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SYNTHESIS OF A BRANCHED GLYCOPEPTIDE DERIVATIVE CONTAINING TERMINAL D-MANNOSE 6-PHOSPHATE RESIDUES

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ABSTRACT

A branched glycopeptide derivative incorporating two D-mannose 6-phosphate residues was prepared by coupling 6-aminohexyl 6-*O*-[bis(2,2,2-trichloroethoxy)phosphinyl]-α-D-mannopyranoside with *N*-acetyl-L-tyrosyl-L-aspart-oyldi-L-alanine followed by reductive deprotection of the phosphate group.

INTRODUCTION

D-Mannose 6-phosphate (Man-6-P) is the essential component of a recognition marker for the targeting of lysosomal enzymes[1,2]. Intracellular transport to the lysosomes is mediated by receptors in the Golgi apparatus or on the cell surface that bind Man-6-P residues with high affinity. Man-6-P residues are present at the terminal and/or subterminal positions of asparagine-linked oligosaccharides of the high-mannose type in newly biosynthesized lysosomal enzymes. In the largest of these oligosaccharides (**1**), phosphorylation is known[3,4] to occur on two or more of the mannose residues marked *. Natowicz *et al.*[5] and Kornfeld *et al.*[6] have suggested that the underlying oligosaccharide structures, as well as the Man-6-P residues, play a critical role in the specific recognition of the phosphorylated oligosaccharide by Man-6-P receptors. Model compounds for examining these interactions have been synthesized by Srivastava and Hindsgaul[7–11].

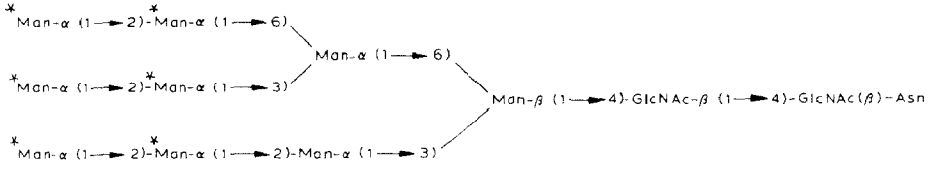
We have studying the structure-binding relationships between sugars and their binding proteins (lectins) by using a variety of synthetic ligands[12]. Some of the ligands we have prepared are doubly or triply branched, and these multivalent compounds have proved to be extremely useful in demonstrating the enhanced binding, caused by multiple bonding possibilities, often seen in animal lectins ("cluster effect")[12]. In the recognition of Man-6-P residue, such a cluster effect also appears to exist. We reasoned that this effect could be probed by a branched ligand containing Man-6-P at its termini. We have therefore synthesized a new ligand, **2**, containing two Man-6-P residues attached to a radio-iodinatable peptide moiety, TyrAsp(Ala)$_2$, *via* a 6-aminohexyl "spacer". Since the only sugar units in this ligand are the terminal groups, the compound will enable us also to assess the role of

underlying oligosaccharide structures for the recognition of Man-6-P receptors. During this synthetic study we also synthesized a 4,6-cyclic phosphate derivative related to **2**.

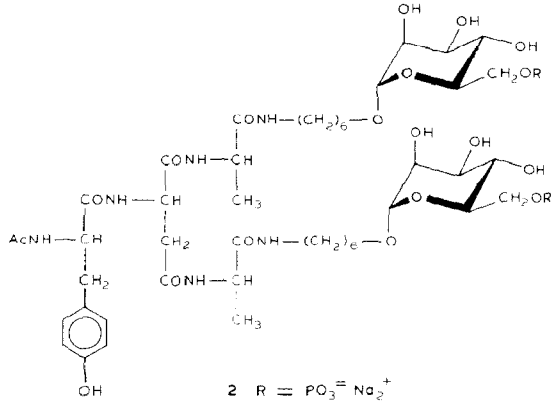
RESULTS AND DISCUSSION

The synthesis of **2** comprised the following steps (Scheme 1): (i) glycosidation of *N*-protected 6-aminohexanol with D-mannose pentaacetate (**5**), (ii) *O*-deacetylation, (iii) regioselective 6-*O*-phosphorylation of the *O*-deprotected mannoside (**7**), (iv) *N*-deprotection of the resulting **8**, (v) coupling of the 6-aminohexyl mannoside (**9**) with the peptide (**13**), and (vi) phosphate deprotection. In this strategy care is required in the choice of protective groups for the amino and phosphate functions because the *N*-protecting group has to be removed without affecting the phosphate moiety, and a protective group on the phosphate function should not only be stable during *N*-deprotection and coupling to the peptide but also easily removable at the final stage. We first chose the benzyloxycarbonyl group for *N*-protection, as it can be removed under specific hydrogenolysis conditions (Pd–C), and the phenyl group, which can be removed under another set of hydrogenolysis conditions (PtO_2), for phosphate protection. We also chose not to protect the 2-, 3-, and 4-hydroxyl groups of the sugar in order to reduce the number of protection/deprotection steps.

6-(Benzyloxycarbonylamino)hexanol[13] reacted with α-D-mannopyranose pentaacetate (**5**) in the presence of tin(IV) chloride[14] in dichloromethane to give a

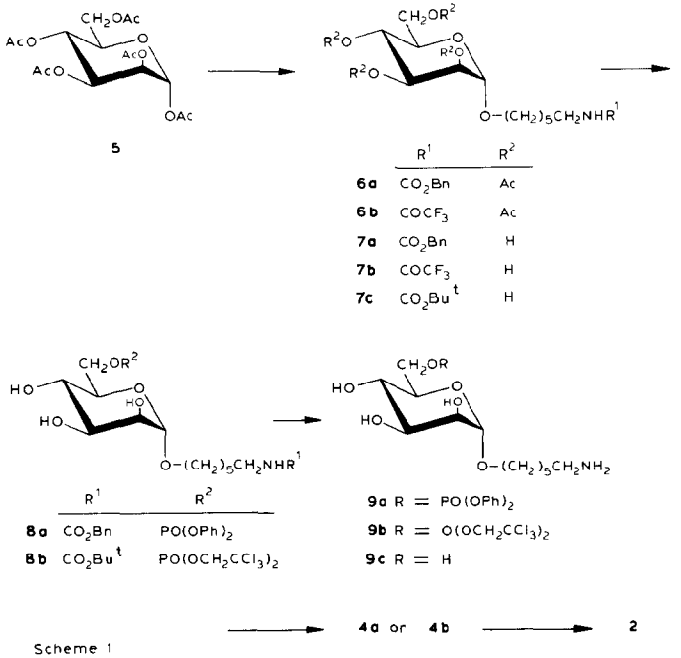


† (* indicates residue that may be phosphorylated)



2 R = $PO_3^{=}Na_2^{+}$
3 R = H
4a R = $PO(OPh)_2$
4b R = $PO(OCH_2CCl_3)_2$

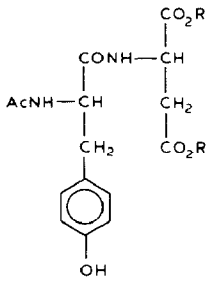
6-(Benzyloxycarbonylamino)hexanol[13] reacted with α-D-mannopyranose pentaacetate (**5**) in the presence of tin(IV) chloride[14] in dichloromethane to give a 52% yield of mannoside **6a** as a single product after column chromatography on Sephadex LH-20. Removal of the *O*-acetyl groups of **6a** gave **7a**, which was treated[15] with two molar equivalents of diphenyl phosphorochloridate in pyridine at 0° to afford an 83% yield of the 6-*O*-diphenoxyphosphinyl mannoside **8a**. In the ^{1}H-n.m.r. spectrum of **8a** the signals for H-6a and -6b appeared as broad triplets shifted down to δ 4.43 and 4.58 p.p.m., respectively, in contrast to those of the nonphosphorylated mannosides such as **7b**, which were found at δ 3.68–3.82 and 3.87. The *N*-benzyloxycarbonyl group of **8a** was removed by hydrogenolysis over 10% Pd–C without affecting the diphenoxyphosphinyl moiety.



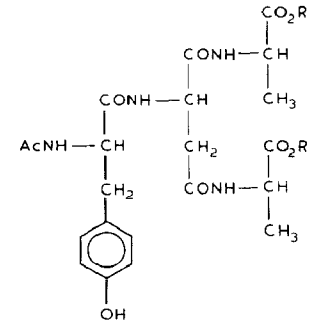
Scheme 1

Peptide **13** was prepared as follows. *N*-Acetyl-L-tyrosine was coupled with L-aspartic acid dibenzyl ester in the presence of 2-ethoxy-*N*-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) in absolute ethanol to give **10** in 63% yield. Removal of the benzyl groups by hydrogenolysis yielded **11**, which was condensed with L-alanine benzyl ester in the presence of 1-hydroxybenzotriazole (HOBT) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDAC) in *N,N*-dimethylformamide (DMF). Hydrogenolysis of the product afforded **13**.

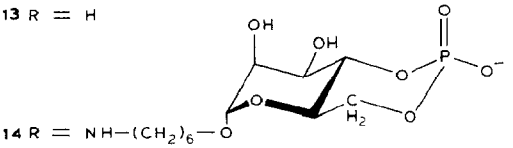
To investigate the coupling of 6-aminohexyl mannosides with peptide **13**, **9c** was used as a model compound. We tested several peptide-activating reagents,



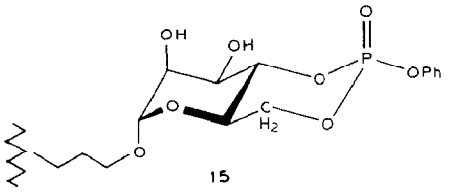
10 R = Bn
11 R = H



12 R = Bn
13 R = H



14 R = NH—$(CH_2)_6$—O



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such as (i) methyl chloroformate, (ii) *N*-hydroxysuccinimide and EDAC, and (iii) HOBT and EDAC. The mixed anhydride method using methyl chloroformate was troublesome because of the low temperature (−70°) required[12b], and it gave a low yield. The employment of *N*-hydroxysuccinimide and EDAC caused some racemization (10%) as judged by the ^{1}H-n.m.r. spectrum of the product. However, the coupling reaction proceeded smoothly in the presence of HOBT and EDAC in DMF at room temperature and provided a 63% yield of **3** without racemization. For the purification of the product, Sephadex G-25 or LH-20 column chromatography was effective, with the coupling product eluting first, free of contamination. 6-Aminohexyl 6-*O*-(diphenoxyphosphinyl)-α-D-mannopyranoside, **9a**, was successfully condensed with peptide **13** in the presence of HOBT and EDAC in DMF to give **4a** in 93% yield after Sephadex LH-20 chromatography.

For phosphate deprotection, catalytic hydrogenolysis was first attempted. The 6-*O*-(diphenoxyphosphinyl)mannoside derivative **4a** contains a tyrosine residue which tends to be reduced by hydrogenation over PtO_2 in acidic media[16]. Therefore, hydrogenation was attempted for one day in a neutral solvent (ethanol–water), but deprotection was only partial, as shown by the ^{1}H-n.m.r. spectrum in which two phenyl groups were evident. Complete reaction was accomplished by prolonging the hydrogenation time to four days, or employing aqueous acetic acid

as the solvent, but the absence of any aromatic proton signals in the spectrum of the product showed that the tyrosine had been saturated.

Upon treatment with aqueous triethylamine[17] **4a** gave as the only identifiable product, in 29% yield, a substance containing an intact tyrosyl moiety but no other phenyl group as judged by its ^{1}H-n.m.r. spectrum, and having two phosphate groups per molecule by phosphate analysis[18]. However, a treatment of the product with alkaline phosphatase *(E. coli)*, known to cleave phosphomonoester linkages[19], liberated no inorganic phosphate. The ^{31}P-n.m.r. spectrum of the compound showed a single peak at −1.99 p.p.m. All these data suggested that the product had phosphodiester groups. On careful analysis of the ^{1}H-n.m.r. spectrum the signals for H-4, -6a, and -6b were seen at much lower field than in the spectrum of the nonphosphorylated congener **3** (Table I), and these signals appeared to show coupling with the phosphorous atom. In the ^{13}C-n.m.r. spectrum, the signals for

TABLE I

SELECTED N.M.R. DATA FOR LIGANDS **2**, **3**, AND **14**[a]

Nucleus[b]	*Chemical shift in p.p.m. (J in Hz)*		
	2	**3**	**14**
H-1,1′ ($J_{1,2}$)	4.85 (1.57)	4.80 (1.76)	4.84 (0.97)
H-2,2′ ($J_{2,3}$)	3.92 (3.27)	3.87 (3.44)	3.93–3.95 (m)
H-3,3′ ($J_{3,4}$)	3.79 (9.06)	3.73 (9.04)	3.92 (10.01)
H-4,4′ ($J_{4,5}$)	3.83 (9.96)	3.60 (9.04)	4.19 (9.79)
($J_{4,P}$)			(9.79)
H-5	3.66–3.76 (m)	3.55–3.61 (m)	3.83–3.88 (m)
H-6a,6′a ($J_{5,6a}$)	4.10 (3.81)	3.83 (2.15)	4.08 (2.01)
($J_{6a,6b}$)	(11.26)	(12.01)	(10.50)
($J_{6a,P}$)	(6.29)		(10.50)
H-6b,6′b ($J_{5,6b}$)	4.02 (2.12)	3.71 (5.60)	4.12–4.17 (m)
($J_{6b,P}$)	(5.28)		[c]
CH_3CONH	1.99	1.94	1.94
α-H of Tyr	4.47 (7.72)	4.41 (7.81)	4.41 (7.92)
α-H of Asp	4.58	4.52	4.53
	(6.53, 7.33)	(6.06, 8.21)	(5.88, 8.03)
α-H of Ala	4.21 (7.25)	4.15 (7.17)	4.09–4.18 (m)
C-1	102.56	102.11	103.09
C-1′	102.70	102.29	103.40
C-2,2′	73.09	72.62[d]	71.26
C-3,3′	73.09	73.21[d]	71.26
C-4,4′	68.84	69.33	72.64[e]
C-5,5′	74.92	75.26	78.48
C-6,6′	65.46[e]	63.46	69.26[e]
OCH_2	70.65	70.34	70.79
P,P′	+4.25		−1.99

[a]Experimental conditions and reference standards are described in the Experimental section. [b]Locants 1–6 and 1′–6′ refer to the mannose residues. [c]Not determined due to spectral overlap. [d]Interchangeable. [e]Phosphorus-carbon coupling was not observed due to peak broadening.

both C-4 and C-6 were moved to lower field. Thus, the product was judged to be a 4,6-cyclic phosphate derivative, **14**. For the formation of this 4,6-cyclic phosphate from **4a** the following mechanism can be suggested. Triethylamine catalyzed the cyclization of the 6-*O*-(diphenoxyphosphinyl) group onto the 4-hydroxy group[20] to give the 4,6-cyclic phosphotriester derivative **15**, which is then hydrolyzed under the basic conditions to produce **14**.

As a phosphate-protecting group we next employed the 2,2,2-trichloroethyl group[21], which is removable by reductive elimination with zinc. This necessitated a change to the *tert*-butoxycarbonyl (BOC) group as an *N*-protective group because the 2,2,2-trichloroethyl group is susceptible to the hydrogenolysis conditions[22] used for the cleavage of benzyloxycarbonyl groups. 6-(*tert*-Butoxycarbonylamino)hexyl α-D-mannopyranoside (**7c**) was prepared from the corresponding 6-(trifluoroacetylamino)hexyl α-D-mannopyranoside[23] (**7b**). This in turn was obtained by removal of the *O*-acetyl groups from **6b** with methanolic sodium methoxide. On cleavage of the *N*-trifluoroacetyl group (with aqueous triethylamine) from **7b**, the resulting 6-aminohexyl α-D-mannopyranoside **9c** smoothly reacted with 2-(*tert*-butoxycarbonyloxyimino)-2-phenylacetonitrile[24] (BOC-ON) in the presence of triethylamine to give **7c** in 99% yield.

Phosphorylation of **7c** with bis(2,2,2-trichloroethyl) phosphorochloridate furnished the 6-*O*-[bis(2,2,2-trichloroethoxy)phosphinyl] α-D-mannopyranoside **8b** in 55% yield after silica gel column chromatography. The product gave satisfactory elemental analytical data and a reasonable ^{1}H-n.m.r. spectrum. Removal of the BOC group from **8b** by treatment with 60% trifluoroacetic acid gave **9b**, which was coupled with peptide **13** under the same conditions as for the preparation of **4a** to afford a 28% yield of **4b**. For phosphate deprotection, **4b** was treated with zinc[25] in 90% pyridine in the presence of 2,4-pentanedione for 50 min at 100°, then zinc cation was removed with Chelex resin (NH_4^+ form)[21]. The product was successively purified by silica gel and Bio-Gel P-2 column chromatography to give **2** in 36% yield. The structure was confirmed by its ^{1}H- and ^{13}C-n.m.r. data, which are summarized in Table I. Particularly noteworthy are the resonances due to C-6, which are shifted to lower field than those of **3**; however, $J_{C,P}$ coupling was not observed because of peak broadening. Treatment of **2** with alkaline phosphatase *(E. coli)* produced inorganic phosphate, indicating the presence of phosphomonoester. Also, the chemical shift of the phosphorus atoms of **2** is +4.25 p.p.m., which supports the phosphomonoester structure.

In conclusion, synthesis of a divalent ligand having two D-mannose 6-phosphate residues has been achieved. The results of tests of the binding activity of **2** will be reported elsewhere.

EXPERIMENTAL

General methods. — Melting points were determined with a Fisher–Johns melting point apparatus and are uncorrected. Elemental analyses were performed

by Galbraith Laboratories Inc. (Knoxville, TN). Unless otherwise specified, reactions were carried out at room temperature (22–24°). Solutions were evaporated under diminished pressure; and organic layers from extractions were dried over anhydrous sodium sulfate. Chromatography was performed on columns of Silica Gel 60 (15–40 μm; E. Merck). Thin-layer chromatography was conducted on Silica Gel 60 F_{254} (precoated plates; layer thickness, 0.25 mm; E. Merck). Gel filtration was performed on columns of Sephadex LH-20 or G-25 (Pharmacia) or Bio-Gel P-2 (Bio-Rad). Chelex 100 Resin (Bio-Rad) was successively washed with 0.1M ammonium bicarbonate and water before use. Nuclear magnetic resonance spectra were recorded with a Varian XL-400 or Brucker WH 360 spectrometer, for solutions in $CDCl_3$ otherwise specified. ^{1}H-Chemical shifts are expressed in p.p.m. relative to tetramethylsilane, by internal reference in $CDCl_3$, CD_3OD, and Me_2SO-d_6, or measured from *H*OD (δ 4.778 at 24°) in D_2O. ^{13}C-Chemical shifts, also referenced to Me_4Si, were measured from external 1,4-dioxane (δ 67.4). ^{31}P-Chemical shifts are referenced to external 85% H_3PO_4 in D_2O.

*6-(Benzyloxycarbonylamino)hexyl 2,3,4,6-tetra-*O*-acetyl-α-*D*-mannopyranoside* (**6a**). — Tin(IV) chloride (3.34 g, 12.8 mmol; 1.5 mL) was added dropwise over 20 min to a solution of 1,2,3,4,6-penta-*O*-acetyl-α-D-mannopyranose (**5**, Sigma) (4.55 g, 11.9 mmol) and 6-(benzyloxycarbonylamino)hexanol[13] (2.0 g, 7.96 mmol) in dichloromethane (100 mL), and the mixture was stirred for 10 h. It was then poured into ice-cold aqueous sodium hydrogencarbonate with vigorous stirring, and the resulting precipitate was collected on a Celite bed. The filtrate was thoroughly extracted with chloroform, and the combined extracts, without washing with water, were dried and concentrated. The residual syrup was chromatographed on Sephadex LH-20 (5 × 190 cm), equilibrated and eluted with 95% ethanol, to give **6a** as a syrup (2.40 g, 52%); ^{1}H-n.m.r.: δ 2.008, 2.060, 2.116, 2.172 (4 s, 12 H, CH_3CO), 3.18–3.24 (m, 2 H, CH_2NHZ), 3.42–3.49 (m, 1 H, OCH_2), 3.66–3.72 (m, 1 H, OCH_2), 3.98 (ddd, 1 H, *J* 2.06, 5.28, 10.25 Hz, H-5), 4.13 (dd, 1 H, *J* 2.06, 12.03 Hz, H-6a), 4.30 (dd, 1 H, *J* 5.28, 12.03 Hz, H-6b), 4.812 (s, 1 H, H-1), 5.110 (s, 2 H, $PhCH_2$), 5.24 (br. d, 1 H, *J* 3.61 Hz, H-2), 5.29 (t, 1 H, *J* 10.25 Hz, H-4), and 5.36 (dd, 1 H, *J* 3.56, 10.25 Hz, H-3).

Anal. Calc. for $C_{28}H_{39}NO_{12}$: C, 57.82; H, 6.76; N, 2.41. Found: C, 57.70; H, 6.88; N, 2.31.

*6-(Trifluoroacetylamino)hexyl α-*D*-mannopyranoside* (**7b**). — As for the preparation of **6a**, **5** (8.24 g, 21.1 mmol) and 6-(trifluoroacetylamino)hexanol[13] (3.00 g, 14.1 mmol) were treated with tin(IV) chloride (8.90 g, 34.2 mmol; 4.0 mL) in dichloromethane (100 mL) to give **6b** (5.99 g, 78%); m.p. 52–54° (ethanol–hexane).

Compound **6b** (8.17 g, 15.0 mmol) was treated with methanolic sodium methoxide (0.1M, 3 mL) in methanol (100 mL) for 3 h. The solution was neutralized with Dowex 50W-X8 (H^+) resin, the resin was filtered off, and the filtrate was concentrated. The residue was chromatographed on silica gel, with 65:25:5 chloroform–ethyl acetate–methanol as eluant, to give **7b** (4.5 g, 92%); m.p. 105–106° (methanol–hexane); ^{1}H-n.m.r. (D_2O): δ 1.30–1.48, 1.53–1.70 (2 m, 4 H, CH_2 of

6-aminohexyl group), 3.26–3.36 (2 H, $CH_2NHCOCF_3$), 3.51–3.58 (m, 1 H, OCH_2), 3.60–3.82 (m, 5 H, 1 H of OCH_2, H-3,4,5,6a), 3.87 (dd, 1 H, *J* 1.60, 11.93 Hz, H-6b), 3.92 (dd, 1 H, *J* 1.66, 3.33 Hz, H-2), and 4.85 (d, 1 H, *J* 1.66 Hz, H-1).

Anal. Calc. for $C_{14}H_{23}F_3NO_{17}$: C, 44.81; H, 6.18; N, 3.73. Found: C, 44.84; H, 6.19; N, 3.69.

6-Aminohexyl α-D-mannopyranoside (**9c**). — Compound **7b** (250 mg, 0.67 mmol) was treated with triethylamine (1.5 mL) and water (8.5 mL) for one d, and the mixture was concentrated. The product showed a single spot on t.l.c. (R_F 0.32 in 3:2:1 ethyl acetate–acetic acid–water), which was ninhydrin-positive. The residue was chromatographed on Sephadex G-25 (2 × 140 cm), equilibrated and eluted with 0.1M acetic acid, to give a syrup (166 mg, 90%). If necessary, the compound could be treated with Dowex 1 (OH^-) resin to remove acetic acid, which sometimes formed a salt with the amino group.

*6-(*tert*-Butoxycarbonylamino)hexyl α-D-mannopyranoside* (**7c**). — A solution of BOC-ON[24] (1.44 g, 5.82 mmol) in 1,4-dioxane (5 mL) was added dropwise over 5 min to a solution of **9c** (740 mg, 2.65 mmol) and triethylamine (402 mg, 3.96 mmol; 0.54 mL) in water (10 mL), and the mixture was stirred for 3 h. The mixture was concentrated and the residual syrup was chromatographed on silica gel, with 9:1 chloroform–methanol containing 0.05% triethylamine as eluant, to give **7c** as a syrup (990 mg, 99%); ^{1}H-n.m.r. (CD_3OD): δ 1.43 [s, 9 H, $C(CH_3)_3$], 3.00–3.05 (2 H, CH_2NH), 3.78 (dd, 1 H, *J* 1.44, 3.20 Hz, H-2), 3.82 (dd, 1 H, *J* 3.20, 11.68 Hz, H-3), and 4.73 (d, 1 H, *J* 1.44 Hz, H-1).

6-(Benzyloxycarbonylamino)hexyl 6-O-(diphenoxyphosphinyl)-α-D-mannopyranoside (**8a**). — To a solution of **6a** (2.25 g, 3.87 mmol) in methanol (80 mL) was added methanolic sodium methoxide (0.1M, 1.0 mL) and the mixture was stirred for 1 h. After neutralization with Dowex 50W-X8 (H^+) resin (10 mL), the suspension was filtered and the filtrate was concentrated, then co-evaporated with pyridine twice. To a cooled solution of the residue in pyridine (80 mL) was added dropwise diphenyl phosphorochloridate (1.15 g, 5.80 mmol; 1.2 mL) at 0–5°, and the mixture was stirred for 15 min with cooling. Water (2 mL) was added to the reaction mixture and the mixture was stirred for 10 min with cooling, then concentrated. The residual syrup was chromatographed on silica gel, with 70:30:2 chloroform–ethyl acetate–methanol as eluant, to give **8a** as a syrup (2.08 g, 83%); ^{1}H-n.m.r.: δ 1.18–1.63 (m, 8 H, CH_2 of aglycon), 3.08–3.12 (m, 2 H, CH_2NHZ), 3.30–3.35 (m, 1 H, OCH_2), 3.53–3.62 (m, 1 H, OCH_2), 4.43 (br. t, 1 H, *J* 10.50 Hz, H-6a), 4.58 (br. dt, *J* 2.24, 9.81 Hz, H-6b), 4.76 (br. s, 1 H, H-1), and 5.09 (s, 2 H, $PhCH_2$).

Anal. Calc. for $C_{32}H_{40}NO_{11}\cdot 0.3\ H_2O$: C, 59.04; H, 6.29; N, 2.15; P, 4.76. Found: C, 59.10; H, 6.52; N, 2.13; P, 5.00.

*6-(*tert*-Butoxycarbonylamino)hexyl 6-O-[bis(2,2,2-trichloroethoxy)phosphinyl]-α-D-mannopyranoside* (**8b**). — Bis(2,2,2-trichloroethyl) phosphorochloridate (1.3 g, 3.43 mmol) was added to a cold solution of **7c** (651 mg, 1.72 mmol) in pyridine (30 mL), with cooling, and the mixture was stirred for 1 h at 0–5°. Water

(2 mL) was added to the mixture and the mixture was stirred for 5 min, then concentrated. The residue was chromatographed on silica gel, with 500:300:15 chloroform–ethyl acetate–methanol as eluant, to give **8b** as a syrup (680 mg, 55%); ^{1}H-n.m.r.: δ 1.44 [s, 9 H, C(CH_3)$_3$], 3.05–3.15 (2 H, CH_2NH), 3.43 (ddd, 1 H, *J* 6.01, 6.01, 9.74 Hz, OCH_2), 4.47 (br. t, 1 H, *J* 8.19 Hz, H-6a), 4.58 (br. t, 1 H, *J* 12.00 Hz, H-6b), 4.65–4.70 (m, 4 H, 2 OCH_2CCl$_3$), and 4.84 (br. s, 1 H, H-1).

Anal. Calc. for $C_{21}H_{36}Cl_6NO_{11}P$: C, 34.93; H, 5.02; N, 1.94; P, 4.29. Found: C, 34.84; H, 5.15; N, 1.91; P, 4.46.

N-*Acetyl*-L-*tyrosyl*-L-*aspartoyldi*-L-*alanine* (**13**). — To a solution of *N*-acetyl-L-tyrosine (2.23 g, 10.0 mmol) and dibenzyl L-aspartate *p*-toluenesulfonate (6.31 g, 13 mmol) were added triethylamine (2 mL, 14 mmol) and EEDQ (3.21 g, 13 mmol), and the mixture was stirred for one day. The mixture was evaporated to dryness and the resulting solid was dissolved in chloroform and water, then the aqueous layer was extracted with chloroform. The combined extracts were successively washed with ice-cold dilute hydrochloric acid, aqueous sodium hydrogencarbonate, and water, dried, and concentrated. The compound was purified by recrystallization from hot 95% ethanol to give dibenzyl *N*-acetyl-L-tyrosyl-L-aspartate (**10**) (3.26 g, 63%); m.p. 177–178°.

The dibenzyl ester **10** (1.56 g, 3.0 mmol) was hydrogenated over 10% Pd–C (170 mg) in absolute ethanol (50 mL) and 60% acetic acid (20 mL). After the hydrogen uptake ceased, the reaction suspension was filtered and the filtrate was concentrated to give solid *N*-acetyl-L-tyrosyl-L-aspartic acid (**11**) (1.0 g) almost quantitatively.

EDAC (906 mg, 4.72 mmol) was added to a cooled solution of **11** (456 mg, 1.35 mmol), L-alanine benzyl ester *p*-toluene sulfonate (1.42 g, 4.05 mmol), and HOBT (547 mg, 4.05 mmol) at 0–5°, and the mixture was stirred for 10 h. The mixture was concentrated and the residue was chromatographed on silica gel, with 4:6 chloroform–ethyl acetate as eluant, to give the dialanine dibenzyl ester **12** (594 mg, 67%), m.p. 232–234° (from ethanol–ethyl acetate).

Compound **12** (300 mg, 0.45 mmol) was hydrogenolyzed over 10% Pd–C (60 mg) in 95% ethanol (10 mL) and 60% acetic acid (5 mL) for 4 h. The catalyst was filtered off and the filtrate was evaporated to give solid **13** (196 mg, 91%); ^{1}H-n.m.r. (Me_2SO-d_6): δ 1.24, 1.25 (2 d, 6 H, *J* 7.38 Hz, CH_3 of Ala), 1.744 (s, 3 H, CH_3CO), 2.46–2.62 (m, 3 H, β-CH_2 of Tyr, 1 of β-CH_2 of Asp), 2.88 (dd, 1 H, *J* 4.13, 14.39 Hz, β-CH_2 of Asp), 4.12–4.26 (m, 2 H, α-C*H* of Ala), 4.32–4.42 (m, 1 H, α-C*H* of Tyr), 4.52–4.58 (m, 1 H, α-C*H* of Asp), 6.62 (d, 2 H, *J* 8.20 Hz, Ph-H of Tyr), 7.02 (d, 2 H, *J* 9.07 Hz, Ph-H of Tyr), 7.94 (d, 1 H, *J* 7.44 Hz, N*H*), 8.05 (d, 1 H, *J* 8.42 Hz, N*H*), and 8.16–8.17 (2 H, N*H*).

N-*Acetyl*-L-*tyrosyl*-L-*aspartoylbis*[N-[6-[[6-O-*(diphenoxyphosphinyl)*-α-D-*mannopyranosyl*]*oxy*]*hexyl*]-L-*alaninamide*] (**4a**). — Compound **8a** (300 mg, 0.46 mmol) was hydrogenolyzed over 10% Pd–C (60 mg) in 95% ethanol (5 mL) and 60% acetic acid (1 mL). After the hydrogen uptake ceased, the mixture was filtered, and the filtrate was concentrated. The product (**9a**) was u.v.-positive and

ninhydrin-positive on t.l.c. (R_F 0.33 in 8:2:1 ethyl acetate–acetic acid–water) and was employed for the next step without further purification. EDAC (74 mg, 0.38 mmol) was added to a cooled solution of **9a**, peptide **13** (70 mg, 0.15 mmol), and HOBT (39 mg, 0.35 mmol) in DMF (5 mL) at −10°, and the mixture was stirred for 10 h. It was then concentrated and the residue was chromatographed on Sephadex LH-20 (2 × 180 cm), equilibrated and eluted with 95% ethanol, to give **4a** as a syrup (210 mg, 93%); ^{1}H-n.m.r. (CD_3OD): δ 1.924 (s, 3 H, CH_3CO), 4.72 (d, 2 H, J 1.20 Hz, H-1,1′ of Man), 6.69 (d, 2 H, J 8.77 Hz, Ph-H of Tyr), 7.05 (d, 2 H, J 8.75 Hz, Ph-H of Tyr), and 7.21 (m, 20 H, C_6H_5OP).

Anal. Calc. for $C_{69}H_{92}N_6O_{25}P_2 \cdot 7\ H_2O$: C, 52.00; H, 6.70; N, 5.27. Found: C, 51.84; H, 6.34; N, 5.39.

N-*Acetyl*-L-*tyrosyl*-L-*aspartoylbis*[N-[*6*-[[*6*-O-[*bis(2,2,2-trichloroethoxy)phosphinyl*]-α-D-*mannopyranosyl*]*oxy*]*hexyl*]-L-*alaninamide*] (**4b**). — Twenty mL of 90% trifluoroacetic acid was added to a cooled solution of **8b** (680 mg, 0.94 mmol) in 95% ethanol (10 mL) at 0–5°, and the mixture was stirred for 1 h with cooling. The mixture was concentrated, then subjected to azeotropic evaporation with toluene twice. The product showed a single, ninhydrin-positive spot on t.l.c., (R_F 0.35 in 8:2:1 ethyl acetate–acetic acid–water).

EDAC (201 mg, 1.05 mmol) was added to a cooled solution of the above residue, peptide **13** (210 mg, 0.44 mmol), and HOBT (130 mg, 0.94 mmol) at 0–5°, and the mixture was stirred for 10 h. After this it was concentrated and the residue was chromatographed on Sephadex LH-20 (2 × 180 cm), equilibrated and eluted with 95% ethanol, to give **4b** as a syrup (186 mg, 26%); ^{1}H-n.m.r. (CD_3OD): δ 1.938 (s, 3 H, CH_3CO), 4.21–4.31 (m, 1 H), 4.40–4.50 (m, 2 H, H-6a of Man, α-C*H* of peptide), 4.50–4.54 (m, 2 H, H-6b of Man, α-C*H* of peptide), 4.56–4.60 (m, 1 H, α-C*H* of peptide), 4.76–4.78 (8 H, Cl_3CCH_2), 4.81 (d, 2 H, J 1.65 Hz, H-1,1′ of Man), 6.71 (d, 2 H, J 7.05 Hz, Ph-H of Tyr), and 7.06 (d, 2 H, J 7.10 Hz, Ph-H).

Anal. Calc. for $C_{53}H_{80}Cl_{12}N_6O_{25}P_2 \cdot 8\ H_2O$: C, 34.73; H, 5.28. Found: C, 34.81; H, 5.18.

N-*Acetyl*-L-*tyrosyl*-L-*aspartoylbis*[N-[*6*-[*(4,6*-O-*phosphinato*-α-D-*mannopyranosyl*]*oxy)hexyl*]-L-*alaninamide*] *disodium salt* (**14**). — Triethylamine (2 mL) was added to a solution of **4a** (100 mg, 0.07 mmol) in 95% ethanol (6 mL) and water (8 mL) and the mixture was stirred for 2 days. During this time more triethylamine (1 mL) was added. The mixture was concentrated and the residue was chromatographed on silica gel, with 2:2:1 ethyl acetate–2-propanol–water as eluant, to give a major product (R_F 0.28 in 2:2:1 ethyl acetate–2-propanol–water). This was passed through a column of Bio-Gel P-2 (ref. 7) (2 × 50 cm), equilibrated and eluted with 10% ethanol, to give a pure fraction, which was concentrated. The residue was dissolved in 10% ethanol and passed through a column of Dowex 50W-X8 (Na^+) resin[7] (1 × 6 cm), equilibrated and eluted with 10% ethanol, and the eluate was concentrated to dryness. The residue was dissolved in water and freeze-dried to give **14** as a white powder (24 mg, 29%).

N-*Acetyl*-L-*tyrosyl*-L-*aspartoylbis*[N-[*6*-[*(6*-O-*phosphonato*-α-D-*mannopyra*-

nosyl]oxy)hexyl]-L-*alaninamide] tetrasodium salt* (**2**). — A suspension of **4b** (160 mg, 0.098 mmol), 2,4-pentanedione (977 mg, 9.75 mmol; 1.0 mL), and zinc (638 mg, 9.75 mmol) in a mixture of pyridine (13 mL) and water (2 mL) was heated for 50 min at 100° with vigorous stirring. After cooling, Chelex 100 (NH_4^+) resin[21] (20 mL) was added and the mixture was stirred for 30 min. The resin was filtered off and the filtrate was concentrated. The residue was chromatographed on silica gel, with 2:2:1.5 ethyl acetate–2-propanol–water as eluant, and the major product was passed through a column of Bio-Gel P-2 (2 × 50 cm), equilibrated and eluted with 10% ethanol, to give a pure fraction. This was concentrated and the residue was passed through a column of Dowex 50W-X8 (Na^+) resin (1 × 8 cm), equilibrated and eluted with 10% ethanol, providing pure material that was dissolved in water and freeze-dried to give **2** as a white powder (43.5 mg, 36%).

Anal. Calc. for $C_{45}H_{72}N_6Na_4O_{25}P_2 \cdot 1.5\ H_2O$: C, 42.29; H, 5.92; N, 6.58. Found: C, 42.34; H, 6.29; N, 6.26.

N-*Acetyl*-L-*tyrosyl*-L-*aspartoylbis*[N-[*6-(α*-D-*mannopyranosyl]oxy)hexyl]*-L-*alaninamide*] (**3**). — EDAC (149 mg, 0.78 mmol) was added to a solution of **9c** (166 mg, 0.60 mmol), **13** (130 mg, 0.27 mmol), and HOBT (88 mg, 0.65 mmol) in DMF (5 mL) at −10°, and the mixture was stirred for one day. The mixture was concentrated and the residual syrup was chromatographed on Sephadex G-25 (2 × 140 cm), equilibrated and eluted with 0.1M acetic acid, furnishing pure material that was dissolved in water and freeze-dried to give **3** (122 mg, 63%).

Anal. Calc. for $C_{45}H_{74}N_6O_{19} \cdot 2\ H_2O$: C, 52.02; H, 7.57; N, 8.09. Found: C, 51.97; H, 7.56; N, 8.06.

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