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Constrained Glycopeptide Ligands for MPRs. Limitations of Unprotected Phosphorylated Building Blocks

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Abstract—A new methodology for the synthesis of cyclic and phosphorylated glycopeptide templates was developed. First, fully protected building blocks containing mannose and mannose disaccharides with bis-trichloroethyl phosphate on Fmoc-Thr-OPfp were synthesized. These were used in solid-phase assembly through side chain anchoring of glycosylated hexa- and octa-peptides protected at the C-terminal carboxylate as the allyl ester. Selective allyl ester cleavage and head-to-tail cyclization under pseudo-dilution conditions gave a high yield of pure cyclic peptide templates. Unprotected phosphate in the building block was evaluated as an alternative to the problematic trichloroethyl group. It was found that one unprotected phosphate is readily incorporated, whereas the second unprotected phosphorylated building block react very slowly due to electrostatic repulsion in the solid-phase synthesis. For comparison with previous binding studies modified glycopeptide templates containing only phosphorylated mannose monosaccharides or templates modified in the peptide part were synthesized. All the structures were tested for their binding to the mannose 6-phosphate receptor, and it was found that although mannose disaccharides are required for optimal interaction, the detailed structure of the peptide template has a strong influence on binding to the receptor. The restricted conformations of the cyclic peptides decreased the binding considerably.

Introduction

Mannose 6-phosphate (6-*O*-PO_3H_2-Man) has been found to inhibit inflammation in the central nervous system,[1] possibly by blocking the interaction between mannose 6-phosphate receptors (MPRs) and transport of lysosomal enzymes.[2–4] The lysosomal enzymes have oligosaccharides attached through N-linkages to asparagine, and these oligosaccharides contain terminal Man-6-P residues[5] to be processed and exported properly in the Golgi apparatus.

In the previous work[6,7] it was demonstrated that simple peptides (tri- to penta-), α-glycosylated with two α(1→2) linked mannose disaccharides act as highly potent bidentate ligands for MPRs. The so far most promising compound, **35**, had anthranilic acid (ABz-OH) attached to a lysine side chain: Ac-Thr[6-*O*-PO_3H_2-Man(1→2)Man]-Lys(ABz)-Thr[6-*O*-PO_3H_2-Man(1→2)Man]-NH_2. However, besides serving as a fluorescent probe, the ABz substituent was also found to contribute to the activity of the peptide, which exhibited approximately 1500-fold better binding to the cation-independent mannose 6-phosphate receptor (^{CI}MPR) compared to the monosaccharide 6-*O*-PO_3H_2-Man itself. A simple enzyme-linked immunosorbent assay (ELISA) has been developed to monitor the IC_{50} for putative glycopeptide inhibitors.[6]

In order to simplify the synthesis, and to investigate whether high-affinity binding to the MPRs is possible with ligands containing only Man-6-P monosaccharide residues, glycopeptide **17** was regarded as a putative efficient ligand based on distance considerations. The five amino acids instead of three (in **35**) separating the 6-*O*-PO_3H_2-Man units in glycopeptide **17** should thus compensate for the omitted mannose residues. The linear glycosylated tripeptide **35**, the tetrapeptide Ac-Thr[6-P-Man(1→2)Man]-Lys(ABz)-Gly-Thr[6-P-Man-(1→2)Man]-NH_2 and the pentapeptide Ac-Thr[6-P-Man(1→2)Man]-Gly-Lys(ABz)-Gly-Thr[6-P-Man(1→2)Man]-NH_2, which all contain two phosphorylated α(1→2)-linked mannose disaccharides, were found to be almost equally potent inhibitors of the binding of a phosphomannan core fragment to the ^{CI}MPR.[6]

Frequently, even better inhibitors can be obtained if the peptide backbone of a peptidic inhibitor is constrained by cyclization, resulting in an optimal spatial arrangement of the parts interacting with the receptor.[8–11] Thus, the cyclic glycopeptides **28**, **29** and **32** based upon the most potent tripeptide **35** were likely to provide the necessary restriction of the conformational space. In glycopeptides **28** and **32**, alternate D- and L-amino acids were chosen to enhance cyclization[12] and to yield an equatorial arrangement of side chains, whereas L-Lys present in glycopeptide **29** (cf. **28**) might test the effect of such a substitution on the affinity for the ^{CI}MPR.

Previously, it was demonstrated[6] that 6′-*O*-phosphorylated α(1→2)-linked disaccharides were conveniently incorporated into glycopeptides when utilizing phosphorylated, glycosylated threonine (or serine) building blocks in solid-phase peptide synthesis. Furthermore, 2,2,2-trichloroethyl (Tce) protection of

the phosphate group proved suitable for the synthesis of glycopeptide **35** although its removal by treatment with zinc and silver carbonate in 10% acetic acid:pyridine was problematic in large-scale synthesis.[6] However, only this method, among several attempted, gave satisfactory deprotection of both Tce groups without formation of by-products. A possible solution to the problems associated with phosphate protection might be to leave it unprotected during peptide synthesis. In the present work, this concept was investigated in an alternative synthesis of glycopeptide **35**.

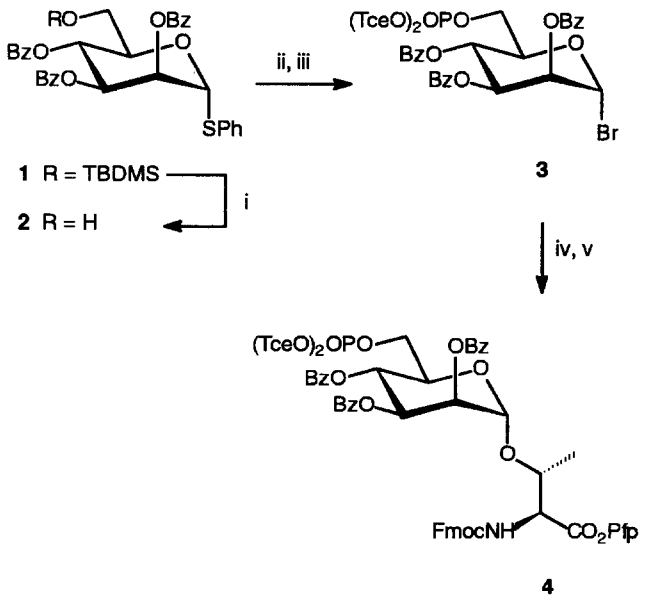
Results and Discussion

Synthesis of building blocks

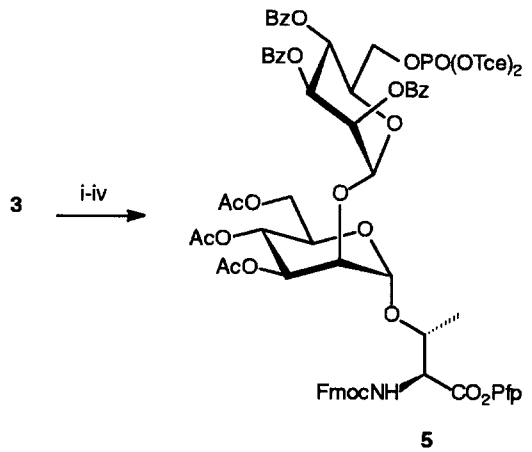
The glycosyl donor **3** was prepared in three steps from thioglycoside **1** as previously reported[7] (see Scheme 1). Subsequently, silver triflate-promoted glycosylation of N^{α}-(fluoren-9-ylmethoxycarbonyl)-threonine pentafluorophenyl ester (N^{α}-Fmoc-Thr-OPfp) with the donor **3** afforded the protected 6-*O*-PO_3H_2-Man building block **4** in 60% yield.

Similarly, the Tce-protected 6-*O*-PO_3H_2-Man(1→2) Man building block **5** was obtained by condensation of N^{α}-Fmoc-Thr-OPfp and a fully protected phosphorylated dimannosyl donor, using silver triflate as promoter as previously reported[7] (see Scheme 2).

Thus, in both the above syntheses of threonine building blocks, the Tce group was employed for the protection of the phosphate moiety. However, owing to the rather extensive loss of material during the Tce-deprotection from the fully protected tripeptide **33**, an alternative synthetic strategy would be desirable. Among the other two major methods currently available: (i) phosphorylation after completion of chain elongation and (ii) use of side chain-unprotected phosphorylated building blocks, the former one proved difficult and the latter approach was considered promising for our purpose due to its demonstrated success in the synthesis of phosphotyrosine[13,14] and phosphoserine[15] containing peptides.



Scheme 1. Reagents and conditions: i, H^+, H_2O, 93%; ii, ClPO$(OCH_2CCl_3)_2$, pyridine, CH_2CL_2, 3 Å MS, 98%, iii, Br_2, CH_2Ckl_2, 4 Å MS, darkness, 100%; iv, Fmoc-Thr-OPfp, AgOTf, CH_2Cl_2, 3 Å MS, −40 °C; v, 2,4,6-collidine, 60%.



Scheme 2. Reagents and conditions: i, 1,3,4,6-Ac_4-β-Manp, AgOTf, CH_2Cl_2, 4 Å MS, −40 °C; ii, 2,4,6-collidine, 69%; iii, HBr–HOAc, 88%; iv, Fmoc-Thr-OPfp, AgOTf; CH_2Cl_2, 3 Å MS, −60 °C; v, 2,4,6-collidine, 82%.

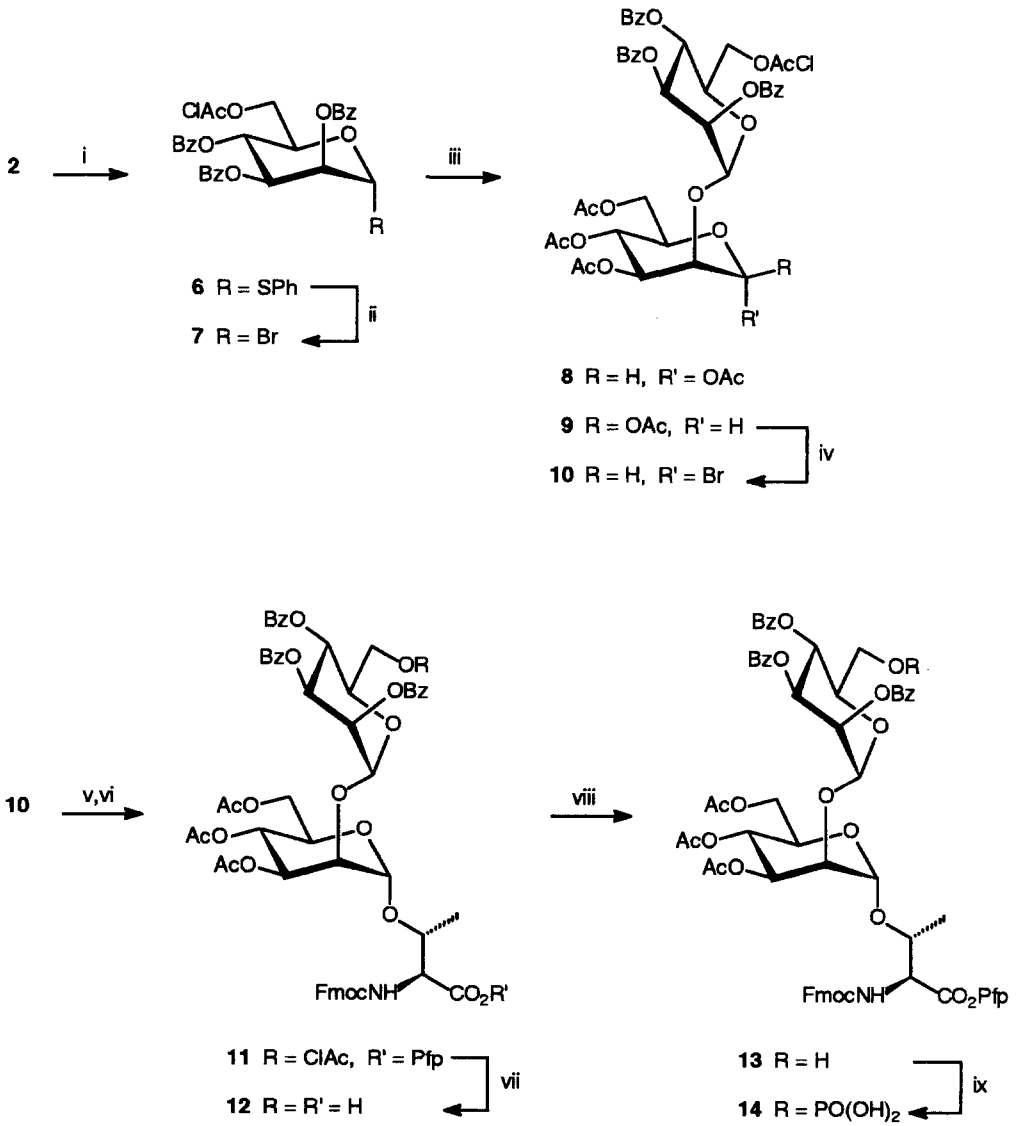
The initial investigation of such a pathway showed that an unprotected phosphate group is incompatible with the usual glycosylation conditions, which implies that the phosphorylation preferably might be performed as the last step in the building block synthesis. Any unanticipated problems with a free phosphate during the sequence of other reactions is also avoided when employing this strategy.

Thioglycoside **2** was found to be a convenient starting material as it allows the introduction of a temporary orthogonal protecting group at the 6′-position of the final dimannosylated building block (see Scheme 3). The chloroacetyl (ClAc) group possesses the necessary properties of being stable towards glycosylation conditions as well as being easily cleavable in the presence of acetates and benzoates by treatment with hydrazinedithiocarbonate[16] (HDTC). Hydrolysis of the crystalline[17] TBDMS-ether **1** was performed by an alternative method: treatment with two equivalents of *p*-toluenesulfonic acid in an acetonitrile:water mixture. The yield of compound **2** (94%) was comparable to that obtained with amorphous[7] TBDMS-ether **1** in an acetic acid:water:tetrahydrofuran[7] mixture (93%), but with a reaction time of a few hours instead of several days. Moreover, in contrast to the acetic acid procedure no interference by precipitating educt was observed with the method presented here.

The product **2** with an unprotected primary OH group was then subjected to acylation by treatment with chloroacetic anhydride and pyridine in dichloromethane. Compound **6** was isolated in 87% yield by crystallization of the residue. A quantitative conversion

of the thioglycoside **6** into bromide **7** was achieved by reaction with bromine in dichloromethane shielded from light. Glycosylation of 1,3,4,6-tetra-*O*-acetyl-β-mannose[17] with the donor **7** was performed using silver triflate as promoter. As previously found,[17] addition of the hindered base 2,6-di-*tert*-butyl-4-methyl-pyridine was necessary to prevent extensive anomerization of the 1-*O*-β-acetate and acyl migration to the 2-position of the acceptor. Purification by VLC on silica gel afforded a mixture of anomers **8/9** (78%), which upon crystallization gave pure 1-*O*-β-acetate **9** (60%) (see Table 1 for ^{1}H NMR data).

The glycosyl bromide **10** was obtained from disaccharide **9** by treatment with hydrogen bromide in acetic acid, and subsequent silver triflate-promoted glycosylation of N^{α}-Fmoc-Thr-OPfp afforded the corresponding dimannosylated protected amino acid **11** (86%). In principle, the remaining part of the synthesis should merely involve an exchange of the 6′-*O*-chloroacetyl group for an unprotected phosphate, however, it was demonstrated (unpublished results) that the Pfp ester is unstable towards weakly nucleophilic reagents, e.g. thiourea, which may also be used to accomplish the selective choroacetyl removal at elevated temperature. Hence, it seemed most straightforward to selectively cleave the Pfp ester prior to the treatment with hydrazinedithiocarbonate. Hydrolysis of the Pfp ester in compound **11** was effected by treatment overnight with 1-hydroxybenzotriazole (HOBt) and water in an acetonitrile:dimethylformamide mixture. The resulting crude carboxylic acid was then directly (without any purification) subjected to selective deprotection at the 6′-position. Hydrazinedithiocarbonate (HDTC) was prepared in situ[16] and added to the reaction mixture containing the intermediate acid together with 4-ethylmorpholine (NEM). After stirring at room temperature for 1.5 h the reaction (involving an initial displacement



Scheme 3. Reagents and conditions: i, $(ClAc)_2O$, pyridine, CH_2Cl_2, cryst., 90%; ii, Br_2 CH_2Cl_2, darkness, 100%; iii, AgOTf, 2,6-di-Bu′-4-Me-pyridine, 1,3,4,6-Ac_4-β-D-Manp, CH_2Cl_2, −30 °C, VLC, 78% **8/9**, cryst., 60% **9**; iv, HBr–HOAc, CH_2Cl_2, 3 Å MS, 91%; v, AgOTf, Fmoc-Thr-OPfp, CH_2Cl_2, −20 to 10 °C; vi, 2,6-di-Bu′-4-Me-pyridine, 86%; vii, HOBt, H_2O, MeCN–DMF overnight, then HDTC-soln, 4-ethylmorpholine, 93%; viii, Pfp-OH, DCCI, CH_2Cl_2, 3 Å MS, 0 °C, 81%; ix, 4-ethylmorpholine, $POCl_3$, CH_2Cl_2, 3 Å MS, then H_2O–Me_2CO; 75%.

Table 1. ^{1}H NMR data (500 MHz, except for **10** recorded at 250 MHz, $CDCl_3$) of monosaccharides **6**/**7** and disaccharides **9**/**10**. Chemical shifts in ppm (J in Hz)

Proton	6	7	9	10
1-H[a]	—	—	5.34 (1.7)	5.13 (1.5)
2-H[a]	—	—	5.77 (2.6)	5.60 (2.0)
3-H[a]	—	—	6.05 (10.1, 2.6)	5.83 (10.0, 3.5)
4-H[a]	—	—	6.09 (9.7)	5.78 (10.0)
5-H[a]	—	—	4.78 (9.4, 3.0)	4.43
6-H[a]	—	—	4.52 (12.2, 4.1)	4.37
			4.40 (12.2, 2.4)	
1-H[b]	5.82 (1.5)	6.61	5.91	6.59 (1.0)
2-H[b]	5.99 (3.1, 1.5)	5.92 (3.5)	4.32 (2.4)	4.35 (3.0, 1.0)
3-H[b]	5.90 (10.0, 3.2)	6.30 (10.0, 3.5)	5.25 (9.7, 2.9)	5.65 (10.0, 3.0)
4-H[b]	6.04 (10.0)	6.10 (10.0)	5.50 (9.9)	5.49 (10.0)
5-H[b]	4.92 (5.6, 2.6)	4.60	3.91 (4.9, 2.2)	4.15
6-H[b]	4.56 (12.2, 5.6)	4.56 (12.5, 3.9)	4.41 (12.5, 4.4)	4.30 (12.5, 4.0)
	4.42 (12.2, 2.6)	4.47 (12.5, 2.0)	4.29 (12.5, 2.4)	4.14 (12.5, 2.0)
$ClCH_2CO$	4.11–4.03	4.17	4.16–4.08	4.06
Ar-H	8.15–7.28	8.17–7.30	8.17–7.29	8.10–7.25

[a,b]Designates the non-reducing and the reducing sugar unit, respectively.

of the chloride by sulfur and subsequent intramolecular attack of the hydrazide amino group on the carbonyl group) was completed, and the hydroxy-acid **12** was obtained in an overall yield of 93% after purification by HPLC.

Normally, Pfp esters are readily prepared by an overnight reaction using dicyclohexylcarbodiimide (DCCI) as the condensation agent in tetrahydrofuran, but in this particular case (compound **12**) the conversion into the corresponding Pfp ester **13** appeared to be extremely slow (>3 days) and a large excess of DCCI (2.5 equiv) was necessary for the completion of the reaction. Whether this was caused by solubility problems or an inherent lower reactivity of the acid component was not investigated in detail, however, it prompted us to attempt a change of the solvent to dichloromethane in which the educt was more soluble. Use of the reverse DCCI procedure[18] where the pentafluorophenol (3 equiv) was dissolved in dichloromethane and reacted with DCCI at 0 °C for 30 min prior to the addition of the hydroxy-acid **12** seemed indeed more satisfactory, since the reaction time was reduced to 5 h, and the yield 81% (of the Pfp ester **13**) after HPLC was in the expected range. In addition it was shown that extremely careful removal of residual trifluoroacetic acid (TFA) present in the hydroxy-acid **12**, due to its purification by HPLC using acetonitrile-:water:TFA mixtures, was crucial for obtaining high yields of the Pfp ester, since the 6′-hydroxy group otherwise was converted into the corresponding TFA ester (confirmed by NMR and MS) in up to 25% yield. Repeated concentration with toluene followed by lyophilization overnight proved to be sufficient.

The final phosphorylation was performed in dry dichloromethane using freshly distilled $POCl_3$ and NEM as the reagents. The reaction was easily followed by analytical HPLC as the products were considerably more polar than the starting material. Initially, only 2 equiv of $POCl_3$ and 3 equiv of NEM were employed, but less than 30% conversion was reached after reaction overnight. Upon addition of a large excess (16 equiv of $POCl_3$ and 12 equiv of NEM, respectively) of reagents to the mixture, >80% conversion was obtained within 2 h. In a second attempt using 20 equiv of $POCl_3$ and 18 equiv of NEM complete formation of phosphorylated species was achieved within 15 h. HPLC of the reaction mixture revealed the presence of both the initial putative dichlorophosphate product and the partially hydrolysed monochlorophosphate product, which predominated, probably because of ready hydrolysis on the column. Treatment with water in acetone for 30 min at room temperature afforded an approximately 2:1 mixture of the desired phosphate product **14** and the monochlorophosphate compound; the latter surprisingly being the most polar component according to HPLC. Repeated concentration with water:acetone gave a mixture in which the desired phosphate **14** accounted for >80%, and after purification by preparative HPLC a 75% yield of the building block **14** was obtained.

Again, the 6′-*O*-TFA ester was seen as a by-product if the starting material was not free from residual TFA, (see Tables 2 and 3 for NMR data).

Synthesis of a glycopeptide with two 6-PO_3H_2-Man residues

Synthesis of the protected glycopeptide **15** was performed in DMF by the syringe method[19] using the PEGA 1900/130 resin[20,21] derivatized with the Rink-linker.[22] The glycosylated building block **4** was employed, and glycopeptide **15** was obtained in a yield of 49%. The Tce groups were removed from the phosphate moiety with zinc and silver carbonate, as previously reported,[7] to give the partially deprotected glycopeptide **16** in 99% yield after HPLC purification. Deacylation was performed with hydrazine hydrate in methanol:chloroform (4:1) to give the fully depro-

Table 2. ^{1}H NMR data (500 MHz, $CDCl_3$ except for **14** recorded in DMSO-d_6) of building blocks **4**, **13** and **14** and the intermediates **11/12**. Chemical shifts in ppm (selected J in Hz)

Proton	4	11	12	13	14
1-H[a]	—	5.22 (1.3)	5.34	5.21	5.30
2-H[a]	—	5.67	5.72 (3.1)	5.68	5.64 (2.7, 1.9)
3-H[a]	—	5.85 (10.0, 2.6)	6.01 (10.0, 3.1)	5.90 (10.0, 2.9)	5.72 (10.0, 3.0)
4-H[a]	—	5.92 (9.8)	5.71 (10.4)	5.76 (10.1)	5.80 (10.0)
5-H[a]	—	4.44	4.26 (9.8, 1.8)	4.27	4.45 (10.1)
6-H[a]	—	4.43	4.00 (12.5, 9.5)	3.71 (12.2, 5.5)	4.03–3.96
		4.18 (10.8)	3.90 (12.5, 1.8)	3.66 (12.2)	
1-H[b]	5.34 (1.8)	5.23 (1.8)	5.15	5.30 (1.6)	5.35
2-H[b]	5.62 (3.2)	4.09 (2.7)	4.14	4.12	4.07
3-H[b]	5.92	5.39	5.42	5.38	5.41 (9.8, 2.9)
4-H[b]	5.92	5.36	5.42	5.36	5.26 (9.9)
5-H[b]	4.48	4.11	4.14	4.11	4.31
6-H[b]	4.50	4.36 (12.3, 5.7)	4.37 (12.0, 5.3)	4.36 (12.2, 5.7)	4.23 (12.0, 4.9)
		4.27 (12.3, 1.8)	4.29 (12.0)	4.27 (12.2, 2.2)	4.12 (12.0)
H$^\alpha$	4.97	4.86 (9.7, 1.3)	4.53 (9.6, 1.5)	4.85 (9.5)	4.76 (8.1, 3.8)
H$^\beta$	4.72	4.64 (6.3)	4.58 (6.4, 1.5)	4.61 (6.2)	4.58
H$^\gamma$	1.58	1.50 (6.3)	1.39 (6.4)	1.46 (6.2)	1.40 (6.2)
NH	5.92	5.77 (9.7)	5.56 (9.6)	5.96 (9.5)	8.54 (8.1)
Fmoc-CH	4.38	4.23 (6.9)	4.30	4.23 (6.6)	4.28 (7.2)
Fmoc-CH_2	4.59	4.52 (10.6, 6.9)	4.48–4.41	4.52 (10.6, 6.7)	4.38 (7.2)
		4.42		4.43 (10.6, 6.5)	
Ar-H	8.14–7.27	8.14–7.29	8.14–7.27	8.06–7.29	
AcO	—	2.22, 2.19	2.24, 2.22	2.21, 2.19	2.06, 2.03
		2.13	2.11	2.13	1.99
$ClCH_2CO$	—	4.06–3.97	—	—	—
CH_2CCl_3	4.69–4.62	—	—	—	—

[a,b]Designates the non-reducing and the reducing sugar unit, respectively.

tected glycopeptide **17** in an overall yield of 47% (see Scheme 4).

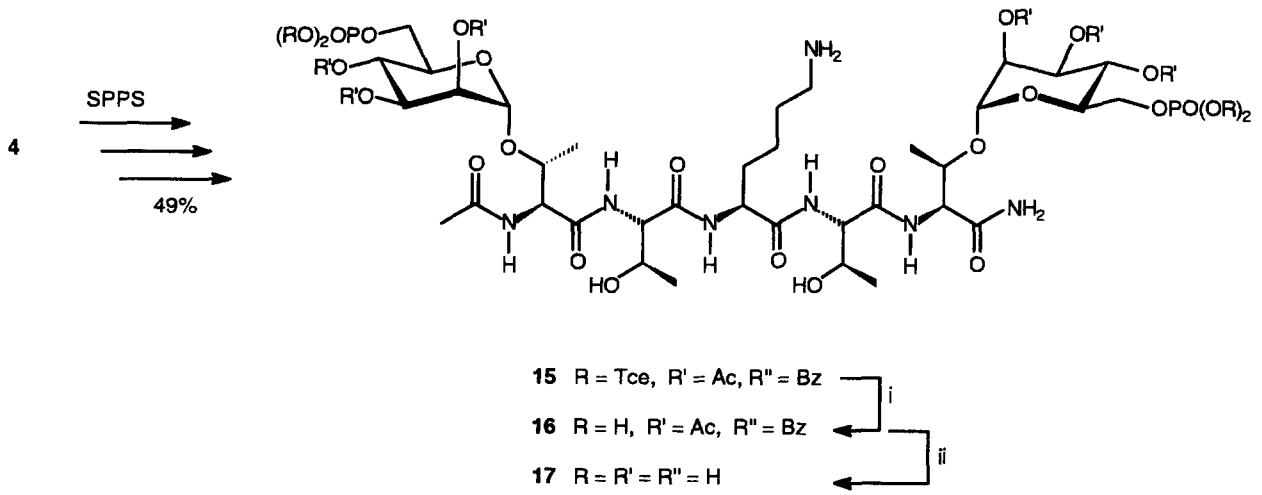
The applicability of the 2-(4-nitrophenyl)ethyl (NPE) group for phosphate protection in Fmoc solid-phase peptide synthesis (SPPS) was briefly tested in an analogous synthesis of a glycopeptide similar to compound **15** using the corresponding glycosylated building block with NPE protection.

In contrast to the Tce group, the NPE group may be cleaved from the phosphate using homogeneous condi-

Table 3. ^{13}C NMR data (125 MHz except for **6,9** and **10** recorded at 62.9 MHz, $CDCl_3$) of protected monosaccharide **6** and disaccharides **9/10**, and protected amino acid derivatives **4/11–14**. Chemical shifts in ppm

Carbon	6	9	10	4	11	12	13	14
C-1[a]	—	98.8	99.7	—	98.2	98.6	98.6	98.2
C-2[a]	—	71.0	70.4	—	70.2	70.8	70.6	69.8
C-3[a]	—	69.5	68.9	—	69.1[c]	68.6	69.0	70.0
C-4[a]	—	67.0	66.9	—	66.7	67.9	67.2[e]	66.3
C-5[a]	—	69.4	69.8	—	69.3	71.6	71.9	69.6
C-6[a]	—	64.2	64.3	—	63.9	63.4	61.5	63.7
C-1[b]	86.2	91.5	84.7	99.0	100.0	100.7	99.6	98.8
C-2[b]	72.2	75.1	80.4	70.1	75.7	74.9	76.0	76.0
C-3[b]	70.6	72.5	69.1	69.3	69.1[c]	70.3	69.3	69.5
C-4[b]	67.3	66.3	65.2	66.5	67.3[d]	66.9	67.2[e]	66.4
C-5[b]	69.9	73.7	73.0	70.1	69.6	69.1	69.4	68.2
C-6[b]	64.6	62.3	61.2	67.2	62.5	62.4	62.6	62.0
$ClCH_2CO$	40.9	40.9	40.7	—	40.4	—	—	—
CH_2CCl_3	—	—	—	77.0	—	—	—	—
C$^\alpha$	—	—	—	58.5	58.6	58.3	58.5	58.8
C$^\beta$	—	—	—	76.8	76.5	77.6	76.1	75.7
C$^\gamma$	—	—	—	18.3	18.5	18.4	18.4	17.4
Fmoc-*C*H	—	—	—	47.2	47.0	47.1	47.1	46.6
Fmoc-CH_2	—	—	—	67.8	67.3[d]	67.6	67.2[e]	66.1
Pfp-C	—	—	—	142.0–136.0	142.0–136.0	—	142.0–136.0	142.5–137.5

[a,b]Designates the non-reducing and the reducing sugar unit, respectively.
[c,d]Signals of double intensity.
[e]Signal corresponding to three carbon atoms.



Scheme 4. Reagents and conditions: i, Zn, Ag_2CO_3, Pyr, HOAc; ii, hydrazine·H_2O, $CHCl_3$–MeOH.

tions. Thus, removal of the NPE groups was attempted on resin-bound peptide by treatment with DBU[23,24–27] in DMF for 8 h. Subsequent cleavage of the peptide from the resin resulted in several products. Analysis by ^{1}H NMR showed that the major glycopeptides contained fully and partially NPE protected phosphates, respectively. From oligonucleotide chemistry it is known that NPE groups may be removed from a phosphortriester by treatment with DBU in an aprotic solvent within 1 h.[23] This corresponds to the observation that the first NPE group is relatively labile, whereas the second NPE group is a part of a phosphordiester, and therefore is much more persistent. Similarly, the cleavage of the Tce groups supports the trend that removal of the second protecting group is more difficult irrespective of the nature of the protecting group and method of cleavage, as the cationic phosphordiester generally is a poor leaving group. Moreover, the NPE group of the phosphortriester proved to exhibit an inherent lability towards 20% piperidine in DMF, making this protection scheme less promising in solid-phase glycopeptide synthesis.

Synthesis of cyclic glycopeptides

The cyclic glycopeptides were also synthesized by the syringe method.[19] The first amino acid, Fmoc-Asp-OAll,[28,29] was attached to the Rink-amide linker[22] on the resin via the β-carboxylic functionality.

Chain elongation was obtained by addition of Fmoc-L-amino acid-OPfp esters, or by activation with *O*-(1H-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) and diisopropylethylamine (DIPEA) in the case of D-amino acids.

After assembly of the linear peptide chains, the allyl group, which had been selected as an orthogonal protection for the α-carboxylic functionality of Asp, was cleaved by the method developed by Kates et al.[30] Here, the resin was treated with tetrakis(triphenylphosphine) palladium(0) in a chloroform suspension, containing 2.5% NEM and 5% acetic acid, for four hours under argon. After washing, the resin was N^{α}-Fmoc deprotected in 20% piperidine:DMF. Cyclization was performed on the resin-bound peptide to take advantage of the pseudo-dilution effect,[31,32] which minimizes interchain side reactions during the cyclization step. The coupling reagent TBTU was utilized, however, the normal procedure with mixing of TBTU (or HBTU) and the free carboxylic acid prior to addition to the resin is not possible in an on-resin cyclization. Recently, Story et al.[33] reported an attempt to form a cyclic peptide on the resin via side chain to side chain cyclization using *O*-(1H-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU), but only linear products were obtained. The two major products were the tetramethyl guanidinium derivative of the linear precursor and the corresponding dimeric linear tetramethyl guanidinium-containing peptide. This may be explained by the fact that HBTU was added to the resin prior to addition of diisopropylethyl amine (DIPEA), allowing the free amino groups to react with HBTU before the carboxylic acids were converted into the acylating HOBt esters. These observations emphasize the necessity of adding the tertiary amine to the mixture containing free amino and carboxyl groups *before* TBTU (or HBTU) is added.

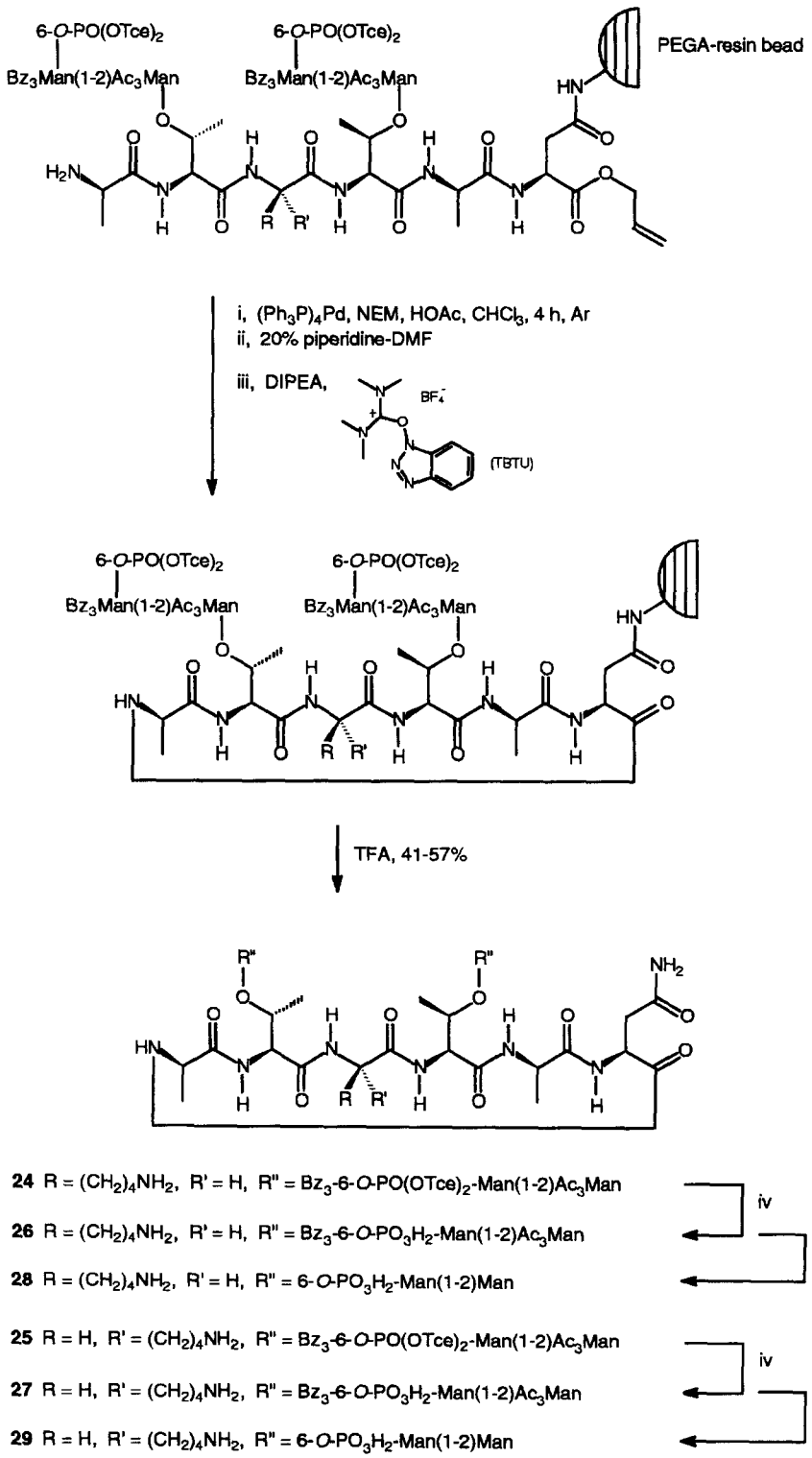
Thus, to prepare cyclic peptides **24**, **25** and **30** (see Schemes 5 and 6), DIPEA was added to the resin-bound peptide to form the carboxylate ions prior to addition of TBTU, which subsequently was added to the resin in small portions over a period of one hour. After an additional 3 h, the resin was washed, and the cyclization procedure was repeated.

The crude linear peptides were analysed by HPLC (after cleavage from the resin) prior to cyclization, and by comparison with the crude cyclization mixtures it was shown that only insignificant formation of di- or oligomers had occurred. This low level of di- and oligo-

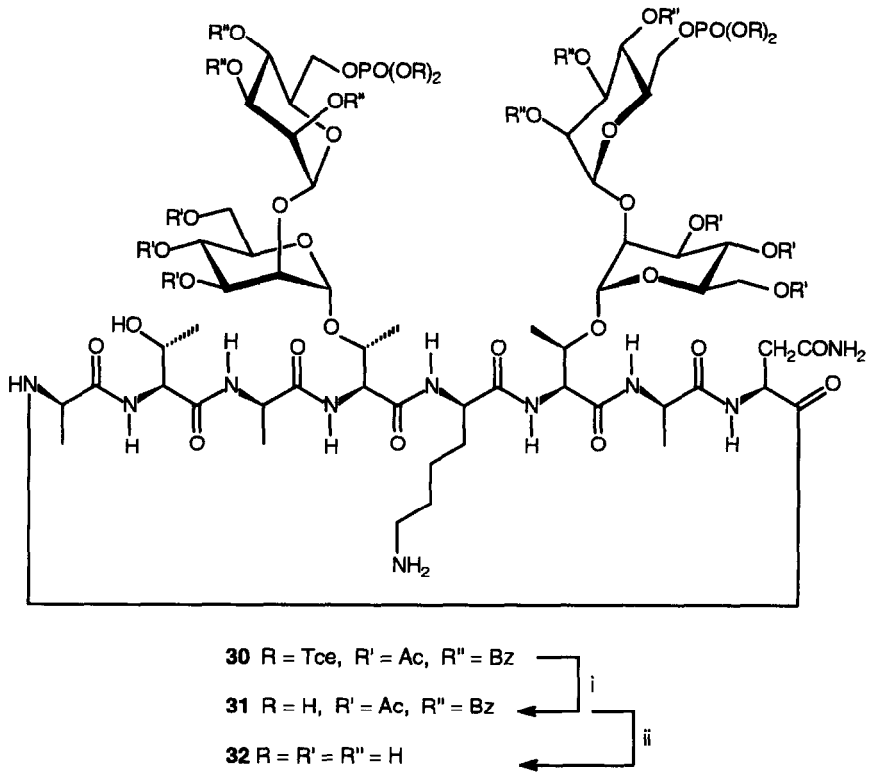
merization may be ascribed to the low frequency of encounter between the resin-bound peptides.

The cyclic peptides were cleaved from the resin with 95% TFA and purified by preparative HPLC, affording the protected, cyclic peptides **24**, **25** and **30** in 41, 57 and 51% yield, respectively. The corresponding protected, linear peptides were obtained in 57, 88 and 77% yield, respectively, and were merely synthesized for comparison and were not further deprotected.

Cleavage of the Tce groups from the cyclic glycopeptides **24**, **25** and **30**, using zinc and silver carbonate in pyridine containing 10% acetic acid, gave the



Scheme 5. Synthesis of cyclic glycopeptides, reagents and condition: iv, Zn, Ag_2CO_3, Pyr, HOAc; v, Hydrazine·H_2O, $CHCl_3$–MeOH, 21–35% overall.



Scheme 6. Reagents and conditions: i, Zn, Ag_2CO_3, Pyr, HOAc; ii, Hydrazine·H_2O, $CHCl_3$–MeOH.

partially deprotected glycopeptides **26**, **27** and **31** in an average yield of 70% after HPLC purification. Deacylation of compounds **26**, **27** and **31** was accomplished with hydrazine hydrate in methanol-chloroform (4:1), giving the fully deprotected, cyclic glycopeptides **28**, **29** and **32** in overall yields of 21, 35 and 21%, respectively (for ^{1}H NMR data see Tables 4–8).

Glycopeptide synthesis using a building block with an unprotected phosphate

As already mentioned, the previously reported[6] synthesis of the biologically most potent glycopeptide **35** involves a difficult phosphate deprotection step (see Scheme 7) on the fully protected glycopeptide **33**.

Table 4. ^{1}H NMR data (500 MHz, DMSO-d_6) for amino acids in protected cyclic glycopeptides **24**, **25** and **30**. Chemical shifts in ppm

	$N^{\alpha}H$	H^{α}	H^{β}	H^{γ}	H^{δ}	H^{ε}	$N^{\varepsilon}H$ or NH_2
24							
D-Ala-1	7.59	4.22	1.19	—	—	—	—
L-Thr-2	8.34	4.31	3.95	1.22	—	—	—
D-Lys-3	8.38	4.38	1.69/1.56	1.32	1.54	2.74	7.62
L-Thr-4	7.96	4.56	4.01	1.16	—	—	—
D-Ala-5	8.24	4.22	1.19	—	—	—	—
L-Asn-6	8.20	4.52	2.56/2.28	—	—	—	—
25							
D-Ala-1	8.35	4.35	1.21	—	—	—	—
L-Thr-2	8.51	4.20	4.33	1.23	—	—	—
L-Lys-3	7.91	4.38	1.78/1.68	1.37	1.52	2.75	7.65
L-Thr-4	8.24	4.23	4.11	1.26	—	—	—
D-Ala-5	8.67	4.11	1.26	—	—	—	—
L-Asn-6	7.90	4.51	2.49/2.43	—	—	—	—
30							
D-Ala-1	7.87	4.32	1.24–1.17	—	—	—	—
L-Thr-2	7.74	4.19	3.95	0.99	—	—	—
D-Ala-3	7.78	4.39	1.24–1.17	—	—	—	—
L-Thr-4	8.19	4.56	3.92	1.22	—	—	—
D-Lys-5	8.34	4.55	1.64–1.54	1.32	1.53	2.74	7.68
L-Thr-6	8.28	4.72	4.02	1.19	—	—	—
D-Ala-7	8.27	4.39	1.24–1.17	—	—	—	—
L-Asn-8	8.15	4.82	2.53/2.33	—	—	—	—

Table 5. ^{1}H NMR data (500 MHz, DMSO-d_6) for amino acids in protected glycopeptide **15** and partially protected glycopeptide **36**. Chemical shifts in ppm

	$N^{\alpha}H$	H^{α}	H^{β}	H^{γ}	H^{δ}	H^{ε}	$N^{\varepsilon}H$
15							
L-Thr-1	8.32	4.56	4.32	1.31	—	—	—
L-Thr-2	7.79	4.28	3.99	1.00	—	—	—
L-Lys-3	7.99	4.40	1.70/1.54	1.33	1.52	2.76	7.67
L-Thr-4	7.87	4.47	4.09	1.05	—	—	—
L-Thr-5	7.91	4.46	4.37	1.28	—	—	—
36							
L-Lys(ABz)[a]							
major conf.	8.05	4.17	1.68/1.57	1.31	1.48	3.17	8.25
minor conf.	7.81	4.34	1.68/1.57	1.31	1.48	3.17	8.25
L-Thr[b]							
major conf.	8.19	4.45	4.06	1.24	—	—	—
minor conf.	8.12	4.24	4.26	1.19	—	—	—

[a]ABz chemical shifts: δ 7.47, 7.17, 6.76 and 6.62.
[b] C-terminal amide chemical shifts: δ 7.33/7.01 and 7.43/7.34, ~2:1 ratio of conformers.

Although the reaction proceeds very cleanly to the desired product **34**, only an approximately 60% yield can be obtained, which may be due to the large excess of zinc and silver carbonate, necessary for a quantitative conversion. Apparently a large amount of product is bound irreversibly to either of these reagents or some pyridine complexes hereof.

Therefore, a new synthetic strategy using a building block without phosphate protection was tested. In an initial attempt, the direct synthesis of glycopeptide **34** was carried out by the syringe method[19] using DMF as solvent and a PEGA resin as the solid phase. After derivatization with the Rink-linker,[22] glycosylated building block **14** was coupled in the presence of Dhbt-OH, however, the normal colour reaction seemed to be disturbed by the free phosphate moiety, as the pronounced yellow colour (complex between free amino groups on the resin and Dhbt-OH) disappeared instantly upon addition of compound **14**. To ensure complete coupling when using only 1.1 equiv of building block **14** an extended reaction time of 2 days was considered sufficient. The Fmoc-Lys[ABz(Boc)]-OPfp coupling (also with addition of Dhbt-OH) was

Table 6. ^{1}H NMR data (500 MHz, DMSO-d_6) for sugar units in protected glycopeptides **15**, **24**, **25** and **30**, and partially protected glycopeptide **36**. Chemical shifts in ppm

		1-H	2-H	3-H	4-H	5-H	6-H
15							
L-Thr-1	Man(1→Thr)	5.26	5.66	5.75	5.75	4.53	4.47–4.37
L-Thr-5	Man(1→Thr)	5.28	5.61	5.76	5.78	4.51	4.47–4.37
24							
L-Thr-2	Man(1→2)	5.46[a]	5.65	5.79	5.96	4.60	4.45
	Man(1→Thr)	5.31	4.18[b]	5.19[c]	5.30	4.00	4.23/4.08
L-Thr-4	Man(1→2)	5.48[a]	5.65	5.79	5.96	4.60	4.45
	Man(1→Thr)	5.31	4.23[b]	5.22[c]	5.30	4.00	4.23/4.08
25							
L-Thr-2	Man(1→2)	5.35	5.65	5.79	5.96	4.57	4.50–4.39
	Man(1→Thr)	5.21	4.02	5.31	5.28	4.21	4.21/4.02
L-Thr-4	Man(1→2)	5.47	5.66	5.78	5.93	4.58	4.50–4.39
	Man(1→Thr)	5.31	4.23	5.22	5.30	3.99	4.24/4.04
30							
L-Thr-4	Man(1→2)	5.46	5.66	5.79	5.98	4.60	4.55–4.43
	Man(1→Thr)	5.32	4.18	5.20	5.30	3.99	4.22/4.06
L-Thr-6	Man(1→2)	5.47	5.66	5.79	5.98	4.60	4.55–4.43
	Man(1→Thr)	5.26	4.21	5.27	5.26	4.01	4.22/4.06
36							
L-Thr	Man(1→2)						
	major conf.	5.36	5.66–5.64	5.73	5.88	4.45	4.24–4.02
	minor conf.	5.34	5.66–5.64	5.72	5.84	4.39	4.24–4.02
	Man(1→Thr)						
	major conf.	5.27–5.23	4.14	5.27–5.23	5.27–5.23	4.24–4.02	4.24–4.02
	minor conf.	5.19	4.14	5.27–5.23	5.27–5.23	4.24–4.02	4.24–4.02

[a–c]May be interchanged vertically.

Table 7. ^{1}H NMR data (500 MHz, CD_3COOD:H_2O, 1:1) for amino acids in deacylated glycopeptide **17**, **28**, **29** and **32**. δ-Values in ppm

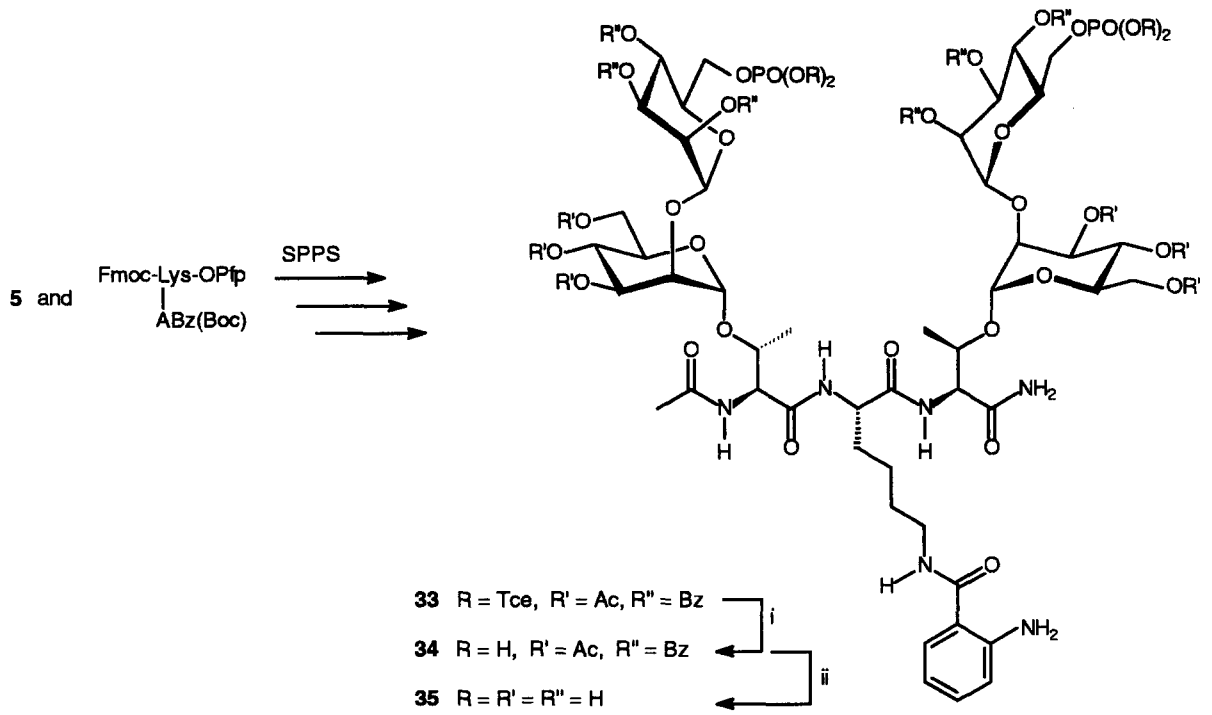
	$N^{\alpha}H$	H^{α}	H^{β}	H^{γ}	H^{δ}	H^{ε}	$H^{\varepsilon}H$
17							
L-Thr-1	8.02	4.60	4.29	1.26	—	—	—
L-Thr-2	8.05	4.43	4.17	1.19	—	—	—
L-Lys-3	8.27	4.52	1.88/1.78	1.45	1.72	3.04	7.48
L-Thr-4	8.22	4.54	4.26	1.19	—	—	—
L-Thr-5	8.03	4.55	4.29	1.27	—	—	—
28							
D-Ala-1	8.06	4.57	1.34	—	—	—	—
L-Thr-2	8.08	4.46	4.27	1.28	—	—	—
D-Lys-3	8.43	4.44	1.84	1.52/1.42	1.77	3.07	7.40
L-Thr-4	8.42	4.43	4.23	1.27	—	—	—
D-Ala-5	8.20	4.40	1.35	—	—	—	—
L-Asn-6	8.33	4.63	2.86/2.72	—	—	—	—
29							
D-Ala-1	8.38	4.50	1.40	—	—	—	—
L-Thr-2	8.38	4.43	4.56	1.31[a]	—	—	—
L-Lys-3	7.91	4.70	1.84	1.56	1.82/1.77	3.09	7.34
L-Thr-4	8.20	4.09	4.18	1.31[a]	—	—	—
D-Ala-5	8.50	4.35	1.42	—	—	—	—
L-Asn-6	8.11	4.73	2.72	—	—	—	—
32							
D-Ala-1	8.18	4.54	1.36	—	—	—	—
L-Thr-2	8.00	4.52	4.37	1.17	—	—	—
D-Ala-3	8.12	4.55	1.40	—	—	—	—
L-Thr-4	8.29	4.62	4.40	1.27	—	—	—
D-Lys-5	8.22	4.54	1.85/1.75	1.48/1.40	1.74	3.06	7.45
L-Thr-6	8.47	4.67	4.32	1.26	—	—	—
D-Ala-7	8.32	4.39	1.40	—	—	—	—
L-Asn-8	8.56	4.83	2.89/2.81	—	—	—	—

[a]Two distinct peaks.

subsequently performed with 2 equiv for 22 h. After the final coupling of compound **14** (1.1 equiv for 2.5 days) and an acetylation step (brief coupling with the Dhbt-ester of acetic acid), the subsequent cleavage from the resin afforded a crude product (50%), which by preparative analytical HPLC proved to contain several components. Analysis by MALDI-TOF MS showed one of the minor peaks (<5%) to contain the desired tripeptide **34**, whereas the more polar major peaks contained dipeptides as judged by their MS spectra. In the above synthesis the standard Fmoc deprotection (i.e. 20% piperidine:DMF) and washing procedures were employed. To examine whether the base used in the Fmoc deprotection step, might have a crucial influence on the product distribution in this particular case, the synthesis was repeated (1.2 equiv of

Table 8. ^{1}H NMR data (500 MHz, CD_3COOD-H_2O 1:1) for sugar units of deacylated glycopeptides **17**, **28**, **29** and **32**. Chemical shifts in ppm

		1-H	2-H	3-H	4-H	5-H	6-H
17							
L-Thr-1	Man (1→Thr)	4.89	3.88	3.90–3.79	3.90–3.79	3.90–3.79	4.20–4.14
L-Thr-5	Man (1→Thr)	4.95	3.90	3.90–3.79	3.90–3.79	3.90–3.79	4.20–4.14
28							
L-Thr-2	Man (1→2)	4.99	4.10	3.87	3.75	3.89	4.22/4.13
	Man (1→Thr)	5.19	3.80	3.91	3.74	3.69	3.87/3.77
L-Thr-4	Man (1→2)	4.97	4.10	3.87	3.75	3.89	4.22/4.13
	Man (1→Thr)	5.12	3.82	3.92	3.75	3.69	3.87/3.77
29							
L-Thr-2	Man (1→2)	4.98	4.14	3.88	3.76	3.81	4.29/4.25
	Man (1→Thr)	5.01	3.83	3.97	3.77	3.71	3.88/3.80
L-Thr-4	Man (1→2)	5.10	4.13	3.88	3.76	3.95	4.27/4.20
	Man (1→Thr)	5.27	4.02	3.92	3.77	3.69	3.86/3.79
32							
L-Thr-4	Man (1→2)	4.96	4.10	3.87	3.76	3.88	4.25/4.13
	Man (1→Thr)	5.17	3.72	3.90	3.74	3.80–3.70	3.90/3.78
L-Thr-6	Man (1→2)	5.01	4.10	3.86	3.76	3.88	4.25/4.13
	Man (1→Thr)	5.20	3.79	3.92	3.75	3.80–3.70	3.90/3.78



Scheme 7. Reagents and conditions: i, Zn, Ag_2CO_3, Pyr, HOAc; ii, Hydrazine·H_2O, $CHCl_3$–MeOH.

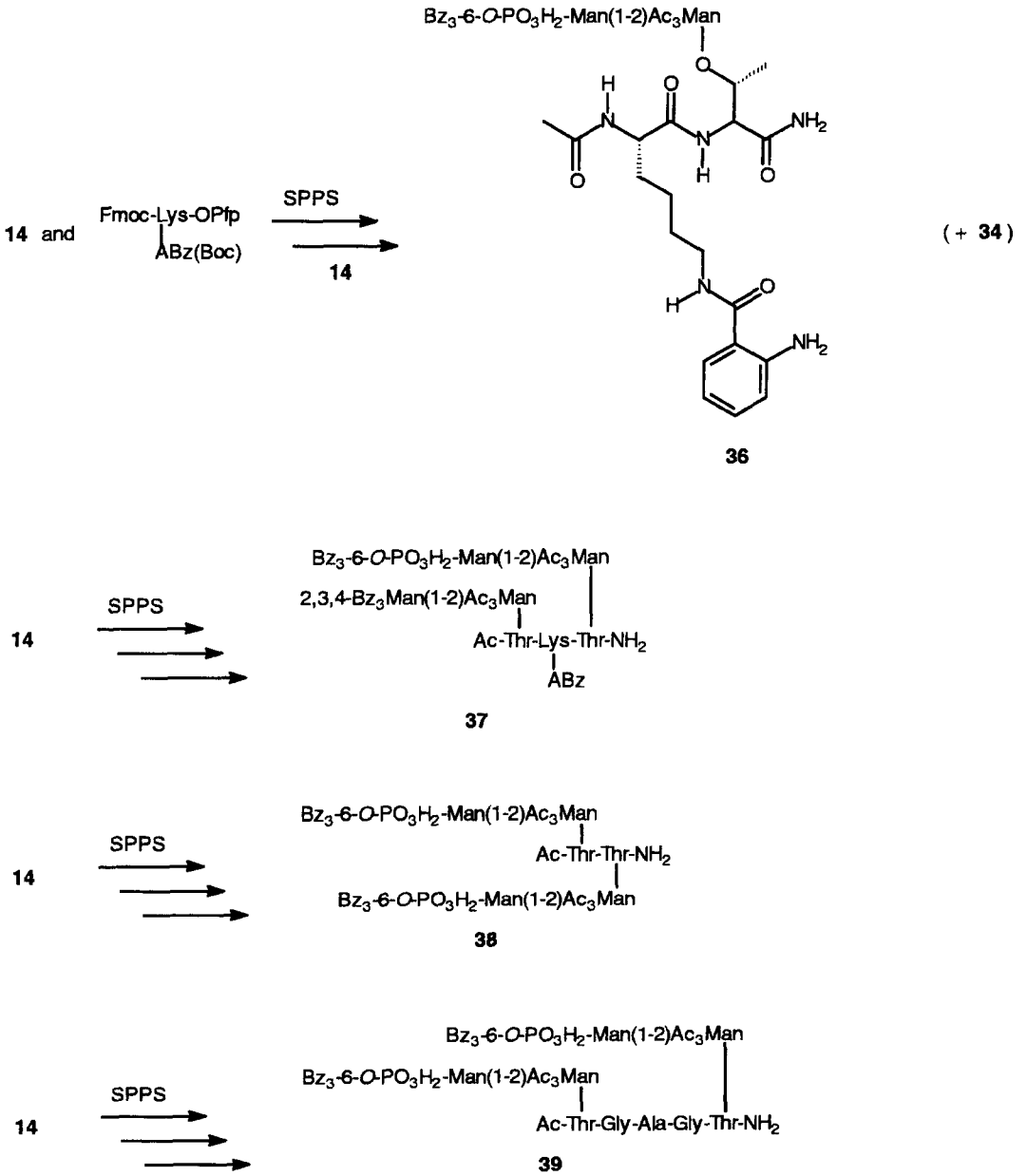
14 and 2.5 equiv of Fmoc-Lys[ABz(Boc)]-OPfp), but now 50% morpholine-DMF was applied for the Fmoc deprotection. The analysis of the reaction mixture, after cleavage from the resin, showed indeed a much more clean reaction providing one major component, which by MALDI-TOF MS gave a single peak: $[M—H]^-$ 1248.3 corresponding to the partially protected dipeptide **36**. Again, the desired tripeptide **34** was only present as a trace (<1%) whereas the dipeptide **36** was isolated in 41% yield. In the ^1H NMR spectrum (see Tables 5 and 6) compound **36** appeared as a mixture of two conformers or as mono- and di-ionization salts in a concentration independent manner. From these findings it was evident that the third building block coupling proceeded either extremely slowly (weeks) or not at all after a certain time when all the building block **14** had been quenched by a putative side reaction. The latter possibility might in fact occur due to a salt formation between the Fmoc deprotection base (piperidine or morpholine) and the phosphate moiety on the resin-bound peptide chain. Addition of a Pfp ester to the resin might then shift the ammonium phosphate salt equilibrium irreversibly if the small amount of the nucleophilic free amine constantly is removed by reaction with the Pfp ester (e.g. **14**). If this really accounts for the low conversion in the third coupling, an intermediate washing (exchange of morpholine) with the stronger tertiary base DIPEA should prevent quenching of the Pfp ester, since only a non-nucleophilic base then participates in the salt formation with the resin-bound phosphate. However, no improvement in the ratio between tri- and dipeptide was obtained by this modification of the synthesis. Clearly, another effect must be responsible for the slow coupling reaction. Plausible explanations might be either steric hindrance or a strong electrostatic repulsion due the negative charge on the resin-bound peptide as well as on the incoming building block **14**. These two hypotheses were investigated by attempts at the multiple column glycopeptide synthesis[34,35] (MCPS) of compounds **37–39** (see Scheme 8). Compound **37** is similar to compound **34** except that the second phosphate is removed. In compound **38** two phosphorylated glycosyl amino acids are directly connected. Compound **39** is similar to compound **34** except for the insertion of the longer sequence Gly-Ala-Gly between the phosphorylated building blocks. The cleaved mixture of crude glycopeptides were separated by semipreparative HPLC, and the fractions were analysed by ES-MS in the negative mode. The results show that the electrostatic repulsion between the unprotected phosphorylated building block and the unprotected phosphate on the resin-bound peptide has a strong influence on the yield of the acylation reaction. A strong dependence on the distance between the phosphate of the growing peptide chain and the phosphate on the incoming building block was observed. A low degree of acylation (<1%) in the formation of **34** was observed even with 7-aza-1-hydroxy-1,2,3-benzotriazol (HOAt) catalysis. In the case of the shorter peptide **38** 16% acylation was achieved while compound **39** gave 51% acylation. The crude product of **37** contained a substantial amount of an unidentified by-product, however, the yield of **37** appeared to be high and was formed without significant steric hindrance. The higher degree of acylation obtained in case of the shorter peptide **38** may seem surprising; however, in the extended conformation the distance between phosphates in the reacting species may well be longer than for tripeptide **34**. The distance relation is confirmed by the enhanced degree of acylation (51%) observed for compound **39**, in which both

the distance and flexibility have been increased. In conclusion, protection of phosphate groups is a requirement for efficient assembly of peptides and glycopeptides containing more than one phosphorylation by sequential solid-phase synthesis. This is particularly important when the distance between the phosphorylation sites is short. To our knowledge, this is the first observation of suppression of reaction due to electrostatic repulsion of charges positioned quite distant from the actual reaction centre. This observation may be of general importance in the selection of adequate protection schemes for complicated syntheses.

Biological evaluation of the glycopeptides

Compounds **17**, **28**, **29** and **32** were all tested as inhibitors of ^{CI}MPR binding to an immobilized phosphomannan core as previously described.[6] The MPR was biotin labelled and employed in a competitive inhibition assay with the synthetic ligands. Binding could be detected with peroxidase labelled streptavidin. Their IC_{50}-values are presented in Table 9 together with the potencies of previously[6,7] reported compounds (see Scheme 9) for comparison.

These results confirm previous results[5,36] indicating α(1→2)-linked mannose disaccharides to be superior inhibitors compared to 6-*O*-PO_3H_2-Man in contrast to (1→6)-linked mannose disaccharides, which were even less efficiently bound to ^{CI}MPRs. Thus, glycopeptides **35**, **43** and **46** with three to five amino acid spacers between the α(1→2)-linked mannose disaccharides exhibited the highest potencies, which all were of the same order of magnitude reflecting the flexible nature of the peptide backbone. Removal of the ABz group



Scheme 8. Attempted syntheses of glycopeptides using building block **14** having an unprotected phosphate group.

Table 9. IC_{50}-values μmmol/L, first column) for compounds **17**, **28**, **29**, **32**, **35** and **40–55** in the binding of ^{CI}MPR to phosphomannan, and values relative to 6-*O*-PO_3H_2-Man (IC_{50} 260 μmmol/L) as a reference (second column)

Linkage type	Tripeptides (IC_{50})			Tetra-peptides (IC_{50})			Penta-peptides (IC_{50})			Cyclic peptides (IC_{50})			Mono and oligsaccharides (IC_{50})		
α(1→2), α(1→2)	**35**	0.09[a]	0.0003	**43**	0.20[a]	0.0008	**46**	0.17[a]	0.0006	**28**	400	1.5	**52**	1.6[a]	0.006
	54	1.8	0.007	—	—	—	—	—	—	**29**	110	0.4	**53**	6.8[a]	0.003
	55	20	0.07	—	—	—	—	—	—	**32**	55	0.2	—	—	—
α(1→2)	**50**	78[a]	0.3	—	—	—	—	—	—	—	—	—	—	—	—
α(1→2), α(1→6)	**40**	4.0[a]	0.15	**44**	7.0[a]	0.03	**47**	5.0[a]	0.02	—	—	—	—	—	—
α(1→6), α(1→2)	**41**	9.0[a]	0.035	**45**	8.0[a]	0.03	**48**	13[a]	0.05	—	—	—	—	—	—
α(1→6), α(1→6)	**42**	1000[a]	3.8	—	—	—	**49**	330[a]	1.3	—	—	—	—	—	—
α(1→6)	**51**	630[a]	2.4	—	—	—	—	—	—	—	—	—	—	—	—
6-*O*-PO_3H_2-Man	—	—	—	—	—	—	**17**	300	1.2	—	—	—	—	—	—

[a]Taken from ref. 6.

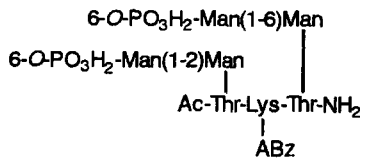
from inhibitor **35** (i.e. to **54**) caused a significant drop in potency (20 times less efficient) revealing that even a small change in the peptide template may have profound effect on the interaction with the receptor.

Furthermore, it can be concluded that the 6′-*O*-phosphorylated mannose containing glycopeptide **17** is distinctly inferior to the similar disaccharide derivative **54** in its affinity for ^{CI}MPRs, and compound **17** is also a slightly poorer inhibitor than 6-*O*-PO_3H_2-Man itself. This demonstrates that in order to exhibit high affinity towards MPRs, the ligands must be bidentate *and* must contain 6′-*O*-phosphorylated α(1→2)-linked mannose *disaccharides*. However, cyclic glycopeptides **28**, **29** and **32** which do contain two 6′-*O*-phosphorylated α(1→2)-linked mannose disaccharides were found to be quite weak inhibitors implying that the ^{CI}MPR is not flexible enough to accommodate these larger cyclic peptides at the binding site. Cyclization has in these cases not resulted in an optimal spatial orientation of the interacting disaccharide. The rigid and bulky cyclic peptide may also interfere sterically with the protein in the binding. The octapeptide **32** was the best inhibitor of the three cyclic glycopeptides tested, which might be due to a higher degree of flexibility than that of the hexapeptides **28**/**29**. Thus, if rigid high-affinity inhibitors are to be designed, information about the actual conformation of a receptor-bound ligand would be necessary. This is currently being investigated by transfer NOEs between the ^{CI}MPR and the most potent linear inhibitor, compound **35**.

Experimental

TLC was performed on Merck Silica Gel 60 F_{254} aluminium sheets with detection by charring with sulfuric acid, and by UV light, when applicable. Mps were measured on a Büchi melting point apparatus and are uncorrected. Vacuum liquid chromatography (VLC)[37] was performed on Merck silica gel 60 H (0.040–0.060 mm), and VLC under dry conditions was performed on dried silica gel (120 °C; >24 h) with solvents dried over molecular sieves. DMF was freshly distilled by fractional distillation at red. pres. and kept over molecular sieves (3 Å). Dichloromethane was distilled from phosphorous pentoxide and kept over 3 Å molecular sieves. Pyridine was distilled and kept over 3 Å molecular sieves. Light petroleum was the 60–80 °C fraction. Concentrations were performed under red. pres. at temperatures <40 °C. Bis-(2,2,2-trichloroethyl)phosphorchloridate was purchased from Aldrich, *tert*-butyldimethylchlorosilane, 4-ethylmorpholine (NEM), 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (Dhbt-OH), phosphorous oxychloride, silver carbonate, tetrakis(triphenylphosphine) palladium(0) [$Pd(PPh_3)_4$] and hydrazine hydrate from Fluka. Commercial zinc powder was 'activated' by washing successively and rapidly with 1 M 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 glycopeptides were hydrolysed with 6 M HCl at 110 °C for 24 h and the amino acid composition was determined on a Pharmacia LKB Alpha Plus amino acid analyzer. Electrospray mass spectrometry was performed in the positive mode for protected glycopeptides and in the negative mode for compounds with unprotected phosphates on a VG Fisons Quattro Instrument. MALDI-TOF MS was performed in the negative mode for compounds with unprotected phosphates on a Finnigan MAT 2000 using a matrix of α-cyano-4-hydroxycinnamic acid. Optical rotations were recorded on a Perkin–Elmer 241 polarimeter, and are given in units of 10^{-1} deg cm^2 g^{-1}. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AM 500 MHz spectrometer. Chemical shifts are given in ppm and referenced to an internal $SiMe_4$ (δ_H, δ_C 0.00) for solns in $CDCl_3$ at 300 K, and to external dioxane (δ_H 3.76, δ_C 67.40) for solns in D_2O at 300 K. For spectra recorded in DMSO-d_6 and CD_3COOD:H_2O the DMSO signal at 2.50 ppm and HOAc signal at 2.03 ppm, respectively, were used as internal references. For the assignment of signals, ^{1}H–^{1}H (COSY) and ^{13}C–^{1}H shift correlation spectroscopy were used. Semipreparative and preparative reverse 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 mL min^{-1} and a Delta PAK C-18 column

(15 μm, 300 Å, 47 mm × 300 mm) with a flow rate of 20 mL min^{-1}, respectively; detection at 215 nm with a photodiode array detector (Waters M 991). Solvent system A: 0.1% TFA; B: 0.1% TFA in 90% aceto-



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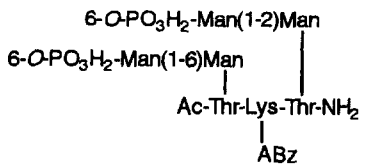

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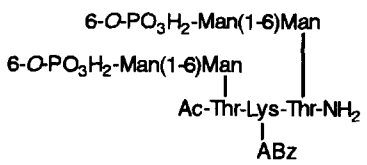

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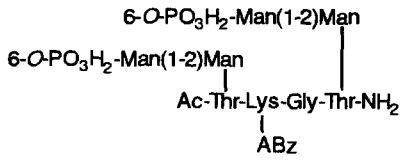

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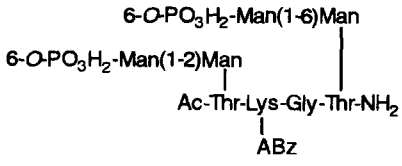

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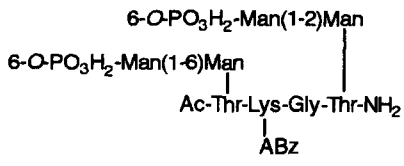

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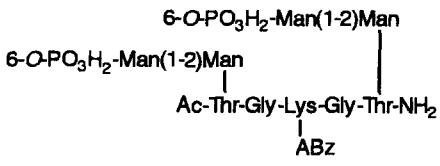

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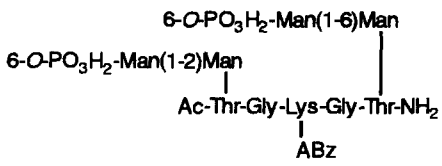

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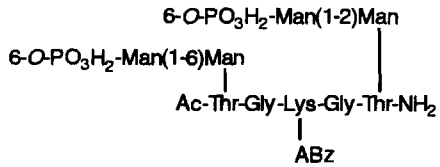

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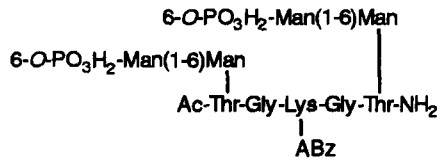

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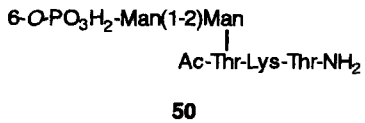

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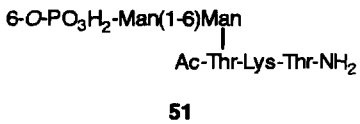

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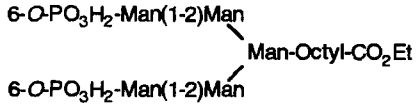

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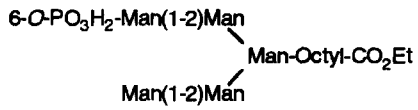

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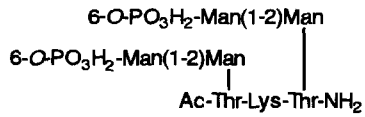

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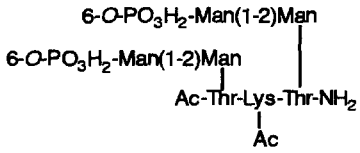

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Scheme 9. Additional compounds tested as inhibitors of ^{CI}MPR.

nitrile:10% water. Gel filtrations were performed on Sephadex G-10 (Pharmacia) with a flow rate of 1 mL min^{-1} and detection at 230 nm.

Phenyl 2,3,4-tri-*O*-benzoyl-1-thio-α-D-mannopyranoside (2). TBDMS-ether **1** (2.47 g, 3.53 mmol), prepared as previously described,[6] and $TsOH \cdot H_2O$ (1.34 g, 7.06 mmol) were stirred in MeCN:H_2O (9:1, 150 mL) for 5.5 h at room temperature. The solution was concd to ~½ vol. and then dild with CH_2Cl_2 (500 mL). The organic layer was subsequently washed with satd $NaHCO_3$ (150 mL) and water (2 × 150 mL). Upon drying ($MgSO_4$) and filtration, the filtrate was concd (to 15 mL) and loaded onto a VLC column (5 × 5 cm). Gradient elution with hexane to hexane:EtOAc (5:1) afforded compound **2** (1.93 g, 94%).

***N*$^{\alpha}$-(Fluoren-9-ylmethoxycarbonyl)-*O*-[2,3,4-tri-*O*-benzoyl-6-*O*-bis(2,2,2-trichloroethoxy)phosphoryl-α-D-mannopyranosyl]-L-threonine pentafluorophenyl ester (4).** Glycosyl bromide[7] **3** (1.32 g, 1.47 mmol) and *N*$^{\alpha}$-Fmoc-Thr-OPfp (1.12 g, 2.21 mmol) were dissolved in dry CH_2Cl_2 (7 mL) and stirred for 1 h under Ar over molecular sieves (3 Å, 0.5 g) at −40 °C. Silver triflate (0.45 g, 1.8 mmol) was added quickly, and the mixture stirred at −40 °C for 2 h. Then 2,4,6-collidine (0.39 mL, 2.94 mmol) was added, and the temperature was slowly raised to 20 °C. After dilution with CH_2Cl_2 and filtration through Celite, the filtrate was washed twice with a mixture of 10% aq. TFA and ice, and once with water. Drying ($MgSO_4$) and concn were followed by VLC on dried silica gel with dry solvents (light petroleum:ethyl acetate, 7:2), which gave compound **4** (1.18 g, 60%) $[\alpha]_D^{25}$ −49.2 (*c* 1.3, CH_2Cl_2); ^1H and ^{13}C NMR data are given in Tables 2 and 3, respectively (Found: C, 50.83; H, 3.41; N, 1.15. $C_{56}H_{43}Cl_6F_5NO_{16}P$ requires C, 50.78; H, 3.27; N,1.06,%; *M*, 1324.64).

Phenyl 2,3,4,-tri-*O*-benzoyl-6-*O*-chloroacetyl-1-thio-α-D-mannopyranoside (6). Compound **2** (5.05 g, 8.64 mmol) was dissolved in dry CH_2Cl_2 (25 mL), and pyridine (2 mL) was added. Upon cooling to 0 °C, chloroacetic anhydride (1.77 g, 10.37 mmol) was added and the mixture stirred at room temperature for 1.5 h. Water (3 mL) was added and the mixture stirred for 30 min. Dilution with CH_2Cl_2 (100 mL) was followed by washing with satd $NaHCO_3$ (100 mL) and water (2 × 100 mL). The organic layer was dried ($MgSO_4$) and concd with toluene (3 × 25 mL). The partially crystalline residue was dissolved in CH_2Cl_2 (10 mL) and then Et_2O (200 mL) was added. The mixture was kept overnight in the refrigerator; the crystals were filtered off and freeze-dried to give the 6-ClAc-derivative **6** (5.13 g, 90%) as yellowish crystals, mp 139–140 °C, $[\alpha]_D^{20}$ +3.0° (*c* 1.0, CH_2Cl_2); ^1H and ^{13}C NMR data are presented in Tables 1 and 3, respectively, (Found: C, 63.4; H, 4.4. $C_{35}H_{29}O_9SCl$ requires C, 63.59; H, 4.42, S, 4.85; Cl, 5.36; *M* 661.12).

2,3,4-Tri-*O*-benzoyl-6-*O*-chloroacetyl-α-D-mannopyranosyl bromide (7). Thioglycoside **6** (176 g, 2.66 mmol) was dissolved in dry CH_2Cl_2 (8 mL) and stirred with molecular sieves (4 Å, 1.5 g) for 30 min. A soln of bromine (0.165 mL, 3.19 mmol) in dry CH_2Cl_2 (1.25 mL) was added, and then the mixture was stirred for 4.5 h shielded from light. After filtration, the mixture was concd and co-distilled with toluene (3 times). The syrup was dried overnight under high vacuum to give crude glycosyl bromide **7** (1.95 g, 100%); ^1H NMR data are presented in Table 1.

1,3,4,6-Tetra-*O*-acetyl-2-*O*-(2,3,4-tri-*O*-benzoyl-6-*O*-chloroacetyl-α-D-mannopyranosyl)-β-D-mannopyranose (9). Glycosyl bromide **7** (1.68 g, 2.66 mmol) was dissolved in dry CH_2Cl_2 (15 mL) with molecular sieves (3 Å, 1.8 g) and 2,6-di-*tert*-butyl-4-methylpyridine (0.49 g, 2.40 mmol) added. Stirring under Ar at −30 °C was continued for 1 h. Silver triflate (0.82 g, 3.19 mmol) was added quickly, and the mixture stirred for an additional 15 min shielded from light. Then 1,3,4,6-tetra-*O*-acetyl-β-D-mannopyranose[17] (1.21 g, 3.47 mmol) was added over a period of 5 min. After stirring for 3 h, an additional amount of 1,3,4,6-tetra-*O*-acetyl-β-D-mannopyranose (0.18 g, 0.52 mmol) was added. Stirring at −30 °C was continued for a further 4 h. Then 2,6-di-*tert*-butyl-4-methylpyridine (0.11 g, 0.53 mmol) was added, and the mixture left overnight at −30 °C. The mixture was allowed to warm to room temperature, and was diluted with dry CH_2Cl_2 (10 mL), filtered through Celite and then concd. Purification by VLC (toluene:EtOAc, 8:1) gave an anomeric mixture of **8/9** (1.88 g, 78%), which upon crystallization (EtOAc-light petroleum) yielded the pure β-anomer **9** (1.43 g, 60%), mp 155–155.5 °C, $[\alpha]_D^{20}$ −102° (*c* 1.0, CH_2Cl_2), ^1H and ^{13}C NMR data are presented in Tables 1 and 3, respectively, (Found: C, 57.3; H, 4.9; Cl, 4.2; ES-MS$^+$ with addition of LiCl: $[M+Li]^+$ 905.78. $C_{43}H_{43}O_{19}Cl$ requires C, 57.43; H, 4.83; Cl, 3.94%; *M* 899.26).

***N*$^{\alpha}$-(Fluoren-9-ylmethoxycarbonyl)-*O*-[3,4,6-tri-*O*-acetyl-2-*O*-(2,3,4-tri-*O*-benzoyl-6-*O*-chloroacetyl-α-D-mannopyranosyl)-α-D-mannopyranosyl]-L-threonine pentafluorophenyl ester (11).** Disaccharide **9** (2.05 g, 2.28 mmol) was treated with HBr:HOAc (4M, 5.7 mL) in dry CH_2Cl_2 (10 mL) with molecular sieves (3 Å, 2.0 g) added. After stirring at room temperature for 1.5 h, the reaction mixture was diluted with CH_2Cl_2 (50 mL) and then poured into ice-water (50 mL). The organic layer was washed with cold water (50 mL), satd $NaHCO_3$ (2 × 50 mL) and water (50 mL), dried ($MgSO_4$) and filtered ($MgSO_4$:Celite). Concentration of the filtrate gave a foam, which was freeze-dried for 3 days to give the dimannosyl bromide **10** (1.92 g, 91%), ^1H NMR data are presented in Table 1. An aliquot of the bromide **10** (1.73 g, 1.88 mmol) and Fmoc-Thr-OPfp (1.05 g, 2.07 mmol) were dissolved in dry CH_2Cl_2 (50 mL) and stirred at −40 to −30 °C for 1 h under Ar over molecular sieves (3 Å, 3.0 g). Then silver triflate (0.58 g, 2.26 mmol) was added quickly, and stirring at −20 °C was continued for 1 h. After stirring for an additional 2 h at −20 to −10 °C, 2,6-di-*tert*-butyl-4-methylpyridine (0.39 g, 2.26 mmol) was added. Upon stirring without cooling for 30 min,

the mixture was filtered ($MgSO_4$:Celite). Concentration (to 15 mL) and purification by VLC (gradient elution with hexane to hexane:EtOAc, 3:1) yielded compound **11** (2.17 g, 86%), $[\alpha]_D^{20}$ −41° (*c* 1.0, CH_2Cl_2), ^1H and ^{13}C NMR data are presented in Tables 2 and 3, respectively, (Found: C, 58.6; H, 4.5; N, 1.0; ES-MS$^+$ with addition of LiCl: 1353.03 $[M+Li]^+$. $C_{66}H_{57}O_{22}NF_5Cl$ requires C, 58.86; H, 4.27; N, 1.04%; *M*, 1346.62.

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-*O*-[3,4,6-tri-*O*-acetyl-2-*O*-(2,3,4-tri-*O*-benzoyl-α-D-mannopyranosyl)-α-D-mannopyranosyl]-L-threonine (12). The Pfp ester **11** (2.07 g, 1.54 mmol) was dissolved in MeCN (17 mL) and DMF (22 mL). HOBt (0.21 g, 1.55 mmol) and water (5.5 mL) were added. Stirring at room temperature overnight.

A stock solution of diisopropylethylammonium hydrazinedithiocarbonate (HDTC, 0.364 M) was prepared: Hydrazine hydrate (0.73 mL, 15.0 mmol) was dissolved in EtOH:water (2:1, 30 mL), DIPEA (2.57 mL, 15.0 mmol) was added, and the soln cooled to 0 °C. Then CS_2 (0.90 mL, 15.0 mmol) in dioxane (filtered through basic Al_2O_3, 7 mL) was added dropwise to the cooled hydrazine solution.

To the above reaction mixture was added NEM (585 µl, 4.62 mmol) and an aliquot (12.7 mL, 4.62 mmol) of the HDTC stock soln, and stirring at room temperature was continued for 1.5 h. Concentration and co-distillation with water (oil pump) gave a residue, which was dissolved in MeCN:water (1:1, 30 mL). Purification by preparative HPLC (2 portions), using first 50% solvent B for 10 min, then the linear gradient 50→75% solvent B during 30 min, and finally 75→100% solvent B over 60 min, gave a product fraction (RT 73 min) which was concd and co-distilled with toluene, and then freeze-dried overnight to give the hydroxy-acid **12** (1.58 g, 93%), $[\alpha]_D^{20}$ −47° (*c* 1.0, CH_2Cl_2), ^1H and ^{13}C NMR data are presented in Tables 2 and 3, respectively, (Found: C, 62.8; H, 5.4; N, 1.1; ES-MS$^+$ 1126.72 $[M+Na]^+$. $C_{58}H_{57}O_{21}N$ requires C, 63.09; H, 5.21; N, 1.27%; *M*, 1104.06).

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-*O*-[3,4,6-tri-*O*-acetyl-2-*O*-(2,3,4-tri-*O*-benzoyl-α-D-mannopyranosyl)-α-D-mannopyranosyl]-L-threonine pentafluorophenyl ester (13). Pentafluorophenol (281 mg, 1.52 mmol) was dissolved in dry CH_2Cl_2 (25 mL) with molecular sieves (3 Å, 2.0 g) added, and the solution was cooled to 0 °C and stirred for 10 min. Then DCCI (157 mg, 0.76 mmol) was added and allowed to react at 0 °C for 30 min. A soln of compound **12** (561 mg, 0.51 mmol) in dry CH_2Cl_2 (15 mL) was added and the temperature kept at 0 °C for 5 h. The reaction mixture was filtered ($MgSO_4$:Celite) and the filtrate concd. The residue was dissolved in MeCN (20 mL), and pptd dicyclohexylurea was filtered off, then water (13 mL) was added to the soln. Purification by preparative HPLC (2 portions), using first 50% solvent B for 10 min, then the linear gradient 50→75% solvent B during 30 min, and finally 75→100% solvent B over 60 min, gave a product fraction (RT 96.5 min) which was partitioned in CH_2Cl_2:water (3:1, 600 mL). The organic layer was washed with water (300 mL), dried ($MgSO_4$), concd, co-distilled with toluene, and then freeze-dried overnight to give the Pfp ester **13** (522 mg, 81%), $[\alpha]_D^{20}$ −42° (*c* 0.6, CH_2Cl_2), ^1H and ^{13}C NMR data are presented in Tables 2 and 3, respectively, (Found: C, 60.1; H, 4.5; N, 1.1; ES-MS$^+$ with addition of LiCl: $[M+Li]^+$ 1276.70. $C_{64}H_{56}O_{21}NF_5$ requires C, 60.52; H, 4.44; N, 1.10%; M, 1269.76). When the starting material was not completely free from residual TFA a substantial amount of the corresponding 6′-*O*-trifluoroacetyl derivative was obtained, ^1H NMR (500 MHz; CD_3OD): δ 5.94 (*t*, *J* = 9.8 Hz, 4-H^a), 5.66 (dd, *J* = 10.0 and 3.3 Hz, 3-H^a), 5.69 (br s, 2-H^a), 5.44–5.36 (*m*, 2H, 3-H^b and 4-H^b), 5.28 (d, *J* = 1.2 Hz, 1-H^b), 5.21 (br s, 1-H^a), 4.79 (obscured by a residual HOD-peak, H$^{\gamma}$), 4.62 (m, H$^{\beta}$), 4.57 (dt, *J* = 9.4 and 2 × 2.0 Hz, 5-H^a), 4.51 (dd, *J* = 10.2 and 6.1 Hz, Fmoc-CH_2), 4.47 (s, 2H, 2 × 6-H^a), 4.43–4.37 (m, 2H, Fmoc-CH_2 and 6-H^b), 4.23–4.17 (m, 3H, Fmoc-CH, 6-H^b and 5-H^b), 4.16 (br s, 2-H^b), 8.11–7.24 (Ar-H), 2.14–2.09 (3 × 3H, 3 × AcO); where ^{a} and ^{b} designates the non-reducing and the reducing sugar unit, respectively; MALDI-TOF MS $[M+Na]^+$ 1389.4, $C_{66}H_{55}O_{22}NF_8$ requires *M*, 1366.14.

N^{α}-(Fluoren-9-ylmethoxycarbonyl)-*O*-[3,4,6-tri-*O*-acetyl-2-*O*-(2,3,4-tri-*O*-benzoyl-6-*O*-phosphate-α-D-mannopyranosyl)-α-D-mannopyranosyl]-L-threonine pentafluorophenyl ester (14). The Pfp ester **13** (557 mg, 0.44 mmol) was dissolved in dry CH_2Cl_2 (30 mL) with molecular sieves (3 Å, 1.5 g) added. Then NEM (1.00 mL, 7.89 mmol) and $POCl_3$ (0.80 mL, 8.77 mmol) were added quickly. The flask was filled with Ar and stirring was continued at room temperature for 15 h when the reaction mixture showed complete conversion as seen by analytical HPLC; it was then filtered and concd. The residue was dissolved in Me_2CO:water (5:1, 30 mL) and was allowed to stand at room temperature for 30 min. The solution was concd (to 2 mL), and an additional amount of Me_2CO:water (5:1, 30 mL) was added and the soln concd. This was repeated twice, and finally only Me_2CO (25 mL) was added and the soln concd. The residue was dissolved in Me_2CO:water (2:1, 20 mL). Purification by preparative HPLC, using first 50% solvent B for 10 min, then the linear gradient 50→70% solvent B during 20 min, and finally 70→100% solvent B over 70 min, gave a product fraction (RT 76.5 min), which was concd and freeze-dried to give phosphorylated building block **14** (444 mg, 75%), $[\alpha]_D^{20}$ −21° (*c* 0.3, CH_2Cl_2), ^1H and ^{13}C NMR data are presented in Tables 2 and 3, respectively, (Found: C, 57.0; H, 4.6; N, 0.9; ES-MS$^-$ $[M-2H]^{2-}$ 1348.70. $C_{64}H_{57}O_{24}NF_5P$ requires C, 56.93; H, 4.26; N, 1.04%; *M* 1350.12).

Peptide synthesis by the syringe method,[19] general example

Synthesis of glycopeptides was performed in DMF using the resin PEGA 1900/130 [poly(ethyleneglycol) dimethylacrylamide copolymer]. The resin (1.16 g;

loading 0.24 mmol/g) was packed into a 20 mL disposable syringe (Discardit II, Beckton Dickinson) fitted with a teflon filter. The syringe was connected to a suction flask through a teflon tube with a manual 2-way teflon valve; excess reagent, DMF, etc., was removed by applying vacuum. The resin was derivatized with the Rink-linker:[22] the Rink-linker (300 mg, 0.56 mmol), TBTU (160 mg, 0.45 mmol) and NEM (0.14 mL, 1.11 mmol) were dissolved in DMF, and after 10 min the soln was added to the resin. After 2 h the soln was removed, and the resin was washed with DMF (3 × 10 mL), and a soln (10 mL) of acetic anhydride:DMF (1:7) was added. After 20 min the resin was washed thoroughly with DMF (15 × 15 mL) and the solvent removed from the resin. This washing procedure was repeated after each coupling/deprotection step. N^{α}-Fmoc deprotection was then effected by successive 1 and 30 min treatments of the resin with 20% piperidine in DMF (10 mL).

The protected amino acids (1.6–3.1 equiv) and Dhbt-OH (1 equiv) were dissolved in DMF (7.5 mL), and the soln added to the resin. The suspension was agitated twice and then left for 24 h. After washing, the N^{α}-Fmoc group was removed and the resin washed as described above. The synthesis cycle was repeated with the remaining amino acids. After the final N^{α}-Fmoc deprotection, the resin was treated with a soln (10 mL) of acetic anhydride:DMF (1:7) for a period of 20 min. The resin was washed successively with DMF (15 × 5 mL) and diethyl ether (10 × 5 mL) and dried.

The resin was removed from the syringe, and cleavage of the glycopeptide from the linker and simultaneous side chain deprotection were performed by treatment with 95% TFA (20 mL) for 2 h, followed by filtration and washing of the resin with 95% TFA and CH_2Cl_2. Acetic acid (3 mL) was added to the filtrate and after concn, the glycopeptide was purified by preparative HPLC, using the linear gradient 85–100% solvent B for 30 min.

Deprotection of glycopeptides. General example

The fully protected glycopeptide (e.g. 10 mg) was dissolved in pyridine containing 10% acetic acid (3 mL). Zinc (17-fold the weight of glycopeptide, e.g. 170 mg) and silver carbonate (sixfold the weight of glycopeptide, e.g. 60 mg) were added, and the suspension stirred at 50–60 °C for 18 h. The suspension was then filtered and directly purified by HPLC, using first solvent A (100%) for 20 min, then the linear gradient 0–100% solvent B for 100 min. The partially deprotected glycopeptide was then dissolved in $CHCl_3$ (0.2 mL), and MeOH (0.8 mL) and hydrazine hydrate (0.2 mL) were added. After 2.5 h the reaction mixture was directly purified by gel filtration followed by HPLC, using the same gradient as above.

Ac-L-Thr[6-*O*-PO_3H_2-α-D-Man]-L-Thr-L-Lys-L-Thr-L-Thr[6-*O*-PO_3H_2-α-D-Man]-NH_2 (17). Synthesis of glycopeptide **15** was performed as described in the general example. The PEGA resin (0.30 g; loading 0.24 mmol/g) derivatized with the Rink-linker was used. The amino acids used were: glycosylated building block **4** (144 mg, 0.11 mmol), Fmoc-Thr(Bu^t)-OPfp (98 mg, 0.18 mmol) and Fmoc-Lys(Boc)-OPfp (115 mg, 0.18 mmol). Dhbt-OH (12 mg, 0.07 mmol) was added for each coupling reaction. Couplings, washings, N^{α}-Fmoc deprotections, acetylation after the final N^{α}-Fmoc deprotection and cleavage of the peptide from the resin were performed as described in the general example. Purification after cleavage from the resin was performed by semipreparative HPLC, using the linear gradient 85–100% solvent B for 50 min. This gave protected glycopeptide **15** (78.5 mg, 49%, RT 28 min). ^{1}H NMR data are presented in Tables 5 and 6. Amino acid analysis (theoretical values in parentheses): Thr 3.93 (4) and Lys 1.07 (1).

Cleavage of the Tce groups from compound **15** (11.9 mg) and purification (RT 82 min) were performed as described in the general procedure to yield compound **16** (9.1 mg, 99%).

Deacylation of compound **16** (5.2 mg) was performed as described in the general procedure, and the reaction mixture was directly purified by gel filtration and HPLC to yield glycopeptide **17** (3.2 mg, 97%). ^{1}H NMR data are presented in Tables 7 and 8. ES-MS$^-$ 1074.1 $[M-H]^-$. $C_{36}H_{67}N_7O_{26}P_2$ requires *M*, 1075.36].

Synthesis of cyclic glycopeptides 24, 25 and 30. General procedure

Synthesis of the glycopeptides was performed in DMF using the PEGA 1900/130 resin, similarly to the synthesis of glycopeptide **15**. The first three amino acids were identical for all three glycopeptides, and the first three coupling reactions were thus performed on a larger amount of resin.

The resin (0.88 g; loading 0.24 mmol/g) was derivatized using Rink-linker (229 mg, 0.42 mmol), TBTU (113 mg, 0.38 mmol) and NEM (0.11 mL, 0.85 mmol) as described in the general example. Washing and N^{α}-Fmoc deprotections were performed as described for glycopeptide **15**.

The resin was washed, N^{α}-Fmoc deprotected and washed, and the first amino acid, Fmoc-Asp-OAll (175 mg, 0.42 mmol), TBTU (113 mg, 0.38 mmol) and NEM (0.11 mL, 0.84 mmol) were dissolved in DMF (5 mL), and after 10 min added to the resin. After 2.5 h the resin was washed with DMF (3 × 5 mL), acetylated and washed with DMF as described in the general procedure. The peptide assembly was performed as described above for compound **15**, and after N^{α}-Fmoc-deprotection and washing, Fmoc-D-Ala-OH (145 mg, 0.47 mmol) was coupled with TBTU (123 mg, 0.38 mmol) and NEM (0.11 mL, 0.85 mmol). The resin was washed, N^{α}-Fmoc-deprotected and washed and the synthesis cycle was repeated with glycosylated building block **5** (414 mg, 0.25 mmol) with addition of Dhbt-OH (35 mg, 0.21 mmol). After washing, N^{α}-Fmoc-deprotection and washing as described above, amino acid

analysis of the resin showed a loading of only 0.15 mmol/g. The resin was then divided into three portions for synthesis of glycopeptides **24**, **25**, and **30**, respectively. The synthesis cycles were repeated as described above for the three resin portions. L-Amino acids were coupled as Fmoc-amino acid-OPfp esters (2 equiv) with addition of DhbtOH (1 equiv), D-amino acids as Fmoc-amino acids (2.2 equiv) with TBTU (2 equiv) and NEM (4 equiv).

For glycopeptide **24**, amino acids number 4–6 in the couplings were Fmoc-D-Lys(Boc)-OH, glycosylated building block **5**, and Fmoc-D-Ala-OH, respectively. For glycopeptide **25**, amino acids no. 4–6 were Fmoc-L-Lys(Boc)-OPfp, glycosylated building block **5**, and Fmoc-D-Ala-OH, respectively. For glycopeptide **30**, amino acids nos. 4–8 were Fmoc-D-Lys(Boc)-OH, glycosylated building block **5**, Fmoc-D-Ala-OH, Fmoc-L-Thr(Bu^t)-OPfp, and Fmoc-D-Ala-OH, respectively.

At the end of peptide assembly, the resin was washed successively with DMF (10 × 5 mL) and Et_2O and dried. The resin (e.g. 302 mg, 0.07 mmol) was then transferred to a round-bottomed flask (25 mL) and suspended in $CHCl_3$ (25 mL) containing 5% acetic acid and 2.5% NEM. The suspension was purged with Ar for 30 min, and removal of the allyl group was effected by addition of $Pd(PPh_3)_4$ (e.g. 251 mg, 0.21 mmol). Slow purging with Ar was continued for 4 h, the resin was then transferred to a filter and washed successively with $CHCl_3$ (3 × 10 mL), DMF (5 × 10 mL), a soln of 5% DIPEA and sodium diethyldithiocarbamate in DMF (3 × 10 mL), and DMF (10 × 10 mL). N^{α}-Fmoc-deprotection and washing were then performed as described above.

Cyclization of glycopeptides. General procedure

After cleavage of the allyl group and the final N^{α}-Fmoc deprotection, cyclization was performed as follows: a soln of DIPEA (e.g. 50 µL, 0.26 mmol) in DMF (3 mL) was added to the resin (e.g. 275 mg, 0.066 mmol). A soln of TBTU (e.g. 22 mg, 0.066 mmol) in DMF (0.24 mL) was added in portions (40 µL) to the resin over a period of 1 h. After a further 3 h, the resin was washed twice with DMF (7 mL), and the cyclization procedure was repeated. The resin was then washed thoroughly with DMF (10 × 7 mL) and Et_2O (5 × 7 mL) and dried.

Cleavage of the glycopeptide from the resin was performed by treatment with 95% TFA (20 mL) for 2.5 h, followed by filtration and washing of the resin with 95% TFA and CH_2Cl_2. After concn, the glycopeptide was purified by preparative HPLC, using first 75% solvent B for 5 min, then the linear gradient 75–100% solvent B for 50 min.

Cyclo {D-Ala-L-Thr[6-*O*-PO_3H_2-α-D-Man(1→2)-α-D-Man]-D-Lys-L-Thr[6-*O*-PO_3H_2-α-D-Man(1→2)-α-D-Man]-D-Ala-L-Asn} (28). Solid-phase synthesis, cyclization, cleavage from 200 mg of the resin and purification (RT 53 min) as described in the general procedures gave compound **24** (41 mg, 47%). ^{1}H NMR data are presented in Tables 4 and 6. Amino acid analysis (theoretical values in parentheses): Ala 2.12(2), Asp 1.03(1), Lys 1.01(1), and Thr 1.85(2). ES-MS$^+$ 2799.0 $[M+H]^+$. $C_{110}H_{124}Cl_{12}N_8O_{47}P_2$ requires *M*, 2797.60].

Cleavage of the Tce groups from compound **24** (8.9 mg) and purification (RT 95 min) as described in the general procedure gave compound **26** (4.4 mg, 61%).

Deacylation of compound **26** (4.4 mg) and purification (RTr 35 min) were performed as described in the general procedure to yield glycopeptide **28** (2.0 mg, 73%). ^{1}H NMR data are presented in Tables 7 and 8. ES-MS$^-$ 1393.6 $[M-H]^-$; $C_{48}H_{84}N_8O_{35}P_2$ requires *M*, 1394.45.

Cyclo {D-Ala-L-Thr[6-*O*-PO_3H_2-α-D-Man(1→2)-α-D-Man]-L-Lys-L-Thr[6-*O*-PO_3H_2-α-D-Man(1→2)-α-D-Man]-D-Ala-L-Asn} (29). Solid-phase synthesis, cyclization, cleavage from 76 mg of the resin and purification (RT 53 min) as described in the general procedures gave compound **25** (19 mg, 57%). ^{1}H NMR data are presented in Table 4 and 6. Amino acid analysis (theoretical values in parenthesis): Ala 2.04(2), Asp 1.05(1), Lys 1.00(1), and Thr 1.92(2). ES-MS$^+$ 2799.0 $[M+H]^+$. $C_{110}H_{124}Cl_{12}N_8O_{47}P_2$ requires *M*, 2797.60.

Cleavage of the Tce groups from compound **25** (4.1 mg) and purification (RT 100 min) as described in the general procedure gave compound **27** (2.6 mg, 78%).

Deacylation of compound **27** (2.6 mg) and purification (RT 36 min) were performed as described in the general procedure to yield glycopeptide **29** (1.3 mg, 79%). ^{1}H NMR data are presented in Tables 7 and 8. ES-MS$^-$ 1393.0 $[M-H]^-$. $C_{48}H_{84}N_8O_{35}P_2$ requires *M*, 1394.45.

Cyclo {D-Ala-L-Thr-D-Ala-L-Thr[6-*O*-PO_3H_2-α-D-Man(1→2)-α-D-Man]-D-Lys-L-Thr[6-*O*-PO_3H_2-α-D-Man(1→2)-α-D-Man]-D-Ala-L-Asn} (32). Solid-phase synthesis, cyclization, cleavage from 287 mg of the resin and purification (RT 52 min) as described in the general procedures gave compound **30** (69 mg, 51%). ^{1}H NMR data are presented in Tables 4 and 6. Amino acid analysis (theoretical values in parenthesis): Ala 3.10(3), Asp 1.05(1), Lys 0.99(1), and Thr 2.87(3). ES-MS$^+$ 2970.6 $[M+H]^+$. $C_{117}H_{136}Cl_{12}N_{10}O_{50}P_2$ requires *M*, 2969.78.

Cleavage of the Tce groups from compound **30** (14.5 mg) and purification (RT 94 min) as described in the general procedure gave compound **31** (9.5 mg, 80%).

Deacylation of compound **32** (9.5 mg) and purification (RT 36 min) were performed as described in the general procedure to yield glycopeptide **32** (3.1 mg, 51%). ^{1}H NMR data are presented in Tables 7 and 8. ES-MS$^-$ 1565.2 $[M-H]^-$. $C_{55}H_{96}N_{10}O_{38}P_2$ requires *M*, 1566.54.

Ac-L-Thr[6-*O*-PO_3H_2-α-D-Bz_3Man (1→2)-α-D-Ac_3Man]-L-Lys(ABz)-L-Thr[6-*O*-PO_3H_2-α-D-Bz_3Man 1→2)-α-D-

Ac_3Man] (34) and Ac-L-Lys(ABz)-L-Thr[6-*O*-PO_3H_2-α-D-Bz_3Man(1→2)-α-D-Ac_3Man] (36). Synthesis of partially protected glycopeptide **34** was attempted by the syringe method[19] essentially as described in the general procedure using a PEGA 1900 resin (0.376 g, loading: 0.18 mmol/g) derivatized with the Rink-linker. Glycosylated building block **14** (100 mg, 0.0744 mmol, 1.1 equiv) was used for the first and third coupling whereas Fmoc-L-Lys[ABz(Boc)]-OPfp (102 mg, 0.135 mmol, 2.0 equiv) was employed in the second coupling. Dhbt-OH (12 and 22 mg, i.e. same equiv as amino acid derivative) was added before each coupling reaction. Couplings, washing, N^{α}-Fmoc deprotection (20% piperidine:DMF) and cleavage from the resin were performed as described in the general procedure. However, the acetylation step (before resin cleavage) was carried out by a 2 h coupling with Dhbt-OAc (28 mg, 0.135 mmol, 2.0 equiv).

The coupling times for the three other couplings were: 2 days, 22 h and 2.5 days, respectively. The crude product (73 mg, 50%) was shown to contain several components by analytical and semipreparative HPLC. MALDI-TOF MS of the fractions revealed the main components to be mixtures of dipeptides (*M* 1300–1200); the desired tripeptide[6] **34** was only present as a small fraction and less than 1 mg was isolated by semipreparative HPLC.

The above synthesis was repeated with a similar PEGA resin (0.14 g, loading: 0.36 mmol/g) derivatized with the Rink-linker. Glycosylated building block **14** (80 mg, 1.2 equiv) was used for the first and third coupling whereas Fmoc-L-Lys[ABz(Boc)]-OPfp (95 mg, 2.5 equiv) was employed in the second coupling. Dhbt-OH (9 mg, 1.1 equiv) was added before each coupling reaction. The following coupling times were employed: 14, 18 and 33.5 h. For Fmoc deprotection 50% morpholine:DMF was employed. The final acetylation with Dhbt-OAc (21 mg, 2.0 equiv) was performed twice (2 × 1 h). After cleavage from the resin (95% TFA), the reaction mixture contained one major compound, which by MALDI-TOF MS^- showed one sharp peak: 1248.3 $[M-H]^-$, $C_{58}H_{67}N_5O_{24}P$ requires *M*, 1249.16. ^1H NMR (see Tables 5 and 6) supported the structure **36** of the dipeptide (22 mg, 41%, RT 58 min) isolated by semipreparative HPLC using first 20% solvent B for 10 min, then the linear gradient 20–45% solvent B during 25 min and finally the linear gradient 45–100% solvent B over 90 min.

A modified synthesis of compound **34** was attempted: PEGA resin (28 mg, loading: 0.36 mmol/g) derivatized with the Rink-linker was used. Glycosylated building block **14** (15.3 mg, 1.2 equiv) was used for the first and third coupling whereas Fmoc-L-Lys[ABz(Boc)]-OPfp (19 mg, 2.5 equiv) was employed in the second coupling. Dhbt-OH (1.64 mg, 1.1 equiv) was added before each coupling reaction. The following coupling times were employed: 22, 28 and 15.5 h. For Fmoc deprotection 50% morpholine:DMF was employed, but before the coupling reaction was performed, the resin was successively washed with 10% DIPEA:DMF (3 times) and DMF (8 times). The final acetylation with Dhbt-OAc (4.14 mg, 2.0 equiv) was performed twice (2 × 30 min). After cleavage from the resin (95% TFA), the reaction mixture was shown to be mixture of dipeptides by preparative analytical HPLC and MALDI-TOF MS.

MCPS. General procedure

An attempt at the synthesis of the protected glycopeptides **34** and **37–39** was performed in DMF, using the PEGA 1900/130 resin[38,39] with a loading of 0.36 mmol/g. The resin (400 mg) was derivatized with the Rink-linker[22] as described above for the syringe method. The resin was divided into the 20 wells of the manual multiple-column peptide synthesizer[34,35] fitted with teflon filters.

Fmoc deprotection was performed by two successive treatments with 50% morpholine × DMF (0.6 mL in each well), each for 10 min. Then the resin was washed with DMF (8 × 0.75 mL); this washing procedure was repeated after each coupling/deprotection. Upon removal of reagents (by suction) and subsequent DMF-wash, the resin was ready for the sequence of couplings. Glycosylated building block **13** (18.3 mg, 0.014 mmol, 2.0 equiv) and glycosylated phosphorylated building block **14** (19.5 mg, 0.014 mmol, 2.0 equiv) in DMF (0.25 mL) were dispensed into each well, containing 20 mg resin, at the appropriate time. The non-glycosylated building blocks Fmoc-Lys[ABz(Boc)]-OPfp (13.6 mg, 0.018 mmol, 2.5 equiv), Fmoc-Gly-OPfp (10.0 mg, 0.0216 mmol, 3.0 equiv) and Fmoc-Ala-OPfp (10.3 mg, 0.0216 mmol, 3.0 equiv) were likewise employed.

Each glycopeptide was synthesized in two wells (2 × 20 mg resin) whereas the remaining wells were left with DMF during couplings. Before each coupling, Dhbt-OH (1.2 mg per 20 mg resin, 2.0 equiv) in DMF (50 μL per 20 mg resin) was added to the resin giving a very distinct yellow colour of the resin. The first amino acid derivative in DMF (0.25 mL per 20 mg resin) was then added to the wells, and the synthesizer shaken gently for 20.5 h. After DMF-wash, the Fmoc group was removed and the resin was washed as described above. The synthesis cycle was repeated until the end of each peptide. The coupling times were as follows: 12 h (second coupling), 20 h (third coupling), 17 h (fourth coupling) and 26 h for the fifth coupling. After the last N^{α}-Fmoc deprotection for each peptide, the resin was removed from the well(s), and transferred to a syringe (2 mL) fitted with a teflon filter, and was acetylated for 8 h using Dhbt-OAc (4.5 mg, 0.0216 mmol, 3.0 equiv). Upon suction, the resin was washed with DMF (8 × 1 mL) and Et_2O (3 × 1 mL). Upon freeze-drying overnight the glycopeptides were cleaved from the linker by treatment with 95% aq. TFA (2 mL per 20 mg resin) for 2 h, followed by filtration and washing of the resin with 95% TFA and 95% HOAc. The filtrates were concentrated and an aliquot of each of the residues fractionated by preparative analytical HPLC (elution: 50% solvent B for 5 min followed by the

linear gradient 50–100% solvent B during 45 min). ES-MS$^-$ analysis of the major fractions from synthesis of compounds: **34** (**36**: 1248.7 $[M-H]^-$), **37** (**37**: 2112.3 $[M-H]^-$, unidentified by-product: 1282.8), **38** (**38**: 1944.6 $[M-H]^-$, Ac-Thr[6-*O*-PO_3H_2-Bz_3Man(1→2) Ac_3Man]-NH_2: 1001.6 $[M-H]^-$), **39** (**39**: 2130.1 $[M-H]^-$, Ac-Gly-Ala-Gly-Thr[6-*O*-PO_3H_2-Bz_3Man(1→2) Ac_3Man]-NH_2: 1186.7 $[M-H]^-$).

Biological assays

Purification of the ^{CI}MPR, biotinylation of the receptor and the ELISA assay employed for evaluation of the inhibitor potency of glycopeptides **17**, **28**, **29**, **32**, **54** and **55** were performed as previously reported.[6]

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