Comparison of *N*-Dts and *N*-Aloc in the solid-phase syntheses of *O*-GlcNAc glycopeptide fragments of RNA-polymerase II and mammalian neurofilaments

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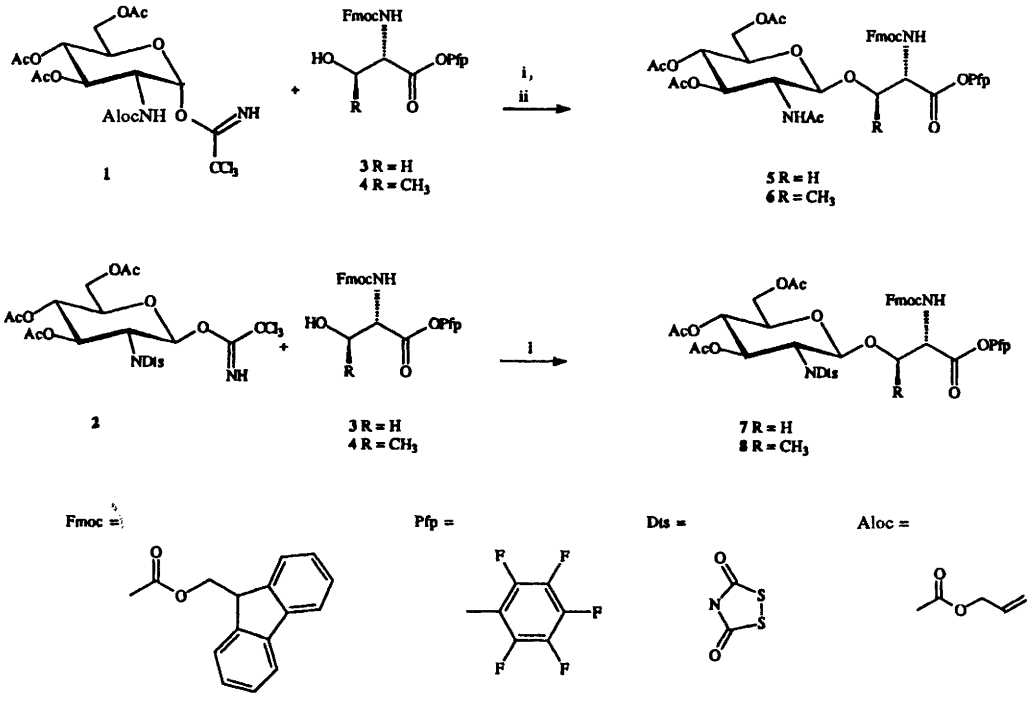
The application of the glycosylated building blocks N^{α}-Fmoc-Ser(Ac_3-β-D-GlcNAc)-OPfp **5**, N^{α}-Fmoc-Thr(Ac_3-β-D-GlcNAc)-OPfp **6**, N^{α}-Fmoc-Ser(Ac_3-β-D-GlcNDts)-OPfp **7** and N^{α}-Fmoc-Thr(Ac_3-β-D-GlcNDts)-OPfp **8** in the solid-phase synthesis of *O*-GlcNAc glycopeptides from the C-terminal domain repeating unit of RNA-polymerase II and the NF-L chain of mammalian neurofilaments is described. The removal of the *N*-dithiasuccinoyl (*N*-Dts) amino-protecting group was achieved rapidly and quantitatively by thiolysis with 2-sulfanylethanol or dithiothreitol after the incorporation of the building blocks into the resin-bound peptide. The *O*-GlcNAc glycopeptides **9–11**, **13** and **15–21** were synthesised in good yields by comparative studies employing any of the building blocks **5–8**. The *O*-GlcNAc glycopeptides were fully characterised by 1D- and 2D-^{1}H NMR spectroscopy and ES-MS.

Introduction

2-Acetamido-2-deoxy-β-D-glucopyranose (β-D-GlcNAc) *O*-glycosidically linked to serine or threonine has been reported as a novel type of post-translational glycosylation.[1,2] Unlike other forms of post-translational glycosylation the *O*-GlcNAc moiety occurs as a simple unmodified monosaccharide O-linked to serine or threonine and often occurring at clustered multiple sites in the same protein. The O-linked GlcNAc residues are found predominantly on intracellular proteins within the nucleoplasmic and cytoplasmic compartments of the cell.[3,4] Among others, *O*-GlcNAc occurs on nuclear pore proteins, viral proteins, chromatin proteins, transcriptions factors and cytoskeletal proteins. The functions of *O*-GlcNAc still remain enigmatic but there is evidence that it may play a vital role in gene transcription regulation and nucleocytoplasmic transport. Furthermore, the *O*-GlcNAc glycosylation and deglycosylation appears to be highly dynamic and responsive to cellular stimuli in a fashion analogous to phosphorylation. Post-translational *O*-GlcNAc modification may be crucial in blocking site-specific phosphorylation.[5–7] Studies suggested a reciprocal, a so-called 'yen-yang', relationship between *O*-GlcNAc modification and phosphorylation.[8] Furthermore the *O*-GlcNAc residue may provide a binding ligand for intracellular lectins. Recent studies have shown the presence of *O*-GlcNAc-specific lectins in the nucleus.[9] The addition of *O*-GlcNAc may alter the protein conformation, resulting in activation or inactivation of the corresponding enzyme or in the formation of multimeric complexes. So far only a few *O*-GlcNAc glycopeptides have been characterised in order to identify the nature of the glycosylation sites. A better understanding of the vital role of *O*-GlcNAc requires accurate structural and functional studies, including determination of *O*-GlcNAc site occupancy, sequencing of glycosylated peptides and confirming whether the *O*-GlcNAc moiety may be modified in any way. For all these investigations the chemical synthesis of a wide range of *O*-GlcNAc glycopeptides is therefore an essential and versatile tool.

In this paper the synthesis of *O*-GlcNAc glycopeptide fragments from the carboxy-terminal domain (CTD) repeating unit of RNA-polymerase II and the light polypeptide chain of mammalian neurofilament (NF-L, 61 kDa) is described. Eukaryotic RNA polymerase II is part of a complex multisubunit enzyme system which is responsible for the regulated synthesis of messenger RNA. RNA polymerase II contains two structural domains: a catalytic core region and a unique extension ('tail') of the CTD consisting of 52 tandem repeats of the consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser.[10] This repetitive CTD is essential for the polymerase function *in vivo*. The evolutionary conservation of the CTD in eukaryotic RNA polymerase II suggests that it plays a fundamental role in the transcription process. Deletions that remove all or most of the repeats in yeast or the mouse CTD are lethal.[11] During the active transcription process RNA polymerase II is known to be highly phosphorylated on this repetitive domain. It has also been shown that the CTD is modified by *O*-GlcNAc glycosylation.[5] The glycosylation occurs at multiple sites throughout the CTD, similar to the phosphorylation of this domain. The *O*-GlcNAc glycosylation, however, is not found on the phosphorylated form of the enzyme. The fact that only the unphosphorylated form of RNA polymerase II contains *O*-GlcNAc suggests that phosphorylation and glycosylation are separate events and that the CTD of RNA polymerase II appears to exist in three distinct conformational states: unmodified, phosphorylated or glycosylated with *O*-GlcNAc. The differential modification may play a role in the regulation of the expression of genes transcribed by RNA polymerase II.[5] The predominant site for the *O*-GlcNAc attachment of CTD *in vivo* appears to be the fourth position (Thr) of the repeat.[5] There seemed to be direct evidence of multiple glycosylation along the CTD. The synthesis of the non-glycosylated heptapeptide **12** and the glycopeptide **13**, which is *O*-GlcNAc-modified on the fourth position (Thr) of the repeating unit, is described in this paper in order to allow a study of the influence of the sugar moiety on biological function and structure.

Virtually all *O*-GlcNAc-modified peptides form reversible multimeric complexes with other proteins. Another good example are neurofilaments (NF), the most abundant cytoskeletal elements in neuronal cells. The neurofilaments are composed of three subunits, NF-L, NF-M and NF-H. There is evidence that NFs play a critical role in the growth and maintenance of the calibre of large myelinated axons.[12]



Scheme 1 Syntheses of the building blocks **5**–**8** employed in the multi-column solid-phase *O*-GlcNAc glycopeptide synthesis. *Reagents and conditions*: i, AgOTf, 3 Å molecular sieves, CH_2Cl_2; ii, $Pd(PPh_3)_4$, Bu_3SnH, Ac_2O, CH_2Cl_2.

Abnormalities in NF distribution are also implicated in some neuropathogenic processes such as motor neuron disease.[13] G. W. Hart and co-workers have recently demonstrated that neurofilaments are modified by at least three *O*-GlcNAc attachment sites on NF-L.[14] They might be involved in a lectin-type interaction among neurofilaments. Deletion of *O*-GlcNAc sites causes abnormal filament formation. Although the function and significance of *O*-GlcNAc glycosylation remains to be established, specific identification of *O*-GlcNAc attachment sites would give more detailed information about the function of *O*-GlcNAc on neurofilaments. To understand the enigmatic role of the *O*-GlcNAc modification on neurofilaments determination of occupancy sites on the peptide is essential. The precise location of the glycosylation sites may be determined by comparison of glycosylated peptides obtained by sequential cyanogen bromide or trypsin digestion of natural *O*-GlcNAc-containing glycopeptides with synthetic *O*-GlcNAc glycopeptides. The synthetic glycopeptides may influence filament formation.

One of the currently most versatile approaches to the multiple syntheses of *O*-glycopeptides is the active ester method employing appropriately protected glycosylated Fmoc-amino acid esters as building blocks.[15] However, this approach requires the direct glycosylation of N^{α}-Fmoc-amino acid OPfp esters. In the preparation of the desired building blocks N^{α}-Fmoc-Ser(Ac_3-β-D-GlcNAc)-OPfp and N^{α}-Fmoc-Thr(Ac_3-β-D-GlcNAc)-OPfp particular problems are observed depending on the kind of the 2-amino protecting group used in the corresponding glycosyl donor. Synthesis employing the *N*-acetyl group generally requires multistep procedures including reprotection schemes and activation of the carboxy group after glycosylation of the amino acid. Nevertheless, it has been simplified by performing direct glycosylation of the N^{α}-Fmoc-Ser and N^{α}-Fmoc-Thr amino acids.[16] In two previous papers we have described alternative strategies which simplify the direct glycosylation of the active esters derivatives N^{α}-Fmoc-Ser-OPfp **3** and N^{α}-Fmoc-Thr-OPfp **4** with the imidate **1**[17] or **2**[18] (Scheme 1). In the first approach N^{α}-Fmoc-Ser(Ac_3-β-D-GlcNAc)-OPfp **5** and N^{α}-Fmoc-Thr(Ac_3-β-D-GlcNAc)-OPfp **6** have been synthesised by stereoselective glycosylation with the 2-allyloxycarbonylamino glycosyl donor **1** of N^{α}-Fmoc-Ser-OPfp **3** and N^{α}-Fmoc-Thr-OPfp **4**, followed by Pd^0-catalysed allyl transfer and cleavage of the *N*-allyloxycarbonyl (Aloc) group in the presence of acetic anhydride to yield the *N*-acetamido group.[17] In an alternative approach the dithiasuccinimido (Dts) group was applied as an amino-protecting group in the syntheses of the β-linked building blocks N^{α}-Fmoc-Ser(Ac_3-β-D-GlcNDts)-OPfp **7** and N^{α}-Fmoc-Thr(Ac_3-β-D-GlcNDts)-OPfp **8**.[18] The *O*-(3,4,6-tri-*O*-acetyl-2-deoxy-2-dithiasuccinimido-β-D-glucopyranosyl) trichloroacetimidate **2** was successfully employed in the glycosylation of N^{α}-Fmoc-Ser-OPfp **3** and N^{α}-Fmoc-Thr-OPfp **4** in the presence of silver trifluoromethanesulfonate (AgOTf) as a promoter. The N-Dts protecting group was found to be compatible with conditions of the Fmoc/Pfp[18] strategy for the synthesis of glycopeptides and was conceived to be removable rapidly under mild conditions by thiolysis by employing 2-sulfanylethanol or dithiothreitol (DTT) during solid-phase glycopeptide synthesis.[19] In the present work the efficient application of the four building blocks in the syntheses of a wide range of *O*-GlcNAc glycopeptides is described.

Results and discussion

The versatility of the four building blocks **5**–**8**[17,18] in the synthesis of different *O*-GlcNAc-bearing glycopeptides was investigated. The protecting-group pattern of the four building blocks was designed for the solid-phase syntheses of *O*-GlcNAc glycopeptides. The use of the Fmoc group allows deprotection of the α-amino group under mild conditions with piperidine or morpholine to occur without β-elimination of the carbohydrate.[20] O-Acetyl groups were employed for protection of the carbohydrate hydroxy groups, allowing deprotection with a catalytic amount of sodium methoxide in methanol. The carboxy group is highly activated as the pentafluorophenyl ester for aminolysis and formation of peptide bond.[21] The

reactivity of the Pfp ester was further enhanced by addition of 3-hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine (Dhbt-OH). The use of Dhbt-OH as an auxiliary nucleophile allows the progress of the formation of peptide to be followed visually.[22] In the case of utilisation of the building blocks **7** and **8** the *N*-Dts groups could be removed during the solid-phase synthesis by thiolysis with 2-sulfanylethanol or DTT.

9 Ac-Val-Ser*-Gly-NH_2
10 Ac-Val-Thr*-Gly-NH_2
11 Ac-Thr-Ser-Pro-Thr*-Ser-Pro-Ser-NH_2
12 Ac-Tyr-Ser-Pro-Thr-Ser-Pro-Ser-NH_2
13 Ac-Tyr-Ser-Pro-Thr*-Ser-Pro-Ser-NH_2
14 Ac-Tyr-Ser-Ala-Pro-Val-Ser-Ser-Ser-Leu-Ser-Val-NH_2
15 Ac-Tyr-Ser-Ala-Pro-Val-Ser-Ser-Ser*-Leu-Ser-Val-NH_2
16 Ac-Tyr-Ser-Ala-Pro-Val-Ser-Ser*-Ser-Leu-Ser-Val-NH_2
17 Ac-Tyr-Ser-Ala-Pro-Val-Ser*-Ser-Ser-Leu-Ser-Val-NH_2
18 Ac-Tyr-Ser-Ala-Pro-Val-Ser-Ser*-Ser*-Leu-Ser-Val-NH_2
19 Ac-Tyr-Ser-Ala-Pro-Val-Ser*-Ser-Ser*-Leu-Ser-Val-NH_2
20 Ac-Tyr-Ser-Ala-Pro-Val-Ser*-Ser*-Ser-Leu-Ser-Val-NH_2
21 Ac-Tyr-Ser-Ala-Pro-Val-Ser*-Ser*-Ser*-Leu-Ser-Val-NH_2

* GlcNAc

O-GlcNAc glycopeptides **9**–**11**, **13** and **15**–**21** synthesised by the syringe or the multi-column technique.

In order to investigate the applicability of the Dts-concept, the *N*-Dts-protected glycosylated building blocks **7** and **8**, obtained exclusively as the β-anomers,[18] were used in a solid-phase synthesis of the three model glycopeptides **9**, **10** and **11**. In a series of comparative glycopeptide syntheses, which were carried out manually in a 20-column Teflon block on PEGA resin[23] modified with the acid-labile Rink-amide-linker,[24] different conditions for the thiolytic removal of the Dts group with DTT or 2-sulfanylethanol were investigated. Throughout the syntheses the removal of N^{α}-Fmoc was accomplished with piperidine in dimethylformamide (DMF). Couplings of all N^{α}-Fmoc-amino acids were achieved using their Pfp esters in the presence of Dhbt-OH in DMF. After incorporation of the glycosylated serine and threonine building blocks **7** and **8**, the *N*-Dts protecting group was cleaved either by thiolysis with 2-sulfanylethanol–triethylamine or alternatively with DTT–diisopropylethylamine (DIPEA) in dichloromethane. N-Acetylation was then effected by treatment of the resin-bound peptide with acetic anhydride in DMF. After coupling of the last amino acid the N^{α}-Fmoc group was removed, followed by N-acetylation, cleavage of the glycopeptide from the resin with aq. trifluoroacetic acid (TFA) and finally removal of the O-acetyl groups with a catalyic amount of sodium methoxide in methanol. The model glycopeptides **9**–**11** were then purified by preparative HPLC. The results of utilising the different building blocks and *N*-Dts deprotection conditions were compared and are shown in Table 1. The compared results showed that rapid and quantitative deprotection of the *N*-Dts amino protective group could be achieved during the solid-phase synthesis both by thiolysis with 2-sulfanylethanol and with DTT. On account of the higher yields of the obtained glycopeptides **9**–**11** (Table 1) the Dts deblocking procedure employing the DTT–DIPEA thiolysis procedure was preferable and consequently was used in the following glycopeptide syntheses.

For more complicated synthetic studies the building blocks **5**–**8** were then used in multiple solid-phase syntheses of naturally occurring *O*-GlcNAc glycopeptides. The glycopeptide **13** represents the repeating heptapeptide unit in the CTD of eukaryotic RNA polymerase II,[10] and compounds **14**–**21** correspond to various glycosylated peptide sequences 43–53 of the head domain of NF-L of mammalian neurofilaments.[14]

For the synthesis of the glycopeptide **13** the *N*-Dts-containing building block **8** was used. The syntheses of the peptide **12** and the glycopeptide **13** were carried out manually by use of a plastic syringe technique.[23] As a key step the deprotection of the Dts amino-protecting group was performed by thiolysis with DTT–DIPEA in dichloromethane. After subsequent N-acetylation with acetic anhydride in DMF the peptide synthesis was continued. It should be mentioned that the N^{α}-Fmoc amino-protecting group cannot be removed with piperidine or morpholine in the presence of the *N*-Dts group because of the instability of Dts towards nucleophiles and bases. The *N*-Dts has to be removed before continuation of the peptide synthesis on the solid support. After coupling of the last amino acid (Tyr), Fmoc cleavage, N^{α}-acetylation, cleavage from the resin with 95% TFA, O-deacetylation and preparative HPLC purification the glycopeptide **13** could be obtained in 75% overall yield. The purity of the protected and of the deprotected glycopeptide **13** was excellent as indicated by analytical HPLC (Fig. 2; see later). Compounds **12** and **13** were fully characterised by ES-MS and 1D- and 2D-^{1}H NMR spectroscopy (Tables 4 and 5; see later). The chemical shifts of the anomeric protons vary significantly in comparison to the other carbohydrate protons. Connectivities of the α, β, γ, δ and ε protons were accessed by ^{1}H–^{1}H 2D homonuclear chemical shift correlation (COSY) and ^{1}H–^{13}C correlation experiments. The glycosylated threonine in compound **13** could be distinguished from Thr in the peptide **12** by a significant shift of the α and β protons of threonine, which were shifted 0.168 ppm and 0.094 ppm downfield, respectively (see Table 4). The α and β carbons were shifted 1.47 ppm upfield and 7.72 ppm downfield, respectively (see Table 5).

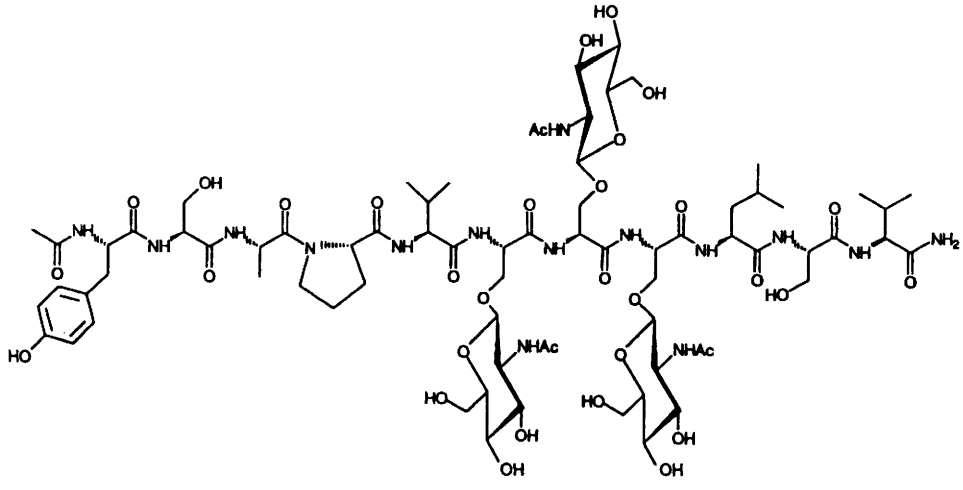
To demonstrate further the use of the Dts-concept in multiple glycopeptide synthesis the seven *O*-GlcNAc glycopeptides **15**–**21** from neurofilaments (NF) in neuronal cells were synthesised. The multiple glycosylated glycopeptides **15**–**21** correspond to residue 43–53 of the parent NF-L undecapeptide **14** of mammalian neurofilaments. Based on the undecapeptide **14** a series of seven *O*-GlcNAc glycosylated and terminally N-acetylated peptide carboxamides were synthesised (compounds **15**–**21**). In the natural protein it is not known which of the three serine residues in the triad are glycosylated. All three serines are potential, yet not confirmed glycosylation sites. Manual Edman degradation failed to identify the sites of *O*-GlcNAc attachment due to low repetitive yield of serine residues.[14] This may be an indication that one or more Ser residues on the N-terminus of Leu are likely to be glycosylated and this raises the question of which serine residues are modified. So we decided to shift the *O*-GlcNAc glycosylation between the three successive serine residues in compound **14** to get all possible glycosylation combinations for comparative studies.

The synthesis of the *O*-GlcNAc glycopeptides **15**–**21** was carried out utilising both building block N^{α}-Fmoc-Ser(Ac_3-β-D-GlcNAc)-OPfp **5** and the corresponding building block N^{α}-Fmoc-Ser(Ac_3-β-D-GlcNDts)-OPfp **7** in two Teflon synthesis blocks each equipped with 20 columns for comparative studies. Each column was equipped with a Teflon filter and a bottom outlet for removal of reagents by suction through a vacuum and pressure chamber. The columns were each packed with 80 mg of resin. All the glycopeptides were synthesised in two neighbouring wells. The resins were washed thoroughly after removal of Fmoc and after each acylation step. Fig. 1 outlines the principle of the parallel solid-phase glycopeptide synthesis performed on the PEGA resin. The acid-labile linker 4-(α-amino-2,4-dimethoxybenzyl)phenoxyacetic acid (Rink amide-linker) was selected. The Fmoc-protected Rink linker was coupled to the resin by activation with *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) and 4-ethylmorpholine (NEM). Unchanged amino groups were capped with acetic anhydride. All Fmoc cleavages were effected with 20% piperidine in DMF. After removal of the Fmoc group the first amino acid, valine, was coupled to the linker as Pfp

Table 1 Results obtained by use of the building blocks **5–8** and the different *N*-Dts deprotection conditions in the syntheses of glycopeptides **9–11**, **13** and **15–21**. Comparison of expected and observed molecular mass for the individual glycopeptides analysed by ES-MS

Compound	Molecular formula	Relative molecular mass	ES-MS[a,b]	Yields (%) obtained using building block (after preparative reversed-phase HPLC)			
				5	**6**	**7**	**8**
9	$C_{20}H_{35}N_5O_{10}$	505.529	528.34[a]	78		51[c]/68[d]	
10	$C_{21}H_{37}N_5O_{10}$	519.556	542.35[a]		91		66[c]/77[d]
11	$C_{37}H_{61}N_9O_{18}$	919.948	943.55[a]		95		71[c]/81[d]
13	$C_{42}H_{63}N_9O_{18}$	982.019	1004.52[a]				75[d]
15	$C_{58}H_{93}N_{13}O_{23}$	1340.461	1341.10[b]	57		46[d]	
16	$C_{58}H_{93}N_{13}O_{23}$	1340.461	1341.11[b]	61			
17	$C_{58}H_{93}N_{13}O_{23}$	1340.461	1341.14[b]	51			
18	$C_{66}H_{106}N_{14}O_{28}$	1542.658	1565.30[a]	75		68[d]	
19	$C_{66}H_{106}N_{14}O_{28}$	1542.658	1565.31[a]	25			
20	$C_{66}H_{106}N_{14}O_{28}$	1542.658	1565.34[a]	61			
21	$C_{74}H_{119}N_{15}O_{33}$	1746.854	1769.65[a]	34		28[d]	

[a] Glycopeptides were detected as $[glycopeptideNa]^+$ ions. [b] Glycopeptides were detected as $[glycopeptideH]^+$ ions. [c] Thiolysis conditions: 2-sulfanylethanol (0.2 mol dm^{-3})–triethylamine (0.5 mol dm^{-3}) in dichloromethane. [d] Thiolysis conditions: DTT (0.2 mol dm^{-3})–DIPEA (0.1 mmol dm^{-3}) in dichloromethane.



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ester. The following Fmoc amino acids were coupled as Pfp esters. All the Pfp esters including the glycosylated serine building blocks **5** and **7** were used with the addition of Dhbt-OH as auxiliary nucleophile. In the case of use of the Dts-protected building block **7** the removal of the Dts group was achieved by thiolysis with DTT–DIPEA in dichloromethane and subsequent N-acetylation with acetic anhydride in DMF. The progress of the peptide-coupling reactions could be followed by the gradual disappearance of the bright yellow colour. The side chain of non-glycosylated serine residue was protected as its Bu^t ether. After coupling of the last amino acid the Fmoc group was cleaved with piperidine in DMF and the N-termini were acetylated with acetic anhydride in DMF. Cleavage of the glycopeptides from the resin was performed with 95% TFA with concurrent removal of the Bu^t-protecting groups. Finally, the O-acetyl groups of the *O*-GlcNAc moieties were removed with catalytic amounts of sodium methoxide in methanol at pH = 9 (measured on dry pH-paper). All deacetylations were finished after 4 h even in the case of the multiple glycosylated peptides **18–21**. The glycopeptides were purified by preparative reversed-phase HPLC. After lyophilisation the pure *O*-GlcNAc glycopeptides **15–21** were obtained in yields from 25–75% based on the loading of the resin (Table 1). The syntheses of the glycopeptides **15–21** were achieved without any assembly problems even for the construction of the more crowded sequences as, for instance, in structures **18**, **20** and **21**.

The glycopeptides **15–21** were fully characterised by ES-MS and 1D- and 2D-^{1}H NMR spectroscopy (Tables 1, 6 and 7, see later). The chemical shifts of the anomeric protons vary significantly in comparison to the other carbohydrate protons. The connectivities between the α, β, γ, δ and ε protons were in all cases accessed by ^{1}H–^{1}H-COSY and ^{1}H–^{13}C correlation experiments. The glycosylated serines could be distinguished from the non-glycosylated serines in structures **15–21** by significant shift differences for the β-carbon atom of serine, which were shifted approximately 5.6–5.8 ppm downfield upon glycosylation (Table 7). In the ^{1}H NMR spectra significant shifts of the α and the β protons of the glycosylated serine residues could be determined. The shift differences for the α protons were approximately 0.127 ppm and for the β protons 0.170 ppm downfield due to glycosylation of serine (Table 6). These shift differences are most likely due to substitution effects rather than to conformational effects.

Conclusions

In conclusion, our studies demonstrated that any of the building blocks **5–8** are well suited for the multiple-column solid-phase syntheses of β-*O*-GlcNAc glycopeptides. The *N*-

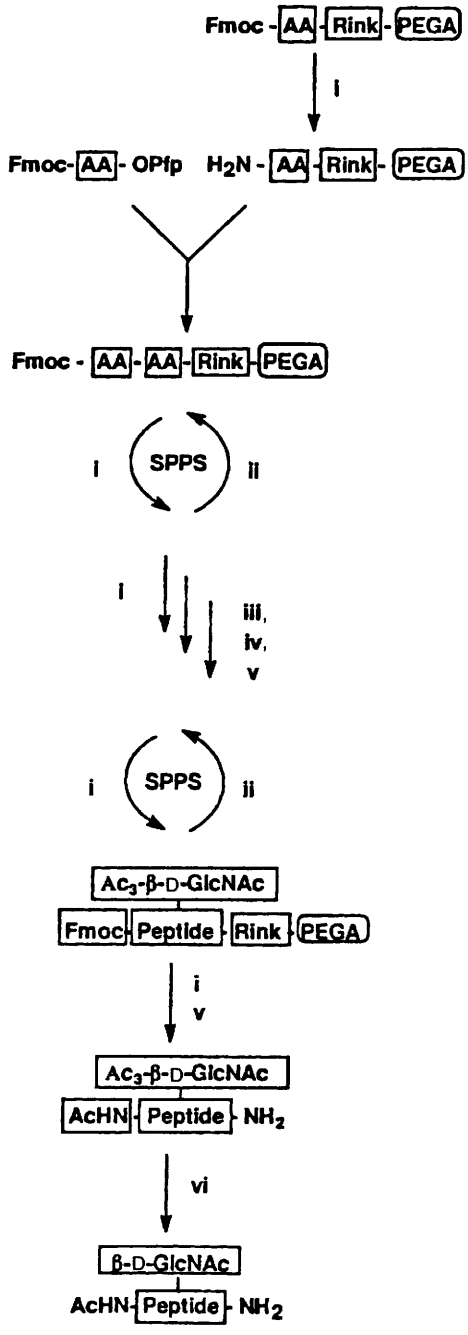

Fig. 1 Principle of the *O*-GlcNAc glycopeptide syntheses using building blocks **7** and **8**. *Reagents*: i, 20% piperidine in DMF; ii, Fmoc-AA-OPfp (3 mol equiv.), Dhbt-OH (1 mol equiv.); iii, **7** or **8** (2 mol equiv.), Dhbt-OH (1 mol equiv.); iv, 2-sulfanylethanol (0.2 mol dm^{-3}), triethylamine (0.5 mol dm^{-3}) or DTT (0.2 mol dm^{-3}), DIPEA (0.1 mmol dm^{-3}) in CH_2Cl_2; v, 15% Ac_2O in DMF; vi, 95% aq. TFA; vii, 1% NaOMe in MeOH (pH = 9).

Dts amino-protecting group possesses favourable properties, leading exclusively to the β-linked building blocks **7** and **8** and allows an easy conversion of the N-Dts into the desired N-acetyl function during the solid-phase syntheses. Possibly the most salient feature of the Dts-concept may lie in the mildness of the cleavage conditions. On the one hand the thiolysis reaction does not induce any decomposition of the base-sensitive glycopeptides and on the other hand it offers additionally the possibility of further modification of the sugar amino function in the syntheses of branched glycopeptides or *N*-acyl glycopeptides.

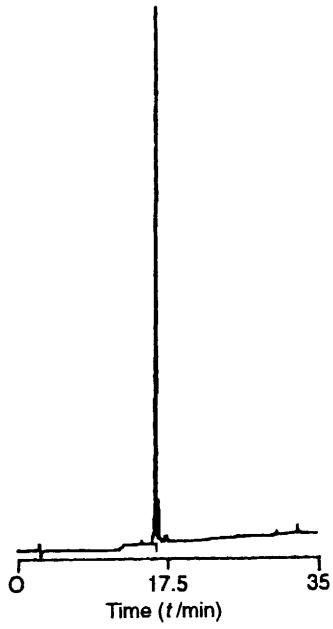

Fig. 2 Analytical RP-HPLC trace of crude glycopeptide **13**

Experimental

Vacuum liquid chromatography (VLC) was performed on Merck Silica Gel 60 H and chromatography under dry conditions was performed on dried Silica Gel (120 °C; > 24 h) with distilled solvents kept over appropriate molecular sieves. TLC was performed on Merck Silica Gel 60 F_{254} with detection by charring with sulfuric acid, or by UV light when applicable. DMF was freshly distilled by fractional distillation at reduced pressure. All organic solvents were of p.a. quality or better. Concentrations were performed under reduced pressure at temperatures < 30 °C. 4-[α-(Fluoren-9-ylmethoxycarbonylamino)-2,4-dimethoxybenzyl]phenoxyacetic acid (Rink-amide-linker) and suitable protected N^{α}-Fmoc-amino acids were purchased from Nova Biochem (Switzerland), TBTU and Dhbt-OH from Fluka (Switzerland), NEM from Merck (Germany), DTT (dithiothreitol or *threo*-1,4-disulfanylbutane-2,3-diol) (99%) from Aldrich (Germany), 2-sulfanylethanol from Sigma (USA). Optical rotations were measured with a Perkin-Elmer 241 Polarimeter and are given in units of 10^{-1} deg cm^3 g^{-1}. All NMR spectra were recorded on a Bruker AM 500 MHz spectrometer. The ^1H and ^{13}C resonances were assigned by ^1H, ^{13}C, ^1H–^1H COSY, ^1H–^1H double quantum filtered phase-sensitive COSY, NOE in rotating frame (ROESY) and ^1H–^{13}C correlation experiments. NMR spectra were recorded in $CDCl_3$, $(CD_3)_2SO$, D_2O or water–CD_3CO_2D mixtures and pH (pD) values of NMR samples were measured at room temperature by using a PHM63 digital pH meter (Radiometer) equipped with an Ingold electrode with no correction for isotope effects. Chemical shifts are given in ppm and referenced to internal $SiMe_4$ (δ_H, δ_C 0.00) for solutions in $CDCl_3$ at 300 K, to external 1,4-dioxane (δ_H 3.76, δ_C 67.40) for solutions in D_2O and for solutions in $(CD_3)_2SO$ at 300 K. HPLC was performed on a Waters system with a 600 controller, a 410 differential refractometer or a 991 photodiode array detector, both equipped with preparative flow cells, and a model 600 pump with modified 80 cm^3 min^{-1} pump heads. The system was fitted with switchable analytical RCM (8 × 100 mm) and Deltapak (19 × 300 mm) columns packed with reversed-phase C-18. Solvent system A: 0.1% TFA in water and B: 0.1% TFA in 90% acetonitrile–10% water, was employed both for analytical

Table 2 ^{1}H NMR chemical-shift assignments for the β-*O*-GlcNAc-glycosylated tripeptides **9** and **10** measured at 500.138 MHz on solutions in D_2O at 300 K [internal reference DOH (δ 4.75)]

Amino acid		Chemical shifts (δ) α-H	β-H	γ-H
Val	**9**	4.096	2.061	0.931/0.919
	10	4.093	2.065	0.941/0.928
Ser*	**9**	4.555	4.074/3.931	
Thr*	**10**	4.507	4.285	1.139
Gly	**9**	3.904		
	10	3.889		

O-GlcNAc data of glycopeptides **9** and **10**

	1-H	2-H	3-H	4-H	5-H	6-H[a]	6-H[b]
9	4.526	3.657	3.508	3.401	3.439	3.913	3.714
10	4.526	3.639	3.505	3.401	3.433	3.917	3.718

Others:	NH*Ac*	NH*Ac*
9	2.02	2.00
10	2.02	2.02

Table 3 ^{13}C-NMR chemical-shift assignments for the β-*O*-GlcNAc-glycosylated tripeptides **9** and **10** measured at 125.759 MHz on solutions in D_2O relative to dioxane (δ_C 67.4) at 300 K

Amino acid		Chemical shifts (δ) C-α	C-β	C-γ	C-γ′
Val	**9**	60.36	30.89	19.08	18.21
	10	60.74	30.65	19.18	18.37
Ser*	**9**	54.45	68.68		
Thr*	**10**	58.45	75.36	16.53	
Gly	**9**	43.03			
	10	43.04			

O-GlcNAc data of **9** and **10**

	C-1	C-2	C-3	C-4	C-5	C-6
9	101.68	56.16	74.58	70.60	76.72	61.50
10	100.37	56.27	74.42	70.60	76.67	61.53

Others:	NHCO*Me*	NHCO*Me*
9	23.01	22.41
10	22.96	22.39

(1 cm^3 min^{-1}) and preparative separations (10 cm^3 min^{-1}) and UV detection at 215 and 280 nm.

Solid-phase synthesis. Procedure used for the comparative studies in the syntheses of compounds 9–11

Syntheses of the glycopeptides **9–11** were performed in DMF with a custom-made Teflon block with 20 wells or by the plastic syringe technique[23] using the PEGA-resin.[24] Amino acids were coupled as their Pfp esters (3 mol equiv.) with Dhbt-OH (1 mol equiv.) added as an auxiliary nucleophile. The side chains were protected with Bu^t for serine, threonine and tyrosine. N^{α}-Fmoc cleavage was effected by treatment with 20% piperidine in DMF. After coupling of the Rink-amide-linker[25] by the TBTU procedure,[26] the first amino acid was coupled and residual amino groups were capped by addition of acetic anhydride before coupling of the next amino acid. Incorporation of the glycosylated serine and threonine amino acids into the glycopeptides was accomplished by utilising N^{α}-Fmoc-Ser/Thr(Ac_3-β-D-GlcNAc)-OPfp **5** and **6** or N^{α}-Fmoc-Ser/Thr(Ac_3-β-D-GlcNDts)-OPfp **7** and **8** (2 mol equiv.) in the presence of Dhbt-OH (1 mol equiv.). After coupling of the *N*-Dts-containing building blocks, cleavage of the Dts group was performed on the solid-phase by thiolysis, alternatively with 2-sulfanylethanol (0.2 mol dm^{-3})–triethylamine (0.5 mol dm^{-3}) in dichloromethane or with DTT (0.2 mol dm^{-3})–DIPEA (0.1 mmol dm^{-3}) in dichloromethane in three portions. The first portion was sucked quickly through the resin, followed by two additional portions, which were each removed after 10 min of reaction. After a thorough rinse with DMF (10 vol.) the amino group was acetylated with acetic anhydride in DMF (15%). After completion of the syntheses the resin was washed with dichloromethane and dried. Cleavage of the glycopeptides from the linker was performed by two successive treatments with 95% TFA for 2 h, followed by filtration, concentration under reduced pressure, precipitation by addition of ether, and purification by preparative HPLC. The purified acetylated glycopeptides were dissolved in dry methanol (1 mg cm^{-3}) and sodium methoxide in methanol was added until pH 9 was reached (dry pH paper). After neutralisation with solid CO_2, and evaporation under reduced pressure, the residue was dissolved in water and purified by preparative HPLC using 100% solvent A for 10 min, followed by a linear gradient of 0–50% B during 50 min. Structures of **9–11** were confirmed by ^{1}H and ^{13}C NMR spectroscopy and ES-MS. ^{1}H and ^{13}C NMR data are presented in Tables 2 and 3, respectively. Yields and ES-MS data are presented in Table 1.

Solid-phase synthesis: plastic syringe technique

The peptide Ac-Tyr-Ser-Pro-Thr-Ser-Pro-Ser-NH_2 **12** and the glycopeptide Ac-Tyr-Ser-Pro-Thr-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-Ser-Pro-Ser-NH_2 **13** were synthesised by use of the plastic syringe technique. A 10 cm^3 disposable plastic syringe A (without piston) was fitted with a sintered Teflon filter (pore size 70 μm) and the outlet was connected to the outlet of a 50 cm^3 plastic syringe B *via* a Teflon tube with female Luer adaptors. Syringe B was used as a waste syringe to remove solvents and unused reagents. PEGA-resin (250 mg; 0.36 mmol g^{-1}) was placed in syringe A and allowed to swell in DMF (5 cm^3). After a thorough rinse with piperidine in DMF (20%) the resin was rinsed with DMF (10 volumes), Rink-linker (97.14 mg, 0.18 mmol), TBTU (57.79 mg, 0.18 mmol) and NEM (45.5 mm^3, 0.36 mmol) were dissolved in DMF (2 cm^3) and after 10 min added to the resin. After 2 h the resin was rinsed with DMF (10 volumes). After cleavage of N^{α}-Fmoc with 20% piperidine in DMF (1 volume, 2 × 10 min) and thorough DMF washing (10 volumes), the first amino acid, Fmoc-Ser(Bu^t)-OPfp (148 mg, 0.27 mmol) and Dhbt-OH (14.68 mg, 0.09 mmol) were dissolved in DMF (2 cm^3) and the mixture was added to the resin. The acylation time was determined by observation of the yellow colour formed between Dhbt-OH and unchanged amino groups. After coupling, the resin was rinsed with DMF (10 volumes) before cleavage of N^{α}-Fmoc with 20% piperidine in DMF. The following amino acids were coupled as their Pfp esters (3 mol equiv.) with Dhbt-OH (14.68 mg, 1 mol equiv.) The glycosylated building block **8** was incorporated into the peptide chain by using the Pfp ester (2 mol equiv.) with Dhbt-OH (14.68 mg, 1 mol equiv.) added as an auxiliary nucleophile. After a careful wash of the resin with dichloromethane (5 volumes) the *N*-Dts cleavage on the solid-phase was performed by DTT (0.2 mol dm^3)–DIPEA (0.1 mmol dm^{-3}) in dichloromethane in three portions (1 volume each). The first portion was sucked quickly through the resin, followed by two additional portions, which were each removed after 10 min of reaction. After a thorough rinse with DMF (10 volumes) the amino group was acetylated with acetic anhydride in DMF (15%). After cleavage of N^{α}-Fmoc the residual amino acids were coupled as described above. The terminal amino group was finally acetylated with acetic anhydride in DMF (15%) for 30 min. The resin was then washed successively with DMF (10 volumes) and dichloromethane (5 volumes) and dried before cleavage of the glycopeptide from the resin with 95% TFA to

Table 4 ^{1}H NMR chemical shift assignments for the β-*O*-GlcNAc-glycosylated peptide **13** and the unglycosylated peptide **12** measured at 500 MHz on solutions in D_2O at 300 K [internal reference DOH (δ 4.75)]

		Chemical shifts (δ)				
Amino acid[a]		α-H	β-H	γ-H	δ-H	ε-H
Tyr	**13**	4.513	2.921/2.906		7.061	6.738
	12	4.503	2.940/2.895		7.087	6.788
Ser	**13**	4.774	3.871			
	12	4.767	3.853			
Pro	**13**	4.421	2.315/1.934	1.987	3.717/3.579	
	12	4.454	2.295/1.927	1.994	3.724/3.546	
Thr	**13**	4.471	4.283	1.131		
	12	4.303	4.189	1.169		
Ser	**13**	4.731	3.751			
	12	4.679	3.798			
Pro	**13**	4.468	2.315/1.934	1.987	3.734/3.578	
	12	4.407	2.295/1.927	1.994	3.731/3.548	
Ser	**13**	4.382	3.871			
	12	4.369	3.783			

O-GlcNAc data of glycopeptide **13**:

1-H	2-H	3-H	4-H	5-H	6-H[a]	6-H[b]
4.512	3.637	3.505	3.402	3.407	3.847	3.731

Others:		NH*Ac*	NH*Ac*
	13	2.027	1.952
	12		1.943

[a] Assignments of protons of those amino acids of which there are more than one in the molecular entity may be interchanged.

Table 5 ^{13}C NMR chemical shift assignments of the β-*O*-GlcNAc-glycosylated peptide **13** and the unglycosylated peptide **12** measured at 125 MHz in D_2O relative to external dioxane (δ_C 67.4)

		Chemical shifts (δ)					
Amino acid[a]		C-α	C-β	C-γ	C-δ	C-ε	C-ζ
Ac-Tyr	**13**	56.29	37.17	127.22	131.23	116.93	157.74
	12	56.12	37.15	128.74	131.33	116.21	155.26
Ser	**13**	56.11	61.46				
	12	55.56	61.53				
Pro	**13**	61.63	30.35	25.41	48.93		
	12	61.69	30.23	25.37	48.91		
Thr	**13**	58.21	75.44	16.98			
	12	59.68	67.72	19.69			
Ser	**13**	53.70	61.53				
	12	54.36	61.81				
Pro	**13**	61.54	30.15	25.30	48.85		
	12	61.42	30.13	25.28	48.84		
Ser	**13**	53.44	61.70				
	12	53.50	61.70				

Carbonyl carbons:	175.57	175.15	175.08	174.79	173.97	171.72	170.86	170.08
	175.16	175.09	174.76	173.86	172.49	170.98	170.49	

O-GlcNAc data of glycopeptide **13**:

C-1	C-2	C-3	C-4	C-5	C-6
100.39	56.40	74.51	70.60	76.62	61.53

Others:	NHCO*Me*	NHCO*Me*
	23.00	22.43
		22.37

[a] Assignments of carbons to those amino acids of which there are more than one in the molecular entity may be interchanged.

yield the crude O-acetylated glycopeptide **13**. The glycopeptide solution was filtered through a glass filter, concentrated and triturated with diethyl ether. The crude O-acetylated glycopeptide **13** was dissolved in water–DMF mixtures and analysed by analytical HPLC using 100% solvent A for 5 min, followed by a linear gradient of 0–100% solvent B during 30 min. Protected compound **13** was then dissolved in methanol (1 mg cm^{-3}) and subsequently O-deacetylated by addition of a catalytic amount of sodium methoxide in methanol until pH 9 was reached (dry pH paper). The resulting analytical RP-HPLC trace of the glycopeptide **13** showed essentially one peak (> 90%; t_R 16.38 min, Fig. 2). Final purification was achieved

Table 6 ^{1}H NMR chemical shift assignments and coupling constants (Hz, in parentheses) of the undecapeptide **14** and of the β-*O*-GlcNAc-glycosylated undecapeptides **15**–**21** measured at 500 MHz on solutions in $(CD_3)_2SO$ at 300 K [internal reference $\delta_C(CD_3)_2SO$ 2.90]

	Chemical shifts (δ)							
	14	**15**	**16**	**17**	**18**	**19**	**20**	**21**
Tyr								
NH	8.008 (8.0)	8.003 (8.0)	8.004 (8.0)	8.005 (8.2)	8.003 (8.1)	8.004 (8.0)	8.004 (8.0)	8.005 (8.1)
α-H	4.457	4.443 (3.4/8.4)	4.437 (3.3/8.3)	4.454 (3.3/8.4)	4.445 (3.3/8.4)	4.451 (3.4/8.4)	4.453 (3.4/8.4)	4.448 (3.3/8.5)
β-H	2.900 (3.9/13.9)	2.901 (3.8/13.9)	2.900 (3.8/13.9)	2.897 (3.7/13.9)	2.897 (3.8/13.8)	2.903 (3.7/13.8)	2.901 (3.8/13.8)	2.901 (3.7/13.8)
β-H′	2.589 (10.6)	2.587 (10.4)	2.585 (10.5)	2.587 (10.3)	2.582 (10.6)	2.581 (10.5)	2.590 (10.6)	2.584 (10.4)
δ-H, δ-H′	7.036 (8.1)	7.036 (8.4)	7.035 (8.5)	7.038 (8.4)	7.032 (8.2)	7.035 (8.4)	7.036 (8.3)	7.035 (8.4)
ε-H, ε-H′	6.622	6.624	6.623	6.622	6.619	6.623	6.624	6.624
Ser								
NH	8.028 (8.9)	8.017 (8.9)	8.027 (8.8)	8.025 (8.9)	8.019 (8.9)	8.022 (9.0)	8.018 (9.0)	8.028 (8.8)
α-H	4.296	4.285	4.288	4.281	4.286	4.288	4.279	4.278
β-H	3.595	3.593	3.587	3.584	3.583	3.584	3.589	3.579
β-H′	3.547	3.543	3.527	3.537	3.528	3.521	3.517	3.521
Ala								
NH	7.998 (7.6)	7.996 (7.6)	7.987 (7.6)	7.992 (7.6)	7.989 (7.6)	7.994 (7.7)	7.997 (7.5)	7.994 (7.7)
α-H	4.523	4.524	4.522	4.526	4.519	4.522	4.525	4.522 (7.1)
β-H_3	1.205 (6.7)	1.204 (6.8)	1.203 (6.8)	1.203 (6.8)	1.200 (6.7)	1.204 (6.8)	1.206 (6.7)	1.205 (6.6)
Pro								
NH								
α-H	4.438	4.439	4.438	4.449	4.438	4.441	4.437	4.424
β-H	1.992	1.987	1.985	1.986	1.983	1.987	1.985	1.981
β-H′	1.881	1.889	1.881	1.880	1.880	1.873	1.872	1.879
γ-H_2	1.841	1.843	1.847	1.857	1.846	1.843	1.843	1.841
δ-H	3.623	3.607	3.613	3.667	3.661	3.659	3.613	3.603
δ-H′	3.508	3.503	3.511	3.515	3.514	3.514	3.507	3.504
Val								
NH	7.774 (8.6)	7.779 (8.5)	7.777 (8.6)	7.798 (8.6)	7.793 (8.7)	7.798 (8.7)	7.798 (8.7)	7.799 (8.4)
α-H	4.154 (6.5/8.3)	4.156 (6.5/8.3)	4.168 (6.4/8.3)	4.172 (6.4/8.2)	4.166 (6.4/8.2)	4.170 (6.4/8.3)	4.173 (6.3/8.3)	4.174 (6.6/8.4)
β-H	1.996	1.997	1.996	1.995	1.998	1.992	1.998	1.998
γ-H_3	0.858	0.857	0.851	0.848	0.851	0.852	0.851	0.853
γ-H_3′	0.815	0.812	0.813	0.814	0.813	0.814	0.813	0.811
Ser								
NH	8.021	8.012	8.011	7.837	8.017	7.821	7.831	7.834
α-H	4.311	4.304	4.297	4.414	4.307	4.413	4.417	4.421
β-H	3.607	3.613	3.653	3.752	3.679	3.748	3.738	3.728
β-H′	3.589	3.598	3.591	3.707	3.598	3.703	3.699	3.700
Ser								
NH	7.892	7.994	7.870	7.991	7.821	7.993	7.878	7.894
α-H	4.289	4.287	4.401	4.362	4.414	4.361	4.417	4.418
β-H	3.615	3.612	3.791	3.610	3.789	3.607	3.724	3.723
β-H′	3.543	3.543	3.681	3.520	3.704	3.521	3.698	3.696
Ser								
NH	8.017	7.873	8.013	8.071	7.886	7.857	8.075	7.887
α-H	4.317	4.389	4.314	4.290	4.394	4.398	4.297	4.409
β-H	3.623	3.823	3.601	3.651	3.824	3.841	3.631	3.703
β-H′	3.538	3.705	3.545	3.602	3.713	3.708	3.548	3.698
Leu								
NH	7.915	7.958	7.927	7.918	7.914	7.918	7.992	8.017
α-H	4.287	4.283	4.299	4.314	4.312	4.303	4.279	4.276
β-H_2	1.487	1.462	1.503	1.479	1.488	1.461	1.509	1.494
γ-H	1.609	1.589	1.613	1.614	1.623	1.587	1.611	1.617
δ-H_3	0.863	0.862	0.861	0.862	0.863	0.864	0.873	0.863
δ-H_3′	0.829	0.831	0.819	0.824	0.828	0.821	0.821	0.820
Ser								
NH	7.901 (7.6)	7.909 (7.5)	7.919 (7.3)	7.912 (7.4)	7.913 (7.3)	7.918 (7.3)	7.937 (7.3)	7.939 (7.3)
α-H	4.328	4.314	4.324	4.328	4.307	4.318	4.312	4.318
β-H	3.587	3.581	3.579	3.576	3.581	3.579	3.583	3.579
β-H′	3.537	3.537	3.532	3.541	3.532	3.537	3.518	3.532

Table 6 (*continued*)

	Chemical shifts (δ)							
	14	**15**	**16**	**17**	**18**	**19**	**20**	**21**
Val								
NH	7.512 (8.7)	7.495 (8.8)	7.542 (8.7)	7.523 (8.7)	7.513 (8.5)	7.503 (8.6)	7.557 (8.7)	7.584 (8.6)
α-H	4.095 (5.9/8.7)	4.096 (5.8/8.7)	4.095 (5.7/8.7)	4.099 (5.8/8.8)	4.098 (5.9/8.7)	4.097 (5.8/8.8)	4.102 (6.0/8.7)	4.104 (5.7/8.5)
β-H	2.017	2.014	2.018	2.013	2.011	2.012	2.011	2.014
γ-H_3	0.851	0.857	0.851	0.848	0.853	0.854	0.854	0.857
γ-H_3′	0.823	0.823	0.824	0.823	0.824	0.821	0.820	0.824
*Glc*NAc								
1-H		4.351			4.358	4.356		4.356
2-H		3.411			3.417	3.411		3.410
3-H		3.291			3.295	3.298		3.304
4-H		3.056			3.091	3.071		3.073
5-H		3.121			3.147	3.148		3.142
6-H[a]		3.693			3.681	3.694		3.707
6-H[b]		3.501			3.499	3.504		3.500
*Glc*NAc								
1-H			4.319		4.381		4.334	4.361
2-H			3.418		3.428		3.425	3.421
3-H			3.291		3.314		3.19	3.317
4-H			3.087		3.104		3.088	3.100
5-H			3.117		3.141		3.147	3.157
6-H[a]			3.704		3.708		3.713	3.709
6-H[b]			3.493		3.501		3.500	3.510
*Glc*NAc								
1-H				4.346		4.386	4.351	4.348
2-H				3.412		3.421	3.417	3.418
3-H				3.280		3.288	3.310	3.298
4-H				3.082		3.101	3.102	3.097
5-H				3.113		3.117	3.138	3.117
6-H[a]				3.706		3.748	3.713	3.709
6-H[b]				3.480		3.501	3.500	3.510
NH		7.823 (8.2)	7.783 (8.5)	7.777 (8.0)	7.781 (8.5)	7.765 (8.5)	7.766 (8.8)	7.787
					7.844 (8.1)	7.857 (9.2)	7.858 (9.5)	7.799 7.837
NHCO*Me*								
GlcNAc		1.862	1.848	1.832	1.834, 1.843	1.831, 1.847	1.838, 1.847	1.831, 1.841, 1.855
Tyr-NHAc	1.742	1.742	1.741	1.742	1.737	1.742	1.743	1.743
CONH	7.281	7.282	7.292	7.288	7.289	7.289	7.299	7.312
CONH′	7.085	7.084	7.084	7.083	7.078	7.080	7.088	7.075

by preparative HPLC using 100% solvent A for 1 min, followed by a linear gradient of 0–50% solvent **B** during 50 min (t_R 39 min). Structures of compounds **12** and **13** were confirmed by ^1H and ^{13}C NMR spectrometry and electrospray mass spectroscopy (ES-MS). ^1H and ^{13}C data are presented in Tables 4 and 5, respectively. Yield and ES-MS data are presented in Table 1.

Solid-phase synthesis. Multiple-column glycopeptide syntheses of glycopeptides 15–21

Synthesis of the glycopeptides **15–21** was performed in a custom-made block of Teflon with 20 columns. Each column was equipped with a Teflon filter and a bottom outlet for removal of reagents by suction. Each column was packed with PEGA resin (50 mg; 0.36 mmol g^{-1}), allowed to swell in DMF for 30 min, then treated with 20% piperidine in DMF for 10 min and washed with 10 volumes of DMF. The 4-[α-(fluoren-9-ylmethoxycarbonylamino)-2,4-dimethoxybenzyl]phenoxyacetic acid (Rink-amide-linker) (0.036 mmol dm^{-3}; 19.5 mg; 2 mol equiv.), TBTU (0.036 mmol dm^{-3}; 11.5 mg, 2 mol equiv.) and NEM (0.072 mmol dm^{-3}; 9 mm^3, 4 mol equiv.) were dissolved in DMF (600 mm^3) and added to the resin after 10 min. After 3 h the resin was washed with DMF (5 volumes) and treated with 20% acetic anhydride in DMF for 30 min, followed by thorough rinsing with DMF (10 volumes). Throughout the syntheses the cleavage of the N^{α}-Fmoc moiety was achieved by treatment with 20% piperidine in DMF (2 ×) for 15 min and washing of the resin with DMF (10 volumes). All amino acids were then coupled to the peptide as their Pfp esters (3 mol equiv. each) with Dhbt-OH (29 mg, 1 mol equiv. each) added as an auxiliary nucleophile. Acylation times were *ca.* 2–4 h throughout the syntheses. Incorporation of the glycosylated serine amino acid into the glycopeptide was achieved by utilising either the building block N^{α}-Fmoc-Ser(Ac_3-β-D-GlcNAc)-OPfp **5** (30 mg, 2 mol equiv.) or N^{α}-Fmoc-Ser(Ac_3-β-D-GlcNDts)-OPfp **7** (33 mg, 2 mol equiv.) in the presence of Dhbt-OH (29 mg, 1 mol equiv.). In the case of the glycosylated building blocks **5** and **7** the reaction times were elongated to 6–8 h. After thorough rinsing of the resin with dichloromethane (5 volumes) the cleavage of the *N*-Dts group on the solid phase was achieved by adding DTT (0.1 mol dm^{-3})–DIPEA (0.1 mmol dm^{-3}) in dichloromethane in three portions. The first volume was sucked through the resin, followed by two additional portions, which were each removed after 15 min of reaction time. The amino group was then N-acetylated with 15% acetic

Table 7 ^{13}C NMR chemical shift assignments of the undecapeptide **14** and of the β-*O*-GlcNAc-glycosylated undecapeptides **15–21** measured at 125 MHz on solutions in $(CD_3)_2SO$ at 300 K [internal reference $\delta_C(CD_3)_2SO$ 39.5]

	Chemical shifts (δ)							
	14	**15**	**16**	**17**	**18**	**19**	**20**	**21**
Tyr								
C-α	59.17	59.17	59.01	59.13	58.99	59.11	59.11	59.09
C-β	36.72	36.74	36.57	36.76	36.58	36.74	36.74	36.73
C-γ	128.12	128.12	127.96	128.14	127.96	128.12	128.13	128.09
C-δ	130.01	130.02	129.85	130.03	129.85	130.02	130.03	130.00
C-ε	114.74	114.76	114.96	114.77	114.59	114.76	114.76	114.75
C-ζ	155.63	155.65	155.48	155.66	155.48	155.65	155.65	155.66
Ser								
C-α	54.95	55.03	55.09	55.13	54.83	54.79	54.77	54.81
C-β	61.38	61.14	61.30	61.22	61.52	61.46	61.48	61.46
Ala								
C-α	46.40	46.41	46.25	46.42	46.25	46.40	46.40	46.37
C-β	17.06	17.08	16.91	17.11	16.92	17.10	17.09	17.09
Pro								
C-α	54.20	53.58	53.01	52.95	53.02	53.47	52.95	52.90
C-β	28.71	28.73	28.58	28.71	28.59	28.70	28.72	28.71
C-γ	24.47	24.48	24.33	24.52	24.33	24.51	24.51	24.49
C-δ	46.60	46.62	46.46	46.63	46.46	46.62	46.62	46.60
Val								
C-α	57.41	57.41	57.27	57.44	57.23	57.40	57.43	57.41
C-β	30.53	30.56	30.37	30.67	30.40	30.70	30.69	30.72
C-γ	19.21	19.13	19.06	19.24	19.10	19.22	19.22	19.26
C-γ′	17.83	17.83	17.04	17.83	17.64	17.82	17.81	17.78
Ser								
C-α	54.78	54.82	54.77	54.95	54.64	54.79	54.78	54.81
C-β	61.45	61.68	61.52	67.30	61.52	67.44	67.44	67.35
Ser								
C-α	55.37	54.59	54.04	54.80	54.44	55.27	54.21	54.24
C-β	61.38	61.29	67.44	61.48	67.38	61.46	67.10	67.16
Ser								
C-α	54.20	54.22	54.05	54.00	54.06	54.22	54.21	54.24
C-β	61.66	67.54	61.63	61.70	67.13	67.29	61.48	67.16
Leu								
C-α	51.18	51.20	51.07	51.18	51.19	51.20	51.21	51.36
C-β	40.60	40.50	40.39	40.50	40.48	40.54	40.50	40.71
C-γ	24.05	24.03	23.88	24.06	24.00	24.02	24.01	23.96
C-δ	23.07	23.28	22.93	23.09	23.08	23.01	23.06	23.03
C-δ′	22.49	22.45	22.29	22.46	22.29	22.45	22.44	22.44
Ser								
C-α	54.20	54.00	54.17	54.22	53.92	54.22	54.21	54.24
C-β	61.38	60.99	60.79	60.54	60.81	60.97	60.99	60.96
Val								
C-α	57.49	57.60	57.27	57.50	57.23	57.39	57.43	57.41
C-β	30.10	30.13	29.93	30.13	29.98	30.14	30.13	30.11
C-γ	19.21	19.22	19.06	19.24	19.10	19.22	19.21	19.20
C-γ′	17.51	17.53	17.37	17.54	17.36	17.51	17.52	17.51
*Glc*NAc								
C-1		100.88			100.72	100.82		100.84
C-2		55.27			54.99	55.27		54.81
C-3		74.39			74.00	74.45		74.20
C-4		70.53			70.31	70.51		70.44
C-5		76.92			76.92	77.06		77.05
C-6		61.45			61.60	61.85		61.67
*Glc*NAc								
C-1			101.02		100.72		100.98	100.84
C-2			55.09		55.00		54.96	54.90
C-3			74.17		74.00		74.22	74.20
C-4			70.49		70.31		70.45	70.44
C-5			77.10		76.84		77.09	77.05
C-6			61.52		61.52		61.69	61.46
*Glc*NAc								
C-1				101.76		100.71	100.67	100.64
C-2				55.44		55.00	54.88	54.99
C-3				74.22		74.22	74.22	74.13
C-4				70.45		70.46	70.45	70.44
C-5				77.13		77.06	77.07	77.05
C-6				61.38		61.69	61.48	61.39

Table 7 (*continued*)

	Chemical shifts (δ)							
	14	**15**	**16**	**17**	**18**	**19**	**20**	**21**
NHCO*Me*	21.41	23.02, 21.50	22.93, 21.31	23.09, 21.45	22.86, 22.29, 21.51	23.01, 22.45, 21.53	23.13, 22.80, 21.50	23.10, 23.09, 23.03, 21.44

anhydride in DMF after rinsing of the resin with DMF. After incorporation of the last amino acid, tyrosine, the terminal N^{α}-Fmoc group was cleaved with 20% piperidine in DMF and N-acetylated with 15% acetic anhydride in DMF. After being washed with DMF (10 volumes) and dichloromethane (10 volumes) the resin was dried. Cleavage of the glycopeptides **15–21** from the solid support was performed by treatment with 95% TFA for 2 h. After cleavage the resin was poured onto a glass filter and washed three times with TFA followed by 95% aq. acetic acid. The combined filtrates were concentrated and the glycopeptides **15–21** were precipitated by several triturations with diethyl ether. Residual solvent was removed under reduced pressure and the glycopeptides were purified by preparative HPLC. The purified acetylated glycopeptides were dissolved in dry methanol (1 mg cm^{-3}) and O-deacetylated by addition of a catalytic amount of sodium methoxide in methanol until dry pH paper indicated pH 9. The mixture was then stirred at ambient temperature for 4 h, neutralised with solid CO_2 and concentrated. The crude glycoproteins **15–21** were dissolved in water and purified by preparative HPLC with 100% solvent A for 10 min, followed by a linear gradient of 0–50% B during 50 min. This procedures afforded the pure title compounds **15–21** in yields from 25 to 75%. The ^1H and ^{13}C NMR data of compounds **15–21** are presented and compared in Tables 6 and 7, respectively. Yields and ES-MS data are presented in Table 1.

Acknowledgements

This research was supported by the EC Science Program (M. M., grant SCI*-CT92-0765).

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Paper 5/01609F
Received 14*th March* 1995
Accepted 25*th April* 1995