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Synthesis of glycopeptides with the T_N and T antigen structures, and their coupling to bovine serum albumin

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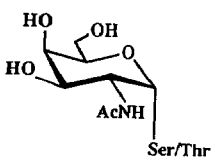
ABSTRACT

Glycopeptides with T_N and T antigen structures that represent the N-terminal tripeptide of asialoglycophorin with blood-group M specificity have been synthesized using fluorenylmethoxycarbonyl (Fmoc) and 2-pyridylethoxycarbonyl (Pyoc) groups for amino protection and the benzyl ester as the carboxyl-blocking function. The Fmoc and the Pyoc groups could be removed by treatment with the weak base morpholine under conditions where the base-sensitive *O*-glycosyl-serine and -threonine linkages were stable. Ester groups were removed from the carbohydrate moieties with methanolic hydrazine, to give the T_N and T antigen glycopeptides which were coupled to bovine serum albumin (BSA) via a carbodi-imide procedure and without any spacer groups. The resulting conjugates contained an average of >20 glycopeptides per protein molecule. They are not microheterogeneous in the carbohydrate part as is commonly found for glycoproteins isolated from biological sources.

INTRODUCTION

Oligosaccharide side-chains of the glycoproteins on cell surfaces have decisive functions in intercellular recognition and the regulation of cell growth[1]. Normal and tumor cells have different profiles of glycoproteins on their cell membranes[2], and those of tumor membranes may be tumor-associated antigens as has been reported[3] for glycoproteins with the Thomsen–Friedensreich antigen structure (T) **2** and its biological precursor, the T_N antigen structure **1**.

In continuing our investigations on the selective removal of single functional groups on polyfunctional glycopeptides[4], we have studied glycopeptides with T_N and T antigen structures, since their coupling to carrier proteins could give conjugates for the induction of antibodies against the tumor-associated antigen structures **1** and **2**.



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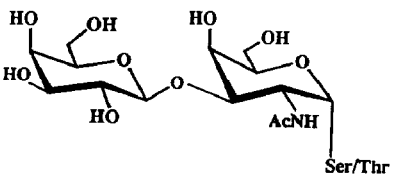

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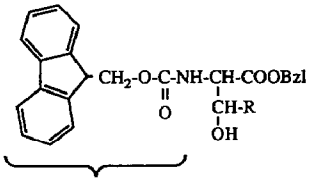
The *O*-glycosyl-serine and -threonine bonds, characteristic of T_N and T antigen glycoproteins, are sensitive to acid-catalyzed anomerization or cleavage and base-catalyzed β-elimination of the carbohydrate moieties.

The selective removal of protecting groups has long been a problem in the synthesis of glycopeptides[5]. One of the first methods we developed for this aim involves the N-terminal fluorenylmethoxycarbonyl (Fmoc) group[6] in conjunction with the C-terminal benzyl ester[7]. This method allows reliable and selective removal of the Fmoc group, even from sensitive *O*-glycopeptides, if the reaction is carried out with morpholine instead of the more strongly basic piperidine. The latter base is commonly used in peptide synthesis and was also used by Pavia *et al.*[8], who independently described the application of the Fmoc group. We have applied the morpholine procedure in the synthesis of glycopeptides with the T_N and T antigen structure[9]. At that time, Sinaÿ *et al.*[10] had reported a synthesis of the T antigen glycopeptide, using the benzyloxycarbonyl group in conjunction with the *tert*-butyl ester in order to achieve selective deprotection. Recently, the selective removal of the Fmoc group with morpholine[7,9] was adopted by Paulsen *et al.*[11], who also described syntheses of glycopeptides with T_N and T antigen structures.

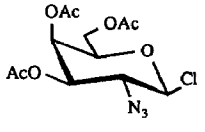
RESULTS AND DISCUSSION

Synthesis of a glycopeptide with the T_N antigen structure. — Pavia *et al.*[8] used benzylated carbohydrates combined with the Fmoc group for amino protection in syntheses of a glycopeptide with the T_N antigen structure. The amino acids were introduced as activated esters and, thus, selective C- or N-terminal deblocking was circumvented. In our approach, use is made of the Fmoc group in conjunction with the



3 R = H

4 R = CH_3



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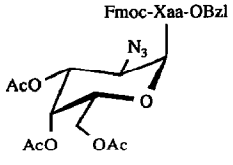
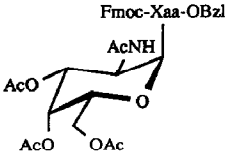
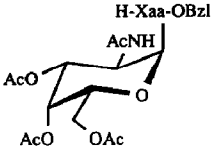






6 Xaa = Ser 75% (α/β-ratio 7:1)

7 Xaa = Thr 64% (α/β-ratio 15:1)

8 Xaa = Ser (86%)

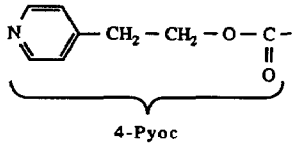
9 Xaa = Thr (85%)

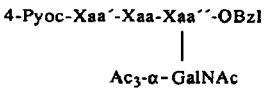
10 Xaa = Ser

11 Xaa = Thr

C-terminal benzyl ester in the glycosylation reactions, and the peptide chain is extended using the 2-(4-pyridyl)ethoxycarbonyl (Pyoc) group[12], which confers solubility and reactivity. Thus, Fmoc-serine benzyl ester (**3**) and Fmoc-threonine benzyl ester (**4**) were each glycosylated, using the glycosyl donor[13] **5**, to give the corresponding *α* glycosides **6** and **7** as the major products. The accompanying *β* anomers were removed by chromatography. The azido functions of **6** and **7** were reduced with sodium borohydride–nickel chloride[14]. Subsequent acetylation of the resulting 2-amino compounds with acetic anhydride in pyridine gave the fully protected T_N-antigen structural elements **8** and **9**, respectively.

The Fmoc group was removed selectively from **8** and **9** by treatment with morpholine. Thus, the basicity of the solution reliably remained below the sensitivity-level of the *O*-glycosylic linkage. The amino derivatives **10** and **11**, formed in almost quantitative yields, are relatively unstable, due to their own basic character. Therefore, the subsequent chain-lengthening reactions have to be carried out immediately and rapidly. Fmoc-protected carboxy derivatives often react sluggishly. However, the Pyoc-protected amino acid **12** and peptides **13–15**, prepared according to the methods described[12], reacted smoothly with **8** and **9** to give the corresponding glycopeptides **16–19** (Table I) in good overall yields. Ethyl 2-ethoxy-1,2-dihydroquinoline-1-carboxylate[15] (EEDQ) was an effective and mild condensing reagent and, during the coupling reactions, it was transformed into quinoline which did not affect the base-sensitive *O*-glycosylic bonds.





12 Xaa′-Xaa = Ser

13 Xaa′ = Tyr(OAllyl), Xaa = Ile

14 Xaa′ = Thr, Xaa = Ile

15 Xaa′ = Xaa = Ala

16 Xaa′-Xaa = Ser, Xaa′′ = Ser

17 Xaa′ = Tyr(OAllyl),Xaa = Ile,Xaa′′ = Ser

18 Xaa′ = Thr, Xaa = Ile, Xaa′′ = Ser

19 Xaa′ = Xaa = Ala, Xaa′′ = Thr

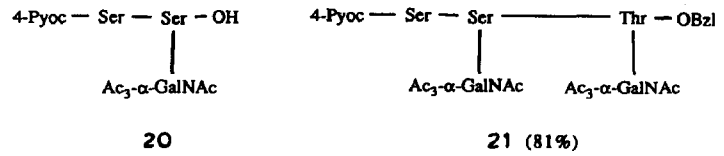
The Pyoc group has an advantage over the Fmoc group[16], in that it is stable towards hydrogenation. Thus, the Pyoc-glycodipeptide benzyl ester **16** was cleaved selectively by hydrogenolysis (Pd–C) to give **20**, which was condensed (EEDQ) with the glycosylthreonine ester **11**. Due to the influence of the polar Pyoc group, the diglycosylated tripeptide **21** was obtained pure and in high yield. The Pyoc group, which is stable to acids and bases, was removed selectively from the sensitive glycopeptide **21** by reaction with methyl iodide in dichloromethane to give the pyridinium form **22** which, with morpholine in dichloromethane, gave **23**. Treatment of **23** with acetic anhydride in pyridine gave **24** in high overall yield, acetylated at the N-terminus and the terminal serine hydroxy group.

TABLE I

Synthesis of Pyoc-glycopeptides

	Component		*Product*	*Yield*[b] (%)	$[\alpha]_D$ (c *1*, $CHCl_3$)	Formula	Elemental analysis		
	Carboxylic	*Amino*					*C*	*H*	*N*
12	Pyoc-Ser[a]	**10**/Ser	**16**	75	+48°	$C_{35}H_{44}N_4O_{15}\cdot 0.1H_2O$	Calc: 54.36	5.92	7.24
							Found: 54.43	6.05	6.95
13	Pyoc-Tyr(All)–Ile[c]	**10**/Ser	**17**	61	+43°	$C_{50}H_{63}N_5O_{16}$	Calc: 59.57	6.50	6.95
							Found: 59.67	6.52	6.70
14	Pyoc-Thr–Ile[c]	**10**/Ser	**18**	80	+36.5°	$C_{42}H_{57}N_5O_{10}$	Calc.: 56.81	6.47	7.89
							Found: 56.62	6.63	8.07
15	Pyoc-Ala–Ala	**11**/Thr	**19**	82	+26°	$C_{39}H_{51}N_5O_{16}$	Calc.: 55.25	6.30	8.26
							Found: 55.23	6.32	7.96

[a] Obtained from the corresponding *tert*-butyl ester by treatment of **12** with CF_3COOH. [b] Overall yield of Fmoc removal and peptide condensation. [c] Used as the trifluoracetate salts with equimolar amounts of triethylamine.



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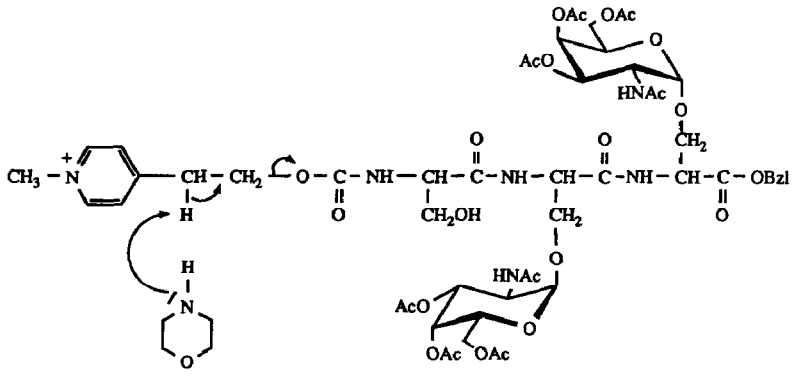
21 (81%)



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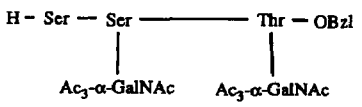

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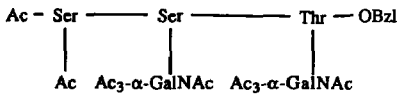
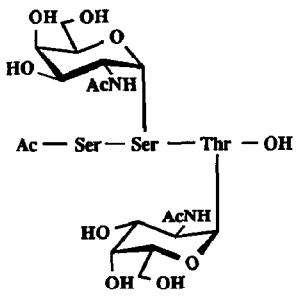


24 (74%)

Hydrogenolysis of the benzyl ester group of **24** followed by treatment with methanolic hydrazine[7], which removed the *O*-acetyl groups, gave **25**. G.l.c. of the latter reaction mixture revealed transesterfication catalysed by the weak base hydrazine (pK_a 8.2) rather than hydrazinolysis. Under these mild conditions, the ester groups are removed from the carbohydrate (and from the terminal serine hydroxy group) without destroying the sensitive glycopeptide.

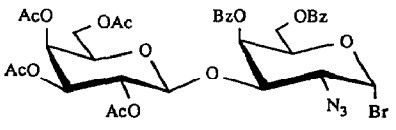
The glycopeptide **25** with the T_N antigen structure, which was isolated pure (^{13}C-n.m.r. and f.a.b.-mass-spectral data) in quantitative yield as a hygroscopic solid,



25

represents the acetylated N-terminal tripeptide of glycophorin with blood-group M-activity[17].

Synthesis of a glycopeptide with the T antigen structure. — The strategy developed for the synthesis of **25** was used to construct a glycopeptide **38** with the disaccharide T-antigen structure[9]. Thus, the Fmoc-serine benzyl ester **3** and Fmoc-threonine benzyl ester **4** were each glycosylated with the disaccharide bromide[18] **26**. From the resulting $\alpha\beta$-mixtures, the desired α-glycosyl-serine (**27**) and -threonine (**28**) derivatives were isolated by flash chromatography. Compounds **27** and **28** were each subjected to azide reduction and then acetylation to give the fully protected T-antigen structural elements **29** and **30**, respectively. The Fmoc groups were removed selectively by treatment with morpholine[7,9], to give **31** and **32**, respectively, in high yields. The serine derivative **31** was condensed immediately with Pyoc-serine (**12**) to give the glycopeptide **33**. Hydrogenolysis of **33** gave **34**, which was coupled (EEDQ) with the disaccharide threonine ester **32** to give **35**.



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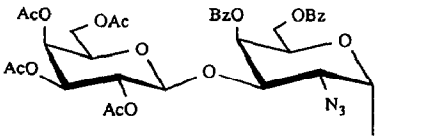


27 Xaa = Ser 71% (α/β ratio 5 : 2)
28 Xaa = Thr 51% (α/β ratio 3 : 2)

Ac_4-β-Gal-(1→3)-Bz_2-α-GalNAc
Fmoc-Xaa-OBzl

29 Xaa = Ser (70%)
30 Xaa = Thr (60%)

Ac_4-β-Gal-(1→3)-Bz_2-α-GalNAc
H- Xaa-OBzl

31 Xaa = Ser
32 Xaa = Thr

Ac_4-β-Gal-(1→3)-Bz_2-α-GalNAc
Pyoc-Ser-Ser-OBzl

33 (51% from **29**)

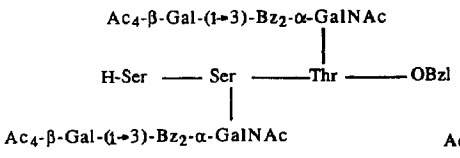
Ac_4-β-Gal-(1→3)-Bz_2-α-GalNAc
Pyoc-Ser-Ser-OH

34 (90%)

Ac_4-β-Gal-(1→3)-Bz_2-α-GalNAc
Pyoc-Ser-Ser-Thr-OBzl
Ac_4-β-Gal-(1→3)-Bz_2-α-GalNAc

35 (44%)

Due to the increased steric demands, the above reactions of the disaccharide derivatives required prolonged reaction times. Consequently, considerable decomposition of the free amino compounds **31** and **32** occurred, and the yields of products were lower than those of the corresponding monosaccharide analogues. Selective removal of the Pyoc group from **35**, by transformation into the methyl pyridinium salt followed by treatment with morpholine–dichloromethane, gave **36**, which was acetylated immediately to give **37**. Hydrogenolysis of the benzyl ester group in **37** and then deacetylation gave the T antigen glycopetide **38** (76% from **35**).



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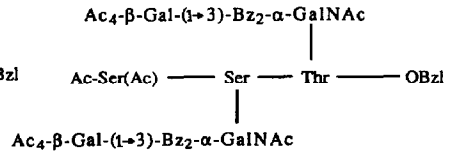

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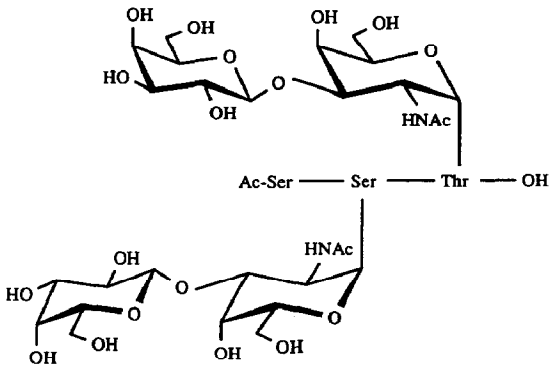
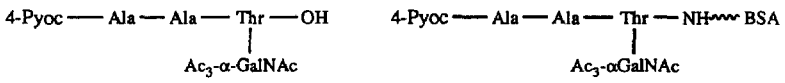


38 (76% from **35**)

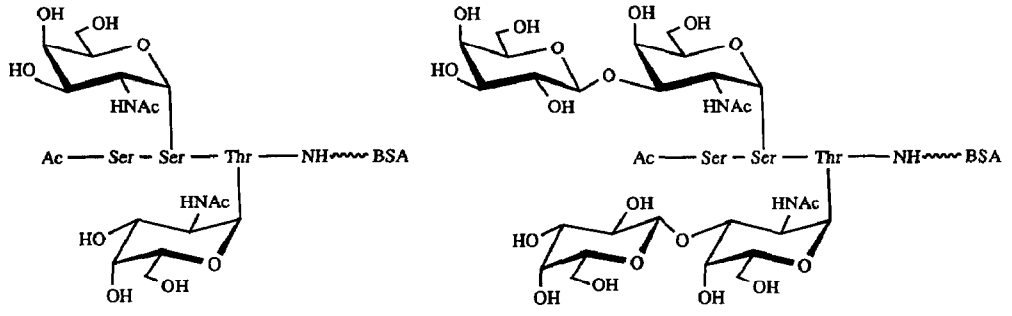
In the foregoing transformations, neither base-catalyzed β-elimination of the carbohydrate moieties nor racemization in the peptide moieties were oberserved. The structure and purity of **38** was demonstrated by ^{13}C-n.m.r. spectroscopy and f.a.b.-mass spectrometry.

Coupling of the synthetic glycopeptides to bovine serum albumin: preparation of synthetic T_N and T antigens. — The binding of β-Gal-(1→3)-GalNAc to carrier proteins has been accomplished by using artificial spacers[18,19]. Bovine serum albumin (BSA, mol. wt. ~67 000) contains 59 lysine residues and one terminal amino group. The coupling with the glycopeptides **25** and **38** can be carried out only in water. Preliminary experiments were performed with the sterically less-demanding glycopeptide **19**, which is a derivative of the repeating unit of the antifreeze glycoproteins of arctic fish[20].

Hydrogenolysis of the benzyl ester group of **19** gave the glycotripeptide derivative **39**. Attachment of **39** to BSA was achieved best in water at pH 6 and 4° by using the 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide hydrochloride in the presence of 1-hydroxybenzotriazole[21] for 6 days. The conjugate **40** was purified by dialysis against water and contained 66 μg of carbohydrate per mg of protein.



39 **40** (66 μg of carbohydrate / mg of protein)



41 (130 μg of carbohydrate / mg of protein) **42** (280 μg of carbohydrate / mg of protein)

Application of the foregoing procedure to the glycopeptides **25** and **38** gave the conjugates **41** and **42**, respectively. Analysis[22] of the T_N antigen **41** revealed 130 μg of GalNAc.mg^{-1} of protein, corresponding to ~25 molecules of **25** per molecule of protein. For the more complex structure **38**, the reaction time was extended to 14 days, and the resulting T antigen **42** contained 280 μg of Gal/GalNAc per mg of protein, corresponding to ~38 molecules of **38** per molecule of protein.

Preliminary results[23] showed that the antibody (IgM) raised against the T antigen **42** reacted with all epithelial tumors tested but also bound in a modified manner to normal cells of the same tissues.

EXPERIMENTAL

General methods. — N.m.r. spectra were recorded with Jeol JMN 60 (60 MHz, ^{1}H), Bruker WP 80 (20.15 MHz, ^{13}C), WP 90 (90 MHz, ^{1}H; 22.63 MHz, ^{13}C), and AM 400 (400 MHz, 1H; 100.6 MHz ^{13}C) spectrometers. I.r. spectra were recorded with a Beckman Acculab-2 instrument and optical rotations with a Perkin–Elmer Polarimeter 241. All melting points are uncorrected. T.l.c. was carried out on Silica Gel $60F_{254}$ (Merck) and detection with u.v. light, 0.3% ninhydrin in methanol–acetic acid (97:3), or

0.1% dihydroxynaphthalene in ethanol–M H_2SO_4 (1:1) and heating. Column chromatography was performed with Silica Gel 63–200 (0.063–0.200 mm) (Woelm). Flash chromatography was carried out with Silica Gel MN-60 (0.04–0.063) (Machery Nagel). *N*-(9-Fluorenylmethoxycarbonyl)serine benzyl ester[7] (**3**), *N*-(9-fluorenylmethoxycarbonyl)threonine benzyl ester[7] (**4**), 3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-*β*-D-galactopyranosyl chloride[24] (**5**), 2-azido-4,6-di-*O*-benzoyl-2-deoxy-3-*O*-(2,3,4,6-tetra-*O*-acetyl-*β*-D-galactopyranosyl)-*α*-D-galactopyranosyl bromide[18] (**26**), and 2-(4-pyridyl)ethoxycarbonylserine[12] (**12**) were obtained as reported. The 2-(4-pyridyl)ethoxycarbonyl-dipeptides **13–15** were synthesized from the corresponding esters by analogy with the method described[12].

N-[*2-(4-Pyridyl)ethoxycarbonyl]-dipeptide* tert-*butyl esters or benzyl esters.* — To a solution of 3 mmol of 2-(4-pyridyl)ethoxycarbonyl amino acid[12] and amino acid *tert*-butyl ester or benzyl ester, respectively, in *N,N*-dimethylformamide (20 mL) was added ethyl 2-ethoxy-1,2-dihydroquinoline-1-carboxylate (EEDQ, 3.3 mmol). The mixture was stirred for 4 days at room temperature, the solvent was evaporated *in vacuo*, and a solution of the oily residue in dichloromethane (150 mL) was washed with water (3 × 50 mL), dried ($MgSO_4$), and concentrated to dryness. Flash chromatography of the residue on silica gel (100 g) with light petroleum–acetone mixtures gave the analytically pure Pyoc-dipeptide esters.

Common n.m.r. characteristics ($CDCl_3$): ^1H (60 MHz), δ 8.5 (d, 2 H, J 4 Hz, pyridine *α*-H), 7.2 (d, 2 H, J 5 Hz, pyridine *β*-H), 6.5 (d, 1 H, J 7 Hz, amide NH), 5.45 (d, 1 H, J 7 Hz, urethane NH), 4.25 (t, 2 H, J 6 Hz, CH_2O), 3.05 (t, 2 H, J 6 Hz, CH_2-pyridine), 1.35 (s, 9 H, ^{t}Bu) or 5.15 (s, 2 H, CH_2Ph).

N-[2-(4-Pyridyl)ethoxycarbonyl]-L-(*O*-allyl)tyrosyl-L-isoleucine *tert*-butyl ester (91%), amorphous solid, $[\alpha]_D^{22}$ +19° (*c* 1, chloroform).

Anal. Calc. for $C_{30}H_{41}N_3O_6$: C, 66.77; H, 7.66; N, 7.79. Found: C, 66.66; H, 7.58; N, 7.82.

N-[2-(4-Pyridyl)ethoxycarbonyl]-L-threonyl-L-isoleucine *tert*-butyl ester (75%), amorphous solid, $[\alpha]_D^{22}$ +16° (*c* 1, chloroform).

Anal. Calc. for $C_{22}H_{35}N_3O_6$: C, 60.39; H, 8.06; N, 9.60. Found: C, 60.34; H, 8.16; N, 9.80.

N-[2-(4-Pyridyl)ethoxycarbonyl]-L-alanyl-L-alanine benzyl ester (61%), m.p. 112°, $[\alpha]_D^{22}$ −14° (*c* 1, chloroform).

Anal. Calc. for $C_{21}H_{25}N_3O_5$: C, 63.15; H, 6.31; N, 10.52. Found: C, 62.92; H, 6.17; N, 10.46.

N-[*2-(4-Pyridyl)ethoxycarbonyl]-dipeptide trifluoroacetates: selective acidolysis of* tert-*butyl esters.* — A solution of 4-Pyoc-dipeptide *tert*-butyl ester (1 mmol) in trifluoroacetic acid (5 mL) was stirred for 30 min, then concentrated *in vacuo*, to give the trifluoroacetate salts of **13** and **14** as amorphous solids. Their n.m.r. spectra are similar to those of the corresponding *tert*-butyl esters, except for the absence of the signal for ^{t}Bu.

N-[2-(4-Pyridyl)ethoxycarbonyl]-L-(*O*-allyl)tyrosyl-L-isoleucine trifluoroacetate

(**13**, quantitative), $[\alpha]_D^{22}$ +5.4° (*c* 1, methanol).

Anal. Calc. for $C_{28}H_{34}F_3N_3O_8$: C, 54.63; H, 5.89; N, 6.83. Found: C, 54.39; H, 5.89; N, 6.97.

N-[2-(4-Pyridyl)ethoxycarbonyl]-L-threonyl-L-isoleucine trifluoroacetate (**14**, 71% after chromatography on 40 g of silica gel with acetone), $[\alpha]_D^{22}$ +1.6° (*c* 1, chloroform).

Anal. Calc. for $C_{18}H_{27}N_3O_6 \cdot 2/3CF_3COOH$: C, 50.76; H, 6.10; N, 9.19. Found: C, 50.73; H, 6.28; N, 9.15.

N-(9-*Fluorenylmethoxycarbonyl*)-O-(*3,4,6-tri-O-acetyl-2-azido-2-deoxy-α-D-galactopyranosyl*)-L-*β-hydroxyamino acid benzyl ester*. — A solution of Fmoc-hydroxyamino acid benzyl ester[7] (**3** or **4**, 10.5 mmol) in toluene (30 mL) and dichloromethane (50 mL) was stirred for 30 min at −5° under argon in the dark with silver carbonate (3.5 g), Drierite (9 g), and molecular sieves type 4A (9 g). Silver perchlorate (0.35 g) in toluene (15 mL) was added and, after 20 min, a solution of **5** (ref. 24) (3.5 g, 10 mmol) in toluene–dichloromethane (1:1, 90 mL) was added slowly. The mixture was stirred for 20 h at room temperature, diluted with dichloromethane (50 mL), filtered with Hyflo-Cel, and washed with saturated aqueous $NaHCO_3$ (2 × 100 mL) and water (2 × 100 mL), dried ($MgSO_4$), and concentrated *in vacuo*. Further purification was performed by flash chromatography on 150 g of silica gel, using light petroleum–ethyl acetate (2:1).

N-(9-Fluorenylmethoxycarbonyl)-*O*-(3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-*α*-D-galactopyranosyl)-L-serine benzyl ester (**6**; 4.45 g, 65%), amorphous solid, $[\alpha]_D^{22}$ +86.5° (*c* 1, chloroform). N.m.r. data ($CDCl_3$): ^1H (90 MHz): δ 7.92–7.17 (m, 13 H, aromatic H), 5.97 (d, 1 H, *J* 8 Hz, NH), 4.86 (d, 1 H, $J_{1,2}$ 3.5 Hz, H-1), 3.56 (dd, 1 H, $J_{2,3}$ 10.9 Hz, H-2); ^{13}C (20.15 MHz), δ 169.84–168.99 (4 s, 4 C=O), 155.5 (urethane), 98.77 (C-1), 61.42 (C-6), 57.04 (C-2), 54.22 (Ser *α*-CH), 46.66 (fluorenyl C-9).

Anal. Calc. for $C_{37}H_{38}N_4O_{12}$: C, 60.82; H, 5.24; N, 7.67. Found: C, 60.75; H, 5.07; N, 7.49.

β Anomer (0.9 g, 9%), amorphous, $[\alpha]_D^{22}$ −7.1° (*c* 1, chloroform). ^{13}C-N.m.r. data (100.6 MHz, $CDCl_3$): δ 170.28–169.32 (4 s, 4 C=O), 155.91 (urethane), 102.31 (C-1), 61.05 (C-6), 60.72 (C-2), 54.19 (Ser *α*-CH), 46.99 (fluorenyl C-9).

Anal. Found: C, 60.75; H, 5.21; N, 7.94.

N-(9-Fluorenylmethoxycarbonyl)-*O*-(3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-*α*-D-galactopyranosyl)-L-threonine benzyl ester (**7**; 4.6 g, 60%), amorphous, $[\alpha]_D^{22}$ +63° (*c* 1, chloroform). N.m.r. data ($CDCl_3$): ^1H (400 MHz), δ 7.79–7.24 (m, 13 H, aromatic H), 5.42 (d, 1 H, $J_{3,4}$ 2.7 Hz, H-4), 4.88 (d, 1 H, $J_{1,2}$ 3.6 Hz, H-1), 3.57 (dd, 1 H, $J_{2,3}$ 11.4 Hz, H-2), 1.32 (d, 3 H, *J* 6.0 Hz, Thr CH_3); ^{13}C (100.6 MHz), δ 170.24–169.74 (4 s, 4 C=O), 156.79 (urethane), 99.13 (C-1), 76.70 (Thr *β*-C), 58.80–57.81 (C-2, Thr *α*-C), 47.16 (fluorenyl C-9), 18.53 (Thr CH_3).

Anal. Calc. for $C_{38}H_{40}N_4O_{12}$: C, 61.28; H, 5.41; N, 7.52. Found: C, 61.10; H, 5.46; N, 7.41.

β Anomer (0.3 g, 4%), amorphous, $[\alpha]_D^{22}$ −14° (*c* 1, chloroform). ^{13}C-N.m.r. data (100.6 MHz): δ 170.27–169.98 (4 s, 4 C=O), 156.68 (urethane), 100.00 (C-1), 75.10 (Thr *β*-C), 60.71 (C-2), 58.40 (Thr *α*-C), 47.10 (fluorenyl C-9), 16.88 (Thr CH_3).

Anal. Found: C, 61.45; H, 5.59; N, 7.62.

Glycosylation of **3** *and* **4** *with* **26**. — A solution of **3** or **4** (1.73 mmol) in toluene (10 mL) and dichloromethane (15 mL) was stirred under argon at room temperature in the dark with silver carbonate (0.52 g) and molecular sieves type 4A (2 g). After 1 h, a solution of silver perchlorate (52 mg) in toluene (5mL) was added and, after 20 min, a solution of 2-azido-4,6-di-*O*-benzoyl-2-deoxy-3-*O*-(2,3,4,6-tetra-*O*-acetyl-*β*-D-galactopyranosyl)-*α*-D-galactopyranosyl bromide[24] (**26**; 1.4 g, 1.73 mmol) in toluene–dichloromethane (1:1, 30 mL) was added. The mixture was stirred for 24 h, diluted with dichloromethane (70 mL), filtered with Hyflo-Cel, washed with saturated aqueous $NaHCO_3$ (2 × 50 mL) and water (2 × 50 mL), dried $MgSO_4$), and concentrated *in vacuo*. Flash chromatography of the residue on 100 g of silica gel gave the following products.

O-[2-Azido-4,6-di-*O*-benzoyl-2-deoxy-3-*O*-(2,3,4,6-tetra-*O*-acetyl-*β*-D-galactopyranosyl)-*α*-D-galactopyranosyl]-*N*-9-fluorenylmethoxycarbonyl)-L-serine benzyl ester (**27**, 51%), amorphous, $[\alpha]_D^{22}$ +64° (*c* 1, chloroform). N.m.r. data ($CDCl_3$): ^1H (400 MHz): *δ* 8.10–7.20 (m, 23 H, aromatic H), 5.89 (d, 1 H, *J* 8.2 Hz, NH), 5.72 (d, 1 H, $J_{3,4}$ 3.3 Hz, H-4), 5.27 (d, 1 H, $J_{3',4'}$, 3.1 Hz, H-4'), 5.22 (s, 2 H, CH_2Ph), 5.08 (dd, 1 H, $J_{1',2'}$ 7.8, $J_{2',3'}$ 10.4 Hz, H-2'), 4.94 (dd, 1 H, H-3'), 4.91 (d, 1 H, $J_{1,2}$ 3.5 Hz, H-1), 4.68 (d, 1 H, H-1'), 3.71 (dd, 1 H, $J_{2,3}$ 10.7 Hz, H-2); ^{13}C (100.6 MHz), *δ* 170.28–169.25 (5 s, C=O), 166.07, 165.51 (benzoyl C=O), 155.83 (urethane), 101.46 (C-1'), 99.07 (C-1), 54.47 (Ser *α*-C).

Anal. Calc. for $C_{59}H_{58}N_4O_{20}\cdot H_2O$: C, 61.03; H, 5.21; N, 4.83. Found: C, 61.33; H, 5.23; N, 4.53.

β Anomer (20%), amorphous, $[\alpha]_D^{22}$ +12° (*c* 1, chloroform). N.m.r. data ($CDCl_3$): ^1H (400 MHz), *δ* 5.64 (d, 1 H, $J_{3,4}$ 3.5 Hz, H-4), 5.28 (dd, 1 H, $J_{3',4'}$ 3.3, $J_{4',5'}$ 1.0 Hz, H-4'), 4.69 (d, 1 H, $J_{1,2}$ 7.7 Hz, H-1'), 4.62 (dt, 1 H, *J* 8.2 Hz, Ser *α*-CH), 4.47–4.25 [m, 5 H, H-6b, C*HC*H_2 (Fmoc), H-1]; ^{13}C (100.6 MHz), *δ* 156.0 (urethane), 102.43 (C-1'), 101.57 (C-1), 54.29 (Ser *α*-C).

Anal. Found: C; 61.01; H; 5.18; N, 4.72.

O-[2-Azido-4,6-di-*O*-benzoyl-2-deoxy-3-*O*-(2,3,4,6-tetra-*O*-acetyl-*β*-D-galactopyranosyl)-*α*-D-galactopyranosyl]-*N*-(9-fluorenylmethoxycarbonyl)-L-threonine benzyl ester (**28**, 30%), amorphous, $[\alpha]_D^{22}$ +55° (*c* 1, chloroform). N.m.r. data ($CDCl_3$): ^1H (400 MHz), *δ* 8.10–7.18 (m, 23 H, aromatic H), 5.78 (dd, 1 H, $J_{3,4}$ 3.3, $J_{4,5}$ 0.9 Hz, H-4), 5.65 (d, 1 H, *J* 9.3 Hz, NH), 5.28 (dd, 1 H, $J_{3',4'}$ 3.4, $J_{4',5'}$ 0.8 Hz, H-4'), 4.88 (d, 1 H, $J_{1,2}$ 3.6 Hz, H-1), 4.75 (d, 1 H, $J_{1',2'}$ 7.5 Hz, H-1'), 1.26 (d, 3 H, *J* 6 Hz, Thr CH_3); ^{13}C (100.6 MHz), *δ* 170.37–169.34 (4 s, C=O), 166.18, 165.60 (C=O, benzoyl), 156.76 (urethane), 101.59 (C-1'), 99.34 (C-1), 18.66 (Thr CH_3).

Anal. Calc. for $C_{60}H_{60}N_4O_{20}\cdot 1.5\ H_2O$: C, 60.85; H, 5.36; N, 4.73. Found: C, 60.92; H, 5.14; N, 4.86.

β Anomer (21%), amorphous, $[\alpha]_D^{22}$ +30° (*c* 1, chloroform). N.m.r. data ($CDCl_3$): ^1H (400 MHz), *δ* 5.61 (d, 1 H, $J_{3,4}$ 3.4 Hz, H-4), 5.28 (d, H, $J_{3',4'}$ 3.3 Hz, H-4'), 4.70 (d, 1 H, $J_{1',2'}$ 7.7 Hz, H-1'), 3.74 (dd, 1 H, $J_{1,2}$ 8.0, $J_{2,3}$ 10.2 Hz, H-2), 1.33 (d, 3 H, *J* 5.4 Hz, Thr CH_3); ^{13}C (100.6 MHz), *δ* 101.62 (C-1'), 99.84 (C-1), 16.46 (Thr CH_3).

Anal. Calc. for $C_{60}H_{60}N_4O_{20}\cdot 2H_2O$: C, 60.40; H, 5.41; N, 4.70. Found: C, 60.14; H, 5.33; N, 4.75.

Reduction and acetylation of **6**, **7**, **27** *and* **28**. — To a solution of $NiCl_2\cdot 6H_2O$ (2.4 g), boric acid (1.2 g), and 1.2 mmol of **6**, **7**, **27**, or **28** in ethanol (60 mL) was added a suspension of sodium borohydride (122 mg, 3.23 mmol) in ethanol (10 mL) under argon. Each mixture was stirred until the starting material had completely disappeared (t.l.c., light petroleum–ethyl acetate mixtures). The solvent was distilled off *in vacuo*, the residue was stirred with pyridine (10 mL) and acetic anhydride (2 mL) for 2 h, and then the mixture was concentrated *in vacuo*. Toluene (2 × 10 mL) was distilled *in vacuo* from the residue, a solution of which in dichloromethane (50 mL) was extracted with aqueous 3% $KHSO_4$ (50 mL) and water (2 × 50 mL), dried ($MgSO_4$), and concentrated *in vacuo*. The residue was purified by flash chromatography with light petroleum–acetone (2:1).

O-(2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-galactopyranosyl)-*N*-(9-fluorenylmethoxycarbonyl)-L-serine benzyl ester (**8**, 86%), amorphous, $[\alpha]_D^{22}$ +60° (*c* 1, chloroform). N.m.r. data ($CDCl_3$): ^1H (90 MHz), δ 5.86 (d, 1 H, *J* 8 Hz, NH), 5.60 (d, 1 H, *J* 9 Hz, NHAc), 4.76 (d, 1 H, $J_{1,2}$ 3.2 Hz, H-1); ^{13}C (22.63 MHz), δ 170.32–169.80 (4 s, 5 C=O), 155.76 (urethane), 99.09 (C-1), 54.51 (Ser α-C), 23.05 (CH_3CONH).

Anal. Calc. for $C_{39}H_{42}N_2O_{13}\cdot H_2O$: C, 61.25; H, 5.80; N, 3.66. Found: C, 61.45; H, 5.84; N, 3.55.

O-(2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-galactopyranosyl)-*N*-(9-fluorenylmethoxycarbonyl-L-threonine benzyl ester (**9**, 85%), amorphous, $[\alpha]_D^{22}$ +50° (*c* 1, chloroform). N.m.r. data ($CDCl_3$): ^1H (400 MHz), δ 5.82 (d, 1 H, *J* 9.8 Hz, AcNH), 5.68 (d, 1 H, *J* 9.7 Hz, Thr NH), 5.42 (d, 1 H, $J_{3,4}$ 2.7 Hz, H-4), 4.78 (d, 1 H, $J_{1,2}$ 3.7 Hz, H-1), 4.51 (ddd, 1 H, $J_{2,3}$ 12.5 Hz, H-2); ^{13}C (20.15 MHz), δ 156.39 (urethane), 99.74 (C-1), 76.67 (Thr β-C), 58.67 (Thr α-C), 23.03 (CH_3CONH), 18.18 (Thr CH_3).

Anal. Calc. for $C_{40}H_{44}N_2O_{13}$: C, 63.15; H, 5.83; N, 3.68. Found: C, 63.03; H, 6.03; N, 3.84.

O-[2-Acetamido-4,6-di-*O*-benzoyl-2-deoxy-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-α-D-galactopyranosyl]-*N*-(9-fluorenylmethoxycarbonyl)-L-serine benzyl ester (**29**, 70%), amorphous, $[\alpha]_D^{22}$ 53° (*c* 1, chloroform). N.m.r. data ($CDCl_3$): ^1H (400 MHz), δ 5.85 (d, 1 H, *J* 7.9 Hz, AcNH), 5.79 (d, 1 H, *J* 7.8 Hz, Ser NH), 5.65 (d, 1 H, $J_{3,4}$ 2.9 Hz, H-4), 5.30 (d, 1 H, $J_{3',4'}$ 3.4 Hz, H-4'), 5.00 (d, 1 H, $J_{1,2}$ 3.2 Hz, H-1), 4.92 (dd, 1 H, $J_{2',3'}$ 10.2 Hz, H-3'); ^{13}C (100.6 MHz), δ 170.29–169.40 (5 s, C=O), 166.08, 165.75 (benzoyl C=O), 155.81 (urethane), 100.18 (C-1'), 98.72 (C-1), 54.57 (Ser α-C), 49.22 (C-2), 23.14 (CH_3CONH).

Anal. Calc. for $C_{61}H_{62}N_2O_{21}\cdot H_2O$: C, 62.24; H, 5.48; N, 2.38. Found: C, 62.55; H, 5.55; N, 2.18.

O-[2-Acetamido-4,6-di-*O*-benzoyl-2-deoxy-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-α-D-galactopyranosyl]-*N*-(9-fluorenylmethoxycarbonyl)-L-threonine benzyl ester (**30**, 60%), amorphous, $[\alpha]_D^{22}$ +63° (*c* 1, chloroform). N.m.r. data ($CDCl_3$): ^1H (400 MHz), δ 5.95 (d, 1 H, *J* 9 Hz, AcNH), 5.69 (d, 1 H, $J_{3,4}$ 3.0 Hz, H-4), 5.64 (d, 1 H, *J* 10 Hz, Thr NH), 5.30 (d, 1 H, $J_{3',4'}$ 3.0 Hz, H-4'), 1.23 (d, 3 H, *J* 6 Hz, Thr CH_3); ^{13}C (100.6 MHz), δ 170.63–169.2 (6 s, C=O), 166.12, 165.85 (benzoyl C=O), 156.40

(urethane), 100.38 (C-1'), 99.90 (C-1), 73.37 (Thr β-C), 58.65 (Thr α-C), 49.16 (C-2), 23.21 (CH_3CONH), 18.44 (Thr CH_3).

Anal. Calc. for $C_{62}H_{64}N_2O_{21}\cdot 2.5H_2O$: C, 61.13; H, 5.71; N, 2.30. Found: C, 61.08; H, 5.73; N, 2.43.

Selective removal of the Fmoc group from **8, 9, 29,** *and* **30**, *and peptide condensation with EEDQ.* — A solution of **8, 9, 29,** or **30** (0.5 mmol) in morpholine (5 mL) was stirred at room temperature for 30 min, then concentrated *in vacuo*. Ether (10 mL) was distilled from the residual N-terminal deblocked derivative (**10, 11, 31,** or **32**), a solution of which in dichloromethane (100 mL) was washed with aqueous 5% NaCl (4 × 50 mL), dried ($MgSO_4$), and concentrated *in vacuo*. Each crude amino-deblocked compound was not purified further but used for the N-terminal chain extension.

To a solution of each crude product in dichloromethane (30 mL) was added 0.5 mmol of **12, 13, 14, 15, 20,** or **34**. The trifluoroacetate salts of **13** and **14** were used together with equimolar quantities of triethylamine. For the preparation of **15**, see below. If necessary, a few drops of methanol were added to give a clear solution. At 0°, a solution of ethyl 2-ethoxy-1,2-dihydroquinoline-1-carboxylate (EEDQ; 135 mg, 0.55 mmol) in dichloromethane was added, and each mixture was stirred for 3 days. For the disaccharide derivatives, the reaction time was 7–10 days. Dichloromethane (100 mL) was added and each solution was extracted with aqueous 5% NaCl (3 × 50 mL), dried ($MgSO_4$), and concentrated *in vacuo*. Further purification was carried out by flash chromatography.

N-[2-(4-Pyridyl)ethoxycarbonyl]-L-seryl-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-galactopyranosyl)-L-serine benzyl ester (**16**); eluent, light petroleum–acetone (1:1)→ acetone. For the yield and analytical data, see Table I. N.m.r. data ($CDCl_3$): ^1H (400 MHz), δ 8.46 (d, 2 H, *J* 5 Hz, pyridine α-H), 7.13 (d, 2 H, pyridine β-H), 5.90 (d, 1 H, *J* 8 Hz, urethane), 4.74 (d, 1 H, $J_{1,2}$ 3.4 Hz, H-1), 4.46 (m, 1 H, H-2), 2.90 (t, 2 H, *J* 6 Hz, CH_2-pyridine); ^{13}C (100.6 MHz), δ 149.71 (pyridine C-2,6), 147.08 (pyridine C-4), 124.28 (pyridine C-3,5), 98.34 (C-1), 47.64 (C-2), 34.65 (pyridine α-CH_2).

O-Allyl-*N*-[2-(4-pyridyl)ethoxycarbonyl]-L-tyrosyl-L-isoleucyl-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-galactopyranosyl)-L-serine benzyl ester (**17**); eluent, light petroleum–acetone (1:1→1:2). For the yield and analytical data, see Table I. N.m.r. data ($CDCl_3$): ^1H (90 MHz): δ 8.65–8.40 (m, 2 H, pyridine α-H), 6.1 (m, 1 H, $CH_2=CH$-), 4.79 (d, 1 H, $J_{1,2}$ 3.5 Hz, H-1); ^{13}C (22.63 MHz), δ 157.71, 156.02 (urethane, Tyr *p*-C), 149.52 (pyridine C-2,6), 133.15 ($CH=CH_2$), 98.38 (C-1), 58.28 (Ile α-C), 56.39 (Tyr α-C), 52.75 (Ser α-C), 15.38, 10.84 (Ile CH_3).

N-[2-(4-Pyridyl)ethoxycarbonyl]-L-threonyl-L-isoleucyl-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-galactopyranosyl)-L-serine benzyl ester (**18**); eluent, light petroleum–acetone (1:2→acetone). For the yield and analytical data, see Table I. N.m.r. data ($CDCl_3$): ^1H (400 MHz), δ 8.47 (d, 2 H, *J* 6 Hz, pyridine, α-H), 5.28 (d, 1 H, $J_{3,4}$ 3.2 Hz, H-4), 4.73 (d, 1 H, $J_{1,2}$ 3.0 Hz, H-1), 4.51 (ddd, 1 H, $J_{2,3}$ 12.5 Hz, $J_{CH,NH}$ 9.7 Hz, H-2), 1.17 (d, 3 H, *J* 6 Hz, Thr CH_3), 0.90 (d, 3 H, *J* 8 Hz, Ile $CHCH_3$), 0.85 (t, 3 H, *J* 7 Hz, Ile CH_2CH_3); ^{13}C (20.15 MHz), δ 156.18 (urethane), 149.47 (pyridine C-2,6), 98.23 (C-1), 77.13 (Thr β-C), 58.32 (Thr and Ile α-C), 52.49 (Ser α-C), 17.96 (Thr CH_3), 15.44, 10.99 (Ile CH_3).

N-[2-(4-Pyridyl)ethoxycarbonyl]-L-threonyl-L-isoleucyl-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-*α*-D-galactopyranosyl)-L-serine benzyl ester (**18**); eluent, light petroleum–acetone (1:2→acetone).For the yield and analytical data, see Table I. N.m.r. data ($CDCl_3$): ^1H (400 MHz), δ 8.47 (d, 2 H, J 6 Hz, pyridine, *α*-H), 5.28 (d, 1 H, $J_{3,4}$ 3.2 Hz, H-4), 4.73 (d, 1 H, $J_{1,2}$ 3.0 Hz, H-1), 4.51 (ddd, 1 H, $J_{2,3}$ 12.5 Hz, $J_{CH,NH}$ 9.7 Hz, H-2), 1.17 (d, 3 H, J 6 Hz, Thr CH_3), 0.90 (d, 3 H, J 8 Hz, Ile CHCH_3), 0.85 (t, 3 H, J 7 Hz, Ile CH_2CH_3); ^{13}C (20.15 MHz), δ 156.18 (urethane), 149.47 (pyridine C-2,6), 98.23 (C-1), 77.13 (Thr *β*-C), 58.32 (Thr and Ile *α*-C), 52.49 (Ser *α*-C), 17.96 (Thr CH_3), 15.44, 10.99 (Ile CH_3).

N-[2-(4-Pyridyl)ethoxycarbonyl]-L-alanyl-L-alanyl-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-*α*-D-galactopyranosyl)-L-threonine benzyl ester (**19**); eluent, light petroleum–acetone (1:2). For the yield and analytical data, see Table I. N.m.r. data ($CDCl_3$): ^1H (400 MHz): δ 8.46 (d, 2 H, J 5 Hz, pyridine *α*-H), 5.30 (dd, 1 H, $J_{3,4}$ 3.4 Hz, $J_{4,5}$ 1.2 Hz, H-4), 4.69 (d, 1 H, $J_{1,2}$ 3.6 Hz, H-1), 1.4–1.1 (m, 9 H, Thr and Ala CH_3); ^{13}C (100.6 MHz), δ 156.03 (urethane), 149.66 (pyridine C-2,6), 98.52 (C-1), 47.3 (C-2), 19.11 (Thr CH_3), 15.55 (Ala CH_3).

N-[2-(4-Pyridyl)ethoxycarbonyl]-L-seryl-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-*α*-D-galactopyranosyl)-L-seryl-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-*α*-D-galactopyranosyl)-L-threonine benzyl ester (**21**, 81%), obtained from **20** (see below) and **11** [reaction time, 5 days; eluent, light petroleum–acetone 2:1→acetone]; amorphous, $[\alpha]_D^{22}$ +68° (*c* 1, chloroform). N.m.r. data ($CDCl_3$): ^1H (400 MHz), δ 8.46 (d, 2 H, J 5 Hz, pyridine *α*-H), 7.77 (d, 1 H, J 9 Hz, Thr NH), 7.45 (d, 1 H, J 9 Hz, Ser-CHO NH), 6.42, 6.37 (2 d, 2 H, J 9 Hz, 2 AcNH), 6.00 (d, 1 H, J 9 Hz, urethane), 4.91 [d, 1 H, $J_{1,2}$ 3.6 Hz, H-1 (Ser)], 4.74 [d, 1 H, $J_{1,2}$ 3.6 Hz, H-1(Thr)], 1.20 (d, 3 H, J 5 Hz, Thr CH_3); ^{13}C (100.6 MHz), δ 156.18 (urethane), 149.76 (pyridine C-2,6), 99.57, 98.51 (2 C-1), 47.76, 47.73 (2 C-2), 56.79, 56.02, 53.37 (Thr *α*-C, 2 Ser *α*-C), 18.72 (Thr CH_3).

Anal. Calc. for $C_{53}H_{70}N_6O_{25}\cdot 2.5H_2O$: C, 51.50; H, 6.11; N, 6.79. Found: C, 51.51; H, 6.14; N, 6.52.

N-[2-(4-Pyridyl)ethoxycarbonyl]-L-seryl-*O*-[2-acetamido-4,6-di-*O*-benzoyl-2-deoxy-3-*O*-(2,3,4,6-tetra-*O*-acetyl-*β*-D-galactopyranosyl)-*α*-D-galactopyranosyl]-L-seryl-*O*-[2-acetamido-4,6-di-*O*-benzoyl-2-deoxy-3-*O*-(2,3,4,6-tetra-*O*-acetyl-*β*-D-galactopyranosyl)-*α*-D-galactopyranosyl]-L-threonine benzyl ester (**35**, 44%), obtained from **34** (see below) and **32** [reaction time, 12 days; eluent, dichloromethane–methanol (10:1)], amorphous, $[\alpha]_D^{22}$ +56° (*c* 1, chloroform), R_F 0.36 (CH_2Cl_2–MeOH, 10:1). According to its 400-MHz ^1H-n.m.r. and 100.6-MHz ^{13}C-n.m.r. spectra, **35** was accompanied by derivatives having less than four *O*-acetyl groups in the two terminal galactosyl units. The compound was identified via its completely deprotected derivative **38** (see below).

Hydrogenolysis of the benzyl ester groups from N-*(4-Pyoc)peptides and glycopeptides.* — The compounds 4-Pyoc-Ala–Ala-OBzl (see above, 2.45 mmol in 20 mL of methanol), **16** (0.9 mmol in 20 mL of methanol), **33** (0.3 mmol in 10 mL of methanol), and **19** (0.22 mmol in 5 mL of methanol) were each hydrogenolysed at atmospheric pressure over 5% Pd/C for 15 h. If precipitates were formed, they were dissolved by the

addition of methanol. The catalyst was removed, and the filtrate was concentrated *in vacuo*.

N-[2-(4-Pyridyl)ethoxycarbonyl]-L-alanyl-L-alanine (**15**, 95%), m.p. 153°, $[\alpha]_D^{22}$ +21° (*c* 1, methanol). N.m.r. data ($CDCl_3/CD_3OD$): ^1H (90 MHz), δ 8.48–8.39 (m, 2 H, pyridine α-H), 7.3 (d, 2 H, *J* 5.9 Hz, pyridine β-H), 2.99 (t, 2 H, *J* 6.5 Hz, pyridine CH_2), 1.42 (d, 3 H, *J* 7.0 Hz, Ala CH_3), 1.35 (d, 3 H, *J* 6.8 Hz, Ala CH_3).

Anal. Calc. for $C_{14}H_{19}N_3O_5$: C, 54.36; H, 6.19; N, 13.95. Found: C, 54.28; H, 6.03; N, 13.72.

N-[2-(4-Pyridyl)ethoxycarbonyl]-L-seryl-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-galactopyranosyl)-L-serine (**20**, quantitative), amorphous, $[\alpha]_D^{22}$ +88° (*c* 0.5, chloroform). N.m.r. data [$(CD_3)_2SO$]: ^1H (400 MHz), δ 8.48 (d, 2 H, *J* 5 Hz, pyridine α-H), 8.13, 7.73, 7.17 each (d, 1 H, *J* 8 Hz, NH), 5.30 (d, 1 H, $J_{3,4}$ 2.7 Hz, H-4), 4.99 (dd, 1 H, $J_{2,3}$ 11.7 Hz, H-3), 4.84 (d, 1 H, $J_{1,2}$ 3.4 Hz, H-1), 2.90 (t, 2 H, *J* 6 Hz, pyridine CH_2); ^{13}C (100.6 MHz), δ 155.82 (urethane), 148.66 (pyridine C-2,6), 97.37 (C-1), 56.99, 52.01 (2 Ser α-C), 46.97 (C-2).

Anal. Calc. for $C_{28}H_{38}N_4O_{15}\cdot 1.5\ H_2O$: C, 48.21; H, 5.92; N, 8.03. Found: C, 48.42; H, 5.85; N, 7.86.

N-[2-(4-Pyridyl)ethoxycarbonyl]-L-seryl-*O*-[2-acetamido-4,6-di-*O*-benzoyl-2-deoxy-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-α-D-galactopyranosyl]-L-serine (**34**, 90%); purified by elution from a short column of 10 g of silica gel with dichloromethane–methanol (10:1)→methanol; amorphous, $[\alpha]_D^{22}$ +70° (*c* 1.5, methanol). N.m.r. data (CD_3OD): ^1H (400 MHz), δ 8.45 (d, 2 H, *J* 5 Hz, pyridine α-H), 5.82 (d, 1 H, $J_{3,4}$ 3 Hz, H-4), 5.32 (d, 1 H, $J_{3',4'}$ 3.2 Hz, H-4'); ^{13}C (100.6 MHz), δ 173.36–171.02 (6 s, C=O), 167.83, 167.79 (benzoyl C=O), 158.20 (urethane), 149.89 (pyridine C-2,6), 102.63 (C-1'), 99.35 (C-1), 58.42, 55.59 (2 Ser α-C), 50.55 (C-2), 35.63 (pyridine α-CH_2).

Anal. Calc. for $C_{50}H_{58}N_4O_{23}\cdot 3H_2O$: C, 52.82; H, 5.67; N, 4.93. Found: C, 52.46; H, 5.50; N, 4.88.

N-[2-(4-Pyridyl)ethoxycarbonyl]-L-alanyl-L-alanyl-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-galactopyranosyl)-L-threonine (**39**, quantitative), amorphous, $[\alpha]_D^{22}$ +53° (*c* 0.6, methanol). N.m.r. data (CD_3OD): ^1H (400 MHz), δ 8.50 (d, 2 H, *J* 4.2 Hz, pyridine α-H), 5.43 (d, 1 H, $J_{3,4}$ 3.1 Hz, H-4), 5.02 (d, 1 H, $J_{1,2}$ 3.7 Hz, H-1), 1.44, 1.37 (2 d, 3 H, *J* 7.2 Hz, Ala CH_3), 1.30 (d, 3 H, *J* 6.4 Hz, Thr CH_3); ^{13}C (100.6 MHz), δ 158.01 (urethane), 149.7 (pyridine C-2,6), 100.51 (C-1), 77.55 (Thr β-C), 19.18 (Thr CH_3), 18.48, 17.30 (Ala CH_3).

Anal. Calc. for $C_{32}H_{45}N_5O_{15}$: C, 50.72; H, 6.25; N, 9.24. Found: C, 51.04; H, 6.26; N, 8.97.

Removal of the 4-Pyoc-group via transformation into the methylpyridinium form and immediate N-acetylation. — A solution of **21** (200 mg, 0.17 mmol) in dry dichloromethane (5 mL) was treated with methyl iodide (0.5 mL) for 12 h at room temperature, then concentrated *in vacuo*. A solution of the residual methoiodide **22** in dichloromethane (5 mL) and morpholine (1 mL) was kept for 1 h. T.l.c. ($CHCl_3$–MeOH, 10:1) then revealed **23**, R_F 0.21. The mixture was diluted with dichloromethane (50 mL) and extracted with water (5 × 50 mL), dried ($MgSO_4$), and concentrated *in vacuo*. To a

solution of crude **23** in dichloromethane (5 mL) at 0° were added acetic anhydride (3 mL) and pyridine (4 mL). After 12 h at room temperature, the volatile components were evaporated *in vacuo*. Toluene (3 × 10 mL) was distilled from the residue. Column chromatography on 60 g of silica gel with dichloroethane–methanol (20:1) then gave *N,O*-diacetyl-L-seryl-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-galactopyranosyl)-L-seryl-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-galactopyranosyl)-L-threonine benzyl ester (**24**), isolated as an amorphous solide (140 mg, 74%), $[\alpha]_D^{22}$ +63° (*c* 0.7, chloroform). N.m.r. data ($CDCl_3$): δ 7.75 (d, 1 H, *J* 9 Hz, Thr NH), 7.69 (d, 1 H, *J* 7 Hz, Ser-CHO NH), 7.07 (d, 1 H, *J* 7 Hz, Ser NH), 6.93 and 6.45 (2 d, H, *J* 9 Hz, AcNH), 4.92 [d, 1 H, $J_{1,2}$ 3.5 Hz, H-1 (Ser)], 4.75 [d, 1 H, $J_{1,2}$ 3.8 Hz, H-1 (Thr)], 1.23 (d, 3 H, *J* 6 Hz, Thr CH_3); ^{13}C (100.6 MHz), δ 171.55–169.67 (11 s, C=O), 134.57 [ipso-C (Bzl)], 99.27, 98.66 (2 C-1), 75.56 (Thr β-C), 56.66, 53.45, 53.16 (Thr, 2 Ser α-C), 47.65, 47.60 (2 C-2), 19.03 (Thr CH_3).

Anal. Calc. for $C_{49}H_{67}N_5O_{25}$: C, 50.64; H, 6.16; N, 6.03. Found: C, 50.61; H, 6.00; N, 5.94.

Hydrogenolysis of the benzyl ester and removal of the ester protecting groups of **24** *by treatment with hydrazine–methanol.* — A solution of **24** (120 mg, 0.106 mmol) in methanol (10 mL) was hydrogenolysed over 5% Pd/C for 18 h. The catalyst was collected and washed with 20 mL of methanol, and the combined filtrate and washings were concentrated *in vacuo*. A solution of the residue in methanol (5 mL) and hydrazine hydrate (100%, 1 mL) was stirred for 1 h, then cooled to 0°, and acetone (20 mL) was added dropwise. The mixture was concentrated *in vacuo* and a solution of the residue in methanol (1 mL) was diluted with ethyl acetate to give *N*-acetyl-L-seryl-*O*-(2-acetamido-2-deoxy-α-D-galactopyranosyl)-L-seryl-*O*-(2-acetamido-2-deoxy-α-D-galactopyranosyl)-L-threonine (**25**) as a hygroscopic amorphous solid (80 mg, quantitative), $[\alpha]_D^{22}$ +123° (*c* 1.2, water). F.a.b.-mass spectrum: *m/z* $(M+1)^+$ and 764 $(M+Na)^+$. ^{13}C-N.m.r. data (100.6 MHz, D_2O; CS_2 as the internal standard, δ 192.7): δ 174.54, 174.4, 173.72, 171.99, 171.12 (C=O), 98.90 [C-1 (Thr)], 97.92 [C-1(Ser)], 76.46 (Thr β-C), 71.34, 71.30 (2 C-5), 57.86, 55.49, 53.49 (Thr, 2 Ser α-C), 49.99, 49.77 (2 C-2), 22.27, 22.12, 21.82 (3 CH_3CONH), 17.90 (Thr CH_3).

N-*Acetyl*-L-*seryl*-O-*(2-acetamido-2-deoxy-3*-O-β-D-*galactopyranosyl*-α-D-*galactopyranosyl)*-L-*seryl-O-(2-acetamido-2-deoxy-3*-O-β-D-*galactopyranosyl*-α-D-*galactopyranosyl)*-L-*threonine* (**38**). — As described above for **21**, **35** (100 mg, 0.05 mmol) was subjected in sequence to modification and removal of the Pyoc group, *N*-acetylation, hydrogenolysis, and treatment with hydrazine in methanol. The morpholine was removed from the residue by washing with ether. Compound **38** was obtained as an amorphous hygroscopic solid (80 mg, 76%), $[\alpha]_D^{22}$ +81° (*c* 0.8, water), R_F 0.09 (dichloromethane–methanol, 1:1). F.a.b.-mass spectrum: *m/z* 1067 $(M+1)^+$ and 1089 $(M+Na)^+$. ^{13}C-N.m.r. data (100.6 MHz, D_2O; CS_2 as the internal standard, δ 192.7): δ 174.78, 174.30, 174.17, 173.92, 171.60, 170.28 (C=O), 104.32, 104.24 (2 C-1'), 98.58 [C-1 (Thr)], 97.58 [C-1 (Ser)], 58.63 (Thr α-C), 55.13, 53.21 (2 Ser α-C), 48.33, 48.05 (2 C-2), 22.05, 21.78, 21.44 (CH_3CONH), 17.49 (Thr CH_3).

Synthesis of synthetic T_N *and T antigens.* — To a solution of BSA (Behring Inst.

ORHD 20/21; 20 mg, 0.3 mol) and 1-hydroxybenzotriazole monohydrate (10 mg, 0.074 mmol) in water (3 mL) at 4° was added a solution of **39**, **25**, or **38** (10 mg) in water (2 mL, 4°). The pH of the mixture was adjusted to 6 by addition of $KHCO_3$ and 3-(3-dimethylaminopropyl)-1-ethylcarbodi-imide hydrochloride (50 mg, 0.26 mmol) was added. The mixture was kept for 6 days (for **39** and **25**) or 14 days (for **38**), then dialysed against water. The solution obtained was used for carbohydrate analysis[22] with 2-acetamido-2-deoxy-D-galactose as the standard. In the analysis of **42**, D-galactose was also used as a standard and identical results were obtained: **40** (protected T_N antigen, from **39**), 66 μg of carbohydrate per mg of protein; **41** (T_N antigen, from **25**), 130 μg of carbohydrate per mg of protein; **42** (T antigen, from **38**), 280 μg of carbohydrate per mg of protein.

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