Synthesis of Mannose 6-Phosphate-containing Disaccharide Threonine Building Blocks and their Use in Solid-phase Glycopeptide Synthesis

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The preparation of 6′-*O*-phosphorylated α(1,2)- and α(1,6)-linked mannose disaccharides is described, starting from phenyl 2,3,4-tri-*O*-benzoyl-1-thio-α-D-mannopyranoside and using bis-(2,2,2-trichloroethyl)phosphorochloridate as phosphorylating reagent. The mannobiosides were converted into glycosyl donors and used in glycosylations of N^{α}-Fmoc-Thr-OPfp. The resulting glycosylated building blocks, **11** and **12**, were used in solid-phase glycopeptide synthesis of *O*-mannosylated tripeptides.

Mannose 6-phosphate (Man-6-*P*) has been shown to be an inhibitor of inflammation in the central nervous system.[1] The anti-inflammatory effect of Man-6-*P* may be due to inhibition of the interaction between Man-6-*P* receptors (MPRs) and lysosomal enzymes, as these interactions are partly responsible for intracellular transport and binding of lysosomal enzymes to the cell surface.[2-4]

Oligosaccharides containing Man-6-*P* have been isolated from lysosomal enzyme mixtures. Structural analysis has revealed these oligosaccharides to contain the Man-6-*P* units as terminal or subterminal residues on either, or both, antennary arms of *N*-linked oligomannosides.[5]

Binding-inhibition studies have shown that mannobiosides phosphorylated at the terminal position and linked α(1,2) are better inhibitors at the MPRs than is Man-6-*P* itself, and also than the corresponding α(1,6)- and α(1,3)-linked isomers. Branched divalent ligands with two terminal Man-6-*P* residues gave much stronger inhibitory effects (6–20 times higher) than the corresponding monophosphorylated compounds.[5,6] It would therefore be interesting to prepare glycopeptides containing mannobiosides phosphorylated at the terminal position and linked α(1,2) or α(1,6)

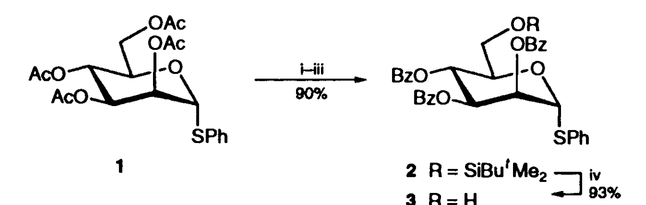
We report here the synthesis of N^{α}-(fluoren-9-ylmethoxycarbonyl)-L-threonine pentafluorophenyl ester (Fmoc-Thr-OPfp) glycosylated with 6′-*O*-phosphorylated α(1,2)- and α(1,6)-linked mannose disaccharides and their incorporation into two model tripeptides.

Our strategy for *O*-glycosylations of amino acids and solid-phase glycopeptide synthesis implies protection of the hydroxy and phosphate groups on the carbohydrate moieties prior to glycosylation.[7,8] *O*-Glycosylated β-hydroxy α-amino acid derivatives are sensitive to both strong acid and strong base,[9] and it is therefore necessary to employ protective groups that can be removed under mild conditions. The hydroxy groups on the carbohydrates were therefore protected with either acetyl or benzoyl groups. The acyl groups confer stability to the bond between the disaccharide and the peptide during the peptide synthesis and are easily removed with sodium methoxide in methanol.[10] The phosphate group was protected with the 2,2,2-trichloroethyl (TCE) group, which is easily introduced and is stable during peptide synthesis. In addition to conventional methods to cleave this group by reductive elimination with zinc[11-14] or zinc–copper couples,[15,16] other methods for its removal have been reported with reagents such as radical anions,[17] fluoride anion,[18] cobalt phthalocyanine,[19] zinc–methanol–ammonium chloride,[20] or catalytic hydrogenation over palladium catalyst.[21,22] The α-amino group was protected with the Fmoc group, which is sensitive to mild organic bases such as piperidine.[23] The Pfp ester serves the dual purpose of protecting the carboxylic function during glycosylation and activating it in the acylation step.[7,8,24] The Pfp ester was therefore chosen as protection for the carboxylic group of the glycosylated threonines, as it was also stable to purification on silica gel under anhydrous conditions.[8] For peptide synthesis the nonglycosylated amino acids were either Fmoc-amino acid 3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl (Dhbt) esters[25] or Fmoc-amino acid Pfp esters with Dhbt-OH added as an auxiliary nucleophile, which also allowed visual monitoring of the acylation reaction. Using this technique, tripeptides were synthesized on a 5 mg scale. Multiple-column peptide synthesis of linear and cyclic peptides containing two or more of the glycosylated building blocks, simulating ligands to the MPRs with the possibility of achieving the aforementioned cluster effect, is currently under investigation and will be reported elsewhere.

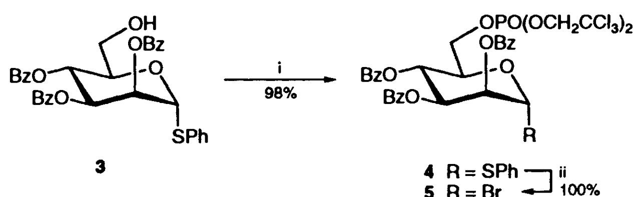
Results and Discussion

Mannose 6-phosphate is the nonreducing carbohydrate unit in both the α(1,2)- and α(1,6)-mannobiosides and we found it most efficient to synthesize a protected, phosphorylated monosaccharide unit, which could be incorporated into both disaccharides by glycoside synthesis. We therefore initially prepared 2,3,4-tri-*O*-benzoyl-6-*O*-diallyloxyphosphinoyl-α-D-mannopyranose, but conversion of this compound into a glycosyl donor resulted in partial loss of the allyl groups. An alternative strategy was to phosphorylate a thioglycoside derivative of D-mannose, as thioglycosides are easily converted into glycosyl bromides by addition of bromine.[26] Therefore phenyl 2,3,4,6-tetra-*O*-acetyl-1-thio-α-D-mannopyranoside **1**[27] was deacetylated, silylated with *tert*-butylchlorodimethylsilane (ᵗBDMSCl) and benzoylated to yield compound **2**, from which the ᵗBDMS group was hydrolysed to give phenyl 2,3,4-tri-*O*-benzoyl-1-thio-α-D-mannopyranoside **3** in 84% overall yield. Reaction of compound **3** with phosphorus trichloride oxide and then allyl alcohol gave phenyl 2,3,4-tri-*O*-benzoyl-6-*O*-diallyloxyphosphinoyl-1-thio-α-D-mannopyranoside. However, when this compound was treated with 1 mole equivalent of bromine, addition occurred solely at the allyl groups, and no conversion of the thioglycoside took place, as analysed by ^{1}H NMR spectroscopy. Consequently, we abandoned the use of the allyl groups for phosphate protection and turned instead to the use of 2,2,2-trichloroethyl groups.

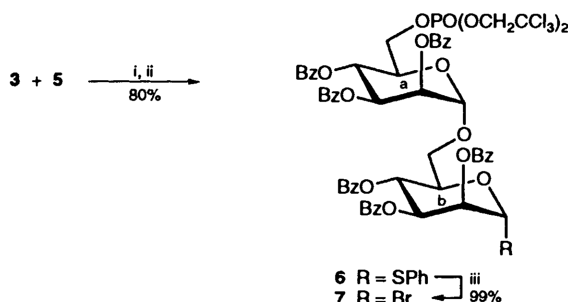
Reaction of compound **3** with bis-(2,2,2-trichloroethyl) phosphorochloridate in pyridine yielded compound **4** in almost quantitative yield (98%). Conversion of compound **4** into the



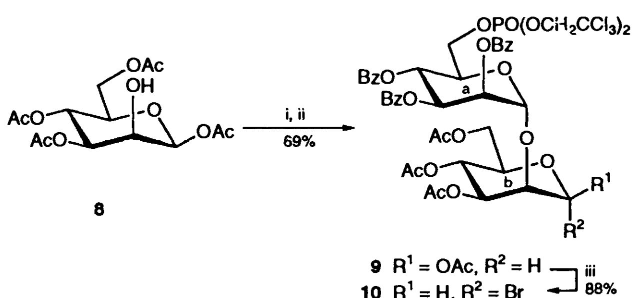
Reagents: i, NaOMe, MeOH; ii, Bu^tMe_2SiCl, pyridine; iii, BzCl, pyridine; iv, H^+



Reagents: i, $ClPO(OCH_2CCl_3)_2$, pyridine; ii, Br_2



Reagents and conditions: i, $AgOSO_2CF_3$, −40 °C; ii, 2,4,6-collidine; iii, Br_2

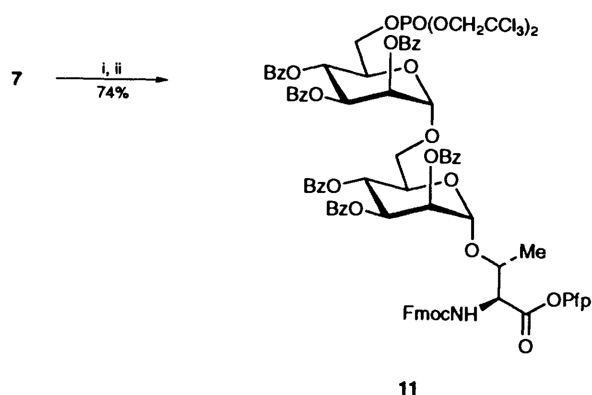


Reagents and conditions: i, **5**, $AgOSO_2CF_3$, −40 °C; ii, 2,4,6-collidine; iii, HBr

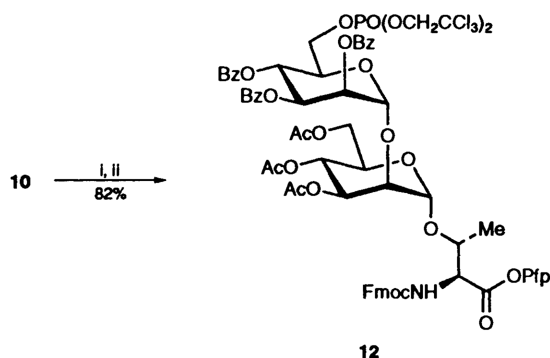
corresponding bromide **5** by reaction with bromine proceeded smoothly. Silver trifluoromethanesulfonate (silver triflate) promoted reactions of the bromide **5** with either compound **3** or 1,3,4,6-tetra-*O*-acetyl-β-D-mannopyranose **8** [28] gave the disaccharides **6** and **9** in 80 and 69% yield, respectively. Disaccharide **6** was converted into the bromide **7** by addition of bromine, and disaccharide **9** was converted into the bromide **10** by reaction with hydrogen bromide in acetic acid. Condensation of N^α-Fmoc-Thr-OPfp with the glycosyl bromides **7** and **10**, using silver triflate as promoter, afforded the protected, glycosylated building blocks **11** and **12** in 74 and 82% yield, respectively.

The presence of phosphorus and chlorine has been verified by elemental analysis of the phosphorylated monosaccharide **5** and the disaccharides **6** and **9**. The content of phosphorus was further established by ^{13}C NMR spectroscopy, which showed a coupling constant of 8 Hz between C-5 and phosphorus for all the phosphorylated compounds.

The glycosylated amino acid derivatives **11** and **12** were then used in solid-phase glycopeptide synthesis of the tripeptides **13** and **15**, respectively. PEGA 1900/130 [poly(ethylene glycol) dimethylacrylamide copolymer] [29] was used as the solid phase and *N*,*N*-dimethylformamide (DMF) as the solvent. The resin was derivatized with the Rink-linker [30] [4-(Fmoc-amino)-2,4-dimethoxybenzylphenoxyacetic acid] by the *O*-(1*H*-benzotriazol-1-yl)-*N*,*N*,*N*′-*N*′-tetramethyluronium tetrafluoroborate (TBTU) procedure.[31] The loading of the resin was determined, by amino acid analysis after coupling of the first amino acid, to be 0.14 mmol g^{-1}. The first amino acid (Thr) was coupled to the resin as its N^α-Fmoc-protected Dhbt-ester with *tert*-butyl (Bu^t) as acid-labile side-chain protection. The synthesis was continued with Lys as its N^α-Fmoc-protected Pfp-ester with *tert*-butoxycarbonyl (Boc) as side-chain protection and Dhbt-OH was added as an auxiliary nucleophile. The acylations with the two first amino acids were complete within 1 h, as judged by the fading of the yellow colour resulting from formation of an ion-pair between Dhbt-OH and unchanged amino groups,[25] but were allowed to proceed for 24 h. The last amino acid, **11** or **12**, was coupled as its N^α-Fmoc-protected Pfp-ester without addition of Dhbt-OH, which gave the possibility of recovering the excess of the glycosylated building blocks. The acylation of compounds **11** and **12** was allowed to proceed for 3 days, after which both building blocks could be recovered by preparative HPLC (73%). Deprotection of the α-amino groups was achieved by treatment with 20% piperidine in DMF. The *N*-terminus was acetylated after the final N^α-Fmoc-deprotection. The *O*-glycopeptides were cleaved from the resin by treatment with 95% trifluoroacetic acid (TFA) with simultaneous deprotection of the side chains, and solidified by treatment with diethyl ether. The crude yields of the TFA-salts **13** and **15** after precipitation were 90% and quantitative, respectively, and, after preparative HPLC, 54 and 76%, respectively, based on the loading of the resin. HPLC diagrams of the crude products **13** and **15** are shown in Fig. 1.



Reagents and conditions: i, Fmoc-Thr-OPfp, $AgOSO_2CF_3$, −40 °C; ii, 2,4,6-collidine



Reagents and conditions: i, Fmoc-Thr-OPfp, $AgOSO_2CF_3$, −60 °C; ii, 2,4,6-collidine

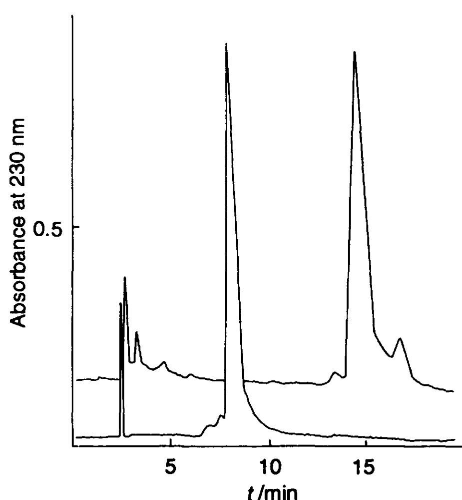


Fig. 1 The analytical HPLC chromatograms (215 nm) of crude glycopeptides **13** (upper trace) and **15** (lower trace), using a linear gradient of 85–95% buffer B in 20 min (see Experimental section)

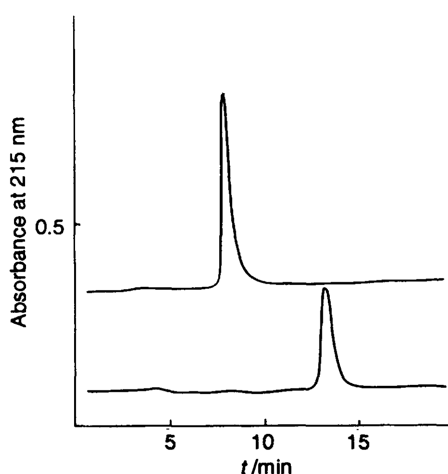
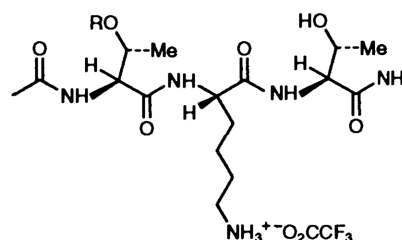


Fig. 2 The analytical HPLC chromatograms (230 nm) of crude glycopeptides **14** (upper trace) and **16** (lower trace), using 100% buffer A (see Experimental section)

Since its introduction by Eckstein,[32] the 2,2,2-trichloroethyl group has been widely employed as a phosphate-protecting group; however, one occasional problem with its use is difficulty in its quantitative removal. To avoid nucleophilic attack on the phosphate function by sodium methoxide or the deprotected hydroxy groups of the disaccharides, we chose to deprotect the phosphate group first. This was effected by hydrogenolysis in aqueous ethanol,[21] which also resulted in a slight reduction of the benzoyl groups. *O*-Deacylation was then performed by treatment with sodium methoxide in methanol. The deprotected glycopeptides, **14** and **16**, were purified by ion-exchange chromatography and gel filtration, in 18 and 36% yield, respectively. In both cases a by-product (30 and 15%, respectively) was isolated, in which the second 2,2,2-trichloroethyl group had not been removed. The complete deprotection of the phosphate function of glycopeptides **14** and **16** was verified by ^1H and ^{13}C NMR spectroscopy, which showed the absence of the 2,2,2-trichloroethyl groups, and by FAB–MS, which showed a molecular ion of 793, corresponding to the free phosphoric acid, and of 839, corresponding to the disodium salt. HPLC diagrams of the purified, deprotected glycopeptides are shown in Fig. 2. Characterization of the glycopeptides was performed by 1D- and 2D-NMR spectroscopy (see Tables 4 and 5).



13 R = Bz$_3$-α-D-Man*p*-6-(TCE)$_2$*p*-(1→6)-Bz$_3$-α-D-Man*p*-(1-
14 R = α-D-Man*p*-6-*p*-(1→6)-α-D-Man*p*-(1-
15 R = Bz$_3$-α-D-Man*p*-6-(TCE)$_2$*p*-(1→2)-Ac$_3$-α-D-Man*p*-(1-
16 R = α-D-Man*p*-6-*p* (1→2)-α-D-Man*p*-(1-

The cleavage of the 2,2,2-trichloroethyl ester groups was optimized to a quantitative reaction by using the model compound Ac-[Bz$_3$-α-D-Man*p*-6-(TCE)$_2$*P*-(1 → 6)-Bz$_3$-α-D-Man*p*-(1→)]-ThrNH$_2$, as will be described in detail in a forthcoming publication. The best conditions were obtained by addition of silver carbonate to the reductive-cleavage medium with zinc in pyridine containing 10% acetic acid.

In conclusion, a simple method for the syntheses of glycopeptides containing phosphorylated carbohydrate moieties has been developed.

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. M.p.s were measured on a Büchi melting point apparatus and are uncorrected. Vacuum liquid chromatography (VLC)[33] was performed on Merck silica gel 60 H (0.040–0.060 mm), and VLC under anhydrous conditions was performed on dried silica gel (120 °C; > 24 h) with solvents dried over molecular sieves 3 Å. DMF was freshly distilled by fractional distillation at reduced pressure and kept over 3 Å molecular sieves. Dichloromethane was distilled from phosphorus pentaoxide 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 reduced pressure at temperatures < 40 °C. Bis(2,2,2-trichloroethyl) phosphorochloridate and 10% Pd-on-charcoal catalyst were purchased from Aldrich, *tert*-butylchlorodimethylsilane from Fluka and ion-exchange resin Amberlite IR C-50 from Merck. Suitably protected N^{α}-Fmoc amino acids and Pfp esters were purchased from MilliGen (Taastrup, Denmark) or Bachem (Bubendorf, Switzerland), and the Dhbt esters were prepared according to the method of Atherton *et al.*[25] The glycopeptides were hydrolysed with 6 mol dm^{-3} HCl at 110 °C for 24 h and the amino acid composition was determined on a Pharmacia LKB Alpha Plus amino acid analyser. Nomenclature is according to IUPAC recommendations. Negative fast-atom-bombardment mass spectra (FAB–MS) were recorded on a JEOL JMS-NX/HX 110A Tandem Mass Spectrometer. Ions were produced by a beam of xenon atoms, 6 keV, from a matrix of glycerol. Optical rotations were recorded on a Perkin-Elmer 241 polarimeter, and are given in units of 10^{-1} deg cm^2 g^{-1}. ^1H and ^{13}C NMR spectra were recorded on a Bruker AM 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$_2$O at 310 K. The coupling constants $^3J_{H^{\alpha}-N^{\alpha}H}$ were measured in (CD$_3$)$_2$SO. For the assignment of signals, proton–proton and carbon–proton shift correlation spectroscopy were used. ^1H NMR chemical shifts of overlapping signals were obtained from the centre of the cross-peaks in the proton–proton 2D homonuclear chemical-shift correlation (COSY)

Table 1 Selected ^1H and ^{13}C NMR shifts (ppm) for compounds **2–5** measured at 500 MHz and 125.77 MHz, respectively, on solutions in $CDCl_3$ at 300 K. Coupling constants (Hz) in parentheses

	1-H	2-H	3-H	4-H	5-H	6-H	6-H′
2	5.79 (1.2)	5.98 (3.5)	5.84 (10.0)	6.09 (10.0)	4.71	3.96 (4.5, 12.0)	3.89 (2.2)
3	5.83 (0.8)	5.99 (3.0)	5.97 (10.0)	6.00 (10.0)	4.63	3.86	3.86
4	5.79 (1.5)	5.98 (3.2)	5.90 (9.8)	6.02 (9.8)	4.94	4.48	4.48
5	5.62 (1.2)	5.92 (3.2)	6.31 (9.8)	6.07 (9.8)	4.61	4.50	4.50

	C-1	C-2	C-3	C-4	C-5	C-6
2	86.10	72.19[a]	70.87[a]	66.93[a]	72.75[a]	62.26
3	86.02	72.01	70.00	67.28	72.13	61.33
4	86.14	71.84	70.12	66.58	70.26[b]	67.01[c]

[a] Assignments may be reversed. [b] J_{CP} 8 Hz. [c] J_{CP} 5 Hz.

Table 2 Selected ^1H NMR chemical shifts (ppm) and coupling constants (Hz) for compounds **6–12**[a] measured at 500 MHz on solutions in $CDCl_3$ at 300 K

	6	7	9	10	11	12
1ᵃ-H	5.16 (1.5)	5.15 (1.8)	5.35 (1.8)	5.28 (1.8)	5.16 (1.8)	5.24 (1.8)
2ᵃ-H	5.81 (3.0)	5.77 (3.1)	5.77 (3.0)	5.71 (3.6)	5.73 (3.5)	5.69 (3.3)
3ᵃ-H	6.01 (10.2)	6.01 (9.8)	6.05 (10.4)	5.99 (10.0)	5.99 (10.0)	5.97 (9.8)
4ᵃ-H	5.97 (10.2)	5.93 (9.8)	6.07 (10.4)	5.93 (10.0)	5.94 (10.0)	5.84 (9.8)
5ᵃ-H	4.23	4.22	4.78	4.52	4.28–4.32	4.52
6ᵃ-H	4.24	4.22	4.45	4.47	4.28–4.32	4.40
6ᵃ-H′	4.24	4.22	4.45	4.47	4.28–4.32	4.40
1ᵇ-H	5.84 (1.3)	6.64 (1.5)	5.91 (1.0)	6.68 (1.8)	5.34 (1.8)	5.32 (2.2)
2ᵇ-H	5.81 (3.2)	5.97 (3.0)	4.34 (3.0)	4.47 (3.0)	5.67 (3.5)	4.12 (3.0)
3ᵇ-H	5.93 (10.1)	6.31 (10.0)	5.25 (9.6)	5.78 (9.8)	5.92 (9.2)	5.39 (9.6)
4ᵇ-H	6.20 (10.1)	6.26 (10.0)	5.51 (9.6)	5.59 (9.8)	5.99 (9.2)	5.42 (9.6)
5ᵇ-H	4.96	4.60	3.91	4.29	4.49	4.13
6ᵇ-H	4.17 (4.7, 10.8)	4.11 (2.2, 11.8)	4.41 (5.4, 12.0)	4.42 (2.0, 13.0)	4.13 (2.0, 12.0)	4.28 (2.0, 12.0)
6ᵇ-H′	3.80 (1.7)	3.80 (3.6)	4.29 (2.2)	4.24 (4.2)	3.78 (5.4)	4.23 (5.2)
Fmoc-CH					4.34	4.27
Fmoc-CH_2					4.54, 4.46	4.46 (7.3), 4.38
NH					4.92 (9.2)	6.20 (9.5)
H^α					4.97 (3.0)	4.84
H^β					4.79 (6.5)	4.58
H^γ					1.73	1.49
CH_2CCl_3	4.50–4.62	4.47–4.60	4.53–4.67	4.50–4.62	4.50–4.62	4.50–4.62

[a] Rings a and b are indicated on structures **6–10**.

Table 3 Selected ^{13}C NMR chemical shifts (ppm) for compounds **6**, **9**, **11** and **12** measured at 125.77 MHz on solutions in $CDCl_3$ at 300 K

	6	9	11	12
C-1ᵃ	98.10	98.42	97.86	99.14
C-2ᵃ	70.12	70.75	70.23	70.53
C-3ᵃ	69.86	68.99	69.60	68.94
C-4ᵃ	66.07	66.37	66.16	66.73
C-5ᵃ	69.09[a]	69.63[a]	69.27[a]	70.06[a]
C-6ᵃ	66.68[b]	67.16	66.81[b]	67.60
C-1ᵇ	86.38	91.04	99.16	99.90
C-2ᵇ	71.96	74.97	70.20	76.75
C-3ᵇ	70.53	72.15	69.70	69.60
C-4ᵇ	66.91	65.85	66.92	66.73
C-5ᵇ	70.60	73.32	70.42	69.43
C-6ᵇ	67.15	61.86	67.09	62.43
Fmoc-CH			47.11	57.09
Fmoc-CH_2			67.86	67.60
C^α			58.53	58.85
C^β			76.85	76.75
C^γ			18.47	18.29
Pfp			136.0–142.0	136.0–142.0
CH_2CCl_3	77.03	77.08	77.07	77.09

[a] J_{CP} 8 Hz. [b] J_{CP} 5 Hz.

spectra. Preparative HPLC separations were performed on a Waters HPLC system using a NOVA PAK HR C-18 column (60 Å; 25 mm × 100 mm) with a flow rate of 10 cm^3 min^{-1} and detection at 215 and 280 nm with a photodiode array detector (Waters M 991). Solvent system A: 0.1% TFA; B: 0.1% TFA in 90% acetonitrile–10% water. Gel filtrations were performed on Sephadex G-10 (Pharmacia) with a flow rate of 1 cm^3 min^{-1} and detection at 230 nm. Ion-exchange chromatography was performed on DEAE-Sepharose (Pharmacia) with a flow-rate of 1 cm^3 min^{-1} and detection at 230 nm. Elemental analysis were carried out at LEO Pharmaceutical Products, Denmark.

Solid-phase Peptide Synthesis. General Procedure.—Synthesis of the glycopeptides was performed in DMF, using the resin PEGA 1900/130 [poly(ethylene glycol) dimethylacrylamide copolymer]. The resin (0.3 g) was packed into a 10 cm^3 disposable syringe (Discardit II, Beckton Dickinson), fitted with a sintered Teflon filter. The syringe was connected to a 50 cm^3 syringe through a Teflon tube; excess of reagent, DMF *etc.* was removed with the second syringe. The resin was derivatized with the Rink-linker (97 mg, 0.18 mmol), TBTU (58 mg, 0.18 mmol) and *N*-ethylmorpholine (46 mm^3, 0.36 mmol). After 2 h, acetic anhydride (50 mm^3) was added, and after 10 min the resin was washed thoroughly with DMF (15 × 15 cm^3). The solvent was removed from the resin with the waste syringe. This washing procedure was repeated after each coupling/deprotection. N^α-Fmoc-deprotection was then effected by treatment of the resin with 20% piperidine in DMF (3 cm^3) for 50 min. The first amino

Table 4 (*a*) Selected ^{1}H NMR chemical shifts (ppm) measured at 500 MHz for compounds **13** and **15** on solutions in $(CD_3)_2SO$ and for compounds **14** and **16** on solutions in D_2O–CD_3CO_2D (1:1) at 300 K. [Ref. $\delta_H(CD_2H)_2SO$ 2.50, δ_H CD_2HCO_2D 2.03]

	Amino acid	$N^{\alpha}H$	H^{α}	H^{β}	H^{γ}	H^{δ}	H^{ϵ}	$N^{\epsilon}H$
13								
	Thr-1	8.23	4.56	4.23	1.36			
	Lys-2	8.13	4.36	1.74/1.62	1.39	1.54	2.74	7.62
	Thr-3	7.69	4.11	4.01	1.00			
15								
	Thr-1	8.22	4.41	3.92	1.24			
	Lys-2	8.29	4.29	1.71/1.58	1.33	1.53	2.75	7.63
	Thr-3		4.05	4.02	1.01			
14								
	Thr-1		4.51	4.26	1.29			
	Lys-2		4.49	1.88/1.58	1.46	1.72	3.05	
	Thr-3		4.38	4.22	1.21			
16								
	Thr-1		4.48	4.34	1.26			
	Lys-2		4.53	1.90/1.77	1.49	1.74	3.05	
	Thr-3		4.36	4.24	1.21			

(*b*) ^{1}H NMR data for the mannose disaccharides in tripeptides **13**, **14**, **15** and **16**

		1-H	2-H	3-H	4-H	5-H	6-H	6-H′
13								
	Man(1 → 6)	5.36	5.74	5.75	5.83	4.12	4.12	4.12
	Man(1 → Thr)	5.33	5.68	5.77	6.03	4.54	4.12	3.86
15								
	Man(1 → 2)	5.43	5.66	5.78	5.99	4.55	4.55	4.41
	Man(1 → Thr)	5.29	4.12	5.22	5.26	4.05	4.22	4.11
14								
	Man(1 → 6)	4.90	3.97	3.84	3.76	3.82	4.19	4.19
	Man(1 → Thr)	4.895	3.84	3.84	3.80	3.79	3.94	3.74
16								
	Man(1 → 2)	4.99	4.11	3.86	3.82	3.87	4.24	4.20
	Man(1 → Thr)	5.12	3.83	3.93	3.74	3.69	3.85	3.78

acid, N^{α}-Fmoc-Thr(Bu^{t})-ODhbt (122 mg, 0.23 mmol), was dissolved in DMF (1.5 cm^3) and the solution was added to the resin. The suspension was agitated twice and then left for 24 h. N^{α}-Fmoc-deprotection was carried out as described above. After the deprotection an aliquot (15 mg) of the resin was removed, washed, and dried, and the loading of the resin was determined by amino acid analysis. The second amino acid, N^{α}-Fmoc-Lys(Bos)-OPfp (143 mg, 0.23 mmol), and DhbtOH (37 mg, 0.23 mmol) were dissolved in DMF (1.5 cm^3) and the solution was added to the resin. The suspension was agitated twice and then left for 24 h. N^{α}-Fmoc-deprotection was carried out as described above. The last amino acid, which was either the glycosylated building block **11** (333 mg, 0.19 mmol) or its analogue **12** (303 mg, 0.19 mmol), was then dissolved in DMF (1.5 cm^3) and the solution was added to the resin. The suspension was agitated twice and then left for 3 days. The excess of the glycosylated amino acids was recovered by removal with a clean syringe and evaporation, and analysed by HPLC. N^{α}-Fmoc-deprotection was performed as above, and the resin was treated with acetic anhydride–DMF (1:7; 3 cm^3) for 20 min. The resin was then washed successively with DMF (15 × 5 cm^3) and diethyl ether (10 × 5 cm^3) and dried by lyophilization. The resin was removed from the syringe, and cleavage of the glycopeptide from the linker and simultaneous side-chain deprotection was performed by treatment with 95% TFA (20 cm^3) for 2 h, followed by filtration. Acetic acid (3 cm^3) was added to the filtrate and, after concentration under reduced pressure, the residue was solidified by treatment with diethyl ether (2 × 25 cm^3). After decantation and evaporation the glycopeptide was purified by preparative HPLC.

Deprotection of the Glycopeptides. General Procedure.—The purified protected glycopeptide was dissolved in ethanol–water (9:1) (4 mg cm^{-3}) and Pd catalyst (10%) was added. The mixture was stirred under H_2 for 24 h. The mixture was filtered and the solvents were evaporated off. The residue was dissolved in methanol (2 mg cm^{-3}) and a solution of sodium methoxide in methanol (1 mol dm^{-3}) was added until a wetted pH-paper indicated pH 12. The mixture was stirred at this pH for 1.5 h, neutralized with small pieces of solid CO_2, filtered, and lyophilized. The residue was dissolved in water (10 mg cm^{-3}) and purified first by gel filtration, then by ion-exchange chromatography by elution with 0.01 mol dm^{-3} NaCl. The product was finally desalted by gel filtration.

Phenyl 2,3,4-Tri-O-benzoyl-6-O-tert-butyldimethylsilyl-1-thio-α-D-mannopyranoside **2**.—Phenyl 2,3,4,6-tetra-*O*-acetyl-1-thio-α-D-mannopyranoside **1** (13.2 g, 29.9 mmol) was deacetylated using sodium methoxide in methanol. After neutralization (Amberlite IR C-50) and concentration, the resulting syrup was lyophilized. The product (phenyl 1-thio-α-D-mannopyranoside) (8.1 g, 29.7 mmol) and ^{t}BDMSCl (4.6 g, 30.0 mmol) were dissolved in pyridine (24 cm^3) and the solution was stirred at room temperature overnight. Benzoyl chloride (19.4 cm^3, 166.3 mmol) was added, and the mixture was diluted with dichloromethane (20 cm^3) and toluene (25 cm^3). After 24 h the mixture was filtered, diluted with toluene (50 cm^3), and the solvents were evaporated off, the residue was diluted with toluene (50 cm^3), and filtered, and the solvent was evaporated off. The resulting syrup was subjected to VLC on dried silica gel with dry solvents [light petroleum–ethyl acetate (20.1)] to give *title compound* **2** (18.7 g, 90%), $[\alpha]_D^{25}$ −7.6 (*c* 2.1, CH_2Cl_2); ^{1}H and ^{13}C NMR data are presented in Table 1 (Found: C, 66.95; H, 6.15; S, 4.6. $C_{39}H_{42}O_8SSi$ requires C, 67.02; H, 6.06; S, 4.59%; M, 698.90).

Table 5 (*a*) Selected ^{13}C NMR chemical shifts (ppm) measured at 125.77 MHz for compounds **13** and **15** on solutions in $(CD_3)_2SO$ and for compounds **14** and **16** on solutions in D_2O–CD_3CO_2D (1:1) at 300 K. [Ref. $\delta_C(CD_3)_2SO$ 39.50, δ_C CD_3CO_2D 20.0]

Amino acid	C^α	C^β	C^γ	C^δ	C^ϵ
13					
Thr-1	57.20	76.17	18.28		
Lys-2	52.68	31.07	22.15	26.55	38.71
Thr-3	57.94	66.31	19.93		
15					
Thr-1	57.87	76.46	19.89		
Lys-2	52.73	30.76	22.10	26.54	38.69
Thr-3	57.78	66.34	18.16		
14					
Thr-1	59.03	76.56	18.17		
Lys-2	54.01	31.18	22.64	26.84	39.87
Thr-3	58.48	67.71	19.24		
16					
Thr-1	59.10	75.97	18.35		
Lys-2	54.01	31.06	22.56	26.71	39.86
Thr-3	59.26	67.65	19.22		

(*b*) ^{13}C NMR data for the mannose disaccharides in tripeptides **13**, **14**, **15** and **16**

	C-1	C-2	C-3	C-4	C-5	C-6
13						
Man(1 → 6)	97.12	70.64	70.64	65.28	68.50	65.62
Man(1 → Thr)	98.51	70.06	69.81	66.31	68.91	66.31
15						
Man(1 → 2)	98.12	69.91	69.78	65.34	68.27	66.34
Man(1 → Thr)	99.09	75.77	69.78	66.34	68.25	62.06
14						
Man(1 → 6)	101.87	70.60	70.87	67.33	71.94	65.24
Man(1 → Thr)	100.01	71.23	71.94	66.90	71.36	66.32
16						
Man(1 → 2)	103.04	70.55	70.90	66.79	72.42	65.03
Man(1 → Thr)	100.30	79.94	70.61	67.60	73.61	61.45

Phenyl 2,3,4-*Tri*-O-*benzoyl*-1-*thio*-α-D-*mannopyranoside* **3**.—Compound **2** (8.22 g, 11.76 mmol) was dissolved in tetrahydrofuran (6 cm^3), then water (6 cm^3) and acetic acid (18 cm^3) were added. The mixture was stirred at room temperature for 5 days, the solvents were evaporated off, and the resulting syrup was subjected to VLC [light petroleum–ethyl acetate (10:1)] to give *title compound* **3** (6.42 g, 93%), $[\alpha]_D^{25}$ −14.9 (*c* 2.4, CH_2Cl_2). ^1H and ^{13}C NMR data are presented in Table 1 (Found: C, 67.7; H, 5.1; S, 5.2. $C_{33}H_{28}O_8S$ requires C, 67.80; H, 4.83; S, 5.48%; M, 584.64).

Phenyl 2,3,4-*Tri*-O-*benzoyl*-1-*thio*-6-O-*bis*(2,2,2-*trichloroethoxy*)*phosphoryl*-α-D-*mannopyranoside* **4**.—Compound **3** (6.2 g, 10.6 mmol) was dissolved in dichloromethane (10 cm^3) and pyridine (2.15 cm^3, 26.5 mmol) was added. The solution was stirred over molecular sieves (3 Å; 0.5 g) for 30 min, and then cooled to −40 °C. Bis(2,2,2-trichloroethyl) phosphorochloridate (10.07 g, 26.5 mmol) was added, and the mixture was stirred for 1 h at −40 °C. The mixture was kept at −20 °C overnight, and was then filtered, diluted with toluene (20 cm^3), and concentrated. The residue was diluted with toluene (20 cm^3) and concentrated. The resulting syrup was subjected to VLC [light petroleum–ethyl acetate (7:1)] to give *title compound* **4** (9.66 g, 98%), m.p. 105–106 °C; $[\alpha]_D^{25}$ −3.84 (*c* 3.7, CH_2Cl_2). ^1H and ^{13}C NMR data are presented in Table 1 (Found: C, 48.0; H, 3.3; Cl, 22.7; P, 3.3; S, 3.7. $C_{37}H_{31}Cl_6O_{11}PS$ requires C, 47.92; H, 3.37; Cl, 22.94; P, 3.34; S, 3.46%; M, 927.40).

Phenyl 2,3,4-*Tri*-O-*benzoyl*-1-*thio*-6-O-[2,3,4-*tri*-O-*benzoyl*-6-O-*bis*-(2,2,2-*trichloroethoxy*)*phosphoryl*-α-D-*mannopyranosyl*]-α-D-*mannopyranoside* **6**.—To a solution of compound **4** (2.22 g, 2.39 mmol) in dry dichloromethane (5 cm^3) containing molecular sieves (4 Å; 0.5 g) was added a solution of bromine (0.13 cm^3, 2.39 mmol), in dry dichloromethane (2.6 cm^3) in the dark. After 4 h the mixture was filtered, and diluted with toluene (10 cm^3), and the filtrate was concentrated. Dilution with toluene, and concentration, was repeated 4 times. The remaining toluene was removed at 35 °C and 0.1 Pa to give the bromide **5** as a syrup (2.15 g, 100%). ^1H NMR data are presented in Table 1.

The resulting bromide **5** (2.15 g, 2.39 mmol) and compound **3** (0.93 g, 1.59 mmol) were dissolved in dry dichloromethane (7 cm^3) and the solution was stirred under Ar over molecular sieves (3 Å; 0.5 g) at −40 °C for 0.5 h. Silver triflate (0.74 g, 2.89 mmol) was added quickly, and the mixture was stirred at −40 °C for 1.5 h. 2,4,6-Trimethylpyridine (2,4,6-collidine) (0.64 cm^3, 4.78 mmol) was then added, and the temperature was slowly raised to room temperature. Dilution with dry dichloromethane, filtration through Celite, and concentration of the filtrate gave a syrup, which was purified by VLC [light petroleum–ethyl acetate (4:1)]. This afforded pure *disaccharide* **6** (1.78 g, 80%), $[\alpha]_D^{25}$ −27.9 (*c* 2.7, CH_2Cl_2); ^1H and ^{13}C NMR data are presented in Tables 2 and 3, respectively (Found: C, 54.8; H, 4.0; Cl, 15.1; P, 1.9; S, 2.4. $C_{64}H_{53}Cl_6O_{19}PS$ requires C, 54.83; H, 3.81; Cl, 15.17; P. 2.21; S, 2.29%; M, 1401.86).

1,3,4,6-*Tetra*-O-*acetyl*-2-O-[2,3,4-*tri*-O-*benzoyl*-6-O-*bis*-(2,2,2-*trichloroethoxy*)*phosphoryl*-α-D-*mannopyranosyl*]-β-D-*mannopyranose* **9**.—The preparation of the bromide **5** was performed as described above from compound **4** (5.2 g, 5.61 mmol). The resulting syrup (5.04 g, 5.61 mmol) was dissolved in dry dichloromethane (18 cm^3) and stirred under Ar over molecular sieves (3 Å; 0.5 g) at −60 °C for 0.5 h. Silver triflate (1.73 g, 6.73 mmol) and then 1,3,4,6-tetra-*O*-acetyl-β-D-mannopyranose **8** (1.30 g, 3.74 mmol) were added, and the mixture was stirred at −60 °C for 1.25 h. 2,4,6-Collidine (1.5

cm^3, 11.2 mmol) was then added, and the temperature was slowly raised to 20 °C. Dilution with dry dichloromethane, filtration through Celite, and concentration were followed by purification by VLC [light petroleum–ethyl acetate (1.85:1)] which gave *disaccharide* **9** (3.02 g, 69%), $[\alpha]_D^{25}$ −72.8 (*c* 2.5, CH_2Cl_2); ^1H and ^{13}C NMR data are presented in Tables 2 and 3, respectively (Found: C, 46.7; H, 3.8; Cl, 18.55; P, 2.4. $C_{45}H_{45}Cl_6O_{21}P$ requires C, 46.37; H, 3.89; Cl, 18.25; P, 2.66%; M, 1165.53).

N$^\alpha$(*Fluoren-9-ylmethoxycarbonyl*)-O-{2,3,4-*tri*-O-*benzoyl*-6-O-[2,3,4-*tri*-O-*benzoyl*-6-O-*bis*-(2,2,2-*trichloroethoxy*)*phosphoryl*-α-D-*mannopyranosyl*]-α-D-*mannopyranosyl*}-L-*threonine Pentafluorophenyl Ester* **11**.—To a solution of compound **6** (1.34 g, 0.95 mmol) in dichloromethane (5 cm^3) containing molecular sieves (4 Å; 0.5 g) was added a solution of bromine (0.05 cm^3, 0.95 mmol) in dry dichloromethane (1.0 cm^3) in the dark. After 2 h the mixture was filtered, diluted with dry toluene (10 cm^3), and concentrated. Dilution with toluene, and concentration, was repeated 4 times. The remaining toluene was removed at 35 °C and 0.1 Pa to give the bromide **7** as a syrup (1.30 g, 99%). ^1H NMR data are presented in Table 2.

The resulting bromide **7** (1.30 g, 0.95 mmol) and N^α-fluoren-9-ylmethoxycarbonyl)-L-threonine pentafluorophenyl ester **10** (0.48 g, 0.95 mmol) (see below) were dissolved in dry dichloromethane (9 cm^3) and the solution was stirred for 0.5 h under Ar over molecular sieves (3 Å; 0.5 g) at −60 °C. Silver triflate (0.30 g, 1.14 mmol) was added quickly and the mixture was stirred at −60 °C for 1 h. 2,4,6-Collidine (0.25 cm^3, 1.89 mmol) was then added, and the temperature was slowly raised to 20 °C. After dilution with dry dichloromethane and filtration through Celite, the filtrate was extracted three times with a mixture of 10% aq. TFA (aq., 10%) and ice, and once with water. Drying ($MgSO_4$) and concentration were followed by VLC on dried silica gel with dry solvents [light petroleum–ethyl acetate (3:1)], which gave *title compound* **11** (1.26 g, 74%), $[\alpha]_D^{25}$ −46.6 (*c* 1.3, CH_2Cl_2); ^1H and ^{13}C NMR data are presented in Tables 2 and 3, respectively (Found: C, 55.2; H, 3.8; N, 0.8. $C_{83}H_{65}Cl_6F_5NO_{24}P$ requires C, 55.41; H, 3.64; N, 0.78%; M, 1799.10).

N$^\alpha$-(*Fluoren-9-ylmethoxycarbonyl*)-O-{3,4,6-*tri*-O-*acetyl*-2-O-[2,3,4-*tri*-O-*benzoyl*-6-O-*bis*-(2,2,2-*trichloroethoxy*)*phosphoryl*-α-D-*mannopyranosyl*]-α-D-*mannopyranosyl*}-L-*threonine Pentafluorophenyl Ester* **12**.—To a solution of disaccharide **9** (2.67 g, 2.29 mmol) in dry dichloromethane (12 cm^3) was added 4 mol dm^{-3} hydrogen bromide in acetic acid (5.7 cm^3, 22.9 mmol). The solution was stirred at room temperature for 30 min, diluted with dichloromethane, and then poured onto ice. The organic layer was washed successively with cold water, saturated aq. sodium hydrogen carbonate, and water. Drying ($MgSO_4$) and concentration gave the bromide **10** (2.40 g, 88%).^1H NMR data are presented in Table 2.

The resulting bromide **10** (2.33 g, 1.97 mmol) and N^α-(fluoren-9-ylmethoxycarbonyl)-L-threonine pentafluorophenyl ester (1.0 g, 1.97 mmol) were dissolved in dry dichloromethane (13 cm^3) and stirred for 0.5 h under Ar over molecular sieves (3 Å; 0.5 g) at −40 °C. Silver triflate (0.62 g, 2.41 mmol) was added quickly and the mixture was stirred at −40 °C for 1 h. 2,4,6-Collidine (0.54 cm^3, 4.04 mmol) was then added, and the temperature was slowly raised to 20 °C. After dilution with dry dichloromethane, and filtration through Celite, the filtrate was extracted three times with a mixture of 10% aq. TFA and ice, and once with water. Drying ($MgSO_4$) and concentration were followed by VLC on dried silica gel with dry solvents [light petroleum–ethyl acetate (3:1)], which gave *title compound* **12** (2.59 g, 82%), $[\alpha]_D^{25}$ −34.3 (*c* 1.4, CH_2Cl_2); ^1H and ^{13}C NMR data are presented in Tables 2 and 3, respectively (Found: C, 49.7; H, 3.7; N, 0.9. $C_{68}H_{59}Cl_6F_5NO_{24}P$ requires C, 50.64; H, 3.69; N, 0.87%; M, 1612.89).

Ac-Thr[α-D-*Man*-6-P-(1,6)-α-D-*Man*]-*Lys-Thr-NH*$_2$ **14**.—The solid-phase synthesis was carried out according to the general procedure described above. After coupling of the first amino acid, the loading of the resin was found to be 0.143 mmol g^{-1}. A large portion (73%) of the glycosylated building block **11** could be recovered. After cleavage from the resin, compound **13** was purified by preparative HPLC using, first, the linear gradient 50–65% solvent A during 30 min, and then 50–90% solvent B during 80 min (t_R 70 min). The yield of the protected tripeptide **13** was 68 mg (54%). ^1H and ^{13}C NMR data are presented in Tables 4 and 5, respectively. Amino acid analysis (theoretical values in parentheses): Thr 1.97(2) and Lys 1.02(1).

Compound **13** (34 mg, 0.02 mmol) was deprotected according to the general procedure described above, to yield the deprotected peptide **14** (3 mg, 18%). ^1H and ^{13}C NMR data are presented in Tables 4 and 5, respectively; *m*/*z* 793 ($C_{28}H_{52}N_5O_{19}P$ requires M, 793.30); *m*/*z* 839 ($C_{28}H_{50}N_5Na_2O_{19}P$ requires M, 839.28).

Ac-Thr[α-D-*Man*-6-P-(1,2)-α-D-*Man*]-*Lys-Thr-NH*$_2$ **16**.—The solid-phase synthesis was carried out according to the general procedure described above. After coupling of the first amino acid, the loading of the resin was found to be 0.148 mmol g^{-1}. A large portion (73%) of the glycosylated building block **12** could be recovered. After cleavage from the resin, compound **15** was purified by preparative HPLC using the linear gradient 50–90% solvent B during 80 min (t_R 20 min). The yield of the protected tripeptide **15** was 55 mg (76%). ^1H and ^{13}C NMR data are presented in Tables 4 and 5, respectively. Amino acid analysis (theoretical values in parentheses): Thr 2.01(2) and Lys 0.99(1).

Compound **15** (35 mg, 0.02 mmol) was deprotected according to the general procedure described above, to yield the deprotected peptide **16** (7 mg, 36%). ^1H and ^{13}C NMR data are presented in Tables 4 and 5, respectively; *m*/*z* 793 ($C_{28}H_{52}N_5O_{19}P$ requires M, 793.30); *m*/*z* 839 ($C_{28}H_{50}N_5Na_2P$ requires M, 839.28).

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