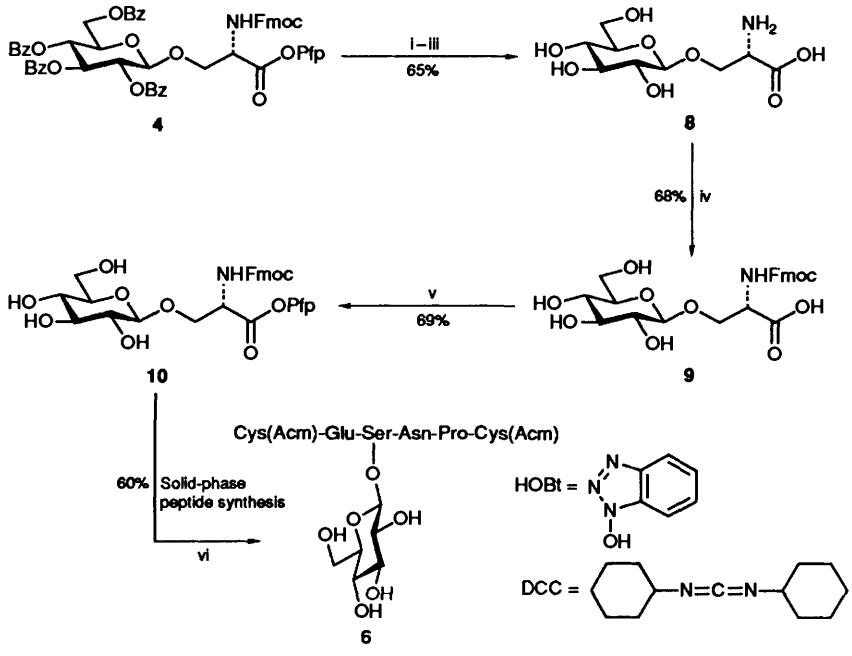
Results and Discussion

Synthetic compound **1** was available[9] only in a small quantity (~20 mg), therefore the glycopeptide with the more readily available glucose conjugate of serine was first prepared. This allowed the investigation of the chemistry of protection and peptide synthesis, as well as affording the glycopeptide with a truncated oligosaccharide for comparison in biological testing. Glycosylation of N^{α}-Fmoc-Ser-OPfp **3**,[2,10] with the donor 2,3,4,6-tetra-*O*-benzoyl-α-D-glucopyranosyl bromide[11] **2**, under silver trifluoromethanesulfonate promotion in dichloromethane (−30 °C), afforded the fully protected building block **4** in 72% yield (Scheme 1). The product could be isolated directly from the crude syrup by trituration from an ethyl acetate–diethyl ether–hexane mixture. The glycosylated serine derivative **4** was then used in an automated continuous-flow solid-phase peptide synthesis of a hexapeptide portion (residues 51–56) of the human blood-clotting factor IX, HBF-IX (51–56) (see the Experimental section for details of the peptide synthesis). The benzoyl groups of glycopeptide **5** could be removed by sodium methoxide-catalysed transesterification in methanol. The sample was dissolved in methanol and made basic (pH ~12 by moist pH paper) by the dropwise addition of 1 mol dm^{-3} sodium methoxide. The progress of the reaction was followed by analytical reversed-phase HPLC, and after 4 h was found to be complete. When the reaction mixture was allowed to become too basic, β-elimination of the glucose residue occurred; therefore the reaction conditions were



Scheme 1 *Reagents and conditions:* i, AgO_3SCF_3, CH_2Cl_2, −30 °C, 2 h; then recrystallization from Et_2O–EtOAc–hexane; ii, **4** (3.8 mol equiv. relative to the amount of peptide); iii, NaOMe, MeOH; iv, $Tl(OCOCF_3)_3$, PhOMe, TFA



Scheme 2 *Reagents and conditions:* i, HOBt, aq. DMF; ii, morpholine; iii, NaOMe, MeOH; iv, Fmoc–Su, 1,4-dioxane, sat. aq. $NaHCO_3$; v, Pfp-OH, DCCI, THF; vi, **10** (1.5 mol equiv. relative to amount of peptide)

monitored carefully. The partially deprotected glycopeptide **6** was isolated after reversed-phase HPLC in 71% yield. The glycopeptide was characterized with the cysteine residues protected as their acetamidomethyl (Acm) derivatives, in order to protect them from oxidation.

It is not known if the natural protein sequence contains a disulfide bridge between residues 51 and 56; such a bridge is possible since there is a proline in position 55 facilitating the formation of such a structure. In light of this it was decided to prepare the cyclic glycopeptide **7** as well. Additionally, a cyclic structure would likely be conformationally more well defined and therefore more suitable for conformational analysis by NMR spectroscopy studies.

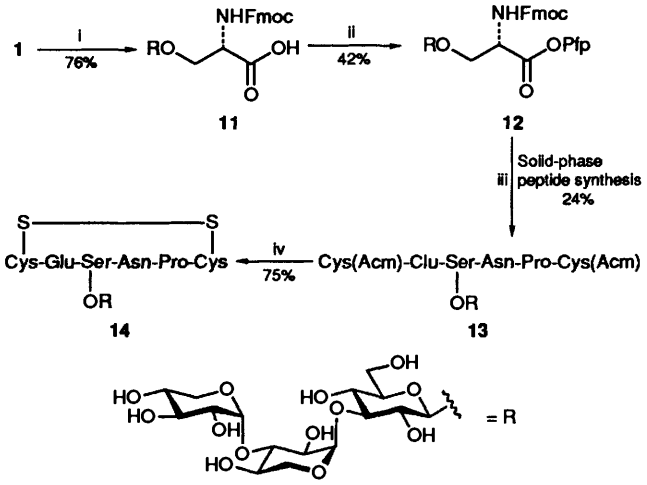
The cyclic peptide was prepared by an intramolecular oxidation procedure,[12] using thallium(III) trifluoroacetate in trifluoroacetic acid (TFA). Preparative HPLC afforded glycopeptide **7** in 83% yield. The cyclic nature of the peptide was confirmed by amino acid analysis which gave an analysis for half-cystine very close to the theoretical value, whereas the preceding linear peptide **6** gave a much reduced amount, which is commonly observed for Cys(Acm) and Cys not involved in a disulfide bond.

An analogue of compound **1** was prepared by removal of all the blocking groups of the ester **4** to give the fully deprotected glycosylated serine derivative **8** (Scheme 2). The deprotection was achieved by hydrolysis of the Pfp-ester, catalysed by 1-hydroxybenzotriazole (HOBt) in aq. dimethylformamide (DMF), followed by the removal of the Fmoc group using 50% morpholine in DMF, and finally removal of the benzoyl esters by sodium methoxide-catalysed transesterfication as before. The deprotection reactions were carried out without purification of the intermediate products, and the only work-up involved evaporation of the reaction mixtures between steps. The final deprotected compound **8** was obtained in 65% overall yield after purification.

Compound **8** was then used as a model compound for the more complex compound **1**. The first step was protection of the free amino group of the serine moiety with the Fmoc group. The reaction was carried out by a procedure described previously,[13] and the Fmoc-protected derivative **9** was obtained in 68% yield. It was decided to prepare the Pfp-ester to serve as both a protection and a source of activation of the carboxyl terminus. This had the advantage of giving a compound which was stable enough to be purified by reversed-phase HPLC, as well as being reactive enough to serve directly as a building block for glycopeptide synthesis.[2] The semiprotected serine derivative **10** was obtained in 69% yield. It was decided to leave the hydroxy groups of the sugar residue unprotected to limit the number of chemical steps in the overall synthesis of the target glycopeptides. There are examples in the literature of couplings of glycosylated amino acids where the hydroxy groups of the sugar residues have been left unprotected.[14,15] The building block **10** was then used in a solid-phase peptide synthesis using only 1.5 mol equiv. of compound **10** relative to the amount of functionalized resin used; the other amino acid building blocks were added in 6-fold excess. The yield of the glycopeptide **6** was 60% after purification. This result compared favourably to the results obtained earlier using the fully blocked glycosylated building block **4**. The overall yield of product **6** including the peptide synthesis and the deprotection step was approximately 56%, whereas the same compound **6** was prepared in 60% yield when using a partially protected building block. Based on these results it was decided to use a partially protected derivative of compound **1** rather than a fully protected building block.

A sequence of protection, analogous to that carried out for compound **8**, was performed using trisaccharide **1** as the starting material. The amino group of the serine portion of the molecule was protected with the Fmoc group, to give protected acid **11** in 76% yield (Scheme 3). The next reaction, protection of the carboxylic acid group as its Pfp-ester, did not proceed as smoothly as with the analogous glucose derivative. The starting material did not dissolve completely in tetrahydrofuran (THF) and some DMF was added to bring the compound completely into solution for the activation with *N,N'*-dicyclohexylcarbodiimide (DCCI) and Pfp-OH. Addition of DMF to increase solubility has frequently been reported in the formation of symmetrical anhydrides although is also well documented that activation of carboxylic acids by DCCI is favoured in apolar media.[16] Following purification by HPLC, compound **12** was obtained in only 42% yield. This yield may be improved by the reversed-addition procedure[17] or by the use of fully protected intermediates. This partially protected building block was then used directly in a solid-phase peptide synthesis protocol.

Normally, during a solid-phase synthesis, the acylating reagent (the activated amino acid) is added in excess, usually 2–3 or more mol. equiv. In this particular case the acylating reagent, compound **12**, was available in only a very small amount so it was decided to make it the limiting reagent, using only 0.37 mol equiv. to afford a greater incorporation of



Scheme 3 *Reagents and conditions:* i, Fmoc-Su, 1,4-dioxane, sat. aq. Na_2CO_3; ii, Pfp-OH, DCCI, THF–DMF; iii, **12** (0.37 mol. equiv. relative to amount of peptide); iv, $Tl(OCOCF_3)_3$, PhOMe, TFA

glycosylated amino acid with less being wasted. With this approach the desired product is not the major product. In order to avoid acetylation of hydroxy groups, it was decided not to cap the unchanged amino groups after incorporation of compound **12** but instead to continue directly with the peptide synthesis. This led to a large amount of an impurity comprising a pentapeptide sequence lacking the glycosylated serine residue. It was assumed that separation of this unglycosylated peptide impurity from the desired glycosylated product would be a simple matter due to the presence of the oligosaccharide structure on the target peptide. Unfortunately this was not the case, but the desired compound could be purified by using a combination of gel filtration chromatography and repeated reversed-phase HPLC. After chromatography a pure sample of compound **13** was obtained in 24% yield (based on the amount of building block **12** consumed). The incorporation of the building block **12** into glycopeptide product was lower than with the corresponding unit **10**. This may be due to the greater effect of the unprotected hydroxy groups with the trisaccharide relative to a monosaccharide.

The effect of having unprotected *versus* protected hydroxy groups on larger oligosaccharides both in the protection scheme and in the peptide synthesis is currently being investigated with the analogous maltotriose conjugates of serine. Thus the acylation conditions are being investigated with derivatives of Fmoc-protected serine bearing either a maltotriose or a glucose unit with the hydroxy groups either protected or unprotected.

Compound **13** was finally cyclized, in a similar manner to its analogue **6**, to give the cyclic glycopeptide **14** in 75% yield. Studies are currently being performed using the molecular modelling force-field[18] and NMR techniques to assess the conformational preferences of the glycopeptides, synthesized in the present work.

Experimental

General Methods.—Analytical TLC was performed on Merck silica gel 60 F_{254} aluminium plates, with detection by UV light and/or charring with sulfuric acid. Medium-pressure chromatography was performed on Merck Kieselgel 60 (0.015–0.040 mm) with a Büchi B-680 chromatographic system. Solvents were purchased from Labscan, were distilled before use, and were dried, as required, by literature procedures. *N*-(Fluoro-9-ylmethoxycarbonyl)succinimide (Fmoc-Su) and Fmoc-amino acid derivatives were purchased from Bachem or Novabiochem, while DCCI, Pfp-OH and 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-

benzotriazine (Dhbt-OH) were from Fluka. Extracts were evaporated under reduced pressure, and below 40 °C. Semi-preparative HPLC separations were performed on a Waters-600E HPLC system using a DELTA PAK C-18 column, except where noted (300 Å; 25 mm × 10 cm), with a flow rate of 10 cm^3 min^{-1} with detection at 200, 215, 235 and 270 nm using a photodiode array detector (Waters–991); solvents systems A: 0.1% TFA in water, B: 0.1% TFA in 90% acetonitrile–10% water. Gel exclusion chromatography was carried out by using a column of Biogel P-2 (fine) (58 cm × 1.5 cm) with a flow rate of 21 cm^3 h^{-1} with water as the eluent, and UV detection at 235 nm. Elemental analyses were carried out at LEO Pharmaceutical Products Denmark. Mass spectra were kindly performed at the Department of Chemistry, Royal Veterinary and Agricultural University of Denmark. Mass spectra of compounds in a matrix of *o*-nitrobenzyl alcohol were recorded by liquid secondary-ion mass spectrometry (LSIMS) on a VEGA 70SE instrument with a Cs ion source calibrated with caesium iodide. Quantitative amino acid analyses were performed on a Pharmacia LKB Alpha Plus amino acid analyser following hydrolysis with 6 mol dm^{-3} HCl at 110 °C for 24 h. Asn was determined as Asp, and Cys was determined as half-cystines. Nomenclature is according to the IUPAC recommendations. Optical rotations were measured with a Perkin-Elmer 241 polarimeter and are given in units of 10^{-1} deg cm^2 g^{-1}. ^1H and ^{13}C NMR spectra were recorded with a Bruker AM-500 or AMX-600 spectrometer at 500.13 or 600.13 MHz for proton, and 125.76 or 150.91 for carbon, respectively. All spectra were recorded at 300 °C. Chemical shifts are given in ppm with the solvent peaks for $CDCl_3$, D_2O, $(CD_3)_2SO$ and CD_3OD set to δ_H 7.26, 4.75, 2.50 and 3.35, respectively. The pD of D_2O solutions was adjusted by using 0.2 mol dm^{-3} Na_2CO_3 in D_2O and then reading the value on a standard pH instrument. The coupling constants for the amide protons were measured in 90% water–10% D_2O. The assignment of the proton chemical shifts was made by examination of the 2D homonuclear chemical-shift correlation (COSY) spectra; in addition, phase-sensitive two-dimensional total correlation (TOCSY) spectra were obtained for compounds **5**, **6** and **13**, using the pulse sequence *d*1–90°–*d*0–MLEV spinlock-FID, with a solvent presaturation pulse of 1 s during *d*1. For the 2D spectra an initial data set of 512 2K experiments was acquired, and zero-filled once in the F_1-direction with no zero filling in F_2, to give a final data set of 1K × 1K real data points. The spin lock (45 μs 90°) was applied for a period of 200 ms. ^1H NMR chemical shifts of overlapping signals were obtained from the centre of the cross-peaks in either the COSY or TOCSY spectra.

Solid-phase Peptide Synthesis, General Procedure.—Synthesis of the glycopeptides was carried out on a Kieselguhr-supported poly(dimethylacrylamide) resin (Macrosorb SPR-250 purchased from Sterling Organics) which was converted into the amine form with ethylenediamine.[19] The linker, 4-(hydroxymethyl)phenoxyacetic acid, was attached using 4-(hydroxymethyl)phenoxyacetic acid 3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl (Dhbt) ester. The first amino acid was attached to the resin-bound linker (2.5 g of resin) by esterification of N^{α}-Fmoc-Cys(Acm)-OH (0.52 g 1.25 mmol) promoted by 1-mesitylsulfonyl-3-nitro-1,2,4-triazole (MSNT) (0.37 g, 1.25 mmol) in dichloromethane with the presence of *N*-methylimidazole[20] (0.075 cm^3, 0.94 mmol). The esterification procedure was repeated using the above amounts of reagents to ensure quantitative functionalization of the linker. A sample of the resin with the first amino acid attached was deblocked and acylated with N^{α}-Fmoc-Ala-Dhbt by using a double coupling procedure to ensure complete acylation, and the level of incorporation of the first amino acid (Cys) was estimated based on the level of incorporation of Ala, which was in turn determined by quantitative amino acid analysis (0.165 mmol g^{-1} resin). The glycopeptides were synthesized in DMF, with the amino acids being added as their N^{α}-Fmoc-protected Dhbt-esters, where the side chains of Cys and Glu were protected with Acm and *tert*-butyl groups, respectively. The glycosylated amino acids were added as their Pfp-esters with the addition of Dhbt-OH as an acylation catalyst as well as an indicator of the end-point of the acylation. Deprotection of the Fmoc groups was achieved with a solution of 50% morpholine in DMF for 30 min. The syntheses were carried out using either a custom-made, fully automatic, continuous-flow peptide synthesizer, with monitoring of the acylation reactions using a solid-phase spectrophotometer operating at 440 nm (Procedure A); or manually using a sintered glass funnel, and monitoring of the acylation times visually by the decrease in the yellow colour of the resin as the acylation proceeded (Procedure B). The latter procedure was better suited to small-scale syntheses. After the final removal of the Fmoc-group the resin was washed successively with DMF and diethyl ether and lyophilized. The peptide-resin was then treated 2 h with 95% aq. TFA (30 cm^3 g^{-1} resin) for the simultaneous removal of *tert*-butyl protection and cleavage of the peptide from the resin. The TFA was removed under reduced pressure and the residue was triturated with diethyl ether. The crude peptides were purified by HPLC as described below.

N^{α}-(*Fluoren-9-ylmethoxycarbonyl*)-O-(2,3,4,6-*tetra*-O-*benzoyl*-β-D-*glucopyranosyl*)-L-*serine Pentafluorophenyl Ester* **4**.—A sample of N^{α}-Fmoc-L-Ser-OPfp **3** (0.95 g, 1.9 mmol), together with silver trifluoromethanesulfonate (0.82 g, 3.2 mmol) and 3 Å molecular sieves, was dissolved in dry dichloromethane (23 cm^3). The solution was cooled to −30 °C, and a solution of 2,3,4,6-tetra-*O*-benzoyl-α-D-glucopyranosyl bromide[11] **2** (1.8 g, 2.7 mmol) in dichloromethane (20 cm^3) was added. The reaction mixture was stirred in the dark under argon and was allowed to warm to room temperature. After 2 h TLC [hexane–ethyl acetate (2:1)] indicated that the reaction was complete. The reaction mixture was then filtered, and the filtrate was washed with 5% aq. sodium hydrogen carbonate, dried ($MgSO_4$), and concencentrated to a syrup. The syrup was triturated with a mixture of ethyl acetate–diethyl ether–hexane (~1:1:1) to give *title compound* **4** as a powder (1.14 g, 1.05 mmol). The mother liquor was chromatographed [hexane–ethyl acetate (2:1) as eluent] and crystallized as above to give a second batch (0.32 g, 0.30 mmol) of compound to give an overall yield of 72%, $[\alpha]_D^{21}$ +0.01 (*c* 1.27, $CHCl_3$); ^1H and ^{13}C NMR data are presented in Tables 1 and 5, respectively (Found: C, 65.0; H, 4.1; N, 1.2. $C_{58}H_{42}F_5NO_{14}$ requires C, 64.99; H, 3.95; N, 1.31%).

[*Bis*-5-*acetamidomethyl-Cys*^{51}, *Cys*^{56}-O-(2,3,4,5-*tetra*-O-*benzoyl*-β-D-*glucopyranosyl*)-*Ser*^{53}]-*HBF-IX* (51–56) **5**.—The solid-phase peptide synthesis (procedure A), and the coupling of the first amino acid to the resin, was carried out as described above. The loading of the first amino acid onto the resin was estimated by quantitative amino acid analysis to be 0.165 mmol g^{-1}. The peptide synthesis was carried out using resin (0.464 g, equivalent to 0.0765 mmol of peptide). In the acylation step with the glycosylated serine **4** an excess of 3.76 mol equiv. (0.309 g, 0.288 mmol) was used. The acylation times varied from 30 min (Pro^{55}) to 13 h (Asn^{54}) with the acylation time for substrate **4** being 25 h. After cleavage of the protected glycopeptide **5** from the resin it was purified by semipreparative HPLC using isocratic solvent A for 5 min, followed by a linear gradient of 0–20% solvent B over a period of 40 min, followed by a linear gradient of 20–100% solvent B over a period of 40 min (retention time of 72 min). The yield of glycopeptide **5** was 82.4 mg, 78.5%; ^1H NMR data are presented in Table 2.

Table 1 ^{1}H NMR data[a] for compounds **4** and **8**–**10**

	4	8	9	10
1-H	4.88 (8.0)	4.48 (8.0)	4.29 (8.5)	4.35 (8.5)
2-H	5.52 (8.0, 9.2)	3.31 (8.0, 9.0)	3.21 (18.5)[b]	3.24 (8.5, 9.0)
3-H	5.95 (19.5)[b]	3.48 (18.5)[b]	3.37 (18.5)[b]	3.39 (18.5)[b]
4-H	5.68 (19.5)[b]	3.37 (18.5)[b]	3.28	3.28
5-H	4.18	3.34	3.27	3.30
6-H	4.66 (3.0, 12.0)	3.89 (2.0, 12.5)	3.87 (2.0, 12.0)	3.88 (2.0, 12.0)
6-H′	4.45 (5.0, 12.0)	3.70 (6.0, 12.5)	3.67 (5.5, 12.0)	3.65 (6.0, 12.0)
Ser-α-H	4.87	3.97	4.44	4.90
Ser-β-H	4.50 (3.5, 10.0)	4.28 (5.5, 11.5)	4.38 3.79	3.90 (3.5, 10.5)
	4.03 (3.5, 10.0)	4.04 (3.5, 11.5)		3.65 (5.5, 10.5)
Ser NH	5.71 (8.0)			
Fmoc CH	4.14 (14.0)[b]		4.25 (14.0)[b]	4.25 (13.5)[b]
Fmoc CH_2	4.40 (7.5, 10.5)		4.40 (7.0, 10.5)	4.46 (6.8, 10.5)
	4.27 (7.5, 10.5)		4.36 (7.0, 10.5)	4.41 (6.8, 10.5)
ArH	8.1–7.2 (28 H)		7.80 (2 H)	7.79 (2 H)
			7.69 (2 H)	7.68 (2 H)
			7.39 (2 H)	7.38 (2 H)
			7.32 (2 H)	7.30 (2 H)

[a] Compound **4** in $CDCl_3$; compound **8** in D_2O, pD 7.1; compounds **9** and **10** in CD_3OD. The numbers in parentheses denote coupling constants in Hz. [b] The values are the sums of the individual coupling constants, $J_{AX} + J_{BX}$.

[*Bis*-5-*acetamidomethyl*-*Cys*^{51}, *Cys*^{56}-O-(β-D-*glucopyranosyl*)-*Ser*^{53}]*HBF-IX* (51–56) **6**.—Compound **5** was debenzoylated as follows. A sample (26.0 mg, 18.9 mmol) was dissolved in methanol (3.0 cm^3) and to this solution was added 1 mol dm^{-3} sodium methoxide until a wetted pH paper indicated pH 12. After 4 h the pH of the solution was adjusted by the addition of solid CO_2. The solvent was removed by evaporation and the resulting residue was purified by semipreparative HPLC using a linear gradient of 0–20% solvent B over a period of 40 min (t_R 21 min). The HPLC fractions were lyophilized to give compound **6** as a solid (12.9 mg, 71.4%); ^{1}H and ^{13}C NMR data are presented in Tables 2 and 6, respectively. Amino acid analyses (theoretical values in parentheses): Asn 1.10 (1), Ser 0.95 (1), Glu 1.05 (1), Cys 0.74 (2) and Pro 0.89 (1); *m/z* 956 $(M + H)^+$ and 978 $(M + Na)^+$ ($C_{35}H_{57}N_9O_{18}S_2$ requires *M*, 955.3).

Cyclic [O-(β-D-*Glucopyranosyl*)-*Ser*^{53}]*HBF-IX* (51–56) **7**.—A sample of compound **6** (4.4 mg, 0.0046 mmol) was dissolved in TFA (2.4 cm^3), together with anisole (10 mm^3, 0.092 mmol). The solution was cooled to 0 °C, and a solution of thallium(III) trifluoroacetate in TFA (0.8 cm^3; 7.5 μmol dm^{-3}) was added. After 75 min the reaction mixture was evaporated to dryness and diethyl ether (2 cm^3) was added to the residue. After the mixture had settled for 30 min, the ether was decanted off, and the residue was purified by semipreparative HPLC using isocratic solvent A (t_R 11 min). Compound **7** was obtained, after lyophilization, as solid (3.1 mg, 83%); ^{1}H and ^{13}C NMR data are presented in Tables 2 and 6, respectively. Amino acid analyses (theoretical values in parentheses): Asn 1.07 (1), Ser 0.95 (1), Glu 1.03 (1), Cys 1.99 (2) and Pro 0.95 (1); *m/z* 812 $(M + H)^+$ and 834 $(M + Na)^+$ ($C_{29}H_{45}N_7O_{16}S_2$ requires *M*, 811.2).

O-(β-D-*Glucopyranosyl*)-L-*serine* **8**.—A sample of the fully protected serine derivative **4** (1.43 g, 1.33 mmol) was dissolved in DMF (20 cm^3). To the stirred solution were added HOBt (67.8 mg, 0.502 mmol) and water (2.0 cm^3). The solution was stirred at room temperature for 20 h and then morpholine (10 cm^3) was added. After a further 20 min the solution was evaporated to dryness, and the residue was taken up in methanol and the solution made basic (pH 12) towards moist pH paper by the addition of 1 mol dm^{-3} sodium methoxide. The solution was kept at room temperature for 18 h, and then neutralized by the addition of solid CO_2. The solution was then evaporated to dryness and the residue was purified by silica gel chromatography using ethyl acetate–methanol–water–acetic acid (6:2:1:1) as the eluent to afford the title compound (231 mg, 64.8%). The ^{1}H NMR data are given in Table 1 and are in accord with previously published data.[21]

N$^{\alpha}$-(*Fluoren*-9-*ylmethoxycarbonyl*)-O-(β-D-*glucopyranosyl*)-L-*serine* **9**.—A sample of the fully deblocked serine derivative **8** (26.1 mg, 0.0977 mmol) was dissolved in saturated aq. sodium hydrogen carbonate (5.0 cm^3). The solution was cooled to 0 °C and a solution of Fmoc-Su (136 mg, 0.403 mmol) in 1,4-dioxane (5.0 cm^3) was added dropwise. The solution was stirred for 2 h at 0 °C, and for 1 h further at room temperature. To the reaction mixture was added water (50 cm^3) and the solution was acidified to pH 4 by the addition of 1 mol dm^{-3} hydrochloric acid. The solution was then extracted with ethyl acetate (3 × 30 cm^3) and the aqueous layer was lyophilized to give a residue, which was purified by semipreparative HPLC using isocratic solvent A for 20 min, followed by a linear gradient of 0–90% solvent B over a period of 30 min (t_R 40 min). Compound **9** (32.2 mg, 67.8%) was obtained, after lyophilization, as a solid; $[\alpha]_D^{21.5}$ +0.01 (*c* 1.06, MeOH); ^{1}H and ^{13}C NMR data are presented in Tables 1 and 5, respectively; *m/z* 490 $(M + H)^+$ and 512 $(M + Na)^+$ ($C_{24}H_{27}NO_{10}$ requires *M*, 489.1).

N$^{\alpha}$-(*Fluoren*-9-*ylmethoxycarbonyl*)-O-(β-D-*glucopyranosyl*)-L-*serine Pentafluorophenyl Ester* **10**.—A sample of compound **9** (32.2 mg, 0.657 mmol) was dissolved in dry THF (4.0 cm^3). To this solution was added DCCI (17.1 mg, 0.0828 mmol). The solution was stirred at room temperature and a solution of Pfp-OH (22.0 mg, 0.119 mmol) in THF (2.0 cm^3) was added. The reaction mixture was stirred for 20 h and was then evaporated to dryness, the residue was taken up in ethyl acetate, and the solution was filtered. The filtrate was concentrated and the residue was purified by semipreparative HPLC using a linear gradient of 35–55% solvent B over a period of 40 min (t_R 34 min) to give compound **10** (0.0297 g, 68.9%); $[\alpha]_D^{21.5}$ −0.16 (*c* 0.90, MeOH); ^{1}H and ^{13}C NMR data are presented in Tables 1 and 5, respectively; *m/z* 656 $(M + H)^+$ and 678 $(M + Na)^+$ ($C_{30}H_{26}F_5NO_{10}$ requires *M*, 655.1).

[O-(β-D-*Glucopyranosyl*)-*Ser*^{53}]*HBX-IX* (51–56) **6**.—The solid-phase peptide synthesis was carried out by method B as described above, using resin (113 mg, equivalent to 0.0186 mmol of peptide). The loading of the first amino acid onto the resin was estimated by quantitative amino acid analysis to be 0.165 mmol g^{-1}. The activated amino acids were added in six-fold excess, except for the glycosylated serine derivative **10** which was added in 1.5-fold excess. The acylation times, as judged by the decrease in the yellow colour of the resin during the acylation, varied from 73 min (Pro^{55}) to 4 h (Asn^{54}), with the acylation time for compound **10** being 16 h. Following cleavage from the resin the glycopeptide **6** was purified by semipreparative HPLC using the same solvent system as for compound **6** from the tetrabenzoyl precursor **5** to give title compound **6** as a solid (10.6 mg, 59.6%). The NMR data of the purified product were identical with those obtained above.

Table 2 ^{1}H NMR data[a] for compounds **5–7**

		NH	α-H	β-H_2	γ-H_2	δ-H_2
Cys[51]	**5**		4.09	3.02 (4,5, 14.5), 2.81 (9.0, 14.5)		
	6		4.30	3.17 (5.5, 14.5), 3.03 (8.0, 14.5)		
	7		4.40 (5.5, 7.4)	3.48, 3.37 (7.4, 14.5)		
Glu[52]	**5**	8.43 (9.0)	4.36	1.90	2.26	
	6	8.97 (7.0)	4.46	2.00, 2.12	2.48 (15.0)[b]	
	7		4.34 (6.5, 8.5)	2.16, 2.07	2.47	
Ser[53]	**5**	8.08 (10.5)	4.46	3.89 (8.0, 10.0), 3.82 (5.0, 10.0)		
	6	8.64 (7.0)	4.60	4.13 (6.5, 10.5), 3.88 (5.0, 10.5)		
	7		4.74	4.00 (6.5, 10.5), 3.88 (7.0, 10.5)		
Asn[54]	**5**	8.28 (8.0)	4.71	2.62 (7.5, 16.5), 2.35 (6.0, 16.5)		
	6	8.48 (7.5)	5.01	2.83 (6.5, 15.5), 2.67 (8.0, 15.5)		
	7		4.76	2.72 (5.5, 15.5), 2.62 (8.5, 15.0)		
Pro[55]	**5**		4.37	1.95	1.85	3.57
	6		4.46	2.22	1.99	3.75
	7		4.71	2.36, 2.20	1.96	3.64, 3.49
Cys[56]	**5**	8.07 (11.0)	4.35	2.98 (4.5, 14.0), 2.86 (9.0, 14.0)		
	6	8.34 (7.5)	4.55	3.13 (4.5, 14.0), 2.96 (8.5, 14.0)		
	7		4.60 (4.5, 9.0)	3.37 (4.5, 14.0), 3.03 (9.0, 14.0)		

		NH	CH_2	Me
Acm	**5**	8.57, 8.46	4.28, 4.16 (2 H, 4.12)	1.86 (3 H), 1.84 (3 H)
	6	8.59, 8.57	4.38, 4.29, 4.26, 4.25	2.00 (3 H), 1.95 (3 H)

		1-H	2-H	3-H	4-H	5-H	6-H_2
β-*Glc*-(1–3)-Ser	**5**	5.24 (8.0)	5.33 (8.0, 9.5)	5.95 (19.0)[b]	5.60 (19.0)[b]	4.48–4.55[c]	4.48–4.55[c]
	6	4.45 (8.0)	3.25 (8.0, 9.0)	3.48 (18.5)[b]	3.35 (19.0)[b]	3.43	3.90 (2.5, 12.5), 3.68 (6.0, 12.5)
	7	4.46 (8.0)	3.32 (8.0, 9.5)	3.50	3.39 (19.0)[b]	3.47	3.94 (2.5, 12.5), 3.74 (6.0, 12.5)

[a] Compound **5** in $(CD_3)_2SO$; compound **6** in 90% water–10% D_2O, pH 2.8; compound **7** in D_2O, pD ~ 6.0. The numbers in parentheses denote coupling constants in Hz. [b] The values are the sums of the individual coupling constants, $J_{AX} + J_{BX}$. [c] The signal is contained within the indicated range.

Table 3 ^{1}H NMR data[a] for compounds **11** and **12**

		1-H	2-H	3-H	4-H	5-H	6-H_2
β-*Glc*-(1-3)-Ser	**11**	4.32 (8.0)	3.32	3.50 (18.0)[b]	3.57	3.19	3.85 (2.0, 12.0), 3.67
	12	4.39 (8.0)	3.35	3.52	3.54	3.32	3.87, 3.66 (6.0, 12.0)
α-*Xyl*-(1-3)-Glc	**11**	5.19 (2.5)	3.54	3.73	3.70	3.91 (21.5),[b] 3.56	
	12	5.21 (3.5)	3.54	3.74 (18.0)[b]	3.71	3.88 (21.0),[b] 3.56 (21.5)[b]	
α-*Xyl*-(1-3)-Xyl	**11**	5.17 (2.5)	3.42 (2.5, 9.5)	3.63 (18.5)[b]	3.47	3.86 (21.5),[b] 3.55	
	12	5.17 (3.5)	3.42 (3.5, 9.5)	3.63 (18.5)[b]	3.48	3.86, 3.55	

		Ser α-H	Ser β-H_2	Fmoc CH	Fmoc CH_2	ArH
Other signals	**11**	4.42	4.38, 3.81	4.25 (14.0)[b]	4.38, 4.35	7.80, 7.69, 7.39, 7.32
	12	4.90	4.55, 3.93	4.25 (13.5)[b]	4.46, 4.41	7.80, 7.68, 7.38, 7.31

[a] Solvent CD_3OD. The numbers in parentheses denote coupling constants in Hz. [b] The values are the sums of the individual coupling constants, $J_{AX} + J_{BX}$.

N$^{\alpha}$-(*Fluoren-9-ylmethoxycarbonyl*)-O-[α-D-*xylopyranosyl*-(1-3)-(α-D-*xylopyranosyl*-(1-3)-β-D-*glucopyranosyl*]-L-*serine* **11**.—A sample of *O*-[α-D-xylopyranosyl-(1-3)-α-D-xylopyranosyl-(1-3)-β-D-glucopyranosyl]-L-serine **1**[6] (20.4 mg, 0.0384 mmol) was dissolved in a mixture of 1,4-dioxane (4.0 cm^3) and 10% aq. sodium carbonate (3.0 cm^3). The solution was cooled to 0 °C, and a solution of Fmoc-Su (19.4 mg, 0.0575 mmol) in 1,4-dioxane (1.0 cm^3) was added dropwise during 25 min. After 30 min the flask was removed from the ice-bath and the mixture was stirred for an additional 90 min before being transferred to a separatory funnel by using portions of water and ethyl acetate. The ethyl acetate layer was extracted with portions of water and the combined aqueous fractions were lyophilized. The resulting residue was purified by semipreparative HPLC, using isocratic solvent A for 15 min followed by a linear gradient of 0–50% solvent B over a period of 50 min (t_R 54 min). Following lyophilization, compound **11** was obtained as a solid (22.0 mg, 76.0%); $[\alpha]_D^{21.4}$ +1.06 (*c* 0.15, MeOH); ^{1}H and ^{13}C NMR data are presented in Tables 3 and 5, respectively; *m/z* 754 $(M + H)^+$ and 776 $(M + Na)^+$ ($C_{34}H_{43}NO_{18}$ requires *M*, 753.2).

N$^{\alpha}$-(*Fluoren-9-ylmethoxycarbonyl*)-O-[α-D-*xylopyranosyl*-(1-3)-α-D-*xylopyranosyl*-(1-3)-β-D-*glucopyranosyl*]-L-*serine Pentafluorophenyl Ester* **12**.—Compound **11** (20.5 mg, 0.0272 mmol) was dissolved in THF (3.0 cm^3), with the addition of DMF (0.5 cm^3) to bring the sample into solution. To the stirred solution, at room temperature, was added DCCI (9.5 mg, 0.046 mmol). The solution was stirred at room temperature and a solution of Pfp-OH (10 mg, 0.054 mmol) in THF (0.2 cm^3) was

Table 4 ^{1}H NMR data[a] for compounds **13** and **14**

		1-H	2-H	3-H	4-H	5-H	6-H_2
β-*Glc*-(1-3)-Ser	**13**	4.47 (8.0)	3.37	3.60	3.63	3.45	3.90, 3.71
	14	4.46	3.41	3.60	3.61	3.46	3.91, 3.72
α-*Xyl*-(1-3)-Glc	**13**	5.27 (4.0)	3.65	3.78	3.82	3.88, 3.62	
	14	5.29 (3.8)	3.64	3.80	3.79	3.92, 3.61	
α-*Xyl*-(1-3)-Xyl	**13**	5.30 (4.0)	3.53 (4.0, 10.0)	3.66	3.59	3.82, 3.64	
	14	5.31 (3.8)	3.53 (4.0, 9.5)	3.66	3.58	3.84, 3.64	

		NH	α-H	β-H_2	γ-H_2	δ-H_2
Cys^{51}	**13**		4.29	3.18 (6.0, 15.0) 3.04 (8.0, 15.0)		
	14		4.38	3.45, 3.33		
Glu^{52}	**13**	8.77 (7.0)	4.45	2.12, 2.01	2.44 (15.0)[b] (2 H)	
	14		4.29	2.11, 1.99	2.36 (2 H)	
Ser^{53}	**13**	8.58 (8.0)	4.61	4.14 (6.0, 11.0), 3.91		
	14		4.72	3.99 (6.5, 10.5), 3.85		
Asn^{54}	**13**	8.45 (8.0)	5.02	2.84 (7.0, 15.0), 2.68 (8.0, 15.0)		
	14		4.74	2.70 (6.5, 15.0), 2.60 (8.0, 15.0)		
Pro^{55}	**13**	8.05 (8.0)	4.46	2.25 (2 H)	2.03 (2 H)	3.76 (2 H)
	14		4.69	2.32, 2.18	1.93 (2 H)	3.62, 3.47
Cys^{56}	**13**		4.42	3.11 (5.0, 14.0), 2.97 (8.0, 14.0)		
	14		4.56	3.34, 2.99 (9.0, 14.0)		

		NH	CH_2	Me
Acm	**13**	8.55, 8.54	4.42 (2 H), 4.30 (2 H)	2.01 (3 H), 1.98 (3 H)

[a] Compound **13** in 90% water–10% D_2O, pH 3.6; compound **14** in D_2O, pD 4.2. The numbers in parentheses denote coupling constants in Hz. [b] The value is the sum of the individual coupling constants, $J_{AX} + J_{BX}$.

Table 5 ^{13}C NMR data[a] for compounds **4** and **9–12**

		C-1	C-2	C-3	C-4	C-5	C-6
β-*Glc*-(1-3)-Ser	**4**	101.1	71.8	72.5	69.4	72.5	62.8
	9	104.7	75.0	77.9	71.5	78.1	62.6
	10	104.2	75.1	78.0	71.7	78.2	62.9
	11	104.9	73.7	86.0	71.4	77.6	62.4
	12	104.5	73.8	86.0	71.5	77.8	62.6
α-*Xyl*-(1-3)-Glc	**11**	101.9	72.5	83.2	71.5	63.4	
	12	101.9	72.5	83.1	71.6	63.4	
α-*Xyl*-(1-3)-Xyl	**11**	101.6	74.1	75.1	71.5	63.6	
	12	101.6	74.1	75.1	71.6	63.6	

		Ser C-α	Ser C-β	Fmoc CH	Fmoc CH_2	Fmoc aromatic C	Pfp C	CO_2Pfp	OCONH
Other signal	**4**	54.2	68.6	47.1	67.3	143.7, 143.6, 141.3, 127.8, 127.1, 125.1, 120.0	140.8 (250), 139.7 (250), 137.7 (245)		155.8
	9	55.7	70.8	48.4	68.2	145.3, 142.6, 128.8, 128.2, 126.3, 120.9			
	10	55.8	69.8	48.4	68.2	145.2, 145.1, 142.6, 128.7, 128.2, 126.2, 120.9	142.5 (253), 139.4 (197)	168.2	158.6
	11	55.9	71.0	48.4	68.1	145.3, 142.6, 128.8, 128.2, 126.3, 120.9		168.3	
	12	55.9	70.0	48.4	68.2	145.2, 145.1, 142.6, 128.8, 128.2, 126.2, 120.9	142.4 (259), 139.3 (244)		158.6

[a] Compound **4** in $CDCl_3$, compounds **9–12** in CD_3OD. [b] These values are the one-bond $^{19}F-^{13}C$ coupling constants [$J(^{19}F-^{13}C)$] in Hz.

added. After 24 h the mixture was evaporated to dryness, and the residue was purified by semipreparative HPLC using a linear gradient of 30–60% solvent B over a period of 60 min (t_R 40 min). Compound **12** was obtained as a solid (10.6 mg, 42.4%); ^{1}H and ^{13}C NMR data are presented in Tables 3 and 5, respectively; *m/z* 920 $(M + H)^+$ and 942 $(M + Na)^+$ ($C_{40}H_{42}F_5NO_{18}$ requires *M*, 919.2).

{*Bis-5-acetamidomethyl-Cys*^{51}, *Cys*^{46}-O-[α-*xylopyranosyl*-(1-3)-α-D-*xylopyranosyl*-(1-3)-β-D-*glucopyranosyl*]-*Ser*^{53}}-*HBF-IX* (51–56) **13**.—The solid-phase peptide synthesis was carried out using method B as described above, using resin (178 mg, equivalent to 0.0294 mmol of peptide). The loading of the first amino acid onto the resin was estimated by quantitative amino acid analysis to be 0.165 mmol g^{-1}. The activated amino

Table 6 ^{13}C NMR data[a] for compounds **6** and **7**

		C-α	C-β	C-γ	C-δ
Cys^{51}	**6**	52.9	31.8		
	7	52.8	39.4		
Glu^{52}	**6**	53.9	27.0	30.7	
	7	54.3	26.7	32.5	
Ser^{53}	**6**	54.3	69.2		
	7	55.8	69.6		
Asn^{54}	**6**	49.4	37.1		
	7	49.6	37.5		
Pro^{55}	**6**	61.6	30.2	25.2	48.7
	7	60.9	31.1	22.8	48.5
Cys^{56}	**6**	53.7	32.8		
	7	52.8	39.6		
Acm	**6**	41.9/41.5	22.9/22.9		

		C-1	C-2	C-3	C-4	C-5	C-6
β-*Glc*-(1-3)-Ser	**6**	103.1	73.9	76.4	70.5	76.7	61.7
	7	103.3	73.9	76.3	70.5	76.7	61.6

[a] Compound **6** in 90% water–10% D_2O, pH 2.8; compound **7** in D_2O, pD ~ 6.0.

acids were added in 3.5-fold excess, except for the glycosylated serine derivative **12** which was added in only 0.37 mol equiv. (10.1 mg, 0.110 mmol). The end-points of the acylations were estimated by the decrease in the yellow colour of the resin during the acylation. The end-point of the acylation for the glycosylated serine derivative **12** could not be determined by the decrease in the yellow colour of the resin (compound **12** was added in only 0.37 mol equiv.), therefore the reaction mixture was left for 43 h to ensure consumption of substrate **12**. Purification of compound **13** was complicated by the presence of the deletion peptide which was a result of the expected incomplete acylation during the coupling of the glycosylated serine derivative. This impurity was expected to have a much different HPLC mobility than the glycosylated peptide; however, the chromatographic mobilities of the two products were almost identical on C-18 and C-8 reversed-phase columns as well as on a Biogel P-2 column. The desired compound was finally purified by repeated HPLC on a semipreparative C-8 column using a linear gradient of 0–20% solvent B over a period of 80 min (t_R 31 min, with the deletion peptide eluting earlier at 27 min). Compound **13** was finally obtained as a solid (3.2 mg, 24% yield based on the amount of compound **12** incorporated into peptide product). ^1H NMR data are presented in Table 4; *m/z* 1220 $(M + H)^+$ ($C_{45}H_{73}N_9O_{26}S_2$ requires *M*, 1219.4).

Cyclic {O-[α-D-*Xylopyranosyl*-(1-3)-α-D-*xylopyranosyl*-1-3)-β-D-*glucopyranosyl*]-*Ser*^{53}}*HBF-IX* (51–56) **14**.—A sample of compound **13** (3.0 mg, 0.0024 mmol) was dissolved in TFA acid (3.0 cm^3) containing anisole (10 mm^3, 0.092 mmol). The solution was cooled to 0 °C, and a solution of thallium(III) trifluoroacetate in TFA (0.25 cm^3; 15.5 μmol dm^{-3}) was added. After 105 min the reaction mixture was evaporated to dryness and diethyl ether (2 cm^3) was added to the residue. After the mixture had been stirred for 40 min the ether was decanted off, and the residue was purified by semipreparative HPLC using a linear gradient of 0–20% solvent B over a period of 80 min (t_R 18 min). The product was further purified by passage through a Biogel P-2 column with water as the eluent. The cyclic glycopeptide **14** was obtained, after lyophilization, as a solid (2.0 mg, 75%); ^1H NMR data are presented in Table 4. Amino acid analyses (theoretical values are in parentheses); Asn 1.04 (1), Ser 0.91 (1), Glu 1.08 (1), Cys 1.97 (2) and Pro 1.00 (1); *m/z* 1076 $(M + H)^+$ and 1098 $(M + Na)^+$ ($C_{39}H_{61}N_7O_{24}S_2$ requires *M*, 1075.3).

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