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Synthesis of the glycosyl amino acids N^{α}-Fmoc-Ser[Ac_4-β-D-Gal p-(1 → 3)-Ac_2-α-D-$GalN_3$ p]-OPfp and N^{α}-Fmoc-Thr[Ac_4-β-D-Gal p-(1 → 3)-Ac_2-α-D-$GalN_3$ p]-OPfp and the application in the solid-phase peptide synthesis of multiply glycosylated mucin peptides with T^n and T antigenic structures.

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Abstract

Two new glycosyl amino acids N^{α}-Fmoc-Ser[Ac_4-β-D-Gal p-(1 → 3)-Ac_2-α-D-$GalN_3$ p]-OPfp and N^{α}-Fmoc-Thr[Ac_4-β-D-Gal p-(1 → 3)-Ac_2-α-D-$GalN_3$ p]-OPfp were synthesized. Glycosylation of N^{α}-Fmoc-Ser-OPfp or N^{α}-Fmoc-Thr-OPfp with protected β-D-Gal-(1 → 3)-D-$GalN_3$ donors afforded the glycosyl amino acids containing an activated C-terminus which could be utilized directly for solid-phase glycopeptide synthesis. The transformation of the 2-azido group into the acetamido derivative was achieved quantitatively at the end of the synthesis by treatment of the polymer-bound glycopeptide with thioacetic acid. The versatility of this strategy was demonstrated by the assembly of eight triply glycosylated mucin peptides which were synthesized simultaneously by multiple column techniques. The glycopeptides were prepared in order to investigate the substrate specificity of a galactosyltransferase.

Keywords: Glycopeptides; Synthesis; Carbohydrates; Solid-phase glycopeptide synthesis

1. Introduction

The carbohydrates of glycoproteins have been subjected to an increasing interest during the last few years because of their role in many biological processes, e.g., cell adhe-

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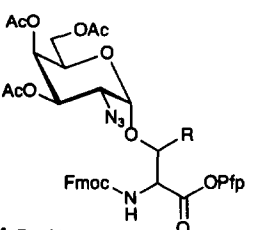
sion [1,2]. Because of the diversity and the heterogeneity of the oligosaccharides attached to the protein backbone, the accessibility of well defined glycoproteins from natural sources is limited. However, techniques for the synthesis of glycopeptides have recently been advanced substantially and a great variety of different N- and O-linked glycopeptides have been synthesized as model compounds for biochemical and structural investigations [3,4].

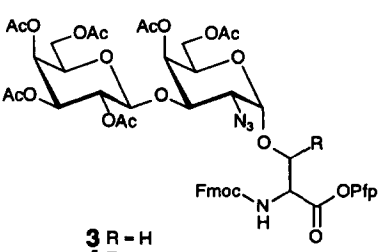
We have recently described an efficient strategy for the solid-phase peptide synthesis of mucin glycopeptides carrying GalNAc residues α-linked to L-serine or L-threonine [5,6]. As a building block for the introduction of the carbohydrate moiety into the peptide, the N^{α}-fluoren-9-ylmethoxycarbonyl (Fmoc)-protected pentafluorophenyl (Pfp) esters **1** and **2**, glycosylated with a 3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-α-D-galactopyranose unit, were synthesized. The protection scheme Fmoc/Pfp [7] is particularly useful in glycopeptide assembly because the Pfp ester is stable under the conditions of glycoside bond formation and can therefore act as a C-terminal protecting group [8]. At the same time, the Pfp ester is highly activated for peptide bond formation. Thus glycosylation of Fmoc/Pfp-protected hydroxy amino acids with 2-azido-2-deoxy-D-galactose derivatives affords building blocks which can be introduced directly into a peptide chain. The transformation of the azido group into the desired acetamido derivative can be performed at the end of the peptide assembly by treating the resin-bound glycopeptide with thioacetic acid. It has been demonstrated by multiple column techniques that this methodology is compatible with all naturally occurring amino acids [5,6].

2. Results and discussion

In the present paper, the synthesis of the building blocks **3** and **4** carrying the disaccharide 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1 → 3)-4,6-di-*O*-acetyl-2-azido-2-deoxy-D-galactopyranose α-linked to N^{α}-Fmoc-Thr-OPfp and N^{α}-Fmoc-Ser-OPfp and the application of **4** and the monosaccharide building block **2** in solid-phase synthesis of multiply glycosylated peptides are described [9].



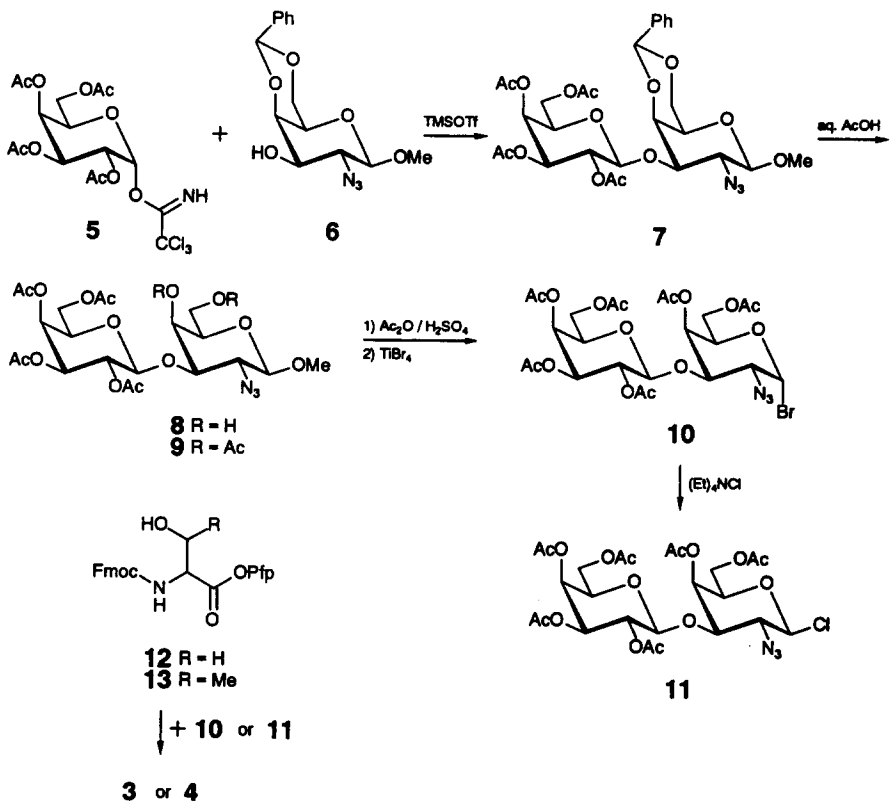
1 R = H
2 R = Me



3 R = H
4 R = Me

The formation of the α-glycosidic linkage between serine or threonine and GalNAc saccharides can only be obtained in a glycosylation reaction with a nonparticipating substituent at C-2 of the glycosyl donor. The azido group was found to be efficient for such stereoselective α-glycosylations and subsequently it can be converted into the naturally occurring acetamido group [10–12]. A suitable glycosyl donor is compound **10** [13], which was synthesized starting from the 4,6-*O*-benzylidene-protected methyl glycoside **6**

[14]. Compound **6** was glycosylated with the peracetylated galactose trichloroacetimidate **5** [15] affording exclusively the β-(1 → 3)-linked disaccharide **7** in 90% isolated yield. The benzylidene group was removed by treatment with aq 80% acetic acid to provide compound **8** which was acetylated to afford **9**. Cleavage of the methyl glycoside **9** was achieved according to a published [13] procedure using 1:40 H_2SO_4–Ac_2O to give the mixture of anomeric acetates which was converted into the α-bromide **10** with titanium tetrabromide. Furthermore, the bromide **10** was converted into the β-chloride **11** by inversion using tetraethylammonium chloride in acetonitrile [16]. Both halides **10** and **11** are suitable glycosyl donors to establish an α-glycosidic linkage to serine and threonine derivatives.



The glycosylation reaction of the bromide **10** and N$^{\alpha}$-Fmoc-Thr-OPfp **13** [7] was performed under catalysis with silver perchlorate. The best α-selectivity and yield were obtained at a reaction temperature of -40°C. The ratio of α to β anomer was found to be 5:1 in the crude product, calculated by integration of the NH signals of Thr in the ^{1}H NMR spectrum. The anomers were separated by preparative reversed-phase HPLC to yield 55% of the pure α anomer **4**. In addition, 30% of a fraction containing both anomers in an α:β ratio of 1.1:1 was isolated. The anomers could also be separated completely by medium-pressure liquid chromatography on dry silica gel. An improved α-selectivity

(α:β = 7.5:1) was obtained in the glycosylation reaction using the β-chloride **11** as glycosyl donor. However, during the conversion of the bromide **10** into the chloride **11** some of the valuable material was lost, and overall the application of the bromide **10** was preferred even though the stereoselectivity was lower. The glycosylation reactions with N^{α}-Fmoc-Ser-OPfp **12** [7] generally resulted in less α-selectivity. In this case, the application of the β-chloride affording an α:β ratio of 3.2:1 was preferred. The mixture of anomers of the serine building block **3** was separated by silica gel chromatography, yielding 49% of the α anomer **3**.

We have previously synthesized series of mucin glycopeptides containing GalNAc residues α-linked to serine and threonine [6,17]. These glycopeptides were used as in vitro substrates for glycosyltransferases and especially a β-3-galactosyltransferase which transfers a galactose to the 3-hydroxy group of the GalNAc. It was found that the activity of the enzyme was influenced by the peptide substitution pattern [18,19]. It was of interest, in addition, to investigate how neighbouring carbohydrate residues would influence the enzyme activity. As a model compound the triply glycosylated peptide **14** [17] was treated with UDP-Gal in presence of the β-3-Gal-transferase. This reaction can form up to six possible intermediates **15–20** until all three potential glycosylation sites are glycosylated, providing compound **21**. Therefore it was attractive to synthesize all the possible enzyme products **15–21** by solid-phase synthesis in order to obtain reference compounds and possible intermediate substrates.

14 Ac-Pro-Thr-Thr-Thr-Pro-Ile-Ser-Thr-NH_2

15 Ac-Pro-Thr-Thr-Thr-Pro-Ile-Ser-Thr-NH_2

16 Ac-Pro-Thr-Thr-Thr-Pro-Ile-Ser-Thr-NH_2

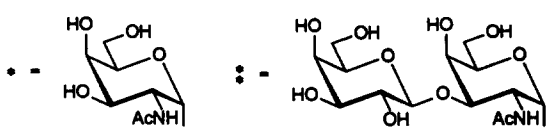
17 Ac-Pro-Thr-Thr-Thr-Pro-Ile-Ser-Thr-NH_2

18 Ac-Pro-Thr-Thr-Thr-Pro-Ile-Ser-Thr-NH_2

19 Ac-Pro-Thr-Thr-Thr-Pro-Ile-Ser-Thr-NH_2

20 Ac-Pro-Thr-Thr-Thr-Pro-Ile-Ser-Thr-NH_2

21 Ac-Pro-Thr-Thr-Thr-Pro-Ile-Ser-Thr-NH_2



The principle of the solid-phase glycopeptide syntheses is illustrated in Fig. 1. A kieselguhr-supported poly(dimethylacrylamide) resin (P) **23** was derivatized with the internal standard norleucine (Nle), a peptide amide linker (PAL) [20,21], and the first amino acid Thr(*t*-Bu). The loading of the resin was determined by amino acid analysis in order to calculate exactly the amount of reagents needed for the coupling reactions. To introduce the second amino acid, N^{α}-Fmoc-Ser(*t*-Bu)-ODhbt **22** was coupled to afford compound **24**. The use of 3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl (Dhbt) [22] esters allowed the progress of the acylation reaction to be followed visually by a colour reaction [23], resulting from deprotonation of 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (Dhbt-OH) by unreacted amino groups. At the beginning of the reaction the resin had a

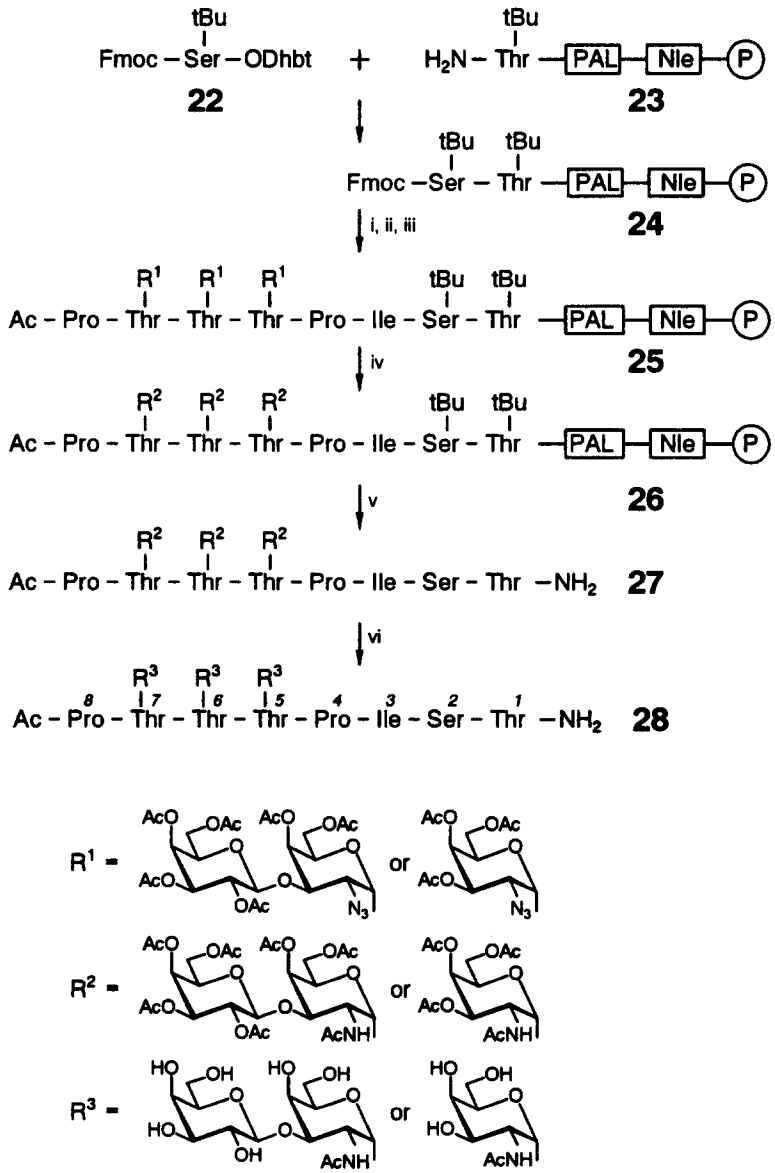


Fig. 1. Principle for the solid-phase glycopeptide synthesis: *i*, 50% morpholine–DMF; *ii*, coupling of the respective N^{α}-Fmoc amino or glycosyl amino acid; *iii*, repeat of *i* and *ii* until completion of the glycopeptide sequence; *iv*, AcSH; *v*, 95% CF_3CO_2H–H_2O; *vi*, NaOH–MeOH.

bright yellow colour which faded away with completion of the acylation. If an amino acid was not accessible as the Dhbt ester, the corresponding Pfp ester was used instead and an equimolar amount of Dhbt-OH was added as an acylation catalyst, thus providing the same colour reaction. The Fmoc group in **24** was removed with 50% morpholine [24] in *N,N*-dimethylformamide (DMF) and the next amino acid or glycosyl amino acid was

coupled. This cycle was repeated until completion of the desired glycopeptide sequence. After removal of the terminal Fmoc group, the resin was acetylated to afford compound **25**. The transformation of the azido group into the acetamido derivative was performed with thioacetic acid. Treatment of the resin **26** with trifluoroacetic acid afforded the protected glycopeptide **27** which was deacetylated and purified by reversed-phase HPLC to provide the final O-glycopeptide **28**.

It was expected that glycopeptide **21** carrying three vicinal disaccharides would be the most difficult compound to be synthesized. In order to investigate the required acylation times it was decided to prepare glycopeptide **21** as the first compound in a custom-made, fully automatic, continuous-flow peptide synthesizer [25] equipped with a solid-phase spectrophotometer [26] which measured the peptide bond formation by the above-mentioned colour reaction. Coupling of the nonglycosylated amino acids was performed with 3 equivalents of the N^{α}-Fmoc amino acid Dhbt esters whereas the glycosylated amino acid **4** was coupled using a 1.5 molar excess in the presence of Dhbt-OH. Fmoc cleavages were effected with 50% morpholine in DMF and monitored by the UV-absorption of the effluent at 320 nm. The coupling of the first glycosylated amino acid Thr-5 to Pro-4 required 22 h and was rather slow compared to the other coupling reactions which were all quantitative after 4–14 h. Surprisingly the coupling of glycosylated Thr-6 and Thr-7 did not require extended reaction times. The synthesis of the glycopeptide was continued according to Fig. 1 until completion of the peptide sequence. The transformation of the three azido groups into the acetamido derivatives was performed with thioacetic acid on the resin-bound glycopeptide. The thioacetic acid was distilled several times and analyzed by gas chromatography to be free of dithioacetic acid in order to avoid the formation of thioacetamido byproducts [6]. The progress of the reduction was followed by IR spectroscopy [6]. After 48 h the azide absorption band had disappeared completely. The glycopeptide was cleaved from the polymer with aq 95% trifluoroacetic acid with concurrent removal of the *t*-Bu side-chain protecting groups and purified by preparative HPLC to yield the protected glycopeptide **21**. Deacetylation of the carbohydrate was performed with a sodium methoxide solution at a pH of 8–8.5 measured on dry pH paper. The reaction was monitored by analytical HPLC and was completed after 4 h. The product was purified by preparative HPLC to afford glycopeptide **21** which was pure according to analytical HPLC, ^{1}H NMR spectroscopy, and mass spectrometry. The analytical data were in agreement with the structure. The complete set of ^{1}H NMR data with sequential assignment of all protons performed by NOESY experiments is presented in Table 1.

The triply glycosylated peptides **14–20** carrying the α-linked GalNAc- or the Gal-β-(1 → 3)-GalNAc saccharides were synthesized simultaneously in a manually operated 20-well multiple-column peptide synthesizer[27] following the reaction scheme in Fig. 1. This time, the glycosylated amino acids **2** and **4** were added in a twofold excess. The active esters were allowed to react for 19–24 h, except Thr-5 which was coupled for 48 h. The solid-phase reduction of the three azido groups in each sequence was monitored by IR spectroscopy and in all cases the azide band disappeared completely within 80 h. The glycopeptides were cleaved from the resin with aq 95% trifluoroacetic acid and purified by HPLC. Deacetylation was performed with a sodium methoxide solution at pH 8–8.5 measured on dry pH paper. The final triply O-glycosylated peptides **14–20** were

Table 1
^{1}H NMR chemical shifts (ppm) and selected coupling constants (Hz, in parentheses) of compound **21** measured at 600 MHz on a solution of 30% CD_3CO_2D in H_2O at 300 K. Reference: internal acetic acid at δ 2.03

						GalNAc								Gal					
	NH	H^{α}	H^{β}	H^{γ}	H^{δ}	NH	Ac	H-1	H-2	H-3	H-4	H-5	H-6	H-1	H-2	H-3	H-4	H-5	H-6
Pro-8		4.58	2.31	2.01	3.65														
			2.01																
Thr-7	8.53	4.75	4.40	1.34		7.63	2.07	4.82	4.24	4.01	4.22	4.05	3.77	4.45	3.52	3.61	3.90	3.61	3.78
	(8.9)					(10.1)								(7.8)					3.79
Thr-6	8.75	4.81	4.38	1.27		7.55	2.03	4.89	4.23	3.98	4.21	3.99	3.75	4.43	3.54	3.61	3.90	3.61	3.78
	(9.1)					(10.1)		(3.7)						(7.8)					3.73
Thr-5	8.63	4.68	4.33	1.30		7.72	2.04	5.03	4.27	4.00	4.23	4.06	3.78	4.42	3.55	3.61	3.90	3.61	3.78
	(9.3)					(9.2)		(3.7)						(7.8)					3.73
Pro-4		4.49	2.29	2.02	3.68														
			1.93																
Ile-3	8.00	3.98	1.78	1.60	0.93														
	(5.6)			1.20	0.91														
Ser-2	8.28	4.55	3.93																
	(7.0)		3.85																
Thr-1	8.03	4.36	4.33	1.20															
	(8.0)																		
NH_2	7.50																		
	7.10																		

Table 2
Yields and FABMS data of the synthesized glycopeptides

	Yield a		Calcd *M*	Found *M* + H	Formulae
	(mg)	(%)			
15	11.7	37	1628.7	1630.0	$C_{67}H_{112}N_{12}O_{34}$
16	11.8	37	1628.7	1629.8	$C_{67}H_{112}N_{12}O_{34}$
17	11.0	34	1628.7	1629.8	$C_{67}H_{112}N_{12}O_{34}$
18	11.8	33	1790.8	1792.1	$C_{73}H_{122}N_{12}O_{39}$
19	9.8	28	1790.8	1792.2	$C_{73}H_{122}N_{12}O_{39}$
20	10.7	30	1790.8	1791.9	$C_{73}H_{122}N_{12}O_{39}$
21	6.1	44	1952.9	1953.9	$C_{79}H_{132}N_{12}O_{44}$
29	5.2	41	1222.6	1224.2	$C_{51}H_{86}N_{10}O_{24}$
30	5.4	42	1222.6	1224.0	$C_{51}H_{86}N_{10}O_{24}$
31	4.0	31	1222.6	1223.6	$C_{51}H_{86}N_{10}O_{24}$
33	5.0	40	1182.6	1183.9	$C_{48}H_{82}N_{10}O_{24}$
34	6.1	49	1182.6	1183.7	$C_{48}H_{82}N_{10}O_{24}$
35	3.9	31	1182.6	1184.2	$C_{48}H_{82}N_{10}O_{24}$
36	4.4	36	1182.6	1183.6	$C_{48}H_{82}N_{10}O_{24}$
37	4.8	39	1182.6	1183.9	$C_{48}H_{82}N_{10}O_{24}$
38	3.4	29	1222.6	1223.6	$C_{51}H_{86}N_{10}O_{24}$
39	3.8	33	1222.6	1223.6	$C_{51}H_{86}N_{10}O_{24}$
40	2.1	18	1222.6	1223.6	$C_{51}H_{86}N_{10}O_{24}$
42	3.4	30	1182.6	1184.0	$C_{48}H_{82}N_{10}O_{24}$
43	4.7	42	1182.6	1183.5	$C_{48}H_{82}N_{10}O_{24}$
44	2.3	20	1182.6	1183.7	$C_{48}H_{82}N_{10}O_{24}$
45	3.0	27	1182.6	1182.8	$C_{48}H_{82}N_{10}O_{24}$
46	2.0	18	1182.6	1184.2	$C_{48}H_{82}N_{10}O_{24}$

[a] Yields are calculated based on the substitution of the resin.

obtained in yields of 28–37% with excellent purity according to analytical HPLC and ^{1}H NMR spectroscopy. The FABMS and ^{1}H NMR data presented in Tables 2 and 3 are in agreement with the structures. The analytical data for compound **14** were in agreement with the previously published [17] data.

In order to establish whether the mixture of anomers of building block **4** could be used in solid-phase glycopeptide synthesis, the additional monoglycosylated peptide sequences **29–46** were designed and synthesized. It was planned to separate the α- and β-linked O-glycopeptides after completion of the peptide synthesis by HPLC. These sequences, containing a varying glycosylation pattern and Gly to Pro exchanges, were assembled in parallel with the other sequences **14–20** during the multiple column synthesis. The mixture of anomers of building block **4** (α:β = 1.1:1) was used. After completion of the peptide assembly, the glycopeptides were cleaved from the resin and analyzed by analytical HPLC. The acetylated compounds appeared as single peaks in the analytical HPLC and therefore a separation of α and β anomers was not feasible. However, after deacetylation of the carbohydrate, the HPLC retention times of the α and β anomers **29–46** showed a large difference with the exception of sequence **32/41** which appeared as a single peak in both analytical and preparative HPLC. Thus it was possible to isolate the 16 glycopeptides **29–46** (except **32** and **41**) in pure form. In most of the cases, the α anomer had the shortest retention time, but for **29/38** and **35/44** the β

Table 3
Selected 1H NMR chemical shifts [a] (ppm) and coupling constants (Hz,in parentheses) for synthesized glycopeptides

	GalNAc H-1	Gal H-1	Thr-H$^{\alpha}$	Ser-H$^{\alpha}$	Pro-H$^{\alpha}$	Thr-H$^{\gamma}$	Ile-H$^{\gamma}$	Ile-H$^{\delta}$	Ac
15	5.07 (3.8)	4.51 (7.8)	4.85 (1.6)	4.58 (5.6, 6.2)	4.65 (5.0, 8.8)	1.41 (6.4)	1.00 (6.8)	0.98 (7.4)	2.18
	4.91 (3.8)		4.80		4.56 (4.6, 8.6)	1.34 (6.4)			2.10
	4.88 (3.8)		4.73			1.32 (6.4)			2.09
			4.45–4.37			1.27 (6.0)			
16	5.06 (3.8)	4.49 (7.8)	4.88	4.58 (5.8, 6.4)	4.64 (5.0, 8.6)	1.42 (6.4)	1.00 (6.6)	0.98 (7.2)	2.18
	4.92 (3.8)		4.80		4.55 (4.6, 8.4)	1.34 (6.4)			2.12
	4.88 (3.8)		4.72			1.32 (6.4)			2.10
			4.49–4.33			1.27 (6.0)			2.06
17	5.07 (3.8)	4.49 (7.8)	4.86 (2.0)	4.58 (5.8, 6.4)	4.64 (5.0, 8.8)	1.40 (6.4)	1.00 (6.8)	0.98 (7.4)	2.18
	4.91 (3.6)		4.80		4.55 (4.6, 8.2)	1.35 (6.6)			2.12
	4.87 (3.8)		4.75			1.33 (6.4)			2.08
			4.49–4.35			1.27 (6.0)			
18	5.07 (3.4)	4.50 (7.8)	4.88	4.58 (5.6, 6.2)	4.64 (4.8, 6.8)	1.41 (6.2)	1.00 (6.6)	0.98 (7.2)	2.18
	4.93 (3.8)	4.49 (7.8)	4.80		4.54 (4.4, 8.2)	1.35 (6.4)			2.12
	4.86 (3.6)		4.74			1.33 (6.4)			2.08
			4.52–4.34			1.27 (6.0)			2.06
19	5.08 (3.6)	4.51 (7.8)	4.86	4.58	4.66 (5.0, 8.8)	1.41 (6.2)	1.00 (6.6)	0.99 (7.2)	2.18
	4.91 (3.6)	4.49 (7.8)	4.86		4.54 (3.6, 8.6)	1.35 (6.4)			2.10
	4.86		4.80			1.33 (6.4)			2.08
			4.75			1.27 (6.0)			
20	5.07 (3.6)	4.52 (7.8)	4.89–4.85	4.58 (5.8, 6.4)	4.66 (5.0, 8.6)	1.42 (6.2)	1.01 (6.6)	0.99 (7.2)	2.18
	4.92 (3.6)	4.49 (7.8)	4.89–4.85		4.54 (3.8, 8.6)	1.35 (6.4)			2.10
			4.82 (1.8)			1.32 (6.4)			2.09
			4.72			1.27 (6.0)			2.07
29	5.11 (3.6)	4.50 (7.8)	4.77 (1.8)	4.58 (5.6, 6.4)	4.64–4.52	1.38 (6.4)	1.00 (7.0)	0.97 (7.4)	2.18
			4.62 (5.0)		4.64–4.52	1.29 (6.4)			2.05
			4.49 (4.4)			1.29 (6.4)			
			4.41–4.20			1.27 (6.4)			
30	4.84 (3.8)	4.49 (7.8)	4.72 (1.8)	4.58 (5.6, 6.0)	4.53 (4.8, 8.8)	1.34–1.25	1.00 (7.0)	0.95 (7.4)	2.18
			4.64 (5.6)		4.51–4.45	1.34–1.25			2.09
			4.61 (5.2)			1.34–1.25			
			4.51–4.45			1.27 (6.4)			

31	4.98 (3.8)	4.51 (7.8)	4.71 (2.0)	4.62 (5.6, 6.2)	4.66–4.59	1.39 (6.4)	1.00 (6.8)	0.94 (7.6)	2.17
			4.61 (6.0)		4.55–4.43	1.33 (6.4)			2.06
			4.50 (4.4)			1.27 (6.4)			
			4.39 (3.4)			1.25 (6.4)			
33	4.97 (3.8)	4.50 (7.8)	4.74 (2.2)	4.62 (6.0, 6.2)	4.53 (4.8, 8.8)	1.34 (6.4)	0.98 (6.8)	0.93 (7.6)	2.18
			4.59 (5.2)			1.32 (6.4)			2.06
			4.43 (4.4)			1.29 (6.4)			
			4.40–4.23			1.27 (6.4)			
34	4.97 (3.8)	4.51 (7.8)	4.70 (2.4)	4.62 (5.6, 6.0)	4.65–4.60	1.38 (6.4)	0.98 (6.8)	0.92 (7.4)	2.17
			4.56 (4.4)			1.28 (6.4)			2.07
			4.41 (4.8)			1.27 (6.4)			
			4.40–4.22			1.26 (6.4)			
35	5.10 (3.8)	4.50 (7.8)	4.77 (1.6)	4.58 (5.8, 6.4)	4.57 (5.0, 8.4)	1.38 (6.4)	0.99 (7.0)	0.97 (7.4)	2.12
			4.62 (5.0)			1.29 (6.4)			2.05
			4.51 (4.4)			1.27 (6.4)			
			4.41–4.23			1.27 (6.4)			
36	4.85 (3.8)	4.49 (7.6)	4.73 (1.8)	4.62 (6.0, 6.0)	4.52–4.34	1.38–1.21	1.00 (6.8)	0.95 (7.4)	2.12
			4.63 (5.2)			1.38–1.21			2.09
			4.62 (4.4)			1.38–1.21			
			4.52–4.34			1.27 (6.2)			
37	5.00 (3.8)	4.51 (7.8)	4.73 (2.4)	4.62 (5.6, 6.2)	4.55–4.49	1.37 (6.4)	1.00 (6.8)	0.94 (7.4)	2.12
			4.62(6.2)			1.32 (6.4)			2.06
			4.51 (4.4)			1.27 (6.2)			
			4.41–4.34			1.26 (6.0)			
38	4.62 (8.6)	4.49 (7.8)	4.63 (6.4)	4.62	4.58–4.46	1.30 (6.4)	1.00 (6.8)	0.93 (7.6)	2.18
			4.51 (4.4)		4.58–4.46	1.28 (6.4)	2.08		
			4.49 (3.6)			1.27 (6.4)			
			4.42–4.15			1.25 (6.2)			
39	4.61 (8.4)	4.49 (7.8)	4.66 (6.6)	4.62 (6.0, 6.0)	4.58–4.47	1.32 (6.6)	1.00 (6.8)	0.94 (7.4)	2.17
			4.64 (4.2)		4.58–4.57	1.30 (6.0)			2.09
			4.50 (4.4)			1.27 (6.4)			
			4.41–4.33			1.20 (6.0)			
40	4.64 (8.4)	4.54	4.66 (3.2)	4.62 (5.6, 5.6)	4.54–4.48	1.37–1.20	0.99 (6.8)	0.94 (7.4)	2.18
			4.66 (6.6)		4.54–4.48	1.37–1.20			2.10
			4.48 (4.4)			1.37–1.20			
			4.43–4.35			1.32 (6.2)			

Table 3 (continued)

	GalNAc H-1	Gal H-1	Thr-H$^{\alpha}$	Ser-H$^{\alpha}$	Pro-H$^{\alpha}$	Thr-H$^{\gamma}$	Ile-H$^{\gamma}$	Ile-H$^{\delta}$	Ac
42	4.65 (8.4)	4.50 (7.6)	4.72-4.69	4.62 (6.2, 6.4)	4.57–4.50	1.30 (6.4)	0.98 (7.0)	0.92 (7.4)	2.17
			4.72-4.69			1.27 (6.4)			2.09
			4.51 (4.4)			1.22 (6.4)			
43	4.65 (8.4)	4.50 (7.8)	4.66 (3.6)	4.62 (6.0, 6.2)	4.55–4.48	1.29 (6.4)	0.98 (6.8)	0.92 (7.4)	2.18
			4.52 (4.8)			1.27 (6.4)			2.10
			4.43 (4.4)			1.26 (6.4)			
			4.43–4.28						
44	4.65–4.60	4.50 (7.8)	4.65–4.60	4.65–4.60	4.55–4.47	1.33–1.20	1.00 (6.8)	0.93 (7.4)	2.12
			4.53 (4.6)			1.33–1.20			2.08
			4.49 (4.8)			1.33–1.20			
			4.41–4.34			1.30 (6.4)			
45	4.6 (8.4)	4.50 (7.8)	4.65 (6.2)	4.62 (6.2, 6.2)	4.53 (6.2, 8.4)	1.32 (6.4)	0.99 (6.8)	0.94 (7.4)	2.12
			4.63 (3.6)			1.27 (6.4)			2.09
			4.51 (4.6)			1.27 (6.4)			
			4.41–4.32			1.21 (6.4)			
46	4.62 (8.4)	4.50 (7.6)	4.66 (6.0)	4.63	4.52 (6.0, 8.0)	1.32 (6.4)	0.99 (6.8)	0.94 (7.4)	2.12
			4.65 (3.6)			1.27 (6.4)			2.10
			4.48 (4.6)			1.27 (6.4)			
			4.41–4.21			1.22 (6.4)			

[a] Measured in D2O at 300 K, internal reference DOH = 4.8 ppm.

anomer was eluted first. The assignment of the configuration was performed by ^{1}H NMR spectroscopy where the α-glycosides showed a small coupling constant of 3.8 Hz for the H-1 GalNAc whereas the products of β configuration had a corresponding coupling constant of 8.4–8.6 Hz.

29 Ac-Pro-Thr-Thr-Thr-Pro-Ile-Ser-Thr-NH_2 **38** Ac-Pro-Thr-Thr-Thr-Pro-Ile-Ser-Thr-NH_2

30 Ac-Pro-Thr-Thr-Thr-Pro-Ile-Ser-Thr-NH_2 **39** Ac-Pro-Thr-Thr-Thr-Pro-Ile-Ser-Thr-NH_2

31 Ac-Pro-Thr-Thr-Thr-Pro-Ile-Ser-Thr-NH_2 **40** Ac-Pro-Thr-Thr-Thr-Pro-Ile-Ser-Thr-NH_2

32 Ac-Pro-Thr-Thr-Thr-Gly-Ile-Ser-Thr-NH_2 **41** Ac-Pro-Thr-Thr-Thr-Gly-Ile-Ser-Thr-NH_2

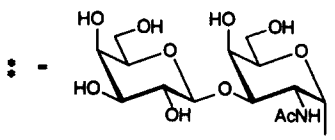
33 Ac-Pro-Thr-Thr-Thr-Gly-Ile-Ser-Thr-NH_2 **42** Ac-Pro-Thr-Thr-Thr-Gly-Ile-Ser-Thr-NH_2

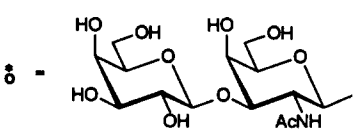
34 Ac-Pro-Thr-Thr-Thr-Gly-Ile-Ser-Thr-NH_2 **43** Ac-Pro-Thr-Thr-Thr-Gly-Ile-Ser-Thr-NH_2

35 Ac-Gly-Thr-Thr-Thr-Pro-Ile-Ser-Thr-NH_2 **44** Ac-Gly-Thr-Thr-Thr-Pro-Ile-Ser-Thr-NH_2

36 Ac-Gly-Thr-Thr-Thr-Pro-Ile-Ser-Thr-NH_2 **45** Ac-Gly-Thr-Thr-Thr-Pro-Ile-Ser-Thr-NH_2

37 Ac-Gly-Thr-Thr-Thr-Pro-Ile-Ser-Thr-NH_2 **46** Ac-Gly-Thr-Thr-Thr-Pro-Ile-Ser-Thr-NH_2





3. Experimental

General methods.—TLC was performed on Silica Gel 60 F_{254} (Merck). Medium pressure liquid chromatography (MPLC) was performed on Silica Gel Silitech 12-26, 6 nm (ICN) under a pressure of 1.5–6 bar with distilled solvents. HPLC separations were performed on a Merck/Hitachi HPLC system using a Lichrospher RP-18 column (250 × 25 mm, 7 nm) with a flow rate of 10 mL/min. For analytical HPLC separations a Lichrospher RP-18 column (250 × 4 mm, 10 nm) with a flow rate of 2 mL/min was applied. Peak detection was performed with a photodiode array detector at 215 nm. Solvent system: *A*, 0.1% CF_3CO_2H in water; *B*, 0.1% CF_3CO_2H in MeCN. ^{1}H NMR spectra were recorded on a Bruker AMX 400 or AMX 600 spectrometer at 300 K. Chemical shifts are given in ppm with internal Me_4Si ($\delta = 0.00$ ppm) as reference for solutions in $CDCl_3$. Coupling constants are given in Hz. Melting points were determined on a Leitz heating-table instrument and are not corrected. Positive-ion FAB mass spectra were recorded on a double-focused VG-Analytical 70-250S mass spectrometer with a *m*-nitrobenzyl alcohol or a glycerol/thioglycerol matrix. IR spectra were recorded on a Perkin–Elmer FTIR 1720 X spectrometer. All solvents used for syntheses were distilled at the appropriate pressure. DMF was freshly distilled before use and analyzed for free

amines by addition of Dhbt-OH prior to use. Suitably protected N^{α}-Fmoc amino acids and Pfp esters were purchased from Novabiochem, and the Dhbt esters were synthesized according to the method of Atherton et al. [23]. The Macrosorb resin was from Sterling Organics and derivatized with ethylenediamine, norleucine, the peptide amide linker (PAL) [20,21], and the first amino acid [N^{α}-Fmoc-Thr(*t*-Bu)] as previously described [17]. Glycosylation reactions were carried out under N_2 with dry solvents.

*Methyl 2-azido-2-deoxy-4,6-*O*-benzylidene-β-*D*-galactopyranoside* (**6**).—The synthesis was performed according to the methods of Paulsen and Paal [14] and Sabesan and Lemieux [28]. Methyl 2-azido-2-deoxy-β-D-galactopyranoside [14] (2.4 g, 11 mmol) was dissolved in MeCN (50 mL) and treated with dimethoxytoluene (2.71 mL) and *p*-toluenesulfonic acid (44 mg) for 2 h at room temperature. The mixture was neutralized with Et_3N and concentrated, when the product (1.9 g) began to crystallize. The crystals were collected and the mother liquors purified by chromatography on silica gel (3:1 toluene–acetone), affording a more crystalline product. The total yield was 4.7 g (70%); mp 174–176°C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.53–7.48 (m, 2 H, Ar), 7.40–7.36 (m, 3 H, Ar), 5.58 (s, 1 H, PhC*H*), 4.36 (dd, 1 H, $J_{5,6a}$ 1.6, $J_{6a,6b}$ 12.6 Hz, H-6a), 4.20 (d, 1 H, $J_{1,2}$ 7.6 Hz, H-1), 4.18 (dd, 1 H, $J_{3,4}$ 3.2, $J_{4,5}$ 1.0 Hz, H-4), 4.09 (dd, 1 H, $J_{5,6b}$ 2.0 Hz, H-6b), 3.64–3.59 (m, 2 H, H-2,3), 3.60 (s, 3 H, OCH_3), 3.45 (ddd, 1 H, H-5), 2.26 (d, 1 H, $J_{3,OH}$ 10.4 Hz, OH-3). Anal. Calcd for $C_{14}H_{17}N_3O_5$: C, 54.72; H, 5.58; N, 13.67. Found: C, 54.78; H, 5.55; N, 13.62.

*Methyl (2,3,4,6-tetra-*O*-acetyl-β-*D*-galactopyranosyl)-(1 → 3)-2-azido-4,6-*O*-benzylidene-2-deoxy-β-*D*-galactopyranoside* (**7**).—The acceptor **6** (1.0 g, 3.25 mmol), the trichloroacetimidate **5** [15] (2.0 g, 4.06 mmol), and molecular sieves (0.4 nm, 1.0 g) were dissolved in 1,2-dichloroethane (30 mL) and stirred at room temperature for 1 h. The mixture was cooled to −18°C and a solution of Me_3Si-triflate (TMSOTf) (60 μL, 0.33 mmol) in 1,2-dichloroethane (5.4 mL) was added. TLC (2:1 toluene–EtOAc) indicated a quantitative reaction after 10 min. The mixture was warmed to room temperature, diluted with CH_2Cl_2 (20 mL), and neutralized with Et_3N. The reaction product was purified by MPLC on silica gel (20:1 toluene–acetone) to give **7** (1.89 g, 91%) as a colourless foam; $[\alpha]_D^{25}$ +7.7° (*c* 1, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.56–7.52, 7.41–7.31 (m, 5 H, 5 × Ar), 5.55 (s, 1 H, PhC*H*), 5.39 (dd, 1 H, $J_{4',5'}$ 0.8 Hz, H-4'), 5.26 (dd, 1 H, $J_{2',3'}$ 10.4 Hz, H-2'), 5.04 (dd, 1 H, $J_{3',4'}$ 3.6 Hz, H-3'), 4.81 (d, 1 H, $J_{1',2'}$ 7.8 Hz, H-1'), 4.34 (dd, 1 H, $J_{5,6a}$ 1.4, $J_{6a,6b}$ 12.4 Hz, H-6a), 4.23 (dd, 1 H, $J_{4,5}$ 0.6 Hz, H-4), 4.20 (d, 1 H, $J_{1,2}$ 8.0 Hz, H-1), 4.20 (dd, 1 H, $J_{5',6'a}$ 6.2, $J_{6'a,6'b}$ 11.2 Hz, H-6'a), 4.11 (dd, 1 H, $J_{5',6'b}$ 6.8 Hz, H-6'b), 4.06 (dd, 1 H, $J_{5,6b}$ 1.6 Hz, H-6b), 3.90 (ddd, 1 H, H-5), 3.79 (dd, 1 H, $J_{2,3}$ 10.4 Hz, H-2), 3.58 (s, 3 H, $COCH_3$), 3.48 (dd, 1 H, $J_{3,4}$ 3.4 Hz, H-3), 3.38 (br s, 1 H, H-5), 2.16, 2.07, 2.04, 1.98 (4 s, 12 H, 4 × $COCH_3$). Anal. Calcd for $C_{28}H_{35}N_3O_{14}$: C, 52.75; H, 5.53; N, 6.59. Found: C, 52.79; H, 5.55; N, 6.62.

*Methyl (2,3,4,6-tetra-*O*-acetyl-β-*D*-galactopyranosyl)-(1 → 3)-2-azido-2-deoxy-β-*D*-galactopyranoside* (**8**).—Compound **7** (1.35 g, 2.12 mmol) was dissolved in aq 80% AcOH (60 mL) and the solution was stirred at 45°C for 18 h (TLC, 1:4 toluene–EtOAc). The solvent was concentrated to dryness and the residue was coevaporated with toluene several times. The crude product was purified by MPLC on silica gel (1:2 toluene–EtOAc) and the main fractions were collected and concentrated. The product was dissolved in CH_2Cl_2 (10 mL) and Et_2O (20 mL) and precipitated with *n*-pentane (100 mL)

to yield **8** (1.07 g, 92%) as a white amorphous powder; mp 215°C; $[\alpha]_D^{20}$ −3.2° (*c* 1, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$): δ 5.40 (dd, 1 H, $J_{4',5'}$ 0.8 Hz, H-4'), 5.26 (dd, 1 H, $J_{2',3'}$ 10.6 Hz, H-2'), 5.04 (dd, 1 H, $J_{3',4'}$ 3.4 Hz, H-3'), 4.69 (d, 1 H, $J_{1',2'}$ 8.0 Hz, H-1'), 4.18 (dd, 1 H, $J_{5',6'a}$ 7.0, $J_{6'a,6'b}$ 11.4 Hz, H-6'a), 4.17 (d, 1 H, $J_{1,2}$ 8.0 Hz, H-1), 4.09 (dd, 1 H, $J_{5',6'b}$ 6.0 Hz, H-6'b), 4.04–3.95 (m, 2 H, H-4,6a), 3.93 (ddd, 1 H, H-5'), 3.84 (dd, 1 H, $J_{5,6a}$ 5.0, $J_{6a,6b}$ 11.6 Hz, H-6b), 3.60 (dd, 1 H, $J_{2,3}$ 10.4 Hz, H-2), 3.58 (s, 3 H, OCH_3), 3.50 (m, 1 H, H-5), 3.41 (dd, 1 H, $J_{3,4}$ 3.2 Hz, H-4). 2.72 (br s, 1 H, OH-4), 2.28 (dd, 1 H, $J_{OH,6a}$ 4.0, $J_{OH,6b}$ 8.0 Hz, OH-6), 2.17, 2.10, 2.05, 2.00 (4 s, 12 H, 4 × $COCH_3$). Anal. Calcd for $C_{21}H_{31}N_3O_{14}$: C, 45.90; H, 5.69; N, 7.65. Found: C, 45.83; H, 5.73; N, 7.61.

*Methyl (2,3,4,6-tetra-*O*-acetyl-β-*D*-galactopyranosyl)-(1 → 3)-4,6-di-*O*-acetyl-2-azido-2-deoxy-β-*D*-galactopyranoside* (**9**).—Compound **7** (1.0 g, 1.57 mmol) was dissolved in aq 80% AcOH (50 mL) and stirred at 45°C for 20 h. The mixture was concentrated to dryness under vacuum and coevaporated several times with toluene. The residue was dissolved in 2:1 Ac_2O–pyridine (15 mL) and kept for 6 h at room temperature. The solvent was removed under vacuum and the residue was concentrated five times with toluene. The crude product was purified by MPLC on silica gel (2:5:1 toluene–EtOAc) to give the product **9** in a yield of 884 mg (89%); ^1H NMR data and optical rotation were in agreement with published data [13].

*2,3,4,6-Tetra-*O*-acetyl-β-*D*-galactopyranosyl-(1 → 3)-4,6-di-*O*-acetyl-2-azido-2-deoxy-β-*D*-galactopyranosyl chloride* (**11**).—The bromide **10** [13] (437 mg, 0.64 mmol) was dissolved in MeCN (43 mL). Tetraethylammonium chloride (218 mg) was added and the solution was agitated for 1 min. A 1-mL sample of the mixture was filled into a cuvette and the progress of the inversion was followed by the decrease of the optical rotation. After 21 min, the optical rotation reached the minimum value and the reaction was stopped by addition of toluene (80 mL). The organic layer was washed carefully four times with cold water, dried over Na_2SO_4, filtered, and concentrated. The crude product was purified by MPLC on silica gel (2:1 petroleum ether–EtOAc) and the main fractions were collected and concentrated to yield **11** (245 mg, 60%) as a syrup; $[\alpha]_D^{20}$ +12° (*c* 1, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$): δ 5.41 (dd, 1 H, $J_{4,5}$ 0.8 Hz, H-4), 5.37 (dd, 1 H, $J_{4',5'}$ 0.8 Hz, H-4'), 5.16 (dd, 1 H, $J_{2',3'}$ 10.6 Hz, H-2'), 5.02 (d, 1 H, $J_{1,2}$ 9.0 Hz, H-1), 5.01 (dd, 1 H, $J_{3',4'}$ 3.4 Hz, H-3'), 4.73 (d, 1 H, $J_{1',2'}$ 7.8 Hz, H-1'), 4.20 (dd, 1 H, $J_{5,6a}$ 4.8, $J_{6a,6b}$ 11.8 Hz, H-6a), 4.16 (dd, 1 H, $J_{5',6'a}$ 6.2, $J_{6'a,6'b}$ 11.8 Hz, H-6'a), 4.09 (dd, 1 H, $J_{5',6'b}$ 7.0 Hz, H-6'b), 4.02 (dd, 1 H, $J_{5,6b}$ 7.4 Hz, H-6b), 3.89 (ddd, 1 H, H-5'), 3.84 (ddd, 1 H, H-5), 3.77 (dd, 1 H, $J_{2,3}$ 10.0 Hz, H-2), 3.56 (dd, 1 H, $J_{3,4}$ 3.4 Hz, H-3), 2.17, 2.16. 2.04, 2.03, 2.01, 1.99 (6 s 18 H, 6 × $COCH_3$). Anal. Calcd for $C_{24}H_{32}ClN_3O_{15}$: C, 45.18; H, 5.06; N, 6.59; Cl, 5.56. Found: C, 45.01; H, 5.12; N, 6.49; Cl, 5.43.

*N$^{\alpha}$-(Fluoren-9-ylmethoxycarbonyl)-3-*O*-[2,3,4,6-tetra-*O*-acetyl-β-*D*-galactopyranosyl-(1 → 3)-4,6-di-*O*-acetyl-2-azido-2-deoxy-α-*D*-galactopyranosyl]-*L*-serine pentafluorophenyl ester* (**3**).—N$^{\alpha}$-Fmoc-Ser-OPfp **12** (23 mg, 47 μmol) [7] and powdered molecular sieves (0.4 nm) were suspended in 1:1 toluene–CH_2Cl_2 (2 mL) and cooled to −20°C. The mixture was stirred for 1 h and $AgClO_4$ (14 mg, 67 μmol) was added. The β-chloride **11** (44 mg, 69 μmol), dissolved in 1:1 toluene–CH_2Cl_2 (2 mL), was then added dropwise. The reaction was complete after 2 h. The mixture was diluted with

$CHCl_3$ and extracted with water. The organic layer was dried with $MgSO_4$, filtered, and concentrated. The ^1H NMR spectrum of the crude product indicated the α/β ratio to be 3.2:1. The α anomer was isolated by MPLC on dry silica gel (3:2 petroleum ether–EtOAc). Yield 25 mg (49%); $[\alpha]_D^{20}$ +54° (*c* 1, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$): δ 7.81–7.75, 7.65–7.57, 7.45–7.38, 7.35–7.29 (4 m, 8 H, Ar), 6.04 (d, 1 H, $J_{NH,CH\alpha}$ 8.6 Hz, NH-Ser), 5.46 (dd, 1 H, $J_{3,4}$ 3.4, $J_{4,5}$ 0.8 Hz, H-4), 5.35 (dd, 1 H, $J_{4',5'}$ 0.8 Hz, H-4'), 5.17 (dd, 1 H, $J_{2',3'}$ 11.4 Hz, H-2'), 4.99 (dd, $J_{3',4'}$ 3.4 Hz, H-3'), 4.99–4.92 (m, 2 H, CHα-Ser, H-1), 4.66 (d, 1 H, $J_{1',2'}$ 7.8 Hz, H-1'), 4.54–4.48 (m, 2 H, CH_2-Fmoc), 4.34 (dd, 1 H, $J_{CH\alpha,CH\beta a}$ 3.2, $J_{CH\beta a,CH\beta b}$ 11.2 Hz, CHβa-Ser), 4.28–4.22 (m, 1 H, CH-Fmoc), 4.21–3.99, 3.95–3.83 (2 m, 8 H, H-5, 2 × H-6, 2 × H-6', CHβb-Ser, H-3,5'), 3.69 (dd, 1 H, $J_{1,2}$ 3.6, $J_{2,3}$ 10.8 Hz, H-2), 2.15, 2.14, 2.05, 2.02, 1.99, 1.98 (6 s, 18 H, 6 × $COCH_3$). Mass spectrum (FAB): (M + H) 1095.3; Calcd mass for $C_{48}H_{47}F_5N_4O_{20}$: 1094.3.

N^{α}-*(Fluoren-9-ylmethoxycarbonyl)-3-*O-*[2,3,4,6-tetra-*O-*acetyl-*β-D-*galactopyranosyl-(1 → 3)-4,6-di-*O-*acetyl-2-azido-2-deoxy-*α-D-*galactopyranosyl]-*L-*threonine pentafluorophenyl ester* (**4**).—The glycosyl bromide **10** [13] (1.0 g, 1.47 mmol), N^{α}-Fmoc-Thr-OPfp **13** [7] (572 mg, 1.13 mmol), and powdered molecular sieves (0.4 nm, 1.3 g) were dissolved in 1:1 toluene–CH_2Cl_2 (65 mL) and stirred for 1 h. The mixture was cooled to −40°C and $AgClO_4$ (304 mg, 1.47 mmol) was added. After reacting for 1.5 h the bromide **10** could no longer be detected by TLC (1:4 toluene–EtOAc). The mixture was warmed to room temperature, diluted with $CHCl_3$, filtered, and extracted with water. The organic layer was dried with $MgSO_4$, filtered, and concentrated. Purification of the crude product was performed by preparative HPLC [MeCN–water 50:50 → 35:65 (30 min) → 15:85 (20 min) → 5:95 (20 min)]. The first fraction gave 620 mg (55%) of the pure α anomer; a second fraction yielded 370 mg (30%) of an α,β-mixture with a 1:1.1 ratio between the α and β anomers as calculated by integration of the NH-Thr protons in the ^1H NMR spectra. Alternatively, the anomers can be separated by rapid silica gel MPLC (2:1 petroleum ether–EtOAc). The pure α anomer was dissolved in Et_2O (20 mL) and precipitated with *n*-pentane (60 mL) to give a white, amorphous powder; mp 103°C; $[\alpha]_D^{23}$ +51° (*c* 1, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$): δ 7.80–7.75, 7.64–7.59, 7.44–7.37, 7.34–7.28 (4 m, 8 H, Ar), 5.82 (d, 1 H, $J_{NH,CH\alpha}$ 9.2 Hz, NH-Thr), 5.49 (d, 1 H, $J_{1,2}$ 3.0 Hz, H-1), 5.37 (dd, 1 H, $J_{4',5'}$ 0.6 Hz, H-4'), 5.19 (dd, 1 H, $J_{2',3'}$ 10.4 Hz, H-2'), 5.14 (dd, 1 H, $J_{4,5}$ 0.6 Hz, H-4), 5.01 (dd, 1 H, $J_{3',4'}$ 3.4 Hz, H-3'), 4.75 (dd, 1 H, $J_{CH\alpha,CH\beta}$ 2.4 Hz, CHα-Thr), 4.73 (d, 1 H, $J_{1',2'}$ 7.8 Hz, H-1'), 4.58 (dd, 1 H, $J_{CH,CH2a}$ 7.0 Hz, $J_{CH2a,CH2b}$ 10.4 Hz, CH_{2a}-Fmoc), 4.58–4.53 (m, 1 H, CHβ-Thr), 4.43 (dd, 1 H, $J_{CH,CH2b}$ 7.2 Hz, CH_{2b}-Fmoc), 4.28 (t, 1 H, CH-Fmoc), 4.22–4.13 (m, 3 H, H-5,6a,6'a), 4.09 (dd, 1 H, $J_{5',6'a}$ 7.0 Hz, $J_{6'a,6'b}$ 11.2 Hz, H-6'b), 4.02 (dd, 1 H, $J_{2,3}$ 10.6 Hz, H-2), 3.98 (dd, 1 H, $J_{5,6b}$ 11.0, $J_{6a,6b}$ 12.8 Hz, H-6b), 3.92 (ddd, 1 H, H-5'), 3.71 (dd, 1 H, $J_{3,4}$ 3.8 Hz, H-3), 2.16, 2.14, 2.04, 2.00 (6 s, 18 H, 6 × $COCH_3$), 1.43 (d, 3 H, $J_{CH\beta,CH\gamma}$ 6.4 Hz, CHγ-Thr). Mass spectrum (FAB): (M + H) 1109.6; Calcd mass for $C_{49}H_{49}F_5N_4O_{20}$: 1108.3.

Solid-phase synthesis of glycopeptide **21**.—Compound **21** was synthesized on a custom-made, fully automatic, continuous-flow peptide synthesizer with real time monitoring, feedback control, and in-line dissolution of amino acids. The Macrosorb resin **23** (450 mg, substitution 97 mmol/g), which had been derivatized with ethylenediamine,

norleucine, the PAL-linker, and the first amino acid Thr(*t*-Bu), was placed in a glass column through which the reagents were pumped or circulated with a flow rate of 1.44 mL/min. Fmoc deprotections were effected with 50% morpholine in DMF for 60 min. The suitably protected nonglycosylated amino acids (3 equiv) were activated as Dhbt esters; the glycosyl amino acid Pfp ester **4** (1.5 equiv) was coupled with the addition of Dhbt-OH (1.5 equiv). The acylation reactions were monitored by a solid-phase spectrophotometer following the disappearance of the yellow ion pair of Dhbt-OH and unreacted amino groups [26]. After each Fmoc deprotection or coupling reaction, the resin was washed with DMF for 20 min. The assembly of the glycopeptide was continued according to the peptide sequence starting with Ser-2 at the C-terminal end with the following acylation times: Ser-2, 10 h; Ile-3, 3 h; Pro-4, 3.7 h; Thr-5, 22 h; Thr-6, 13 h; Thr-7, 5,3 h; Pro-8, 14 h. After completion of the peptide synthesis, the Fmoc group was removed and the terminal amino groups were acetylated with 7:1 DMF–Ac_2O (40 min). Washing with DMF, Et_2O, and CH_2Cl_2 and drying afforded 550 mg of resin. The azide reduction was performed after transferring the solid support into a reaction flask. An aliquot (269 mg) of the polymer was treated with freshly distilled (GC > 99.9%) thioacetic acid (4 mL). The reaction was followed by IR spectroscopy through the disappearance of the azide absorption band at νKBr 2117 cm^{-1}. After 48 h the azide band had disappeared completely. The resin was carefully washed with CH_2Cl_2, MeOH, toluene, and CH_2Cl_2 (5 × 5 mL of each solvent) and dried. The protected O-glycopeptide was cleaved from the polymer with concurrent removal of the *t*-Bu side-chain protecting groups by treating the resin with aq 95% CF_3CO_2H (6 mL) for 2 h. The resin was washed extensively with CF_3CO_2H (6 × 5 mL, 2 min each). The combined filtrates were evaporated and the residue was coevaporated several times with toluene. The protected compound was purified by preparative HPLC [Buffer *A*–Buffer *B*, 70:30 → 50:50 (40 min) → 20:80 (30 min)]. Yield 27 mg (47%). The protected glycopeptide **21** (9 mg, 3.3 μmol) was dissolved in MeOH (2 mL) and treated with a 1 M solution of NaOMe in MeOH (33 μL). The deacetylation was followed by analytical HPLC [Buffer *A*–Buffer *B*, 95:5 → 85:15 (20 min) → 50:50 (10 min)]. After 4 h a single peak indicated the end of the reaction. The final product **21** was purified by preparative HPLC [Buffer *A*–Buffer *B*, 95:5 → 85:15 (20 min) → 50:50 (30 min)]. Yield 6.1 mg (94% in the last reaction, the overall yield for the glycopeptide assembly was 44%). ^{1}H NMR and FABMS data are presented in Tables 1 and 2.

Multiple-column peptide synthesis of the glycopeptides **14–20** *and* **29–46**.—The glycopeptides **14–20** and **29–46** were synthesized simultaneously in a 20-well multiple-column peptide synthesizer (MCPS) [27]. The custom-made synthesizer consisted of a Teflon block with 20 columns for parallel syntheses. Each column was equipped with a sintered Teflon filter at the bottom and the outlet was connected to a chamber beneath for either vacuum or N_2 pressure. The delivery of solvents and reagents was performed manually using an Eppendorf Multipette. Solvents were removed from the columns by applying a vacuum for 2 min. The derivatized resin **23** (100 mg/column; substitution, 0.202 mmol/g) was distributed into the columns. Each sequence was synthesized in a separate well except for compound **14** which was needed in a larger quantity and therefore synthesized in 4 wells. Fmoc deprotections were effected with 50% morpholine in DMF (600 μL, 2 min + 2 × 30 min). A small amount of the red dye azorubin (0.01%) was added

to the deprotection reagent in order to indicate complete removal of the base. The resin was washed with DMF (750 μL) 8 times after Fmoc deprotections and 5 times after coupling reactions. N^{α}-Fmoc-Ser(*t*-Bu) and N^{α}-Fmoc-Thr(*t*-Bu) were activated as Dhbt esters (3 equiv); all other amino acids (3 equiv) or the glycosyl amino acids **2** and **4** (1.5 equiv) were coupled as Pfp esters with the addition of equimolar amounts of Dhbt-OH. For the assembly of the sequences **29–46**, the α,β-mixture of the building block **4** (α:β = 1.1:1) was used. The coupling reactions were each carried out in a volume of 600 μL of DMF. The parallel synthesis started at the C-terminus with Ser-2 and was continued until the N-terminus by the application of the respective activated amino acids. During the acylation reactions the Teflon block was placed on a rocker table and gently shaken while a slight N_2 overpressure in the chamber below the wells was established to keep the solvent in the columns. The activated amino acids were allowed to react overnight (19–24 h), Thr-5 was coupled for 48 h. The disappearance of the yellow Dhbt anion indicated a complete acylation reaction for all couplings. After coupling of the last amino acid, the Fmoc group was removed and the terminal amino groups were acetylated with 7:1 DMF–Ac_2O (500 μL, 2 min + 2 × 20 min). The resin was washed with DMF, Et_2O, and CH_2Cl_2 (750 μL, 5 times each) and dried for 1 h by air suction. The reduction of the azido groups was performed with thioacetic acid (-ci1 mL, 80 h) as described above. The thioacetic acid was renewed every 20 h and the resin was washed carefully before the addition with CH_2Cl_2 (750–1000 μL, 6 × 2 min). The IR spectra of each resin-bound glycopeptide (3 mg in 110 mg KBr) indicated a quantitative reduction. The resin was washed with CH_2Cl_2 (750–1000 μL, 5 × 2 min), MeOH, toluene, and CH_2Cl_2 (750–1000 μL, 10 × 2 min each) and dried. The glycopeptides were cleaved from the polymer outside the synthesizer in separate reaction flasks by a 2-h treatment with aq 95% CF_3CO_2H (compound **14**, 12 mL; all other sequences, 3 mL). The solutions were filtered and the resins were carefully washed 5 times with CF_3CO_2H. The combined CF_3CO_2H fractions of each sequence were concentrated under reduced pressure, and the residues were concentrated twice with toluene and 1:1 toluene–MeOH. The crude products were dissolved in MeOH, filtered, and purified by HPLC [Buffer *A*–Buffer *B*, 70:30 → 50:50 (40 min) → 20:80 (30 min)]. The fractions of each glycopeptide were collected and concentrated. The separation of the α,β anomers of the glycopeptides **29–46** was not possible at this step. The deacetylation of the carbohydrate moieties was performed with a solution of NaOMe in MeOH. The glycopeptides were dissolved in MeOH (compound **14**, 10 mL; all other sequences, 4 mL) and treated with a 1 M solution of NaOMe in MeOH (47 μL; sequence **14**, 105 μL) at room temperature for 3.5–4 h. The reactions were controlled by TLC (1:2 H_2O–MeCN) or by analytical HPLC [Buffer *A*–Buffer *B*, 95:5 → 80:20 (20 min) → 50:50 (10 min)]. The solutions were neutralized with AcOH (47 μL; compound **14**, 105 μL), filtered, and concentrated. The final purification of the glycopeptides **14–20** and the separation of the α,β anomers of compounds **29–46** was performed by HPLC [compounds **14–20**, Buffer *A*–Buffer *B* 95:5 → 80:20 (30 min) → 50:50 (10 min); compounds **29–46**, Buffer *A*–Buffer *B*, 90:10 → 80:20 (30 min) → 40:60 (10 min)]. The fractions of each sequence were collected, concentrated, and lyophilized. All glycopeptides were pure according to analytical HPLC, ^1H NMR spectroscopy, and FABMS. Yields, FABMS data, and ^1H NMR data are presented in Tables

2 and 3; compound **14** was synthesized previously and the analytical data were as reported [17].

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