110. Synthesis and Anaphylactogenicity of Monohaptenic Carbohydrate Conjugates

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Summary

Peptidic model conjugates carrying a single 2-carboxy-4,6-dinitrophenyl haptenic group, and as carbohydrate moieties D-gluconoyl, β-D-mannopyranosyl, 2-deoxy-β-D-glucopyranos-2-yl, or lactobionoyl residues, including the pseudo-carbohydrate residue 1,3,4,5-tetrahydroxycyclohexane-1-carbonyl, were synthesized. Conjugates carrying the lactobionoyl of the bis(2-deoxy-β-D-glucopyranos-2-yl) moiety were anaphylactogenic in the guinea pig, passively sensitized against 2-carboxy-4,6-dinitrophenyl antigen.

According to earlier work [1], penicilloylated oligosaccharide and polysaccharide conjugates retain their capacity to elicit penicilloyl-specific anaphylactic reactions even when the average degree of substitution falls considerably below one penicilloyl haptenic group per carbohydrate molecule. The findings were later extended [2], and it became clear that monopenicilloylated raffinose, maltotriose, dextran and inulin molecules (but not glucose) are quite generally to be regarded as anaphylactogens with the carbohydrate moiety displaying some kind of auxiliary function. Since the penicilloyl-carbohydrate conjugates are not stable and get split at neutral pH into penicilloic acid and carbohydrate [3] [4], further study with this class of compounds appeared difficult. Furthermore, the preparations were presumably mixtures of conjugates differing in the location of haptenic group attachment and thus lacked full definition.

We therefore set out to prepare a series of defined and stable monohaptenic model conjugates involving monosaccharide and disaccharide derivatives for further evaluation of the anaphylactogenic capacities of monohaptenic carbohydrates. As haptenic group, the 2-carboxy-4,6-dinitrophenyl (Dncp) residue [5] was selected and β-alanylglutamic acid, 1,6-hexanediamine and 6-aminohexanoic acid were used as bridges between haptenic group and carbohydrate moiety *(Table)*. This communication reports details on the synthesis of the conjugates and some

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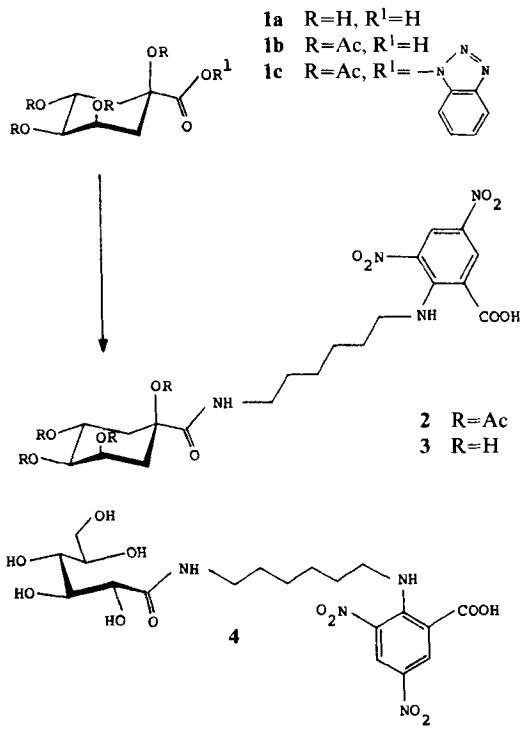
data on their capacity to elicit passive cutaneous anaphylaxis (PCA) in anti-Dncp sensitized guinea pigs. The findings corroborate and extend the results with the less well-defined penicilloylated carbohydrates.

Synthesis. – The synthesis of the tetrahydroxycyclohexanecarbonyl derivative **3**, a rather stable pseudocarbohydrate conjugate, was included to extend the scope of the biological evaluation *(cf. Scheme 1)*. Acetylation of D(−)-1,3,4,5-tetrahydroxycyclohexane-1-carboxylic acid (**1a**) afforded D(−)-1,3,4,5-tetraacetoxycyclohexane-1-carboxylic acid (**1b**), which could be converted to the ester derivative **1c** of 1,2,3-benzotriazol-1-ol and reacted with N^1-(2-carboxy-4,6-dinitrophenyl)-1,6-hexanediamine (= 2-(6-aminohexylamino)-3,5-dinitrobenzoic acid) to **2**. The required hexanediamine derivative was obtained from 2-chloro-3,5-dinitrobenzoic acid and 1,6-hexanediamine according to [6]. Compound **2** was finally de-acetylated with sodium methylate in MeOH to give **3**.

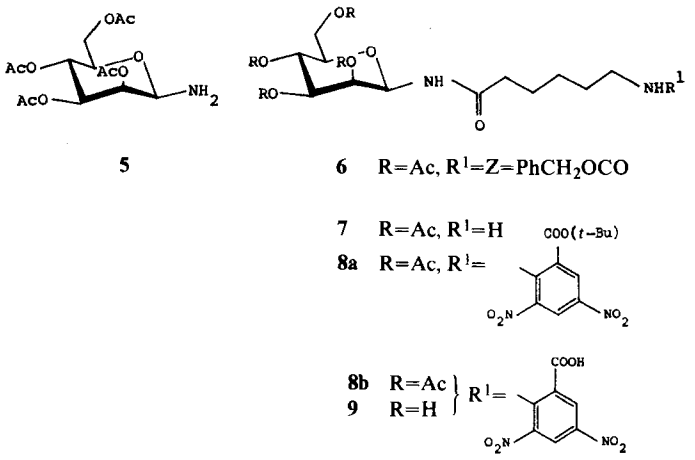
The preparation of the gluconic acid derivative **4** was carried out according to [7], using aminolysis of D(+)-glucono-1,5-lactone with N^1-(2-carboxy-4,6-dinitrophenyl)1,6-hexanediamine.

The synthesis of the mannopyranose derivative **9** *(cf. Scheme 2)* required the key intermediate **7** which was obtained *via* **6** by attaching 6-(benzyloxycarbonyl) amino)hexanoic acid to 2,3,4,6-tetra-*O*-acetyl-*β*-D-mannopyranosylamine (**5**) [8]

Scheme 1



Scheme 2



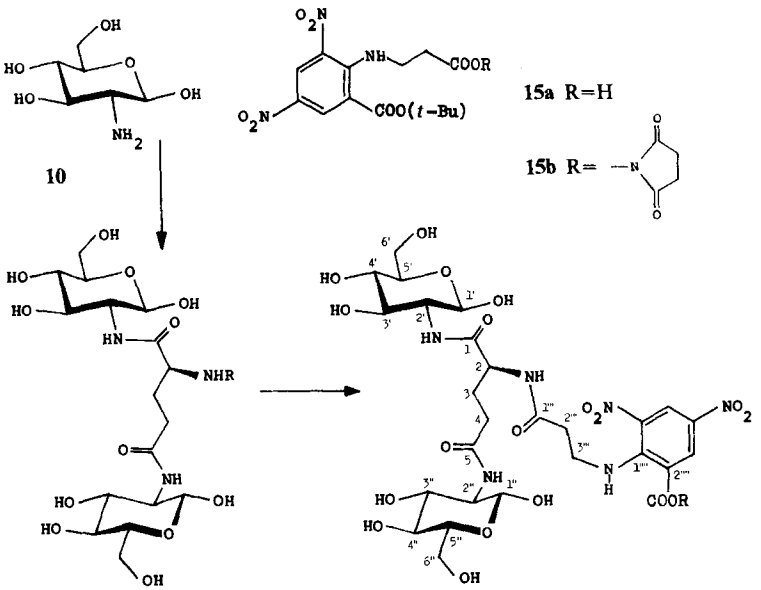
using *N*-ethyl-*N'*-(3-(dimethylamino)propyl)carbodiimide hydrochloride and 1,2,3-benzotriazol-1-ol [9], and by removing the benzyloxycarbonyl protecting group from **6** by hydrogenolysis. Incorporation of the Dncp haptenic group into **7** using *tert*-butyl 2-chloro-3,5-dinitrobenzoate in 1,4-dioxane gave **8a**. Cleavage of the *tert*-butyl ester with trifluoroacetic acid gave **8b** which was deacetylated with sodium methylate in MeOH to **9**. The *tert*-butyl 2-chloro-3,5-dinitrobenzoate which enabled relatively smooth incorporation of the Dncp hapten was obtained by transesterification from *tert*-butyl acetate and 2-chloro-3,5-dinitrobenzoic acid in the presence of $HClO_4$.

The glycopeptide **14** was synthesized according to *Scheme 3*. The intermediate **11** was obtained according to *Jones et al.* [10] from unprotected 2-amino-2-deoxy-D-glucopyranose (**10**) and *N*-(benzyloxycarbonyl)glutamic acid in aqueous pyridine using *N,N'*-dicyclohexylcarbodiimide. The product was obtained in low yield, and the procedure proved somewhat less satisfactory than in the hands of the Canadian workers, who prepared 2-amino-*N*-(*N*-(benzyloxycarbonyl)threonyl)-2-deoxy-D-glucopyranose. Removal of the benzyloxycarbonyl group from **11** by hydrogenolysis gave **12**, which was reacted with the activated ester **15b** to yield **13**. After removal of the *tert*-butyl ester protection **14** was obtained. For the preparation of the intermediate **15b**, *tert*-butyl 2-chloro-3,5-dinitrobenzoate was reacted with *β*-alanine to **15a**, which was esterified with *N*-hydroxysuccinimide in the presence of *N,N'*-dicyclohexylcarbodiimide.

The lactobionic acid conjugate **17** *(cf. Scheme 4)* was prepared by aminolysis of lactobionolactone (**16**) with N^1-(2-carboxy-4,6-dinitrophenyl)-1,6-hexanediamine in MeOH. The required lactone **16** was obtained according to [11] by repeated evaporation at 50° of an aqueous solution of lactobionic acid to which EtOH was added.

Anaphylactogenicity. – According to the *Table*, monosaccharide derivatives incorporated into the monohaptenic conjugates are not sufficient for anaphy-

Scheme 3



Scheme 4

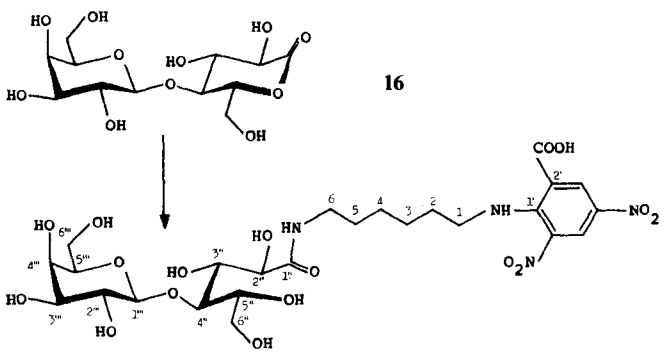


Table. *Anaphylactogenicity of mono-Dncp-carbohydrate conjugates*

Conjugate	PCA-testing[a])		
	Antiserum	Dose [μmol]	Response [mm]
N^1-Dncp-N^6-(D-1,3,4,5-tetrahydroxy-cyclohexane-1-carbonyl)-1,6-hexanediamine (**3**)	1/40–1/1280	1	neg.
		2	neg.
		4	neg.
N^1-Dncp-N^6-D-gluconoyl-1,6-hexane-diamine (**4**)	1/40–1/640	4	neg.
N-[6-(Dncp-amino)hexanoyl]-β-D-manno-pyranosylamine (**9**)	1/40–1/1280	0.1	neg.
		1	neg.
		4	neg.
N^1,N^5-Bis[2-deoxy-β-D-glucopyranos-2-yl]-N^2-[N^3-Dncp-β-alanyl]glutaminamide (**14**)	1/40	0.1	26
	1/1280	0.1	11
	1/1280	2	16
N^1-Dncp-N^6-lactobionoyl-1,6-hexane-diamine (**17**)	1/40	0.1	18
	1/320	0.1	11
	1/320	2	15

[a]) For details *cf. Exper. Part.*

lactogenicity. This applies to open-chain carbohydrates as in the gluconic acid conjugate **4** as well as to mannopyranosylamine ring structures as in **9**. The gluco-pyranosylamine analog of **9** (not reported here) was also negative. On the other hand, the bis(2-deoxyglucopyranos-2-yl) arrangement of **14** provides considerable anaphylactic potency, and this applies also to the lactobionoyl residue of **17**. For comparison, fully Dncp-substituted deca-L-lysine, an efficient, multivalent anaphylactogen, shows a PCA response of 28 mm at a dose of 1 μmol and an anti-Dncp antiserum dilution of 1:1280.

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Experimental Part

General. Melting points (m.p.) were determined on a heatable microscope (*Reichert*, Vienna) and are uncorrected. Solvents were removed under vacuum at 35–40° in a rotary evaporator. Preparative separations of mixtures were carried out using column, dry column or thin layer chromatography (TLC.) with silica gel *60* (0.063 – 0.2 mm, *Merck*). For dry columns, nylon tubing (30 mm diameter, *ICN Pharmaceuticals*) and silica gel of 40% relative humidity were used. – Intermediates and final products were controlled by TLC. on silica gel *60, F 254, Merck;* amounts of 50–100 μg were spotted and run with adequate solvents. A: acetone/cyclohexane 1:1; B: $CHCl_3$/MeOH 9:1; C: BuOH/AcOH/H_2O 4:1:5; D: 1,4-dioxane/H_2O 5:1; E: $CHCl_3$/EtOAc 1:1; F: propanol/H_2O/EtOAc 7:2:1; G: BuOH/pyridine/H_2O 6:4:3; H: BuOH/H_2O/EtOAc 9:4:5; J: MeOH/EtOAc 3:7; K: $CHCl_3$/MeOH 3:1. Detection of spots in the case of peptides and amino-acid derivatives was with *Fluram* (*F;* = 4-phenyl-spiro[furan-2(3*H*),1′-phthalan]-3,3′-dione; *Roche*), ninhydrin (Nh) and Cl_2-vapour followed by KI/*o*-toluidine according to *Reindel & Hoppe* [12] *(RH)*. Carbohydrate derivatives were detected with ethanolic H_2SO_4-solution [13] (H_2SO_4) or sodium periodate/benzidine [14] (IO_4). Quenching of fluorescence under 254 nm radiation (UV.) was also observed. Color reactions of the spots are indicated

by '+' (positive) or '−' (negative). Electrophoresis at 50 Volt/cm was carried out on paper *(MN 214, Machery & Nagel)* or on other carriers, as indicated, in a *Camag* electrophoresis apparatus. – IR. spectra were run on a *Beckman-IR-4210* spectrometer, $\tilde{\nu}_{max}$ in cm^{-1}. Bands are characterized as strong (*s*), medium (*m*) and weak (*w*). – The ^{1}H-NMR. spectrum (internal standard tetramethylsilane (= 0 ppm)) was obtained on a *Varian-A-60* (60 MHz), ^{13}C-NMR. spectra (internal reference D_6-DMSO (= 39,7 ppm)) on a *Varian-XL-100* spectrometer. – Elementary analyses were carried out by *H. Frohofer*, Institute for Organic Chemistry, University of Zürich. – Abbreviations: Boc, *tert*-butoxycarbonyl; Z, benzyloxycarbonyl; *t*-Bu, *tert*-butyl; DMF, dimethylformamide; DMSO, dimethylsulfoxide; THF, tetrahydrofuran; DMAP, 4-(dimethylamino)pyridine; r.t., room temperature; *i.v.*, in vacuo; PCA, passive cutaneous anaphylaxis.

Passive cutaneous anaphylaxis. Outbred guinea pigs (250–300 g) were sensitized intradermally with 0.1 ml of anti-Dncp-bov. gamma globulin antiserum from the rabbit [6], appropriately diluted with 0.01M phosphate-buffered saline (pH 7.4). Elicitation of reactions was 18–20 h later by intravenous injection of 0.5 ml of aq. *Evans* blue together with the Dncp-conjugate in up to 1 ml of phosphate-buffered saline. The size of the blue spots after 20 min on the shaven skin is given as the mean diameter in millimeters. Values given are averages from 2–4 animals.

D*(−)-1,3,4,5-tetrahydroxycyclohexane-1-carboxylic acid* (**1a**) and simple carbohydrate derivatives were from *Fluka*, CH-9470 Buchs.

Synthesis of N^1*-(2-carboxy-4,6-dinitrophenyl)-*N^6*-(*D*-1,3,4,5-tetrahydroxycyclohexane-1-carbonyl)-1,6-hexanediamine (=3,5-dinitro-2-[6-(*D*-1,3,4,5-tetrahydroxycyclohexane-1-carbonylamino)hexylamino]benzoic acid;* **3**). D*(−)-1,3,4,5-Tetraacetoxycyclohexane-1-carboxylic acid* (**1b**). To 961 mg (5 mmol) of **1a** in 12 ml of Ac_2O/pyridine 1:2, 20 mg (0.16 mmol) of DMAP was added at 5°. After 24 h at r.t. the solution was added dropwise to 30 ml of ice water, acidified to pH 3 with 3M HCl and extracted with CH_2Cl_2 (4 × 50 ml). The combined extracts were dried (Na_2SO_4), and the solvent was removed: 1.77 g (98%) of oil. TLC. (C): Rf 0.65 (H_2SO_4, +); TLC. (G): Rf 0.69 (H_2SO_4, +). – IR. (CH_2Cl_2): 3470*w*, 3300*m* br., 1750*m*, 1370*s*, 1220*s*, 1120*s*, 1060*s*, 930*m*.

1,2,3-Benzotriazol-1-yl D*(−)-1,3,4,5-tetraacetoxycyclohexane-1-carboxylate* (**1c**). To 1.2 g (3.33 mmol) of **1b** and 490 mg (3.33 mmol) of 1,2,3-benzotriazol-1-ol (92%) in 30 ml of CH_2Cl_2/THF 1:1, 755 mg (3.66 mmol) of *N,N'*-dicyclohexylcarbodiimide was added at 5°. The resulting suspension was stirred at 5° for 1 h and at r.t. for 2 h, filtered and the filtrate evaporated. The oil solidified upon trituration with 2-propanol and was dried *i.v.*: 1.48 g (93%) of **1c**. TLC. (A): Rf 0.45 (UV., +), and trace at start (UV., +); TLC. (B): Rf 0.88 (UV., +), and trace at Rf 0.09 (UV., +).

N^1*-(2-Carboxy-4,6-dinitrophenyl)-*N^6*-(*D*(−)-1,3,4,5-tetraacetoxycyclohexanecarbonyl)-1,6-hexanediamine (=3,5-dinitro-2-[6-(1,3,4,5-tetraacetoxycyclohexane-1-carbonylamino)hexylamino]benzoic acid;* **2**). To 460 mg (0.96 mmol) of **1c** in 8 ml of CH_2Cl_2, 330 mg (0.91 mmol) of N^1-(2-carboxy-4,6-dinitrophenyl)-1,6-hexanediamine hydrochloride [6] in 3 ml of DMF and triethylamine to pH 9 was added, and the base was used to keep the pH at 9. After 5 h, the solution was diluted with 200 ml of CH_2Cl_2, extracted with 2000 ml of 0.1M HCl, and washed with H_2O to pH 7. The org. phase was dried (Na_2SO_4) and evaporated. The crude product (530 mg) was chromatographed on a silica-gel (40 g) column with solvent K to give 490 mg (76%) of **2**. TLC. (K): Rf 0.66 (UV., +; Nh, −), traces at Rf 0.22, 0.74, and 0.87 (UV., +). – IR. (CH_2Cl_2): 3430*w*, 3060*w*, 2930*m*, 2860*w*, 1750*m*, 1670*m*, 1620*s*, 1530*m*, 1430*m*, 1370*s*, 1320*s*, 1220*s*, 1110*m*, 1060*m*, 815*w*.

Conversion to **3**. A solution of 490 mg (0.73 mmol) of **2** in 10 ml of CH_3OH was mixed with 4 mg of CH_3ONa in 2 ml of CH_3OH and stirred for 2 h at r.t. and for another 30 min after addition of 1 g of moist ion-exchange resin (*Amberlyst 15*, H^+-form, pretreated with MeOH for 24 h). Filtration and evaporation of the filtrate left 330 mg (90%) of viscous oil. An aliquot of 80 mg was twice chromatographed on silica-gel columns with solvent K to give 65 mg of amorphous material. TLC. (C): Rf 0.59, homogeneous (UV., +; Nh, −); TLC. (D): Rf 0.84, homogeneous (UV., +; Nh, −). Electrophoresis (Al_2O_3 plate; 0.05M PO_4^{3-}, pH 7.4; 30 min): 18 mm, anodic, homogeneous. – IR. (KBr): 3400*m* br., 2930*m*, 2860*w*, 1625*s*, 1580*m*, 1540*s*, 1435*m*, 1360*m*, 1320*s*, 1170*w*, 1090*m*, 915*w*, 815*w*, 740*w*, 710*w*.

$C_{20}H_{28}N_4O_{11}$ (500.46) Calc. C 47.99 H 5.63 N 11.19% Found C 47.88 H 6.12 N 11.46%

Synthesis of N^1*-(2-Carboxy-4,6-dinitrophenyl)-*N^6*-*D*-gluconoyl-1,6-hexanediamine (=2-[6-(*D*-gluconoylamino)hexylamino]-3,5-dinitrobenzoic acid;* **4**). To 535 mg (3 mmol) of D(+)-glucono-1,5-lactone in 16 ml of CH_3OH, a solution of 1.0 g (2.8 mmol) of N^1-(2-carboxy-4,6-dinitrophenyl)-1,6-

hexanediamine hydrochloride [6] in 15 ml of 0.2M NaOH in MeOH was added at 60–65°. The solution was stirred under reflux for 3 h and then mixed with 3 g of moist ion-exchange resin (*Amberlyst 15*, H^+-form, pretreated with MeOH for 24 h). After 30 min, the resin was filtered off, the filtrate evaporated and the residual oil dissolved in hot 2-propanol and treated with charcoal. After removal of charcoal and solvent, the oil crystallized. It was purified by TLC. with solvent D to give 270 mg of **4**. TLC. (D): Rf 0.83, homogeneous (UV., +; H_2SO_4, +; Nh, –). – IR. (KBr): 3600–3400*m* br., 2940*m*, 2860*m*, 1770*w*, 1750*m*, 1630*s*, 1610*s*, 1575*s*, 1525*s*, 1435*s*, 1360*s*, 1320*s*, 1275*s*, 1170*m*, 1090*m*, 940*w*, 915*m*, 820*m*, 740*m*, 730*m*, 715*m*.

$C_{19}H_{28}N_4O_{12} \cdot 1.5\,H_2O$	Calc.	C 42.94	H 5.88	N 10.54%
(531.48)	Found	„ 43.17	„ 6.42	„ 10.55%

Synthesis of N-*[6-(2-carboxy-4,6-dinitrophenylamino)hexanoyl]-β-*D-*mannopyranosylamine* (= *2-[5-(β-*D-*mannopyranosylaminocarbonyl)pentylamino]-3,5-dinitrobenzoic acid;* **9**). tert-*Butyl 2-chloro-3,5-dinitrobenzoate.* A solution of 3.0 g (12.2 mmol) of 2-chloro-3,5-dinitrobenzoic acid in 20 ml of *tert*-butyl acetate was mixed with 1 ml of 70% $HClO_4$ solution and stirred at r.t. for 5 h. After dilution with 100 ml of Et_2O, extractions with 8% $NaHCO_3$-solution (10 times 40 ml) and H_2O (10 times 40 ml) left a solution which was dried (Na_2SO_4) and evaporated. The residual oil (2.12 g, 57%) crystallized at 0°, m.p. 70–71°. TLC. (A): Rf 0.63 (UV., +); TLC. (B): Rf 0.92 (UV., +). – IR. (CH_2Cl_2): 3080*m*, 1725*m*, 1605*m*, 1585*m*, 1540*s*, 1340*s*, 1300*s*, 1145*s*, 1060*m*, 840*m*. – ^{1}H-NMR. ($CDCl_3$): 1.67 (*s*, 9 H); 8.67 (*s*, 2 H).

$C_{11}H_{11}N_2O_6Cl$ (302.67) Calc. C 43.65 H 3.66 N 9.26% Found C 43.77 H 3.83 N 9.00%

*2,3,4,6-Tetra-*O*-acetyl-*N*-[6-(benzyloxycarbonylamino)hexanoyl]-β-*D*-mannopyranosylamine* (= *6-benzyloxycarbonylamino-*N*-(2,3,4,6-tetra-*O*-acetyl-β-*D*-mannopyranosyl)hexanamid* **6**). To 2.65 g (10.3 mmol) of 6-(benzyloxycarbonylamino)hexanoic acid and 1.47 g (10 mmol) of 1,2,3-benzotriazol-1-ol (92%) in 15 ml of THF, 2.11 g (11 mmol) of *N*-ethyl-*N'*-(3-(dimethylamino)propyl)carbodiimide hydrochloride and 1.21 ml (11 mmol) of 4-methylmorpholine were added at 5°, followed by a solution of 3.47 g (10 mmol) of 2,3,4,6-tetra-*O*-acetyl-β-D-mannopyranosylamine[3]) in 15 ml of DMF. The suspension was stirred at r.t. for 20 h, diluted with 400 ml of CH_2Cl_2 and extracted in a spray column extractor [15] with 2000 ml of 0.1M HCl, 500 ml of H_2O, 2000 ml of 0.15M K_2CO_3 and 2000 ml of H_2O. Removal of the org. phase after drying (Na_2SO_4) left 6.02 g of syrup, which was subjected to column chromatography (150 g of silica gel, solvent E). Evaporation of the eluent left 3.79 g (62%) of solid foam. TLC. (E): Rf 0.34 (H_2SO_4, +). – IR. (CH_2Cl_2): 3420*m*, 2950*m*, 2860*w*, 1750*m*, 1500*s*, 1370*s*, 1220*s*, 1050*s*.

*2,3,4,6-Tetra-*O*-acetyl-*N*-[6-(2-carboxy-4,6-dinitrophenylamino)hexanoyl]-β-*D*-mannopyranosylamine* (= *2-[5-(2,3,4,6-tetra-*O*-acetyl-β-*D*-mannopyranosylaminocarbonyl)pentylamino]-3,5-dinitrobenzoic acid;* **8b**). To 2.88 g (6.25 mmol) of **7** in 10 ml of 1,4-dioxane were added 2.18 g (7.2 mmol) of *tert*-butyl 2-chloro-3,5-dinitrobenzoate in 5 ml of 1,4-dioxane and 0.75 ml (6.8 mmol) of 4-methylmorpholine. The mixture was kept at r.t. for 24 h, diluted with 400 ml of CH_2Cl_2 and extracted in a spray column extractor [15] with 2000 ml of 0.1M HCl, 500 ml of H_2O, 2000 ml of 0.3M K_2CO_3 and 2000 ml of H_2O. The org. phase was dried (Na_2SO_4) and evaporated, and the crude product (3 g) purified by column chromatography (solvent E). Further purification by prep. TLC. with $CHCl_3$/MeOH 4:1 gave 1.11 g (24%) of **8a**. TLC. (E): Rf 0.69 (UV., +; H_2SO_4, +), trace at Rf 0.46 (UV., +). – IR. (CH_2Cl_2): 3430*w*, 2940*w*, 2870*w*, 1750*m*, 1690*m*, 1605*s*, 1505*s*, 1370*s*, 1330*s*, 1220*s*, 1140*s*, 1050*s*, 845*w*.

All of **8a** was kept in 10 ml of 90% trifluoroacetic acid at 0° for 45 min. After removal of the trifluoroaceticacid *i.v.*, 10 ml of EtOH was added and removed *i.v.* The EtOH-treatment was repeated twice, whereupon the residue was chromatographed on a column of silica gel (50 g; solvent B) to give 526 mg (51%) of **8b**. TLC. (B): Rf 0.79 (UV., +); TLC. (J): Rf 0.26 (UV., +), and trace at Rf 0.15 (UV., +).

Conversion to **9**. A solution of 525 mg (0.78 mmol) of **8b** in 10 ml of MeOH was mixed with 4 mg of CH_3ONa in 2 ml of MeOH and stirred for 4 h at r.t. and for 1 h after addition of 5 g of moist ion-exchange resin (*Amberlyst 15*, H^+-form, pretreated with MeOH for 24 h). Filtration and evaporation of the filtrate and column chromatography of the residue on silica gel (50 g; solvent C) gave 71 mg (18%)

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[3]) Obtained according to [8]. D-Mannopyranose was fully acetylated and reacted with trimethylsilylazide to 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosylazide. This gave the required intermediate upon catalytic hydrogenation.

of **9**. TLC. (C): Rf 0.43 (UV., +; H_2SO_4, +). Electrophoresis (cellulose plate; 0.05M PO_4^{3-}, pH 7.4; 25 min): 17 mm, anodic, homogeneous. – IR. (KBr): 3380*m* br., 3090*w*, 2930*m*, 2860*w*, 1620*s*, 1575*s*, 1535*s*, 1435*s*, 1360*m*, 1320*s*, 1170*w*, 1075*m*, 940*w*, 915*w*, 810*w*, 790*w*, 720*w*.

$C_{19}H_{26}N_4O_{12}$ (502.43) Calc. C 45.42 H 5.22 N 11.15% Found C 45.24 H 5.49 N 10.62%

Synthesis of N^1,N^5*-bis[2-deoxy-β-*D*-glucopyranos-2-yl]-*N^2*-[*N^3*-(2-carboxy-4,6-dinitrophenyl)-β-alanyl]glutaminamide (= 2-[[N-(1,5-bis(2-deoxy-β-*D*-glucopyranos-2-ylamino)-1,5-dioxopentan-2-yl)-amino]carbonylethylamino]-3,5-dinitrobenzoic acid;* **14**). N-*(2-tert-Butoxycarbonyl-4,6-dinitrophenyl)-β-alanine* (**15a**). *tert*-Butyl 2-Chloro-3,5-dinitrobenzoate (6.4 g, 21.1 mmol) in 30 ml of 1,4-dioxane and 2.26 g (25.4 mmol) of β-alanine in 40 ml of H_2O were mixed, and after addition of 2.55 ml (23.2 mmol) of 4-methylmorpholine stirred for 24 h at r.t. in the dark. The mixture was dried (Na_2SO_4) and evaporated, and the crude product purified on a column (120 g of silica gel; solvent B) giving 3.75 g (50%) of **15a**, m.p. 145–146°. TLC. (A): Rf 0.53 (UV., +; Nh, –). – IR. (CH_2Cl_2): 2860*w*, 1755*m*, 1725*w*, 1690*m*, 1600*s*, 1510*m*, 1330*s*, 1140*s*, 1100*m*, 875*m*.

$C_{14}H_{17}N_3O_8$ (355.30) Calc. C 47.33 H 4.82 N 11.83% Found C 47.16 H 4.81 N 11.96%

N^3*-(2-tert-Butoxycarbonyl-4,6-dinitrophenyl)-β-alanine succinimido ester* (**15b**). A suspension of 711 mg (2 mmol) of **15a**, 254 mg (2.2 mmol) of *N*-hydroxysuccinimide and 413 mg (2 mmol) of *N,N'*-dicyclohexylcarbodiimide in 6 ml of CH_2Cl_2 was stirred for 1 h at 5° and for 2 h at r.t. After filtration, the filtrate was diluted with 200 ml of CH_2Cl_2 and extracted with 0.3M K_2CO_3 (3 times 50 ml) and H_2O (3 times 50 ml). The org. phase was dried (Na_2SO_4) and evaporated leaving an oil which crystallized: 849 mg (94%), m.p. 172–173°. TLC. (C): Rf 0.88 (UV., +). – IR. (CH_2Cl_2): 2940*w*, 1815*w*, 1755*m*, 1690*m*, 1605*s*, 1545*m*, 1510*m*, 1330*s*, 1205*s*, 1140*s*, 845*w*.

$C_{18}H_{20}N_4O_{10}$ (452.38) Calc. C 47.79 H 4.46 N 12.38% Found C 48.30 H 4.38 N 12.65%

N^2*-Benzyloxycarbonyl-*N^1,N^5*-bis[2-deoxy-β-*D*-glucopyranos-2-yl]glutaminamide* (**11**). To an ice-cooled solution of 10.7 g (50 mmol) of 2-amino-2-deoxy-D-glucopyranose hydrochloride (**10** · HCl) in 50 ml of H_2O/2M NaOH 1:1 7.0 g (25 mmol) of *N*-(benzyloxycarbonyl)glutamic acid in 17 ml of pyridine was added. After addition of 15.4 g (75 mmol) of *N,N'*-dicyclohexylcarbodiimide in 50 ml of pyridine and stirring for 20 h at r.t., the mixture was filtered and the filtrate extracted with Et_2O (3 × 70 ml). The aq. phase was evaporated and the residue reprecipitated 3 times from MeOH giving 1.06 g (7%) of **11**. TLC. (C): Rf 0.50 (H_2SO_4, +; *RH*, +); TLC. (F): Rf 0.80 (H_2SO_4, +); TLC. (G): Rf 0.84 (H_2SO_4, +). The combined mother liquors gave another 340 mg of slightly less pure material.

N^1,N^5*-Bis(2-deoxy-β-*D*-glucopyranos-2-yl)glutaminamide* (**12** · *HOAc*). Catalytic hydrogenation of 1.06 g (1.76 mmol) of **11** in 20 ml of 75% AcOH in the presence of 100 mg of Pd on C (10%)/Pt on SiO_2 (5%), 1:1 was carried out for 6 h. After filtering off the catalyst, the filtrate was frozen and lyophilized giving 993 mg of **12** · HOAc. TLC. (C): Rf 0.10 (*F*, +; *RH*, +), trace at Rf 0.23 (*F*, +).

N^1,N^5*-Bis[2-deoxy-β-*D*-glucopyranos-2-yl]-*N^2*-[*N^3*-(2-tert-butoxycarbonyl-4,6-dinitrophenyl)-β-alanyl]glutaminamide (= tert-butyl 2-[[N-(1,5-bis(2-deoxy-β-*D*-glucopyranos-2-ylamino)-1,5-dioxopentan-2-yl)amino]carbonylethylamino]-3,5-dinitrobenzoate;* **13**). A solution of 800 mg (1.51 mmol) of **12** in 4 ml of DMSO was mixed with a solution of 918 mg (1.4 mmol) of **15a** in 5 ml of DMSO and 50 mg of 1,2,3-benzotriazol-1-ol. After 20 h, 200 ml of 2-propanol was added and the precipitate filtered off, washed with 2-propanol and Et_2O and dried: 956 mg (78%) of **13**. TLC. (C): Rf 0.39 (UV., +; H_2SO_4, +); TLC. (H): Rf 0.58 (UV., +; H_2SO_4, +). – ^{13}C-NMR. (25.2 MHz, 75°, D_6-DMSO; s. *Scheme 3*): 27.8 ($C(CH_3)_3$); 28.8, 32.0, 34.6 (C(3), C(4), C(2‴)); 43.6 (C(3‴)); 52.5 (C(2)); 54.5 (C(2′), C(2″,α)); 57.8 (C(2′), C(2″,β)); 61.5 (C(6′), C(6″)); 70.9, 71.5, 72.0 (C(3′), C(4′), C(5′), C(3″), C(4″), C(5″,α)); 74.3, 76.6 (C(3′), C(5′), C(3″), C(5″,β)); 83.9 ($C(CH_3)_3$); 90.8 (C(1′), C(1″,α)); 95.2, 95.3 (C(1′), C(1″,β)); 118.5 (C(2⁗)); 125.9 (C(3⁗)); 130.5 (C(5⁗)); 133.7 (C(6⁗)); 134.6 (C(4⁗)); 147.4 (C(1⁗)); 164.4, 169.7, 171.2, 172.1 (*C*OO*t*Bu, C(1‴), C(5), C(1)).

$C_{31}H_{46}N_6O_{19} + H_2O$ (824.75) Calc. C 45.14 H 5.87 N 10.19% Found C 45.28 H 6.47 N 10.00%

Conversion to **14**. For deprotection, 450 mg (0.56 mmol) of **13** was kept in 5 ml of ice-cold 90% trifluoroacetic acid for 45 min. After evaporation, the residue was suspended 3 times in EtOH, the EtOH being removed together with remaining trifluoroacetic acid *i.v.* This left 389 mg (91%) of crude product which was purified on a silica gel dry column with solvent C, giving 284 mg of **14**. TLC. (C):

Rf 0.17 (UV., +; Nh, −); TLC. (H): Rf 0.66 (UV., +; Nh, −). Electrophoresis (cellulose; 0.05M PO_4^{3-}, pH 7.4; 25 min): 16 mm, anodic, homogeneous. – IR. (KBr): 3340*s* br., 2915*m*, 1620*s*, 1575*m*, 1530*s*, 1440*m*, 1320*s*, 1170*w*, 1060*s*, 915*w*, 815*w*, 740*w*, 710*w*.

$C_{27}H_{38}N_6O_{19} + H_2O$ (768.6) Calc. C 42.19 H 5.24 N 10.93% Found C 42.78 H 5.92 N 10.41%

Synthesis of N^1*-(2-carboxy-4,6-dinitrophenyl)-*N^6*-lactobionoyl-1,6-hexanediamine (=2-[6-(4-O-β-*D*-galactopyranosyl-*D*-gluconoylamino)hexylamino]-3,4-dinitrobenzoic acid;* **17**). Lactobiono lactone (**16**) was obtained by evaporating to dryness (*cf.*[11]) a solution of 10 g of lactobionic acid (=4-*O*-β-D-galactopyranosyl-D-gluconic acid) in 100 ml of H_2O, after addition of 50 ml of EtOH at 50°. EtOH (50 ml) was again added and removed for a total of 9 times, and the residue dried over P_4O_{10}: 8.8 g (93%) of **16**. Thereof 2.0 g (5.9 mmol) in 27 ml of MeOH/H_2O 8:1 was mixed with 1.8 g (5 mmol) of N^1-(2-carboxy-4,6-dinitrophenyl)-1,6-hexanediamine hydrochloride [6] in 20 ml of MeOH and 5.5 ml of 1M NaOH in MeOH and stirred for 2h at 65°. After addition of 10 g of moist ion-exchange resin (*Amberlyst 15*, H^+-form, pretreated with MeOH for 24 h) and further stirring at r.t. for 45 min, the mixture was filtered and the filtrate evaporated. The residue was purified on a column (100 g of silica gel; solvent E) giving 670 mg (19%) of **17**. TLC. (C): Rf 0.37 (UV., +; H_2SO_4, +); TLC. (F): Rf 0.80 (UV., +; H_2SO_4, +). Electrophoresis (cellulose; 0.05M PO_4^{3-}, pH 7.4; 30 min): 21 mm, anodic, homogeneous. – IR. (KBr): 3400*s* br., 2930*m*, 2860*w*, 1750*m*, 1620*s*, 1530*m*, 1430*m*, 1360*m*, 1320*m*, 1080*m*, 815*w*. – ^{13}C-NMR. (25.2 MHz, 75°, D_6-DMSO; s. *Scheme 4*): 25.8, 25.9, 28.8, 29.0 (C(5), C(4), C(3), C(2)); 38.9 (C(6)); 45.3 (C(1)); 60.7, 62.5 (C(6″), C(6‴)); 68.3, 71.1, 71.7, 71.9, 73.0, 73.4, 75.6, 82.2 (C(2″), C(3″), C(4″), C(5″), C(2‴), C(3‴), C(4‴), C(5‴)); 104.2 (C(1‴)); 124.0, 129.2 (C(5′), C(3′)); 125.0, 132.7, 133.8 (C(6′), C(4′), C(2′)); 148.8 (C(1′); 167.7 (C(1″)); 171.9 (COOH).

$C_{25}H_{38}N_4O_{17}$ (666.6) Calc. C 45.05 H 5.75 N 8.40% Found C 45.05 H 6.12 N 8.08%

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