Glycosylation of Fmoc amino acids: preparation of mono- and di-glycosylated derivatives and their incorporation into Arg-Gly-Asp (RGD)-containing glycopeptides

Hiran Vegad,*[a] Charles J. Gray,[a] Peter J. Somers[a] and Anand S. Dutta[b]

[a] *School of Chemistry, The University of Birmingham, Edgbaston, Birmingham, UK B15 2TT*
[b] *ZENECA Pharmaceuticals, Alderley Park, Macclesfield, Cheshire, UK SK10 4TG*

Compounds containing O-glycosidic linkages between carbohydrate species (D-glucopyranose, D-mannopyranose and L-rhamnopyranose) and hydroxy-containing amino acid components (L-threonine, L-serine and L-hydroxyproline) have been synthesized. Significant quantities of disaccharide-containing by-products have been isolated and identified from reactions involving 2,3,4,6-tetraacetyl-α-D-gluco-pyranosyl bromide. With other sugar derivatives (D-mannose and L-rhamnose), the yields of these disaccharide-containing amino acid products are very much reduced.

The glycoamino acids have been incorporated into linear peptides containing the Arg-Gly-Asp (RGD) sequence in high yields by using solid-phase Fmoc-based methodology. In addition, cyclic glycopeptides containing cystine residues have been synthesized.

The synthesized glycopeptides are found to be equipotent with, or slightly less potent than, the corresponding non-glycosylated parent peptides on testing as platelet aggregation inhibitors.

Introduction

Glycopeptides contain one or more carbohydrate chains linked covalently by a glycosidic bond to an amino acid side chain. From the biological point of view, the importance of the amino acid sequence in a protein or a peptide is well established, but the role of the sugar residues linked to the amino acid sequence remains much less defined. However, for both glycopeptides and glycoproteins it has been suggested that the carbohydrate groups confer important physical properties such as extending the biological half-life of the active peptides *in vivo* (proteolytic protection)[1] and giving significantly different overall charges and water-binding capacities.[2] Additional roles supported by experimental evidence include alteration of the peptide backbone conformation,[3] control of molecular recognition and membrane permeability.[4] The carbohydrate moiety in proteins can bind hormones, enzymes and antibodies, but also toxins, viruses and bacteria[2,5] and all this may be important in biological recognition processes. Further properties of peptides which have been altered in a beneficial manner by the addition of sugar residues shown by recent experimental work include improvements in potency,[6] receptor selectivity,[7] duration of action[8] and their oral absorption.[9]

These diverse and important biological functions of the carbohydrate moiety have stimulated considerable efforts towards the synthesis of glycopeptides.[10–12] One approach to the synthesis of glycopeptides is the synthesis of the core peptide containing the desired amino acids followed by glycosylation. This has generally been shown to be unsatisfactory.[13] A more efficient method has been to prepare protected and glycosylated amino acids and to use them in a stepwise synthesis of glycopeptides using solid-phase peptide synthesis.[14]

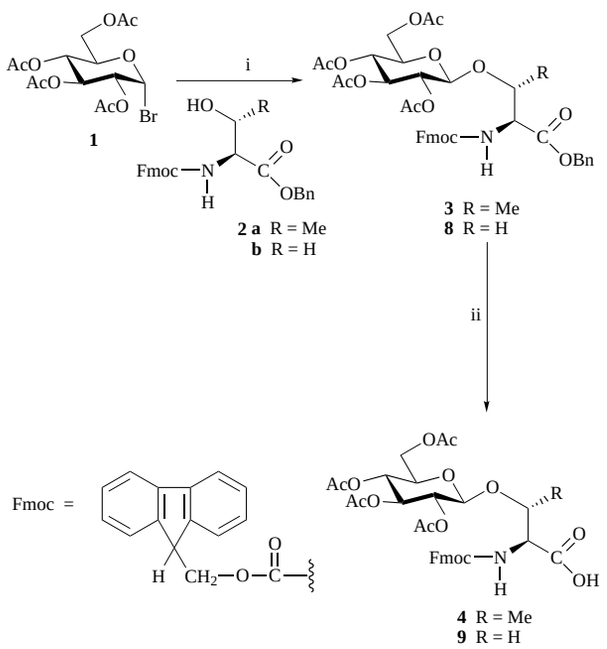
Mono-[15] and di-saccharide[16] O-linked amino acids have been prepared by coupling the appropriately protected glycosyl halides with the protected amino acid in a silver trifluoromethanesulfonate-promoted reaction (Hanessian's modification of the Koenigs–Knorr reaction).[17] For these reactions, the protecting groups used in the synthesis need to be carefully chosen, since the derivatives are sensitive to both strong acid (bond cleavage and anomerization) and strong base (β-elimination and racemization).[18] Other problems encountered by the pioneers in this area include low yields and poor α/β selectivity.

The purpose of the present investigation was to examine and to improve on the silver trifluoromethanesulfonate methodology for the glycosylation of L-serine, L-threonine and L-hydroxyproline derivatives and to investigate suitable methods for the incorporation of these derivatives into peptides. The sugar components used are derivatives of D-glucose, D-mannose and L-rhamnose.

For our reactions, the *N*-fluoren-9-ylmethoxycarbonyl (Fmoc) (removed using weak base, *e.g.* piperidine) and benzyl ester (removed by catalytic hydrogenation) groups were used to protect the amino acid during attachment of the sugar molecule. For protection of the hydroxy groups on the carbohydrate molecule, the acetate ester unit was employed since it also helps to control selectivity at the anomeric position. Subsequently the incorporation of these glycosylated amino acids into peptide chains by solid-phase peptide synthesis (SPPS) using the Fmoc strategy was examined.[19] Peptides containing the arginylglycylaspartic acid (Arg-Gly-Asp) (RGD) sequence, useful as antithrombotic agents,[20,21] were chosen to incorporate the glycosylated amino acids.

One of the main events in the formation of a blood clot is platelet aggregation. The activated platelets contain a cell-surface protein (GP IIb/IIIa) which acts as a receptor for plasma proteins involved in platelet aggregation and thrombus formation [*e.g.* fibrinogen and von Willebrand factor (vWF)]. The binding between the plasma proteins and IIb/IIIa is through the interaction of Arg-Gly-Asp sequences present in these proteins. Therefore, analogues of Arg-Gly-Asp or the non-peptide mimetics of this tripeptide would be expected to interfere with this protein–protein interaction. This has already been found to be the case and a number of publications have appeared in the literature on synthetic and naturally occurring (*e.g.* echistatin) inhibitors of platelet aggregation mediated by this protein–protein interaction.[21–24] We have used this tripeptide sequence to (a) devise suitable chemical methods for the synthesis of glycopeptides, (b) investigate the effects of mono- and di-saccharide derivatives on the conformation and biological activity (platelet aggregation) of the peptides and (c)

study the effects of the sugar residues on the cyclisation of small peptides. The glycoamino acids were incorporated into an RGD peptide at the N-terminal and then later at the C-terminal.

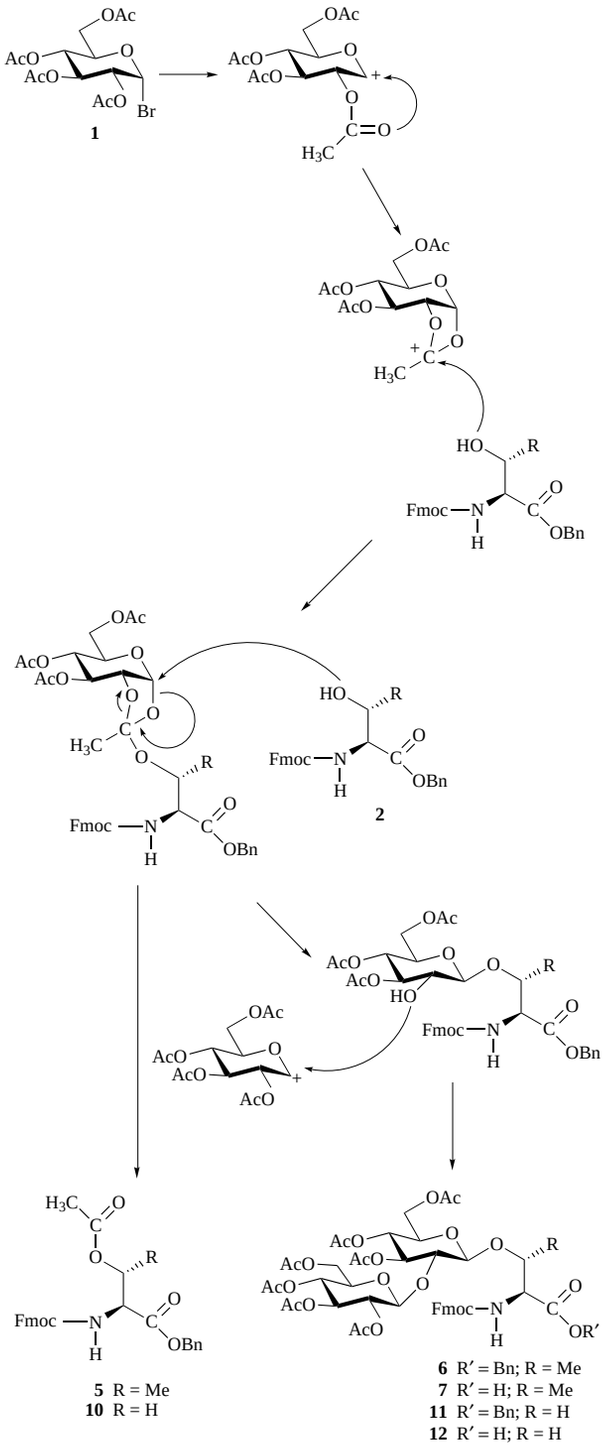


Scheme 1 *Reagents and conditions:* i, AgOTf, CH_2Cl_2, 0 °C; ii, H_2, Pd–C, EtOAc

Results and discussion

The route for the preparation of a glycosylated amino acid derivative such as *N*-Fmoc-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-L-threonine **4** is shown in Scheme 1. Glycosylation of *N*-Fmoc-L-threonine benzyl ester[25] **2a** with 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide[26] **1** (1.2 mol equiv.) was carried out using silver trifluoromethanesulfonate (AgOTf) (1.2 mol equiv.). TLC (EtOAc–toluene 1 : 4 v/v and 2 : 1 v/v) analysis after 2 h showed that, in addition to the desired product, a number of other products were produced during the reaction. After silica gel chromatography, the required product **3** was isolated in 35% yield. Two of the main impurities (Scheme 2) were shown to be *N*-Fmoc-*O*-acetyl-L-threonine benzyl ester **5** (33%) and *N*-Fmoc-*O*-[3,4,6-tri-*O*-acetyl-2-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-β-D-glucopyranosyl]-L-threonine benzyl ester **6** (17%). The final stage in Scheme 1 involved the removal of the benzyl ester protecting group to provide the required glycosylated amino acid **4** for incorporation into a peptide chain. The hydrogenation method used (10% Pd–C; H_2 balloon) was found to remove the Fmoc group to give 9-methylfluorene, a side reaction reported earlier by Martinez *et al.*[27] However, since the benzyl ester group was more sensitive to hydrogenolysis than the Fmoc group, this unwanted deprotection only proceeded in the latter period of the reaction, when almost 90% of the reaction had been completed, and so could be controlled. Like the glucopyranosyl threonine derivative **4**, the corresponding disaccharide derivative **6** could also be hydrogenolysed to give **7**.

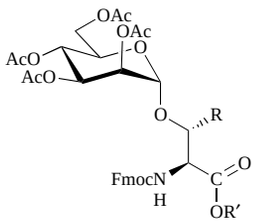
Similar results were obtained when *N*-Fmoc-L-serine benzyl ester **2b** was used in place of the threonine derivative in the reaction with 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide **1**. In addition to the desired product *N*-Fmoc-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-L-serine benzyl ester **8**, which on hydrogenolysis gave the free acid **9**, *N*-Fmoc-*O*-acetyl-L-serine benzyl ester **10** and *N*-Fmoc-*O*-[3,4,6-tri-*O*-acetyl-2-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-β-D-glucopyranosyl]-L-serine benzyl ester **11** were generated as side-products in the reaction. Raising the temperature had very little effect on the composition of the product. Also, the presence of sodium hydrogen carbonate, added during the reaction to keep the medium neutral, had little effect. As in the case of the disaccharide-containing threonine derivative **6**, hydrogenolysis of the serine derivative **11** gave the partially protected serine derivative **12** ready for incorporation into peptides.



Scheme 2 Postulated reaction mechanism to show side-product formation

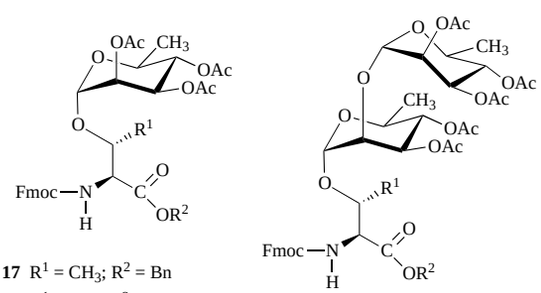
In contrast to the reaction of 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide **1** with *N*-Fmoc-L-threonine benzyl ester or *N*-Fmoc-L-serine benzyl ester derivatives, the side reactions producing the disaccharide-containing amino acid derivatives were not found to occur in significant amounts when other sugar derivatives were used. The reaction of 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl bromide under identical conditions with either L-threonine or L-serine derivatives resulted in

high yields (>70%) of the required monoglycosylated threonine and serine benzyl esters (**13** and **15**) with very little of the acetylated amino acid derivatives (<5%). FAB Mass spectroscopy showed the presence of only very small quantities of disaccharide-containing products. Catalytic hydrogenolysis of the benzyl esters **13** and **15** gave the free acids **14** and **16**.



13 R = Me; R′ = Bn
14 R = Me; R′ = H
15 R = H; R′ = Bn
16 R = H; R′ = H

With 2,3,4-tri-*O*-acetyl-α-L-rhamnopyranosyl bromide (compounds **17**–**22**), the side products were produced again in very small amounts. The ^{1}H NMR spectrum for the isolated disaccharide product containing the L-rhamnose and L-threonine moieties was extremely characteristic of this compound. The spectrum showed 13 H in the aromatic region and five clear singlets (3 H each) for the five acetate ester protecting groups on the disaccharide molecule. Furthermore, three sets of doublets at high field (δ 1.25, 1.21 and 1.10) characterized the two methyl groups on the two attached carbohydrate components and one methyl group on the threonine moiety. A fast-atom bombardment (FAB) mass spectrum confirmed the presence of the disaccharide molecule by showing the $[M + Na]^+$ peak at *m/z* 956.

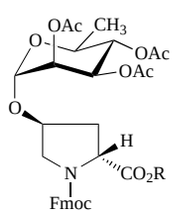


17 $R^1 = CH_3$; R^2 = Bn
18 $R^1 = CH_3$; R^2 = H
20 R^1 = H; R^2 = Bn
21 $R^1 = R^2$ = H

19 $R^1 = CH_3$; R^2 = Bn
22 R^1 = H; R^2 = Bn

L-Rhamnose-containing glycoamino acid derivatives **17**–**22**

Glycosylation of *N*-Fmoc-L-hydroxyproline benzyl ester with the L-rhamnose derivative gave *N*-Fmoc-*O*-(2,3,4-tri-*O*-acetyl-α-L-rhamnopyranosyl)-L-hydroxyproline benzyl ester **23** (63%), which on hydrogenolysis gave the hydroxyproline derivative **24**. There was very little evidence of any side product.

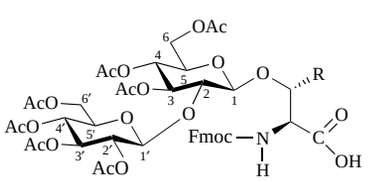


23 R = Bn
24 R = H

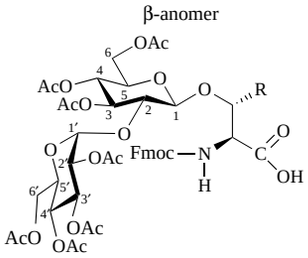
The reason for the difference in product composition between manno, rhamno and gluco derivatives was unclear, but is presumed to involve the position of the acetate ester protecting group at C-2 on the sugar molecule. In the D-glucose derivative this group is in the equatorial position, and in the D-mannose and L-rhamnose derivatives it occupies the axial position.

A reaction mechanism involving an orthoester is postulated to account for the production of these side products (Scheme 2). We assume provisionally that it was the acetate ester group on C-2 which was transferred to the amino acid hydroxy group. The disaccharide-containing amino acid derivatives would then have the second carbohydrate molecule on this C-2 position.

The ^{1}H 2D homonuclear chemical-shift correlation (COSY) spectra for the disaccharide-containing analogues were complicated and did not permit the identification of the position of attachment for the second carbohydrate molecule. At this stage the ^{13}C NMR spectrum of the monoglycosylated threonine derivative (with Fmoc protection) was compared with the ^{13}C NMR spectrum obtained for the disaccharide-containing threonine derivative. All the peaks obtained for the monoglycoamino acid could be correctly assigned. For the disaccharide-containing amino acid derivative, two sets of peaks were to be seen for all the different carbon atoms of the threonine molecule, *e.g.* two CH_3 peaks are seen (δ_C 16.3 and 18.1), when there should have only been one as shown on the monoglycosylated threonine molecule (δ_C 17.5). For the disaccharide moiety, three or four peaks could be assigned for each of the carbon atoms in the carbohydrate ring molecule, when only two were expected (*e.g.* C-1 from one glucose residue and C-1′ from the second attached glucose). This observation suggested that diastereoisomers were present of the disaccharide-containing threonine derivative. The two peaks obtained for each carbon atom on the threonine molecule were very close and indicated very little if any structural difference to cause formation of diastereoisomers. The largest shift was to be seen for C-1 and C-1′ and then for C-2 and C-2′ on the disaccharide molecule. The other set of peaks for the other carbon atoms (C-3 to C-6 and C-3′ to C-6′) on the sugar molecule were grouped fairly close together. This strongly suggested the presence of two different compounds with the main difference being the attachment of the second glucose molecule (α or β position) at its anomeric position (C-1′) to the first glucose molecule at carbon atom C-2.



β-anomer



α-anomer

7 R = Me
12 R = H

Diastereoisomers of disaccharide-containing threonine derivative **7** and serine derivative **12**

Identification of the disaccharide-containing analogues enhances the importance of these reactions, since they would allow mono- and di-saccharide amino acid derivatives to be produced in a one-pot mixture and then, after separation, to be used for incorporation into peptides. Further work to deter-

Table 1 Platelet aggregation activities of the glycopeptides

Compound	Peptide	pA_2 mean ± s.e.
25	H-Ser-Pro-Arg-Gly-Asp-Phe-OH	5.2 ± 0.1
26	H-Thr(α-L-rhamnose)-Pro-Arg-Gly-Asp-Phe-OH	5.0 ± 0.0
27	H-Thr(α-D-mannose)-Pro-Arg-Gly-Asp-Phe-OH	4.8 ± 0.3
28	H-Ser(L-rhamnose)-Pro-Arg-Gly-Asp-Phe-OH	5.2 ± 0.2
29	H-Ser(α-D-mannose)-Pro-Arg-Gly-Asp-Phe-OH	5.0 ± 0.1
30a and **30b**	H-Thr[β-D-glucosyl-(1→2)-D-glucose]-Pro-Arg-Gly-Asp-Phe-OH	5.4 ± 0.1
31	H-Asn-Pro-Arg-Gly-Asp-Phe-OH	6.0 ± 0.0
32	H-Asn-Pro-Arg-Gly-Asp-Phe-Thr(α-L-rhamnose)-OH	5.0 ± 0.4
33	H-Asn-Pro-Arg-Gly-Asp-Phe-Thr(β-D-glucose)-OH	NA
34a	H-Asn-Pro-Arg-Gly-Asp-Phe-Ser(α-L-rhamnose)-OH	NA
34b	H-Asn-Pro-Arg-Gly-Asp-Phe-Ser(β-L-rhamnose)-OH	5.1 ± 0.1
35	H-Asn-Pro-Arg-Gly-Asp-Phe-Hyp(α-L-rhamnose)-OH	5.8 ± 0.3
36	Ac-Asn-Cys-Arg-Gly-Asp-Cys-OH (cyclic peptide; S–S bridge)	6.0 ± 0.1
37	Ac-Thr(α-L-rhamnose)-Cys-Arg-Gly-Asp-Cys-OH (cyclic peptide; S–S bridge)	5.7 ± 0.2
38	Ac-Cys-Thr(α-L-rhamnose)-Pro-Arg-Gly-Asp-Cys-OH (cyclic peptide; S–S bridge)	NA

NA = pA_2 value <4.0.

mine the ideal conditions for the formation of disaccharide-containing derivatives is being investigated.

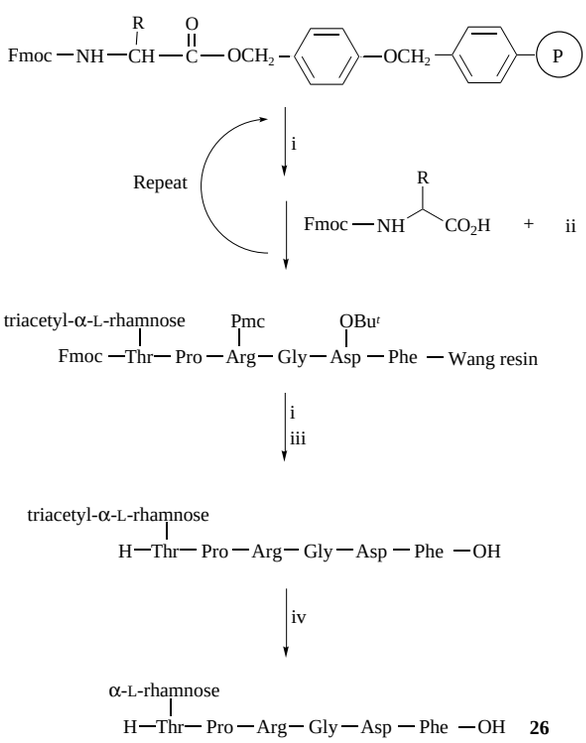
Solid-phase peptide synthesis

As mentioned earlier, Arg-Gly-Asp-containing peptides were used as model peptides to study the incorporation of glycoamino acid derivatives into peptides. All the peptides were synthesized by using the solid-phase peptide methodology.[19] In the Ser-Pro-Arg-Gly-Asp-Phe (**25**) series, the N-terminal Ser residue was replaced by amino acids (Ser or Thr) containing a sugar species in the side chain (compounds **26**–**30**), whereas in the Asn-Pro-Arg-Gly-Asp-Phe (**31**) series, the glycoamino acid residue was incorporated at the C-terminal end (compounds **32**–**35**). To investigate the effect of a protected sugar residue on cyclization (formation of disulfide bridge), acetyl-Asn-Cys-Arg-Gly-Asp-Cys **36** was used as a model peptide and the glycoamino acid residues were incorporated at the N- or C-terminal side of the first cysteine residue involved in the disulfide bridge (see peptides **37** and **38**). The peptides listed in Table 1 were tested as platelet aggregation inhibitors using human platelet rich plasma. Details of the procedure have been reported earlier.[28]

Initially, Wang resin pre-loaded with the first amino acid was used as the solid support. The resin consists of 1% cross-linked polystyrene beads (200–400 mesh) functionalized with the trifluoroacetic acid (TFA)-labile *p*-benzyloxybenzyl alcohol handle. Cleavage from the resin leads to peptides containing a free C-terminal carboxy group.[29]

A series of deblocking and coupling cycles was carried out to generate a fully protected peptide on the resin (Scheme 3). Each cycle consisted of removal of Fmoc, by the action of 20% (v/v) piperidine in dimethylformamide (DMF) solution. Coupling of the appropriate Fmoc-amino acid to the exposed amino terminal was effected by 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and diisopropylethylamine (DIPEA) activation in DMF (0.5 M). Four-fold excesses of the Fmoc-amino acid and HBTU (compared with the resin) were used with an eight-fold excess of the base DIPEA. At the end of the chain assembly, the deprotection reagent mixture of TFA–water (95 : 5% v/v) with triisopropylsilane (TIPS) was employed to detach the peptide from the resin support and simultaneously to remove all the side-chain-protecting groups from the amino acid residues. Removal of the acetate ester groups from the carbohydrate portion of the *O*-linked glycopeptide was achieved using hydrazine hydrate in methanol.[30] This was carried out on the detached glycopeptide, in order to avoid formation of a C-terminal hydrazide.

Incorporation of glycoamino acid at the N-terminal. Glycopeptides **26–30** were synthesized with the glycoamino acid at the N-terminal. For the monoglycoamino acids, a three-fold excess of the amino acid derivative was used and coupling was



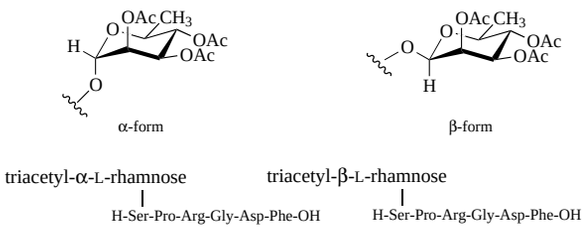
Scheme 3 *Reagents:* i, piperidine, DMF; ii, HBTU, DIPEA; iii, TFA–water, TIPS; iv, $H_2NNH_2 \cdot H_2O$, MeOH

complete within 30 min. Incorporation of a glycosylated threonine derivative into peptides **26** and **27** presented no problems. Analytical high-performance liquid chromatography (HPLC) analysis of the crude acetylated peptides exhibited one principal peak and the same was observed on complete removal of the acetate groups. The formation of the free hydroxy groups on the carbohydrate molecule caused the peptides to become more hydrophilic and a more shallow eluent gradient (less acetonitrile) was required to observe the peak on the chromatogram.

Reversed-phase HPLC analysis of the cleaved acetylated peptide with an incorporated serine (L-rhamnose) derivative exhibited two principal peaks (75 and 15%). On removal of the acetate groups the two peaks could not be separated by HPLC. However, when the purified peptide **28** was analysed using ion-exchange chromatography and capillary electrophoresis, two major products could be seen (80 and 18%). Using reversed-phase HPLC, separation of the peptides could only be achieved with the acetate protection on the sugar moiety. Both peptides gave a similar amino acid analysis, and MALDI mass spectroscopy showed that both had the same molecular mass (950).

This indicated the presence of two isomeric forms of the peptide.

Further analysis of the two peptides using ^{1}H NMR spectroscopy showed a major difference in the positioning of the anomeric proton (H-1 on the carbohydrate molecule), with the rest of both spectra being almost identical. The doublet ($J_{1,2}$ 1.0 Hz) representing the anomeric proton was shown to have a chemical shift of δ 4.94 and 4.86, respectively, for the two peptides. This gave strong evidence to suggest that the two peptides differed in the way in which the rhamnose moiety was attached to the serine residue (α = 80% and β = 18%).



Two anomeric forms of acetylated glycopeptide **28**

The likely explanation appears to be that the glycosylated serine derivative was present in two diastereomeric forms before being inserted into the peptide sequence. Although this could not easily be seen from the ^{1}H NMR spectrum of the single amino acid derivative, incorporation into a peptide has caused the anomeric forms to be more distinguishable as shown by the shift in the position of the anomeric proton. This was further confirmed by treatment of the glycosylated peptide with piperidine, TFA and hydrazine hydrate to test whether their action could cause the formation of the isomer, but all seemed to show little effect.

The presence of two peptides was also seen on HPLC when an L-serine(tetraacetyl-D-mannose) residue was incorporated. The separation of these two peaks was very difficult since they were very close together, and only one of the peptides (attachment of a mannose molecule in the α-form) could be isolated in a reasonable yield to be later deacetylated to give peptide **29**. Since peptides incorporating the glycosylated threonine derivatives existed as only one anomeric form, the importance of the methyl group on the threonine molecule in controlling the stereochemistry of the glycosidic linkage can be noted.

For the disaccharide-containing glycoamino acid derivative **7**, only 1.25 mol equiv. was used to incorporate the residue into the peptide, since very little of the derivative had been prepared. The coupling was complete in 30 min. Peptide obtained after cleavage with TFA was shown to exhibit a single peak on HPLC. Upon removal of all the acetate groups, two major peaks could now be seen (41 and 54%). Attempted separation of the two peaks gave a mixture (29%) and 6% yields of each isolated product **30a** and **30b**. The presence of two different peptides was confirmed from ion-exchange chromatography. This was expected since the glycoamino acid residue used for the coupling was shown earlier to be a mixture of diastereoisomers.

Incorporation of glycoamino acid at the C-terminal. The synthesis of a peptide which contained a glycoamino acid at the C-terminal end would require several treatments with piperidine. To establish whether the glycosidic linkage and the protecting groups were stable to repetitive piperidine treatments, glycopeptides containing a glycosylated amino acid at the C-terminal end of the peptide were synthesized. This also enabled us to investigate the ease of attachment of the glycoamino acid derivative to the resin which is made *via* an ester bond; this is often a difficult process which, unless carried out under controlled conditions, can lead to low substitution, racemization or formation of a dipeptide.[31]

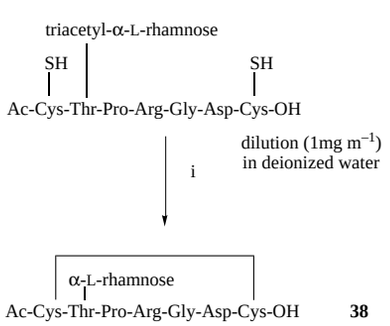
Attachment of the amino acid derivative to the hydroxymethylphenyl resin was achieved using 2,6-dichlorobenzoyl chloride in dichloromethane.[32] The residual, unchanged hydroxy groups of the polymer were acetylated with acetic anhydride and pyridine. Glycopeptides **31–34b** shown in Table 1 were synthesized. L-Hydroxyproline(triacetyl-α-L-rhamnose) residue was also used and was found to couple very well to the Wang resin, but problems were met during the build-up of the peptide sequence. At the dipeptide stage, after the removal of the Fmoc group, a loss of resin peptide was observed in the reactor vessel. This loss of peptide from the resin at the dipeptide stage is likely to have been caused by cyclization, with formation of a diketopiperazine.[33] To overcome this, 2-chlorotrityl chloride resin[34] was used. The extreme steric hindrance of the resin prevents formation of a diketopiperazine. The L-hydroxyproline residue was attached and the peptide chain was elaborated without any problems. For the removal of the acetate protection from the glycosylated residue, two routes were used. One method was to treat the peptide with hydrazine hydrate–DMF (1:4 v/v) whilst it remained anchored to the resin and an alternative route was to cleave the peptide from the resin and then to treat it with hydrazine hydrate. Both methods successfully gave the required peptide **35** and there was no formation of hydrazide at the C-terminal as seen when using Wang resin.

Synthesis of cystine-containing peptides. The peptides were assembled on the resin using procedures similar to those described above. Formation of disulfide bonds from free cysteine residues was performed using either air oxidation[35] or dimethyl sulfoxide (DMSO) (10% v/v) in TFA.[36,37] Both methods were successfully used to synthesize the cystine-containing peptide **36**. To monitor the progress of oxidation of the crude peptide and to assess reaction completion, reversed-phase HPLC, MALDI mass spectroscopy (change of 2 units in mass) and capillary electrophoresis were used.

The two peptides (**37** and **38**) synthesized had the glycosylated amino acid residue either outside or within the sequence incorporating the disulfide bridge.

Oxidation of the acetylated form of the peptide **37** using the DMSO–TFA method, followed by treatment with hydrazine hydrate, gave a final product which exhibited two peaks on HPLC (55 and 30%). The larger peak was shown to be the required oxidized peptide **37** and the other peak was found to be the reduced form of the peptide. The use of hydrazine hydrate had apparently reduced the oxidized peptide to a certain extent.

Oxidation of the acetylated peptide using the air oxidation method gave a peptide which was shown to have a molecular mass of 840. This was surprising since this was to be the molecular mass of the final fully deprotected oxidized peptide **37**. The few drops of ammonia solution added to the mixture (change in pH from 5.3 to 8.5) for the air-oxidation process were apparently responsible for removing the acetate groups from the sugar moiety. Both formation of cystine and acetate removal were achieved in one single, simple step and the use of hydrazine hydrate for deprotection was avoided. Using this air-oxidation method, a disulfide bridge was also formed with the glycosylated threonine derivative between two cysteine residues (Scheme 4).



Scheme 4 Formation of cystine and deprotection of acetate ester. *Reagents and conditions:* i, ammonia solution (pH 8.5), 2–3 days.

Both peptides were purified by preparative HPLC and a single peak was observed in all the chromatographic techniques used (>98% pure).

Platelet aggregation inhibitory activity of glycopeptides. In comparison with the parent peptides **25** and **31**, the incorporation of a glycoamino acid residue at the N- or C-terminal end of the peptides did not lead to an improvement in potency. All the glycopeptide analogues were either equipotent or slightly less potent than the corresponding parent peptide. The nature of the sugar residue when incorporated at the N-terminal end does not appear to make any significant difference to the biological activity. Glycopeptides **26–30** containing either a monosaccharide (rhamnose or mannose) or a disaccharide (glucosylglucose) were similar in potency to the parent peptide **25**. This indicates that the sugar residues at the N-terminal end of the peptide do not interfere with the biological activity. In contrast, the incorporation of the sugar residues at the C-terminal end appears to reduce potency. Two of the rhamnose- and glucose-containing peptides, **33** and **34a**, were at least 100-fold less potent ($pA_2 < 4.0$) than the non-glycosylated hexapeptide **31**. Only one of the peptides, **35**, was comparable in potency to the non-glycosylated hexapeptide.

In the case of the cyclic peptides **37** and **38**, the glycoamino acid residues did not have any deleterious effect on the cyclization of the peptides. The three disulfide bridge-containing peptides listed in Table 1 were obtained in comparable yield and purity. However, the biological activities of the cyclic peptides were significantly changed depending on the position of the sugar residue. Replacement of the asparagine residue in peptide **36** by a Thr(α-L-rhamnose) residue resulted in an equipotent compound, but when the same glycoamino acid was incorporated within the ring structure the resulting compound **38** was much less potent. The loss in activity is not just due to additional amino acid residues in the ring structure because the corresponding non-glycosylated peptide Ac-Cys-Asn-Pro-Arg-Gly-Asp-Cys (cyclic peptide; S–S bridge) has been reported to be very active.[23] It would, therefore, appear that the incorporation of a glycoamino acid residue in the conformationally restricted cyclic ring structure of the peptide changes the overall conformation very significantly in comparison to the peptide when the same glycoamino acid is present just outside the ring structure.

Experimental

Preparation of glycosylated amino acids

General procedures. All solvents were freshly distilled and dried over 3 Å molecular sieves prior to use. For flash chromatography, 400–230 mesh silica gel 60 (E.Merck No. 9385) was employed. TLC analysis was carried out on silica gel plates (Merck 1.05554 Kieselgel 60 F254). Compounds were visualized by UV light (254 nm) and by dipping with conc. H_2SO_4–methanol (1:10 v/v) and subsequent charring with heat. ^1H and ^{13}C NMR spectroscopy was performed on a Bruker AC300 (300 and 75 MHz, respectively) FT-NMR spectrometer. Chemical shifts are given in parts per million (δ) and referenced to internal $SiMe_4$. *J* Values are given in Hz (±0.5 Hz). For all final glycoamino acids, the assignment of ^1H NMR spectra was based on COSY spectra. ^{13}C NMR spectra were recorded using the JMOD pulse sequence. Elemental analyses were performed on a Perkin-Elmer 240 Elemental Analyzer or were obtained from Sheffield University, Sheffield. FAB mass spectra were obtained on a Kratos MS-80 RF or a VG.ZABSPEC mass spectrometer using a Sun workstation for data processing. All mps were measured on a Hoover capillary melting point apparatus and are uncorrected.

General description of glycosylation reactions. The glycosyl acceptor (18 mmol, 1 mol equiv.), glycosyl donor (22 mmol, 1.2 mol equiv.) and oven-dried 4 Å molecular sieves (10 g) in CH_2Cl_2 (100 ml) were stirred at 0 °C under nitrogen for 10 min. A solution of silver triflate (22 mmol, 1.2 mol equiv.) in toluene (20 ml) was added at 0 °C in portions over a period of 20 min and stirring was continued for 2 h in the dark. The reaction mixture was quenched with *N*-methylmorpholine to change pH from 2.0 to 7.0 and was then filtered through Celite. The organic layer was washed with water (3 × 50 ml) and dried ($MgSO_4$). Rotary evaporation, followed by flash chromatography with toluene–EtOAc (4:1 v/v) provided the glycosylated product.

General description for removal of benzyl ester group from derivatives. The glycosylated derivative (9.0 g) was dissolved in ethyl acetate (100 ml) and deoxygenated for 5–10 min under hydrogen gas. 10% Pd–C (1.8 g, 20% mass of glycosylated derivative) was then added and the mixture was flushed with hydrogen and then stirred (under H_2 balloon). The reaction was monitored by TLC (EtOAc–toluene, 2:1 v/v) and stopped at a stage (after 2 h) when the Fmoc group had begun to be eliminated to produce 9-methylfluorene (R_f 0.89). To stop the reaction, the mixture was flushed with nitrogen gas and the Pd–C was filtered off using Celite. The solvents were then evaporated off and the residue was chromatographed, initially with dichloromethane to extract 9-methylfluorene, followed by elution with CH_2Cl_2–EtOH (10:1 v/v) to isolate the required product. The organic phase was dried ($MgSO_4$) and evaporated to yield the required glycoamino acid.

N-*Fmoc*-O-(2,3,4,6-*tetra*-O-*acetyl*-β-D-*glucopyranosyl*)-L-*threonine benzyl ester* **3**.—The glycosylation reaction was carried out using *N*-Fmoc-L-threonine benzyl ester[25] **2** (7.76 g, 18.0 mmol) and 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide[26] **1** (8.88 g, 21.6 mmol). Chromatography on silica gel with toluene–EtOAc (4:1 v/v; R_f 0.20) as the eluent yielded title compound **3** as a foam (4.79 g, 35%) (Found: C, 62.5; H, 5.61; N, 1.81. $C_{40}H_{43}NO_{14}$ requires C, 63.1; H, 5.65; N, 1.84%); δ_H(300 MHz; $CDCl_3$) 1.22 (3 H, d, *J* 7.0, CH_3), 2.00–2.05 (4 × 3 H, s, OAc), 3.43–3.50 (1 H, m, 5-H), 4.05 (1 H, dd, $J_{6,6'}$ 12.0, $J_{5,6}$ 4.0, 6-H), 4.18–4.29 (2 H, m), 4.35–4.50 (5 H, m), 4.92 (1 H, t, $J_{3,4}$ 10.0, $J_{4,5}$ 10.0, 4-H), 5.03 (1 H, t, $J_{2,3}$ 10.0, $J_{3,4}$ 10.0, 3-H), 5.10–5.24 (3 H, m, 1-H, CH_2Ph), 5.70 (1 H, d, *J* 9.0, NH exchanges with D_2O), 7.27–7.43 (9 H, m, ArH), 7.63 (2 H, d, *J* 8.0, ArH) and 7.77 (2 H, d, *J* 8.0, ArH); δ_C(75 MHz; $CDCl_3$) 17.9 (CH_3), 20.5, 20.7 (CH_3 on OAc), 47.1 (CH in Fmoc), 58.5 (NHCH), 62.6 (CH_2 in Fmoc), 66.4 (*C*HCH_3), 67.1 (C-6), 67.7 (*C*H_2Ph), 68.7, 69.2 and 69.8 (C-3, -4 and -5), 76.9 (C-2), 98.9 (C-1), 120.1, 125.3, 127.1, 127.7, 128.3, 128.6 and 128.7 (CH aromatic), 135.1, 140.7 and 143.9 (aromatic), 156.7 (C=O in Fmoc), 169.5 and 169.7 (C=O in OAc) and 170.4 (C=O on benzyl ester); *m/z* (FAB) 762 [$(M + H)^+$, 6%], 331 [(sugar residue)$^+$, 24] and 178 [(9-methylfluorene)$^+$, 100].

N-*Fmoc*-O-(2,3,4,6-*tetra*-O-*acetyl*-β-D-*glucopyranosyl*)-L-*threonine* **4**.—Removal of the benzyl ester group from compound **3** (4.57 g, 6 mmol), followed by silica gel chromatography (R_f 0.59; CH_2Cl_2–EtOH, 10:1 v/v) provided *acid* **4** as a foam (2.82 g, 70%) (Found: C, 58.8; H, 5.45; N, 2.06. $C_{33}H_{37}NO_{14}$ requires C, 59.0; H, 5.55; N, 2.09%); δ_H(300 MHz; [$^2H_6$]DMSO) 1.12 (3 H, d, *J* 6.5, CH_3), 1.91, 1.92, 1.93 and 1.94 (4 × 3 H, s, OAc), 3.20–3.50 (1 H, br s, CO_2H exchanges with D_2O), 3.79–4.07 (3 H, br m), 4.19–4.34 (5 H, m), 4.67 (1 H, t, $J_{2,3}$ 10.0, $J_{1,2}$ 9.5, 2-H), 4.78 (1 H, d, $J_{1,2}$ 9.5, 1-H), 4.87 (1 H, t, $J_{3,4}$ 10.0, $J_{4,5}$ 10.0, 4-H), 5.19 (1 H, t, $J_{3,4}$ 10.0, $J_{3,2}$ 10.0, 3-H), 6.04–6.13 (1 H, br m, NH exchanges with D_2O), 7.32 (2 H, t, *J* 7.5, ArH), 7.40 (2 H, t, *J* 7.5, ArH), 7.68 (2 H, d, *J* 7.5, ArH) and 7.86 (2 H, d, *J* 7.5, ArH); δ_C(75 MHz; [$^2H_6$]DMSO) 17.5 (CH_3), 20.3 and 20.5 (CH_3 in OAc), 46.7 (CH in Fmoc), 59.6 (NHCH), 61.7 (CH_2 in Fmoc), 65.7 (C-6), 68.1 (*C*HCH_3), 70.4, 71.0 and 72.1 (C-3, -4 and -5), 75.4 (C-2), 97.5 (C-1), 120.1, 125.2, 127.1 and 127.6 (CH aromatic), 140.7 and 143.9 (aromatic), 156.1 (C=O in Fmoc), 169.1, 169.2, 169.5 and 170.0 (4 × C=O in OAc) and 176.3 (CO_2H); *m/z* (FAB) 694 [$(M + Na)^+$, 38%] and 331 [(sugar residue)$^+$, 100].

O-*Acetyl*-N-*Fmoc*-L-*threonine benzyl ester* **5**.—Compound **5**

was isolated from the reaction mixture during the synthesis of compound **3**, by silica gel chromatography, as a solid (2.81 g, 33%), R_f 0.54 (toluene–EtOAc, 4:1 v/v) (Found: C, 71.2; H, 5.66; N, 2.91. $C_{28}H_{27}NO_6$ requires C, 71.0; H, 5.71; N, 2.96%); δ_H(300 MHz; $CDCl_3$) 1.26 (3 H, d, *J* 7.0, CH_3), 1.89 (3 H, s, OAc), 4.25 (1 H, t, *J* 8.0, CH in Fmoc), 4.45 (2 H, d, *J* 8.0, CH_2 in Fmoc), 4.55 (1 H, dd, *J* 8.0 and 3.0, NHC*H*), 5.12 (1 H, d, *J* 13.0, OCH^a*H*^{b}Ph), 5.19 (1 H, d, *J* 13.0, OC*H*^{a}H^{b}Ph), 5.40–5.46 (1 H, m, C*H*CH_3), 5.48 (1 H, d, *J* 9.0, NH exchanges with D_2O), 7.27–7.43 (9 H, m, ArH), 7.60 (2 H, d, *J* 8.0, ArH) and 7.76 (2 H, d, *J* 8.0, ArH); *m/z* (FAB) 496 [(M + Na)$^+$, 2%], 474 [(M + H)$^+$, 8] and 178 [(9-methylfluorene)$^+$, 100].

N-*Fmoc*-O-[3,4,6-*tri*-O-*acetyl*-2-O-(2,3,4,6-*tetra*-O-*acetyl*-β-D-*glucopyranosyl*)-β-D-*glucopyranosyl*]-L-*threonine benzyl ester* (*mixture of* α- *and* β-*anomers*) **6**.—Compound **6** was isolated from the reaction mixture during the synthesis of compound **3** by silica gel chromatography as a foam (1.96 g, 17%), R_f 0.75 (toluene–EtOAc, 1:2 v/v) (Found: C, 58.4; H, 5.56; N, 1.28. $C_{52}H_{59}NO_{22}$ requires C, 59.5; H, 5.62; N, 1.33%); δ_H(300 MHz; $CDCl_3$) 1.30 (3 H, d, *J* 7.0, CH_3), 1.98–2.12 (21 H, br m, 7 × OAc), 3.62–3.71 (1 H, m), 3.80–3.85 (1 H, m), 4.02–4.50 (11 H, m), 4.90–5.58 (8 H, m), 6.15 (1 H, m, NH exchanges with D_2O) and 7.22–7.80 (13 H, ArH); characteristic δ_C(75 MHz; $CDCl_3$) 17.3 and 17.8 (α- and β-CH_3), 20.1, 20.2, 20.3, 20.4, 20.6, 20.7 and 20.9 (OAc), 46.5 and 47.0 (α- and β-CH in Fmoc), 62.1 and 62.6 (α- and β-CH_2 in Fmoc), 66.7 and 67.1 (α- and β-C-6), 76.3, 76.6, 76.8 and 77.0 (α- and β-C-2, -C-2′), 97.7, 98.2, 99.0 and 100.1 (α- and β-C-1, -C-1′) 120.1, 125.2, 127.1, 127.7, 128.3, 128.5 and 128.7 (CH aromatic), 135.9, 140.7, 143.7 and 143.8 (aromatic), 156.3 and 156.6 (α- and β-C=O in Fmoc), 168.2, 169.3, 169.4, 169.5, 169.7, 169.8, 169.9 and 170.0 (C=O in OAc) and 170.1 and 170.3 (C=O of benzyl ester); *m/z* (FAB) 1050 [(M + H)$^+$, 14%], 619 [(disaccharide residue)$^+$, 9] and 331 [(monosaccharide residue)$^+$, 100].

N-*Fmoc*-O-[3,4,6-*tri*-O-*acetyl*-2-O-(2,3,4,6-*tetra*-O-*acetyl*-β-D-*glucopyranosyl*)-β-D-*glucopyranosyl*]-L-*threonine* (*mixture of* α- *and* β-*anomers*) **7**.—Removal of the benzyl ester group from compound **6** (1.80 g, 1.7 mmol), followed by silica gel chromatography (R_f 0.53; EtOH–hexane, 3:1 v/v) provided *acid* **7** as a foam (1.06 g, 65%) (Found: C, 55.9; H, 5.54; N, 1.47. $C_{45}H_{53}NO_{22}$ requires C, 56.3; H, 5.57; N, 1.46%); δ_H(300 MHz; [$^2H_6$]DMSO) 1.25 (3 H, d, *J* 6.5, CH_3), 1.93–2.10 (21 H, br m, 7 × OAc), 3.74–4.38 (14 H, m), 4.74–5.43 (7 H, m), 7.33 (2 H, t, *J* 7.5, ArH), 7.42 (2 H, t, *J* 7.5, ArH), 7.72 (2 H, d, *J* 7.5, ArH) and 7.90 (2 H, d, *J* 7.5, ArH); characteristic δ_C(75 MHz; [$^2H_6$]DMSO) 16.3 and 18.1 (α- and β-CH_3), 46.5 and 46.6 (α- and β-CH in Fmoc), 61.6 and 61.8 (α- and β-CH_2 in Fmoc), 65.9 and 66.1 (α- and β-C-6), 76.2, 77.3, 77.6 and 77.7 (α- and β-C-2, -C-2′), 96.9, 98.5, 99.0 and 100.3 (α- and β-C-1, -C-1′) 120.1, 125.2, 125.3, 125.6, 127.1 and 127.6 (CH aromatic), 140.6, 140.7, 143.7 and 143.8 (aromatic), 156.0 and 156.3 (α- and β-C=O in Fmoc), 168.7, 169.1, 169.2, 169.3, 169.4, 169.5, 169.6 and 170.0 (C=O in OAc) and 170.2 and 170.3 (α- and β-CO_2H); *m/z* (FAB) 982 [(M + Na)$^+$, 75%], 960 [(M + H)$^+$, 19], 619 [(disaccharide residue)$^+$, 18] and 331 [(monosaccharide residue)$^+$, 100].

N-*Fmoc*-O-(2,3,4,6-*tetra*-O-*acetyl*-β-D-*glucopyranosyl*)-L-*serine benzyl ester* **8**.—The glycosylation reaction was carried out using *N*-Fmoc-L-serine benzyl ester[25] (7.51 g, 18.0 mmol) and 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide **1** (8.88 g, 21.6 mmol). Chromatography on silica gel with toluene–EtOAc (4:1 v/v; R_f 0.25) as the eluent yielded *title compound* **8** as a foam (4.30 g, 32%) (Found: C, 62.8; H, 5.44; N, 1.82. $C_{39}H_{41}NO_{14}$ requires C, 62.7; H, 5.49; N, 1.87%); δ_H(300 MHz; $CDCl_3$) 1.98–2.05 (4 × 3 H, s, OAc), 3.58–3.63 (1 H, m, 5-H), 3.87 (1 H, dd, $J_{5,6}$ 4.0, $J_{6,6'}$ 12.0, 6-H), 4.09 (1 H, dd, $J_{5,6'}$ 4.0, $J_{6,6'}$ 12.0, 6′-H), 4.18–4.58 (8 H, m), 4.95 (1 H, t, $J_{3,4}$ 10.0, $J_{4,5}$ 10.0, 4-H), 5.05 (1 H, t, $J_{2,3}$ 10.0, $J_{3,4}$ 10.0, 3-H), 5.20 (2 H, s, CH_2Ph), 5.62 (1 H, d, *J* 9.0, NH exchanges with D_2O), 7.28–7.44 (9 H, m, ArH), 7.61 (2 H, d, *J* 8.0, ArH) and 7.77 (2 H, d, *J* 8.0, ArH); δ_C(75 MHz; $CDCl_3$) 20.7 and 20.8 (CH_3 in OAc), 47.1 (CH in Fmoc), 56.5 (NHCH), 62.5 (CH_2 in Fmoc), 66.2 (NHC*H*CH_2), 67.2 (C-6), 67.7 (*C*H_2Ph), 68.7, 69.3, 69.4 and 69.5 (C-2, -3, -4 and C-5), 98.8 (C-1), 120.1, 125.2, 127.1, 127.8, 128.3, 128.5 and 128.7 (CH aromatic), 135.1, 140.9 and 143.7 (aromatic), 156.4 (C=O in Fmoc), 169.5, 169.6, 169.7 and 169.9 (4 × C=O in OAc) and 170.5 (C=O on benzyl ester); *m/z* (FAB) 748 [(M + H)$^+$, 4%], 331 [(sugar residue)$^+$, 24] and 178 [(9-methylfluorene)$^+$, 100].

N-*Fmoc*-O-(2,3,4,6-*tetra*-O-*acetyl*-β-D-*glucopyranosyl*)-L-*serine*[15] **9**.—Removal of the benzyl ester group from compound **8** (4.15 g, 5.6 mmol), followed by silica gel chromatography (R_f 0.57; CH_2Cl_2–EtOH, 10:1 v/v) provided acid **9** as a foam (2.65 g, 72%) (Found: C, 58.3; H, 5.26; N, 2.09. $C_{32}H_{35}NO_{14}$ requires C, 58.4; H, 5.36; N, 2.13%); δ_H(300 MHz; [$^2H_6$]DMSO) 1.87, 1.93, 1.98 and 2.04 (4 × 3 H, s, OAc), 3.20–3.50 (1 H, br s, CO_2H exchanges with D_2O), 3.74–4.06 (5 H, m), 4.16–4.32 (4 H, m), 4.69 (1 H, t, $J_{2,3}$ 10.0, $J_{1,2}$ 10.0, 2-H), 4.78 (1 H, d, $J_{1,2}$ 10.0, 1-H), 4.88 (1 H, t, $J_{3,4}$ 10.0, $J_{4,5}$ 10.0, 4-H), 5.24 (1 H, t, $J_{3,4}$ 10.0, $J_{3,2}$ 10.0, 3-H), 6.44–6.52 (1 H, br m, NH exchanges with D_2O), 7.34 (2 H, t, *J* 7.5, ArH), 7.42 (2 H, t, *J* 7.5, ArH), 7.70 (2 H, d, *J* 7.5, ArH) and 7.90 (2 H, d, *J* 7.5, ArH); δ_C(75 MHz; [$^2H_6$]DMSO) 20.8 and 21.5 (CH_3 in OAc), 46.9 (CH in Fmoc), 60.6 (NHCH), 61.2 (CH_2 in Fmoc), 65.1 (C-6), 68.3, 70.8, 70.9 and 71.1 (C-3, -4, -5 and CH*C*H_2), 75.4 (C-2), 96.7 (C-1), 120.4, 125.2, 127.5 and 127.6 (CH aromatic), 141.8 and 142.9 (aromatic), 155.1 (C=O in Fmoc), 169.7, 169.9, 170.0 and 170.3 (4 × C=O in OAc) and 176.0 (CO_2H); *m/z* (FAB) 680 [(M + Na)$^+$, 100%] and 331 [(sugar residue)$^+$, 76].

O-*Acetyl*-N-*Fmoc*-L-*serine benzyl ester* **10**.—*Compound* **10** was isolated from the reaction mixture during the synthesis of compound **8**, by silica gel chromatography, as a solid (2.48 g, 30%), R_f 0.57 (EtOAc–toluene, 4:1 v/v) (Found: C, 70.5; H, 5.47; N, 3.02. $C_{27}H_{25}NO_6$ requires C, 70.6; H, 5.45; N, 3.05%); δ_H(300 MHz; $CDCl_3$) 1.96 (3 H, s, OAc), 4.22 (1 H, t, *J* 8.0, CH in Fmoc), 4.35 (1 H, dd, *J* 3.0 and 11.0, CH^a*H*^{b}OAc), 4.41 (2 H, d, *J* 8.0, CH_2 in Fmoc), 4.55 (1 H, dd, *J* 3.0 and 11.0, C*H*^{a}H^b-OAc), 4.70 (1 H, m, NHC*H*), 5.17 (1 H, d, *J* 13.0, OCH^a*H*^{b}Ph), 5.23 (1 H, d, *J* 13.0, OC*H*^{a}H^{b}Ph), 5.61 (1 H, d, *J* 9.0, NH exchanges with D_2O), 7.27–7.43 (9 H, m, ArH), 7.60 (2 H, d, *J* 8.0, ArH) and 7.76 (2 H, d, *J* 8.0, ArH); (FAB) *m/z* 460 [(M + H)$^+$, 5%] and 178 [(9-methylfluorene)$^+$, 100].

N-*Fmoc*-O-[3,4,6-*tri*-O-*acetyl* -2-O-(2,3,4,6-*tetra*-O-*acetyl*-β-D-*glucopyranosyl*)-β-D-*glucopyranosyl*]-L-*serine benzyl ester* (*mixture of* α- *and* β-*anomers*) **11**.—*Compound* **11** was isolated from the reaction mixture during the synthesis of compound **8**, by silica gel chromatography, as a foam (1.94 g, 17%), R_f 0.66 (toluene–EtOAc, 1:2 v/v) (Found: C, 58.8; H, 5.46; N, 1.31. $C_{51}H_{57}NO_{22}$ requires C, 59.1; H, 5,51; N, 1.35%); δ_H(300 MHz; $CDCl_3$) 1.95–2.12 (21 H, br m, 7 × OAc), 3.62–3.80 (2 H, m), 3.97–4.67 (12 H, m), 4.94–5.30 (8 H, m), 6.46–6.50 (1 H, m, NH exchanges with D_2O) and 7.22–7.81 (13 H, ArH); characteristic δ_C(75 MHz; $CDCl_3$) 20.0, 20.1, 20.3, 20.5, 20.7, 20.8 and 21.0 (CH_3 in OAc), 46.9 and 47.3 (α- and β-CH in Fmoc), 62.3 and 62.6 (α- and β-CH_2 in Fmoc), 66.6 and 67.0 (α- and β-C-6), 76.5, 76.6, 76.9 and 77.1 (α- and β-C-2, -C-2′), 98.1, 98.3, 99.4 and 100.4 (α- and β-C-1, -C-1′) 120.0, 125.1, 126.9, 127.2, 128.1 128.5 and 128.8 (CH aromatic), 134.9, 141.7, 143.7 and 143.9 (aromatic), 156.6 and 156.8 (α- and β-C=O on Fmoc), 168.4, 168.6, 169.1, 169.4, 169.7, 169.8, 170.1 and 170.2 (C=O in OAc) and 170.6 and 170.7 (C=O in benzyl ester); (FAB) *m/z* 1036 [(M + H)$^+$, 9%], 814 [(M − Fmoc)$^+$, 6], 619 [(disaccharide residue)$^+$, 15] and 331 [(monosaccharide residue)$^+$, 100].

N-*Fmoc*-O-[3,4,6-*tri*-O-*acetyl*-2-O-(2,3,4,6-*tetra*-O-*acetyl*-β-D-*glucopyranosyl*)-β-D-*glucopyranosyl*]-L-*serine* (*mixture of* α- *and* β-*anomers*) **12**.—Removal of the benzyl ester group from compound **11** (1.75 g, 1.7 mmol), followed by silica gel chromatography (R_f 0.53; EtOH–hexane, 3:1 v/v) provided *acid* **12** as a foam (1.09 g, 68%) (Found: C, 55.6; H, 5.35; N, 1.45. $C_{44}H_{51}NO_{22}$ requires C, 55.9; H, 5.43; N, 1.48%); δ_H(300 MHz;

[$^2H_6$]DMSO) 1.86–2.07 (21 H, br m, 7 × OAc), 3.20–3.50 (1 H, br s, CO_2H exchanges with D_2O), 3.71–4.36 (12 H, m), 4.69–4.99 (5 H, m), 5.06–5.29 (3 H, m), 6.71–6.80 (1 H, br m, NH exchanges with D_2O), 7.33 (2 H, t, *J* 7.5, ArH), 7.42 (2 H, t, *J* 7.5, ArH), 7.73 (2 H, d, *J* 7.5, ArH) and 7.90 (2 H, d, *J* 7.5, ArH); characteristic δ_C(75 MHz; [$^2H_6$]DMSO) 46.2 and 46.4 (α- and β-CH in Fmoc), 61.3 and 61.5 (α- and β-CH_2 in Fmoc), 66.0 and 66.1 (α- and β-C-6), 76.4, 77.4, 77.6 and 77.9 (α- and β-C-2, -C-2′), 97.1, 98.2, 99.1 and 100.6 (α- and β-C-1, -C-1′) 120.5, 125.1, 125.6, 125.9, 127.5 and 127.8 (CH aromatic), 140.3, 140.5, 143.1 and 143.8 (aromatic), 156.1 and 156.4 (α- and β-C=O in Fmoc), 168.4, 169.6, 169.7, 169.9, 170.0, 170.1, 170.2 and 170.3 (C=O in OAc) and 170.5 and 170.7 (α- and β-CO_2H); (FAB) *m/z* 968 [(M + Na)$^+$, 61%], 946 [(M + H)$^+$, 17], 619 [(disaccharide residue)$^+$, 18] and 331 [(monosaccharide residue)$^+$, 100].

N-*Fmoc*-O-(2,3,4,6-*tetra*-O-*acetyl*-α-D-*mannopyranosyl*)-L-*threonine benzyl ester*[16] **13**.—The glycosylation reaction was carried out using *N*-Fmoc-L-threonine benzyl ester (7.76 g, 18.0 mmol) and 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl bromide[26] (8.88 g, 21.6 mmol). Chromatography on silica gel with toluene–EtOAc (4:1 v/v; R_f 0.25) as the eluent yielded title compound **13** as a foam (9.73 g, 71%) (Found: C, 63.6; H, 5.70; N, 1.79. $C_{40}H_{43}NO_{14}$ requires C, 63.1; H, 5.65; N, 1.84%); δ_H(300 MHz; $CDCl_3$) 1.32 (3 H, d, *J* 7.0, CH_3), 2.04, 2.08, 2.09 and 2.17 (4 × 3 H, s, OAc), 4.01–4.56 (10 H, m), 4.85 (1 H, d, $J_{1,2}$ 2.0, 1-H), 5.08 (1 H, m), 5.05 (2 H, s, CH_2Ph), 5.65 (1 H, d, *J* 9.0, NH exchanges with D_2O), 7.27–7.43 (9 H, m, ArH), 7.64 (2 H, d, *J* 8.0, ArH) and 7.77 (2 H, d, *J* 8.0, ArH); δ_C(75 MHz; $CDCl_3$) 18.0 (CH_3), 20.7 and 20.9 (CH_3 in OAc), 47.2 (CH in Fmoc), 58.7 (NHCH), 62.6 (CH_2 in Fmoc), 66.4 (*C*HCH$_3$), 67.5 (C-6), 67.8 (*C*H$_2$Ph), 68.8, 69.2 and 69.8 (C-3, -4 and -5), 76.7 (C-2), 98.8 (C-1), 120.0, 125.3, 127.2, 127.8, 128.3, 128.5 and 128.7 (CH aromatic), 135.1, 141.3 and 143.8 (aromatic), 156.7 (C=O in Fmoc), 169.7 and 169.8 (C=O of OAc) and 170.5 (C=O of benzyl ester); *m/z* 784 [(M + Na)$^+$, 14%], 762 [(M + H)$^+$, 4] and 331 [(sugar residue)$^+$, 100].

N-*Fmoc*-O-(2,3,4,6-*tetra*-O-*acetyl*-α-D-*mannopyranosyl*)-L-*threonine* **14**.—Removal of the benzyl ester group from compound **13** (9.50 g, 12.5 mmol), followed by silica gel chromatography (R_f 0.48; CH_2Cl_2–EtOH, 10:1 v/v) provided *acid* **14** as a foam (6.11 g, 73%) (Found: C, 58.8; H, 5.44; N, 1.96. $C_{33}H_{37}NO_{14}$ requires C, 59.0; H, 5.55; N, 2.09%); δ_H(300 MHz; [$^2H_6$]DMSO) 1.19 (3 H, d, *J* 7.0, CH_3), 1.90, 2.01, 2.02 and 2.04 (4 × 3 H, s, OAc), 3.84 (1 H, br s, CO_2H exchanges with D_2O), 4.01–4.35 (8 H, m), 5.03 (1 H, d, $J_{1,2}$ 1.5, 1-H), 5.03 (1 H, t, $J_{3,4}$ 10.0, $J_{4,5}$ 10.0, 4-H), 5.11 (1 H, dd, $J_{2,3}$ 3.5, $J_{1,2}$ 1.5, 2-H), 5.16 (1 H, dd, $J_{3,4}$ 10.0, $J_{2,3}$ 10.0, 3-H), 6.55–6.68 (1 H, br m, NH exchanges with D_2O), 7.33 (2 H, t, *J* 7.5, ArH), 7.42 (2 H, t, *J* 7.5, ArH), 7.72 (2 H, d, *J* 7.5, ArH) and 7.90 (2 H, d, *J* 7.5, ArH); δ_C(75 MHz; $CDCl_3$) 18.3 (CH_3), 20.6 and 20.9 (CH_3 of OAc), 47.1 (CH in Fmoc), 60.1 (NHCH), 62.5 (CH_2 in Fmoc), 66.2 (C-6), 67.3, 68.9, 69.5 and 70.8 (C-3, -4, -5 and *C*HCH$_3$), 71.7 (C-2), 98.3 (C-1), 119.9, 125.4, 127.1 and 127.6 (CH aromatic), 141.2 and 144.0 (aromatic), 157.4 (C=O in Fmoc), 169.1, 169.2, 169.7 and 170.5 (4 × C=O of OAc) and 177.1 (CO_2H); (FAB) *m/z* 694 [(M + Na)$^+$, 17%], 672 [(M + H)$^+$, 4], 331 [(sugar residue)$^+$, 21] and 178 [(9-methylfluorene)$^+$, 100].

N-*Fmoc*-O-(2,3,4,6-*tetra*-O-*acetyl*-α-D-*mannopyranosyl*)-L-*serine benzyl ester*[16] **15**.—The glycosylation reaction was carried out using *N*-Fmoc-L-serine benzyl ester (7.51 g, 18.0 mmol) and 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl bromide (8.88 g, 21.6 mmol). Chromatography on silica gel with toluene–EtOAc (4:1 v/v; R_f 0.30) as the eluent yielded title compound **15** as a foam (9.04 g, 68%) (Found: C, 62.9; H, 5.54; N, 1.88. $C_{39}H_{41}NO_{14}$ requires C, 62.7; H, 5.49; N, 1.87%); δ_H(300 MHz; $CDCl_3$) 2.01, 2.03, 2.06 and 2.16 (4 × 3 H, s, OAc), 3.90–4.28 (6 H, m), 4.40 (2 H, d, *J* 8.5), 4.61 (1 H, m), 4.74 (1 H, d, $J_{1,2}$ 2.0, 1-H), 5.16–5.29 (5 H, m), 5.95 (1 H, d, *J* 9.0, NH exchanges with D_2O), 7.27–7.43 (9 H, m, ArH), 7.63 (2 H, d, *J* 8.0, ArH) and 7.77 (2 H, d, *J* 8.0, ArH); δ_C(75 MHz; $CDCl_3$) 20.7 and 20.8 (CH_3 of OAc), 47.2 (CH in Fmoc), 54.6 (NHCH), 62.4 (CH_2 in Fmoc), 66.0 (C-5), 67.4 (NHC*H*CH_2), 67.8 (C-6), 68.9, 69.3 and 69.4 (C-2, -3 and -4), 69.7 (*C*H_2Ph), 98.6 (C-1), 120.0, 125.2, 127.1, 127.8, 128.3, 128.4 and 128.7 (CH aromatic), 135.1, 141.3 and 143.8 (aromatic), 155.9 (C=O in Fmoc), 169.5, 169.7, 169.9 and 170.0 (4 × C=O of OAc) and 170.3 (C=O of benzyl ester); (FAB) *m/z* 770 [(M + Na)$^+$, 2%], 748 [(M + H)$^+$, 17] and 331 [(sugar residue)$^+$, 100].

N-*Fmoc*-O-(2,3,4,6-*tetra*-O-*acetyl*-α-D-*mannopyranosyl*)-L-*serine* **16**.—Removal of the benzyl ester group from compound **15** (8.50 g, 11.4 mmol), after silica gel chromatography (R_f 0.56; CH_2Cl_2–EtOH, 10:1 v/v) provided *acid* **16** as a foam (5.00 g, 67%) (Found: C, 58.3; H, 5.26; N, 2.09. $C_{32}H_{35}NO_{14}$ requires C, 58.5; H, 5.36; N, 2.13%); δ_H(300 MHz; [$^2H_6$]DMSO) 1.92, 1.93, 2.01 and 2.10 (4 × 3 H, s, OAc), 3.79–4.38 (10 H, m), 4.86 (1 H, d, $J_{1,2}$ 0.5, 1-H), 5.03–5.10 (2 H, m, 2- and 4-H), 5.14 (1 H, dd, $J_{3,4}$ 10.0, $J_{2,3}$ 10.0, 3-H), 6.83–6.87 (1 H, br m, NH exchanges with D_2O), 7.35 (2 H, t, *J* 7.5, ArH), 7.42 (2 H, t, *J* 7.5, ArH), 7.72 (2 H, d, *J* 7.5, ArH) and 7.90 (2 H, d, *J* 7.5, ArH); δ_C(75 MHz; $CDCl_3$) 20.8 and 21.9 (4 × CH_3 of OAc), 48.1 (CH in Fmoc), 60.1 (NHCH), 61.2 (CH_2 in Fmoc), 66.2 (C-6), 66.8, 68.9, 69.8 and 70.8 (C-3, -4, -5 and C*H*CH_2), 72.5 (C-2), 97.8 (C-1), 120.6, 125.4, 126.9 and 127.8 (CH aromatic), 141.6 and 144.0 (aromatic), 156.9 (C=O in Fmoc), 168.6, 169.0, 169.1 and 170.3 (4 × C=O in OAc) and 176.3 (CO_2H); (FAB) *m/z* 680 [(M + Na)$^+$, 87%], 658 [(M + H)$^+$, 2], 458 [(M − Fmoc + Na)$^+$, 60], 331 [(sugar residue)$^+$, 42] and 178 [(9-methylfluorene)$^+$, 100].

N-*Fmoc*-O-(2,3,4-*tri*-O-*acetyl*-α-L-*rhamnopyranosyl*)-L-*threonine benzyl ester* **17**.—The glycosylation reaction was carried out using *N*-Fmoc-L-threonine benzyl ester (7.76 g, 18.0 mmol) and 2,3,4-tri-*O*-acetyl-α-L-rhamnopyranosyl bromide[26] (7.70 g, 21.8 mmol). Chromatography on silica gel with toluene–EtOAc (4:1 v/v; R_f 0.36) as the eluent yielded *title compound* **17** as an orange foam (10.0 g, 79%) (Found: C, 64.9; H, 5.88; N, 1.98. $C_{38}H_{41}NO_{12}$ requires C, 64.9; H, 5.83; N, 2.00%); δ_H(300 MHz; $CDCl_3$) 1.14 (3 H, d, *J* 6.5, CH_3), 1.26 (3 H, d, *J* 6.5, CH_3), 2.01, 2.02 and 2.17 (3 × 3 H, s, OAc), 3.68–3.80 (1 H, m, 5-H), 4.23–4.48 (4 H, m), 4.56 (1 H, dd, $J_{3,4}$ 10.0, $J_{2,3}$ 2.5, 3-H), 4.85 (1 H, s, $J_{1,2}$ 1.5, 1-H), 5.05 (1 H, t, $J_{3,4}$ 10.0, $J_{4,5}$ 10.0, 4-H), 5.14–5.26 (4 H, m), 5.61 (1 H, d, *J* 9.5, NH exchanges with D_2O), 7.27–7.44 (9 H, m, ArH), 7.63 (2 H, d, *J* 7.5, ArH) and 7.77 (2 H, d, *J* 7.5, ArH); δ_C(75 MHz; $CDCl_3$) 15.2 (CH_3), 17.4 (CH_3), 20.7, 20.8 and 20.9 (3 × CH_3 on OAc), 47.2 (CH in Fmoc), 58.7 (NHCH), 67.1 (*C*HCH$_3$), 67.6 (CH_2 in Fmoc), 67.7 (*C*H$_2$Ph), 69.1, 70.1 and 70.8 (C-3, -4 and -5), 72.2 (C-2), 94.4 (C-1), 120.0, 125.2, 127.2, 127.7, 128.2, 128.6 and 128.7 (CH aromatic), 135.0, 141.3, 143.7 and 144.0 (aromatic), 156.7 (C=O in Fmoc), 169.7 and 169.8 (C=O on OAc) and 170.2 (C=O in benzyl ester); (FAB) *m/z* 726 [(M + Na)$^+$, 5%], 704 [(M + H)$^+$, 12], 273 [(sugar residue)$^+$, 80] and 178 [(9-methylfluorene)$^+$, 100].

N-*Fmoc*-O-(2,3,4-*tri*-O-*acetyl*-α-L-*rhamnopyranosyl*)-L-*threonine* **18**.—Removal of the benzyl ester group from compound **17** (9.70 g, 13.8 mmol), followed by silica gel chromatography (R_f 0.60; CH_2Cl_2–EtOH, 10:1 v/v) provided *acid* **18** as a foam (5.84 g, 69%) (Found: C, 60.6; H, 5.74; N, 2.28. $C_{31}H_{35}NO_{12}$ requires C, 60.7; H, 5.71; N, 2.28%); δ_H(300 MHz; [$^2H_6$]DMSO) 1.08 (3 H, d, *J* 6.5, CH_3), 1.14 (3 H, d, *J* 6.5, CH_3), 1.94, 1.95 and 2.11 (3 × 3 H, s, OAc), 3.35 (2 H, br s, NH and CO_2H exchanges with D_2O), 3.38–3.99 (1 H, m, 5-H), 4.09–4.30 (5 H, br m), 4.81–4.88 (2 H, m, 1- and 4-H), 5.05–5.11 (2 H, m, 3- and 2-H), 7.35 (2 H, t, *J* 7.5, ArH), 7.42 (2 H, t, *J* 7.5, ArH), 7.72 (2 H, d, *J* 7.5, ArH) and 7.90 (2 H, d, *J* 7.5, ArH); δ_C(75 MHz; [$^2H_6$]DMSO) 15.3 (CH_3), 17.2 (CH_3), 20.5, 20.6 and 20.7 (3 × CH_3 in OAc), 46.7 (CH in Fmoc), 58.5 (NHCH), 66.0 (*C*HCH$_3$), 68.7, 69.7 and 70.4 (C-3, -4 and -5), 71.6 (C-2), 93.9 (C-1), 120.1, 125.5, 127.2 and 127.7 (CH aromatic), 140.8 and 143.8 (aromatic), 156.8 (C=O in Fmoc), 169.4, 169.7 and 171.8

(3 × C=O of OAc) and 175.3 (CO_2H); (FAB) *m/z* 636 [$(M + Na)^+$, 4%], 273 [(sugar residue)$^+$, 34] and 178 [(9-methylfluorene)$^+$, 100].

O-[3,4-*Di*-O-*acetyl*-2-O-(2,3,4-*tri*-O-*acetyl*-L-*rhamnopyranosyl*)-α-L-*rhamnopyranosyl*]-N-*Fmoc*-L-*threonine benzyl ester* (*mixture of* α- *and* β-*anomers*) **19**.—Compound **19** was isolated from the reaction mixture during the synthesis of compound **17**, by silica gel chromatography, as a foam (0.41 g, 4%) R_f 0.47 (toluene–EtOAc, 2:1 v/v) (Found: C, 62.4; H, 5.95; N, 1.46. $C_{48}H_{55}NO_{18}$ requires C, 61.7; H, 5.89; N, 1.50%); δ_H(300 MHz; $CDCl_3$) 1.10 (3 H, d, *J* 6.5, CH_3), 1.21 (3 H, d, *J* 6.5, CH_3), 1.25 (3 H, d, *J* 6.5, CH_3), 1.98, 2.06, 2.13, 2.15 and 2.21 (5 × 3 H, s, OAc), 3.57–3.64 (1 H, m), 3.86–3.98 (3 H, m), 4.23–4.58 (5 H, m), 4.83–4.89 (2 H, d, *J* 13.0, C*H*$_2$Ph), 5.02–5.30 (6 H, m), 5.48 (1 H, d, *J* 9.5, NH exchanges with D_2O), 7.22–7.41 (9 H, ArH), 7.63 (2 H, d, *J* 7.5, ArH) and 7.77 (2 H, d, *J* 7.5, ArH); characteristic δ_C(75 MHz; $CDCl_3$) 15.1, 15.3, 15.4, 15.5, 17.3 and 17.5, (6 × CH_3), 20.7, 20.8, 20.9, 21.1, 21.3, 21.4 and 21.5 (CH_3 of OAc), 47.1 and 47.5 (α- and β-CH in Fmoc), 66.5 and 67.2 (α- and β-CH_2 in Fmoc), 72.3, 72.6, 72.8 and 73.0 (α- and β-C-2, -C-2′), 94.7, 95.2, 97.0 and 99.0 (α- and β-C-1, -C-1′) 120.1, 125.2, 127.2, 127.7, 128.3, 128.6 and 128.7 (CH aromatic), 135.4, 141.7, 143.7 and 143.8 (aromatic), 156.3 and 156.7 (α- and β-C=O on Fmoc), 168.5, 169.1, 169.3, 169.5, 169.7 and 169.8 (C=O of OAc) and 170.4 and 170.5 (C=O on benzyl ester); (FAB) *m/z* 956 [$(M + Na)^+$, 15%], 503 [(disaccharide residue)$^+$, 34] and 273 [(monosaccharide residue)$^+$, 100].

N-*Fmoc*-O-(2,3,4-*tri*-O-*acetyl*-α-L-*rhamnopyranosyl*)-L-*serine benzyl ester* **20**.—The glycosylation reaction was carried out using *N*-Fmoc-L-serine benzyl ester (7.51 g, 18.0 mmol) and 2,3,4-tri-*O*-acetyl-α-L-rhamnopyranosyl bromide (7.70 g, 21.8 mmol). Chromatography on silica gel with toluene–EtOAc (4:1 v/v; R_f 0.36) as the eluent yielded *title compound* **20** as an orange foam (9.30 g, 75%) (Found: C, 64.1; H, 5.69; N, 2.05. $C_{37}H_{39}NO_{12}$ requires C, 64.4; H, 5.66; N, 2.03%); δ_H(300 MHz; $CDCl_3$) 1.12 (3 H, d, *J* 6.5, CH_3), 2.00, 2.04 and 2.15 (3 × 3 H, s, OAc), 3.63–3.73 (2 H, m), 4.18–4.29 (2 H, m), 4.37–4.42 (2 H, m), 4.64–4.70 (1 H, br m), 4.74 (1 H, $J_{1,2}$ 2.5, 1-H), 5.05 (1 H, $J_{3,4}$ 10.0, $J_{4,5}$ 10.0, 4-H), 5.18 (1 H, dd, $J_{3,4}$ 10.0, $J_{2,3}$ 3.0, 3-H), 5.26 (2 H, s, *J* 6.0, C*H*$_2$Ph), 5.29 (1 H, br m, 2-H), 5.75 (1 H, d, *J* 8.5, NH exchanges with D_2O), 7.28–7.43 (9 H, m, ArH), 7.65 (2 H, d, *J* 7.5, ArH) and 7.77 (2 H, d, *J* 7.5, ArH); δ_C(75 MHz; $CDCl_3$) 17.3 (CH_3), 20.7, 20.8 and 20.9 (3 × CH_3 of OAc), 47.2 (CH in Fmoc), 54.3 (NHCH), 66.9 (C-5), 67.5 (CH_2 in Fmoc), 67.7 (*C*H$_2$Ph), 68.0 (NHCH*C*H$_2$), 69.0, 69.5 and 70.9 (C-4, -3 and -2), 97.8 (C-1), 120.0, 125.3, 127.2, 127.7, 128.3, 128.6 and 128.7 (CH aromatic), 135.0, 141.3, 143.8 and 143.9 (aromatic), 156.0 (C=O in Fmoc), 169.5, 169.8 and 169.9 (3 × C=O of OAc), 170.1 (C=O in benzyl ester); (FAB) *m/z* 712 [$(M + Na)^+$, 100%].

N-*Fmoc*-O-(2,3,4-*tri*-O-*acetyl*-α-L-*rhamnopyranosyl*)-L-*serine* **21**.—Removal of the benzyl ester group from compound **20** (9.00 g, 13.1 mmol), followed by silica gel chromatography (R_f 0.60; CH_2Cl_2–EtOH, 10:1 v/v) provided *acid* **21** as a foam (5.16 g, 66%) (Found: C, 60.2; H, 5.56; N, 2.29. $C_{30}H_{33}NO_{12}$ requires C, 60.1; H, 5.51; N, 2.34%); δ_H(300 MHz; [$^2H_6$]DMSO) 1.10 (3 H, d, *J* 6.5, CH_3), 1.92, 1.93 and 2.10 (3 × 3 H, s, OAc), 3.35 (2 H, br s, NH and CO_2H exchanges with D_2O), 3.69–3.91 (3 H, m), 4.12–4.30 (4 H, br m), 4.80 (1 H, d, $J_{1,2}$ 0.5, 1-H), 4.86 (1 H, t, $J_{3,4}$ 10.0, $J_{4,5}$ 10.0, 4-H), 5.04 (1 H, dd, $J_{3,4}$ 10.0, $J_{2,3}$ 3.5, 3-H), 5.15 (1 H, dd, $J_{2,3}$ 3.5, $J_{1,2}$ 0.5, 2-H), 7.35 (2 H, t, *J* 7.5, ArH), 7.42 (2 H, t, *J* 7.5, ArH), 7.72 (2 H, d, *J* 7.5, ArH) and 7.90 (2 H, d, *J* 7.5, ArH); δ_C(75 MHz; [$^2H_6$]DMSO) 17.3 (CH_3), 20.4, 20.5 and 20.6 (3 × CH_3 of OAc), 46.8 (CH in Fmoc), 56.6 (NHCH), 65.8 (C-5), 65.9 (CH_2 in Fmoc), 67.3 (NHCH*C*H$_2$), 68.8 and 69.0 (C-3 and -4), 70.2 (C-2), 96.5 (C-1), 120.1, 125.4, 127.2 and 127.7 (CH aromatic), 141.8 and 144.0 (aromatic), 156.1 (C=O in Fmoc), 168.5, 169.5 and 169.7 (3 × C=O in OAc) and 172.0 (CO_2H); (FAB) *m/z* 622 [$(M + Na)^+$, 24%] and 178 [(9-methylfluorene)$^+$, 100].

O-[3,4-*Di*-O-*acetyl*-2-O-(2,3,4-*tri*-O-*acetyl*-L-*rhamnopyranosyl*)-α-L-*rhamnopyranosyl*]-N-*Fmoc*-L-*serine benzyl ester* (*mixture of* α- *and* β-*anomers*) **22**.—Isolated from the reaction mixture during the synthesis of compound **20**, by silica gel chromatography, as a foam (0.30 g, 3%) R_f 0.49 (toluene–EtOAc, 2:1 v/v) (Found: C, 62.3; H, 5.82; N, 1.50. $C_{47}H_{53}NO_{18}$ requires C, 61.4; H, 5.77; N, 1.52%); δ_H(300 MHz; $CDCl_3$) 1.14 (3 H, d, *J* 6.5, CH_3), 1.25 (3 H, d, *J* 6.5, CH_3), 1.97, 1.98, 2.11, 2.16 and 2.19 (5 × 3 H, s, OAc), 3.51–4.00 (4 H, br m), 4.12–4.45 (4 H, br m), 4.63–4.82 (3 H, m), 5.01–5.28 (7 H, m), 5.64–5.69 (1 H, br s, NH exchanges with D_2O), 7.22–7.41 (9 H, ArH), 7.65 (2 H, d, *J* 7.5, ArH) and 7.77 (2 H, d, *J* 7.5, ArH); characteristic δ_C(75 MHz; $CDCl_3$) 17.3, 17.5, 17.6 and 17.7 (CH_3), 20.4, 20.6, 20.7, 21.1, 21.2, 21.3 and 21.5 (CH_3 of OAc), 47.2 and 47.4 (α- and β-CH in Fmoc), 66.2 and 66.6 (α- and β-CH_2 in Fmoc), 71.9, 72.5, 72.8 and 73.4 (α- and β-C-2, -C-2′), 92.7, 94.2, 96.0 and 98.8 (α- and β-C-1, -C-1′), 120.1, 124.9, 127.3, 127.7, 128.1, 128.3 and 128.7 (CH aromatic), 135.7, 141.8, 143.1 and 143.8 (aromatic), 156.4 and 156.8 (α- and β-C=O in Fmoc), 167.5, 168.1, 168.3, 168.8, 169.1 and 169.2 (C=O in OAc) and 170.2 and 170.4 (C=O of benzyl ester); (FAB) *m/z* 942 [$(M + Na)^+$, 13%], 503 [(disaccharide residue)$^+$, 40] and 273 [(monosaccharide residue)$^+$, 100].

N-*Fmoc*-O-(2,3,4-*tri*-O-*acetyl*-α-L-*rhamnopyranosyl*)-L-*hydroxyproline benzyl ester* **23**.—The glycosylation reaction was carried out using *N*-Fmoc-L-hydroxyproline benzyl ester [25] (2.31 g, 5.26 mmol) and 2,3,4-tri-*O*-acetyl-α-L-rhamnopyranosyl bromide (2.23 g, 6.31 mmol). Chromatography on silica gel with toluene–EtOAc (2:1 v/v; R_f 0.42) as the eluent yielded *title compound* **23** as a yellow syrup (2.33 g, 63%) (Found: C, 65.9; H, 5.80; N, 1.95. $C_{39}H_{41}NO_{12}$ requires C, 65.5; H, 5.73; N, 1.96%); δ_H(300 MHz; $CDCl_3$) 1.19 (3 H, d, *J* 7.0, CH_3), 2.00–2.20 (11 H, m, 3 × OAc, 2 H on hydroxyproline ring), 2.40–2.48 (1 H, m), 3.70–3.85 (3 H, m), 4.25–4.57 (4 H, m), 4.82 (1 H, dd, $J_{1,2}$ 1.5, $J_{2,3}$ 1.5, 2-H), 5.01–5.28 (5 H, m), 7.24–7.43 (9 H, m, ArH), 7.61 (2 H, d, *J* 7.5, ArH) and 7.76 (2 H, d, *J* 7.5, ArH); δ_C(75 MHz; $CDCl_3$) 17.4 (CH_3), 20.7 and 20.9 (CH_3 of OAc), 35.7 (CH_2), 47.2 (CH in Fmoc), 52.0 (CH_2), 57.8 (*C*HCO$_2$Ph), 67.0 (*C*H$_2$Ph), 67.1 (C-5), 67.8 (CH_2 in Fmoc), 68.8, 68.9 and 70.9 (C-4, -3 and NCH$_2$*C*H), 75.7 (C-2), 97.0 (C-1), 120.0, 125.2, 127.2, 127.8, 128.2, 128.3 and 128.6 (CH aromatic), 141.3, 143.7 and 144.1 (aromatic), 154.3 (C=O in Fmoc), 169.9 (C=O in OAc) and 170.4 (*C*O$_2$Ph); (FAB) *m/z* 716 [$(M + H)^+$, 15%], 273 [(sugar residue)$^+$, 15] and 178 [(9-methylfluorene)$^+$, 100].

N-*Fmoc*-O-(2,3,4-*tri*-O-*acetyl*-α-L-*rhamnopyranosyl*)-L-*hydroxyproline* **24**.—Removal of the benzyl ester group from compound **23** (2.00 g, 2.80 mmol), followed by silica gel chromatography (R_f 0.43; CH_2Cl_2–EtOH, 10:1 v/v) provided *acid* **24** as a foam (1.12 g, 67%) (Found: C, 61.5; H, 5.56; N, 2.23. $C_{32}H_{35}NO_{12}$ requires C, 61.4; H, 5.60; N, 2.24%); δ_H(300 MHz; [$^2H_6$]DMSO) 1.15 (3 H, d, *J* 7.0, CH_3), 1.90–2.19 (11 H, m, 3 × OAc, 2 H on hydroxyproline ring), 3.26–3.70 (3 H, m), 3.74–3.85 (1 H, m, 5-H), 4.10–4.41 (5 H, m), 4.82–5.11 (4 H, m), 7.33 (2 H, t, *J* 7.5, ArH), 7.41 (2 H, t, *J* 7.5, ArH), 7.72 (2 H, d, *J* 7.5, ArH) and 7.91 (2 H, d, *J* 7.5, ArH); δ_C(75 MHz; [$^2H_6$]DMSO) 17.2 (CH_3), 20.5, 20.6 and 20.7 (3 × CH_3 of OAc), 34.7 (CH_2), 46.7 (CH in Fmoc), 52.0 (CH_2), 58.0 (*C*HCO$_2$H), 66.3 (C-5), 66.6 (CH_2 in Fmoc), 68.6, 69.2 and 70.1 (C-4, -3 and NCH$_2$*C*H), 75.2 (C-2), 96.0 (C-1), 120.2, 125.4, 127.2 and 127.7 (CH aromatic), 140.8 and 143.9 (aromatic), 154.1 (C=O in Fmoc), 169.7 (C=O on OAc) and 174.4 (CO_2H); (FAB) *m/z* 648 [$(M + Na)^+$, 62%] and 178 [(9-methylfluorene)$^+$, 100].

Solid-phase peptide synthesis

General procedures. Fmoc-amino acids, resins and HBTU were purchased from Novabiochem. All chiral amino acids used were of the L-configuration. Amino acid side-chain protection was as follows: arginine (2,2,5,7,8-pentamethyl-

chromane-6-sulfonyl, Pmc), asparagine (trityl, Trt), aspartic acid (*tert*-butyl, OBu^t), cysteine (trityl, Trt).

Elongation of peptide on Wang resin. Peptide assembly *via* Fmoc chemistry was performed manually, starting with Wang resin loaded with the first amino acid. Removal of Fmoc was accomplished with 20% piperidine in DMF (10 ml g^{-1} resin) and carried out in two stages (1 × 3 min, followed by 1 × 12 min). The ninhydrin (Kaiser) colour test[38] should be positive and, if not, the deprotection was repeated. All co-products and excess of reagents were removed by thorough washing with DMF (5 × 10 ml).

Couplings were achieved by adding the following reagents dissolved in DMF (0.5 M) to the washed (DMF) resin assembly; 4.0 mol equiv. of Fmoc-amino acid, 3.9 mol equiv. of HBTU, and 8 mol equiv. of DIPEA. When the glycoamino acid derivative was coupled, 3.0 mol equiv. were used. In some peptides the N-terminal was acetylated and this was achieved by using 4.0 mol equiv. of acetic anhydride and 8 mol equiv. of DIPEA in DMF.

The mixture was agitated with a spatula every 5 min for 30 min and then the resin assembly was analysed by the colour test for completion of reaction. If a positive test was observed, the acylation step was repeated with fresh activated derivative.

Excess reagents were removed with 5 × 10 ml DMF washes.

Cleavage and deprotection of the assembled peptide. The peptide–resin was prepared for cleavage by being thoroughly washed on a sintered peptide vessel under suction successively with DMF, CH_2Cl_2 and then finally diethyl ether to shrink the assembly. The peptide–resin was removed and dried in a high-vacuum oven overnight. Typically, the peptide–resin (0.5–1.0 g) was treated with 95% aq. TFA (10 ml) with TIPS (500 μl) for 1 h and then was filtered in a round bottomed flask. Treatment with TFA was repeated twice, excluding the addition of TIPS. The filtrates were combined and left for a further 1 h (total reaction time was now 4 h). After evaporation, the peptide was triturated with diethyl ether. The precipitated peptide was collected on a sintered glass filter under a light vacuum and was dried in a high-vacuum oven overnight.

Removal of acetate groups from cleaved peptide. Typically, the dry peptide (100–200 mg) was dissolved in hydrazine hydrate–methanol (1:4 v/v) (25 ml) and the solution was stirred for 3 h at room temperature. Acetone (20 ml) was dropped into the mixture (at 0 °C) which, after a further 30 min, was evaporated to dryness. Water (100 ml) was added and the solution was lyophilized to obtain crude peptide.

Purification and characterization of peptides

Analytical reversed-phase HPLC. The crude peptide (1 mg) was dissolved in water (1 ml). A sample of this solution (20 μl) was eluted through an analytical reversed-phase HPLC column (Beckmann System Gold) equipped with a Vydac (218TP54) protein and peptide C_{18} column (5 μ; 4.6 × 250 mm), with detection at 220 nm (Beckmann Diode Array Detector Module 168). Elution was performed using a linear gradient of reagent B, at a flow rate of 1 ml min^{-1}, where reagent A was 0.1% aq. TFA and reagent B was 0.1% TFA in acetonitrile.

Preparative reversed-phase HPLC. The crude peptide (50–80 mg) was dissolved in water (5 ml). The filtered sample was eluted through a preparative-scale reversed-phase HPLC column (Beckmann System Gold) equipped with a Vydac (218TP1022) protein and peptide C_{18} column (10 μ; 22 × 250 mm), flow rate 10.0 ml min^{-1}, with detection at 220 nm (Beckmann Diode Array Detector Module 166). The major peak fractions were collected (Waters Fraction Collector 126) and the purity of each fraction was assessed by analytical HPLC before being combined and lyophilized.

Ion-exchange chromatography. A solution containing the peptide (1 mg ml^{-1} of water) was analysed using a Gilson Pump Model 302 with detection at 230 and 280 nm (Gilson Detector Model 116). Two strong cation-exchange columns were used: 1. Hydropore-SCX (83-C03-E5TI) (5 μ; 4.6 × 100 mm), 2. S Hyper D 10 – Biosepra column (200422). Samples were eluted with a linear gradient of 0–100% B in 20 min, where A was 8 mmol phosphate (pH 3) in 1:4 acetonitrile–water and B was 0.4 mmol NaCl in A, at a flow rate of 1 ml min^{-1}.

Capillary electrophoresis. A solution containing the peptide (1 mg ml^{-1} of water) was analysed using the Beckmann System Gold P/ACE System 2100 equipped with a capillary cartridge (50 cm × 75 μm ID × 375 μm OD), held at 30 °C throughout the analysis. The capillary was flushed for 1 min with 0.1 M NaOH followed by equilibration for 1 min with the electrolyte. The liquid electrolyte used was either 100 mM phosphate buffer (pH = 2.5) or 100 mM borate buffer (pH = 8) and separation was carried out at 7.3 μA constant current (10 kV) for 20 min. Detection was at 230 nm.

Amino acid analysis. Analysis were performed using a Pharmacia Biochrom Tech Ltd LKB Alpha Plus Sodium Amino Acid Analyser (4151). The free peptide was hydrolysed in constant-boiling 6 M HCl at 130 °C in the presence of phenol for 24 or 16 h in an evacuated, sealed tube.

Other analysis. The ^1H and ^{13}C NMR spectra were obtained on a Bruker WM 400 MHz spectrometer. Mass spectroscopy was carried out on a Kratos Analytical Kompact Maldi II with Sun Workshop.

Incorporation of glycoamino acid at the N-terminal

H-Ser-Pro-Arg-Gly-Asp-Phe-OH 25. Synthesis, cleavage of the resin-bound peptide (0.25 mmol scale), and purification by HPLC [t_R = 17.11 min (100%), 5–25% B gradient in 30 min, C_{18} column] gave title compound **25** (100 mg, 59%); ion-exchange chromatography [t_R = 9.98 min (99.8%), 0–100% B gradient in 20 min, Hydropore-SCX column]; capillary electrophoresis [t_R = 3.82 min (98.7%), phosphate buffer pH 2.5]; electrospray MS $[M + H]^+$, *m/z* 679 (Calc. 679); amino acid analysis (6 M HCl; 24 h; 130 °C): Arg 1.00 (1), Asp 0.96 (1), Gly 1.05 (1), Phe 0.96 (1), Pro 1.03 (1), Ser 0.60 (1).

H-Thr(α-L-rhamnose)-Pro-Arg-Gly-Asp-Phe-OH 26. Synthesis, and cleavage of the resin-bound peptide (0.20 mmol scale), gave the tri-*O*-acetyl derivative of title compound **26** (167 mg); HPLC [t_R = 20.59 min (98.7%), 10–40% B gradient in 30 min, C_{18} column]; MALDI MS $[M + H]^+$, *m/z* 965 (Calc. 965).

The crude peptide (165 mg) was treated with hydrazine hydrate according to the general procedure and purification by HPLC [t_R = 18.77 min (100%), 5–25% B gradient in 30 min, C_{18} column] gave title compound **26** (69 mg, 42% yield based on starting resin); ion-exchange chromatography [t_R = 9.29 min (97.2%), 0–100% B gradient in 20 min, Hyper-D SCX column]; capillary electrophoresis [t_R = 4.28 min (99.2%), phosphate buffer pH 2.5]; electrospray MS $[M + H]^+$, *m/z* 838 (Calc. 838); amino acid analysis (6 M HCl; 16 h; 130 °C): Arg 1.00 (1), Asp 1.02 (1), Gly 0.99 (1), Phe 1.00 (1), Pro 0.99 (1), Thr 0.95 (1).

H-Thr(α-D-mannose)-Pro-Arg-Gly-Asp-Phe-OH 27. Synthesis, and cleavage of the resin-bound peptide (0.20 mmol scale), gave the tetra-*O*-acetyl derivative of compound **27** (170 mg); HPLC [t_R = 20.66 min (75.4%), 10–40% B gradient in 30 min, C_{18} column]; MALDI MS $[M + H]^+$, *m/z* 1023 (Calc. 1023).

The crude peptide (168 mg) was treated with hydrazine hydrate according to the general procedure, and purification by HPLC [t_R = 16.27 min (100%), 5–25% B gradient in 30 min, C_{18} column] gave title compound **27** (58 mg, 35% yield based on starting resin); ion-exchange chromatography [t_R = 9.96 min (96.1%), 0–100% B gradient in 20 min, Hyper-D SCX column]; capillary electrophoresis [t_R = 4.33 min (97.6%), phosphate buffer pH 2.5]; electrospray MS $[M + H]^+$, *m/z* 855 (Calc. 855); amino acid analysis (6 M HCl; 16 h; 130 °C): Arg 0.97 (1), Asp 1.01 (1), Gly 1.00 (1), Phe 1.00 (1), Pro 1.02 (1), Thr 0.99 (1).

H-Ser(L-rhamnose)-Pro-Arg-Gly-Asp-Phe-OH 28. Synthesis,

and cleavage of the resin-bound peptide (0.33 mmol scale), gave the tri-*O*-acetyl derivative of compound **28** (389 mg) as a mixture of α- and β-anomeric forms; HPLC [t_R = 19.96 min (75%) (α-form), t_R = 21.06 min (15%) (β-form), 10–40% B gradient in 30 min, C_{18} column]. A small amount of each peptide was isolated by using preparative HPLC and was analysed; α-form: MALDI MS $[M + H]^+$, *m/z* 951 (Calc. 951); characteristic δ_H(400 MHz; [$^2H_6$]DMSO) 4.94 (1 H, s, *J* 1.0, anomeric H-1); β-form: MALDI MS $[M + H]^+$, *m/z* 951 (Calc. 951); characteristic δ_H(400 MHz; [$^2H_6$]DMSO) 4.86 (1 H, s, *J* 1.0, anomeric H-1).

The crude mixture of peptides (373 mg) was treated with hydrazine hydrate according to the general procedure, and purification by HPLC [t_R = 17.74 min (100%), 5–25% B gradient in 30 min, C_{18} column] gave title compound **28** (162 mg, 60% yield based on starting resin) as a mixture of α- and β-anomeric forms; ion-exchange chromatography [t_R = 5.39 min (17.9%), t_R = 5.85 min (80.8%), 0–100% B gradient in 20 min, Hydropore SCX column]; capillary electrophoresis [t_R = 4.31 min (81.0%), t_R = 4.52 min (18.3%), phosphate buffer pH 2.5]; electrospray MS $[M + H]^+$, *m/z* 825 (Calc. 825); amino acid analysis (6 M HCl; 16 h; 130 °C): Arg 1.08 (1), Asp 0.97 (1), Gly 1.00 (1), Phe 0.99 (1), Pro 0.95 (1), Ser 0.95 (1).

H-Ser(α-D-mannose)-Pro-Arg-Gly-Asp-Phe-OH 29. Synthesis, and cleavage of the resin-bound peptide (0.2 mmol scale), gave the tetra-*O*-acetyl derivative of compound **29** (239 mg) as a mixture of α- and β-anomeric forms; HPLC [t_R = 11.55 min (73%) (α-form), t_R = 12.49 min (23%) (β-form), 20–30% B gradient in 30 min, C_{18} column]. Since the two peaks were very close together, only the peptide with the sugar in the α-anomeric form was able to be isolated by preparative HPLC; MALDI MS $[M + H]^+$, *m/z* 1009 (Calc. 1009); characteristic δ_H(400 MHz; [$^2H_6$]DMSO) 4.8 (1 H, s, *J* 1.0, anomeric H-1).

The peptide (60 mg) was treated with hydrazine hydrate according to the general procedure, and purification by HPLC [t_R = 14.87 min (100%), 5–25% B gradient in 30 min, C_{18} column] gave compound **29** (25 mg, 15% yield based on starting resin); capillary electrophoresis [t_R = 3.57 min (96.7%), phosphate buffer pH 2.5]; electrospray MS $[M + H]^+$, *m/z* 841 (Calc. 841); amino acid analysis (6 M HCl; 16 h; 130 °C): Arg 1.05 (1), Asp 1.01 (1), Gly 1.04 (1), Phe 0.99 (1), Pro 0.98 (1), Ser 0.98 (1).

H-Thr[β-D-glucosyl-(1→2)-D-glucose]-Pro-Arg-Gly-Asp-Phe-OH 30 (mixture of 30a and 30b). Peptide was synthesized by using 1.25 mol equiv. of disaccharide-containing glycoamino acid **7**. Synthesis, and cleavage of the resin-bound peptide (0.1 mmol scale) gave the hepta-*O*-acetyl derivative of compounds **30** (105 mg); HPLC [t_R = 20.11 min (89.0%), 10–60% B gradient in 30 min, C_{18} column]; MALDI MS $[M + H]^+$, *m/z* 1311 (Calc. 1311).

The crude peptide (103 mg) was treated with hydrazine hydrate according to the general procedure, and analysis by HPLC exhibited two major peaks [t_R = 14.90 (41.0%) **30a**, t_R = 15.56 min (54%) **30b**, 5–25% B gradient in 30 min, C_{18} column]. Attempted separation of these two main peaks by preparative HPLC gave compounds **30** (mixture, 29 mg), **30a** (6 mg) and **30b** (7 mg).

Isomer **30a**: capillary electrophoresis [t_R = 3.42 min (97.3%), phosphate buffer pH 2.5]; MALDI MS $[M + H]^+$, *m/z* 1017 (Calc. 1017); amino acid analysis (6 M HCl; 16 h; 130 °C): Arg 1.01 (1), Asp 0.97 (1), Gly 0.96 (1), Phe 0.95 (1), Pro 0.98 (1), Thr 1.05 (1).

Isomer **30b**: capillary electrophoresis [t_R = 3.46 min (96.5%), phosphate buffer pH 2.5]; MALDI MS $[M + H]^+$, *m/z* 1017 (Calc. 1017); amino acid analysis (6 M HCl; 16 h; 130 °C): Arg 0.97 (1), Asp 1.04 (1), Gly 0.98 (1), Phe 0.99 (1), Pro 0.96 (1), Thr 1.06 (1).

H-Asn-Pro-Arg-Gly-Asp-Phe-OH 31. Synthesis, cleavage of the resin-bound peptide (0.25 mmol scale) and purification by HPLC [t_R = 17.28 min (100%), 5–25% B gradient in 30 min, C_{18} column] gave title compound **31** (128 mg, 72%); ion-exchange chromatography [t_R = 4.41 min (97.6%), 0–100% B gradient in 20 min, Hydropore-SCX column]; capillary electrophoresis [t_R = 3.93 min (98.6%), phosphate buffer pH 2.5]; electrospray MS $[M + H]^+$, *m/z* 706 (Calc. 706); amino acid analysis (6 M HCl; 24 h; 130 °C): Arg 1.03 (1), Asp 1.90 (2), Gly 1.06 (1), Phe 0.97 (1), Pro 1.06 (1).

Incorporation of glycoamino acid at the C-terminal

General procedures. Attachment of amino acid on to Wang resin[32] and 2-chlorotrityl chloride resin[34] was performed as described in the literature. The substitution of the resin can be measured by spectrophotometric determination of Fmoc following treatment of a weighed sample with 50% piperidine in DMF. This was achieved by using the method outlined below; 1–2 mg of resin and the standard (Fmoc-NH_2) were weighed out accurately into 1 ml sample vials. DMF (200 μl) and piperidine (200 μl) were pipetted into each vial and samples were left for 30 min. An aliquot (40 μl) was taken from each vial and diluted with MeCN (1.8 ml) and TFA (160 μl) (total volume = 2.0 ml). The solutions (20 μl) were then analysed by reversed-phase HPLC (30–70% B gradient in 10 min, C_{18} column) with detection at 265 nm. The area count on the chromatograms for the Fmoc residue were incorporated in equation (1) to determine the Fmoc content (resin substitution value).

$$\text{Fmoc content (mmol g}^{-1}) = \frac{\text{Wt std}}{\text{Wt resin}} \times \frac{\text{AC resin}}{\text{AC std}} \times \frac{1000}{M_r \text{ std (239)}} \quad (1)$$

where Wt = weight
std = standard (Fmoc-NH_2)
AC = area count

H-Asn-Pro-Arg-Gly-Asp-Phe-Thr(α-L-*rhamnose*)-*OH* **32**.—Attachment of the threonine derivative **18** (0.57 g, 0.94 mmol) on the Wang resin (0.6 g, 0.47 mmol) was achieved with a substitution value of 0.41 mmol g^{-1} resin (73%). The amino acid–resin (0.836 g) was then used for the synthesis of peptide **32**. Synthesis, and cleavage of the resin-bound peptide (0.34 mmol scale), gave the tri-*O*-acetyl derivative of compound **32** (373 mg); HPLC [t_R = 20.13 (78.0%), 10–40% B gradient in 30 min, C_{18} column]; MALDI MS $[M + H]^+$, *m/z* 1079 (Calc. 1079).

The crude peptide (370 mg) was treated with hydrazine hydrate according to the general procedure, and purification by HPLC [t_R = 16.48 min (100%), 5–25% B gradient in 30 min, C_{18} column] gave compound **32** (138 mg, 43% yield based on starting resin); ion-exchange chromatography [t_R = 8.69 min (96.2%), 0–100% B gradient in 20 min, Hyper-D SCX column]; capillary electrophoresis (t_R = 4.12 min (95.1%), phosphate buffer pH 2.5]; electrospray MS $[M + H]^+$, *m/z* 953 (Calc. 953); amino acid analysis (6 M HCl; 16 h; 130 °C): Arg 1.00 (1), Asp 1.96 (2), Gly 1.02 (1), Phe 1.02 (1), Pro 1.04 (1), Thr 0.96 (1).

H-Asn-Pro-Arg-Gly-Asp-Phe-Thr(β-D-*glucose*)-*OH* **33**.—Attachment of the threonine derivative **4** (0.52 g, 0.78 mmol) on the Wang resin (0.5 g, 0.39 mmol) was achieved with a substitution value of 0.52 mmol g^{-1} resin (96%). The amino acid–resin (0.72 g) was then used for the synthesis of peptide **33**. Synthesis, and cleavage of the resin-bound peptide (0.37 mmol scale), gave the tetra-*O*-acetyl derivative of compound **33** (338 mg); HPLC [t_R = 21.26 (89.3%), 10–40% B gradient in 30 min, C_{18} column]; MALDI MS $[M + H]^+$, *m/z* 1137 (Calc. 1137).

The crude peptide (335 mg) was treated with hydrazine hydrate according to the general procedure, and purification by HPLC [t_R = 13.78 min (100%), 5–25% B gradient in 30 min, C_{18} column) gave title compound **33** (122 mg, 34% yield based on starting resin); capillary electrophoresis [t_R = 3.69 min (99.2%), phosphate buffer pH 2.5]; electrospray MS $[M + H]^+$, *m/z* 969 (Calc. 969); amino acid analysis (6 M HCl; 16 h; 130 °C): Arg

0.98 (1), Asp 1.95 (2), Gly 1.06 (1), Phe 1.08 (1), Pro 1.00 (1), Thr 0.97 (1).

H-Asn-Pro-Arg-Gly-Asp-Phe-Ser(L-*rhamnose*)-*OH* (α-*form* **34a**, β-*form* **34b**).—Attachment of the serine derivative **21** (0.56 g, 0.94 mmol) on the Wang resin (0.6 g, 0.47 mmol) was achieved with a substitution value of 0.48 mmol g^{-1} resin (86%). The amino acid–resin (0.839 g) was then used for the synthesis of peptide **34**. Synthesis, and cleavage of the resin-bound peptide (0.40 mmol scale), gave the tri-*O*-acetyl derivative of compound **34** (as a mixture of α- and β-anomeric forms) (366 mg); HPLC [t_R = 19.80 min (50.1%), t_R = 21.78 min (29%), 10–40% B gradient in 30 min, C_{18} column]; MALDI MS $[M + H]^+$, *m/z* 1065 (Calc. 1065).

The crude peptide (363 mg) was treated with hydrazine hydrate according to the general procedure, and analysis by HPLC exhibited two major peaks [t_R = 14.86 min (58.9%) **34a**, t_R = 16.69 min (29.4%) **34b**, 5–25% B gradient in 30 min, C_{18} column]. Attempted separation of these two main peaks by preparative HPLC gave isomers **34a** (100 mg) and **34b** (42 mg).

H-Asn-Pro-Arg-Gly-Asp-Phe-Ser(α-L-*rhamnose*)-*OH* **34a**: ion-exchange chromatography [t_R = 8.60 min (98.6%), 0–100% B gradient in 20 min, Hyper-D SCX column]; capillary electrophoresis [t_R = 3.94 min (99.1%), phosphate buffer pH 2.5]; MALDI MS $[M + H]^+$, *m/z* 939 (Calc. 939); amino acid analysis (6 M HCl; 16 h; 130 °C): Arg 0.98 (1), Asp 1.97 (2), Gly 1.02 (1), Phe 1.04 (1), Pro 1.03 (1), Ser 0.95 (1).

H-Asn-Pro-Arg-Gly-Asp-Phe-Ser(β-L-*rhamnose*)-*OH* **34b**: ion-exchange chromatography [t_R = 8.42 min (97.1%), 0–100% B gradient in 20 min, Hyper-D SCX column]; capillary electrophoresis (t_R = 4.34 min (96.4%), phosphate buffer pH 2.5); MALDI MS $[M + H]^+$, *m/z* 939 (Calc. 939); amino acid analysis (6 M HCl; 16 h; 130 °C): Arg 0.95 (1), Asp 1.96 (2), Gly 0.96 (1), Phe 0.98 (1), Pro 1.01 (1), Ser 0.94 (1).

H-Asn-Pro-Arg-Gly-Asp-Phe-Hyp(α-L-*rhamnose*)-*OH* **35**.—Attachment of the hydroxyproline derivative **24** (0.3 g, 0.47 mmol) on the 2-chlorotrityl chloride resin (0.5 g, 0.3 mmol) was achieved with a substitution value of 0.43 mmol g^{-1} resin (97%). The amino acid–resin (0.67 g) was then used for the synthesis of peptide **35** (0.3 mmol scale). Synthesis, and cleavage of the resin-bound peptide (537 mg, 0.2 mmol scale), gave the tri-*O*-acetyl derivative of compound **35** (207 mg); HPLC [t_R = 22.07 (100%), 10–40% B gradient in 30 min, C_{18} column]; MALDI MS $[M + H]^+$, *m/z* 1091 (Calc. 1091).

Two methods were employed to remove the acetate ester protection on the sugar moiety, both giving the required peptide **35**.

Method 1: The tri-*O*-acetyl derivative of compound **35** (205 mg) was treated with hydrazine hydrate according to the general procedure, and purification by HPLC [t_R = 17.64 min (100%), 5–25% B gradient in 30 min, C_{18} column] gave title compound **35** (75 mg, 39% yield based on starting resin); capillary electrophoresis [t_R = 3.70 min (90.5%), phosphate buffer pH 2.5]; electrospray MS $[M + H]^+$, *m/z* 965 (Calc. 965); amino acid analysis (6 M HCl; 24 h; 130 °C): Arg 0.96 (1), Asp 1.92 (2), Gly 1.03 (1), Hyp 0.97 (1), Phe 1.07 (1), Pro 1.05 (1).

Method 2: 2-Chlorotrityl chloride resin–peptide (0.1 mmol) was suspended in hydrazine hydrate–DMF (1:4 v/v; 20 ml) in a sintered reaction vessel. After 4 h, the solution was filtered and the resin–peptide was washed with DMF, CH_2Cl_2 and finally diethyl ether. Cleavage of the resin-bound peptide gave compound **35** (33 mg, 36% yield based on starting resin).

Synthesis of cystine-containing peptides

General procedures. Formation of disulfide from free cysteine residues was performed using either air oxidation[35] or DMSO–TFA oxidation.[36,37]

Ac-Asn-Cys-Arg-Gly-Asp-Cys-OH (*cyclic peptide; S–S bridge*) **36**.—Synthesis, and cleavage of the resin-bound peptide (0.33 mmol scale), gave the reduced form of peptide **36** (200 mg); HPLC [t_R = 22.93 min (73.5%), 2–10% B gradient in 30 min, C_{18} column]; capillary electrophoresis [t_R = 1.42 min (65%), borate buffer pH 8.0]; MALDI MS $[M + H]^+$, *m/z* 710 (Calc. 710). Oxidation of the peptide using both oxidation methods gave the required peptide **36**.

Method 1: The crude peptide (100 mg) was oxidized (air), and purification by HPLC [t_R = 16.38 min (94.3%), 2–10% B gradient in 30 min, C_{18} column] gave compound **36** (23 mg, 20% yield based on starting resin); ion-exchange chromatography [t_R = 7.39 min (97.0%), 0–100% B gradient in 20 min, Hyper-D SCX column]; capillary electrophoresis [t_R = 1.18 min (92.5%), borate buffer pH 8.0]; electrospray MS $[M + H]^+$, *m/z* 708 (Calc. 708); amino acid analysis (6 M HCl; 24 h; 130 °C): Arg 0.94 (1), Asp 1.97 (2), Gly 1.07 (1), CySS 0.68 (1); amino acid analysis (1 h performic acid oxidation; 6 M HCl; 24 h; 130 °C) Arg 0.97 (1), Asp 2.03 (2), Gly 1.03 (1), Cys 1.97 (2).

Method 2: The crude peptide (100 mg) was oxidized with TFA–DMSO, and purification by HPLC gave compound **36** (29 mg, 25% yield based on starting resin).

*Ac-Thr(*α-L-*rhamnose)-Cys-Arg-Gly-Asp-Cys-OH* (*cyclic peptide; S–S bridge*) **37**.—Synthesis, and cleavage of the resin-bound peptide (0.30 mmol scale), gave the tri-*O*-acetyl derivative (reduced form) of compound **37** (180 mg); HPLC [t_R = 19.60 min (41.8%), 10–40% B gradient in 30 min, C_{18} column]; MALDI MS $[M + H]^+$, *m/z* 969 (Calc. 969).

The crude peptide (90 mg) was oxidized (air) and the method used removed the acetate groups from the sugar moiety. Purification by HPLC [t_R = 13.55 min (98.1%), 2–15% B gradient in 20 min, C_{18} column] gave title compound **37** (29 mg, 23% yield based on starting resin); ion-exchange chromatography [t_R = 6.49 min (98.2%), 0–100% B gradient in 20 min, Hyper-D SCX column]; capillary electrophoresis [t_R = 6.75 min (100%), phosphate buffer pH 2.5]; electrospray MS $[M + H]^+$, *m/z* 841 (Calc. 841); amino acid analysis (6 M HCl; 16 h; 130 °C): Arg 0.98 (1), Asp 1.04 (1), CySS 0.75 (1), Gly 1.05 (1), Thr 0.96 (1); amino acid analysis (1 h performic acid oxidation; 6 M HCl; 16 h; 130 °C) Arg 1.02 (1), Asp 1.04 (1), Cys 1.91 (2), Gly 1.09 (1), Thr 0.95 (1).

Ac-Cys-Thr(α-L-*rhamnose*)-*Pro-Arg-Gly-Asp-Cys-OH* (*cyclic peptide; S–S bridge*) **38**.—Synthesis, and cleavage of the resin-bound peptide (0.30 mmol scale), gave the tri-*O*-acetyl derivative (reduced form) of compound **38** (190 mg); HPLC [t_R = 19.56 min (51.3%), 10–40% B gradient in 30 min, C_{18} column]; Maldi MS $[M + H]^+$, *m/z* 1066 (Calc. 1066).

The crude peptide (188 mg) was oxidized (air) and the method used removed the acetate groups from the sugar moiety. Purification by HPLC [t_R = 16.28 min (98.3%), 2–15% B gradient in 20 min, C_{18} column] gave compound **38** (56 mg, 20% yield based on starting resin); ion-exchange chromatography [t_R = 6.88 min (97.6%), 0–100% B gradient in 20 min, Hyper-D SCX column]; capillary electrophoresis [t_R = 7.14 min (95.6%), phosphate buffer pH 2.5]; electrospray MS $[M + H]^+$, *m/z* 938 (Calc. 938); amino acid analysis (6 M HCl; 16 h; 130 °C): Arg 1.04 (1), Asp 1.05 (1), CySS 0.71 (1), Gly 0.96 (1), Pro 0.94 (1), Thr 0.96 (1); amino acid analysis (1 h performic acid oxidation; 6 M HCl; 16 h; 130 °C) Arg 1.06 (1), Asp 1.00 (1), Cys 1.90 (2), Gly 1.03 (1), Pro 1.08 (1), Thr 0.92 (1).

Acknowledgements

We would like to thank Peter Ashton and Nick May of the Mass Spectroscopy facility and Malcolm Tolley of the NMR facility for providing spectroscopic data for this work. Grateful acknowledgement is also made to Jim Gormley for his invaluable help and guidance. The funding for this project was provided by EPSRC and Zeneca Pharmaceuticals Ltd.

References

1 N. Jentoft, *Trends Biochem. Sci.*, 1990, **15**, 291.
2 J. C. Paulson, *Trends Biochem. Sci.*, 1989, **14**, 272.

3 R. J. Ivatt, *The Biology of Glycoproteins*, Plenum, New York, 1984.
4 P. J. Winterburn and C. F. Phelps, *Nature* (*London*), 1972, **236**, 147.
5 J. Montreuil, *Adv. Carbohydr. Chem. Biochem.*, 1980, **37**, 157.
6 L. Rocchi, F. Biondi and E. Filira, *Peptides: Chemistry and Biology*, Proceedings of the 12th American Peptide Symposium, 1992, p. 881.
7 J. Horvat, *Int. J. Pept. Protein Res.*, 1988, **31**, 499.
8 G. L. Bundy, *J. Med. Chem.*, 1990, **33**, 2276.
9 D. Bellamy, *J. Med. Chem.*, 1993, **36**, 898.
10 H. Kunz, *Angew. Chem., Int. Ed. Engl.*, 1987, **26**, 294.
11 H. Kunz, *Pure Appl. Chem.*, 1993, **65**, 1223.
12 H. Paulsen, *Angew. Chem., Int. Ed. Engl.*, 1990, **29**, 823.
13 J. M. Lacombe, A. A. Pavia and J. M. Rocheville, *Can. J. Chem.*, 1981, **59**, 482.
14 M. Meldal, *Glycopeptide Synthesis*. In *Neoglycoconjugates: Preparation and Applications*, ed. Y. C. Lee and R. T. Lee, Academic Press, San Diego, 1994, 145.
15 R. Polt, L. Szabo, J. Treiberg, Y. Li and V. J. Hruby, *J. Am. Chem. Soc.*, 1992, **114**, 10249.
16 A. Helander, L. Kenne, S. Oscarson, T. Peters and J. R. Brisson, *Carbohydr. Res.*, 1992, **230**, 299.
17 S. Hanessian and J. Banoub, *Carbohydr. Res.*, 1977, **53**, C13.
18 K. Wakabayashi and W. Pigman, *Carbohydr. Res.*, 1974, **35**, 3.
19 E. Atherton and R. C. Sheppard, *Solid Phase Peptide Synthesis: a Practical Approach*, Oxford University Press, Oxford, 1989.
20 W. S. Craig, S. Cheng, D. G. Mullen, J. Blevitt and M. D. Pierschbacher, *Biopolymers*, 1995, **37**, 157.
21 N. S. Cook, G. Kottirsch and H. G. Zerwes, *Drugs Fut.*, 1994, **19(2)**, 135.
22 Z. R. Gan, R. J. Gould, J. W. Jacobs, P. A. Friedman and M. J. Polokoff, *J. Biol. Chem.*, 1988, **263**, 19 827.
23 R. F. Nutt, S. F. Brady, J. T. Sisko, T. M. Ciccarone, C. D. Colton, M. R. Levy, R. J. Gould, G. Zhang, P. A. Friedman and D. F. Veber, *Peptides*, ed. E. Giralt and D. Andreu, ESCOM Science Publishers B. V., Leiden, 1990, p. 784.
24 J. Samanen, F. Ali, T. Romoff, R. Calvo, E. Sorenson, J. Vasko, B. Storer, D. Berry, D. Bennett, M. Strohsacker, D. Powers, J. Stadel and A. Nichols, *J. Med. Chem.*, 1991, **34**, 3114.
25 C. D. Chang, M. Waki, M. Ahmad, J. Meienhofer, E. O. Lundell and J. D. Haug, *Int. J. Pept. Protein Res.*, 1980, **15**, 59.
26 K. P. Ravindranathan and H. J. Jennings, *J. Carbohydr. Chem.*, 1990, **9**, 777.
27 J. Martinez, J. C. Tolle and M. Bodanszky, *J. Org. Chem.*, 1979, **44**, 3596.
28 R. P. Brownlie, N. J. Brownrigg, H. M. Butcher, R. Garcia, R. Jessup, V. J. Lee, S. Tunstall and M. G. Wayne, *Br. J. Pharmacol.*, 1993, **110**, 1600.
29 S.-S. Wang, *J. Am. Chem. Soc.*, 1973, **95**, 1328.
30 P. Schultheiss-Reimann and H. Kunz, *Angew. Chem., Int. Ed. Engl.*, 1983, **22**, 62.
31 E. Atherton, C. J. Logan and R. C. Sheppard, *J. Chem. Soc., Perkin Trans. 1*, 1981, 538.
32 P. Sieber, *Tetrahedron Lett.*, 1987, **28**, 6147.
33 R. Steinauer and P. White, Poster 73 at 3rd Solid Phase Synthesis Conference, Oxford, 1993.
34 K. Barlos, O. Chatzi, D. Gatos and G. Stavropoulos, *Int. J. Pept. Protein Res.*, 1991, **37**, 513.
35 V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts and P. G. Katsoyanis, *J. Am. Chem. Soc.*, 1954, **76**, 3115.
36 A. Otaka, T. Koide, A. Shide and N. Fujii, *Tetrahedron Lett.*, 1991, **32**, 1223.
37 T. Koide, A. Otaka and N. Fujii, *Chem. Pharm. Bull.*, 1993, **41**, 1030.
38 E. Kaiser, R. L. Colescott, C. D. Bossinger and P. I. Cook, *Anal. Biochem.*, 1970, **84**, 595.

Paper 6/04372K
Received 24*th June* 1996
Accepted 20*th December* 1996