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Piperidine is Preferred to Morpholine for Fmoc Cleavage in Solid Phase Glycopeptide Synthesis as Exemplified by Preparation of Glycopeptides Related to HIV gp120 and Mucins

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Abstract: Protected derivatives of the Tn antigens [Fmoc-Ser/Thr(Ac$_3$GalNAcα)-OH, compounds **5** and **8**] have been prepared by glycosylation of Fmoc-Ser/Thr-OAllyl with 3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-D-galactopyranosyl chloride (**2**), followed by conversion of the azido group to an acetamide and deallylation. The derivatives **5** and **8** were used for solid phase synthesis of glycopeptides related to HIV gp120 and mucins. In these syntheses piperidine was found to give efficient Fmoc removal whereas deprotection with morpholine was slow and incomplete for some steps. In contrast to previous concerns β-elimination and epimerization of glycopeptide stereocenters was not encountered when piperidine was used for Fmoc deprotection. However, it was found that for glycopeptides which contained cysteine residues, de-*O*-acetylation with methanolic ammonia had to be performed before side-chain deprotection and cleavage from the solid phase. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Viral envelope proteins are of major importance for attachment of viruses to host cells and for penetration into the cells. The envelope proteins also serve as primary targets for the immune system of the host in its efforts to neutralize viral infections. Most viral envelope proteins are glycosylated and the glycans have important roles in induction and maintenance of protein conformation and in shielding of antigenic peptide epitopes.[1,2] The human immunodeficiency virus, HIV, expresses the envelope glycoprotein gp120 which has a carbohydrate content of 50% and is one of the most heavily glycosylated viral glycoproteins known to date. Gp120 contains a large number of potential *N*-glycosylation sites which are relatively conserved and carry a heterogeneous population of glycans.[3] In addition, gp120 also carries a smaller number

of short *O*-linked glycans identified as the Tn (GalNAcα1→*O*-Ser/Thr) and sialyl Tn (NeuAcα2→6GalNAcα1→O-Ser/Thr) antigens.[4-6] Binding of gp120 to CD4 molecules on T helper lymphocytes of the host is a key event in the infectious cycle of HIV.

Efforts to develop vaccines that provide protection against infections by HIV have so far been unsuccessful. One reason for this failure is that the polypeptide part of gp120 is highly variable, which allows the virus to escape neutralization by antibodies of infected individuals.[7] The carbohydrate moieties of gp120 constitute another potential target for the immune system. Since glycosylation of gp120 is performed by the glycosylation apparatus of the host cell, the viral glycan structures are similar to those of the host and in general not immunogenic. The *O*-linked Tn and sialyl Tn antigens found on gp120[4-6] constitute an exception since they are immature structures produced by cancer cells but not by normal cells.[8,9] Recently, anti-Tn antibodies were found to neutralize HIV that had undergone mutations in the variable peptide part of gp120, and the Tn antigen may therefore be a potential target for prevention and therapy of HIV infections.[10]

Approximately 4-5 *O*-linked glycans have been estimated to exist on gp120[6] but specific sites of *O*-glycosylation have not yet been elucidated by direct structural analysis. However, *in vitro* analysis of the specificity of a polypeptide GalNAc-transferase extract for peptide fragments from gp120 has indicated the location of 3-4 sites, with one or more sites in the hypervariable V3-loop.[11] Interestingly, despite large sequence variation in the V3-loop, *in vitro* analysis of *O*-glycosylation indicated that the glycosylation sites were maintained in the V3 region. In order to enable further characterization of these putative *O*-glycosylation sites of gp120, and to study the immune response to potential glycopeptide fragments from gp120, we now describe the preparation of glycopeptides **12**, **14** and **16**. Glycopeptides **12** and **14** correspond to amino acids 303-320 and 312-327 of the V3-loop of the HIV-3B isolate, whereas **16** corresponds to residues 489-503.[12] In **14** and **16** a cysteine residue has been added at the *C*- and *N*-terminus, respectively, to allow conjugation to carrier proteins.

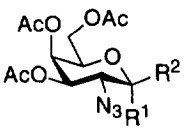
The predicted *O*-glycosylation sites of gp120 are single serine/threonine sites, however, *O*-glycosylation often occurs in clusters, as found in mucins. Immunization of rabbits with desialylated ovine submaxillary mucin (AOSM), which contains high amounts of the Tn antigen, gave a polyclonal immune response with significant cross-reactivity to HIV.[13] Glycopeptides **18** and **20** were therefore prepared to evaluate the immune response to HIV obtained with well-defined, synthetic glycopeptides containing clustered Tn antigens, as compared to HIV-derived glycopeptides. Glycopeptide **18** is derived from a partial sequence of ovine submaxillary mucin,[14] and **20** from the 23 amino acid tandem repeat of the human intestinal mucin Muc2.[15]

Stepwise synthesis of glycopeptides using glycosylated amino acids as building blocks has been found to be the preferred route for organochemical preparation of glycopeptides, and it is also well suited for synthesis on solid phase.[16] Use of N^{α}-Boc protection during peptide synthesis requires conditions for the final deprotection and cleavage from the resin which are incompatible with acid labile *O*-glycosidic linkages. Therefore, the N^{α}-fluoren-9-ylmethoxycarbonyl (Fmoc) group[17] is predominantly used in solid phase synthesis of glycopeptides since mild acidic conditions can then be used for the final deprotection and cleavage. The hydroxyl groups of the carbohydrate moieties are usually protected with acetyl or benzoyl groups which stabilize the glycosidic bonds during deprotection and cleavage.[18-21] A potential drawback with the Fmoc-acyl protective group combination is the need for removal of these protective groups under basic conditions. This has caused concern[18,22] since carbohydrates linked to serine and threonine residues in

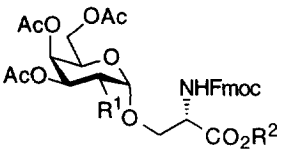
proteins can be removed by base-catalyzed β-elimination,[23] and amino acid residues can undergo base-catalysed epimerization. In order to avoid such side-reactions the weak base morpholine has been recommended for Fmoc-cleavage in synthesis of glycopeptides instead of piperidine which is normally used in peptide synthesis.[18,22] In a preliminary report we have described that piperidine does not give rise to base-catalysed side reactions and that it leads to a more efficient removal of the Fmoc-group.[24] A model study employing a glycosylated tripeptide also revealed that conditons in common use for deacetylation of glycopeptides cause neither β-elimination nor epimerization.[25] We now confirm these observations by synthesis of more complex glycopeptides containing the *O*-linked Tn antigens.

RESULTS AND DISCUSSION

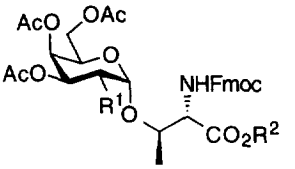
The Tn-building blocks **5** and **8**, can be used directly in solid phase glycopeptide synthesis and were prepared from 3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-D-galactopyranosyl chloride[26] (**2**) and the allyl esters[27] of Fmoc-serine and Fmoc-threonine, respectively. The glycosyl chloride **2** was obtained as a mixture of the α- and β-anomers after treatment of 3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-α-D-galactopyranosyl bromide[26,28] (**1**) with tetraethylammonium chloride in acetonitrile essentially as described previously.[26] Glycosylation of Fmoc-Ser-OAllyl and Fmoc-Thr-OAllyl with **2** under promotion by silver carbonate and silver perchlorate in a mixture of toluene and dichloromethane gave the α-glycosides **3** and **6** in 55 and 50% yields, respectively. The yields of **3** and **6** were found to be independent of the anomeric composition of the glycosyl chloride **2** used as glycosyl donor. Reduction of the azido groups in **3** and **6** was effected with thioacetic acid to give the acetamides **4** and **7** (81 and 82%, respectively), which were deprotected to give the Tn-building blocks **5**[29] and **8**[29,30] by palladium-(0)-catalyzed allyl transfer to *N*-methylaniline[31] (89 and 95%, respectively). A related synthesis of **5** and **8** has been described previously, without inclusion of full experimental details.[31]



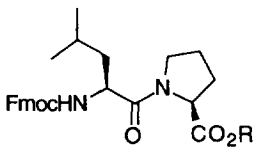
1 R^1 = Br, R^2 = H
2 R^1, R^2 = H, Cl



3 R^1 = N_3, R^2 = Allyl
4 R^1 = NHAc, R^2 = Allyl
5 R^1 = NHAc, R^2 = H



6 R^1 = N_3, R^2 = Allyl
7 R^1 = NHAc, R^2 = Allyl
8 R^1 = NHAc, R^2 = H

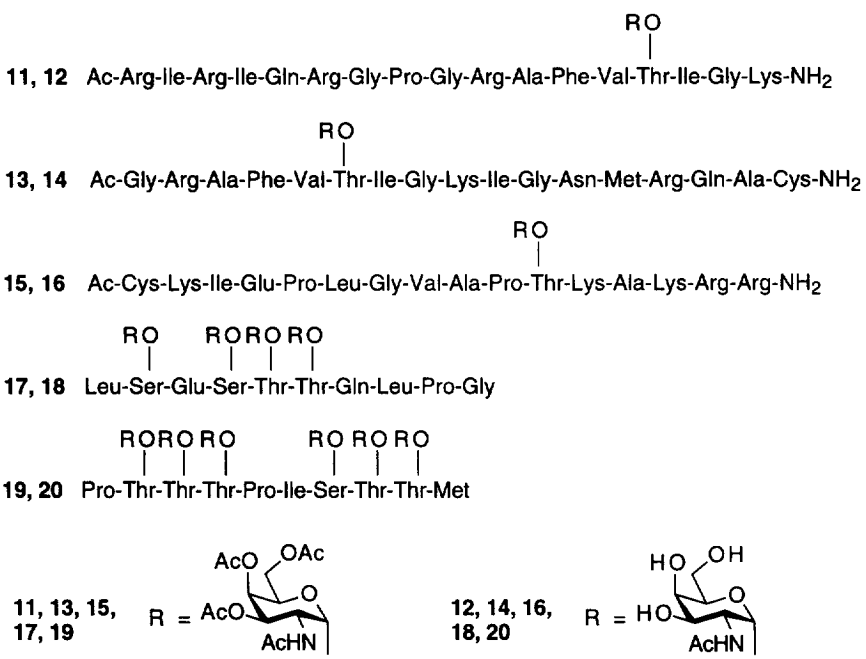


9 R = Allyl
10 R = H

The dipeptide Fmoc-L-leucyl-L-proline (**10**) was prepared in order to avoid anticipated diketopiperazine formation after incorporation of the two *C*-terminal amino acids in the synthesis of glycopeptide **18**. Coupling of Fmoc-L-leucine to L-proline allyl ester hydro-*p*-toluenesulfonate[32] was achieved with *N*,*N*-

diisopropylcarbodiimide and 1-hydroxybenzotriazole[33] to give the fully protected dipeptide **9** (79%). Palladium-(0)-mediated allyl transfer[31] was then used for removal of the allyl ester from **9** and the dipeptide fragment **10** was obtained in 43% yield.

Synthesis of the target glycopeptides **12**, **14**, **16**, **18**, and **20** was performed on a polystyrene resin grafted with polyethylene glycol spacers (TentaGel™ resin). The *C*-terminally amidated glycopeptides **12**, **14**, and **16** were prepared on a resin functionalized with the Rink amide linker,[34,35] using a fully automatic continuous flow peptide synthesizer constructed in our laboratory essentially as described.[36] For glycopeptides **18** and **20**, which have unmodified *C*-termini, *p*-hydroxymethylphenoxyacetic acid[37] was used as linker and synthesis was performed manually in a mechanically agitated reactor. In the synthesis of all glycopeptides N^{α}-Fmoc amino acids (3-4 equivalents) with standard side chain protective groups, as well as the Tn-building blocks **5**[29] and **8**[29,30] (2-3 equivalents), were activated with 1,3-diisopropylcarbodiimide and 1-hydroxybenzotriazole[33] in DMF before being added to the peptide-resin. The *N*-acylations were monitored using the acid-base indicator Bromophenol Blue,[38] and in the manual synthesis also by the ninhydrin test.[39]



In early syntheses of *O*-linked glycopeptides[40,41] piperidine was used for cleavage of the N^{α}-Fmoc group, but fear of β-elimination and epimerization of peptide stereocenters during synthesis[18,22] has since lead to predominant replacement of piperidine (pK_a=11.1) with the weaker base morpholine (pK_a=8.3). In the synthesis of the 17-mer glycopeptide **12** morpholine was therefore used for Fmoc-deprotection after incorporation of the *O*-acetylated α-D-GalNAc-threonine residue. After completion of the solid phase synthesis the glycopeptide was cleaved from the resin with trifluoroacetic acid containing water, thioanisole and ethanedithiol as scavengers to give **11** which still carried the *O*-acetyl protective groups for the carbohydrate moiety. Analytical reversed-phase HPLC revealed crude **11** to consist of one, main product but

also several minor by-products. Some of the by-products had retention times close to that of **11**, but purification with preparative reversed-phase HPLC was still succesfully achieved.

In the synthesis of **14** piperidine was also replaced by morpholine after incorporation of the glycosylated threonine residue.[24] However, spectrophotometric monitoring of the dibenzofulvene-morpholine adduct in the column effluent at 350 nm then revealed that cleavage of the Fmoc group, after coupling of each of the amino acids GalNAcα-Thr^6 through Gly^1, was slow. Most likely this reflects internal aggregation of the peptide chains during the synthesis which results in incomplete Fmoc removal.[42] Consequently, the crude glycopeptide **13**, obtained after cleavage from the resin and side chain deprotection, was found to contain several by-products that were difficult to remove by preparative HPLC (Figure 1a). When the synthesis was repeated using 20% piperidine in DMF, instead of morpholine, fast and complete Fmoc removal was observed and crude **13** of high purity was obtained (Figure 1b). Purification by preparative reversed-phase HPLC was now facile and **13**[24] was obtained in 36% yield, based on the resin capacity (the peptide content was taken into account when yields were calculated). The two side-products eluting immediately before **13** (Figure 1b) were shown by FAB MS to originate from incomplete incorporation of one glycine and oxidation of **13**, respectively. Based on the experiences from the synthesis of **13** piperidine was employed for all Fmoc-deprotection steps in the solid phase synthesis of **16**. After cleavage from the resin analytical reversed-phase HPLC revealed that crude **15**, *i.e.* the *O*-acetylated precursor of **16**, contained only one major by-product (10-15% of the material) which could be removed by preparative HPLC.

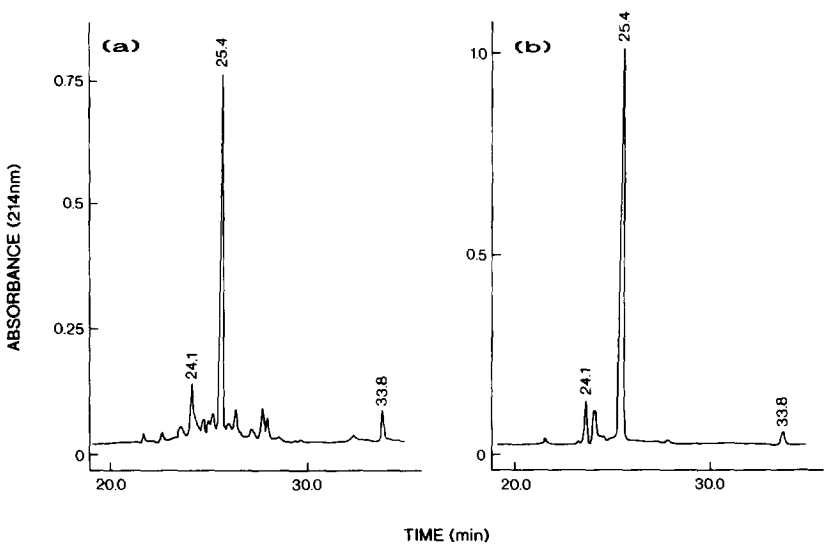


Figure 1. Analytical HPLC chromatograms of the crude glycopeptide **13** (25.4 min) obtained from syntheses in which cleavage of the N^{α}-Fmoc groups of residues Gly^1-Thr^6 was performed with (**a**) 50% morpholine or (**b**) 20% piperidine in DMF. HPLC conditions are described in the experimental part.

Deacetylation of the carbohydrate moiety of **11** was performed with saturated methanolic ammonia and analytical reversed-phase HPLC revealed complete conversion of **11** into **12**. Purification with preparative

HPLC gave **12** in 14% overall yield based on the capacity of the resin. Attempted deacetylation of glycopeptides **13** and **15** with methanolic ammonia led to the formation of a large number of products which were not purified and characterized (cf. deacetylation of **15**, Figure 2a). However, it was assumed that these products had been formed by oxidative dimerization of the cysteine residues in **13** and **15**, as well as by cysteine-catalyzed cleavage of the peptide backbones. Therefore, deacetylation was instead performed before cleavage of the glycopeptides from the solid phase. Subsequent cleavage and deprotection of the amino acid side chains with trifluoroacetic acid and scavengers gave crude glycopeptides (cf. crude **16**, Figure 2b) which were purified by HPLC to give **14** and **16** (45 and 15% yields, respectively). Glycopeptide **16** contains a glutamic acid residue at position 4 which conceivably could have been converted into a glutamine during the deacetylation with methanolic ammonia. The lack of resonances from a CONH_2-group in the 500 MHz ^{1}H NMR spectrum of **16** however confirmed that Glu^4 had not undergone such a modification.

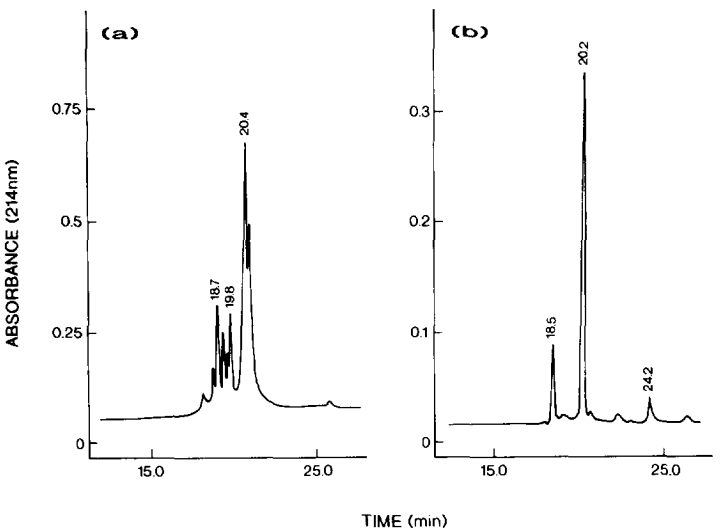


Figure 2. Analytical HPLC chromatograms of (**a**) the mixture of products obtained on attempted deactylation of **15** with a solution of methanolic ammonia for 6 h and (**b**) crude **16** obtained after deacetylation had been performed before cleavage of the glycopeptide from the solid phase. HPLC conditions are described in the experimental part.

The heavily glycosylated glycopeptides **18** and **20** were prepared using piperidine for removal of the Fmoc-groups during assembly on the solid phase. Trifluoroacetic acid catalyzed cleavage from the solid phase and purification by HPLC gave the *O*-acetylated glycopeptides **17** and **19** which were then deprotected with saturated methanolic ammonia. Deacetylation of **17** and **19** was quantitative according to analytical HPLC and glycopeptides **18** and **20** were obtained in 4.5 and 5.1% overall yields, respectively, after purification by HPLC. The low overall yields reflect problems in removal of side-products in the crude **17** and **19**, and most likely also losses due to irreversible retention of **17**-**20** on reversed-phase silica gel columns as encountered previously by others (cf. preparation of compound 13 in reference 43).

In a model study we recently showed that glycopeptide diastereomers which are epimers at an α-carbon stereocenter have significantly different ^{1}H NMR spectra, and that 500 MHz ^{1}H NMR spectroscopy is a sensitive tool for detection of epimerization during glycopeptide synthesis.[25] Similarly, products formed by β-elimination were easily detected by 500 MHz ^{1}H NMR spectroscopy in this model study, and it was also found that they could be removed by preparative reversed-phase HPLC, in contrast to glycopeptide diastereomers. In order to investigate if epimerization or β-elimination had occurred during synthesis of glycopeptides **12**, **14**, **16**, **18** and **20** the glycopeptides were characterized by ^{1}H NMR spectroscopy (cf. Tables 1-5), amino acid analysis, and fast atom bombardment mass spectroscopy. Glycopeptide **13**, the *O*-acetylated precursor of **14**, has previously been characterized in the same manner.[24] For these six glycopeptides all cross peaks in the 500 MHz COSY NMR spectra could be satisfactorily assigned and no impurities were detected. The region containing the peptide NH→H-α and GalNAc NH→H-2 crosspeaks in the COSY spectrum of glycopeptide **20** is shown as an example in Figure 3. The lack of unassigned cross peaks in the COSY spectra of the glycopeptides therefore revealed that neither epimerization nor β-elimination had occured during Fmoc-deprotection with piperidine, or during the subsequent deacetylation with saturated methanolic ammonia. This conclusion was further supported by the fact that D-alloisoleucine, which is formed on epimerization of L-isoleucine, could not be detected in the glycopeptides by amino acid analysis. The lack of epimerization and β-elimination encountered during the synthesis of glycopeptides **12**, **14**, **16**, **18**, and **20** is in full agreement with recent results obtained by us,[24,25] and others,[44] and confirm that previous concerns[18,22] over the extent of epimerization and β-elimination in glycopeptide synthesis were exaggerated.

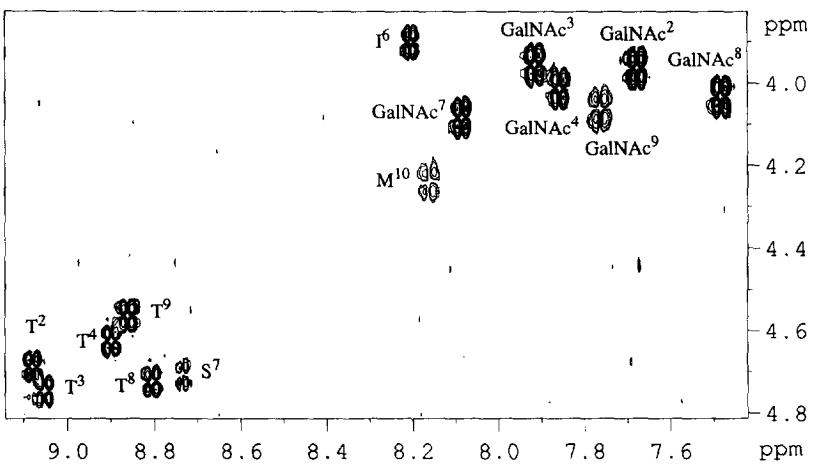


Figure 3. 500 MHz COSY spectrum of **20** obtained in 90% H_2O/10% D_2O at pH 5.8 and 5 °C. The region containing peptide NH→H-α and GalNAc NH→H-2 crosspeaks has been plotted so as to barely include some noise. Crosspeaks have been labelled using one-letter codes for the amino acid residues and the position of each residue in **20**. Note that the crosspeaks belonging to the five GalNAc-Thr residues are all well resolved.

Peptides derived from the third variable region (the V3-loop) of the external envelope glycoprotein gp120 of the human immunodeficiency virus, such as **12**, contain a highly conserved Gly-Pro-Gly-Arg tetrapeptide.[7] Epitopes centered around this tetrapeptide constitute targets for antibodies that neutralize virus infectivity and the conformation of the V3 region of gp 120 has therefore been intensively studied. A β-turn was predicted for the Gly-Pro-Gly-Arg tetrapeptide,[7] and was also observed in solution using ^{1}H NMR spectroscopy when the tetrapeptide was part of long, linear or cyclic peptides.[45,46] Furthermore, the tetrapeptide adopts a type II β-turn when bound by a broadly neutralizing antibody directed towards the HIV virus.[47] Using ^{1}H NMR spectroscopy we were unable to detect any of the NOEs reported[45] as characteristic for a β-turn encompassing residues Gly^7-Pro-Gly-Arg^{10} in **12**, *i.e.* a NH→NH NOE between Gly^9 and Arg^{10} and a Hα→NH NOE between Pro^8 and Arg^{10}. This may be due to insufficient length of the linear glycopeptide **12**, as suggested by previous studies of peptides from the V3-loop,[45] or to an influence from the carbohydrate moiety in **12**. Glycopeptide **12**, **14** and **16** all displayed strong Hα→NH(i,i+1) and weak NH→NH(i,i+1) qualitative NOEs for the peptide backbone. These NOE patterns are compatible with conformationally averaged structures for the glycopeptides but not with an ordered secondary structure.[48]

EXPERIMENTAL

General Methods and Materials.—TLC was performed on Silica Gel 60 F_{254} (Merck) with detection by UV light and charring with sulfuric acid. Flash column chromatography was performed on silica gel (Matrex, 60 Å, 35-70 μm, Grace Amicon) with distilled solvents. Immediately before being used, toluene and THF were dried by distillation from metallic sodium. Dichloromethane was dried by distillation from calcium hydride and acetonitrile was passed through a column of neutral aluminium oxide (activity 1). Organic solutions were dried over Na_2SO_4.

The ^{1}H- and ^{13}C-NMR spectra were recorded with a Bruker DRX-400 or a Bruker ARX-500 spectrometer for solutions in $CDCl_3$ [residual $CHCl_3$ (δ_H 7.25 ppm) as internal standard] at 295 K or in 9:1 mixtures of H_2O and D_2O [H_2O (δ_H 4.98 ppm) as internal standard] at 278 K. The pH of the aqueous NMR samples were adjusted to 5.4-5.8 with solutions of 0.4% NaOD and 0.4% DCl in D_2O. First-order chemical shifts and coupling constants were obtained from one-dimensional spectra and proton resonances were assigned from COSY[49] TOCSY[50] and ROESY[51] experiments. Resonances for aromatic protons and resonances that could not be assigned are not reported. *J* values are given in Hz. Positive fast atom bombardment mass spectra were recorded on a Jeol JMS SX 102 A mass spectrometer. Ions were produced by a beam of Xenon atoms (6 keV) from a matrix of glycerol and thioglycerol. In the amino acid analyses, asparagine and glutamine were determined as aspartic acid and glutamic acid, respectively.

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-L-serine allyl ester[27], N^{α}-(fluoren-9-ylmethoxycarbonyl)-L-threonine allyl ester[27], 3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-D-galactopyranosyl bromide[26,28] (**1**), 3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-D-galactopyranosyl chloride[26] (**2**) and L-proline allyl ester hydro-*p*-toluenesulfonate[32] were prepared according to the indicated literature methods.

General Procedure for Solid-phase Synthesis.—Glycopeptides **12, 14**, and **16** were synthesized in a custom made, fully automatic continuos flow peptide synthesizer constructed essentially as described,[36] whereas glycopeptides **18** and **20** were synthesized manually in a mechanically agitated reactor. A resin consisting of a crosslinked polystyrene backbone grafted with polyethyleneglycol chains (TentaGel™, Rapp Polymere, Germany) was used for the syntheses. The resin was functionalized with the linker *p*-[α-(fluoren-9-ylmethoxyformamido)-2,4-dimethoxybenzyl]phenoxyacetic acid[34,35] (Novabiochem, Switzerland) for synthesis of **12, 14**, and **16** whereas *p*-hydroxymethylphenoxyacetic acid[37] (Novabiochem, Switzerland) was used for **18** and **20**. N^{α}-Fmoc amino acids (Bachem, Switzerland) with the following protective groups were used: 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) for arginine; triphenylmethyl (Trt) for asparagine, cysteine and glutamine; *tert*-butoxycarbonyl (Boc) for lysine; and *tert*-butyl for glutamic acid. DMF was distilled before being used.

In the synthesis of glycopeptides **12, 14**, and **16** 400 mg (70 μmol) of resin, functionalized with the Rink linker,[34,35] was used in the peptide synthesizer. The N^{α}-Fmoc amino acids and the glycosylated amino acid **8**[29,30] were coupled to the peptide-resin as 1-benzotriazolyl (HOBt) esters.[33] These were prepared, *in situ*, by reaction of the appropriate N^{α}-Fmoc amino acid (0.28 mmol), 1-hydroxybenzotriazole (0.42 mmol) and 1,3-diisopropylcarbodiimide (0.27 mmol) in DMF (1.3 mL). The glycosylated amino acid **8** (0.14-0.21 mmol) was activated in the same way. After 45 min bromophenol blue in DMF (0.15 mM, 0.35 mL) was added to the solution of the HOBt ester by the synthesizer, and the solution was recirculated through the column containing the resin. The acylation was monitored[38] using the absorbance of bromophenol blue at 600 nm, and the peptide-resin was automatically washed with DMF after 1 h or when monitoring revealed the coupling to be complete. N^{α}-Fmoc deprotection of the peptide resin was performed by a flow of 20% piperidine in DMF (2 mL/min) through the column for 12.5-17.5 min, and was monitored[52] using the absorbance of the dibenzofulvene-piperideine adduct at 350 nm. Alternatively, a flow of 50% morpholine in DMF (2 mL/min) for 25 min was used. After completion of the N^{α}-Fmoc deprotection the peptide-resin was again washed automatically with DMF. Acetylation of the *N*-terminus of the completed peptide resins was done with acetic anhydride (0.8 mL) in the synthesizer.

In the manual synthesis of **18** and **20** the *C*-terminal amino acids (Fmoc-Gly and Fmoc-Met) were coupled to the linker-functionalized resin (0.35 g, 50 μmol) with 2,4,6-mesitylenesulfonyl-3-nitro-1,2,4-triazolide (MSNT) in the presence of 1-methylimidazole.[53] The N^{α}-Fmoc amino acids (0.15 mmol), the glycosylated amino acids **5**[29] and **8**[29,30] (0.15 mmol), and the dipeptide **10** (0.25 mmol) were coupled to the peptide-resin as 1-benzotriazolyl (HOBt) esters, as described above.[33]. Couplings were monitored by addition of Bromophenol Blue[38] (0.05% of the resin capacity) to the reactor, and by the ninhydrin test.[39] N^{α}-Fmoc deprotection was effected by treatment with 20% piperidine in DMF and washings were done with DMF.

After completion of the synthesis, the resins carrying the protected glycopeptides **12, 18** and **20** were washed with dichloromethane (5 x 5 mL) and dried under vacuum. For each glycopeptide-resin the glycopeptide was then cleaved from the resin (200 mg), and the amino acid side chains were deprotected, by treatment with trifluoroacetic acid-water-thioanisole-ethanedithiol (87.5:5:5:2.5, 20 mL) for 2 h, followed by filtration. Acetic acid (20 mL) was added to the filtrate, the solution was concentrated, and acetic acid (20 mL) was added again followed by concentration. The residue was triturated with diethyl ether (10 mL) which gave a solid, crude glycopeptide which was dissolved in acetic acid - water (1:4, 25 mL) and freeze dried. Purification by preparative HPLC gave *O*-acetylated glycopeptide which was deacetylated in saturated

methanolic ammonia (2 mL/mg of crude glycopeptide) at room temperature for 3-18 h. Concentration of the solution and purification of the residue by preparative HPLC gave glycopeptides **12**, **18** and **20**. Glycopeptide **13** was cleaved from the resin and purified in the same manner as for **12**, **18**, and **20**, with omission of the deacetylation step. For glycopeptides **14** and **16** deacetylation of the carbohydrate moiety was performed on the resin using saturated methanolic ammonia (5 mL/100 mg of glycopeptide-resin). Cleavage from the resin was then performed as above and was followed by purification by preparative HPLC.

The glycopeptides were analyzed on a Beckman System Gold HPLC using a Kromasil C-8 column (100 Å, 4.6 x 250 mm) and a linear gradient of 0 - 80% of *B* in *A* over 60 min with a flow rate of 1.5 mL/min and detection at 214 nm (solvent systems *A*: 0.1% aqueous trifluoroacetic acid and *B*: 0.1% trifluoroacetic acid in acetonitrile). Purification of the crude glycopeptides were performed with the same HPLC system on a Kromasil C-8 column (100 Å, 20 x 250 mm) with a flow rate of 11 mL/min.

In calculating the final yields for the glycopeptides the peptide content of the purified glycopeptide, as determined by amino acid analysis, has been taken into account.

N$^{\alpha}$-*(Fluoren-9-ylmethoxycarbonyl)-3*-O-*(3,4,6-tri*-O-*acetyl-2-azido-2-deoxy*-α-D-*galactopyranosyl)*-L-*serine Allyl Ester* **3**.—Silver perchlorate (325 mg, 1.57 mmol) was added to a mixture of N$^{\alpha}$-(Fluoren-9-ylmethoxycarbonyl)-L-serine allyl ester[27] (2.16 g, 5.88 mmol), silver carbonate (3.24 g, 11.8 mmol) and activated molecular sieves (3.8 g) that had been stirred in dry toluene (31 mL) for 20 min at -9 °C. After a further 30 min at -9 °C, 3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-D-galactopyranosyl chloride[26] (**2**) (1.98 g, 4.90 mmol) in dichloromethane-toluene (5:2, 70 mL) was slowly added. The mixture was allowed to attain room temperature during 14 h and was then filtered through celite. The solution was washed with water (2 x 250 mL), the aqueous phase was extracted with dichloromethane and the combined organic phases were dried, filtered and concentrated. Purification of the residue by flash column chromatography (toluene-ethyl acetate, 5:1) gave **3** (2.12 g, 55%), $[\alpha]_D^{23}$ +91 (*c* 0.97 in $CHCl_3$); δ_H (400 MHz, $CDCl_3$) 5.96 (1 H, ddt, *J* 16.5, 10.4, and 5.8, -$CH_2C\underline{H}$=CH_2), 5.45 (1 H, d, *J* 2.9, H-4), 5.41–5.31 (2 H, m, -CH_2CH=$C\underline{H_2}$), 5.31 (1 H, d, *J* 10.6, H-3), 4.96 (1 H, d, *J* 3.5, H-1), 4.72 (2 H, d, *J* 5.1, -$C\underline{H_2}CH$=CH_2), 4.61 (1 H, t, *J* 2.9, H-α), 4.42 (2 H, d, *J* 7.6, Fmoc-CH_2), 4.27 (1 H, t, Fmoc-CH), 4.20 (1 H, t, *J* 5.6, H-β), 4.15 (1 H, dd, *J* 10.8 and 3.5, H-β'), 4.05 (2 H, d, *J* 6.7, H-5, H-6), 3.64 (1 H, dd, *J* 11.1 and 3.4, H-2), 2.19, 2.09 and 2.02 (each: 3 H, s, Ac); Anal. calcd. for $C_{33}H_{36}N_4O_{11}$: C, 58.2; H, 5.3; N, 8.2. Found: C, 58.6; H, 5.4; N, 7.6.

N$^{\alpha}$-*(Fluoren-9-ylmethoxycarbonyl)-3*-O-*(2-acetamido-3,4,6-tri*-O-*acetyl-2-deoxy*-α-D-*galactopyranosyl)*-L-*serine Allyl Ester* **4**.—Compound **3** (2.12 g, 3.12 mmol) was treated with distilled thioacetic acid (85 mL) for 64 h at room temperature. The solution was then concentrated and purification of the residue by flash column chromatography (heptane-ethyl acetate, 1:2) gave **4** (1.75 g, 81%), $[\alpha]_D^{23}$ +91 (*c* 0.095 in $CHCl_3$); δ_H (400 MHz, $CDCl_3$) 5.92 (1 H, ddt, *J* 17.1, 10.6 and 5.7, -$CH_2C\underline{H}$=CH_2), 5.88 (1 H, d, *J* 10.2, NH), 5.73 (1 H, d, *J* 9.6, AcNH), 5.38 (1 H, d, *J* 17.2, -CH_2CH=$C\underline{H_2}$), 5.37 (1 H, d, *J* 3.1, H-4), 5.32 (1 H, d, *J* 10.4, -CH_2CH=$C\underline{H_2}$), 5.12 (1 H, dd, *J* 11.3 and 2.9, H-3), 4.85 (1 H, d, *J* 3.0, H-1), 4.69 (2 H, d, *J* 5.5, -$C\underline{H_2}CH$=CH_2), 4.61–4.55 (2 H, m, H-α, H-2), 4.47 (2 H, d, *J* 7.4, Fmoc-CH_2), 4.26 (1 H, t, J 6.8, Fmoc-CH), 4.15–4.02 (3 H, m, 5–H, H-6,6'), 3.98 (2 H, br s, H-β,β'), 2.19, 2.03, 2.02 and 1.97 (each: 3 H, s, Ac); Anal. calcd. for $C_{35}H_{40}N_2O_{12}$: C, 60.3; H, 5.8; N, 4.0. Found: C, 60.4; H, 5.5; N, 3.8.

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-3-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-α-D-galactopyranosyl)-L-serine **5**[29].—A solution of **4** (1.65 g, 2.37 mmol) and *N*-methylaniline (0.77 mL, 7.11 mmol) in dry THF (50 mL) was treated with tetrakis(triphenylphosphine) palladium(0) (274 mg, 237 μmol) under nitrogen at room temperature for 1 h. Then the solution was diluted with dichloromethane (100 mL) and washed with water (2 x 100 mL). The aqueous phase was extracted with dichloromethane and the combined organic phases were dried, filtered and concentrated. Purification of the residue by flash column chromatography in two different solvent systems (dichlormethane-methanol, 9:1 and ethyl acetate-acetic acid, 9:1) gave **5** (1.38 g, 89%), $[\alpha]_D^{23}$ +74.4 (*c* 1.14 in $CHCl_3$); ^1H NMR data were in agreement with those reported previously[29].

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-3-O-(3,4,6-tri-O-acetyl-2-azido-2-deoxy-α-D-galactopyranosyl)-L-threonine Allyl Ester **6**.—*N^{α}*-(Fluoren-9-ylmethoxycarbonyl)-L-threonine allyl ester[27] (925 mg, 2.42 mmol) was glycosylated with 3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-D-galactopyranosyl chloride[26] (**2**) (803 mg, 2.02 mmol) under promotion by silver carbonate (1.11 g, 4.04 mmol) and silver perchlorate (84 mg, 0.40 mmol) as described for **3**. Purification of the crude product by flash column chromatography (toluene-acetone, 20:1) gave **6** (699 mg, 50%), $[\alpha]_D^{23}$ +82 (*c* 0.99 in $CHCl_3$); δ_H (400 MHz, $CDCl_3$) 5.96 (1 H, ddt, *J* 16.7, 10.6 and 6.1, $-CH_2C\underline{H}=CH_2$), 5.68 (1 H, d, *J* 9.7, NH), 5.48 (1 H, dd, *J* 3.2 and 0.8, H-4), 5.39 (1 H, dd, *J* 17.2 and 1.4, $-CH_2CH=C\underline{H}_2$), 5.31 (1 H, dd, *J* 10.7 and 3.2, H-3), 5.30 (1 H, dd, *J* 10.2 and 0.9, $-CH_2CH=C\underline{H}_2$), 5.06 (1 H, d, *J* 3.5, H-1), 4.71 (2 H, br d, *J* 5.9, $-C\underline{H}_2CH=CH_2$), 4.51-4.35 (4 H, m, H-α, H-β, H-5, H-6), 4.31-4.26 (2 H, m, Fmoc-CH, H-6), 4.11 (2 H, br d, *J* 6.6, Fmoc-CH_2), 3.68 (1 H, dd, *J* 11.2 and 3.7, H-2), 2.17, 2.09 and 2.06 (each: 3 H, s, Ac), 1.37 (3 H, d, *J* 6.4, H-γ); HRMS $(M + Na)^+$ calcd 717.2384, obsd 717.2401.

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-3-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-α-D-galactopyranosyl)-L-threonine Allyl Ester **7**.—Reduction of **6** (0.97 g, 1.4 mmol) with thioacetic acid (40 mL), as described for **4**, and purification of the crude product by flash column chromatography (heptane-ethyl acetate, 1:2) gave **7** (0.82 g, 82%), $[\alpha]_D^{23}$ +78 (*c* 0.15 in $CHCl_3$); δ_H (400 MHz, $CDCl_3$) 5.89 (1 H, ddt, *J* 16.7, 10.4 and 5.7, $-CH_2C\underline{H}=CH_2$), 5.77 (1 H, d, *J* 9.6, AcNH), 5.57 (1 H, d, *J* 9.6, NH), 5.40 (1 H, br d, *J* 2.4, H-4), 5.37 (1 H, dd, *J* 17.1 and 1.0, $-CH_2CH=C\underline{H}_2$), 5.32 (1 H, dd, *J* 10.3 and 1.0, $-CH_2CH=C\underline{H}_2$), 5.10 (1 H, dd, *J* 11.3 and 3.1, H-3), 4.90 (1 H, d, *J* 3.7, H-1), 4.69 (1 H, dd, *J* 12.6 and 5.8, $-C\underline{H}_2CH=CH_2$), 4.60 (1 H, dd, *J* 12.4 and 5.4, $-C\underline{H}_2CH=CH_2$), 4.57 (1 H, dd, *J* 9.7 and 3.4, H-2), 4.49-4.45 (3 H, m, Fmoc-CH_2, H-α), 4.28 (2 H, br t, *J* 6.6, H-β, Fmoc-CH), 4.24 (1 H, t, *J* 6.4, H-5), 4.13 (1 H, dd, *J* 11.2 and 5.4, H-6), 4.08 (1 H, dd, *J* 11.2 and 7.2, H-6), 2.17, 2.06, 2.02 and 2.01 (each: 3 H, s, Ac), 1.35 (3 H, d, *J* 6.4, H-γ); Anal. calcd. for $C_{36}H_{42}N_2O_{13}$: C, 60.8; H, 6.0; N, 3.9. Found: C, 60.8; H, 6.0; N, 3.8.

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-3-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-α-D-galactopyranosyl)-L-threonine **8**[29,30].—Compound **7** (1.40 g, 1.97 mmol) was deallylated with *N*-methylaniline and tetrakis(triphenylphosphine) palladium(0) in dry THF (40 mL) as described for **5**. Purification of the crude product by flash column chromatography (dichloromethane-ethyl acetate, 1:1 followed by ethyl acetate) gave **8** (1.25 g, 95%), $[\alpha]_D^{23}$ +72 (*c* 0.72 in $CHCl_3$); ^1H NMR data were in agreement with those reported previously[30].

N$^{\alpha}$-*(Fluoren-9-ylmethoxycarbonyl)*-L-*leucyl*-L-*proline Allyl Ester* **9**.—*N*$^{\alpha}$-Fmoc-L-leucine (502 mg, 1.42 mmol) was treated with 1-hydroxybenzotriazole[33] (288 mg, 2.13 mmol) and *N*,*N*-diisopropylcarbodiimide (220 mL, 1.42 mmol) in dichloromethane (5 mL) at room temperature for 1 h and 30 min. L-Proline allyl ester hydro-*p*-toluenesulfonate[32] (490 mg, 1.42 mmol) in dichloromethane (5 mL) was added and after 20 h at room temperature the solution was refluxed for 3 h. The solution was diluted with dichloromethane (50 mL) and washed with water (2 x 100 mL). The aqueous phase was extracted with dichloromethane (100 mL), the combined organic phases were dried, filtered and concentrated. Purification of the residue by flash column chromatography (toluene-ethyl acetate, 9:1) gave **9** (547 mg, 79%), $[\alpha]_D^{22}$ -53 (*c* 1.2 in $CHCl_3$); δ_H (400 MHz, $CDCl_3$) 5.92 (1 H, ddt, *J* 17.1, 10.5 and 5.66, $-CH_2C\underline{H}=CH_2$), 5.51 (1 H, d, *J* 9.1, Leu-NH), 5.35 (1 H, dd, *J* 17.2 and 1.4, $-CH_2CH=C\underline{H}_2$), 5.26 (1 H, dd, *J* 10.4 and 1.1, $-CH_2CH=C\underline{H}_2$), 4.64 (2 H, m, $-C\underline{H}_2CH=CH_2$), 4.62-4.56 (2 H, m, Leu-H-α, Pro-H-α), 4.36 (2 H, m, Fmoc-CH_2), 4.22 (1 H, t, *J* 7.0, Fmoc-CH), 3.79 (1 H, dt, *J* 9.7 and 6.5, Pro-H-δ), 3.64 (1 H, m, Pro-H-δ), 2.26 (1 H, m, Pro-H-β), 2.13-2.00 (3 H, m, Pro-H-β, Pro-H-γ,γ′), 1.80 (1 H, m, *J* 6.8, Leu-H-γ), 1.58 (1 H, dd, *J* 5.2 and 2.0, Leu-H-β), 1.56 (1 H, dd, *J* 5.2 and 1.7, Leu-H-β), 1.03 (3 H, d, *J* 6.5, Leu-H-δ), 0.99 (3 H, d, *J* 6.7, Leu-H-δ); HRMS M^+ calcd 490.2467, obsd 490.2468.

N$^{\alpha}$-*(Fluoren-9-ylmethoxycarbonyl)*-L-*leucyl*-L-*proline* **10**.—Compound **9** (480 mg, 0.978 mmol) was deallylated with *N*-methylaniline and tetrakis(triphenylphosphine) palladium(0) for 4 h 30 min in dry THF (10 mL) as described for **5**. Purification of the residue by flash column chromatography in two different solvent systems (dichlormethane-ethyl acetate, 1:4 and dichlormethane-ethyl acetate, 3:7) gave **10** (191 mg, 43%), $[\alpha]_D^{23}$ -43 (*c* 0.12 in $CHCl_3$); δ_H (400 MHz, $CDCl_3$) 5.53 (1 H, d, *J* 9.0, Leu-NH), 4.57 (1 H, dd, *J* 9.6 and 3.7, Pro-H-α), 4.62 (1 H, dt, *J* 7.8 and 3.6, Leu-H-α), 4.40 (1 H, dd, *J* 10.6 and 7.6, Fmoc-CH_2), 4.36 (1 H, dd, *J* 10.5 and 6.8, Fmoc-CH_2), 4.22 (1 H, t, *J* 7.2, Fmoc-CH), 3.79 (1 H, dt, *J* 9.8 and 7.2, Pro-H-δ), 3.61 (1 H, m, Pro-H-δ). 2.31 (1 H, m, Pro-H-β), 2.18-2.04 (3 H, m, Pro-H-β, Pro-H-γ,γ′), 1.76 (1 H, m, Leu-H-γ), 1.61 (1 H, ddd, *J* 13.9, 10.2 and 4.7, Leu-H-β), 1.50 (1 H, ddd, *J* 14.0, 9.2 and 4.1, Leu-H-β), 1.00 (3 H, d, *J* 6.5, Leu-H-δ), 0.98 (3 H, d, *J* 6.7, Leu-H-δ); HRMS M^+ calcd 450.2155, obsd 450.2145.

N-*Acetyl*-L-*arginyl*-L-*isoleucyl*-L-*arginyl*-L-*isoleucyl*-L-*glutaminyl*-L-*arginyl-glycyl*-L-*prolyl-glycyl*-L-*arginyl*-L-*alanyl*-L-*phenylalanyl*-L-*valyl*-O-*(2-acetamido-2-deoxy-α-D-galactopyranosyl)*-L-*threonyl*-L-*isoleucyl-glycyl*-L-*lysine amide* **12**.—Synthesis, cleavage of the resin-bound glycopeptide (540 mg, 61 μmol), and purification by HPLC (22 % *B* in *A*) according to the general procedure, gave the *O*-acetylated **11** (58 mg). Deacetylation and purification by HPLC (20 % *B* in *A*) gave **12** (34 mg, 54% peptide content, 14% overall yield). For ^1H NMR data, see Table 1. FABMS *m/z* 2169 (M + H) [Calc. *m/z* 2169 (M + H)]. Amino acid analysis: Ala 1.03 (1), Arg 3.91 (4), Glu 1.03 (1), Gly 2.98 (3), Ile 2.99 (3), Lys 0.99 (1), Phe 1.03 (1), Pro 1.02 (1), Thr 0.99 (1), Val 1.03 (1).

Table 1. ^{1}H NMR chemical shifts (δ, ppm) for compound **12** in water containing 10% D_2O[a]

Residue	NH	Hα	Hβ	Hγ	Others
Arg^1	8.37	4.18	1.71[b]	1.57[b]	3.14[b] (Hδ,δ'), 1.96 (Ac)
Ile^2	8.30	4.08	1.78	1.14, 1.43	0.83 (β- and γ-Me)
Arg^3	8.59	4.30	1.68, 1.72	1.49[b]	3.15[b] (Hδ,δ')
Ile^4	8.38	4.06	1.75	1.12, 1.35	0.82 (β- and γ-Me)
Gln^5	8.66	4.30	1.90, 2.02	2.28[b]	6.92 and 7.60 (γ-$CONH_2$)
Arg^6	8.61	4.32	1.70, 1.80	1.58[b]	3.15[b] (Hδ,δ')
Gly^7	8.45	3.95, 4.11			
Pro^8	-	4.38	1.93, 2.22	1.96[b]	3.58[b] (Hδ,δ')
Gly^9	8.59	3.88[b]			
Arg^{10}	8.25	4.23	1.65, 1.72	1.51[b]	3.10[b] (Hδ,δ')
Ala^{11}	8.38	4.20	1.25		
Phe^{12}	8.30	4.58	2.95, 3.04		7.18-7.28 (H-arom)
Val^{13}	8.18	4.21	1.94	0.87[b]	
Thr^{14}	8.72	4.52	4.21	1.22	GalNAcα[c]
Ile^{15}	8.42	4.09	1.77	1.14, 1.44	0.79 (γ-Me), 0.89 (β-Me)
Gly^{16}	8.56	3.74, 3.97			
Lys^{17}	8.50	4.19	1.70, 1.82	1.42[b]	1.64[b] (Hδ,δ'), 2.95[b] (Hε,ε'), 7.20 and 7.79 (α-$CONH_2$)

[a]Obtained at 500.14 MHz, 278 K, and pH 5.7 with H_2O (δ_H 4.98 ppm) as internal standard. [b]Degeneracy has been assumed. [c]Chemical shifts (δ, ppm) for the *N*-acetylgalactosamine moiety in **12**: 4.72 (H-1), 4.02 (H-2), 3.80 (H-3), 3.89 (H-4), 7.82 (NH), 1.97 (Ac).

N-*Acetyl-glycyl*-L-*arginyl*-L-*alanyl*-L-*phenylalanyl*-L-*valyl*-O-*(2-acetamido-3,4,6-tri*-O-*acetyl-2-deoxy-α-D-galactopyranosyl)*-L-*threonyl*-L-*isoleucyl-glycyl*-L-*lysyl*-L-*isoleucyl-glycyl*-L-*asparaginyl*-L-*methionyl*-L-*arginyl*-L-*glutaminyl*-L-*alanyl*-L-*cysteine amide* **13**.—Synthesis, cleavage of the resin-bound glycopeptide (100 mg, 11.5 μmol), and purification by HPLC (25 % *B* in *A*) according to the general procedure, gave **13** (12.5 mg, 73% peptide content, 36% overall yield). ^{1}H NMR, FABMS, and amino acid analysis data for **13** have been reported previously.[24]

N-*Acetyl-glycyl*-L-*arginyl*-L-*alanyl*-L-*phenylalanyl*-L-*valyl*-O-*(2-acetamido-2-deoxy-α-D-galactopyranosyl)*-L-*threonyl*-L-*isoleucyl-glycyl*-L-*lysyl*-L-*isoleucyl-glycyl*-L-*asparaginyl*-L-*methionyl*-L-*arginyl*-L-*glutaminyl*-L-*alanyl*-L-*cysteine amide* **14**.—Synthesis, deacetylation of the resin-bound glycopeptide (200 mg, 22 μmol), followed by cleavage and purification by HPLC (21 % *B* in *A*) according to the general procedure, gave **14** (30 mg, 69% peptide content, 45% overall yield). For ^{1}H NMR data, see Table 2. FABMS *m/z* 2066 (M + H) [Calc. *m/z* 2066 (M + H)]. Amino acid analysis: Ala 2.01 (2), Arg 2.00 (2), Asp 1.04 (1), Cys 0.99 (1), Glu 1.02 (1), Gly 2.97 (3), Ile 1.95 (2), Lys 1.04 (1), Met 0.98 (1), Phe 1.02 (1), Thr 1.00 (1), Val 0.99 (1).

Table 2. ^{1}H NMR chemical shifts (δ, ppm) for compound **14** in water containing 10% D_2O[a]

Residue	NH	Hα	Hβ	Hγ	Others
Gly[1]	8.38	3.86[b]			1.97 (Ac)
Arg[2]	8.35	4.24	1.64, 1.72	1.52[b]	3.11[b] (Hδ,δ'), 7.16 (δ-NH)
Ala[3]	8.38	4.20	1.23		
Phe[4]	8.25	4.57	2.95, 3.05		7.17 and 7.27 (H-arom)
Val[5]	8.16	4.19	1.94	0.86	
Thr[6]	8.71	4.52	4.21	1.21	GalNAcα[c]
Ile[7]	8.39	4.09	1.76	1.16, 1.44	0.78 (γ-Me), 0.90 (β-Me)
Gly[8]	8.52	3.79, 3.96			
Lys[9]	8.41	4.25	1.62, 1.75	1.39[b]	1.62[b] (Hδ,δ'), 2.93[b] (Hε,ε')
Ile[10]	8.36	4.11	1.81	1.16, 1.44	0.81 (γ-Me), 0.86 (β-Me)
Gly[11]	8.59	3.88[b]			
Asn[12]	8.40	4.64	2.70, 2.77		6.95 and 7.66 (β-$CONH_2$)
Met[13]	8.46	4.40	1.97, 2.04	2.47, 2.56	2.04 (Me)
Arg[14]	8.42	4.23	1.72[b]	1.56[b]	3.14[b] (Hδ,δ'), 7.19 (δ-NH)
Gln[15]	8.58	4.23	1.92, 2.04	2.32[b]	6.92 and 7.60 (γ-$CONH_2$)
Ala[16]	8.56	4.25	1.35		
Cys[17]	8.43	4.41	2.87[b]		7.28 and 7.66 (α-$CONH_2$)

[a]Obtained at 500.14 MHz, 278 K, and pH 5.4 with H_2O (δ_H 4.98 ppm) as internal standard. [b]Degeneracy has been assumed. [c]Chemical shifts (δ, ppm) for the *N*-acetylgalactosamine moiety in **14**: 4.71 (H-1), 4.02 (H-2), 3.80 (H-3), 3.89 (H-4), 7.83 (NH), 1.96 (Ac).

N-*Acetyl*-L-*cysteinyl*-L-*lysyl*-L-*isoleucyl*-L-*glutam-1-yl*-L-*prolyl*-L-*leucyl-glycyl*-L-*valyl*-L-*alanyl*-L-*prolyl*-O-*(2-acetamido-2-deoxy-α-D-galactopyranosyl)*-L-*threonyl*-L-*lysyl*-L-*alanyl*-L-*lysyl*-L-*arginyl*-L-*arginine amide* **16**.—Synthesis, deacetylation of the resin-bound glycopeptide (250 mg, 26 μmol), followed by cleavage and purification by HPLC (18 % *B* in *A*) according to the general procedure, gave **16** (13 mg, 59% peptide content, 15% overall yield). For ^{1}H NMR data, see Table 3. FABMS *m/z* 2010 (M + H) [Calc. *m/z* 2011 (M + H)]. Amino acid analysis: Ala 1.97 (2), Arg 2.02 (2), Cys 1.02 (1), Glu 0.97 (1), Gly 1.01 (1), Ile 1.01 (1), Leu 1.02 (1), Lys 2.94 (3), Pro 2.02 (2), Thr 0.99 (1), Val 1.02 (1).

L-*Leucyl*-O-*(2-acetamido-2-deoxy-α*-D-*galactopyranosyl)*-L-*seryl*-L-*glutam-1-yl*-O-*(2-acetamido-2-deoxy-α*-D-*galactopyranosyl)*-L-*seryl*-O-*(2-acetamido-2-deoxy-α*-D-*galactopyranosyl)*-L-*threonyl*-O-*(2-acetamido-2-deoxy-α*-D-*galactopyranosyl)*-L-*threonyl*-L-*glutaminyl*-L-*leucyl*-L-*prolyl-glycine* **18**.—Synthesis, cleavage of the resin-bound glycopeptide (250 mg, 36 μmol), and purification by HPLC (29 % *B* in *A*) according to the general procedure, gave the *O*-acetylated **17** (13.6 mg). Deacetylation and purification by HPLC (9 % *B* in *A*) gave **18** (4.3 mg, 70% peptide content, 4.5% overall yield). For ^{1}H NMR data, see Table 4. FABMS *m/z* 1844 (M + H) [Calc. *m/z* 1845 (M + H)]. Amino acid analysis: Glu 1.93 (2), Gly 1.02 (1), Leu 1.94 (2), Pro 1.03 (1), Ser 1.96 (2), Thr 2.11 (2).

Table 3. ^{1}H NMR chemical shifts (δ, ppm) for compound **16** in water containing 10% D_2O[a]

Residue	NH	Hα	Hβ	Hγ	Others
Cys^1	8.41	4.36	2.82[b]		1.97 (Ac)
Lys^2	8.59	4.28	1.69[b]	1.35[b]	1.61[b] (Hδ,δ'), 2.92[b] (Hε,ε')
Ile^3	8.37	4.10	1.75	1.11, 1.41	0.78 (γ-Me), 0.81 (β-Me)
Glu^4	8.64	4.55	1.80[b]	2.23[b]	
Pro^5	-	4.34	1.85, 2.24	1.96[b]	3.68 and 3.77 (Hδ,δ')
Leu^6	8.48	4.26	1.52[b]	1.63	0.83 and 0.88 (γ,γ'-Me)
Gly^7	8.50	3.84, 3.91			
Val^8	8.03	4.06	1.98	0.86[b]	
Ala^9	8.52	4.53	1.31		
Pro^{10}	-	4.56	1.88, 2.29	1.99[b]	3.62 and 3.80 (Hδ,δ')
Thr^{11}	8.80	4.45	4.23	1.25	GalNAcα[c]
Lys^{12}	8.54	4.17	1.69[b]	1.40[b]	1.63[b] (Hδ,δ'), 2.95[b] (Hε,ε')
Ala^{13}	8.48	4.09	1.31		
Lys^{14}	8.54	-[d]	1.69[b]	1.40[b]	1.63[b] (Hδ,δ'), 2.95[b] (Hε,ε')
Arg^{15}	8.55	4.24	1.72[b]	1.58[b]	3.14[b] (Hδ,δ'), 7.20 (δ-NH)
Arg^{16}	8.61	4.22	1.71[b]	1.60[b]	3.15[b] (Hδ,δ'), 7.21 (δ-NH), 7.19 and 7.75 (α-$CONH_2$)

[a]Obtained at 500.14 MHz, 278 K, and pH 5.8 with H_2O (δ_H 4.98 ppm) as internal standard. [b]Degeneracy has been assumed. [c]Chemical shifts (δ, ppm) for the *N*-acetylgalactosamine moiety in **16**: 4.77 (H-1), 3.98 (H-2), 3.82 (H-3), 3.92 (H-4), 7.76 (NH), 1.98 (Ac). [d]Could not be assigned due to spectral overlap.

Table 4. ^{1}H NMR chemical shifts (δ, ppm) for compound **18** in water containing 10% D_2O[a]

Residue	NH	Hα	Hβ	Hγ	Others
Leu^1	-[b]	3.93	1.63[c]	1.63	0.87 (2 γ-Me)
Ser^2	-[b]	4.57	3.73, 3.88		
Glu^3	8.76	4.33	1.87, 1.95	2.24, 2.27	
Ser^4	8.88	4.68	3.77, 4.00		
Thr^5	8.86	4.70	4.34	1.22	
Thr^6	8.96	4.51	4.19	1.14	
Gln^7	8.53	4.24	1.82, 2.00	2.29[c]	6.93 and 7.76 (γ-$CONH_2$)
Leu^8	8.51	4.37	1.51[c]	1.62	0.91 (2 γ-Me)
Pro^9	-	4.34	2.24[c]	1.82, 2.00	3.58, 3.89 (Hδ,δ')
Gly^{10}	8.07	3.63, 3.73			

	N*H*Ac	H-1	H-2	H-3	H-4,5,6,6'[c]
GalNAcα-Ser^2	8.08	4.82	4.08	3.78	3.81, 3.86, 4.10
GalNAcα-Ser^4	8.01	4.76	4.06	3.81	3.83, 4.03, 4.08
GalNAcα-Thr^5	7.62	4.70	3.95	3.79	3.81, 3.93, 3.97
GalNAcα-Thr^6	7.63	4.80	4.01	-[d]	3.88, 3.99, 4.03

[a]Obtained at 500.14 MHz, 278 K, and pH 5.8 with H_2O (δ_H 4.98 ppm) as internal standard. [b]Not observed. [c]Degeneracy has been assumed. [d]Could not be assigned due to spectral overlap.

L-*Prolyl*-O-*(2-acetamido-2-deoxy-α*-D-*galactopyranosyl)*-L-*threonyl*-O-*(2-acetamido-2-deoxy-α*-D-*galactopyranosyl)*-L-*threonyl*-O-*(2-acetamido-2-deoxy-α*-D-*galactopyranosyl)*-L-*threonyl*-L-*prolyl*-L-*isoleucyl*-O-*(2-acetamido-2-deoxy-α*-D-*galactopyranosyl)*-L-*seryl*-O-*(2-acetamido-2-deoxy-α*-D-*galactopyranosyl)*-L-*threonyl*-O-*(2-acetamido-2-deoxy-α*-D-*galactopyranosyl)*-L-*threonyl*-L-*methionine* **20.**— Synthesis, cleavage of the resin-bound glycopeptide (370 mg, 51 μmol), and purification by HPLC (36.5 % *B* in *A*) according to the general procedure, gave the *O*-acetylated **19** (16.4 mg). Deacetylation and purification by HPLC (8 % *B* in *A*) gave **20** (8.6 mg, 69% peptide content, 5.1% overall yield). For ^{1}H NMR data, see Table 5. FABMS *m/z* 2267 (M + H) [Calc. *m/z* 2268 (M + H)]. Amino acid analysis: Ile 1.01 (1), Met 0.96 (1), Pro 2.14 (2), Ser 1.07 (1), Thr 4.83 (5).

Table 5. ^{1}H NMR chemical shifts (δ, ppm) for compound **20** in water containing 10% D_2O[a]

Residue	NH	Hα	Hβ	Hγ	Others
Pro[1]	-	4.53	2.07, 2.47	1.98[b]	3.34, 3.39 (Hδ,δ')
Thr[2]	9.08	4.68	4.42	1.27	
Thr[3]	9.06	4.73	4.32	1.19	
Thr[4]	8.90	4.62	4.24	1.20	
Pro[5]	-	4.44	1.85, 2.24	1.97[b]	3.62[b] (Hδ,δ')
Ile[6]	8.21	3.90	1.72	1.17, 1.59	0.87 (γ-Me), 0.89 (β-Me)
Ser[7]	8.74	4.70	3.78, 3.97		
Thr[8]	8.81	4.71	4.32	1.25	
Thr[9]	8.86	4.55	4.25	1.15	
Met[10]	8.16	4.23	1.85, 1.99	2.40, 2.48	-[c]

	N*H*Ac	H-1	H-2	H-3,4,5,6,6'[c]	Ac
GalNAcα-Thr[2]	7.68	4.69	3.95	3.80, 3.83, 3.88, 3.96, 3.98	2.00
GalNAcα-Thr[3]	7.92	4.75	3.95	3.79, 3.82, 3.88, 3.97, 3.99	1.97
GalNAcα-Thr[4]	7.86	4.71	4.01	3.81, 3.83, 3.91, 3.99, 4.03	1.97
GalNAcα-Ser[7]	8.09	4.81	4.08	3.82, 3.85, 3.90, 4.01, 4.06	1.99
GalNAcα-Thr[8]	7.48	4.71	4.02	3.82, 3.85, 3.89, 4.01, 4.05	1.99
GalNAcα-Thr[9]	7.77	-[c]	4.06	3.83, 3.85, 3.90, 4.04, 4.09	2.01

[a]Obtained at 500.14 MHz, 278 K, and pH 5.8 with H_2O (δ_H 4.98 ppm) as internal standard. [b]Degeneracy has been assumed. [c]Could not be assigned due to spectral overlap.

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