Synthesis of Glycosylated Peptide Templates Containing 6'-*O*-Phosphorylated Mannose Disaccharides and Their Binding to the Cation-independent Mannose 6-Phosphate Receptor

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The multiple-column peptide synthesis of eleven glycopeptides containing two 6'-*O*-phosphorylated mannose disaccharides linked either α(1→2) or α(1→6) is described. Binding-inhibition studies were performed on the cation-independent mannose 6-phosphate receptor, revealing glycopeptides containing two 6'-*O*-phosphorylated α(1→2)-linked mannose disaccharides to be potent inhibitors of the receptor binding to a phosphomannan core fragment.

Mannose 6-phosphate (Man-6-*P*) has been shown to be an inhibitor of inflammation in the central nervous system[1] and of adjuvant arthritis[2] in rats. The anti-inflammatory effect of Man-6-*P* may be due to an inhibition of the interaction between the mannose 6-phosphate receptors (MPRs) and lysosomal enzymes, as this interaction is involved in intracellular sorting and targeting of lysosomal enzymes.[3–5] However, Man-6-*P* has to be administered in relatively large amounts to exert its anti-inflammatory effect (25 mg kg^{-1} day^{-1} rat^{-1}),[1,2] which may be due to the low affinity of the monovalent Man-6-*P* to the MPRs.[6,7]

Mannose 6-phosphate residues are present at the terminal positions of asparagine-linked oligosaccharides of the high mannose type found in lysosomal enzymes.[6] Binding-inhibition studies have demonstrated that mannose disaccharides phosphorylated at the terminal position and linked α(1→2) are better inhibitors at the MPRs than is Man-6-*P* itself, and also better than the corresponding α(1→6)- or α(1→3)-linked isomers. A branched mannose pentasaccharide containing two terminal Man-6-*P* units, which has been suggested to be one of the natural ligands for the MPRs,[8–10] has been described to be a 100-fold better inhibitor than Man-6-*P*.[6] Branched divalent ligands with two terminal Man-6-*P* units gave stronger inhibitory effects (6–20-times higher) than the corresponding monophosphorylated compounds.[6,7] We have therefore found it interesting to synthesize and investigate glycopeptides containing two 6'-*O*-phosphorylated mannose disaccharides as bidentate ligands for the MPRs. In these glycopeptides the peptide acts as a template which mimics the scaffolding core structure of the natural oligosaccharide ligand.[11] Force field calculations using the GEGOP program[12] were performed on the branched core mannose trisaccharide of the *N*-linked oligosaccharides and this showed the distance between the phosphorylated disaccharides to be 8–13 Å [from O-2 in the α(1→3)-linked mannose unit to either O-6 or O-3 in the α(1→6)-linked mannose unit], which corresponds to three to five amino acids.

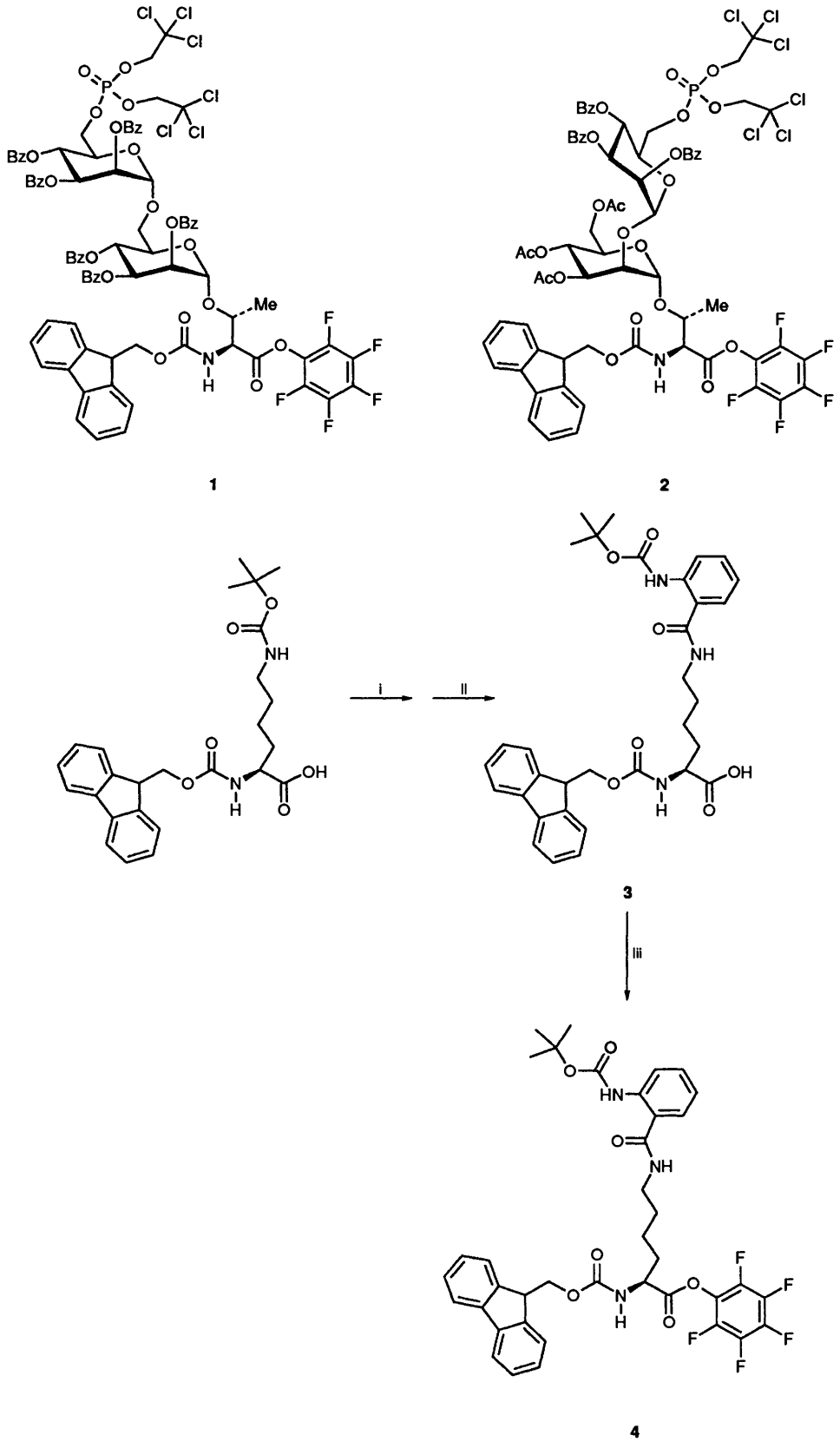
We here report a convenient, multiple-column, solid-phase peptide synthesis (MCPS)[13] of glycopeptides containing two 6'-*O*-phosphorylated mannose disaccharides linked α(1→2) and/or α(1→6), consisting of 3–5 amino acids. The glycopeptides also contain a fluorescence probe (anthranilic acid), which allows easy monitoring in biological assays. Binding-inhibition studies were performed on the cation-independent mannose 6-phosphate receptor (^{Cl}MPR), showing glycopeptides having two α(1→2)-linked phosphorylated mannose disaccharides to be 600–1500-fold better inhibitors than is Man-6-*P* itself.

Results and Discussion

The most convenient method for the synthesis of the glycopeptides containing 6'-*O*-phosphorylated α(1→2)- and/or α(1→6)-linked disaccharides utilizes phosphorylated, glycosylated threonine (or serine) building blocks in MCPS. We have previously described the synthesis of the glycosylated amino acid derivatives **1** and **2** with 2,2,2-trichloroethyl (TCE) protection of the phosphate group, and these have been used in the solid-phase synthesis of two tripeptides.[14] However, removal of the TCE groups from the phosphate moiety by catalytic hydrogenation in aq. ethanol as previously described was not quantitative. The first TCE group was cleaved in a fast reaction but the second was very persistent. To optimize the reaction conditions the model compound Ac-[Bz_3-α-D-Man*p*-6-$(TCE)_2$P-(1→6)-Bz_3-α-D-Man*p*-(1→)]Thr-NH_2 was used. Various reaction conditions were tried such as: (a) catalytic hydrogenation in aq. ethanol at 50 atm., (b) treatment with Zn in glacial acetic acid,[15] (c) treatment with Zn in pyridine containing acetylacetone,[16,17] (d) treatment with a Zn/Cu couple,[18,19] and (e) treatment with Zn and silver carbonate in pyridine containing 10% acetic acid.[20,21] Only the latter reaction conditions resulted in quantitative removal of the TCE groups, and this method was therefore subsequently used in the deprotection of all the glycopeptides.

In order to incorporate a fluorescent moiety into the glycopeptides, commercially available N^{α}-(fluoren-9-ylmethoxycarbonyl)-N^{ε}-(*tert*-butoxycarbonyl)-L-lysine[Fmoc-Lys(Boc)-OH] was treated with trifluoroacetic acid (TFA) to remove the Boc group. The free N^{ε}-amino group was then allowed to react with 3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl 2-(*tert*-butoxycarbonylamino)benzoate (Boc-ABz-ODhbt)[22] to give compound **3** (Scheme 1). Compound **3** was converted into the pentafluorophenyl (Pfp) ester using Pfp-OH and *N*,*N*'-dicyclohexylcarbodiimide (DCCI) to give compound **4** in 64% overall yield.

Synthesis of the glycopeptides **5**–**7** and **9**–**15** was performed in dimethylformamide (DMF) by using the resin PEGA 1900/130 [poly(ethylene glycol)dimethylacrylamide copolymer][23,24] in a 20-well manual synthesizer.[25] The glycopeptide **8** was synthesized by the syringe method[14,26] in a similar manner. The resin was derivatized with 4-[α-(fluoren-9-ylmethoxycarbonylamino)-2,4-dimethoxybenzyl]phenoxyacetic acid (Rink-



Scheme 1 *Reagents and yields:* i, TFA; ii, Boc-ABz-ODhbt (80%); iii, Pfp-OH, DCCI (80%)

linker)[27] by the *O*-(1*H*-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) procedure.[28] The synthesis was carried out with N^{α}-Fmoc-protected amino acid Pfp-esters with addition of Dhbt-OH as an auxiliary nucleophile. Deprotection of the α-amino groups was achieved by treatment with 20% piperidine in DMF. The N-terminus was acetylated after removal of the final N^{α}-Fmoc group. The *O*-glycopeptides were cleaved from the resin by treatment with 95%

TFA, and were purified by preparative HPLC, to give 20–40 mg of the protected glycopeptides **5–15** in 45–92% yield (70% on average). The protected peptides were fully characterized by ^{1}H NMR spectroscopy as presented in Table 1 and the identity of the glycopeptides was confirmed by electron-spray mass spectrometry. The TCE groups were then removed from the phosphate moieties by the method described above, to give the partially deprotected glycopeptides **16–26** in an average 60% yield after purification by preparative HPLC. As the last step of the synthesis the glycopeptides were deacylated. Compound **19** was treated with sodium methoxide in methanol, which is the standard procedure for deacylation of glycopeptides. However, ^{1}H NMR spectroscopy revealed that not only were the acyl groups removed, the ABz group was also partially cleaved. This was quite an unexpected result, since the ABz group is attached to the N^{ε}-amino group of lysine *via* an amide linkage, and as amide linkages are generally stable to the sodium methoxide treatment. The cleavage may, however, be due to anchimeric assistance from the adjacent amino group. Various deacylation conditions were then investigated: (a) sodium methoxide in methanol–water (1:0.1), (b) sodium methoxide in methanol–tetrahydrofuran (THF)–water (1:1:0.2), (c) sodium methoxide in methanol–acetonitrile–water (1:1:0.2), (d) potassium carbonate in methanol, (e) hydrazine hydrate in methanol, (*f*) hydrazine hydrate in methanol–chloroform (4:1). The first five sets of reaction conditions resulted in several products as seen by analytical HPLC, and ^{1}H NMR spectroscopy revealed that some of these had lost one of the disaccharides, presumably by base-catalysed β-elimination. This may be due to the low solubility of the compounds in methanol, resulting in an

Table 1 (*a*) Selected ^{1}H NMR chemical shifts (ppm) measured at 500 MHz for compounds **5–15** in $(CD_3)_2SO$ at 300 K [Ref. $\delta_H(CD_2HSOCD_3)$ 2.50]

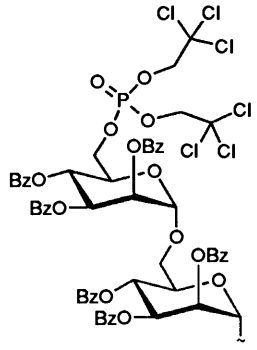
Amino acid	NH^{α}	H^{α}	H^{β}	H^{γ}	H^{δ}	H^{ε}	NH^{ε}
5							
Thr-1	8.22	4.48	3.97	1.25			
Lys-2	8.30	4.38	1.62, 1.74	1.33, 1.39	1.51	3.18	8.26
Thr-3	7.84	4.42	4.31	1.18			
6							
Thr-1	8.24	4.52	4.04	1.28			
Lys-2	8.30	4.43	1.66, 1.81	1.33, 1.41	1.52	3.17	8.20
Thr-3	7.96	4.51	4.42	1.36			
7							
Thr-1	8.21	4.57	4.31	1.33			
Lys-2	8.09	4.42	1.68, 1.80	1.35, 1.43	1.51	3.18	8.23
Thr-3	7.99	4.41	4.28	1.18			
8							
Thr-1	8.22	4.62	4.31	1.37			
Lys-2	8.13	4.43	1.69, 1.83	1.44	1.52	3.18	8.20
Thr-3	8.00	4.48	4.41	1.33			
9							
Thr-1	8.19	4.45	3.99	1.24			
Lys-2	8.13	4.29	1.61, 1.73	1.36, 1.37	1.48	3.17	8.26
Gly-3	8.04	3.84, 3.94					
Thr-4	8.04	4.37	4.30	1.19			
10							
Thr-1	8.20	4.46	4.01	1.25			
Lys-2	8.17	4.31	1.62, 1.74	1.35, 1.40	1.49	3.18	8.27
Gly-3	8.09	3.96					
Thr-4	8.17	4.47	4.41	1.35			
11							
Thr-1	8.22	4.57	4.31	1.36			
Lys-2	8.00	4.33	1.62, 1.76	1.38	1.49	3.18	8.27
Gly-3	8.14	3.81, 3.92					
Thr-4	7.96	4.37	4.29	1.17			
12							
Thr-1	8.20	4.42	4.02	1.25			
Gly-2	8.36	3.75, 3.84					
Lys-3	7.92	4.27	1.54, 1.67	1.25, 1.27	1.45	3.16	8.22
Gly-4	8.15	3.85, 3.88					
Thr-5	8.03	4.36	4.28	1.19			
13							
Thr-1	8.21	4.42	4.02	1.26			
Gly-2	8.38	3.76, 3.86					
Lys-3	7.97	4.31	1.56, 1.69	1.34, 1.36	1.48	3.18	8.36
Gly-4	8.21	3.87, 3.98					
Thr-5	8.16	4.46	4.41	1.35			
14							
Thr-1	8.23	4.55	4.26	1.38			
Gly-2	8.38	3.80, 3.86					
Lys-3	8.01	4.23	1.54, 1.68	1.27, 1.31	1.47	3.17	8.31
Gly-4	8.11	3.81, 3.87					
Thr-5	8.05	4.35	4.27	1.19			
15							
Thr-1	8.22	4.55	4.28	1.37			
Gly-2	8.37	3.81, 3.87					
Lys-3	8.04	4.25	1.55, 1.68	1.30, 1.32	1.47	3.17	8.26
Gly-4	8.14	3.85, 3.97					
Thr-5	8.13	4.46	4.40	1.34			

Table 1 (*contd.*) (*b*) ^{1}H NMR data for the mannose disaccharides in glycopeptides **5–15**

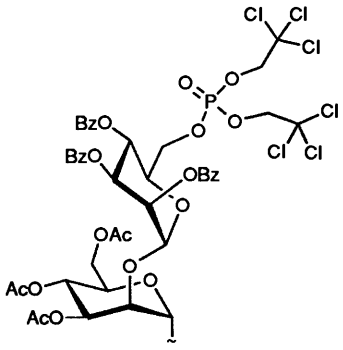
Amino acid		1-H	2-H	3-H	4-H	5-H	6-H	6-H′
5								
Thr-1	Man(1→2)	5.45	5.68	5.79	6.01	4.56	4.42	4.56
	Man(1→Thr)	5.30	4.13	5.24	5.26	4.07	4.11	4.26
Thr-3	Man(1→2)	5.38	5.67	5.77	5.97	4.48	4.47	4.62
	Man(1→Thr)	5.18	4.19	5.29	5.26	4.04	4.11	4.24
6								
Thr-1	Man(1→2)	5.45	5.67	5.79	6.00	4.56	4.42	4.58
	Man(1→Thr)	5.31	4.16	5.24	5.26	4.07	4.08	4.20
Thr-3	Man(1→6)	5.37	5.75	5.75	5.84	4.18	4.18	4.18
	Man(1→Thr)	5.28	5.74	5.75	6.00	4.53	3.88	4.14
7								
Thr-1	Man(1→6)	5.36	5.77	5.78	5.84	4.14	4.14	4.14
	Man(1→Thr)	5.32	5.68	5.80	6.03	4.54	3.87	4.13
Thr-3	Man(1→2)	5.40	5.67	5.77	5.96	4.48	4.48	4.63
	Man(1→Thr)	5.17	4.16	5.30	5.25	4.04	4.13	4.22
8								
Thr-1	Man(1→6)	5.35	5.75	5.75	5.84	4.14	4.14	4.14
	Man(1→Thr)	5.34	5.69	5.81	6.02	4.52	3.85	4.08
Thr-3	Man(1→6)	5.35	5.75	5.75	5.84	4.14	4.14	4.14
	Man(1→Thr)	5.28	5.73	5.80	6.00	4.54	3.85	4.08
9								
Thr-1	Man(1→2)	5.44	5.67	5.78	5.99	4.56	4.41	4.56
	Man(1→Thr)	5.28	4.14	5.31	5.25	4.07	4.10	4.23
Thr-4	Man(1→2)	5.38	5.66	5.76	5.96	4.49	4.47	4.60
	Man(1→Thr)	5.18	4.17	5.31	5.25	4.07	4.10	4.23
10								
Thr-1	Man(1→2)	5.44	5.67	5.79	5.99	4.54	4.42	4.56
	Man(1→Thr)	5.29	4.15	5.25	5.25	4.05	4.09	4.23
Thr-4	Man(1→6)	5.37	5.75	5.75	5.84	4.17	4.17	4.17
	Man(1→Thr)	5.29	5.73	5.79	5.99	4.54	3.89	4.11
11								
Thr-1	Man(1→6)	5.37	5.77	5.78	5.84	4.16	4.16	4.16
	Man(1→Thr)	5.32	5.68	5.78	6.02	4.55	3.87	4.11
Thr-4	Man(1→2)	5.39	5.67	5.77	5.96	4.50	4.45	4.61
	Man(1→Thr)	5.18	4.18	5.30	5.25	4.06	4.09	4.22
12								
Thr-1	Man(1→2)	5.43	5.66	5.77	5.98	4.56	4.42	4.54
	Man(1→Thr)	5.27	4.17	5.29	5.24	4.07	4.08	4.22
Thr-5	Man(1→2)	5.38	5.65	5.76	5.96	4.49	4.47	4.60
	Man(1→Thr)	5.18	4.16	5.28	5.24	4.07	4.08	4.22
13								
Thr-1	Man(1→2)	5.44	5.66	5.79	5.99	4.55	4.41	4.55
	Man(1→Thr)	5.27	4.17	5.26	5.25	4.07	4.09	4.23
Thr-5	Man(1→6)	5.37	5.74	5.75	5.84	4.18	4.11	4.11
	Man(1→Thr)	5.29	5.72	5.79	5.98	4.56	4.11	4.88
14								
Thr-1	Man(1→6)	5.36	5.74	5.75	5.83	4.14	4.12	4.12
	Man(1→Thr)	5.35	5.68	5.76	6.00	4.52	3.87	4.10
Thr-5	Man(1→2)	5.39	5.66	5.76	5.96	4.49	4.46	4.60
	Man(1→Thr)	5.18	4.16	5.30	5.24	4.06	4.09	4.22
15								
Thr-1	Man(1→6)	5.36	5.74	5.76	5.84	4.16	4.09	4.14
	Man(1→Thr)	5.35	5.68	5.75	6.01	4.53	3.88	4.10
Thr-5	Man(1→6)	5.36	5.74	5.76	5.84	4.16	4.09	4.14
	Man(1→Thr)	5.28	5.72	5.79	5.98	4.53	3.88	4.10

excessively long reaction time. Addition of water to convert the sodium methoxide into the weaker base sodium hydroxide resulted in an even lower solubility. In the last set of reaction conditions, however, the glycopeptide was dissolved in chloroform, and then methanol and hydrazine hydrate were added. Using this method, elimination of neither the ABz group nor the carbohydrate moieties was observed, and the glycopeptides **16–26** were deacylated analogously, to give the fully deprotected glycopeptides **27–37** in 70–90% yield after gel filtration and purification by preparative HPLC. It was found necessary to purify the reaction mixture by gel permeation chromatography prior to purification by HPLC, as the glycopeptides decomposed upon evaporation with hydrazine hydrate, and benzoyl hydrazide, formed during the deacylation, co-eluted with the glycopeptides. The fully deprotected glycopeptides were thus obtained in overall yields ranging from 26–67%, with an average yield of 41%. The ^{1}H NMR spectra of the protected and the completely deprotected peptides are presented by examples in Fig. 1, and the ^{1}H NMR data are presented in Table 2. The identity of the glycopeptides was confirmed by ES–MS.

A simple enzyme-linked immunosorbent assay (ELISA) was developed to test the inhibitory potencies of glycopeptides **27–37** against the binding of a phosphomannan core fragment to the ^{CI}MPR. The phosphomannan core fragment obtained by acid hydrolysis from the high-molecular-mass phosphomannan from *Hansenula holstii* (Y.2448)[29] was coupled to Maxisorp microtiter plates. The affinity-purified ^{CI}MPR from foetal calf serum was biotinylated and then added with the potential inhibitor to the microtiter plates. The plates were then



A



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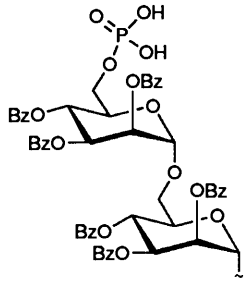

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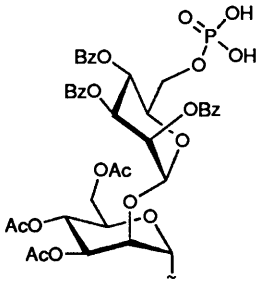

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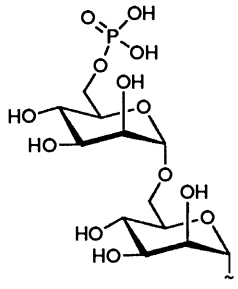

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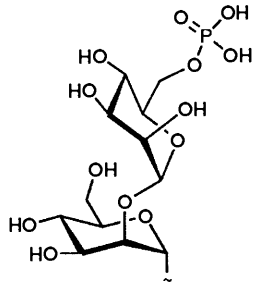

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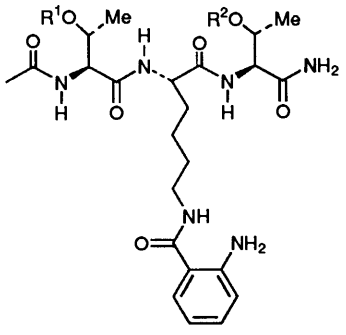
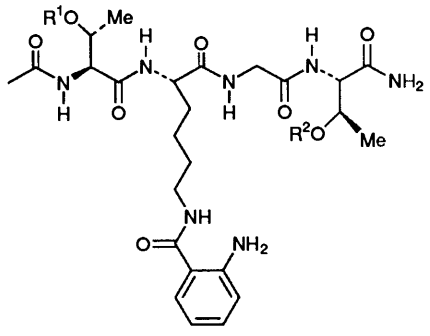
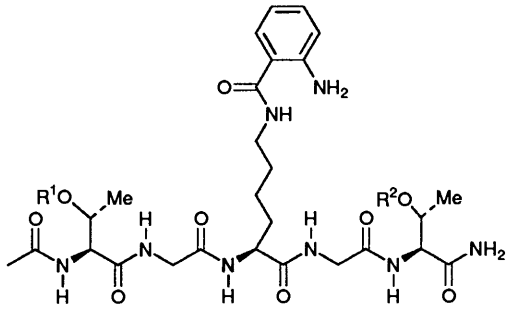




5 $R^1 = R^2 =$ **B**	**16** $R^1 = R^2 =$ **D**	**27** $R^1 = R^2 =$ **F**
6 $R^1 =$ **B**, $R^2 =$ **A**	**17** $R^1 =$ **D**, $R^2 =$ **C**	**28** $R^1 =$ **F**, $R^2 =$ **E**
7 $R^1 =$ **A**, $R^2 =$ **B**	**18** $R^1 =$ **C**, $R^2 =$ **D**	**29** $R^1 =$ **E**, $R^2 =$ **F**
8 $R^1 = R^2 =$ **A**	**19** $R^1 = R^2 =$ **C**	**30** $R^1 = R^2 =$ **E**

9 $R^1 = R^2 =$ **B**	**20** $R^1 = R^2 =$ **D**	**31** $R^1 = R^2 =$ **F**
10 $R^1 =$ **B**, $R^2 =$ **A**	**21** $R^1 =$ **D**, $R^2 =$ **C**	**32** $R^1 =$ **F**, $R^2 =$ **E**
11 $R^1 =$ **A**, $R^2 =$ **B**	**22** $R^1 =$ **C**, $R^2 =$ **D**	**33** $R^1 =$ **E**, $R^2 =$ **F**



12 $R^1 = R^2 =$ **B**	**23** $R^1 = R^2 =$ **D**	**34** $R^1 = R^2 =$ **F**
13 $R^1 =$ **B**, $R^2 =$ **A**	**24** $R^1 =$ **D**, $R^2 =$ **C**	**35** $R^1 =$ **F**, $R^2 =$ **E**
14 $R^1 =$ **A**, $R^2 =$ **B**	**25** $R^1 =$ **C**, $R^2 =$ **D**	**36** $R^1 =$ **E**, $R^2 =$ **F**
15 $R^1 = R^2 =$ **A**	**26** $R^1 = R^2 =$ **C**	**37** $R^1 = R^2 =$ **E**

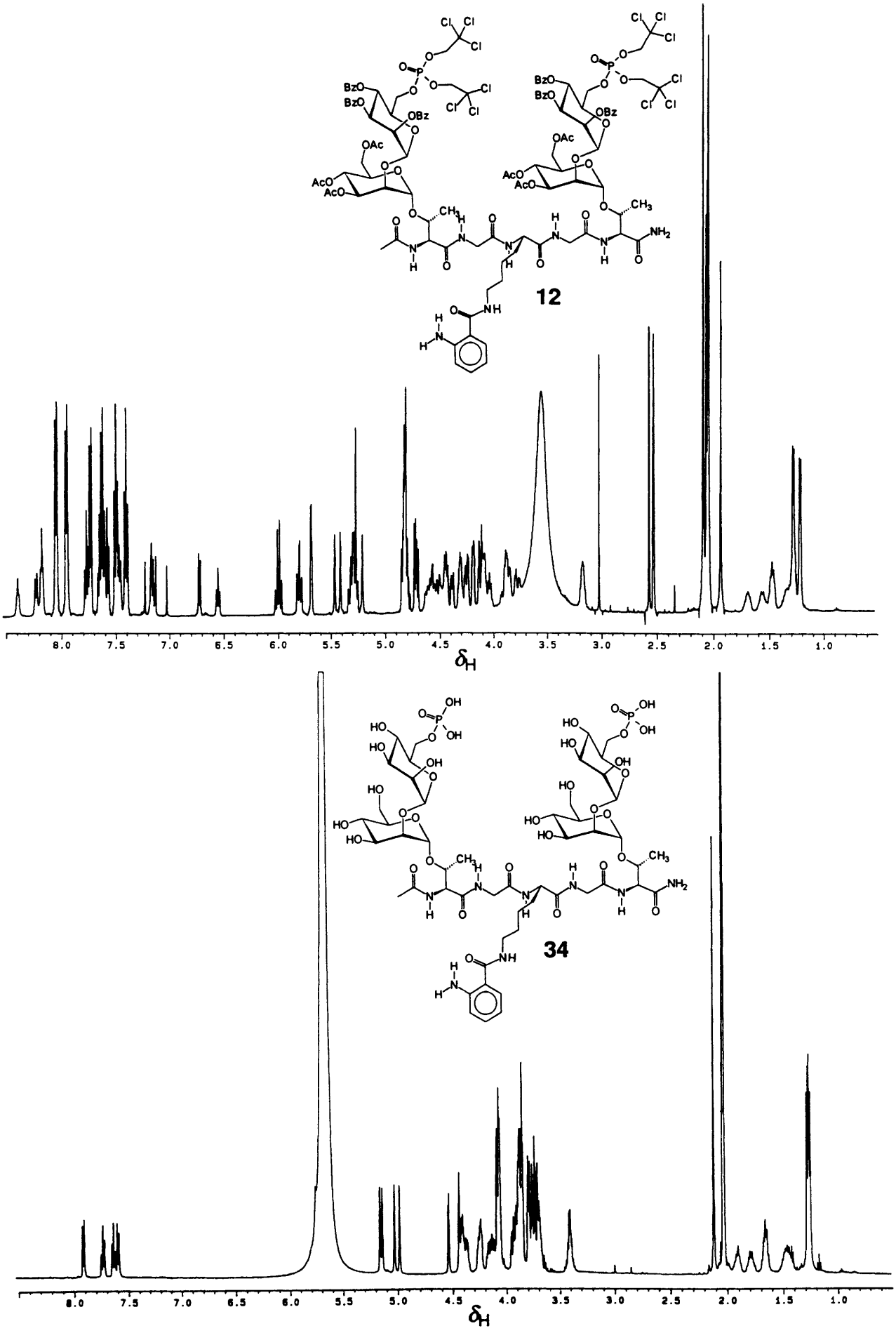


Fig. 1 ^{1}H NMR spectra of protected glycopeptide **12** (top) and fully deprotected glycopeptide **34** (bottom)

Table 2 Selected ^{1}H NMR chemical shifts (ppm) measured at 500 MHz for compounds **27–37** in D_2O–CD_3CO_2D (1:1) at 300 K [ref. δ_H CD_2HCO_2D 2.03]

	Anomeric protons	Phosphorylated 6-Hs	Thr H$^\alpha$	Thr H$^\beta$	Thr H$^\gamma$	Lys H$^\alpha$	Lys H$^\beta$	Lys H$^\gamma$	Lys H$^\delta$	Lys H$^\epsilon$	ABz
27	5.00, 5.02, 5.15, 5.18	4.10–4.12, 4.22–4.30	4.50–4.52	4.22–4.30, 4.36	1.23, 1.27	4.56	1.79, 1.91	1.42, 1.52	1.68	3.42	7.57, 7.61, 7.70, 7.89
28	4.89, 4.92, 5.00, 5.18	4.12–4.31	4.48–4.55	4.12–4.31	1.23, 1.28	4.48–4.55	1.79, 1.90	1.42, 1.52	1.68	3.42	7.56, 7.62, 7.70, 7.89
29	4.90, 4.91, 5.02, 5.17	4.11–4.23	4.51–4.56	4.25, 4.37	1.26, 1.28	4.51–4.56	1.80, 1.90	1.42, 1.49	1.68	3.42	7.54, 7.58, 7.69, 7.86
30	4.90 (2), 4.91, 4.93	4.16–4.25	4.51–4.55	4.26, 4.29	1.23, 1.26	4.50	1.80, 1.89	1.43, 1.51	1.68	3.42	7.56, 7.61, 7.70, 7.88
31	4.99, 5.03, 5.135, 5.14	4.05–4.30	4.42, 4.50	4.31, 4.40	1.23, 1.26	4.47	1.80, 1.90	1.48, 1.51	1.68	3.42	7.56, 7.60, 7.70, 7.88
32	4.89 (2), 4.99, 5.14	4.11–4.25	4.40–4.50	4.28–4.40	1.23, 1.26	4.40–4.50	1.79, 1.90	1.45, 1.52	1.68	3.42	7.54, 7.59, 7.68, 7.87
33	4.87, 4.89, 5.02, 5.15	4.05–4.29	4.44, 4.52	4.05–4.29, 4.40	1.25, 1.29	4.46	1.79, 1.90	1.40–1.53	1.68	3.42	7.57, 7.61, 7.71, 7.88
34	4.97, 5.02, 5.13, 5.16	4.11–4.19, 4.22–4.28	4.43, 4.53	4.36, 4.41	1.24, 1.27	4.41	1.78, 1.90	1.39–1.51	1.66	3.42	7.55, 7.60, 7.70, 7.88
35	4.89 (2), 4.98, 5.15	4.18–4.28, 4.30–4.42	4.45, 4.53	4.30–4.42	1.25, 1.27	4.40	1.78, 1.90	1.38–1.52	1.65	3.41	7.56, 7.61, 7.70, 7.88
36	4.88, 4.91, 5.03, 5.15	4.04–4.27	4.44, 4.51	4.30, 4.40	1.25, 1.29	4.40	1.78, 1.90	1.40–1.52	1.66	3.41	7.56, 7.60, 7.70, 7.88
37	4.88, 4.90 (3)	4.14–4.25	4.46, 4.51	4.27–4.37	1.26, 1.29	4.41	1.78, 1.90	1.40–1.52	1.66	3.42	7.56, 7.60, 7.70, 7.88

incubated with peroxidase-labelled streptavidin, and immobilized peroxidase was detected using the colorigenic substrate *o*-phenylenediamine (OPD) and hydrogen peroxide, and the absorbance A_{490} was measured at the ELISA reader. The binding-inhibition curves are presented in Fig. 2, and the IC_{50}-values are presented in Table 3. As can be seen, the glycopeptides containing two phosphorylated α(1→2)-linked mannose disaccharides are very potent inhibitors. The peptides containing one α(1→2)- and one α(1→6)-linked mannose disaccharide are of intermediate potency, and the peptides containing only α(1→6)-linked mannose disaccharides are weak inhibitors. These results were as expected according to the reported binding specificity of the MPRs,[6,7] showing a 6′-*O*-phosphorylated mannose disaccharide linked α(1→2) to be a 3–5-fold better inhibitor than Man-6-*P*, and a 6′-*O*-phosphorylated mannose disaccharide linked α(1→6) to be a 2–3-fold less potent inhibitor than Man-6-*P*. Two previously synthesized tripeptides[14] **38** and **39**, each containing only one 6′-*O*-phosphorylated mannose disaccharide linked α(1→2) or α(1→6), respectively, were also tested, and showed the tripeptide containing the α(1→2)-linked mannose disaccharide to be a 3-fold better inhibitor than Man-6-*P*, and the tripeptide containing the α(1→6)-linked mannose disaccharide to be a less potent inhibitor than Man-6-*P*, also as expected according to the binding specificity of the MPRs. These results also indicate that there is no significant interaction between the peptide template and the receptor. The results also demonstrate the bidentate nature of the receptor, as for instance divalent glycopeptide **27** is a 839-fold stronger inhibitor than the monovalent glycopeptide **38**.

To test the glycopeptide templates, where the peptide backbone mimicks an oligosaccharide scaffold, against a suggested natural ligand, two pentamannosides[6] **40** and **41**, containing two or one terminal Man-6-*P* units α(1→2)-linked, respectively, were also tested in the ELISA assay. As can be seen in Fig. 2, the glycopeptide templates containing two phosphorylated disaccharides in α(1→2)-linkage, compounds **27**, **31** and **34**, are 17-, 7- and 9-fold better inhibitors, respectively, than is the diphosphorylated pentamannoside **40**, previously suggested to be a natural ligand, clearly demonstrating that the peptide-template concept has great potential for the design of inhibitors.

The most potent inhibitors, glycopeptides **27**, **31** and **34** which all contain two α(1→2)-linked mannose disaccharides, were nearly equally potent, each having submicromolar IC_{50}-values. This indicates that the distance between the two binding sites in the receptor is spanned with only three amino acids between the disaccharides. The fact that the glycopeptides containing four or five amino acids are equally good inhibitors could be due either to folding of the peptide backbone, or to flexibility of the receptor.

The pentamannoside **40** is a much less potent inhibitor than are the tripeptides **27**, **31** and **34**. Furthermore, as indicated by molecular modelling, the tripeptide part of glycopeptide **27** is much larger than the scaffolding mannose unit in compound **40**, for which it is acting as a template. In Fig. 3, a molecular model of **27** is compared with the model of a mannose heptasaccharide containing two terminal Man-6-*P* units, another proposed natural ligand.[8–10] Here the tripeptide part of compound **27** is of the same size as the scaffolding trisaccharide, for which it is acting as a template, resulting in a similar spatial arrangement of the mannose disaccharides. Thus, these results seem to indicate that a larger oligosaccharide than the pentamannoside **40** is likely to be the optimal natural ligand, or at least that larger oligosaccharides have a higher affinity for the receptor.

In conclusion, a simple method for the synthesis of glycosylated peptide templates containing 6′-*O*-phosphorylated mannose disaccharides has been developed. Furthermore, the application of the glycopeptide templates in a binding-inhibition assay has clearly demonstrated that glycopeptide templates can be obtained, which are as good as, or better, inhibitors at the ^{Cl}MPR than are the oligosaccharide ligands they are mimicking.

A diversity of compounds spanning the required distance between the phosphorylated mannose disaccharides in the core mannose trisaccharides of *N*-linked oligosaccharides could easily be obtained by MCPS of a library of glycopeptides. The principle presented here may even be valid for the synthesis of artificial ligands for the many lectins (*e.g.*, C-type lectins) that are involved in binding of complex or high-mannose structures.

The fluorescent moiety in the glycopeptides was not used for

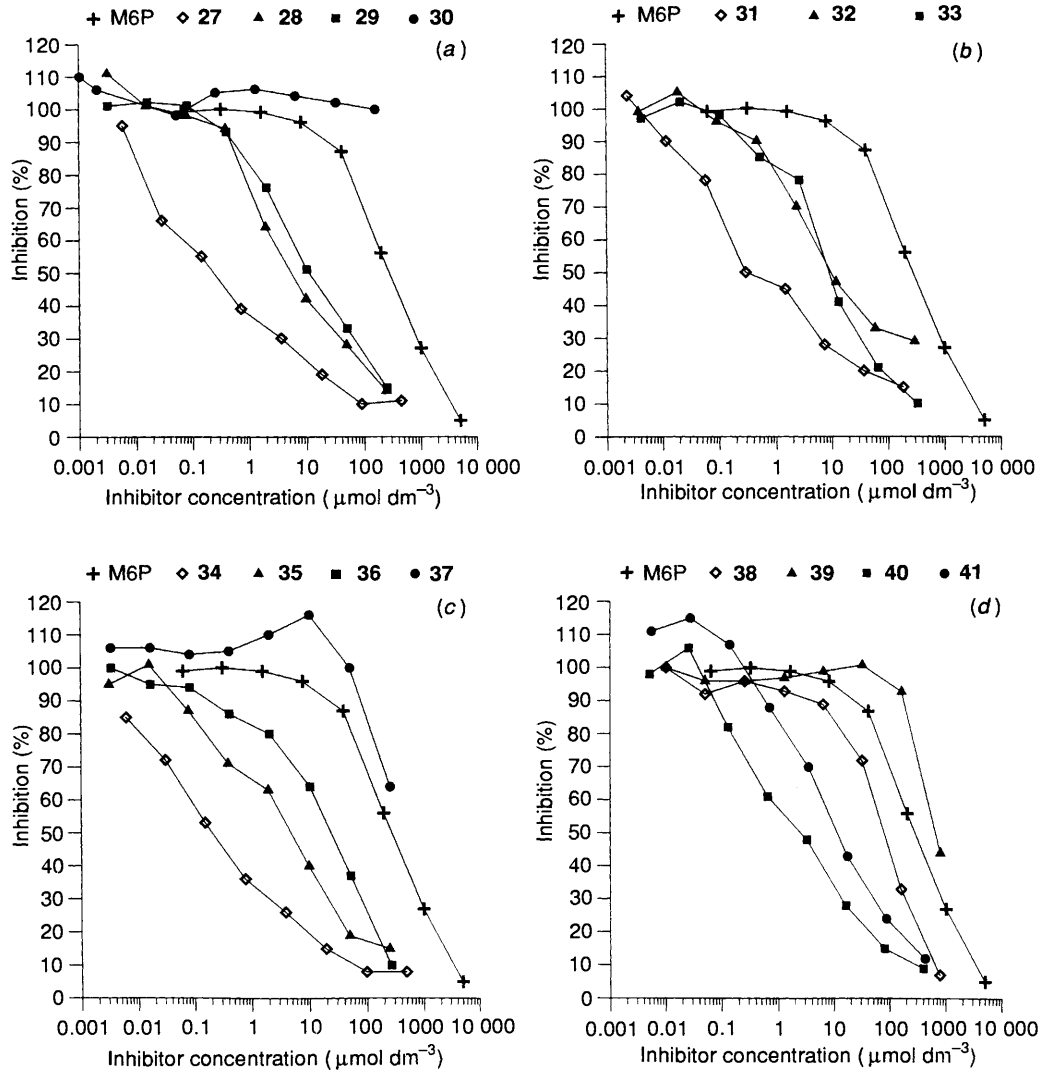


Fig. 2 Inhibition of binding of the ^{CI}MPR to a phosphomannan core fragment, immobilized on polystyrene, by synthetic phosphorylated glycopeptides: (A) diglycosylated tripeptide templates, (B) diglycosylated tetrapeptide templates, (C) diglycosylated pentapeptide templates, and (D) monoglycosylated tripeptides and phosphorylated pentamannosides as control compounds

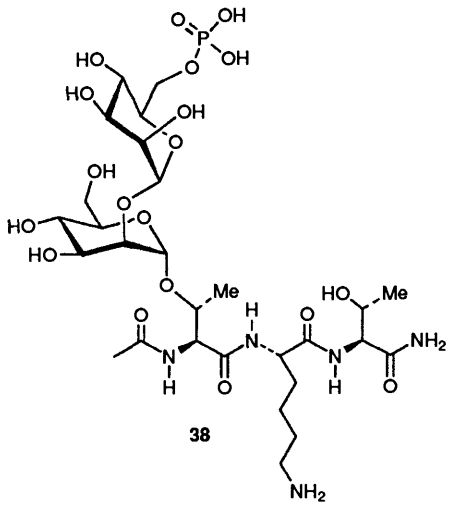
Table 3 IC_{50}-Values (μmol dm^{-3}) for compounds **27**–**41** and Man-6-*P*

Compound	IC_{50}	Compound	IC_{50}	Compound	IC_{50}	Compound	IC_{50}
27	0.093	Man-6-*P*	260	**34**	0.17	**38**	78
28	4.0	**31**	0.23	**35**	5.0	**39**	630
29	9.0	**32**	7.0	**36**	13	**40**	1.6
30	> 1000	**33**	8.0	**37**	330	**41**	6.8

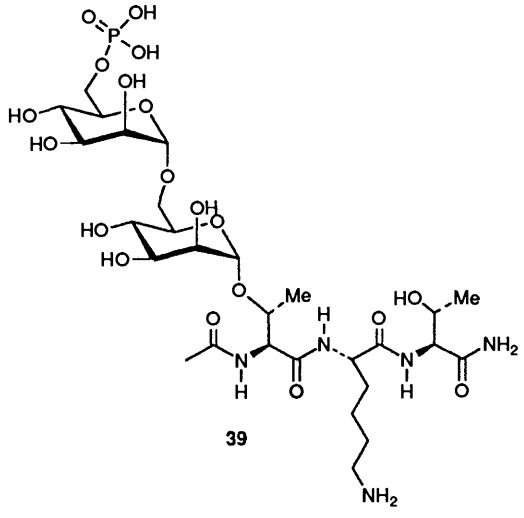
detection in the present ELISA assay, but will be used for labelling of the receptor in further biological studies.

Experimental

Analytical-grade solvents were dried over molecular sieves (3 Å). DMF was freshly distilled by fractional distillation at reduced pressure. Light petroleum was the 60–80 °C fraction. Concentrations were performed under reduced pressure at temperatures < 40 °C. Silver carbonate, *N*-hydroxysuccinimid biotin, bovine serum albumin (BSA), mannose 6-phosphate and glucose 6-phosphate were from Sigma; *N*-ethylmorpholine (NEM), DCCI, 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (Dhbt-OH), pentafluorophenol (Pfp-OH) and hydrazine hydrate were from Fluka. Streptavidine horseradish peroxidase was from Amersham (Arlington Heights, IL) and Maxisorp 96-well microtiter plates were from Nunc (Denmark). To 'activate' zinc, commercial zinc powder was washed successively and rapidly with 1 mol dm^{-3} HCl and water and was dried at 120 °C overnight. Suitably protected N^{α}-Fmoc amino acids and Pfp esters were purchased from MilliGen (Taastrup, Denmark) or Bachem (Bubendorf, Switzerland). The high-molecular-mass phospho-D-mannan from *Hansenula holstii* (Y.2448) was a gift from Dr. Morey Slodki of the Northern Regional Research Center, Peoria, IL, USA. Cyanogen bromide-activated Sepharose was from Pharmacia (Uppsala, Sweden). Vacuum liquid chromatography (VLC)[30] was performed on Merck silica gel 60 H (0.04–0.60 mm). Nomenclature is according to IUPAC recommendations. ESMS was performed in the positive mode for the protected glycopeptides and in the negative mode



38



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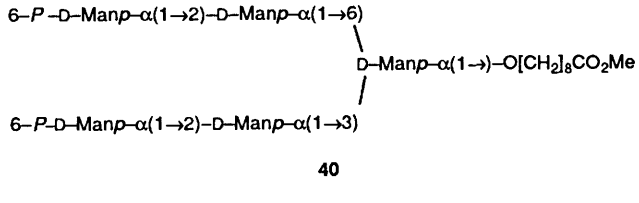
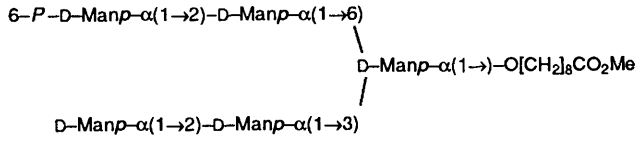


40



41

for the deprotected glycopeptides on a VG Fisons Quattro instrument. Elemental analyses were carried out at LEO Pharmaceutical Products, Denmark. ^1H and ^{13}C NMR spectra were recorded on a Bruker 500 MHz spectrometer. Chemical shifts are given in ppm and referenced to internal $SiMe_4$ (δ_H, δ_C 0.00) for solutions in $CDCl_3$ at 300 K, and to external dioxane (δ_H 3.76, δ_C 67.40) for solutions in D_2O at 300 K. For the assignment of signals proton–proton and carbon–carbon shift-correlation spectroscopy were used. Preparative reversed-phase HPLC separations were performed on a Waters HPLC system using a Delta PAK C-18 column (15 μm; 300 Å; 25 mm × 200 mm) with a flow rate of 10 cm^3 min^{-1}, and

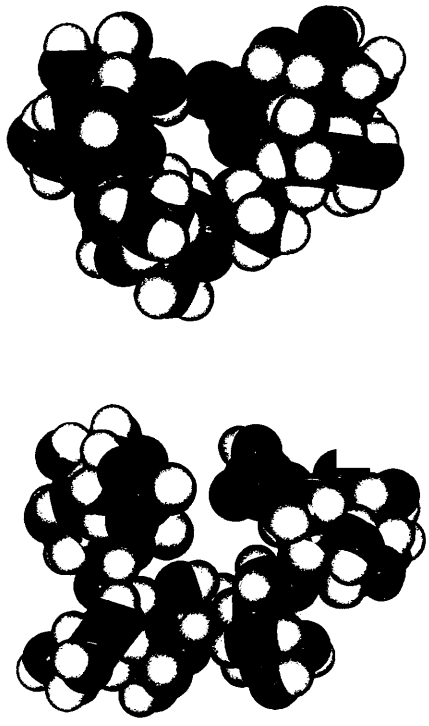


Fig. 3 Molecular models, derived by molecular dynamics calculation/energy minimization, of glycopeptide **27** (top) and the heptasaccharide 6-*P*-D-Man*p*-α(1→2)-D-Man*p*-α(1→3)-D-Man*p*-α(1→6)-[6-*P*-D-Man*p*-α(1→2)-D-Man*p*-α(1→2)-D-Man*p*-α(1→3)]-D-Man*p* (bottom)

detection at 215 nm with a photodiode array detector (Waters M 991). Solvent system A: 0.1% TFA; B: 0.1% TFA in 90% acetonitrile–10% water. Buffers and solutions used in the purification of the ^{Cl}MPR and in the ELISA assays were: PBS (1.9 mmol dm^{-3} NaH_2PO_4, 8.1 mmol dm^{-3} Na_2PO_4 and 154 mmol dm^{-3} NaCl, pH 7.4); Carbonate Buffer (0.1 mol dm^{-3}, pH 9.6); Buffer A (50 mmol dm^{-3} imidazole, pH 6.5, 150 mmol dm^{-3} NaCl, 0.02% NaN_3); Dilution Buffer (PBS, pH 7.4 containing 1% Triton X-100, 1% BSA and Phenol Red); Washing Buffer (PBS, pH 7.4 containing 1% Triton X-100); and OPD (*o*-phenylenediamine 1 mg cm^{-3} in citrate buffer, pH 5.0 and 1 mm^3 30% H_2O_2 cm^{-3}).

Purification of the ^{Cl}*MPR.*—Phosphomannan core fragment (30 mg) was prepared from the high-molecular-mass phosphomannan by the established method[29] and was coupled to cyanogen bromide-activated Sepharose (3 g) by the method of Sahagian *et al.*[31] The resulting phosphomannan-containing affinity matrix was used to purify the ^{Cl}MPR from foetal bovine serum as described by Li *et al.*[32] to give 1 mg of receptor from 250 cm^3 of serum.

Biotinylation of the ^{Cl}*MPR.*—*N*-Hydroxysuccinimid biotin in dimethyl sulfoxide (10 mg cm^{-3}) and the ^{Cl}MPR (1 mg cm^{-3}) in PBS were mixed in a ratio which resulted in 110 μg of ester per mg of receptor. The mixture was incubated end-over-end for 4 h at room temperature. The reaction was stopped by treatment with ammonium chloride at a final concentration of 0.7 mol dm^{-3} for 10 min, and the receptor was dialysed against PBS to remove excess of biotin. The buffer was changed to Buffer A before use in the ELISA assay.

ELISA Assay.—96-Well microtiter plates were coated overnight at 4 °C with phosphomannan core fragment (2 μg cm^{-3}, 100 mm^3 $well^{-1}$ in Carbonate Buffer). Each well was subsequently blocked for 2 h with 1% BSA (200 mm^3) in

Carbonate Buffer at room temperature and washed five times with Washing Buffer. The inhibitors were added to the microtiter wells in 5-fold dilutions in Buffer A and co-incubated for 1 h with 5 μg cm^{-3} biotinylated ^{CI}MPR at room temperature. After being washed five times, the wells were incubated for 1 h at room temperature with a 1:1000 dilution (in Dilution Buffer) of horse radish peroxidase-labelled streptavidin. Detection of immobilized horseradish peroxidase was achieved by addition of 100 mm^3 $well^{-1}$ OPD solution, and the reaction was stopped with 100 mm^3 $well^{-1}$ 1 mol dm^{-3} sulfuric acid. The absorbance was measured at 490 nm.

MCPS. General Procedure.—Synthesis of the glycopeptides was performed in DMF, using the resin PEGA 1900/130[23,24] with a substitution of 0.11 mmol g^{-1}. The resin was dried and measured in portions (60 mg) into the 20 wells of the 20-well manual multiple-column peptide synthesizer,[25] each well being fitted with a sintered Teflon filter (70 μm pore size). After swelling of the resin with DMF the solvent was drained from the resin by suction on the chamber beneath the wells, and a slight overpressure of air was established *via* a 1000 cm^3 min^{-1} gasflow controller. Reagents were dispensed with an Eppendorf multipipette 4780. The resin was derivatized with the Rink-linker:[27] a solution (0.75 cm^3) of the Rink-linker (194 mg, 0.36 mmol), TBTU (104 mg, 0.32 mmol) and NEM (92 mm^3, 0.72 mmol) in DMF (15 cm^3) was added to each well. After 2 h the solution was removed by suction and the wells were washed with DMF (3 × 1 cm^3 each), and a solution (0.75 cm^3) of acetic anhydride–DMF (1:7) was added to each well. After 20 min the solution was removed by suction and the wells were washed thoroughly with DMF (15 × 1 cm^3 each). This washing procedure was repeated after each coupling/deprotection. N^{α}-Fmoc deprotection was then effected by a 1 min and a 25 min treatment of the resin with 20% piperidine in DMF (0.75 cm^3 in each well). The piperidine solution was removed by suction and the resin was washed. The amino acids used were: glycosylated building block **1** (18 mg, 0.010 mmol), glycosylated building block **2** (16 mg, 0.010 mmol), Fmoc-Lys(Boc-ABz)-OPfp **4** (13 mg, 0.017 mmol) or Fmoc-Gly-OPfp (10 mg, 0.021 mmol) in each well. Each peptide was synthesized in two wells. The first amino acid and Dhbt-OH (3 mg, 0.017 mmol) were then added to each well in DMF (0.75 cm^3) and the mixture was left for 24 h. After washing of the resin the N^{α}-Fmoc group was removed and the resin was washed as described above. The synthesis cycle was repeated until the end of each peptide. After the last N^{α}-Fmoc deprotection the resin was treated with acetic anhydride–DMF (1:7) (0.75 cm^3 in each well) for 20 min. The resin was then washed successively with DMF (15 × 1 cm^3 each) and diethyl ether (3 × 1 cm^3 each). The resin was removed from the wells and was dried. Cleavage of the glycopeptides from the linker was performed by treatment with 95% TFA for 2 h, followed by filtration and washing of the resin with TFA and dichloromethane. Acetic acid (2 cm^3) was added to the individual filtrates. After concentration, the glycopeptides were purified by HPLC, using, first, the linear gradient 85–100% solvent B during 30 min, then 100% solvent B for 30 min.

Deprotection of the Glycopeptides. General Procedure.—The fully protected glycopeptide (*e.g.*, 10 mg) was dissolved in pyridine containing 10% acetic acid (3 cm^3). Zinc (17 times the weight of glycopeptide; *e.g.*, 170 mg) and silver carbonate (6 times the weight of glycopeptide; *e.g.*, 60 mg) were added, and the suspension was stirred at 50–60 °C for 18 h. The suspension was then filtered and directly purified by HPLC using, first, solvent A (100%) for 10 min, then the linear gradient 0–100% solvent B during 100 min.

The partially deprotected glycopeptide was then dissolved in chloroform (200 mm^3), and methanol (800 mm^3) and hydrazine hydrate (200 mm^3) were added. After 2 h the reaction mixture was directly purified by gel filtration followed by HPLC using the same gradient as above.

N^{ε}-[2-(tert-*Butoxycarbonylamino)benzoyl*]-N^{α}-(*fluoren*-9-*ylmethoxycarbonyl*)-L-*lysine Pentafluorophenyl Ester* **4**. Fmoc-Lys(Boc)-OH (2.0 g, 4.27 mmol) was dissolved in TFA (100 cm^3) and the solution was concentrated and lyophilized. The resulting oil was dissolved in DMF (10 cm^3), and a solution of Boc-ABz-ODhbt (1.63 g, 4.27 mmol) and NEM (5.4 cm^3, 42.7 mmol) in DMF (20 cm^3) was added. The solution was stirred at room temperature for 1 h, then was kept at −20 °C overnight. The solution was then concentrated, and purified by VLC [first light petroleum–ethyl acetate (1:1) (500 cm^3); then light petroleum–ethyl acetate–acetic acid (10:10:1)] to yield compound **3** (2.02 g, 81%).

Compound **3** (1.28 g, 2.19 mmol) and Pfp-OH (0.40 g, 2.19 mmol) were dissolved in THF (5 cm^3) and the solution was cooled to 0 °C. DCCI (0.45 g, 2.19 mmol) was added, and the solution was stirred at 0 °C for 1 h, then was left at −20 °C overnight. The reaction mixture was then filtered, concentrated, and purified by VLC [light petroleum–ethyl acetate (4:1)] to yield *title compound* **4** (1.30 g, 79%) (Found: C, 62.1; H, 5.0; N, 5.4. $C_{39}H_{36}F_5N_3O_7$ requires C, 62.15; H, 4.81; N, 5.58%); δ_H(500 MHz; $CDCl_3$) 1.51 (9 H, s, Boc), 1.55 (2 H, m, H^{γ}), 1.72 (2 H, m, H^{δ}), 1.93 (1 H, m, H^{β}), 2.08 (1 H, m, H^{β}), 3.45 (2 H, m, H^{ε}), 4.20 (1 H, t, Fmoc), 4.35–4.47 (2 H, m, Fmoc), 4.73 (1 H, m, H^{α}), 5.49 (1 H, d, $N^{\alpha}H$), 6.41 (1 H, t, $N^{\varepsilon}H$), 6.91 (1 H, t, ABz), 7.28 (2 H, t, Fmoc), 7.34–7.41 (5 H, m, ABz and Fmoc), 7.56 (2 H, d, Fmoc), 7.75 (2 H, d, Fmoc) and 8.32 (1 H, d, NH^{ABz}).

Ac-Thr[α-D-*Man*-6-P-(1→2)-α-D-*Man*]-*Lys*(*Abz*)-*Thr*[α-D-*Man*-6-P-(1→2)-α-D-*Man*]-NH_2 **27**. Solid-phase synthesis and purification (t_R 39 min) as described in the general procedure gave compound **5** (22.1 mg, 61%). ^{1}H NMR data are presented in Table 1 [Found: *m/z* 2719.8 (M + 2 H^+). $C_{109}H_{118}Cl_{12}N_6O_{45}P_2$ requires M_{av}, 2717.53].

Cleavage of the 2,2,2-trichloroethyl groups from compound **5** (10.1 mg) and purification (t_R 93 min) as described in the general procedure gave compound **16** (5.0 mg, 62%).

Deacylation of compound **16** (5.0 mg) and purification (t_R 28 min) were performed as described in the general procedure to yield *title compound* **27** (2.8 mg, 93%). ^{1}H NMR data are presented in Table 2 [Found: *m/z*, 1315.6 (M − H^+). $C_{47}H_{48}N_6O_{33}P_2$ requires M, 1316.41].

Ac-Thr[α-D-*Man*-6-P-(1→2)-α-D-*Man*]-*Lys*(*ABz*)-*Thr*[α-D-*Man*-6-P-(1→6)-α-D-*Man*]-NH_2 **28**. Solid-phase synthesis and purification (t_R 46 min) as described in the general procedure gave compound **6** (17.1 mg, 45%). ^{1}H NMR data are presented in Table 1 [Found: *m/z*, 2907.8 (M + 2 H^+). $C_{124}H_{124}Cl_{12}N_6O_{45}P_2$ requires M_{av}, 2905.74].

Cleavage of the 2,2,2-trichloroethyl groups from compound **6** (14.0 mg) and purification (t_R 101 min) as described in the general procedure yielded compound **17** (7.1 mg, 61%).

Deacylation of compound **17** (4.8 mg) and purification (t_R 28 min) were performed as described in the general procedure to yield *title compound* **28** (2.5 mg, 94%). ^{1}H NMR data are presented in Table 2 [Found: *m/z*, 1315.6 (M − H^+). $C_{47}H_{78}N_6O_{33}P_2$ requires M, 1316.41].

Ac-Thr[α-D-*Man*-6-P-(1→6)-α-D-*Man*]-*Lys*(*ABz*)-*Thr*[α-D-*Man*-6-P-(1→2)-α-D-*Man*]-NH_2 **29**. Solid-phase synthesis and purification (t_R 43 min) as described in the general procedure gave compound **7** (27.5 mg, 72%). ^{1}H NMR data are presented in Table 1 [Found: *m/z*, 2907.6 (M + 2 H^+). $C_{124}H_{124}Cl_{12}N_6O_{45}P_2$ requires M_{av}, 2905.74].

Cleavage of the 2,2,2-trichloroethyl groups from compound **7** (11.0 mg) and purification (t_R 99 min) as described in the general procedure gave compound **18** (5.6 mg, 62%).

Deacylation of compound **18** (5.6 mg) and purification by gel filtration alone were performed as described in the general

procedure to yield *title compound* **29** (2.6 mg, 84%). ^{1}H NMR data are presented in Table 2 [Found: *m/z*, 1315.5 (M − H$^+$). $C_{47}H_{78}N_6O_{33}P_2$ requires M, 1316.41].

Ac-Thr[α-D-*Man*-6-P-(1→6)-α-D-*Man*]-*Lys*(*ABz*)-*Thr*[α-D-*Man*-6-P-(1→6)-α-D-*Man*]-NH_2 **30**. Synthesis of the fully protected glycopeptide **8** was performed by the syringe method[14,26] using the PEGA 1900/130 resin (0.3 g). Except for the amount of resin, and concomitantly the amount of amino acids *etc.*, the synthesis was otherwise similar to the general procedure described for MCPS. Purification (t_R 59 min) as described in the general procedure gave compound **8** (85 mg, 92%). ^{1}H NMR data are presented in Table 1 [Found: *m/z*, 3094.0 (M + 2 H$^+$). $C_{139}H_{130}Cl_{12}N_6O_{45}P_2$ requires M_{av}, 3091.95].

Cleavage of the 2,2,2-trichloroethyl groups from compound **8** (10.0 mg) and purification (t_R 105 min) as described in the general procedure yielded compound **19** (6.8 mg, 81%).

Deacylation of compound **19** (4.1 mg) and purification (t_R 28 min) were performed as described in the general procedure to yield *title compound* **30** (1.5 mg, 90%). ^{1}H NMR data are presented in Table 2 [Found: *m/z*, 1315.6 (M − H$^+$). $C_{47}H_{78}N_6O_{33}P_2$ requires M, 1316.41].

Ac-Thr[α-D-*Man*-6-P-(1→2)-α-D-*Man*]-*Lys*(*ABz*)-*Gly*-*Thr*[α-D-*Man*-6-P-(1→2)-α-D-*Man*]-NH_2 **31**. Solid-phase synthesis and purification (t_R 41 min) as described in the general procedure gave compound **9** (29.3 mg, 80%). ^{1}H NMR data are presented in Table 1 [Found: *m/z*, 2778.0 (M + 2 H$^+$). $C_{111}H_{121}Cl_{12}N_7O_{46}P_2$ requires M_{av}, 2776.58].

Cleavage of the 2,2,2-trichloroethyl groups from compound **9** (13 mg) and purification (t_R 92 min) as described in the general procedure gave compound **20** (5.7 mg, 54%).

Deacylation of compound **20** (5.7 mg) and purification (t_R 29 min) were performed as described in the general procedure to yield *title compound* **31** (2.6 mg, 73%). ^{1}H NMR data are presented in Table 2 [Found: *m/z*, 1372.7 (M − H$^+$). $C_{49}H_{81}N_7O_{34}P_2$ requires M, 1373.43].

Ac-Thr[α-D-*Man*-6-P-(1→2)-α-D-*Man*]-*Lys*(*ABz*)-*Gly*-*Thr*[α-D-*Man*-6-P-(1→6)-α-D-*Man*]-NH_2 **32**. Solid-phase synthesis and purification (t_R 41 min) as described in the general procedure gave compound **10** (39.0 mg, 100%). ^{1}H NMR data are presented in Table 1 [Found: 2964.8 (M + 2 H$^+$). $C_{126}H_{127}Cl_{12}N_7O_{46}P_2$ requires M_{av}. 2962.79].

Cleavage of the 2,2,2-trichloroethyl groups from compound **10** (6.6 mg) and purification (t_R 98 min) as described in the general procedure gave compound **21** (3.3 mg, 60%).

Deacylation of compound **21** (3.3 mg) and purification (t_R 28 min) were performed as described in the general procedure to yield *title compound* **32** (1.5 mg, 81%). ^{1}H NMR data are presented in Table 2 [Found: *m/z*, 1372.6 (M − H$^+$). $C_{49}H_{81}N_7O_{34}P_2$ requires M, 1373.43].

Ac-Thr[α-D-*Man*-6-P-(1→6)-α-D-*Man*]-*Lys*(*ABz*)-*Gly*-*Thr*[α-D-*Man*-6-P-(1→2)-α-D-*Man*]-NH_2 **33**. Solid-phase synthesis and purification (t_R 41 min) as described in the general procedure gave compound **11** (36.5 mg, 93%). ^{1}H NMR data are presented in Table 1 [Found: *m/z*, 2964.6 (M + 2 H$^+$). $C_{126}H_{127}Cl_{12}N_7O_{46}P_2$ requires M_{av}, 2962.79].

Cleavage of the 2,2,2-trichloroethyl groups from compound **11** (10.1 mg) and purification (t_R 98 min) as described in the general procedure yielded compound **22** (6.0 mg, 72%).

Deacylation of compound **22** (6.0 mg) and purification (t_R 29 min) were performed as described in the general procedure to yield *title compound* **33** (3.2 mg, 94%). ^{1}H NMR data are presented in Table 2 [Found: *m/z*, 1372.7 (M − H$^+$). $C_{49}H_{81}N_7O_{34}P_2$ requires M, 1373.43].

Ac-Thr[α-D-*Man*-6-P-(1→2)-α-D-*Man*]-*Gly*-*Lys*(*ABz*)-*Gly*-*Thr*[α-D-*Man*-6-P-(1→2)-α-D-*Man*]-NH_2 **34**. Solid-phase synthesis and purification (t_R 37 min) as described in the general procedure gave compound **12** (26.2 mg, 70%). ^{1}H NMR data are presented in Table 1 [Found: *m/z*, 2835.6 (M + 2 H$^+$). $C_{113}H_{124}Cl_{12}N_8O_{47}P_2$ requires M_{av}, 2833.63].

Cleavage of the 2,2,2-trichloroethyl groups from compound **12** (11.5 mg) and purification (t_R 92 min) as described in the general procedure gave compound **23** (5.1 mg, 55%).

Deacylation of compound **23** (5.1 mg) and purification (t_R 30 min) were performed as described in the general procedure to yield *title compound* **34** (2.9 mg, 93%). ^{1}H NMR data are presented in Table 2 [Found: *m/z*, 1429.6 (M − H$^+$). $C_{51}H_{84}N_8O_{35}P_2$ requires M, 1430.45].

Ac-Thr[α-D-*Man*-6-P-(1→2)-α-D-*Man*]-*Gly*-*Lys*(*ABz*)-*Gly*-*Thr*[α-D-*Man*-6-P-(1→6)-α-D-*Man*]-NH_2 **35**. Solid-phase synthesis and purification (t_R 41 min) as described in the general procedure gave compound **13** (39.8 mg, 100%). ^{1}H NMR data are presented in Table 1 [Found: *m/z*, 3021.4 (M + 2 H$^+$). $C_{128}H_{130}Cl_{12}N_8O_{47}P_2$ requires M_{av}, 3019.84].

Cleavage of the 2,2,2-trichloroethyl groups from compound **13** (11.7 mg) and purification (t_R 99 min) as described in the general procedure gave compound **24** (5.2 mg, 54%).

Deacylation of compound **24** (5.2 mg) and purification (t_R 29 min) were performed as described in the general procedure to yield *title compound* **35** (2.9 mg, 97%). ^{1}H NMR data are presented in Table 2 [Found: *m/z*, 1429.7 (M − H$^+$). $C_{51}H_{84}N_8O_{35}P_2$ requires M, 1430.45].

Ac-Thr[α-D-*Man*-6-P-(1→6)-α-D*Man*]-*Gly*-*Lys*(*ABz*)-*Gly*-*Thr*[α-D-*Man*-6-P-(1→2)-α-D-*Man*]-NH_2 **36**. Solid-phase synthesis and purification (t_R 37 min) as described in the general procedure gave compound **14** (32 mg, 80%). ^{1}H NMR data are presented in Table 1 [Found: *m/z*, 3022.0 (M + 2 H$^+$). $C_{128}H_{130}Cl_{12}N_8O_{47}P_2$ requires M_{av}, 3019.84].

Cleavage of the 2,2,2-trichloroethyl groups from compound **14** (11.3 mg) and purification (t_R 97 min) as described in the general procedure to yield compound **25** (5.1 mg, 55%).

Deacylation of compound **25** (5.1 mg) and purification (t_R 29 min) were performed as described in the general procedure to yield *title compound* **36** (2.1 mg, 72%). ^{1}H NMR data are presented in Table 2 [Found: *m/z*, 1429.5 (M − H$^+$). $C_{51}H_{84}N_8O_{35}P_2$ requires M, 1430.45].

Ac-Thr[α-D-*Man*-6-P-(1→6)-α-D-*Man*]-*Gly*-*Lys*(*ABz*)-*Gly*-*Thr*[α-D-*Man*-6-P-(1→6)-α-D-*Man*]-NH_2 **37**. Solid-phase synthesis and purification (t_R 51 min) as described in the general procedure gave compound **15** (26.8 mg, 63%). ^{1}H NMR data are presented in Table 1 [Found: *m/z*, 3207.8 (M + 2 H$^+$). $C_{143}H_{136}Cl_{12}N_8O_{47}P_2$ requires M_{av}, 3206.06].

Cleavage of the 2,2,2-trichloroethyl groups from compound **15** (11.0 mg) and purification (t_R 102 min) as described in the general procedure to yield compound **26** (4.7 mg, 51%).

Deacylation of compound **26** (4.7 mg) and purification (t_R 29 min) were performed as described in the general procedure to yield *title compound* **37** (2.0 mg, 80%). ^{1}H NMR data are presented in Table 2 [Found: *m/z*, 1429.6 (M − H$^+$). $C_{51}H_{84}N_8O_{35}P_2$ requires M, 1430.45].

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