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Solid-phase synthesis and conformational studies of glycosylated derivatives of helper-T-cell immunogenic peptides from hen-egg lysozyme

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ABSTRACT

3-Mercaptopropionic acid and N^{α}-Fmoc serine, both with unprotected carboxyl groups, were stereospecifically glycosylated in 62–82% yields, using saccharide 1,2-*trans* peracetates and Lewis acid catalysis. The resulting glycosylated building blocks were used in the synthesis of derivatives of helper-T-cell stimulating peptides, with the carbohydrate moiety located at the amino terminus, or internally in the peptide chain. ^{1}H NMR spectroscopy in Me_2SO-d_6 showed that the glycopeptides assumed random conformations, which were not influenced by the glycosylation or by single substitutions of amino acids in the peptide moiety.

INTRODUCTION

Peptides have a central role in activation of T cells in the immune system[1]. Foreign protein antigens that enter an organism are internalized and metabolized[2] by macrophages and B cells into peptide fragments, usually composed of 13–17 amino acids[3]. The peptides are bound by class II MHC molecules, and the complexes are presented on the cell surface. Recognition of peptide–MHC II complexes by helper T cells results in stimulation of the T cells and activation of the immune system towards the foreign antigen. Protein antigens produced intracellularily due, for example, to a virus infection are handled in a similar way[1].

Carbohydrates are important antigens on cancer cells, infectious bacteria, and viruses, but they do not bind to MHC molecules[4,5], and give a weaker, T cell independent, immune response. We have therefore started an investigation of the helper T cell response towards *glyco*peptides and have immunized mice with the glycopeptide **10**[6]. Compound **10** has the disaccharide galabiose [α-D-Gal*p*-(1 → 4)-β-D-Gal*p*; part of a tumor-associated antigen in Burkitt Lymphoma[7]] linked to the

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amino terminus of a helper-T-cell stimulating peptide consisting of amino acids 52–61 from hen-egg lysozyme [HEL(52–61)][8]. We now describe the synthesis of nine analogues of **10**, modified in either the carbohydrate or the peptide moiety. These analogues will be used in investigations of the selectivity of helper T cell clones towards different parts of the antigen **10**. A similar investigation by Ishioka et al.[5] was published recently.

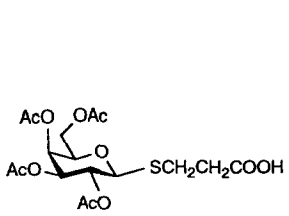
In recent years, there has been an increasing interest in the chemical synthesis of glycopeptides (reviewed in refs 9 and 10, cf. refs 11–17 for leading references to recent work). The direct *O*-glycosylation of peptides is restricted by the solubility[18] of the peptide and yields have often decreased with increasing peptide length[19,20]. Improved yields have been achieved by employment of a large (40-fold) excess of glycosyl donor[11]. The alternative approach, utilizing glycosylated amino acids in the stepwise synthesis of glycopeptides, has been reported as an efficient and reliable method by several groups[12–18].

Protective groups used in glycopeptide synthesis must be removed under mild conditions since glycosides are sensitive to strong acid, and glycosides of serine and threonine can undergo β-elimination and racemization on treatment with base[9,18,21]. The N^{α}-fluoren-9-ylmethoxycarbonyl (Fmoc) group[22] is cleaved by weak bases, such as morpholine, without base-catalyzed side reactions[9]. Protection of the hydroxyl groups of the carbohydrate with acetyl or benzoyl groups stabilizes the glycosidic bonds towards the trifluoroacetic acid used for cleavage from the resin and for side-chain deprotection[9,23]. *O*-Acetyl groups can be removed easily with methanolic potassium cyanide[18], ammonia[18], sodium methoxide[18], or hydrazine[9]. Benzoates are less reactive and require prolonged reaction times for removal, with an accompanying risk of side reactions[18]. In the glycosylation of amino acids, the α-carboxyl group can be temporarily protected[9], preferably as an active ester[14], or even left unprotected[6].

RESULTS AND DISCUSSION

We have described[6] the synthesis of the glycosylated 3-mercaptopropionic acid derivatives **1** and **3** (90% yields) by direct glycosylation of 3-mercaptopropionic acid with β-D-galactose pentaacetate and β-D-galabiose octaacetate[24] (**2**), respectively. By the same procedure, 3-mercaptopropionic acid was glycosylated with the 1,2-*trans* peracetates of lactose and cellobiose (**4** and **6**) in dichloromethane, catalyzed by boron trifluoride etherate[25], to give the building blocks **5** and **7** (82 and 62% yield, respectively). Glycosylation of N^{α}-Fmoc-L-serine (**8**) with **2**, in acetonitrile with boron trifluoride etherate as catalyst, gave **9** (64% yield), after medium-pressure reversed-phase chromatography.

The glycosylated building blocks **1**, **3**, **5**, **7**, and **9** allow activation by a variety of methods and can thus be employed in glycopeptide synthesis without further manipulations of protective groups. Here, we describe their use in the synthesis of glycopeptides **11**–**17**, **19**, and **20**, which are derivatives of helper-T-cell stimulating



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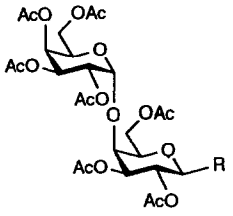

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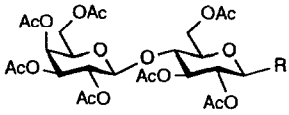

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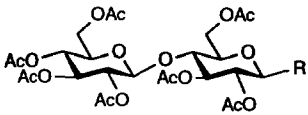

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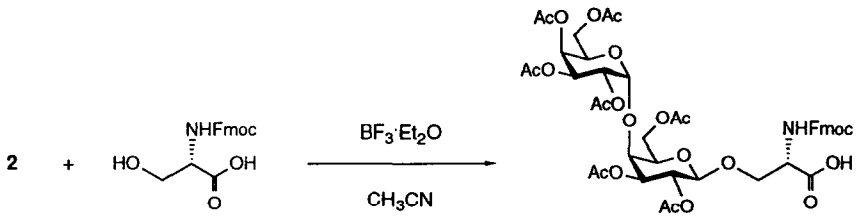


peptides[8] from hen-egg lysozyme (HEL). The glycopeptides were synthesized as *C*-terminal amides on a polystyrene resin functionalized with the Rink linker **21**[26,27], using *N*,*N*-dimethylformamide (DMF) as solvent in a mechanically agitated reactor. N^{α}-Fmoc amino acids, carrying standard side-chain protective groups, and the mercaptopropionic acid glycosides **1**, **3**, **5**, and **7** were coupled to the resin as 1-hydroxybenzotriazole (HOBt) esters[28]. The HOBt-esters were prepared in situ by addition of 1,3-diisopropylcarbodiimide to the appropriate acid and HOBt in DMF. Attempts to couple the glycosylated serine derivative **9** as a HOBt ester failed in the synthesis of **17**. Instead, **9** was converted in situ into the corresponding pentafluorophenyl (Pfp) ester, which was successfully coupled in the presence of HOBt as an auxiliary nucleophile. Three equivalents, relative to the resin capacity, of N^{α}-Fmoc amino acids and of the glycosylated building blocks **1**, **3**, **5**, **7**, and **9**

$RSCH_2CH_2CO$-Asp^{52}-Tyr-Gly-Ile-Leu-Gln-Ile-Asn-Ser-Arg^{61}-NH_2

10 R=A
11 R=B
12 R=C
13 R=D

$RSCH_2CH_2CO$-Asp^{52}-AA1-Gly-Ile-AA2-Gln-Ile-Asn-Ser-Arg^{61}-NH_2

14 R=A, AA1=Tyr, AA2=Phe
15 R=A, AA1=Tyr, AA2=Ala
16 R=A, AA1=Ala, AA2=Leu

Asp^{52}-Tyr-Gly-Ile-Leu-Gln-Ile-Asn-NH-CH(CH_2OR)-CO-Arg^{61}-NH_2

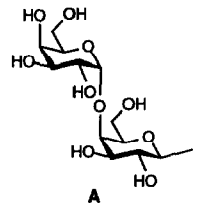
17 R=A
18 R=H

$RSCH_2CH_2CO$-Asn^{46}-Thr-Asp-Gly-Ser-Thr-Asp-Tyr-Gly-Ile-Leu-Gln-Ile-Asn-Ser-Arg^{61}-NH_2

19 R=A

$RSCH_2CH_2CO$-Phe^{34}-Glu-Ser-Asn-Phe-Asn-Thr-Gln-Ala-Thr-Asn-Arg^{45}-NH_2

20 R=A



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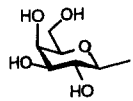

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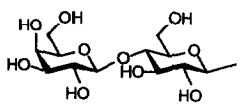

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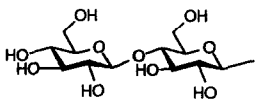

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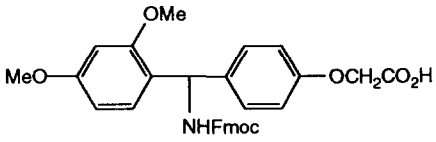
were used in the couplings. The excess of the glycosylated building blocks used in the couplings could be recovered. For instance, 70% of the excess of **9** was recovered after aqueous workup of the reaction solution and flash column chromatography. Acylations were monitored by addition of Bromophenol Blue[29] to the reactor, and by the ninhydrin test[30]. We found that an excess of HOBt was required for reliable monitoring with Bromophenol Blue. N^{α}-Fmoc deprotection was performed with 20% piperidine in DMF or, in the synthesis of **17**, with 50% morpholine in DMF.

The glycopeptides were cleaved from the resin, and the amino acid side chains were simultaneously deprotected, by treatment with trifluoroacetic acid. The



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trifluoroacetic acid contained water, thioanisole, and ethanedithiol as scavengers for the cations generated from the protective groups of the amino acid side chains[31]. Purification by preparative reversed-phase HPLC gave glycopeptides having *O*-acetyl-protected carbohydrate moieties. Deacetylation with saturated methanolic ammonia[18] for 3–18 h and purification by preparative reversed-phase HPLC gave the glycopeptides **11–17**, **19**, and **20** in 10–48% overall yields based on the resin capacity (cf. Table I). Side reactions such as elimination or racemization were not observed by HPLC, nor by ^{1}H NMR spectroscopy, after deacetylation of the glycopeptides. In the synthesis of glycopeptide **19**, very long coupling times were required for the three *N*-terminal amino acids Asn^{46}, Thr^{47}, and Asp^{48}. The coupling of Asn^{46} to Thr^{47} did not reach completion and unreacted Thr^{47} was therefore *N*-acetylated. The prolonged coupling times and the incomplete coupling of Asn^{46} to Thr^{47} could reflect aggregation of the growing peptide chains, which explains the low yield obtained for **19**.

The glycopeptides were characterized by ^{1}H NMR spectroscopy (cf. Tables II and III) as well as by FABMS and amino acid analysis. No significant differences were found for the ^{1}H-chemical shifts and for the qualitative NOEs for the common peptide fragment Asp^{52} → Arg^{61} in the *N*-terminally glycosylated decapeptides (**10** and **11**) and the longer glycopeptide **19**. Glycosylation at the *N*-terminus therefore has no effect on the conformation of the peptide fragment Asp^{52} → Arg^{61}, as compared to an *N*-terminal elongation of the peptide. Comparison of chemical shifts and of qualitative NOEs for the peptide part of glycopeptide **17** (which has galabiose linked to Ser^{60}) and the non-glycosylated **18** showed only a minor downfield shift (~ 0.25 ppm) for the α proton and one of the β protons in Ser^{60} of **17**. These shift differences are expected due to substitution effects[32]. Similarly, substitutions of Tyr^{53} and Leu^{56} by Ala and Phe in glycopeptide **10** (compounds **14–16**) did not significantly influence the observed NMR parameters.

TABLE I

Yields, based on resin capacity, obtained in the synthesis of glycopeptides **11–17**, **19**, and **20**

Compound	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**19**	**20**
Yield (%)	41	30	44	48	48	30	40	10	37

TABLE II

^{1}H NMR chemical shifts [a] (δ, ppm) in Me_2SO-d_6 for the peptide part of peptides **10**, **11**, and **14–19** [b]

Position	Amino acid	Proton	**10** (ref 6)	**11**	**14**	**15**	**16**	**17**	**18**	**19** [c]
52	Asp	NH	8.24	8.28	8.22	8.26	8.32			8.23
		α	4.65	4.65	4.65	4.66	4.69	4.07	3.83	4.68
		β	2.75, 2.51	2.74, 2.49	2.75, 2.52	2.77, 2.52	2.82, 2.60	2.90, 2.67	2.64, 2.40	2.77, 2.55
53	Tyr	NH	7.87	7.86	7.96	7.88		8.67	8.60	7.92
		α	4.45	4.43	4.47	4.48		4.57	4.51	4.45
		β	3.00, 2.82	2.99, 2.82	3.04, 2.85	3.02, 2.83		3.06, 2.79	3.05, 2.81	3.01, 2.82
		Arom	7.07, 6.71	7.07, 6.72	7.09, 6.71	7.09, 6.73		7.16, 6.79	7.13, 6.74	7.10, 6.72
		OH			9.25	9.25		9.30	9.28	9.23
	Ala	NH					8.05			
		α					4.30			
		β					1.34			
54	Gly	NH	8.26	8.24	8.31	8.25	8.13	8.45	8.43	8.26
		α	3.87, 3.80	3.82	3.82	3.83	3.84	3.88	3.90, 3.81	3.83
55	Ile	NH	7.86	7.87	7.85	7.88	7.78	7.92	7.92	7.85
		α	4.30	4.29	4.22	4.32	4.27	4.32	4.30	4.31
		β	1.82	1.81	1.78	1.82	1.82	1.82	1.85	1.85
		γ	1.50, 1.17	1.53, 1.18	1.40, 1.12	1.53, 1.20	1.50, 1.18	1.51, 1.17	1.51, 1.19	1.54, 1.18
		β,γ-CH_3	0.92	0.93	0.83	0.94	0.89, 0.91	0.92	0.92, 0.90	0.98
56	Leu	NH	8.12	8.12			8.09	8.20	8.17	8.12
		α	4.39	4.39			4.38	4.42	4.39	4.42
		β	1.56	1.55			1.56	1.57	1.58	1.71
		γ	1.69	1.69			1.68	1.70	1.69	1.58
		γ-CH_3	0.97, 0.93	0.97, 0.92			0.98, 0.92	0.98	0.98, 0.93	0.99
	Phe	NH			8.20					
		α			4.64					
		β			2.01, 1.88					
		Arom			7.35, 7.27					
	Ala	NH				8.26				
		α				4.39				
		β				1.32				

TABLE II (continued)

Position	Amino acid	Proton	**10** (ref 6)	**11**	**14**	**15**	**16**	**17**	**18**	**19** [c]
57	Gln	NH	8.05	8.05	8.08	8.04	8.04	8.07	8.09	8.05
		α	4.35	4.34	4.40	4.35	4.34	4.38	4.34	4.37
		β	1.95, 1.84	1.95, 1.83	2.01, 1.88	1.98, 1.84	1.96, 1.84	1.96, 1.85	1.97, 1.85	1.98, 1.87
		γ	2.18	2.17	2.24	2.21	2.18	2.19	2.18	2.20
		γ-$CONH_2$	7.33, 6.86	7.33, 6.87	7.39, 7.08	7.33, 6.87	7.35, 6.87	7.38, 6.88	7.38, 6.88	7.33, 6.88
58	Ile	NH	7.81	7.83	7.93	7.84	7.82	7.85	7.84	7.82
		α	4.26	4.25	4.29	4.27	4.26	4.30	4.25	4.30
		β	1.78	1.77	1.82	1.81	1.79	1.81	1.79	1.82
		γ	1.49, 1.15	1.47, 1.13	1.51, 1.19	1.52, 1.17	1.50, 1.14	1.50, 1.16	1.50, 1.15	1.52, 1.20
		β,γ-CH_3	0.89	0.88	0.93	0.93	0.90, 0.89	0.90	0.92, 0.90	0.93
59	Asn	NH	8.30	8.30	8.31	8.30	8.30	8.30	8.33	8.29
		α	4.68	4.68	4.69	4.70	4.69	4.70	4.68	4.71
		β	2.69, 2.55	2.68, 2.54	2.71, 2.58	2.71, 2.55	2.70, 2.55	2.71, 2.55	2.70, 2.56	2.72, 2.57
		β-$CONH_2$	7.59, 7.05	7.58, 7.06	7.59, 7.08	7.59, 7.07	7.60, 7.06	7.57, 7.08	7.60, 7.06	7.59, 7.06
60	Ser	NH	8.00	8.01	8.05	8.02	8.00	8.11	8.05	8.00
		α	4.28	4.28	4.28	4.28	4.28	4.53	4.27	4.31
		β	3.76, 3.62	3.75, 3.62	3.78, 3.63	3.28, 3.15	3.76, 3.62	4.13, 3.63	3.77, 3.63	3.79, 3.66
		OH		5.15	5.17	5.14				5.13
61	Arg	NH	8.14	8.15	8.16	8.15	8.14	8.25	8.12	8.14
		α	4.22	4.22	4.22	4.22	4.22	4.22	4.22	4.23
		β	1.89, 1.64	1.87, 1.64	1.91, 1.64	1.90, 1.63	1.89, 1.64	1.90, 1.63	1.90, 1.65	1.92, 1.63
		γ	1.60	1.57	1.64	1.63	1.57	1.60	1.58	1.63
		δ	3.18	3.18	3.20	3.21	3.19	3.20	3.18	3.22
		δ-NH	7.55	7.54	7.68	7.62	7.56	7.65	7.84	7.53
		α-$CONH_2$	7.20	7.20, 7.08	7.20, 7.08	7.21	7.22, 7.20	7.22, 7.16	7.21	7.20
	SCH_2-CH_2CO		2.88, 2.82, 2.53, 2.48	2.88, 2.48	2.88, 2.75, 2.55	2.85, 2.51	2.92, 2.83, 2.55			2.88, 2.83, 2.57

[a] Obtained at 500 MHz and 300 K with residual Me_2SO-d_5 (δ_H 2.60) as internal standard. [b] Data for **20** are given in the Experimental. [c] Amino acids 46–51 in **19**: δ Asn[46] 8.28 (NH), 7.50 and 7.02 (β-$CONH_2$), 4.77 (Hα), 2.73 (Hβ), 2.54 (Hβ'); Thr[47] 7.86 (NH), 5.06 (OH), 4.27 (Hα), 4.18 (Hβ), 1.15 (Hγ); Asp[48] 8.31 (NH), 4.70 (Hα), 2.85 (Hβ), 2.65 (Hβ'); Gly[49] 8.00 (NH), 3.88 (Hα), Ser[50] 7.98 (NH), 4.53 (Hα), 3.73 (Hβ), 3.68 (Hβ'); Thr[51] 7.90 (NH), 4.42 (Hα), 4.16 (Hβ), 1.15 (Hγ).

TABLE III

^{1}H NMR chemical shifts [a] (δ, ppm) in Me_2SO-d_6 for the carbohydrate part of glycopeptides **10**, **11**, **14–17**, and **19**

Proton	**10** (ref 6)	**11**	**14**	**15**	**16**	**17**	**19**
H-1	4.38	4.34	4.38	4.40	4.37	4.28	4.39
H-2	3.42	3.40	3.43		3.41	3.38	3.46
H-3	3.47	3.50	3.48	3.47	3.47	3.50	3.62
H-4	3.96	3.80	3.96	3.97	3.95	3.91	3.97
H-5	3.59	3.63	3.61	3.61	3.60	3.58	3.58
H-6	3.78, 3.65		3.78, 3.63	3.78		3.82, 3.70	3.77, 3.65
H-1′	4.93		4.92	4.93	4.91	4.92	4.98
H-2′	3.75		3.77	3.76	3.76	3.77	3.76
H-3′	3.66		3.67	3.67	3.66	3.67	3.67
H-4′	3.84		3.85	3.85	3.85	3.85	3.86
H-5′	4.16		4.17	4.17	4.16	4.22	4.17
H-6′	3.63, 3.56		3.60, 3.57	3.57	3.60, 3.56	3.58	3.61, 3.57

[a] Obtained at 500 MHz and 300 K with residual Me_2SO-d_5 (δ_H 2.60) as internal standard.

Consequently, neither glycosylation of Ser^{60} nor amino acid substitution has any influence on the overall peptide conformation for these glycopeptides.

It was suggested that the peptide **18** [HEL(52–61)] was recognized by helper T cells when bound in an α-helical conformation by class II MHC molecules[8]. Circular dichroism studies also showed[33] significant α-helicity for **18** in aqueous trifluoroethanol, but not in water. In Me_2SO-d_6, **18** and its glycosylated derivatives **10**, **11**, **14–17**, and **19** all displayed strong H$\alpha \rightarrow$ NH (i,$i+1$) and weak to medium NH $\rightarrow$ NH (i,$i+1$) qualitative NOEs for all residues in the peptide parts. These NOE patterns are compatible[34] with random conformations for the peptide backbone, but not with an α-helix or β-turns. The coupling constants $^3J_{H\alpha\text{-}NH}$ were previously determined[6] to be 6 to 8 Hz for **10**, further supporting[34] a random structure for the glycopeptides.

The galabiose moieties in the glycopeptides all showed a strong NOE between H-4 and H-1′ as well as a downfield shift of H-5′. These effects were also observed in an investigation of the conformation of methyl β-D-galabioside in Me_2SO-d_6 solution[35]. Although the two effects do not permit a conclusive determination of the galabiose conformation[35], they indicate that the conformation resembles the one adopted in aqueous solution (Φ_H, $\Psi_H = -39, -15$), in contrast to the conformation in the galabiose crystal[36] (Φ_H, $\Psi_H = -18, -35$).

EXPERIMENTAL

General.—TLC was performed on Silica Gel 60 F_{254} (Merck) with detection by UV light and charring with H_2SO_4. Flash column chromatography was performed on Silica Gel 60 (Grace Amicon, 35–70 μm) with distilled solvents. Medium-pressure reversed-phase chromatography was performed on a Lichroprep C-18 column

(40–63 μm, 2.5 × 31 cm). Immediately before use, CH_2Cl_2 was dried by distillation from CaH_2, and MeCN was passed through a column of neutral Al_2O_3 (activity 1). Organic solutions were dried over Na_2SO_4.

The ^1H and ^{13}C NMR spectra were recorded with a Varian XL-300 or a General Electric Ω-500 spectrometer for solutions in $CDCl_3$ [residual $CHCl_3$ (δ_H 7.26) and $CDCl_3$ (δ_C 77.0) as internal standards], acetone-d_6 [residual acetone-d_5 (δ_H 2.05) and acetone-d_6 (δ_C 29.8)] or Me_2SO-d_6 [residual Me_2SO-d_5 (δ_H 2.60)]. First-order chemical shifts and coupling constants were obtained from one-dimensional spectra, and proton resonances were assigned from COSY[37], TOCSY[38], and ROESY[39] experiments. Proton resonances that could not be assigned are not reported. Optical rotations were measured with a Perkin–Elmer 141 polarimeter. Positive fast atom bombardment mass spectra were recorded on a Jeol SX 102 A mass spectrometer. Ions were produced by a beam of Xe atoms (6 keV) from a matrix of glycerol and thioglycerol. In the amino acid analyses, asparagine and glutamine were determined as aspartic acid and glutamic acid, respectively.

3-(2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosylthio)propionic acid[6] (**1**), 1,2,3,6-tetra-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)-β-D-galactopyranose[24] (**2**), and 1,2,3,6-tetra-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-β-D-glucopyranose[40] (**6**) were prepared according to the indicated literature methods.

Compound **9** decomposed on drying and a satisfactory elemental analysis could not be obtained. The purity of **9** was instead established by TLC, HPLC, and NMR spectroscopy. Glycopeptides **12** and **13** were prepared from the peptide resin used in the preparation of **18**. The structure of the non-glycosylated peptide **18** was established by ^1H NMR spectroscopy. The structures and purities of the glycosylated building blocks **5** and **7**, used in the preparation of **12** and **13**, were also established separately, and an NMR study of **12** and **13** was therefore not performed.

General procedure for solid-phase glycopeptide synthesis.—Glycopeptides were synthesized using DMF as solvent in a mechanically agitated reactor, on an aminomethylated polystyrene resin functionalized with the linker *p*-[α-(fluoren-9-ylmethoxyformamido)-2,4-dimethoxybenzyl]phenoxyacetic acid[26,27] (Novabiochem). For each glycopeptide, 0.25 g of resin (0.4 mequiv/g, 0.10 mmol) was used. Reagent solutions and DMF for washing were added manually to the reactor. N^{α}-Fmoc Amino acids (Bachem) with the following protective groups were used: 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) for arginine; triphenylmethyl (Tr) for asparagine and glutamine; and *tert*-butyl for aspartic acid, glutamic acid, serine, threonine, and tyrosine.

The glycosylated N^{α}-Fmoc-L-serine derivative **9** was coupled as the pentafluorophenyl ester. This was prepared, in situ, by addition of 1,3-diisopropylcarbodiimide (45 μL, 0.29 mmol) to **9** (284 mg, 0.30 mmol) and pentafluorophenol (55 mg, 0.30 mmol) in dry CH_2Cl_2 (3 mL) at room temperature. After 1.5 h, the solution was concentrated, the residue and 1-hydroxybenzotriazole (HOBt, 41 mg, 0.30

mmol) were dissolved in DMF (0.7 mL), and the solution was added to the reactor. All other N^{α}-Fmoc amino acids and the mercaptopropionic acid glycosides **1**[6], **3**[6], **5**, and **7** were coupled as HOBt esters[28]. These were prepared, in situ, from the appropriate acid (0.30 mmol), HOBt (61 mg, 0.45 mmol), and 1,3-diisopropylcarbodiimide (45 μL, 0.29 mmol) in DMF (1 mL). After 30–60 min, the solution was added to the reactor. Acylations were monitored by addition of Bromophenol Blue[29] (0.05% of the resin capacity) to the reactor, and by the ninhydrin test[30]. N^{α}-Fmoc deprotection was effected by treatment with 20% piperidine in DMF (2 + 8 min) or, in the synthesis of **17**, with 50% morpholine in DMF (2 + 2 × 15 min).

After completion of the synthesis, the resin was washed with CH_2Cl_2 (5 × 5 mL) and dried under vacuum. The glycopeptide (0.10 mmol) was then cleaved from the resin, and the amino acid side chains were deprotected, by treatment with CF_3CO_2H–H_2O–thioanisole–ethanedithiol (87.5:5:5:2.5, 25 mL) for 2–3 h, followed by filtration. Acetic acid (15 mL) was added to the filtrate, the solution was concentrated, and the crude peptide solidified on trituration with Et_2O (2 × 10 mL). The Et_2O solutions were decanted, the peptide was dried and then dissolved in acetic acid–H_2O (1:1), and the solution was diluted with water and freeze-dried. Purification by preparative HPLC gave *O*-acetylated glycopeptide which was deacetylated in satd methanolic ammonia (1.5 mL/mg of glycopeptide) at room temperature for 3–18 h. Concentration of the solution and purification of the residue by preparative HPLC gave pure glycopeptide.

Preparative HPLC separations were performed on a Beckman System Gold HPLC, using a Kromasil C-8 column (1000 Å, 20 × 250 mm) with a flow rate of 12 or 14 mL/min, detection at 214 nm, and the following solvent systems: *A*, aq 0.1% CF_3CO_2H; and *B*, 0.1% CF_3CO_2H in MeCN.

*3-[2,3,6-Tri-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-α-*D*-galactopyranosyl)-β-*D*-galactopyranosylthio]propionic acid* (**3**).—Compound **3** was prepared as described earlier[6] and had $[\alpha]_D^{25}$ +58° (*c* 0.76, $CHCl_3$). ^{1}H NMR data ($CDCl_3$, 300 MHz): δ 5.56 (d, 1 H, *J* 2.3 Hz, H-4′), 5.47 (dd, 1 H, *J* 11.0, 3.3 Hz, H-3′), 5.37 (t, 1 H, *J* 10.1 Hz, H-2), 5.21 (dd, 1 H, *J* 11.0, 3.6 Hz, H-2′), 4.99 (d, 1 H, *J* 3.6 Hz, H-1′), 4.87 (dd, 1 H, *J* 10.3, 2.6 Hz, H-3), 4.57 (t, 1 H, *J* 7.2 Hz, H-5′), 4.50 (d, 1 H, *J* 9.7 Hz, H-1), 4.42 (dd, 1 H, *J* 11.4, 6.8 Hz, H-6), 3.81 (t, 1 H, *J* 6.4 Hz, H-5), 3.15–3.05 and 2.87–2.76 (2 m, 4 H, $SCH_2CH_2CO_2H$), 2.14, 2.11, 2.08, 2.07, 2.06, 2.05, and 1.99 (7 s, each 3 H, 7 Ac). Anal. Calcd for $C_{29}H_{40}O_{19}S$: C, 48.1; H, 5.6. Found: C, 47.8; H, 5.7.

*3-[2,3,6-Tri-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-*D*-galactopyranosyl)-β-*D*-glucopyranosylthio]propionic acid* (**5**).—Boron trifluoride etherate (139 μL, 1.11 mmol) was added to a solution of 1,2,3,6-tetra-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-*D*-galactopyranosyl)-β-*D*-glucopyranose (**4**; 500 mg, 0.737 mmol) and 3-mercaptopropionic acid (257 μL, 2.95 mmol) in dry CH_2Cl_2 (10 mL) at room temperature. After 3 h, the solution was diluted with CH_2Cl_2 (30 mL) and washed with 1 M HCl (40 mL). The aqueous phase was extracted with CH_2Cl_2 (2 × 20 mL). The

combined organic phases were dried and concentrated. Flash column chromatography (toluene–MeOH–acetic acid, 100:5:2) of the residue gave **5** (436 mg, 82%), $[\alpha]_D^{25}$ −14° (*c* 1.4, $CHCl_3$). NMR data ($CDCl_3$): ^1H (300 MHz), δ 5.35 (dd, 1 H, *J* 3.4, 1.0 Hz, H-4′), 5.22 (t, 1 H, *J* 9.2 Hz, H-3), 5.11 (dd, 1 H, *J* 10.4, 7.8 Hz, H-2′), 4.96 (dd, 1 H, *J* 10.4, 3.4 Hz, H-3′), 4.93 (t, 1 H, *J* 9.7 Hz, H-2), 4.52 (d, 1 H, *J* 9.9 Hz, H-1), 4.50 (1 H, H-6), 4.49 (d, 1 H, *J* 7.8 Hz, H-1′), 3.88 (bt, 1 H, *J* 6.6 Hz, H-5′), 3.78 (t, 1 H, *J* 9.4 Hz, H-4), 3.62 (ddd, 1 H, *J* 9.9, 5.3, and 1.9 Hz, H-5), 3.01-2.70 (m, 4 H, $SCH_2CH_2CO_2H$), 2.16, 2.13, 2.07, 2.05, 2.05, 2.05, and 1.97 (7 s, each 3 H, 7 Ac); ^{13}C (75 MHz), δ 101.0 (C-1′) and 83.9 (C-1). Anal. Calcd for $C_{29}H_{40}O_{19}S$: C, 48.1; H, 5.6. Found: C, 47.3; H, 5.4.

*3-[2,3,6-Tri-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-β-*D*-glucopyranosyl)-β-*D*-glucopyranosylthio]propionic acid* (**7**).—Glycosylation of 3-mercaptopropionic acid (122 μL, 1.41 mmol) with **6**[40] (253 mg, 0.372 mmol) for 1.5 h, as described for **5**, with flash column chromatography (heptane–EtOAc–acetic acid, 95:95:10) of the residue, gave **7** (168 mg, 62%), $[\alpha]_D^{25}$ −34° (*c* 1.5, $CHCl_3$). NMR data ($CDCl_3$): ^1H (300 MHz), δ 5.18 (t, 1 H, *J* 9.3 Hz, H-3′), 5.15 (t, 1 H, *J* 9.4 Hz, H-3), 5.06 (t, 1 H, *J* 9.5 Hz, H-4), 4.92 (t, 1 H, *J* 10.0, 9.7 Hz, H-2), 4.92 (t, 1 H, *J* 9.7, 7.1 Hz, H-2′), 4.54 (m, 1 H, H-6′), 4.52 (d, 1 H, *J* 7.6 Hz, H-1′), 4.51 (d, 1 H, *J* 10.2 Hz, H-1), 4.37 (dd, 1 H, *J* 12.4, 4.1 Hz, H-6), 4.11–4.02 (m, 2 H, H-6,6′), 3.76 (t, 1 H, *J* 9.5 Hz, H-4′), 3.69–3.59 (m, 2 H, H-5,5′), 2.98–2.65 (m, 4 H, $SCH_2CH_2CO_2H$), 2.12, 2.08, 2.03, 2.02, 2.01, 2.00, and 1.97 (7 s, each 3 H, 7 Ac); ^{13}C (75 MHz), δ 100.7 (C-1′) and 83.8 (C-1). Anal. Calcd for $C_{29}H_{40}O_{19}S$: C, 48.1; H, 5.6. Found: C, 48.0; H, 5.6.

N$^{\alpha}$*-(9-Fluorenylmethoxycarbonyl)-3-*O*-[2,3,6-tri-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-α-*D*-galactopyranosyl)-β-*D*-galactopyranosyl]-*L*-serine* (**9**).—Boron trifluoride etherate (261 μL, 2.07 mmol) was added to a solution of *N*$^{\alpha}$-(9-fluorenylmethoxycarbonyl)-L-serine (**8**; 271 mg, 0.828 mmol) and **2**[24] (468 mg, 0.690 mmol) in dry MeCN (8 mL) at room temperature. After 75 min, the reaction was worked up as described for **5**, and medium-pressure reversed-phase chromatography (H_2O–MeCN, 55:45, containing 0.1% of CF_3CO_2H) of the residue gave **9** (420 mg, 64%), $[\alpha]_D^{25}$ +62° (*c* 1.1, $CHCl_3$). NMR data (acetone-d_6): ^1H (300 MHz), δ 6.42 (bd, 1 H, *J* 8.8 Hz, NH), 5.57 (dd, 1 H, *J* 3.2, 1.2 Hz, H-4′), 5.37 (dd, 1 H, *J* 10.9, 3.3 Hz, H-3′), 5.23 (dd, 1 H, *J* 10.9, 3.5 Hz, H-2′), 5.19 (dd, 1 H, *J* 10.4, 7.9 Hz, H-2), 5.14 (d, 1 H, *J* 3.4 Hz, H-1′), 5.05 (dd, 1 H, *J* 10.7, 2.9 Hz, H-3), 4.77 (d, 1 H, *J* 7.8 Hz, H-1), 4.61 (bt, 1 H, *J* 6.8 Hz, H-5′), 4.49 (1 H, Ser-Hα), 3.98 (dd, 1 H, *J* 10.6, 4.0 Hz, Ser-Hβ), 2.13, 2.10, 2.08, 2.03, 1.99, 1.99, and 1.94 (7 s, each 3 H, 7 Ac); ^{13}C (75 MHz), 101.9 (C-1), 99.6 (C-1′), and 54.8 (C-α). FABMS: $(M + H)^+$ 946 (Calcd 946).

3-(β-D-Galactopyranosylthio)propionyl-L-aspart-1-yl-L-tyrosyl-glycyl-L-isoleucyl-L-leucyl-L-glutaminyl-L-isoleucyl-L-asparaginyl-L-seryl-L-arginine amide (**11**).—Synthesis, cleavage of the resin-bound glycopeptide (65 μmol), and purification by HPLC (28% *B* in *A*, 12 mL/min; retention time, 40 min), according to the general procedure, gave *O*-acetylated **11** (47 mg). Deacetylation and purification by HPLC

(20% *B* in *A*, 12 mL/min; retention time, 29 min) gave **11** (38 mg, 41% overall yield). FABMS: $(M + H)^+$ 1427 (Calcd 1427). Amino acid analysis: Asp 1.95 (2), Arg 1.01 (1), Glu 1.01 (1), Gly 0.99 (1), Ile 2.03 (2), Leu 1.03 (1), Ser 0.98 (1), Tyr 1.00 (1).

3-(4-O-β-D-Galactopyranosyl-β-D-glucopyranosylthio)propionyl-L-aspart-1-yl-L-tyrosyl-glycyl-L-isoleucyl-L-leucyl-L-glutaminyl-L-isoleucyl-L-asparaginyl-L-seryl-L-arginine amide (**12**).—Synthesis, cleavage of the resin-bound glycopeptide (33 μmol), and purification by HPLC (32% *B* in *A*, 12 mL/min; retention time, 51 min), according to the general procedure, gave *O*-acetylated **12** (22.5 mg). Deacetylation and purification by HPLC (19.5% *B* in *A*, 12 mL/min; retention time, 33 min) gave **12** (16 mg, 30% overall yield). FABMS: $(M + H)^+$ 1589 (Calcd 1590). Amino acid analysis: Asp 2.00 (2), Arg 1.01 (1), Glu 1.00 (1), Gly 1.00 (1), Ile 1.99 (2), Leu 1.00 (1), Ser 1.00 (1), Tyr 0.99 (1).

3-(4-O-β-D-Glucopyranosyl-β-D-glucopyranosylthio)propionyl-L-aspart-1-yl-L-tyrosyl-glycyl-L-isoleucyl-L-leucyl-L-glutaminyl-L-isoleucyl-L-asparaginyl-L-seryl-L-arginine amide (**13**).—Synthesis, cleavage of the resin-bound glycopeptide (29 μmol), and purification by HPLC (33% *B* in *A*, 14 mL/min; retention time, 33 min), according to the general procedure, gave *O*-acetylated **13** (40 mg). Deacetylation and purification by HPLC (19% *B* in *A*, 14 mL/min; retention time, 38 min) gave **13** (20 mg, 44% overall yield). FABMS: $(M + H)^+$ 1589 (Calcd 1590). Amino acid analysis; Asp 1.97 (2), Arg 1.00 (1), Glu 1.00 (1), Gly 1.01 (1), Ile 1.99 (2), Leu 1.03 (1), Ser 0.98 (1), Tyr 1.02 (1).

3-(4-O-α-D-Galactopyranosyl-β-D-galactopyranosylthio)propionyl-L-aspart-1-yl-L-tyrosyl-glycyl-L-isoleucyl-L-phenylalanyl-L-glutaminyl-L-isoleucyl-L-asparaginyl-L-seryl-L-arginine amide (**14**).—Synthesis, cleavage of the resin-bound glycopeptide (19 μmol), and purification by HPLC (34% *B* in *A*, 14 mL/min; retention time, 30 min), according to the general procedure, gave *O*-acetylated **14** (24 mg). Deacetylation and purification by HPLC (21% *B* in *A*, 14 mL/min; retention time, 26 min) gave **14** (15 mg, 48% overall yield). FABMS: $(M + H)^+$ 1623 (Calcd 1624). Amino acid analysis: Asp 2.02 (2), Arg 0.97 (1), Glu 1.04 (1), Gly 1.06 (1), Ile 1.91 (2), Phe 0.98 (1), Ser 1.01 (1), Tyr 1.00 (1).

3-(4-O-α-D-Galactopyranosyl-β-D-galactopyranosylthio)propionyl-L-aspart-1-yl-L-tyrosyl-glycyl-L-isoleucyl-L-alanyl-L-glutaminyl-L-isoleucyl-L-asparaginyl-L-seryl-L-arginine amide (**15**).—Synthesis, cleavage of the resin-bound glycopeptide (19 μmol), and purification by HPLC (30% *B* in *A*, 14 mL/min; retention time, 30 min), according to the general procedure, gave *O*-acetylated **15** (25 mg). Deacetylation and purification by HPLC (15% *B* in *A*, 14 mL/min; retention time, 28 min) gave **15** (15 mg, 48% overall yield). FABMS: $(M + H)^+$ 1547 (Calcd 1548). Amino acid analysis: Ala 1.02 (1), Asp 2.00 (2), Arg 0.99 (1), Glu 1.00 (1), Gly 1.04 (1), Ile 1.95 (2), Ser 0.98 (1), Tyr 1.00 (1).

3-(4-O-α-D-Galactopyranosyl-β-D-galactopyranosylthio)propionyl-L-aspart-1-yl-L-alanyl-glycyl-L-isoleucyl-L-leucyl-L-glutaminyl-L-isoleucyl-L-asparaginyl-L-seryl-L-arginine amide (**16**).—Synthesis, cleavage of the resin-bound glycopeptide (38 μmol),

and purification by HPLC (31% *B* in *A*, 12 mL/min; retention time, 41 min), according to the general procedure, gave *O*-acetylated **16** (24 mg). Deacetylation and purification by HPLC (17% *B* in *A*, 12 mL/min; retention time, 37 min) gave **16** (17 mg, 30% overall yield). FABMS: $(M + H)^+$ 1497 (Calcd 1498). Amino acid analysis: Ala 1.01 (1), Asp 1.96 (2), Arg 0.99 (1), Glu 0.99 (1), Gly 1.00 (1), Ile 2.06 (2), Leu 1.00 (1), Ser 0.99 (1).

L-Aspart-1-yl-L-tyrosyl-glycyl-L-isoleucyl-L-leucyl-L-glutaminyl-L-isoleucyl-L-asparaginyl-O-(4-O-α-D-galactopyranosyl-β-D-galactopyranosyl)-L-seryl-L-arginine amide (**17**).—Synthesis, cleavage of the resin-bound glycopeptide (64 μmol), and purification by HPLC (31% *B* in *A*, 14 mL/min; retention time, 21 min), according to the general procedure, gave *O*-acetylated **17** (79 mg). Deacetylation and purification by HPLC (18% *B* in *A*, 14 mL/min; retention time, 30 min) gave **17** (39 mg, 40% overall yield). FABMS: $(M + H)^+$ 1501 (Calcd 1502). Amino acid analysis: Asp 2.01 (2), Arg 0.99 (1), Glu 1.00 (1), Gly 1.01 (1), Ile 1.99 (2), Leu 1.02 (1), Ser 0.99 (1), Tyr 0.99 (1).

3-(4-O-α-D-Galactopyranosyl-β-D-galactopyranosylthio)propionyl-L-asparaginyl-L-threonyl-L-aspart-1-yl-glycyl-L-seryl-L-threonyl-L-aspart-1-yl-L-tyrosyl-glycyl-L-isoleucyl-L-leucyl-L-glutaminyl-L-isoleucyl-L-asparaginyl-L-seryl-L-arginine amide (**19**).—Synthesis, cleavage of the resin-bound glycopeptide (13 μmol), and purification by HPLC (29% *B* in *A*, 12 mL/min; retention time, 100 min), according to the general procedure, gave *O*-acetylated **19** (6 mg). Deacetylation and purification by HPLC (19% *B* in *A*, 12 mL/min; retention time, 35 min) gave **19** (3 mg, 10% overall yield). FABMS: $(M + H)^+$ 2166 (Calcd 2165). Amino acid analysis: Asp 4.01 (4), Arg 1.00 (1), Glu 1.01 (1), Gly 2.03 (2), Ile 2.02 (2), Leu 1.02 (1), Ser 2.01 (2), Thr 1.91 (2), Tyr 1.00 (1).

3-(4-O-α-D-Galactopyranosyl-β-D-galactopyranosylthio)propionyl-L-phenylalanyl-L-glutaminyl-L-seryl-L-asparaginyl-L-phenylalanyl-L-asparaginyl-L-threonyl-L-glutaminyl-L-alanyl-L-threonyl-L-asparaginyl-L-arginine amide (**20**).—Synthesis, cleavage of the resin-bound glycopeptide (27 μmol), and purification by HPLC (31% *B* in *A*, 14 mL/min; retention time, 31 min), according to the general procedure, gave *O*-acetylated **20** (37 mg). Deacetylation and purification by HPLC (14.5% *B* in *A*, 14 mL/min; retention time, 33 min) gave **20** (18 mg, 37% overall yield). FABMS: $(M + H)^+$ 1840 (Calcd 1840). Amino acid analysis: Ala 1.00 (1), Asp 3.02 (3), Arg 0.99 (1), Glu 2.00 (2), Phe 1.99 (2), Ser 1.02 (1), Thr 1.98 (2). ^{1}H NMR data (Me_2SO-d_6, 500 MHz): δ α-D-Gal*p* 4.92 (H-1′), 4.17 (H-5′), 3.83 (H-4′), 3.75 (H-2′), 3.59 (H-6′), 3.57 (H-3′), 3.56 (H-6′); β-D-Gal*p* 4.34 (H-1), 3.95 (H-4), 3.75 (H-6,6), 3.57 (H-5), 3.46 (H-3), 3.41 (H-2); SCH_2CH_2CO 2.77, 2.68 and 2.47; Phe^{34} 8.17 (NH), 7.35 (H-arom), 4.64 (Hα), 3.12 (Hβ), 2.87 (Hβ′); Glu^{35} 8.29 (NH), 4.41 (Hα), 2.38 (Hγ), 2.05 (Hβ), 1.90 (Hβ′); Ser^{36} 7.97 (NH), 5.18 (OH), 4.42 (Hα), 3.70 (Hβ), 3.62 (Hβ′); Asn^{37} 8.26 (NH), 7.48 and 7.02 (β-$CONH_2$), 4.63 (Hα), 2.61 (Hβ), 2.49 (Hβ′); Phe^{38} 8.12 (NH), 7.32 (H-arom), 4.55 (Hα), 3.15 (Hβ), 2.88 (Hβ′); Asn^{39} 8.37 (NH), 7.60 and 7.08 (β-$CONH_2$), 4.74 (Hα), 2.75 (Hβ), 2.58 (Hβ′); Thr^{40} 7.80 (NH), 5.03 (OH), 4.26 (Hα), 4.17 (Hβ), 1.19 (Hγ); Gln^{41} 8.05

(NH), 7.31 and 6.87 (γ-$CONH_2$), 4.35 (Hα), 2.24 (Hγ), 2.07 (Hβ), 1.89 (Hβ'); Ala[42] 7.99 (NH), 4.42 (Hα), 1.35 (Hβ); Thr[43] 7.82 (NH), 5.06 (OH), 4.28 (Hα), 4.08 (Hβ), 1.18 (Hγ); Asn[44] 8.10 (NH), 7.57 and 7.08 (β-$CONH_2$), 4.62 (Hα), 2.68 (Hβ), 2.61 (Hβ'); Arg[45] 8.02 (NH), 7.53 (NHδ), 7.40 and 7.20 (α-$CONH_2$), 4.20 (Hα), 3.18 (Hδ), 1.88 (Hβ), 1.61 (Hβ'), 1.61 (Hγ).

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