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Neoglycoconjugates from synthetic tetra- and hexasaccharides that mimic the terminus of the O-PS of *Vibrio cholerae* O:1, serotype Inaba

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Received 26th November 2002, Accepted 6th January 2003
First published as an Advance Article on the web 10th February 2003

A glycosyl acceptor and a glycosyl donor having the *N*-3-deoxy-L-*glycero*-tetronic acid side chain already attached have been prepared and used for the synthesis of the di- through to the hexasaccharide that mimic the upstream terminus of the O-specific polysaccharide of *Vibrio cholerae* O:1, serotype Inaba. The target tetra- and the hexasaccharide, which were obtained in the form of 5-methoxycarbonylpentyl glycosides, were linked to BSA using squaric acid diester chemistry. The conjugation reactions were monitored by surface enhanced laser desorption ionization-time of flight mass spectrometry (SELDI-TOF MS). This allowed the progression of the conjugation of the synthetic oligosaccharides in a controlled way and termination of the reaction when the desired molar hapten/BSA ratio had been reached, yielding neoglycoconjugates with predetermined carbohydrate/carrier ratios. The ability to monitor the conjugation by the SELDI-TOF MS technique made it possible to prepare, from one hapten in a one-pot reaction, several neoglycoconjugates having different, predetermined carbohydrate/carrier ratios.

Introduction

It has been generally accepted that the O-specific polysaccharide (O-PS) domain of lipopolysaccharides (LPS) of gram negative bacteria determines the serological specificity.[1] It should, therefore, be possible to use oligosaccharides that mimic immunologically dominant epitopes of O-polysaccharides as antigenic components of conjugate vaccines. Cholera is a serious enteric disease that remains a persistent problem in the developing and, occasionally, in the developed world.[2] We are currently interested in developing a conjugate vaccine for this disease, and have synthesized a number of oligosaccharides that mimic the structure of the O-PS of the two main causes for cholera, namely *Vibrio cholerae* O:1, serotypes Inaba and Ogawa. Structures of the two O-PSs are shown in Fig. 1. The internal part of the two polysaccharides is identical; they differ in the presence of a methoxyl group at C-2 of the upstream, terminal 4-amino-4,6-dideoxy-α-D-mannopyranose (perosamine) in the Ogawa O-PS. Neoglycoconjugates prepared from the hexasaccharide that represents the upstream terminus of the O-PS of *Vibrio cholerae* O:1, serotype Ogawa have shown immunogenicity for the Ogawa LPS-specific antibodies.[3] To enable further studies of specificity and cross-reactivity involved in the *Vibrio cholerae* O:1 serotypes, we have now prepared neoglycoconjugates from the tetra- and hexasaccharides representing the upstream epitopes of the Inaba O-PS. Preparation of neoglycoconjugates from the analogous disaccharide has been reported.[4]

Results and discussion

In principle, one can envision two fundamentally different strategies to assemble oligosaccharides composed of *N*-acylated perosamine. In the original approach by Bundle *et al.*,[5–7] perosamine-containing oligosaccharides were assembled using mono- or disaccharide building blocks containing azido groups at position 4, that is intermediates lacking the requisite *N*-acyl group. We have adopted the same strategy to synthesize some methyl α-glycosides of fragments of O-PS of *Vibrio cholerae* O:1.[8–10] A different assembly strategy can be based on intermediate building blocks having the *N*-acyl group already in place. Also, the stage at which the linker (spacer) should be attached is an important aspect to consider, when such oligosaccharides are to be used for conjugation to carriers. In our initial synthesis[11] of linker equipped *N*-acylated perosamine-containing haptens we first prepared 2-(trimethylsilylethyl) (SE) glycosides of the requisite oligosaccharides from building blocks lacking the *N*-acyl group. When the targeted oligosaccharide was assembled, the azido groups were reduced to amino groups and the resulting amines were *N*-acylated. Subsequently, the SE functionality was exchanged for an aglycon

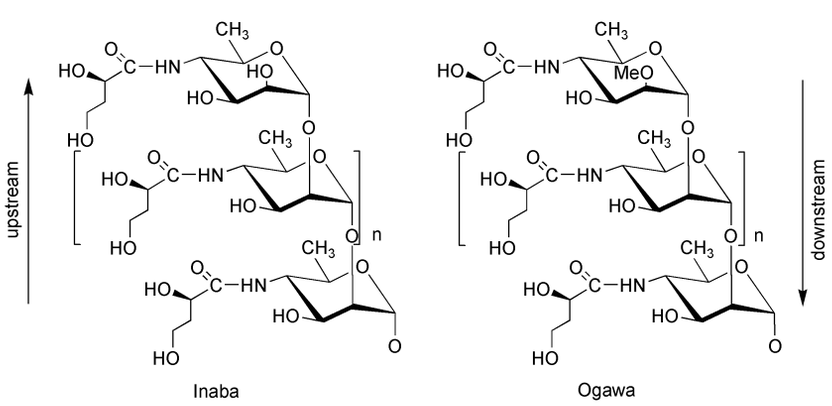
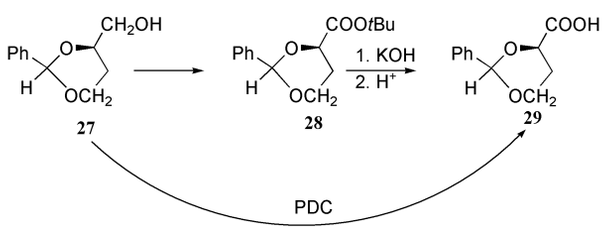


Fig. 1 Structure of the O-PS of *Vibrio cholerae* O1, serotypes Inaba and Ogawa.

DOI: 10.1039/b211660j

(spacer), which made the haptens amenable to chemical linking to a carrier. Generally, the drawback of such an approach is that it requires many chemical manipulations to be performed with the assembled oligosaccharide molecule. We have, in the past, prepared an *N*-acyl-perosamine-containing disaccharide from monosaccharide building blocks that had the requisite *N*-acyl group already in place,[12] and showed that such an approach is feasible. However, since the key glycosyl donor used in the cited work lacked a selectively removable protecting group at the site of further extension of the oligosaccharide chain, the overall reaction scheme was not applicable to synthesizing higher oligosaccharides. Here we describe a new approach to the synthesis of tetra- and hexasaccharide fragments of the O-PS of *Vibrio cholerae* O:1, serotype Inaba, from intermediates having the *N*-acyl side chain already in place, and conjugation thereof to bovine serum albumin (BSA).

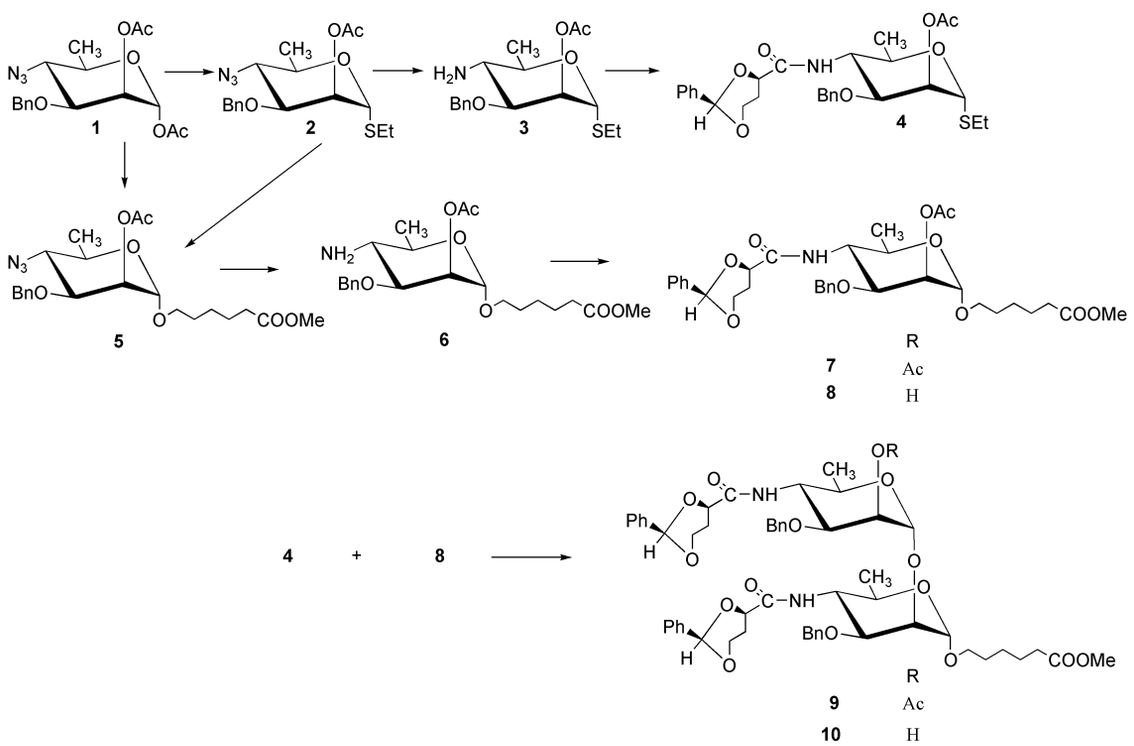
The starting point in the synthesis was the known[5,10] diacetate **1**, which was converted to the amine **3** through the thioglycoside **2**. *N*-Acylation of **3** with 2,4-*O*-benzylidene-L-*glycero*-tetronic acid (**29**) gave the key glycosyl donor **4** having a selectively removable protecting group at O-2 allowing extension of the oligosaccharide chain at that position. The acid **29**, previously obtained by saponification of the corresponding *t*-butyl ester[13] (**28**) has now been obtained directly from the triol (**27**)[13] by oxidation with pyridinium dichromate (Experimental, Scheme 1).



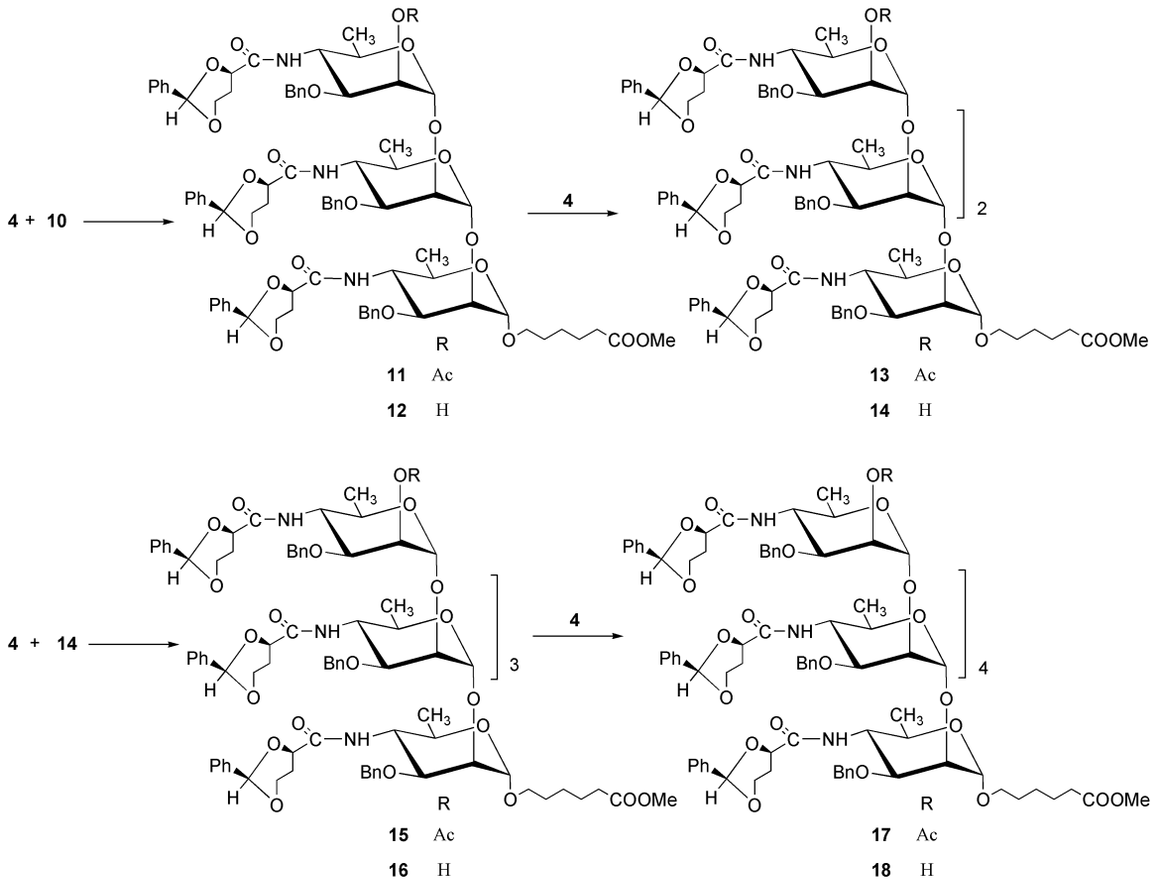
Scheme 1

To obtain the initial glycosyl acceptor (Scheme 2), the diacetate **1** was first converted to 5-methoxycarbonyl glycoside **5** *via* the thioglycoside **2**. The yield (79%) was satisfactory but a more direct route, which gave a comparable yield (75%) of the desired compound **5**, was found when **1** was treated with 5-methoxycarbonyl pentanol[14] in the presence of $SnCl_4$, as described by Banoub and Bundle[15] for similar conversions. Subsequent reduction of the azido group in **5** with H_2S, followed by *N*-acylation of the resulting amine **6**, as described for **3**, gave the fully protected compound **7**, which upon conventional deacetylation (Zemplén) gave the desired alcohol **8**. Having secured the aforementioned key intermediates **4** and **8** we could proceed with oligosaccharide syntheses in the stepwise manner. We opted for the sequential approach, rather than the blockwise approach because, in separate work, we intend to use intermediates obtained in this way to make each of the monothrough to the pentasaccharide–BSA constructs in the serotype Ogawa series. Similar hexasaccharide constructs showed sufficient immunogenicity and protective capacity to have potential as a cholera vaccine.[3] The lower oligosaccharide–BSA constructs will be used to identify the smallest-size hapten that can be used as the antigenic component in the target synthetic vaccine for cholera. Thus, the *N*-iodosuccinimide/silver trifluoromethanesulfonate-mediated (NIS/AgOTf) reaction of **4** with **8** gave the fully protected disaccharide **9**. Deacetylation of **9**, followed by reaction of the formed disaccharide glycosyl acceptor **10** with the donor **4** afforded (Scheme 3) the fully protected trisaccharide **11**. Repetition of this sequence of reaction three more times gave, eventually, the fully protected tetra-, penta-, and the hexasaccharides, **13**, **15**, and **17**, respectively. Deacetylation of the tetrasaccharide **13** (**13** ⟶ **14**) and hydrogenolysis, to simultaneously remove the protecting benzyl and benzylidene groups, gave the fully deprotected methyl ester **19**. The analogous hexasaccharide **23**, which showed characteristics identical to the previously described substance,[11] was obtained in a similar way.

The deprotected tetra- and hexasaccharides **19** and **23** are glycosides whose aglycon allows further conversions to substances amenable to conjugation to amine-containing molecules by squaric acid chemistry.[16] We have studied the efficiency of this method in detail,[4,17,18] and have concluded that, when applied to synthetic oligosaccharides, it is superior to conjugation by reductive amination. Conjugation applying squaric acid chemistry is based on the findings by Tietze *et al.*[19,20] who showed that squaric acid diesters react with amines at different



Scheme 2



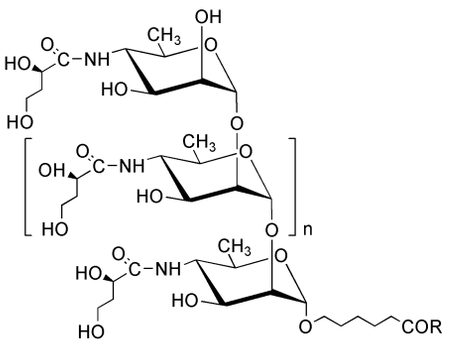
Scheme 3

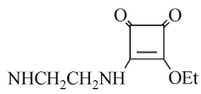
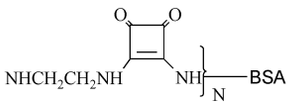
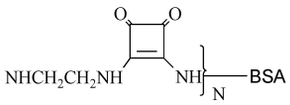
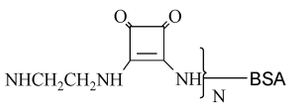
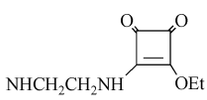
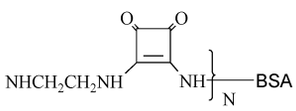
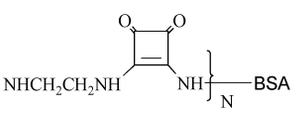
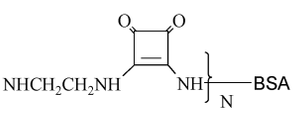
pH to give either symmetrical or unsymmetrical squaric acid diamides. Thus, the 5-methoxycarbonylpentyl glycosides **19** and **23** were first treated with ethylenediamine (⟶**20** and ⟶**24**, respectively), and then with 3,4-diethoxy-3-cyclobutene-1,2-dione (squaric acid diethyl ester) at pH 7. The resulting squaric acid monoamides **21** and **25**, respectively, were isolated by chromatography and treated with BSA at pH 9 to complete the conjugation protocol.[19,20]

Monitoring the conjugation reaction by surface enhanced laser desorption ionization-time of flight mass spectrometry (SELDI-TOF MS) in combination with the ProteinChip® System[21] can furnish valuable data concerning the conjugation process. We have shown[21] that this technique provides virtually real-time information about the increasing molecular weight of the neoglycoconjugate formed, and allows the reaction to be terminated when the desired carbohydrate/BSA ratio had been reached. Thus, the ability to use the ProteinChip® System as a tool to monitor conjugation of synthetic carbohydrates to proteins has become a turning point in the preparation of neoglycoconjugates. During this work, it was interesting to compare the efficiency of conjugation of the hexasaccharide **25** with BSA with that of the corresponding monosaccharide. In the latter case, under optimum conditions,[17] using chicken serum albumin (CSA) as a carrier (containing 46 lysine residues/CSA), a neoglycoconjugate with 29 saccharide residues/CSA was obtained after two days of reaction time. Our first conjugate made from the hexasaccharide **25** was targeted to have the **25**/BSA ratio (loading) of 5:1. Based on the reaction rate observed with the monosaccharide[17] we presumed that the targeted loading should be possible to achieve by conducting the conjugation at the initial **25**/BSA molar ratio of 10:1, namely with a 100% excess of the hapten. However, the conjugation under these conditions was much slower than in the case of the monosaccharide,[17] and no increase in the degree of substitution beyond the final **25**/BSA ratio of 2.9 was noted after 15 days. Further extension of the reaction time was deemed futile, since after 15 days there would have been only little of the monoester **25** left in the reaction mixture, due to the hydrolysis of the reagent.[4]

Based on the above observations, all further conjugations were carried out at the hapten/BSA ratio of 100:1. Under these conditions, reaction of the tetrasaccharide **21** gave conjugate **22B** (**21**/BSA=11.6, see Experimental) after a reaction time of 2 days.

A more efficient conjugation protocol was designed next. As described in the Experimental, two glycoconjugates with predetermined saccharide/carrier ratio were made from the tetrasaccharide **21** and hexasaccharide **25**, each in a one-pot reaction. To wit, for example, our aim was to obtain the hexasaccharide-based conjugates having molar carbohydrate/BSA ratio of 6:1 and 20:1. The reaction was set up so that at the onset of the reaction the molar **25**/BSA ratio was 100:1. With the 59 lysine groups in BSA[22,23] (molecular mass 66,430 Da) we deemed this excess to be sufficient to achieve the required higher carbohydrate-onto-protein loading, while maintaining a reasonable reaction rate. When the molar carbohydrate/BSA ratio of ~6:1 had been reached (3 h, as shown by SELDI-TOF MS), a portion of the reaction mixture was withdrawn and processed. The rest of the material was allowed to react further, until SELDI-TOF MS showed that the molecular mass of the conjugate that was being formed became close to 20 (22 h). After processing and freeze-drying, the products **26A** and **26C** were obtained as white solids whose molecular mass was virtually the same as that to the predetermined ones (see attached formulae and Experimental). Neoglycoconjugates from the tetrasaccharide monoamide **21** (**22A** and **22C**; targeted ratios hapten/BSA of 5 and 15, respectively) were obtained in a similar way (see Experimental). In the conjugations described above, both the tetra- and the hexasaccharide monoamides appeared to be less reactive than the corresponding monosaccharide,[17] but we can not explain the apparent higher reactivity of the hexasaccharide **25**, compared to that of the analogous tetrasaccharide **21**.



	n	N	R
19	2		OCH_3
20	2		$NHCH_2CH_2NH_2$
21	2		
22A	2	4.5	
22B	2	11.6	
22C	2	14.6	
23	4		OCH_3
24	4		$NHCH_2CH_2NH_2$
25	4		
26A	4	2.9	
26B	4	6.6	
26C	4	19	

General methods

Unless stated otherwise, optical rotations were measured at ambient temperature for solutions in chloroform (*c* ~1), with a Perkin-Elmer automatic polarimeter, Model 341. All reactions were monitored by thin-layer chromatography (TLC) on silica gel 60 coated glass slides (Whatman or Analtech). Column chromatography was performed by gradient elution from columns of silica gel. Solvent mixtures less polar than those used for TLC were used at the onset of development. NMR spectra were measured at 300 MHz (^{1}H) and 75 MHz (^{13}C), with a Varian Mercury spectrometer. Assignments of NMR signals were made by first-order analysis of the spectra and, when feasible, the assignments were supported by homonuclear decoupling experiments or homonuclear and heteronuclear 2-dimensional correlation spectroscopy, run with the software supplied with the spectrometers. Spectra of higher oligosaccharides were also analyzed by comparison with spectra of related substances reported previously from this laboratory[8–11,24] or elsewhere.[7] When the latter approach was used, to aid in the ^{13}C NMR signal–nuclei assignments, advantage was taken of variations of line intensity expected for oligosaccharides belonging to the same homologous series.[25,26] Thus, spectra showed close similarity of chemical shifts of equivalent carbon atoms of the internal residues, and an increase in the relative intensity of these signals with the increasing number of *N*-acylated D-perosamine residues in the molecule. When reporting assignments of NMR signals of oligosaccharides, sugar residues in oligosaccharides are serially numbered, beginning with the one bearing the aglycone, and are identified by a Roman numeral superscript in listings of signal assignments. Nuclei-assignments without a superscript notation indicate that those signals have not been individually assigned. Thus, for example, in a spectrum of a pentasaccharide, a resonance denoted H-3 could be that of H-3 of either sugar residue. When reporting NMR data, the nuclei belonging to the 3-deoxy-L-*glycero*-tetronic acid side chain are referred to as primed (′), and those of the spacer aglycon as double primed (″). Surface enhanced laser desorption ionization-time of flight mass spectrometry (SELDI-TOF MS) was done using PBS-II Mass Reader in combination with the ProteinChip® System (Ciphergen Biosystems, Inc.), as previously described.[21] Attempts have been made to obtain correct analytical data for all new compounds. However, some compounds tenaciously retained traces of solvents, despite exhaustive drying, and analytical figures for carbon could not be obtained within 0.3%. The structure of these compounds is evident from the mode of syntheses and *m*/*z* values found in their mass spectra, and the purity was verified by TLC and NMR spectroscopy. These materials were obtained as white solids, which showed mps within 2–3 °C. However, since we were unable to

induce these materials to crystallization from common organic solvents, we describe them below as amorphous solids. Ethylenediamine was freshly distilled, bp 115–116 °C. Palladium-on-charcoal catalyst (5%, ESCAT 103) was a product of Engelhard Industries. 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDAC), (*S*)-1,2,4-butanetriol, and 1-hydroxybenzotriazole (HOBT) were purchased from Aldrich Chemical Co., and used as supplied. *t*-Butanol was dried with molecular sieves 3 Å, and used without distillation. Sep-Pak C18 cartridges were products of Waters Corporation, Inc. *t*-Butyl 2,4-*O*-benzylidene-3-deoxy-L-*glycero*-tetronate (**28**) was made, essentially, as reported. However, the procedure given in the Experimental is described in more detail than previously, and it also lists complete assignment of lines in the ^{13}C NMR spectrum of **28**. 2,4-*O*-Benzylidene-3-deoxy-L-*glycero* tetronic acid (**29**) was prepared (Scheme 1) by saponification of the ester **28**[13] or by oxidation of 1,2,4-(*S*)-butanetriol (**27**), as described below.

Ethyl 4-amino-2-*O*-acetyl-3-*O*-benzyl-4,6-dideoxy-1-thio-α-D-mannopyranoside (3)

Hydrogen sulfide was passed, for 45 min at 40 °C, through a solution of ethyl 2-*O*-acetyl-4-azido-3-*O*-benzyl-4,6-dideoxy-1-thio-α-D-mannopyranoside[7] (**2**, 43 g, 117.6 mmol) in pyridine–water (2:1, v/v, 750 mL). The mixture was stirred at 40 °C in a flask, which was equipped with a rubber septum and a rubber balloon. After 3 h, TLC (30:1 CH_2Cl_2–MeOH) showed that all the starting material was consumed, and that a more polar product was formed. Nitrogen gas was passed through the brown solution until almost no discoloration was noticeable, and the mixture was concentrated. The residue was triturated with EtOAc and the solids were filtered off. The filtrate was concentrated, and crystallization from EtOAc gave **3** (35 g), the mother liquid was chromatographed, to give another crop of **3** (2.9 g), total yield 95%, mp 91–92° (from isopropyl ether), $[\alpha]_D$ +59° (*c* 0.5); ^1H NMR ($CDCl_3$): δ 5.42 (dd, 1 H, $J_{1,2}$ 1.4, $J_{2,3}$ 3.1 Hz, H-2), 5.25 (d, 1 H, H-1), 4.68, 4.38 (2 d, 1 H each, ^2J 11.3 Hz, CH_2Ph), 3.91 (m, 1 H, H-5), 3.53 (dd, 1 H, $J_{3,4}$ 9.8 Hz, H-3), 2.90 (t, 1 H, H-4), 2.62 (m, 2 H, SCH_2), 1.43 (bs, 2 H, NH_2), 1.30 (d, partially overlapped, $J_{5,6}$ ~5.8 Hz, H-6), 1.28 (t, partially overlapped, CH_2CH_3); ^{13}C NMR ($CDCl_3$): δ 82.51 (C-1), 77.96 (C-3), 71.23 (*C*H_2Ph), 69.96 (C-5), 68.86 (C-2), 54.19 (C-4), 25.45 (SCH_2), 20.98 ($COCH_3$), 17.98 (C-6), 14.87 (CH_2CH_3); FABMS: *m*/*z* 340 ($[M + 1]^+$), 362 ($[M + Na]^+$). *Anal.* Calcd for $C_{17}H_{25}NO_4S$: C, 60.15; H, 7.42; N 4.13. Found: C, 59.90; H, 7.34; N, 4.07.

Ethyl 2-*O*-acetyl-3-*O*-benzyl-4-(2,4-*O*-benzylidene-3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-1-thio-α-D-mannopyranoside (4)

A mixture of the foregoing amine **3** (12 g, 35.3 mmol), acid **29** (8.9 g, 42.4 mmol), EDAC (10.0 g, 51.4 mmol), and HOBT (7.2 g, 51.4 mmol) in CH_2Cl_2 (800 mL) was stirred at room temperature for 1 h. TLC (30:1 CH_2Cl_2–MeOH) showed that the starting material was consumed, and that a less polar product was formed. The reaction mixture was concentrated, and the residue was chromatographed (2:1, hexane–acetone) to give **4** (18.1 g, 97%), mp 52.5–53.5° (from isopropyl ether); $[\alpha]_D$ +35.6° (*c* 0.5). ^1H NMR ($CDCl_3$): δ 6.22 (d, 1 H, $J_{4,NH}$ 8.8 Hz, NH), 5.55 (s, 1 H, C*H*Ph), 5.44 (dd, 1 H, $J_{1,2}$ 1.6, $J_{2,3}$ 3.1 Hz, H-2), 5.23 (d, 1 H, H-1), 4.64, 4.36 (2 d, partially overlapped, ^2J 12.0 Hz, CH_2Ph), 4.38–4.32 (m, partially overlapped, H-2′,4′a), 4.13–3.96 (m, 3 H, H-4,5,4′b), 3.70 (dd, 1 H, $J_{3,4}$ 10.1 Hz, H-3), 2.69–2.50 (m, 2 H, SCH_2), 2.15 (s, 3 H, $COCH_3$), 2.06, 1.91 (2 m, 2 H, H-3′), 1.27 (t, partially overlapped, *J* 7.4 Hz, CH_2CH_3), 1.24 (d, partially overlapped, $J_{5,6}$ 5.8, H-6); ^{13}C NMR ($CDCl_3$): δ 101.1 (*C*HPh), 82.25 (C-1), 76.42 (C-2′), 74.15 (C-3), 71.02 (*C*H_2Ph), 69.08 (C-2), 69.0 (C-5), 67.25 (C-4′), 52.36 (C-4), 28.53 (C-3′), 25.49 (SCH_2), 21.06 ($COCH_3$), 17.81 (C-6), 14.80 (CH_2CH_3); CIMS: *m*/*z* 530 ($[M + 1]^+$), 547 ($[M + NH_4]^+$). *Anal.* Calcd for $C_{28}H_{35}NO_7S{\cdot}0.5\ H_2O$: C, 62.43; H, 6.73; N, 2.59. Found: C, 62.39; H, 6.54; N, 2.56.

5-Methoxycarbonylpentyl 2-*O*-acetyl-4-azido-3-O-benzyl-4,6-dideoxy-α-D-mannopyranoside (5)

a. A mixture of ethyl 2-*O*-acetyl-4-azido-3-*O*-benzyl-4,6-dideoxy-1-thio-α-D-mannopyranoside[7] (**2**, 8.0 g, 21.9 mmol), 5-methoxycarbonylpentanol[27] (5.0 g, 34.2 mmol) and 4 Å molecular sieves (6 g)) in dry CH_2Cl_2 (30 mL) was stirred for 15 min at room temperature. The mixture was cooled to −20 °C, and NIS (6.9 g, 32.4 mmol) was added, followed by solid AgOTf (2.1 g, 8.2 mmol, 0.37 equiv.). A few minutes later, the mixture turned red and stirring was continued for another 15 min, when the reaction was quenched by the addition of Et_3N (2 mL). The mixture was partitioned between a mixture of aq. $Na_2S_2O_3$ and $NaHCO_3$, the organic layer was dried, and concentrated. Chromatography (7:1 hexane–EtOAc) gave **5** (7.8 g, 79%), which was identical (TLC, NMR) with the compound described below.

b. $SnCl_4$ (0.35 mL, 2.75 mmol) was added to a solution of 1,2-di-*O*-acetyl-4-azido-3-*O*-benzyl-4,6-dideoxy-α-D-mannopyranose[7] (**1**, 1 g, 2.75 mmol) in dry CH_2Cl_2 (30 mL) followed by a solution of 5-methoxycarbonylpentanol[27] (525 mg, 3.6 mmol) in dry CH_2Cl_2 (2 mL) and the solution was stirred at room temperature until TLC (5:1 hexane–EtOAc) showed that only a little starting material remained (5–7 h). One product, whose chromatographic mobility was very close to that of the starting **1**, was formed. The mixture was neutralized with aqueous $NaHCO_3$, and the product was extracted with CH_2Cl_2. The organic phase was dried, concentrated, and chromatography gave the amorphous **5** (930 mg, 75%), $[\alpha]_D$ +16° (*c* 0.95). ^1H NMR ($CDCl_3$): δ 5.32 (dd, 1 H, $J_{1,2}$ 1.8, $J_{2,3}$ 3.3 Hz, H-2), 4.71 (d, partially overlapped, H-1), 4.69, 4.53 (2 d, 1 H each, ^2J 11.0 Hz, CH_2Ph), 3.81 (dd, 1 H, $J_{3,4}$ 9.8 Hz, H-3), 3.68 (s, partially overlapped, $COOCH_3$), 3.68–3.48 (m, 2 H, H-5,1a″), 3.43–3.34 (m, 2 H, 1b″, incl 3.40, t, partially overlapped, H-4), 2.33 (t, 2 H, *J* 7.5 Hz, H-5″ a,b), 2.12 (s 3 H, $COCH_3$), 1,70–1.53 (m, 4 H, H-4″a,b,2″a,b in that order), 1.44–1.31 (m, 6 H, H-3″a,b, incl 1.32, d, $J_{5,6}$ 6.3 Hz, H-6); ^{13}C NMR ($CDCl_3$): δ 97.60 (C-1), 76.13 (C-3), 71.56 (*C*H_2Ph), 67.66 (C-1″), 67.43 (C-2), 66.83 (C-5), 63.96 (C-4), 51.47 ($COOCH_3$), 33.85 (C-5″ 28.95 (C-2″), 25.61 (C-3″), 24.59 (C-4″), 20.96 $COCH_3$), 18.44 (C-6); FABMS: *m*/*z* 450 ($[M + 1]^+$), 472 ($[M + Na]^+$). *Anal.* Calcd for $C_{22}H_{31}N_3O_7S$: C, 58.78; H, 6.95; N 9.35. Found: C, 58.98; H, 6.87; N, 9.34.

5-Methoxycarbonylpentyl 2-*O*-acetyl-4-amino-3-*O*-benzyl-4,6-dideoxy-α-D-mannopyranoside (6)

Compound **5** (8.0 g, 17.8 mmol) was treated as described for the preparation of **3**. After processing, as described above, chromatography gave amorphous **6** (7.1 g, 94%), $[\alpha]_D$ −2.4° (*c* 0.7). ^1H NMR ($CDCl_3$): δ 5.33 (dd, 1 H, $J_{1,2}$ 1.8, $J_{2,3}$ 3.1 Hz, H-1), 4.75 (d, H-1), 4.71, 4.39 (2 d, 1 H each, ^2J 11.1 Hz, CH_2Ph), 3.69–3.55 (m, 6 H, H-3,5, 1a″ incl 3.68, s, $COOCH_3$), 3.40, 3.37 (2 t, partially overlapped, *J* 6.3 Hz, 1 H, 1b″), 2.88 (t, 1 H, *J* 10.1 Hz, H-4), 2.33 (t, 2 H, *J* 7.5 Hz, H-5″a,b), 2.12 (s 3 H, $COCH_3$), 1.70–1.52 (m, 4 H, H-4″a,b,2″a,b, in that order), 1.47–1.35 (m, 4 H, NH_2, H-3″a,b), 1.28 (d, $J_{5,6}$ 6.3 Hz, H-6); ^{13}C NMR ($CDCl_3$): δ 97.98 (C-1), 77.68 (C-3), 71.26 (*C*H_2Ph), 69.42 (C-5), 67.42 (C-1″), 67.15 (C-2), 53.83 (C-4), 51.47 ($COOCH_3$), 33.89 (C-5″), 29.04 (C-2″), 25.70 (C-3″), 24.64 (C-4″), 21.02 $COCH_3$), 18.04 (C-6). CIMS: *m*/*z* 424 ($[M + 1]^+$). *Anal.* Calcd for $C_{22}H_{33}NO_7$: C, 62.39; H, 7.85; N, 3.31. Found: C, 62.25; H, 7.92; N, 3.31.

5-Methoxycarbonylpentyl 2-O-acetyl-3-*O*-benzyl-4-(2,4-*O*-benzylidene-3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranoside (7)

A mixture of the foregoing amine **6** (4.5 g, 10.6 mmol), 3-deoxy-L-*glycero*-tetronic acid derivative **29** (3.4 g, 16.2 mmol), EDAC

(4.1 g, 32.4 mmol), and HOBT (2.9 g, 32.4 mmol) in CH_2Cl_2 (300 mL) was stirred at room temperature for 1 h. TLC (20:1 CH_2Cl_2–MeOH) showed that the reaction was complete, and that a less polar product was formed. The reaction mixture was concentrated, and the residue was chromatographed, to give amorphous **7** (5.4 g, 83%); $[\alpha]_D$ + 6.0° (*c* 0.6). ^{1}H NMR ($CDCl_3$): δ 6.33 (d, 1 H, $J_{4,NH}$ 9.7 Hz, NH), 5.55 (s, 1 H, C*H*Ph), 5.33 (dd, 1 H, $J_{1,2}$ 1.8, $J_{2,3}$ 3.1 Hz, H-2), 4.74 (d, 1 H, H-1), 4.64 (d, 1 H, ^2J 11.6 Hz, CH_aPh), 4.39 (d, partially overlapped, CH_bPh), 4.39–4.10 (m, partially overlapped, H-2′,4′a), 4.05 (t, partially overlapped, $J_{4,5}$ ~10.5 Hz, H-4), 4.05–3.98 (m, partially overlapped, H-4′b), 3.79 (dd, partially overlapped, H-3), 3.82–3.74 (m, partially overlapped, H-5), 3.64, 3.62 (2 t, partially overlapped, *J* 6.6 Hz, H-1″a), 3.60 (s, partially overlapped, $COOCH_3$), 3.38, 3.35 (2 t, 1 H, H-1″b), 2.29 (t, 2 H, *J* 7.9 Hz, H-5″a,b), 2.14 (s, 3 H, $COCH_3$), 2.08–1.87 (m, 2 H, H-3′a,b), 1.68–1.53 (m, 4 H, H-4″a,b,2″a,b), 1.39–1.30 (m, 2 H, H-3″a,b), 1.24 (d, 3 H, $J_{5,6}$ 6.3 Hz, H-6); ^{13}C NMR ($CDCl_3$): δ 101.20 (*C*HPh), 97.73 (C-1), 76.57 (C-2′), 74.09 (C-3), 71.12 (*C*H_2Ph), 67.55 (C-1″), 67.57, 67.52 (C-2,5), 67.30 C-4′), 52.16 (C-4), 51.42 ($COOCH_3$), 33.87 (C-5″), 28.84 C-2″), 28.56 (C-3′), 24.55 (C-4″), ($COCH_3$) 17.85 (C-6). CIMS: *m*/*z* 614 ([M + 1]$^+$), 631 ([M + NH_4]$^+$). *Anal*. Calcd for $C_{33}H_{43}NO_{10}$: C, 64.58; H, 7.06; N, 2.28. Found: C, 64.64; H, 6.91; N, 2.29.

5-Methoxycarbonylpentyl 3-*O*-benzyl-4-(2,4-*O*-benzylidene-3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-manno-pyranoside (8)

Freshly prepared 1 M methanolic NaOMe (2.5 mL) was added to a solution of **7** (4.6 g, 7.5 mmol) in dry MeOH (100 mL). After 30 min, TLC (2:1 hexane-acetone) showed that no starting material was present, and that a more polar compound was formed. The solution was neutralized with Amberlite IR 120 (H^+) resin, the mixture was filtered, the filtrate was concentrated, and the residue was chromatographed, to give **8** (4.27 g, ~100%), mp 87–87.5 °C, (from EtOAc–hexane), $[\alpha]_D$ −20.4° (*c* 0.7). ^{1}H NMR ($CDCl_3$): δ 6.37 (d, 1 H, $J_{4,NH}$ 9.7 Hz, NH), 5.56 (s, 1 H, C*H*Ph), 4.82 (d, 1 H, $J_{1,2}$ 1.8 Hz, H-1), 4.67, 4.52 (2 d, 1 H each, ^2J 12.0 Hz, CH_2Ph), 4.40–4.31 (m, 2 H, H-2′,4′a), 4.07 (t, partially overlapped, $J_{4,5}$ ~10.2 Hz, H-4), 4.05–4.00 (m, partially overlapped, H-4′b), 3.98 (dd, partially overlapped, H-2), 3.78 (m, partially overlapped, H-5), 3.71 (dd, partially overlapped, H-3), 3.68–3.58 (m, 4 H, H-1″a, incl s at 3.60, $COOCH_3$), 3.40, 3.37 (2 t, *J* 6.5 Hz, 1 H, H-1″b), 2.58 (bs, 1 H, OH), 2.29 (t, 2 H, *J* 7.9 Hz, H-5″a,b), 2.08–1.85 (m, 2 H, H-3′a,b), 1.68–1.53 (m, 4 H, H-2″a,b,4″a,b), 1.39–1.28 (m, 2 H, H-3″a,b), 1.22 (d, 3 H, $J_{5,6}$ 6.2 Hz, H-6); ^{13}C NMR ($CDCl_3$): δ 101.13 (*C*HPh), 99.02 (C-1), 76.52 (C-2′), 76.47 (C-3), 71.42 (*C*H_2Ph), 67.38 (C-1″), 67.25 C-4′), 67.23 (C-2), 66.93 (C-5), 51.69 (C-4), 51.40 ($COOCH_3$), 33.89 (C-5″), 28.87 (C-2″), 28.57 (C-3′), 25.59 (C-3″), 24.56 (C-4″), 17.85 (C-6). CIMS: *m*/*z* 572 ([M + 1]$^+$), 589 ([M + NH_4]$^+$). *Anal.* Calcd for $C_{31}H_{41}NO_9$: C, 65.13; H, 7.23; N, 2.45. Found: C, 64.91; H, 7.20; N, 2.40.

5-Methoxycarbonylpentyl 2-*O*-acetyl-3-*O*-benzyl-4-(2,4-*O*-benzylidene-3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranosyl-(1⟶2)-3-*O*-benzyl-4-(2,4-*O*-benzylidene-3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranoside (9)

To a solution of **4** (9.12 g, 17.2 mmol) and **8** (8.20 g, 14.36 mmol) in dry CH_2Cl_2 (30 mL), were added 4 Å molecular sieves (13 g), and the mixture was stirred for 15 min at room temperature. The mixture was cooled to −20 °C, and NIS (4.5 g, 20.3 mmol) was added, followed by solid AgOTf (1.4 g, 5.6 mmol). When the mixture turned dark red, the mixture was stirred for another 15 min, and the reaction was processed as described for the preparation of **5** (procedure a). Chromatography (3:1⟶1:1 hexane–acetone) gave crystalline **9** (12.8 g, 86%), mp 70.5–71.5 °C (from EtOAc–hexane), $[\alpha]_D$ −23° (*c* 0.6); ^{1}H NMR ($CDCl_3$): δ 6.37, 6.17 (2 d, 1 H each, $J_{4,NH}$ 8.3 and 9.7, respectively, 2 NH), 5.55, 5.54 (2 s, 1 H each, C*H*Ph), 5.49 (dd, 1 H, $J_{1,2}$ 1.8, $J_{2,3}$ 3.1 Hz, H-2^{II}), 4.90 (d, 1 H, H-1^{II}), 4.70–4.40 (4 d, partially overlapped, 2 *C*H_2Ph), 4.66 (d, partially overlapped, $J_{1,2}$ ~1.7 Hz, H-1^I), 4.38–4.30 (m, 4 H, H-2′^{I,II}, 4′a^{I,II}), 4.10 (q, partially overlapped, H-4^{II}), 4.00, 3.99 (2 t, partially overlapped, *J* 11.0 Hz, H-4′b^{I,II}), 3.90–3.71 (m, 6 H, H-2^I,3^I,4^I,5^{I,II}, incl dd at 3.73, $J_{2,3}$ ~3.1, $J_{3,4}$ ~10.7 Hz, H-3^{II}), 3.62–3.54 (m, partially overlapped, H-1″a), 3.60 (s, partially overlapped, $COOCH_3$), 3.32–3.29 (m, 1 H, H-1″b), 2.27 (t, 2 H, *J* 7.4 Hz, H-5″a,b), 2.09 (s, partially overlapped, $COCH_3$), 2.11–1.82 (m, partially overlapped, H-3′a,b^{I,II}), 1.66–1.50 (m, 4 H, H-4″a,b,2″a,b in that order) 1.37–1.29 (m, 2 H, H-3″a,b), 1.20, 1.17 (2 d, 6 H, $J_{5,6}$ 6.2 and 6.0 Hz, H-6^{I,II}). ^{13}C NMR ($CDCl_3$): δ 101.35, 101.11 (2 *C*HPh), 99.81 (C-1^{II}), 98.82 (C-1^I), 76.58 (2 C, C-2′^{I,II}), 75.51 (C-3^I), 74.43 (C-2^I), 73.43 (C-3^{II}), 71.81, 70.99 (2 *C*H_2Ph), 68.52 (C-5), 67.28 (4 C, C-5,4′^{I,II},1″), 67.15 (C-2^{II}), 52.66 (C-4^I), 51.54 (C-4^{II}), 51.40 ($COOCH_3$), 33.83 (C-5″), 28.81 (C-2″), 28.54 (C-3′), 25.51 (C-3″), 24.50 (C-4″), 21.03 ($COCH_3$), 17.96 (2C, C-6^{I,II}). FABMS: *m*/*z* 1039.5 ([M + 1]$^+$), 1062.5 ([M + Na]$^+$). *Anal.* Calcd for $C_{57}H_{70}N_2O_{16}$·0.5H_2O: C, 65.31; H, 6.82; N, 2.67. Found: C, 65.01; H, 6.69; N, 2.65.

5-Methoxycarbonylpentyl 3-*O*-benzyl-4-(2,4-*O*-benzylidene-3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-manno-pyranosyl-(1⟶2)-(3-*O*-benzyl-4-(2,4-*O*-benzylidene-3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranoside (10)

The foregoing fully protected compound **9** (4.6 g) was treated with NaOMe as described for the preparation of **8**. TLC showed complete conversion of the starting material and formation of a more polar product. After processing, as described above, and chromatography, compound **10** (4.4 g, ~100%) was obtained as a white solid showing $[\alpha]_D$ −9° (*c* 0.5); ^{1}H NMR ($CDCl_3$): 6.34, 6.24 (2 d, 1 H each, $J_{4,NH}$ 9.6 Hz, NH), 5.55, 5.54 (2 s, 1 H each, C*H*Ph), 5.07 (d, 1 H, $J_{1,2}$ 1.5 Hz, H-1^{II}), 4.70–4.44 (4 d, partially overlapped, 2 CH_2Ph), 4.68 (d, partially overlapped, $J_{1,2}$ ~1.8 Hz, H-1^I), 4.39–4.29 (m, 4 H, H-2′^{I,II}, 4′a^{I,II}), 4.22 (bdd, 1 H, H-2^{II}), 4.16 (q, partially overlapped, H-4^{II}), 4.03–3.95 (m, 4 H, H-2^I,4^I,H-4′b^{I,II}), 3.77–3.66 (m, 4 H, 5^{I,II}, incl dd at ~3.75, $J_{2,3}$ ~2.7, $J_{3,4}$ ~10.6 Hz for H-3^I, and dd at ~3.68, $J_{2,3}$ ~3.3, $J_{3,4}$ 10.6 Hz for H-3^{II}), 3.62–3.54 (m, partially overlapped, H-1″a), 3.58 (s, partially overlapped, $COOCH_3$), 3.35–3.28 (m, 1 H, H-1″b), 2.60 (bs, 1 H, OH), 2.27 (t, 2 H, *J* 7.4 Hz, H-5″a,b), 2.06, 1.90 (2 m, H-3′a,b^{I,II}), 1.66–1.50 (m, 4 H, H-4″a,b,2″a,b in that order), 1.37–1.28 (m, 2 H, H-3″a,b), 1.20, 1.18 (2 d, 6 H, $J_{5,6}$ 6.4 Hz, H-6^{I,II}); ^{13}C NMR ($CDCl_3$): δ 101.24, 101.07 (2 *C*HPh), 101.92 (C-1^{II}), 98.93 (C-1^I), 76.49, 76.40 (C-2′^{I,II}), 75.94 (C-3^I), 75.64 (C-3^{II}), 73.41 (C-2^I), 71.78, 71.03 (2 *C*H_2Ph), 67.84, 67.58 (C-5^{I,II}), 67.21 (C-1″), 67.18 (2 C, C-4′^{I,II}), 66.57 (C-2^{II}), 52.05 (C-4^I), 51.33 ($COOCH_3$), 51.00 (C-4^{II}), 33.75 (C-5″), 28.72 (C-2″), 28.47 (C-3′^{I,II}), 25.44 (C-3″), 24.42 (C-4″), 17.88, 17.79 (C-6^{I,II}); FABMS: *m*/*z* 997.5 ([M + 1]$^+$), 1019.5 ([M + Na]$^+$). *Anal.* Calcd for $C_{55}H_{68}N_2O_{15}$: C, 66.25; H, 6.87; N, 2.81. Found: C, 65.79; H, 6.85; N, 2.81.

5-Methoxycarbonylpentyl 2-*O*-acetyl-3-*O*-benzyl-4-(2,4-*O*-benzylidene-3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranosyl-(1⟶2)-(3-*O*-benzyl-4-(2,4-*O*-benzylidene-3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranosyl-(1⟶2)-3-*O*-benzyl-4-(2,4-*O*-benzylidene-3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranoside (11)

Compound **4** (6.5 g, 12.3 mmol) when treated with **10** (10.2 g, 10.2 mmol) as described for the preparation of **9** gave, after chromatography, amorphous **11** (11.9 g, 80%), $[\alpha]_D$ −23° (*c* 0.5). ^{1}H NMR ($CDCl_3$): δ 6.40–6.29 (m, 3 H, incl 2 d at 6.35 and 6.31, partially overlapped, *J* ~9.5 Hz, 3 NH), 5.54, 5.52, 5.51 (3 C*H*Ph), 5.47 (dd, 1 H, $J_{1,2}$ 1.9, $J_{2,3}$ 3.0 Hz, H-2^{III}), 4.99 (d,

1 H, $J_{1,2}$ 1.8 Hz, H-1^{II}), 4.81 (d, 1 H, $J_{1,2}$ 1.8 Hz, H-1^{III}), 4.67–4.52 (m, 6 H, 5 C*H*Ph, benzylic, incl. d at ~4.64, partially overlapped, $J_{1,2}$ ~1.8 Hz, H-1^{I}), 4.41–4.22 (m, 7 H, C*H*Ph, benzylic, H-2′$^{I–III}$,4′a$^{I–III}$), 4.13–3.68 (m, 14 H, H-2^{II},3$^{I–III}$,4$^{I–III}$,5$^{I–III}$,4′b$^{I–III}$, incl bdd at 3.87, H-2^{I}), 3.60–3.51 (m, 4 H, H-1″a, incl s at 3.53, $COOCH_3$), 3.32–3.25 (m, 1 H, H-1″b), 2.23 (t, 2 H, *J* 7.5 Hz, H-5″), 2.08–1.78 (m, 9 H, H-3′a,b, incl s at 2.06, $COCH_3$), 1.63–1.47 (m, 4 H, H-2″a,b,4″a,b), 1.34–1.22 (m, 2 H, H-3′$^{I–III}$a,b), 1.18, 1.17, 1.12 (3 d, partially overlapped, 9 H, *J* ~6.1 Hz, H-6$^{I–III}$); ^{13}C NMR ($CDCl_3$): δ 100.96, 100.91, 100.77 (3 *C*HPh), 101.57 (C-1^{II}), 98.96 (C-1^{III}), 98.67 (C-1^{I}), 76.41, 76.31, 76.20 (C-2′$^{I–III}$), 75.41, 74.34, 73.33 (C-3$^{I–III}$), 74.02 (C-2^{I}), 73.46 (C-2^{II}), 71.36, 71.07, 70.72 (3 *C*H_2Ph), 68.28, 68.14, 67.31 (C-5$^{I–III}$),, 67.06 (C-1″), 66.94 (4 C, C-2^{III},C-4′$^{I–III}$), 51.97, 51.69, 51.45 (C-4$^{I–III}$), 51.08 ($COOCH_3$), 33.53 (C-5″), 28.50 (C-2″), 28.33 (3 C, C-3′$^{I–III}$), 25.24 (C-3″), 24.20 (C-4″), 20.75 ($COCH_3$), 17.90, 17.70, 17.65 (C-6$^{I–III}$). FABMS: *m*/*z* 1464.6 ([M + 1]$^+$), 1486.6 ([M + Na]$^+$). *Anal*. Calcd for $C_{81}H_{97}N_3O_{22}$: C, 66.42; H, 6.68; N, 2.87. Found: C, 66.20; H, 6.70; N, 2.89.

5-Methoxycarbonylpentyl 3-*O*-benzyl-4-(2,4-*O*-benzylidene-3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranosyl-(1⟶2)-3-*O*-benzyl-4-(2,4-*O*-benzylidene-3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranosyl-(1⟶2)-3-*O*-benzyl-4-(2,4-*O*-benzylidene-3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranoside (12)

The fully protected compound **11** (11 g) was deacetylated, as described for the preparation of **10**, to give pure (TLC, NMR) **12** (10.6 g, ~100%) as a white solid, $[\alpha]_D$ −19.4° (*c* 0.4); ^{1}H NMR ($CDCl_3$): δ 6.36–6.27 (bs, partially overlapped, NH), 6.30 (d, partially overlapped, *J* 9.3 Hz, NH), 6.2 (d, 1 H, *J* 9.3 Hz, NH), 5.54, 5.52, 5.51 (3 s, 3 C*H*Ph), 5.03 (d, 1 H, $J_{1,2}$ 1.6 Hz, H-1^{III}), 4.98 (d, 1 H, $J_{1,2}$ 1.9 Hz, H-1^{II}), 4.68–4.47 (m, 6 H, 5 C*H*Ph, incl. d at ~4.64, partially overlapped, H-1^{I}), 4.41–4.25 (m, 7 H, C*H*Ph, H-2′$^{I–III}$,4′a$^{I–III}$), 4.20–4.07 (4 H, H-2^{II,III},3$^{I–III}$,4,4), 4.05–3.84 (m, 5 H, 4,4′b$^{I–III}$ incl bt at 3.87, H-2^{I}), 3.76–3.66 (m, 6 H, H-3$^{I–III}$,5$^{I–III}$), 3.60–3.52 (m, 4 H, H-1″a, incl s at 3.58, $COOCH_3$), 3.34–3.25 (m, 1 H, H-1″b), 2.50 (d, 1 H, *J* 1.5 Hz, OH), 2.26 (t, 2 H, *J* 7.4 Hz, H-5″), 2.09–1.79 (m, 6 H, H-3′$^{I–III}$a,b), 1.65–1.48 (m, 4 H, H-2″a,b,4″a,b), 1.35–1.23 (m, 2 H, H-3″a,b), 1.17, 1.14, 1.07 (3 d, 9 H, *J* 6.2 Hz, H-6$^{I–III}$); ^{13}C NMR ($CDCl_3$): δ 101.28, 101.20 (2 *C*HPh), 101.03 (2 C, *C*HPh, C-1^{II}), 100.35 (C-1^{III}), 98.83 (C-1^{I}), 76.51, 76.42 (2 C, C, C-2′$^{I–III}$), 75.78 (C-3^{III}), 75.27, 75.07 (C-3^{I,II}), 74.33 (C-2^{I}), 72.61 (C-2^{II}), 71.46, 71.41, 71.06 (3 *C*H_2Ph), 66.52, 67.84, 67.38 (5$^{I–III}$), 67.20 (3 C, C-4′$^{I–III}$,1″), 66.67, (C-2^{III}), 52.24, 51.53, 51.14 (C-4$^{I–III}$), 51.33 ($COOCH_3$), 33.75 (C-5″), 28.72 (C-2″), 28.49 (C-3″$^{I–III}$), 25.42 (C-3″), 24.42 (C-4″), 18.04, 17.87, 17.70 (C-6$^{I–III}$). FABMS: *m*/*z* 1422.6 ([M + 1]$^+$), 1444.6 ([M + Na]$^+$). HRMS: *m*/*z* 1554.5477. $C_{79}H_{95}N_3O_{21}Cs$ requires 1554.5512.

5-Methoxycarbonylpentyl 2-*O*-acetyl-3-*O*-benzyl-4-(2,4-*O*-benzylidene-3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranosyl-(1⟶2)-bis[3-*O*-benzyl-4-(2,4-*O*-benzylidene-3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranosyl]-(1⟶2)-3-*O*-benzyl-4-(2,4-*O*-benzylidene-3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranoside (13)

Reaction of the glycosyl donor **4** (3.67 g, 6.94 mmol) and the glycosyl acceptor **12** (8.2 g, 5.77 mmol), as described above for similar glycosylations, afforded **13** (8.71 g, 80%), $[\alpha]_D$ −30° (*c* 0.8). Definite signals in the ^{1}H NMR spectrum ($CDCl_3$) were at: δ 6.42–6.17 (m, 4 H, 4 NH), 5.53, 5.52, 5.51, 5.50 (4 s, 4 H, 4 C*H*Ph), 5.44 (dd, 1 H, $J_{1,2}$ 1.8, $J_{2,3}$ 3.0, H-2^{IV}), 5.01, 4.90 (2 d, $J_{1,2}$ 1.7 Hz, H-1^{II,III}), 4.79 (d, 1 H, $J_{1,2}$ 1.6 Hz, H-1^{IV}), 4.65–4.49 (m, 8 H, 3.5 CH_2Ph, incl d at ~4.61, partially overlapped, H-1^{I}), 4.12–4.08 (m, part of a larger m, H-2^{II,III}), ~3.83 (m, part of a larger m, H-2^{IV}), 3.59–3.50 (m, 4 H, H-1″a, incl s at 3.58, $COOCH_3$), 3.30–3.23 (m, 1 H, H-1″b), 2.25 (t, 2 H, *J* 7.6 Hz, H-5″a,b), 2.05–1.81 (m, 8 H, H-3′$^{I–IV}$a,b), 1.63–1.46 (m, 4 H, H-2″a,b,4″a,b), 1.33–1.21 (m, 2 H, H-3″a,b), 1.13, 1.12. 1.08. 1.02 (4 d, partially overlapped, $J_{5,6}$ 6.2 Hz, H-6$^{I–IV}$); ^{13}C NMR ($CDCl_3$): δ 101.28, 101.21, 101.47 (2 C, C, C, 4 *C*HPh), 101.03, 100.25 (C-1^{II,III}), 99.25 (C-1^{IV}), 98.83 (C-1^{I}), 76.64, 76.58, 76.53 (2 C, C, C, C-2′$^{I–IV}$), 75.27, 74.97, 74.71 (C-3$^{I–III}$), 74.14 (C-2^{I}), 73.78 (2 C, C-2,3), 72.83 (C-2), 71.34, 71.06 (3 C, C, 4 *C*H_2Ph), 68.57, 68.30, 67.52 (2 C, C, C, 5$^{I–IV}$), 67.33 (C-1″), 67.26 (5 C, C-2^{IV},C-4′$^{I–IV}$), 52.18, 52.11, 51.69, 51.62 (C-4$^{I–IV}$), 51.41 ($COOCH_3$), 33.82 (C-5″), 28.79 (C-2″), 28.52 (4 C, C-3′$^{I–IV}$), 25.49 (C-3″), 24.49 (C-4″), 21.05 ($COCH_3$), 18.01, 17.92, 17.87 (2 C, C, C, C-6$^{I–IV}$); FABMS: *m*/*z* 1890.81 ([M + 1]$^+$), 1912.81 ([M + Na]$^+$). HRMS: *m*/*z* 2021.7383. $C_{105}H_{124}N_4O_{28}Cs$ requires 2021.7456.

5-Methoxycarbonylpentyl 3-*O*-benzyl-4-(2,4-*O*-benzylidene-3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranosyl-(1⟶2)-bis[3-*O*-benzyl-4-(2,4-*O*-benzylidene-3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranosyl]-(1⟶2)-3-*O*-benzyl-4-(2,4-*O*-benzylidene-3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranoside (14)

Conventional deacetylation of the tetrasaccharide **13** gave **14** as a white solid in virtually theoretical yield, $[\alpha]_D$ −26.4° (*c* 0.4). Definite signals in the ^{1}H NMR spectrum ($CDCl_3$) were at: δ 6.46–6.16 (m, 4 H, 4 NH), 5.53 (s, 2 H, 2 C*H*Ph), 5.52, 5.50 (2 s, 2 H, 2 C*H*Ph), 5.02 (bs, 2 H, H-1^{IV}, H-1), 4.92 (d, 1 H, $J_{1,2}$ 1.7 Hz, H-1), 4.67–4.27 (m, incl d at ~4.63, partially overlapped, H-1^{I}), 3.83 (bt, 1 H, H-2^{I}), 3.59–3.52 (m, 4 H, H-1″a, incl s at 3.58 $COOCH_3$), 3.32–3.24 (m, 1 H, H-1″b), 2.51 (bd, 1 H, OH), 2.25 (t, 2 H, *J* 7.1 Hz, H-5″), 2.05–1.80 (m, 8 H, H-3′$^{I–IV}$a,b), 1.80–1.64 (m, 4 H, H-2″a,b,4″a,b), 1.34–1.23 (m, 2 H, H-3″a,b), 1.14, 1.10, 1.04 (3 d, 12 H, $J_{5,6}$ 6.3 Hz, H-6$^{I–IV}$); ^{13}C NMR ($CDCl_3$): δ 101.23, 101.13, 101.10, 101.08 (4 *C*HPh), 100.88 (C-1), 100.28 (2 C, C-1,1^{IV}), 98.71 (C-1^{I}), 76.57, 76.46, 76.41 (C, C, 2 C, C-2′$^{I–IV}$), 75.87, 75.19, 75.05, 74.60 (C-3$^{I–IV}$), 74.04 (C-2^{I}), 72.80, 72.67 (C-2^{II,III}), 71.26 (3 C), 71.07 (4 *C*H_2Ph), 68.52, 68.45 (C-5^{II,III}), 67.87. 67.43 (C-5^{I,IV}), 67.15 (5 C, C-4$^{I–IV}$,1″), 66.72 (C-2^{IV}), 52.07, 51.65, 51.52, 51.14 (C-4$^{I–IV}$), 51.31 ($COOCH_3$), 33.72 (C-5″), 28.69 (C-2″), 28.45 (C-3′$^{I–IV}$), 25.41 (C-3″), 24.39 (C-4″), 18.00, 17.92, 17.85. 17.70 (C-6$^{I–IV}$); FABMS: *m*/*z* 1847.85 ([M + 1]$^+$), 1869.85 ([M + Na]$^+$). HRMS: *m*/*z* 1979.7346. $C_{103}H_{122}N_4O_{27}Cs$ requires 1979.7351.

5-Methoxycarbonylpentyl 2-*O*-acetyl-3-*O*-benzyl-4-(2,4-*O*-benzylidene-3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranosyl-(1⟶2)-tris[3-*O*-benzyl-4-(2,4-*O*-benzylidene-3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranosyl]-(1⟶2)-3-*O*-benzyl-4-(2,4-*O*-benzylidene-3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranoside (15)

Glycosylation of **14** (5.0 g, 2.70 mmol) with **4** (2.5 g, 4.72 mmol), as described above for similar reactions gave, after chromatography, the pentasaccharide **15** (5 g, ~80%) as a white solid, $[\alpha]_D$ −35° (*c* 0.7). Definite signals in the ^{1}H NMR spectrum ($CDCl_3$) were at δ: 6.40–6.07 (m, 5 H, 5 NH), 5.54, 5.53, 5.52, 5.51, 5.48 (5 s, 5 H, 5 C*H*Ph), 5.45 (bt, 1 H, H-2^{V}), 5.01, 4.94, 4.89 (3 d, $J_{1,2}$ ~1.6 Hz, H-1$^{II–IV}$), 4.80 (d, 1 H, $J_{1,2}$ 1.6 Hz, H-1^{V}), 4.61 (bd, partially overlapped, part of a larger m, H-1^{I}), 4.13 (bt, partially overlapped, H-2), 3.82 (bt, partially overlapped, H-2^{I}), 3.59–3.50 (m, 4 H, m, H-1″a′ incl s at 3.59, $COOCH_3$), 3.30 (m, 1 H, H-1″b), 2.25 (t, 2 H, *J* 7.5 Hz, H-5″a,b), 2.07–1.81 (m, 13 H, H-3′$^{I–V}$, incl s at 2.07, $COCH_3$), 1.64–1.46 (m, 4 H, H-2″a,b,4″a,b), 1.35–1.24 (m, 2 H, H-3″a,b), 1.13–1.03 (5 d, partially overlapped, 15 H, H-6$^{I–V}$); ^{13}C NMR ($CDCl_3$): δ 101.28, 101.20, 101.15 (C, 3 C, C, 5 *C*HPh), 101.02, 100.45, 100.15 (C-1$^{II–IV}$), 99.18 (C-1^{V}), 98.80 (C-1^{I}), 76.62, 76.54 (2 C, 3 C, C-2′$^{I–V}$a,b), 75.18, 74.96, 74.81, 74.56, 74.29, 73.81, 73.60, 72.80, 72.68 (C-2$^{I–IV}$,3$^{I–V}$), 71.36, 71.27, 71.08, 71.04 (C, 2 C, C, C, 5 *C*H_2Ph), 68.63, 68.57, 68.44, 67.57 (2 C, C, C, C, C-5$^{I–V}$), 67.31 (2 C, C-2^{V},1″), 67.26 (5 C, C-4′$^{I–V}$), 52.11, 52.07,

51.77, 51.64, 51.50 (C-4$^{I–V}$), 51.41 (COO*C*H$_3$), 33.82 (C-5″), 28.78 (C-2″), 28.56 (5 C, C-3′$^{I–V}$), 24.48 (C-4″), 21.07 (CO*C*H$_3$), 18.06, 17.97, 17.92 (2 C, 2 C, C, C-6$^{I–V}$); FABMS: *m/z* 2316.17 ([M + 1]$^+$), 2338.16 ([M + Na]$^+$). HRMS: *m/z* 2446.9199. $C_{129}H_{151}N_5O_{34}Cs$ requires 2446.9295.

5-Methoxycarbonylpentyl 3-*O*-benzyl-4-(2,4-*O*-benzylidene-3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranosyl-(1⟶2)-tris[3-*O*-benzyl-4-(2,4-*O*-benzylidene-3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranosyl]-(1⟶2)-3-*O*-benzyl-4-(2,4-*O*-benzylidene-3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranoside (16)

Deacetylation of the fully protected pentasaccharide **15** gave the glycosyl acceptor **16** in virtually theoretical yield as a white solid, $[\alpha]_D$ −24.8° (*c* 0.5). Definite signals in the ^{1}H NMR spectrum ($CDCl_3$) were at *δ*: 6.42, 6.32, 6.27, 6.20, 6.16 (5 d, partially overlapped, 5 H, $J_{4,NH}$ ~9.4 Hz, 5 NH), 5.54, 5.52, 5.49 (3 s, 5 H, 5 C*H*Ph), 5.01, 4.93, 4.89 (bs, 2 bd, 4 H, $J_{1,2}$ ~1.6 Hz, H-1$^{II–V}$), ~4.61 (bd, partially overlapped, H-1^I), 3.81 (bt, 1 H, H-2^I), 3.59 (s, partially overlapped, COOCH_3), 3.56 (m, partially overlapped, H-1′a), 3.29–3.23 (m, 1 H, H-1″b), 2.25 (t, *J* 7.5 Hz, 2 H, H-5″a,b), 2.05–1.80 (m, 2 H, H-3′$^{I–V}$a,b), 1.64–1.46 (m, 4 H, H-2″a,b,4″a,b), 1.32–1.24 (m, 2 H, H-3″a,b), 1.13–1.01 (5 d, partially overlapped, H-6$^{I–V}$); ^{13}C NMR ($CDCl_3$): *δ* 101.18, 101.21, 101.26 (C, 3 C, C, 5 *C*HPh), 100.96, 100.30 (C, 3 C, C-1$^{II–V}$), 98.77 (C-1^I), 76.58, 76.54 (2 C, 3 C, C-2′$^{I–V}$), 75.98, 75.21, 75.15, 74.69, 74.50, 72.91 (2 C), 72.69 (C-2$^{II–IV}$,3$^{II–IV}$), 74.26 (C-2^I), 71.40, 71.22, 71.06 (C, 3 C, C, 5 *C*H$_2$Ph), 68.62, 68.50, 67.95, 67.55 (2 C, C, C, C, C-5$^{I–V}$), 67.33 (C-1″), 67.26 (C-4′$^{I–V}$), 66.84 (C-2^V), 52.08, 51.81, 51.76, 51.21 (C, C, 2 C, C, C, C-4$^{I–V}$), 51.40 (COO*C*H$_3$), 33.81 (C-5″), 28.78 (C-2″), 28.54 (C-3′$^{I–V}$), 25.49 (C-3″), 24.48 (C-4″), 18.02, 17.96, 17.92, 17.79 (2 C, C, C, C, C-6$^{I–V}$). HRMS: *m/z* 2404.9253. $C_{127}H_{149}CsN_5O_{23}$ requires 2404.9189.

5-Methoxycarbonylpentyl 2-*O*-acetyl-3-*O*-benzyl-4-(2,4-*O*-benzylidene-3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranosyl-(1⟶2)-tetrakis[3-*O*-benzyl-4-(2,4-*O*-benzylidene-3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranosyl]-(1⟶2)-3-*O*-benzyl-4-(2,4-*O*-benzylidene-3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranoside (17)

Reaction of **4** (1.4 g, 2.64 mmol) and **16** (3.0 g, 1.32 mmol), as described above for similar reactions, followed by isolation of the main product by chromatography (5:1⟶3:1 toluene–acetone), gave amorphous **17** (2.7 g, 75%), $[\alpha]_D$ −36.4° (*c* 0.66). Definite signals in the ^{1}H NMR spectrum ($CDCl_3$) were at *δ*: 6.42–6.14 (m, 6 H, 6 NH), 5.53, 5.52, 5.51, 5.47 (4 s, partially overlapped, 6 C*H*Ph), 5.45 (bt, partially overlapped, H-2^{VI}), 5.02, 4.93, 4.88, (3 bs, 4 H, H-1$^{II–V}$), 4.80 (bs 1 H, H-1^{VI}), ~4.60 (bd, partially overlapped, H-1^I), 3.57 (COOCH_3), 3.54 (m, partially overlapped, H-1″a), 3.32–3.23 (m, 1 H, H-1″b), 2.24 (t, 2 H, *J* 7.5 Hz, H-5″a,b), 2.07 (s, partially overlapped, COCH_3), 2.10–1.78 (m, partially overlapped, H-3′a,b$^{I–VI}$), 1.63–1.46 (m, 4 H, H-2″a,b,4″a,b), 1.33–1.21 (m, 2 H, H-3″a,b), 1.14–1.01 (6 d, partially overlapped, H-6$^{I–VI}$); ^{13}C NMR ($CDCl_3$): *δ* 101.17, 101.10, 101.06, 101.02, (2 C, 2 C, C, C, 6 *C*HPh), 100.87, 100.30, 100.07 (C, 2 C, C, C-1$^{II–V}$), 99.11 (C-1^{VI}), 98.68 (C-1^I), 76.51, 76.45 (3 C, 3 C, C-2′$^{I–VI}$), 75.08, 74.91, 74.74, 74.48, 74.28, 73.71, 73.65, 72.89, 72.78, 72.71 (C, C, C, C, 2 C, C, C, C, C, C, C-2$^{I–V}$,3$^{I–VI}$), 71.27, 71.23, 71.20, 70.96, 70.92 (C, C, C, C, 2 C, 6 *C*H$_2$Ph), 68.57, 68.53, 68.48, 68.37, 67.49 (C, C, C, C, 2 C, C-5$^{I–VI}$), 67.24 (C-1″), 67.17 (7 C, C-2^{VI},4′$^{I–VI}$), 52.99, 51.93, 51.70, 51.56, 51.37, 51.31 (C, C, C, C, C, 2 C, C-4$^{I–VI}$,-COO*C*H$_3$), 33.73 (C-5″), 28.69 (C-2″), 28.48 (6 C, C-3′$^{I–VI}$), 25.41 (C-3″), 24.40 (C-4″), 20.98 (COCH_3), 18.79, 17.98, 17.91, 17.86 (6 C, C-6$^{I–VI}$). FABMS: *m/z* 2741.09 ([M + 1]$^+$), 2747.3 ([M + Li]$^+$); HRMS: *m/z* 2872.1184. $C_{153}H_{178}N_6O_{40}Cs$ requires 2872.1133.

5-Methoxycarbonylpentyl 3-*O*-benzyl-4-(2,4-*O*-benzylidene-3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranosyl-(1⟶2)-tetrakis[3-*O*-benzyl-4-(2,4-*O*-benzylidene-3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranosyl]-(1⟶2)-3-*O*-benzyl-4-(2,4-*O*-benzylidene-3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranoside (18)

Deacetylation of **17** (Zemplén) gave **18** in virtually theoretical yield as an amorphous solid, $[\alpha]_D$ −29.4 ° (*c* 0.6). Definite signals in the ^{1}H NMR spectrum ($CDCl_3$) were at *δ*: 6.47–6.12 (m, 6 H, 6 NH), 5.44, 5.53, 5.52, 5.51, 5.48 (5 s, 6 H, 6 C*H*Ph), 5.00, 4.92, 4.87 (m, m, bd, 5 H total, H-1$^{II–VI}$), 4.61 (bd, partially overlapped, H-1^I), 3.80 (bt, 1 H, H-2^I), 3.58 (s, partially overlapped, COOCH_3), 3.58–3.30 (m, partially overlapped, H-1″a), 3.28, 3.24 (2 t, 1 H, *J* 6.3 Hz, H-1″b), 2.25 (t, 2 H, *J* 7.5 Hz, H-5″a,b), 2.06–1.82 (m, partially overlapped, H-3′a,b$^{I–VI}$), 1.63–1.46 (m, 4 H, H-2″a,b,4″a,b), 1.33–1.22 (m, 2 H, H-3″a,b), 1.14–1.00 (6 d, partially overlapped, H-6$^{I–VI}$); ^{13}C NMR ($CDCl_3$): *δ* 101.22, 101.09 (5 C, C, 6 *C*HPh), 100.96, 100.29 (C, 4 C, C-1$^{II–V}$), 98.76 (C-1^I), 76.56 (6 C, C-2′$^{I–VI}$), 76.02, 75.26, 75.11, 74.77, 74.51, 74.33 (2 C), 73.04, 72.89, 72.81 72.75 (C-2$^{I–V}$,3$^{I–VI}$), 71.43, 71.25, 71.20, 70.97, (6 C total, *C*H$_2$Ph), 68.58, 68.53, 67.93, 67.55 (2 C, 2 C, C, C, C-5$^{I–VI}$), 67.23 (7 C, C-1″,4′$^{I–VI}$), 66.82 (H-2^{VI}), 52.08, 51.78, 51.66 (3 C), 51.18 (C-4$^{I–VI}$), 51.38 (COO*C*H$_3$), 33.80 (C-5″), 28.77 (C-2″), 28.51 (C-3′$^{I–VI}$), 25.48 (C-3″), 24.47 (C-4″), 17.97, 17.96, 17.92, 17.77 (6 C total, C-6$^{I–VI}$). FABMS: *m/z* 2699.38 ([M + 1]$^+$), 2721.73 ([M + Na]$^+$); HRMS: *m/z* 2830.1067. $C_{151}H_{176}N_6O_{39}Cs$ requires 2830.0760.

5-Methoxycarbonylpentyl 4-(3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranosyl-(1⟶2)-bis[4-(3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranosyl]-(1⟶2)-4-(3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranoside (19)

A mixture of **18** (500 mg) and 5% palladium-on-carbon catalyst (200 mg) in methanol was stirred under hydrogen overnight, when TLC (1:2:0.1 CH_2Cl_2–MeOH–NH_4OH) showed that the reaction was complete. Conventional processing and elution from a small column of silica gel gave amorphous **19** (230 mg, 77%). Definite signals in the ^{1}H NMR (D_2O) spectrum were at: *δ* 5.15, 5.11 (2 d, 1 H each, $J_{1,2}$ 1.7 Hz, H-1^{II,III}), 4.99 (d, 1 H, $J_{1,2}$ 1.9 Hz, H-1^{IV}), 4.82 (d, 1 H, $J_{1,2}$ 1.7 Hz, H-1^I), 4.22–4.16 (m, 4 H, H-2′$^{I–IV}$), 3.66 (s, partially overlapped, COOCH_3), 2.34 (t, 2 H, *J* 7.5 Hz, H-5″a,b), 2.09–1.76 (m, 8 H, H-3′a,b$^{I–IV}$), 1.70–1.57 (m, 4 H, H-4″a,b,2″a,b), 1.47–1.37 (m, 2 H, H-3″a,b), 1.18–1.15 (4 d, partially overlapped, H-6$^{I–IV}$); ^{13}C NMR (D_2O): *δ* 103.83 (C-1^{IV}), 102.73, 102.45 (C-1^{II,III}), 100.20 (C-1^I), 79.90 (C-2^I), 79.09, 78.95 (C-2^{II,III}), 70.96 (C-2^{IV}), 70.67 (4 C, C-2′$^{I–IV}$), 70.02, 69.63, 69.46, 69.35, 68.74 (C, C, 3 C, 2 C, C (C-3$^{I–IV}$,5$^{I–IV}$), 68.42 (C-1″), 59.42 (4 C, C-4′), 54.76, 54.58, 54.51, 54.14 (C-4$^{I–IV}$), 52.10 (COO*C*H$_3$), 38.24 (4 C, C-3′$^{I–IV}$), 34.63 (C-5″), 30.03 (C-2″), 26.71 (C-3″), 25.60 (C-4″), 18.32 (2 C), 18.26, 18.19 (C-6$^{I–IV}$); FABMS: *m/z* 1135.6 ([M + 1]$^+$).

5-Methoxycarbonylpentyl 4-(3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranosyl-(1⟶2)-tetrakis[4-(3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranosyl]-(1⟶2)-4-(3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranoside (23)

This compound was obtained (~90%) by treatment of **18**, as described for tetrasaccharide **14**. The NMR characteristics agreed with those reported previously for the independently synthesized substance.[11]

(2-Aminoethylamido)carbonylpentyl 4-(3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranosyl-(1⟶2)-bis[4-(3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranosyl]-(1⟶2)-4-(3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranoside (20)

In a closed round bottomed flask, a solution of compound **19** (240 mg) in ethylenediamine (3 mL) was heated at 60–70 °C overnight, when TLC (1:2:0.1 CH_2Cl_2–MeOH–NH_4OH) showed that the reaction was complete. After concentration (70° C/133 Pa), the residue was purified by elution from a series of Sep-Pak C18 cartridges. The material was applied as a solution in water, and elution was effected with a gradient of H_2O⟶50% MeOH. Concentration of the relevant fractions, filtration and freeze-drying gave amorphous, hygroscopic amine **20** (140 mg, ~58%). The ^{1}H NMR spectrum of the amine **20** was very similar to that of the ester **19**, and contained 2-proton triplets at δ 3.48 and 3.06 for H-6″a,b and H-7″a,b, respectively. Similarly, the ^{13}C NMR spectrum contained additional signals at δ 42.14 and 41.80 for C-6″ and C-7″, respectively. FABMS: *m*/*z* 1163.51 ([M + 1]$^+$), 1185.51 ([M + Na]$^+$).

1-{(2-Aminoethylamido)carbonylpentyl 4-(3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranosyl-(1⟶2)-bis[4-(3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranosyl]-(1⟶2)-4-(3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranoside}-2-ethoxycyclobutene-3,4-dione (21)

A solution of amine **20** (60 mg, 0.05 mmol) in buffer pH 7 (7 mL) was treated with squaric acid diethylester (13 μL, 0.08 mmol), and kept at room temperature until TLC (1:3 CH_2Cl_2–MeOH) showed complete conversion of the starting material into a less polar UV positive product. After concentration, the residue was eluted from a series of Sep-Pak C18 cartridges to give, after freeze-drying, the squaric acid monoamide **21** (44 mg, 73%). The ^{1}H NMR spectrum of **21** was very similar to that of the amine **20**, and contained multiplets at δ 4.76–4.69 (CH_3CH_2O) and 1.49–1.43 (CH_3CH_2O). The ^{13}C NMR spectrum was very similar to that of the amine **20**. Signals for the OCH_2CH_3 (δ 70.85 and 70.74) and OCH_2CH_3 (16.23 and 16.16) each appeared as doublets, the splitting of these signals being due to the vinylogous amide group, characteristic of squaric acid amide esters.[19] FABMS: *m*/*z* 1287.5 ([M + 1]$^+$), 1309.5 ([M + Na]$^+$).

(2-Aminoethylamido)carbonylpentyl 4-(3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranosyl-(1⟶2)-tetrakis[4-(3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranosyl]-(1⟶2)-4-(3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranoside (24)

Treatment of ester **23** as described for **19** gave the amorphous amine **24** (80%). The ^{1}H NMR spectrum of **24** was very similar to that of the ester **23**, and contained 2-proton triplets at δ 3.26 and 2.74 for H-6″a,b and H-7″a,b, respectively. Similarly, the ^{13}C NMR spectrum contained additional signals at δ 42.58 and 41.91 for C-6″ and C-7″, respectively. FABMS: *m*/*z* 1657.88 ([M + 1]$^+$), 1679.86 ([M + Na]$^+$).

1-{(2-Aminoethylamido)carbonylpentyl 4-(3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranosyl-(1⟶2)-tetrakis-[4-(3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranosyl]-(1⟶2)-4-(3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranoside}-2-ethoxycyclobutene-3,4-dione (25)

Treatment of amine **24** with squaric acid diethylester, as described for the preparation of **21**, gave the monoamide **25** (73%). The NMR spectra of the compound were similar to those of the starting amine **24** and showed signals characteristic of the presence of the squaric acid *O*-ethyl ester group, as in the spectrum of the analogous tetrasaccharide **21**. FABMS: *m*/*z* 1781.74 ([M + 1]$^+$), 1803.74 ([M + Na]$^+$).

***t*-Butyl 2,4-*O*-benzylidene-3-deoxy-L-*glycero*-tetronate (28)**

CrO_3 (41.2 g, 4 equiv.) was added, portionwise with stirring and exclusion of moisture, to pyridine (66.8 mL, 8 equiv.) contained in a 3 L round-bottomed flask, and the stirring was continued for 10 min. With continued stirring, a mixture of CH_2Cl_2–DMF (v/v, 4:1, 1 L) was added and, after 30 min, Ac_2O (78 mL, 8 equiv.) was added portionwise, followed by dry *t*-butanol (197.2 mL, 20 equiv.). A solution of the 2,4-*O*-benzylidene-(*S*)-1,2,4-butanetriol[13] (**27**, 20 g, 103 mmol) in CH_2Cl_2–DMF (v/v, 4:1, 250 mL) was then added dropwise over a period of 1.5 h, and the mixture was stirred until TLC showed that all starting material was consumed (~3 h). One major, fast moving, strongly UV positive product was formed, as shown by TLC. When a silica gel plate was sprayed with 5% H_2SO_4 in EtOH and heated, the spot corresponding to the product turned first red, the color faded on continued heating, and the material charred only very weakly. A number of very minor, more polar by-products were also formed. After cooling (~ 10 °C), solid $NaHCO_3$ (140 g) was added, followed by EtOH (110 mL), and the stirring was continued for 2 h. The mixture was concentrated, and the residue was co-evaporated with pyridine and toluene to remove DMF. The residue was suspended in EtOAc and, after filtration, the solid was washed with EtOAc, and the combined filtrates were concentrated. The residue was purified by chromatography (6:1 to 4:1, hexane–EtOAc) to give the desired product **27** as white solid (22.6 g, 83%), which was identical (^{1}H NMR) to the previously described[13] material. ^{13}C NMR ($CDCl_3$): δ 168.93 (CO), 100.95 (*C*HPh), 81.66 [*C*(CH_3)$_3$], 75.48 (C-2), 66.66 (C-4), 26.15 (C-3), 27.91 [C(*C*H_3)$_3$].

2,4-*O*-Benzylidene-3-deoxy-L-*glycero*-tetronic acid (29)

Pyridinium dichromate (216 g, 4 equiv.) was added at room temperature to a solution of crystalline[13,28] 2,4-*O*-benzylidene-(*S*)-1,2,4-butanetriol (**27**, 30 g, 154.6 mmol) in DMF (400 mL), and the mixture was stirred overnight, when TLC (10:1 CH_2Cl_2–EtOAc) showed absence of the starting material. Water (450 mL) was added, and the mixture was extracted with EtOAc (5×600 mL). The organic layer was washed with water, to remove some colored material, and concentrated to remove EtOAc. DMF was removed by co-evaporation with pyridine or xylene to give acid **29** (28 g, 85%) as a white solid, which showed the same ^{1}H and ^{13}C NMR characteristics as the previously described substance.[13]

Conjugation of squaric acid monoamides to BSA

A. Preparation of the neoglycoconjugate **26A**. Hexasaccharide monoamide **25** (8 mg, 4.5 μmol) was transferred from its weighing container, using 200 μL of phosphate buffer pH 9 divided into three portions (100, 50 and 50 μL), into a suspension of BSA (30 mg, 0.45 μmol) in the same buffer (100 μL), which had been homogenized with the aid of a vortexer, and the mixture was stirred at room temperature. SELDI-TOF analysis after 1, 21, 96 h and 14 d showed that the hapten/BSA ratio was, 0.32, 1.4, 1.5 and 2.8, respectively, which did not increase after 3 more days. The mixture was diluted with phosphate buffer pH 7 (1 mL), and the solution was subjected to ultrafiltration using the Amicon cell equipped with PM-10 membrane (Millipore Copporation, Bedford, MA). The retained material was freeze-dried to give the title conjugate **26A** (25 mg, 83%) having average *m*/*z* 71,464, corresponding to **26A**/BSA ratio of 2.9, as shown by SELDI-TOF MS.

B. Preparation of neoglycoconjugates **26B** and **26C**. Solid hexasaccharide monoamide **25** (27 mg, 15.2 μmol) was transferred from its weighing container (using 600 μL of phosphate buffer pH 9 divided into three 200 μL portions) into a suspension of BSA (10 mg, 0.15 μmol) in the same buffer (400 μL), which had been homogenized with the aid of a vortexer. The mixture was stirred at room temperature and, after 3 h, when SELDI-TOF MS analysis showed that the **25**/BSA ratio in the conjugate being formed was ~6 a portion (500 μL) was withdrawn and processed as described in A. After freeze-drying, SELDI-TOF MS analysis showed that the molecular mass of the product **26B** (5.5 mg, 94%) was 78,080 Da (**25**/BSA = 6.6).

The material remaining in the reaction vessel was allowed to react for a total of 22 h, when SELDI-TOF MS analysis showed that the **25**/BSA ratio in the conjugate formed was ~19. The mixture was processed as described above and, after freeze-drying, the molecular mass of the product **26C** (6.9 mg, 92%) was 99,740 Da, corresponding to **25**/BSA = 19.

C. Preparation of the neoglycoconjugate **22B**. BSA (2.5 mg, ~0.04 μmol) was treated with the tetrasaccharide monoamide **21** (5 mg, ~4 μmol) as described above (A), and the mixture was stirred at room temperature until the molecular mass of the conjugate being formed remained essentially unchanged, as shown by periodical SELDI-TOF analysis (2 d). The mixture was processed as described above in A and, after freeze drying, SELDI-TOF MS analysis showed a peak centered at *m*/*z* 80,850 Da corresponding to the **21**/BSA ratio in the conjugate **22B** thus obtained (2.5 mg, 82%) of 11.6.

D. Preparation of neoglycoconjugates **22A** and **22C**. Solid tetrasaccharide monoamide **21** (20 mg, 15.5 μmol) and a solution of BSA (10 mg, 0.15 μmol) were allowed to react in phosphate buffer pH 9 (1 mL) at room temperature, as described above in B. After 4.5 h, when SELDI-TOF MS analysis showed that the **21**/BSA ratio in the conjugate being formed was 4.9, a portion (500 μL) was withdrawn and processed as described above. After freeze drying, SELDI-TOF MS analysis showed that the molecular mass of the product **22A** (4.8 mg, 89%) was 72,028 Da, corresponding to **21**/BSA ratio of 4.5. The remaining reaction mixture was worked-up after 3.5 d, when SELDI-TOF MS analysis showed that molecular mass of the conjugate being formed was 84,588 Da. After processing, as described above, repeated SELDI-TOF MS analysis showed that the **21**/BSA ratio in the product **22C** (5.8 mg, 90%) was 14.6.

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