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Studies on vaccines against cholera. Synthesis of neoglycoconjugates from the hexasaccharide determinant of *Vibrio cholerae* O:1, serotype Ogawa, by single-point attachment or by attachment of the hapten in the form of clusters

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Abstract

The terminal hexasaccharide of the O-antigen of *Vibrio cholerae* O:1, serotype Ogawa, has been synthesized in the form of a glycoside whose aglycon (linker) allows conjugation to carrier proteins by reductive amination. The conjugate obtained from direct, single-point attachment of the linker-equipped hapten to chicken serum albumin (CSA) contained seven hapten residues/CSA. A neoglycoconjugate containing the carbohydrate antigen in the form of clusters was obtained using, as a hapten subcarrier, an oligopeptide containing 16 amino groups. It was treated with a limited amount of hapten, to give a hapten-carrying subcarrier (HCS). Subsequent conjugation of HCS to CSA, using squaric acid diethyl ester as a conjugation reagent, gave a cross-linked, glycocluster conjugate containing 51% (w/w) of the carbohydrate. Published by Elsevier Science Ltd.

Keywords: Glycoconjugate; Oligosaccharide; Glycocluster; Subcarrier; Synthetic vaccine

1. Introduction

Despite impressive progress that has been made in vaccine development, cholera is one of the diseases for which a satisfactory vaccine is not available. The existing cellular vaccines, as well as those based on the lipopolysaccharide (LPS) of *Vibrio cholerae* O:1, have serious age-related and/or protection limitations. In addition, regardless of the mode of administration, they elicit adverse reactions [1]. Clearly, there is a need for more potent and safe immunogens free from such drawbacks. It is expected that conjugate vaccines, resulting from chemical linking of immunologically dominant epitopes present in LPSs to proteins, will alleviate these problems [2–4].

Part of our effort towards synthetic vaccines is directed towards developing a potent immunogen for protective anti-*V. cholerae* O:1 antibodies using synthetic fragments of the O-polysaccharide (O-PS) as antigen. The internal part of the O-PS of the two main strains of *V. cholerae* O:1, Inaba and Ogawa, consists [5–7] of α-(1→2)-linked D-perosamine (4-amino-4,6-dideoxy-D-mannose) whose amino

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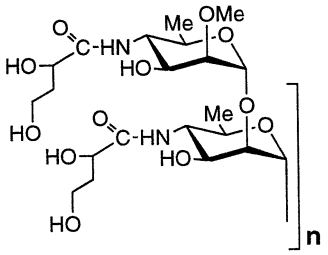
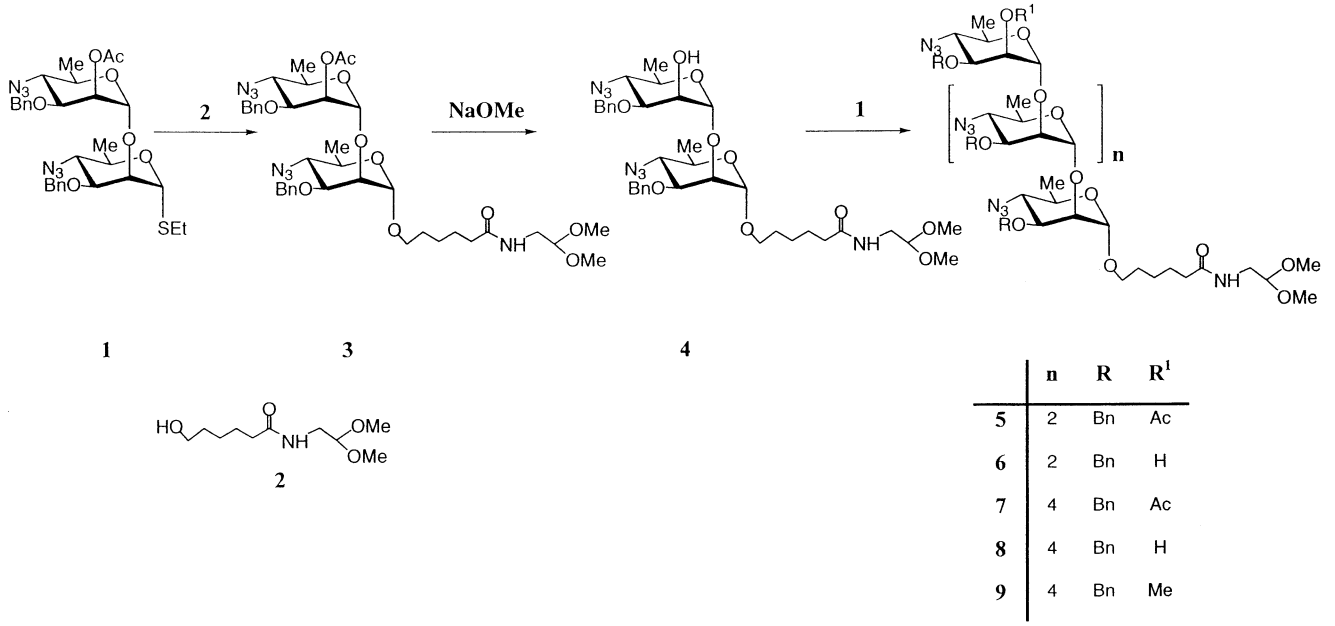


Fig. 1. Structure of the O-PS of *Vibrio cholerae* O:1, serotype Ogawa.

group is acylated with 3-deoxy-L-*glycero*-tetronic acid. The upstream terminal perosamine moiety of the O-PS of the Ogawa strain is methylated at O-2 (Fig. 1). We have previously reported [8,9] on syntheses of neoglycoconjugates from the terminal monosaccharide determinant [10] of the O-PS of *V. cholerae* O:1, serotype Ogawa. Here, we describe syntheses of neoglycoconjugates derived from the terminal hexasaccharide. In addition to conjugating hapten **14** to a carrier protein according to the single-point attachment model [3], we have also prepared a glycoconjugate containing the same hapten in the form of multivalent clusters. The latter was prepared following the concept we have recently introduced [11] and applied to making a conjugate from the corresponding monosaccharide. Enhanced antigenicity resulting from multivalent ligands, compared with their counterparts resulting from single-point attachment of haptens, has been well documented [12]. Our future evaluation of immunogenicity of the two types of conjugates, which differ from each other only in this one fundamental architectonic element, will provide information concerning the merit of this concept.

2. Results and discussion

Synthesis of perosamine-containing oligosaccharides was originally developed by Bundle and co-workers [13–16] in connection with the preparation of methyl glycosides of antigenic determinants of the *Brucella* A antigen. Syntheses of similar but linker-equipped haptens, glycosides whose aglycons allow linking to proteins, is a more formidable task. While other approaches can be envisioned, from the practical point of view it is advantageous to carry out the initial synthetic steps as for the preparation of analogous methyl glycosides [13,17,18]. The blockwise construction of higher oligosaccharides suitable for linking to proteins dictates that the aglycon exchange (from methyl to a linker/spacer, or to a group that could be more readily exchanged for the spacer molecule than the methyl group) be effected at the disaccharide stage. We have demonstrated one such strategy in our synthesis of the hexa- and the dodecasaccharide of the *V. cholerae* O:1, serotype Ogawa [19,20]. There, a glycosyl chloride derived from a disaccharide was used as the key glycosyl donor, and the principal material was carried through the synthesis in the form of the trimethylsilylethyl glycoside until an oligosaccharide of the desired length was obtained. Subsequently, the trimethylsilylethyl aglycon was exchanged for the spacer molecule that allowed linking to carrier proteins using a carbodiimide-type conjugation. Here, larger haptens are prepared using thioglycoside **1** [15,21] as the key glycosyl donor, and they are linked to carriers by reductive amination [22]. Due to their stability during many chemical manipulations, thioglycosides can be used as both glycosyl donors and acceptors, which reduces the total number of synthetic steps towards the target products. Accordingly, the glycosyl donor **1** and the linker-acceptor **2** [23] were condensed to give the fully protected, linker-equipped disaccharide **3** together with a large proportion of the β anomer **15** (see Schemes 1 and 2). Anomeric configurations for the two disaccharides formed were assigned based on the diagnostically significant differences in the $J_{C-1,H-1}$ coupling constants [24,25]. The lack of stereoselectivity observed during this glycosylation was unexpected, since analogous coupling [8] of the same glycosyl acceptor with thioglycosides **16**, which also carry a non-participating group at O-2, gave the corresponding α-glycoside stereospecifically in high yield. Attempts to introduce the hexanamide linker with higher α-stereoselectivity failed. When compound **2** was glycosylated (these unsuc-



	n	R	R^1
5	2	Bn	Ac
6	2	Bn	H
7	4	Bn	Ac
8	4	Bn	H
9	4	Bn	Me

Scheme 1.

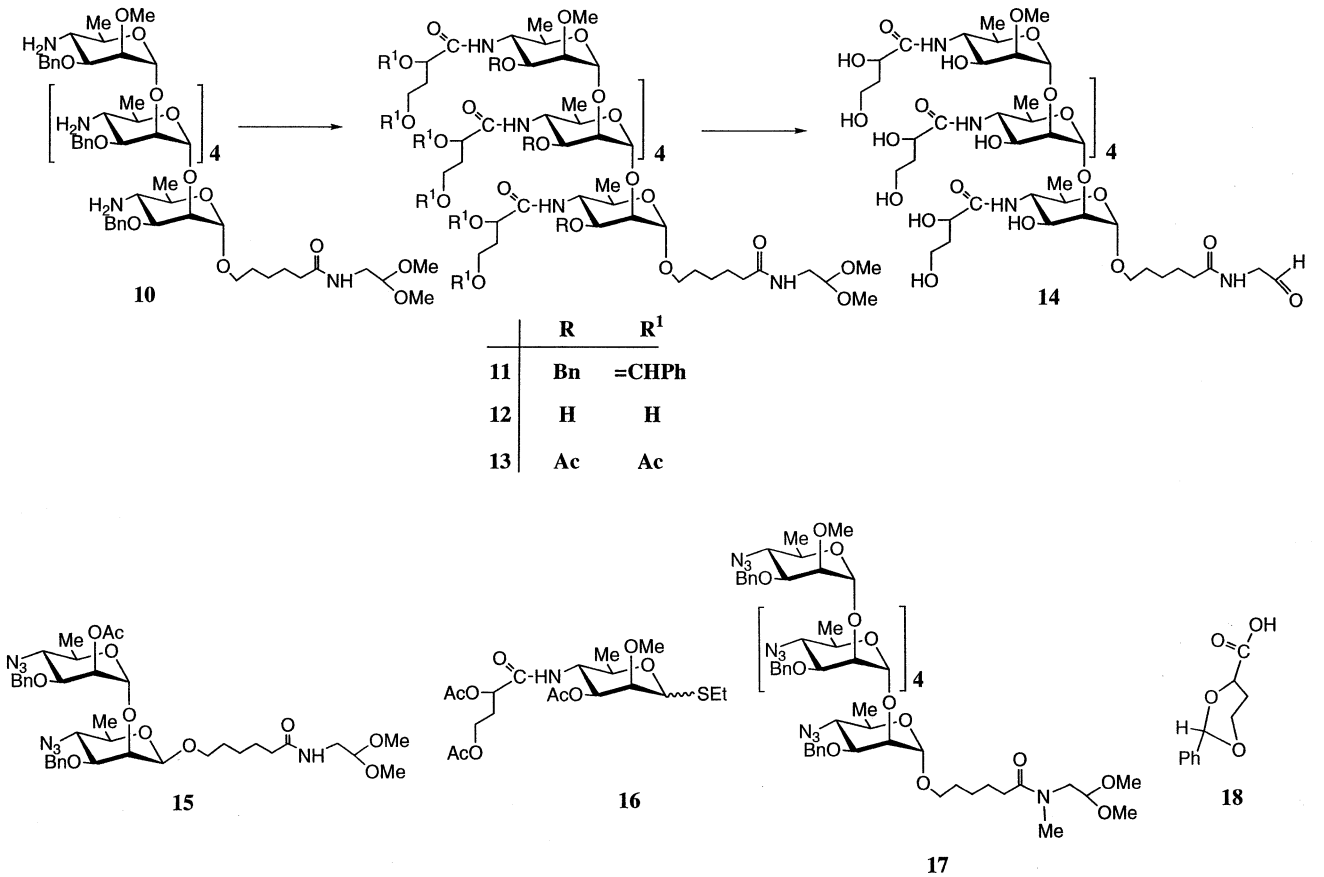
cessful experiments are not described in Section 3) with the monosaccharide thioglycoside ethyl 2-*O*-acetyl-3-*O*-benzyl-4-azido-4,6-dideoxy-α,β-D-mannopyranoside [14], the corresponding orthoester was the sole product. Acid-catalyzed isomerization of the latter to the desired glycoside failed because of the presence of the acid-labile dimethyl acetal function in the aglycon.

To extend the oligosaccharide chain, disaccharide **3** was deacetylated, and product **4** was treated with the thioglycoside glycosyl donor **1** (→**5**) (Scheme 1). This sequence of reactions was repeated (**5**→**6**→**7**), and the hexasaccharide so obtained was deacetylated to give **8**. The latter compound is an important intermediate for the synthesis of hexasaccharide fragments of *V. cholerae* O:1 in both the Ogawa and Inaba series, as well as for syntheses of higher members, since it allows chemical manipulation (methylation or chain extension) at position O-2 in the upstream terminal moiety. For making the title hexasaccharide, introduction of the methyl group at that position was explored. Although this appears to be a simple conversion, the following observations are worth mentioning. No reaction was observed when a solution of **8** in CH_2Cl_2 was treated with MeI and Ag_2O for 24 h. Addition of $(CH_3)_2S$, known to render [26] MeI and Ag_2O a more powerful reagent for methylation, or treatment of a solution of **8** in Me_2SO with MeI in the presence of KOH [27], gave the expected product **9** together with the *N*-methyl derivative **17** (Schemes 1 and 2). The latter structure of the byproduct was readily indicated by the comparison of its ^1H NMR spectrum with that of **9**. In the spectrum of **17**, the typical broad, low-field triplet for NH was no longer present, and the spectrum showed the presence of an additional singlet for the *N*-methyl group. As expected, compound **17** was obtained as a mixture of two isomeric forms (NMR). A clean conversion **8**→**9** was eventually achieved by methylation with neat MeI and Ag_2O.

To introduce the 3-deoxy-L-*glycero*-tetronamido side-chain, the azido group in **9** was reduced with H_2S [28], and the amine **10** thus obtained was treated with acid **18** [29] (Scheme 2) in the presence of *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) [30,31] to give the fully protected hexasaccharide **11**. Simultaneous removal of the benzyl and benzylidene protecting groups by catalytic hydrogenolysis (→**12**), followed by hydrolysis with dilute trifluoroacetic acid, then gave the aldehyde **14**. The latter reaction gave rise to a single product as shown by ^1H NMR spectroscopy (see Section 3).

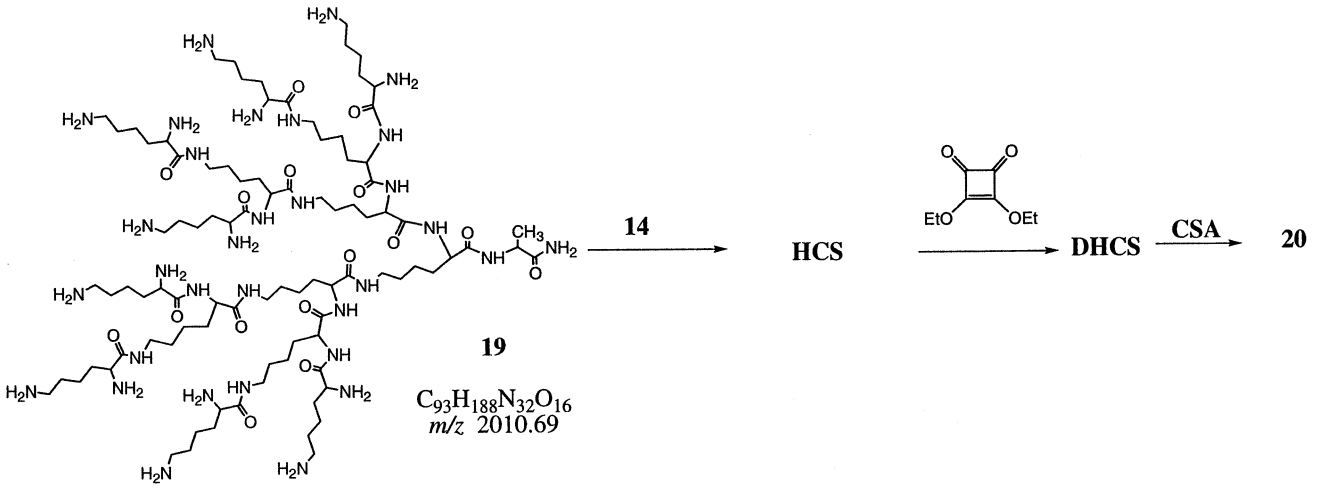
For conjugation according to the single-point attachment model [3], aldehyde **14** was treated with chicken serum albumin (CSA) in the presence of borane–pyridine complex, to reduce the intermediate Schiff base [32]. The reaction conditions applied were such that, based on the study [8] involving the analogous monosaccharide determinant of *V. cholerae* O:1, serotype Ogawa, incorporation of ten hapten residues was expected. The molecular mass value of 78,650 Da found for the neoglycoconjugate obtained (MALDI-TOF mass spectrometry) corresponds, on the average, to incorporation of seven hapten residues/CSA (based on 66,973 Da found for CSA). This suggests, together with our results [8] with the analogous monosaccharide equipped with same linker, that efficiency of conjugation decreases with the size of haptens.

Preparation of the neoglycoconjugate containing the same hapten but in the form of clusters **20** was carried out following our recently described, conceptually new protocol [11] (Scheme 3). It involved coupling, by reductive amination, of a *limited* amount of hapten **14** to the subcarrier molecule **19** containing 16 amino groups. The hapten-carrying subcarrier (HCS) thus obtained was then treated (in phosphate buffer, pH 7) with excess of squaric acid diethyl ester to derivatize [33–35] the amino groups that remained unchanged upon the preceding treatment with **14**. The derivatized HCS (DHCS) thus formed was then treated (in borate buffer, pH 9) with CSA to give a cross-linked, glycocluster conjugate **20**. Owing to the putative polymolecular nature and high molecular weight of the product obtained, and in view of its intended use as an immunogen, the most meaningful characteristic of this material is the content of the antigen **14**. This was determined by the phenol–sulfuric acid colorimetric assay. It showed that the material contained 51% of carbohydrate (expressed as the methyl glycoside analog of **14** [21], which was used as standard material).



	R	R^1
11	Bn	=CHPh
12	H	H
13	Ac	Ac

Scheme 2.



Scheme 3.

3. Experimental

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 glass slides (Whatman or Analtech) coated with silica gel (60 Å pore size). Column chromatography was performed by gradient elution from columns of silica gel. Solvent mixtures slightly less polar than those used for TLC were used at the onset of development. Assignments of NMR signals (300 and 75 MHz for ^1H and ^{13}C, respectively) were made by first-order analysis of the spectra, and when feasible, the assignments were confirmed by homonuclear and heteronuclear 2D correlation spectroscopy, run with the software supplied with the spectrometers. In the case of higher oligosaccharides the assignments were aided by comparison with spectra of related substances previously reported. When the latter approach was used, to assist 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 [36–38]. 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 D-perosamine residues in the molecule. When reporting assignments of NMR signals in oligosaccharides, sugar residues 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. Nuclei associated with the 3-deoxy-L-*glycero*-tetronyl group are denoted with a prime and those associated with the spacer-arm aglycon are denoted with a double prime. When reporting NMR spectral data for **17** (an isomeric mixture), only structurally significant resonances reflecting the presence of the *N*-methyl group in the molecule are given. HATU was purchased from PerSeptive Biosystems, Inc. Palladium-on-charcoal catalyst (5%, ESCAT 103) was a product of Engelhard Industries. The solution of BH_3 in pyridine (8 M) was purchased from Aldrich Chemical Co. The subcarrier **19** was custom synthesized by AnaSpec, Inc. (San Jose, CA). All glycosylations were carried out in the dark under argon. Unless specified otherwise, solutions were concentrated at 40 °C/2 kPa.

N-*(2,2-Dimethoxyethyl)-6-[2-*O*-acetyl-4-azido-3-*O*-benzyl-4,6-dideoxy-α-*D*-mannopyranosyl-(1→2)-(4-azido-3-*O*-benzyl-4,6-*

dideoxy-α- (**3**) *and β-*D*-mannopyranosyl)oxy]-hexanamide* (**15**).—Solid *N*-iodosuccinimide (NIS, 1.24 g, 5.51 mmol), followed by a solution of silver trifluoromethanesulfonate (AgOTf, 0.2 g, 0.78 mmol) in toluene (8 mL), was added at 5–10 °C to a mixture of **1** (2.46 g, 3.92 mmol), **2** (1.03 g, 4.7 mmol) and pulverized 4 Å molecular sieves (1 g) in CH_2Cl_2 (50 mL), which had been stirred at the same temperature for 15 min. The stirring was continued until TLC (2:1 hexane–acetone) showed that all of **1** was consumed. Cooling was terminated, the mixture was neutralized with Et_3N and filtered, and the filtrate was washed successively with aq $NaHCO_3$ and water, dried, and concentrated. Chromatography gave first the desired α isomer **3** (1.29 g, 42%), $[\alpha]_D$ +64° (*c* 1.5). ^{1}H NMR ($CDCl_3$): 5.67 (m, 1 H, NH), 5.40 (dd, 1 H, $J_{1,2}$ 1.9, $J_{2,3}$ 3.0 Hz, H-2^{II}), 4.85 (d, 1 H, H-1^{II}), 4.70, 4.68, 4.63, 4.53 (4 d, partially overlapped, 4 H total, ^2J 11.0 and 11.6 Hz, respectively, 2 CH_2Ph), 4.64 (d, partially overlapped, $J_{1,2}$ 1.8 Hz, H-1^{I}), 4.37 (t, 1 H, *J* 5.1 Hz, H-7″), 3.84 (bdd, 1 H, H-2^{I}), 3.79 (dd, 1 H, $J_{3,4}$ 9.9 Hz, H-3^{II}), 3.73 (dd, 1 H, $J_{3,4}$ 9.7 Hz, H-3^{I}), 3.63–3.53 (m, 2 H, H-5^{II},1″a), 3.49–3.28 (m, 13 H, H-4^{I,II},5^{I},1′b,6″a,b, incl s at 3.39 for 2 OCH_3), 2.18 (t, 2 H, *J* 7.3 Hz, H-5″a,b), 2.07 (s, 3 H, $COCH_3$), 1.70–1.58 (m, partially overlapped, H-4″a,b), 1.62–1.51 (m, partially overlapped, H-2″a,b), 1.38–1.24 (m, 8 H, H-3″a,b, incl 2 d at 1.30 and 2.28, $J_{5,6}$ ~6.1 Hz, H-6^{II} and H-6^{I}, respectively); ^{13}C NMR ($CDCl_3$): 102.58 (C-6″), 99.31 (C-1^{II}, $J_{C,H}$ 171.6 Hz), 98.44 (C-1^{I}, $J_{C,H}$ 169.2 Hz), 77.67 (C-3^{I}), 75.27 (C-3^{II}), 73.93 (C-2^{I}), 71.91, 71.47 (2 *C*H_2Ph), 67.47 (C-5^{II}), 67.43 (C-1″), 67.14 (C-2^{II}), 66.95 (C-5^{I}), 64.09 (C-4^{I}), 63.76 (C-4^{II}), 54.30 (2 C, 2 OCH_3), 40.76 (C-7″), 36.37 (C-5″), 29.04 (C-2″), 25.72 (C-3″), 25.25 (C-4″), 20.86 (OCH_3), 18.50 (C-6^{I}), 18.42 (C-6^{II}); CIMS: *m*/*z* 784 $[M+1]^+$, 801 $[M+18]^+$. Anal. Calcd for $C_{38}H_{53}N_7O_{11}$: C, 58.24; H, 6.77; N, 12.52. Found: C, 57.98; H, 6.77; N, 12.35.

Eluted next was the β isomer **15** (1.23 g, 40%), $[\alpha]_D$ +18°. ^{1}H NMR ($CDCl_3$): 5.68 (m, 1 H, NH), 5.50 (dd, 1 H, $J_{1,2}$ 1.8, $J_{2,3}$ 3.2 Hz, H-2^{II}), 5.04 (d, 1 H, H-1^{II}), 4.73, 4.68, 4.55 (d, 1 H, ^2J 10.5 Hz; s 2 H, d, 1 H, 2 CH_2Ph), 4.36 (t, 2 H, *J* 5.3 Hz, H-7″a,b), 4.28 (s, 1 H, H-1^{I}), 4.16–4.07 (m, 2 H, H-2^{I},5^{II}), 3.3 (dd, 1 H, $J_{2,3}$ 3.3, $J_{3,4}$ 10.0 Hz, H-3^{II}), 3.91–3.83 (m, 1 H, H-1″a), 3.47–3.34 (m, 12 H, H-3^{I},4^{I},^{II},1″b,6″-a,b, 2 OCH_3), 3.16–3.08 (m, 1 H, H-5^{I}), 2.18 (t, 2 H, *J* 7.5 Hz, H-5″), 2.03 (s, 3 H, $COCH_3$), 1.70–1.55 (m, 4 H, H-2″a,b,4″a,b), 1.42–1.34 (m, 4 H, H-3″, incl d at 1.38, $J_{5,6}$ 6.0 Hz, H-6^{I}), 1.27 (d, 3 H, $J_{5,6}$ 6.2 Hz, H-6^{II}); ^{13}C NMR ($CDCl_3$): 102.60 (C-7″), 99.68 (C-1^{I}, $J_{C,H}$ 154.1 Hz), 98.20 (C-1^{II}, $J_{C,H}$ 171.9 Hz), 80.74 (C-3^{I}), 75.99 (C-3^{II}), 71.72, 701.65 (2 *C*H_2Ph), 71.16 (C-5^{I}), 70.69 (C-2^{I}), 69.62 (C-1″), 67.22 (C-2^{II}), 66.71 (C-5^{II}), 63.96 (2 C, C-4^{I,II}), 54.33, 54.29 (2 OCH_3), 40.80 (C-6″), 36.37 (C-5″), 29.26 (C-2″), 25.65 (C-3″), 25.24 (C-4″), 20.86 (CO*C*H_3), 18.41 (C-6^{I}), 18.27 (C-6^{II}); CIMS: *m*/*z* 784 $[M+1]^+$, 801 $[M+18]^+$. Anal. Found: C, 58.33; H, 6.91; N, 12.40.

N*-(2,2-Dimethoxyethyl)-6-[4-azido-3-*O*-benzyl-4,6-dideoxy-α-*D*-mannopyranosyl-(1→2)-(4-azido-3-*O*-benzyl-4,6-dideoxy-α-*D*-mannopyranosyl)oxy]hexanamide* (**4**).—Conventional deacetylation (Zemplén) of **3** gave **4** in virtually theoretical yield, $[\alpha]_D$ +76° (*c* 1.5). ^{1}H NMR ($CDCl_3$): 5.75 (m, 1 H, NH), 4.93 (d, 1 H, $J_{1,2}$ 1.6 Hz, H-1^{II}), 4.72–4.59 (m, 5 H, 2 CH_2Ph, incl d, 4.66, $J_{1,2}$ 1.8 Hz, H-1^{I}), 4.36 (t, 1 H, *J* 5.3, H-7″), 3.98 (m, 1 H, H-2^{II}), 3.88 (bt, 1 H, H-2^{I}), 3.72 (dd, partially overlapped, $J_{2,3}$ 3.0, $J_{3,4}$ 9.9 Hz, H-3^{I}), 3.70 (dd, partially overlapped, $J_{2,3}$ 3.2, $J_{3,4}$ 9.7 Hz, H-3^{II}), 3.65–3.54 (m, 2 H, H-1″a, H-5^{II}), 3.44–3.24 (m, 12 H, H-4^{I},4^{II},5^{I},1″b,6″a,b, incl s, 3.38 for 2 OCH_3), 2.58 (d, 1 H, $J_{2,OH}$ 2.1 Hz, OH), 2.17 (t, 2 H, *J* 7.5 Hz, H-5″), 1.70–1.51 (2 m, partially overlapped, H-4″,2″ in that order), 1.38–1.24 (m, 8 H, H-3″, incl 2 d, 1.29 and 1.28, partially overlapped, $J_{5,6}$ ~6.1 Hz, H-6^{I,II}); ^{13}C NMR ($CDCl_3$): 102.59 (C-7″), 100.82 (C-1^{II}), 98.56 (C-1^{I}), 77.71 (C-3^{I}), 77.43 (C-3^{II}), 73.78 (C-2^{II}), 71.95, 71.85 (2 *C*H_2Ph), 67.39 (C-1″), 67.14 (C-5^{II}), 67.00 (C-2^{II}), 66.89 (C-5^{II}), 64.20 (C-4^{II}), 63.68 (C-4^{II}), 54.25 (2 C, 2 OCH_3), 40.72 (C-6″), 36.30 (C-5″), 28.99 (C-2″), 25.67 (C-3″), 25.21 (C-4″), 18.48 (C-6^{I}), 18.31 (C-6^{II}), CIMS: *m*/*z* 742 $[M+1]^+$, 759 $[M+18]^+$. Anal. Calcd for $C_{36}H_{51}N_7O_{10}$: C, 58.30; H, 6.88; N, 13.23. Found: C, 58.21; H, 6.91; N, 13.10.

N-*(2,2-Dimethoxyethyl)-6-[2-O-acetyl-4-azido-3-O-benzyl-4,6-dideoxy-α-D-mannopyranosyl-(1→2)-bis(4-azido-3-O-benzyl-4,6-dideoxy-α-D-mannopyranosyl)-(1→2)-(4-azido-3-O-benzyl-4,6-dideoxy-α-D-mannopyranosyl)oxy]hexanamide* (**5**).—Compounds **1** and **4** (0.25–3.0 mmol) were treated with NIS and AgOTf, as described for the preparation of **3**. Yields of **5** in several experiments varied from 56 to 72%, $[\alpha]_D$ +78°. ^{1}H NMR ($CDCl_3$): 5.64 (m, 1 H, NH), 5.40 (dd, 1 H, $J_{1,2}$ 1.8, $J_{2,3}$ 3.0 Hz, H-2^{IV}), 4.93, 4.87 (2 d, 1 H each, $J_{1,2}$ 1.6 Hz, H-1^{II,III}), 4.84 (d, 1 H, $J_{1,2}$ 1.5 Hz, H-1^{IV}), 4.73–4.51 (m, 9 H, 4 CH_2Ph, incl d, ~4.60, H-1^{I}), 4.37 (t, 1 H, *J* 5.2, H-7″), 3.87, 3.83 (2 bt, 1 H each, H-2^{II,III}), 3.79–3.75 (m, 2 H, H-2^{I},3^{IV}), 3.71–3.65 (m, 3 H, H-3$^{I–III}$), 3.63–3.14 (m, 18 H, H-4$^{I–IV}$, 5$^{I–IV}$,1″a,b,6″a,b, incl at 3.39, 2 OCH_3), 2.18 (t, 2 H, *J* 7.7 Hz, H-5″a,b), 2.01 (s, 3 H, $COCH_3$), 1.70–1.57 (m, partially overlapped, 2 H, H-4″a,b), 1.59–1.50 (m, partially overlapped, 2 H, H-2″a,b), 1.37–1.23 (m, partially overlapped, H-3″a,b, incl 3 d at 1.26, 1.20, and 1.15, the one at 1.26 showing ~double intensity compared to those at higher fields, H-6$^{I–IV}$); ^{13}C NMR ($CDCl_3$): 172.81, 169.74 (2 CO), 102.62 (C-7″), 100.37, 100.03 (C-1^{II,III}), 99.06 (C-1^{IV}), 98.53 (C-1^{I}), 77.43, 76.76, 76.58 (C-3$^{I–III}$), 75.39 (C-3^{IV}), 73.96 (C-2^{I}), 73.46 (2 C, C-2^{II,III}), 72.17, 72.08, 71.99, 71.50 (4 *C*H$_2$Ph), 67.76 (2 C), 67.62, 67.10, 67.02 (C-2^{IV},5$^{I–IV}$), 67.48 (C-1″), 64.34, 64.21, 64.01 (C-4$^{I–III}$), 63.80 (C-4^{IV}), 54.36 (2 C, 2 OCH_3), 40.81 (C-6″), 36.42 (C-5″), 29.08 (C-2″), 25.74 (C-3″), 25.29 (C-4″), 20.93 (CO*C*H$_3$), 18.57 (2 C), 18.45, 18.34 (C-6$^{I–IV}$), FABMS: *m*/*z* 1328 $[M + Na]^+$; Anal. Calcd for $C_{64}H_{83}N_{13}O_{17}$: C, 58.84; H, 6.40; N, 13.94. Found: C, 58.67; H, 6.47; N, 13.78.

N-*(2,2-Dimethoxyethyl)-6-[4-azido-3-O-benzyl-4,6-dideoxy-α-D-mannopyranosyl-(1→2)-bis(4-azido-3-O-benzyl-4,6-dideoxy-α-D-mannopyranosyl)-(1→2)-(4-azido-3-O-benzyl-4,6-dideoxy-α-D-mannopyranosyl)oxy]-hexanamide* (**6**).—Conventional deacetylation (Zemplén) of **5** gave **6** in virtually theoretical yield, $[\alpha]_D$ +84°. ^{1}H NMR ($CDCl_3$): 5.71 (bt, 1 H, NH), 4.98 (d, 1 H, $J_{1,2}$ 1.3 Hz, H-1^{IV}), 4.94, 4.88 (2 d, $J_{1,2}$ 1.3 Hz, H-1^{II,III}), 4.74–4.55 (m, 9 H, 4 CH_2Ph, incl d at ~4.6 for H-1^{I}), 4.37 (t, 1 H, *J* 5.0 Hz, H-7″), 4.0 (m, 1 H, H-2^{IV}), 3.93, 3.82, 3.78 (3 bt, 1 H each, H-2$^{I–III}$), 3.72–3.65 (m, 4 H, H-3$^{I–IV}$), 3.62–3.14 (m, 18 H, H-4$^{I–IV}$,5$^{I–IV}$,1″a,b,6″a,b, incl s at 3.38 for 2 OCH_3), 2.56 (d, 1 H, *J* 2.0 Hz, OH), 2.17 (t, 2 H, *J* 7.3 Hz, H-5″), 1.69–1.59 (m, partially overlapped, H-4″a,b), 1.59–1.50 (m, partially overlapped, H-2″), 1.38–1.23 (m, partially overlapped, H-3″), 1.25–1.15 (m, partially overlapped, H-6$^{I–IV}$); ^{13}C NMR ($CDCl_3$): 102.51 (C-7″), 100.41 (C-1^{IV}), 100.26, 100.09 (C-1^{II,III}), 98.43 (C-1^{I}), 77.51, 77.35, 76.81, 76.42 (C-3$^{I–IV}$), 73.80 (C-2^{I}), 73.40, 73.00 (C-2^{II,III}), 72.05, 71.98, 71.95, 71.83 (4 *C*H$_2$Ph), 67.37 (C-1″), 67.65 (2 C), 67.23, 66.92 (2 C, C-5$^{I–IV}$,2^{IV}), 64.21, 64.02 (2 C), 63.68 (C-4$^{I–IV}$), 54.28 (2 C, 2 OCH_3), 40.71 (C-6″), 36.31 (C-5″), 29.00 (C-2″), 25.65 (C-3″), 25.21 (C-4″), 18.50 (2 C), 18.40, 18.19 (C-6$^{I–IV}$); CIMS: *m*/*z* 1281 $[M + 18]^+$, FABMS: *m*/*z* 1286 $[M + Na]^+$. Anal. Calcd for $C_{62}H_{81}N_{13}O_{16}$: C, 58.90; H, 6.46; N, 14.40. Found: C, 58.66; H, 6.44; N, 14.26.

N-*(2,2-Dimethoxyethyl)-6-[2-O-acetyl-4-azido-3-O-benzyl-4,6-dideoxy-α-D-mannopyranosyl-(1→2)-tetrakis(4-azido-3-O-benzyl-4,6-dideoxy-α-D-mannopyranosyl)-(1→2)-(4-azido-3-O-benzyl-4,6-dideoxy-α-D-mannopyranosyl)oxy]hexanamide* (**7**).—A mixture of **1** (1.7 g, 2.7 mmol) and **6** (2.0 g, 1.6 mmol) was treated with NIS and AgOTf, as described for the preparation of **3**, to give **7** (1.47 g, 51%), after isolation of the main product by chromatography, $[\alpha]_D$ +76°. ^{1}H NMR ($CDCl_3$): 5.66 (bt, 1 H, NH), 5.41 (dd, 1 H, $J_{1,2}$ 1.8, $J_{2,3}$ 3.2 Hz, H-2^{VI}), 4.95 (d, 1 H, $J_{1,2}$ 1.8 Hz, H-1^{VI}), 4.88, 4.86–4.84 (d, 1 H, $J_{1,2}$ 1.8 Hz, m, 3 H, H-1$^{II–V}$), 4.74–4.51 (m, 13 H, 6 *C*H$_2$Ph, incl d at ~4.60 for H-1^{I}), 4.36 (t, 1 H, *J* 5.8 Hz, H-7″), 3.88, 3.84, 3.81–3.76 (2 bt, 1 H each, m, 4 H, H-2$^{I–VI}$,), 3.74–3.62 (m, 6 H, H-3$^{I–VI}$), 3.54–3.14 (22 H, H-4$^{I–VI}$,5$^{I–VI}$, 1″a,b,6″a,b, incl s at ~3.38 for 2 OCH_3), 2.17 (t, 2 H, *J* 7.3 Hz, H-5″a,b), 2.09 (S, 3 H, $COCH_3$), 1.69–1.61 (m, partially overlapped, H-4″a,b), 1.62–1.50 (m, partially overlapped, H-2″a,b), 1.38–1.12 (m, 20 H, H-6$^{I–VI}$,3″a,b); ^{13}C NMR ($CDCl_3$): 102.52 (C-7″), 102.24 (C-1^{VI}), 100.01 (2 C), 99.93, 98.98 (C-1$^{II–V}$), 98.45

(C-1^{I}), 77.36, 76.72, 76.57, 76.42 (2 C, C-3^{I-V}), 75.32 (C-3^{VI}), 73.86, 73.48, 73.32 (2 C), 73.23 (C-2^{I-V}), 72.05 (3 C), 71.98, 71.92, 71.43 (6 CH_2Ph), 67.39 (C-1′), 67.70 (3 C), 67.62, 67.54, 67.01, 66.92 (C-2^{VI},5^{I-VI}), 64.24, 64.15, 64.06 (2 C), 63.92, 63.69 (C-4^{I-VI}), 54.29 (2 C, 2 OCH_3), 40.72 (C-6′), 36.33 (C-5′), 29.01 (C-2′), 25.67 (C-3′), 25.22 (C-4′), 20.88 ($COCH_3$), 18.51 (2 C), 18.40 (3 C), 18.28 (C-6^{I-VI}); FABMS: m/z 1828 $[M+1]^+$. Anal. Calcd for $C_{90}H_{113}N_{19}O_{23}$: C, 59.10; H, 6.23; N, 14.55. Found: C, 59.15; H, 6.28; N, 14.5.

N-*(2,2-Dimethoxyethyl)-6-[4-azido-3-*O*-benzyl-4,6-dideoxy-α-*D*-mannopyranosyl-(1→2)-tetrakis(4-azido-3-*O*-benzyl-4,6-dideoxy-α-*D*-mannopyranosyl)-(1→2)-(4-azido-3-*O*-benzyl-4,6-dideoxy-α-*D*-mannopyranosyl)oxy]hexanamide* (**8**).—Conventional deacetylation (Zemplén) of **7** gave **8** in virtually theoretical yield, $[\alpha]_D$ 86°. ^{1}H NMR ($CDCl_3$): 5.62 (bt, 1 H, NH), 4.98 (d, 1 H, $J_{1,2}$ 1.4 Hz, H-1^{VI}), 4.96, 4.89, 4.84 (2 bd, 1 H each, bd, 2 H, H-1^{II-V}), 4.74–4.58 (m, 13 H, 6 CH_2Ph, H-1^{I}), 4.36 (t, 1 H, J 5.2 Hz, H-7′), 4.00 (m, 1 H, H-2^{VI}), 3.93, 3.84–3.76 (bt, 1 H, m, 4 H, H-2^{I-V}), 3.73–3.14 (m, 22 H, H-4^{I-VI},5^{I-VI},1′a,b,6′a,b, incl s, at 3.86, 2 OCH_3), 2.37 (d, 1 H, J 1.9 Hz, OH), 2.17 (t, 2 H, J 7.4 Hz, H-5′a,b), 1.69–1.59 (m, 2 H, H-4′a,b), 1.59–1.49 (m, 2 H, H-2′a,b), 1.38–1.12 (2 m, partially overlapped, H-3′a,b, H-6^{I-VI}); ^{13}C NMR ($CDCl_3$): 102.62 (C-7′), 100.46, 100.34, 100.19, 100.09 (2 C, C-1^{I-VI}). 98.53 (C-1^{I}), 77.61, 77.42, 76.92, 76.58, 76.47 (2 C, C-3^{I-VI}), 73.39 (C-2^{I}), 73.54, 73.37 (2 C), 73.14 (C-2^{II-V}), 72.08 (5 C), 71.98 (6 CH_2Ph), 67.44 (C-1′), 67.75 (3 C), 67.67, 67.29, 67.05, 66.96 (C-2^{VI},5^{I-VI}), 64.29, 64.11 (4 C), 63.74 (C-4^{I-VI}), 54.32 (2 C, 2 OCH_3), 40.72 (C-6′), 36.35 (C-5′), 29.02 (C-2′), 25.66 (C-3′), 25.22 (C-4′), 18.49 (2 C), 18.39 (3 C), 18.19 (C-6^{I-VI}); FABMS: m/z 1786 $[M+1]^+$. Anal. Calcd for $C_{88}H_{111}N_{19}O_{22}$: C, 59.15; H, 6.26; N, 14.89. Found: C, 59.22; H, 6.32; N, 14.88.

N-*(2,2-Dimethoxyethyl)-6-[2-*O*-methyl-4-azido-3-*O*-benzyl-4,6-dideoxy-α-*D*-mannopyranosyl-(1→2)-tetrakis(4-azido-3-*O*-benzyl-4,6-dideoxy-α-*D*-mannopyranosyl)-(1→2)-(4-azido-3-*O*-benzyl-4,6-dideoxy-α-*D*-mannopyranosyl)oxy]hexanamide* (**9**) *and* N-*methyl-(2,2-dimethoxyethyl)-6-[2-*O*-methyl-4-azido-3-*O*-benzyl-4,6-dideoxy-α-*D*-mannopyranosyl-(1→2)-tetrakis(4-azido-3-*O*-benzyl-4,6-dideoxy-α-*D*-mannopyranosyl)-(1→2)-(4-azido-3-*O*-benzyl-4,6-dideoxy-α-*D*-mannopyranosyl)oxy]hexanamide* (**17**).—(a) Methylation of **8** with the MeI–Ag_2O–Me_2S reagent [26] or with MeI and KOH in Me_2SO [27] gave, in addition to the desired 2-*O*-methyl derivative **9**, variable amounts of the *N*-methyl derivative **17**. Chromatography (2:1 hexane–acetone) gave first **17** ($\sim$10%), identified by NMR spectroscopy. ^{1}H NMR ($CDCl_3$): 4.49, 4.41 (2 t, 1 H total, J 4.5 Hz, H-7′), 3.06, 2.98 (2 s, 3 H total, NCH_3), 2.38, 2.32 (2 t, 2 H total, J 7.5 Hz, H-5′); ^{13}C NMR ($CDCl_3$): 103.47, 103.12 (1 C total, C-7′), 55.19, 54.62 (2 C total, 2 OCH_3-7′), 52.31, 50.02 (1 C total, C-6′), 37.11, 34.55 (1 C total, NCH_3), 32.24, 32.74 (1 C total, C-5′); FABMS: m/z 1946 $[M+Cs]^+$.

Eluted next was the desired monomethyl derivative **9** ($\sim$85%), $[\alpha]_D$ +94° (c 0.4). ^{1}H NMR ($CDCl_3$): 5.66 (bt, 1 H, NH), 4.93–5.84 (m, 5 H, H-1^{II-VI}), 4.76–4.58 (m, 13 H, 6 CH_2Ph, H-1^{I}), 4.37 (t, 1 H, J 5.1 Hz, H-7′), 3.96, 3.85, 3.82, 3.80–3.76 (3 bt, 1 H each, m 2 H, H-2^{I-V}), 3.72–3.63 (m, 6 H, H-3^{I-VI}), 3.60–3.16 (m, 26 H, H-2^{VI},4^{I-VI},5^{I-VI}, H-1′,6′, incl s at 3.37 for 2 OCH_3-7′ and s at 3.20 for OCH_3-6), 2.18 (t, 2 H, J 7.4 Hz, H-5′), $\sim$1.70–1.59 (m, partially overlapped, H-4′), $\sim$1.60–1.5 (m, partially overlapped, H-2′), $\sim$1.38–1.26 (m, partially overlapped, H-3′), 1.26–1.12 (m, 18 H, H-6^{I-VI}); ^{13}C NMR ($CDCl_3$): 102.56 (C-7′), 100.30, 100.16, 100.05 (2 C, C-1^{II-V}), 98.67 (C-1^{VI}), 98.48 (C-1^{I}), 77.42, 77.36, 77.04, 77.00, 76.58, 76.44 (C-3^{I-VI}), 76.25 (C-2^{VI}), 73.89, 73.48, 73.32, 73.10, 73.02 (C-2^{I-V}), 72.28, 72.06 (3 C), 71.97 (2 C, 6 CH_2Ph), 67.71 (3 C), 67.64 (2 C), 66.91 (C-5^{I-VI}), 67.38 (C-1′), 64.21 (2 C), 64.06 (3 C), 63.98 (C-4^{I-VI}), 58.80 (OCH_3-2), 54.27 (2 C, 2 OCH_3-7′), 40.68 (C-6′), 36.29 (C-5′), 28.96 (C-2′), 25.61 (C-3′), 25.17 (C-4′), 18.45 (2 C), 18.34 (3 C), 18.25 (C-6^{I-VI}); FABMS: m/z 1932 $[M+Cs]^+$. Anal. Calcd for $C_{89}H_{113}N_{19}O_{22}$: C, 59.36; H, 6.32; N, 14.78. Found: C, 59.26; H, 6.31; N, 14.61.

(b) When compound **8** was treated with Ag_2O in MeI as a solvent and reagent overnight, the byproduct **17** was not formed,

and the methyl derivative **9** was obtained in virtually theoretical yield.

N-*(2,2-Dimethoxyethyl)-6-[4-(3-deoxy*-L-glycero-*tetronamido)-4,6-dideoxy-2*-O-*methyl*-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-*mannopyranosyl)oxy]hexanamide* (**12**).—Hydrogen sulfide gas was passed for 30 min through a solution of **9** (370 mg) in 2:1 pyridine–water (6 mL) which was then kept at 40 °C overnight. One product was formed, as shown by TLC (10:1 CH_2Cl_2–MeOH). After concentration, the residue was chromatographed to give *N*-(2,2-dimethoxyethyl)-6-[4-amino-3-*O*-benzyl-4,6-dideoxy-2-*O*-methyl-α-D-mannopyranosyl-(1→2)-tetrakis(4-amino-3-*O*-benzyl-4,6-dideoxy-α-D-mannopyranosyl)-(1→2)-(4-amino-3-*O*-benzyl-4,6-dideoxy-α-D-mannopyranosyl)oxy]hexanamide (**10**, 270 mg, 81%). Structurally diagnostic resonances in the ^{1}H NMR ($CDCl_3$) spectra: 5.74 (m, 1 H, NH″), 5.09, 5.07, 5.00 (bd, m, bd, 5 H, H-1$^{II–VI}$), 4.75–4.36 (m, 14 H, 6 C*H*$_2$Ph, H-1^I, incl t at 4.37, *J* 6.0 Hz, H-7″), 4.12–3.90 (m, 6 H, H-2$^{I–VI}$), 3.39 (s, partially overlapped, 2 OCH_3-7″), 3.28 (s, OCH_3-2), 2.19 (t, 1 H, *J* 7.2 Hz, H-5″), 1.72–1.54 (2 m, partially overlapped, 4 H, H-4″,2″ in that order), 1.40–1.14 (m, H-6$^{I–VI}$,3″); ^{13}C NMR ($CDCl_3$): 102.71 (C-7″), 101.02 (4 C, C-1$^{II–V}$), 99.18 (2 C, C-1^{I,VI}), 79.64, 79.16 (2 C), 78.76, 78.67 (2 C, C-3$^{I–VI}$), 75.86 (C-2^{VI}), 73.04 (3 C), 72.87, 72.62 (C-2$^{I–V}$), 71.65, 71.29, 71.13 (4 C, 6 *C*H$_2$Ph), 70.31 (5 C), 69.62 (C-5$^{I–VI}$), 67.11 (C-1″), 58.89 (OCH_3-2^{VI}), 54.39 (2 C, 2 OCH_3-7″), 53.79, 53.62 (3 C), 53.57 (2 C, C-4$^{I–VI}$), 40.76 (C-6″), 36.49 (C-5″), 29.08 (C-2″), 25.80 (C-3″), 25.32 (C-4″), 18.18 (4 C), 18.10, 17.99 (C-6$^{I–VI}$); FABMS: *m*/*z* 1644 $[M+1]^+$, 1776 $[M+Cs]^+$.

Diisopropylethylamine (0.3 mL, 1.6 mmol) followed by HATU (0.26 g, 1.6 mmol) was added to a solution of the foregoing amine **10** (270 mg, 0.16 mmol) and the acid **18** (342 mg, 1.6 mmol) in CH_2Cl_2 (30 mL). The mixture was stirred at room temperature (rt) for 30 min, when TLC (3:2 toluene–acetone) showed that the starting amine was consumed and that one faster-moving product was formed. After concentration, chromatography gave *N*-(2,2-dimethoxyethyl)-6-{3-*O*-benzyl-4-(2,4-*O*-benzylidene-3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-2-*O*-methyl-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-mannopyranosyloxy}hexanamide (**11**). ^{1}H NMR ($CDCl_3$): 6.42–6.08 (m, 6 H, NH$^{I–VI}$), 5.7 (t, *J* 5.2, NH-7″), 5.55, 5.54, 5.53, 5.52, 5.51, 5.50 (6 s, 6 C*H*Ph), 4.99, 4.95, 4.29, 4.87 (bd, 1 H, bs, 2 H, 2 bd 1 H each, H-1$^{II–VI}$), 4.70–4.27 (m, 20 H, H-1^I,2′$^{I–VI}$,7″ 6 C*H*$_2$Ph), 4.19–3.92 (m, 17 H, H-2$^{I–V}$,4$^{I–VI}$,4′a$^{I–VI}$), 3.84–3.50 (m, 21 H, H-2^{VI},3$^{I–VI}$,5$^{I–VI}$,4′b$^{I–VI}$,1″a,b), 3.36, 3.35 (2 s, 6 H, 2 OCH_3-7″), 3.83, 3.30 (m, 2 H, H-6″a,b), 3.24 (s, 3 H, OCH_3-2^{VI}), 2.12 (t, 2 H, *J* 7.2 Hz, H-5″), 2.09–1.80 (m, 12 H, H-3′$^{I–VI}$), 1.66–1.48 (2 m, 4 H, partially overlapped, H-4″,2″ in that order), 1.35–1.25 (m, 2 H, H-3″), 1.14–1.02 (m, 18 H, H-6$^{I–VI}$); ^{13}C NMR ($CDCl_3$): 102.56 (C-7″), 101.30 (2 C), 101.21, 101.13 (2 C), 101.01 (6 *C*H$_2$Ph), 100.40 (3 C), 100.27 (C-1$^{II–V}$), 99.07 (C-1^I), 98.73 (C-1^{VI}), 76.58 (6 C, C-2′$^{I–VI}$), 76.00, 75.75, 75.5975.04, 74.83, 74.60, 74.48, 74.40 (C-2^{I,VI},3$^{I–VI}$), 72.73 (3 C), 72.58 (C-2$^{II–V}$), 71.99, 71.33, 71.27, 70.97 (3 C, 6 *C*H$_2$Ph), 68.66 (4 C), 68.57 (2 C, C-5$^{I–VI}$), 67.25 (C-4′$^{I–VI}$,1″), 59.02 (OCH_3-2^{VI}), 54.36 (2 C, 2 OCH_3-7″), 52.43, 51.95, 51.78 (2 C), 51.70, 51.42 (C-4$^{I–VI}$), 40.76 (C-6″), 36.44 (C-5″), 28.82 (C-2″), 28.55 (6 C, C-3′$^{I–VI}$), 25.64 (C-3″), 25.19 (C-4″), 17.90, 17.96 (5 C, C-6$^{I–VI}$); FABMS: *m*/*z* 2916 $[M+Cs]^+$, 2784 $[M+1]^+$.

A mixture of the foregoing fully protected compound **11** and 5% Pd–C catalyst in MeOH (50 mL, **Caution:** Extreme fire hazard!) was stirred for 48 h in a hydrogen atmosphere at ambient pressure and temperature, when TLC (2:1:0.4 EtOAc–MeOH–water) showed that the reaction was essentially complete. After filtration and concentration of the filtrate, elution from a small column of silica gel gave pure, hygroscopic dimethyl acetal, *N*-(2,2-dimethoxyethyl)-6-{4-(3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-2-*O*-methyl-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-mannopyranosyl]oxy}hexanamide (**12**, 178 mg, 63% from **10**). ^{1}H NMR (D_2O): 5.20, 5.18, 5.15, 5.13 (bd, 1 H, bd 1 H, bs, 2 H, bs, 1 H, H-1$^{II–VI}$), 4.87 (bs, 1 H, H-1^{I}), 4.51 (t, 1 H, *J* 5.4 Hz, H-7″), 4.30–4.26 (m, 6 H, H-2′$^{I–VI}$), 4.18–4.02 (m, 10 H, H-2$^{II–V}$,3$^{I–VI}$), 3.98–3.82 (m, 13 H, H-2^{I},4$^{I–VI}$,5$^{I–VI}$), 3.77–3.67 (m, 14 H, H-2^{VI},4′$^{I–VI}$,1″a), 3.56–3.50 (m, H-1″b), 3.47 (s, 3 H, OCH_3-2^{VI}), 3.40 (s, 6 H, 2 OCH_3-7″), 3.32 (m, 2 H, H-6″a,b), 2.25 (t, 2 H, *J* 7.2 Hz, H-5″a,b), 2.08–1.78 (2 m, 12 H, H-3′a,b), 1.65–1.56 (m, 4 H, H-2″,4″), 1.40–1.30 (m, 2 H, H-3″a,b), 1.22–1.12 (m, 18 H, H-6$^{I–VI}$); ^{13}C NMR (D_2O): 102.73 (C-7″), 101.00, 100.92 (3 C, C-1$^{II–V}$), 99.14 (C-1^{I}), 98.59 (C-1^{VI}), 79.02 (C-2^{VI}), 77.84, 77.64, 77.31 (2 C), 77.26 (C-2$^{I–V}$), 69.07 (C-2′$^{I–VI}$), 68.38 (3 C), 68.03 (2 C), 67.84, 67.72, 67.62 (3 C), 67.55 (2 C), 67.47 (C-3$^{I–VI}$,5$^{I–VI}$,1″), 58.85 (OCH_3-2^{VI}), 57.94 (C-4′$^{I–VI}$), 54.62 (2 C, 2 OCH_3-7″), 53.26, 53.20, 53.04 (4 C, C-4$^{I–VI}$), 40.81 (C-6″), 36.09 (C-3′$^{I–VI}$), 35.64 (C-5″), 28.24 (C-2″), 25.14 (C-3″), 24.90 (C-4″), 16.90 (6 C, C-6$^{I–VI}$); FABMS: *m*/*z* 1848 $[M + Cs]^+$.

The amorphous, hygroscopic compound **12** was characterized as the corresponding per-*O*-acetyl derivative, *N*-(2,2-dimethoxyethyl)-6-{3-*O*-acetyl-4-(2,4-di-*O*-acetyl-3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-2-*O*-methyl-α-D-mannopyranosyl-(1→2)-tetrakis[3-*O*-acetyl-4-(2,4-di-*O*-acetyl-3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranosyl-(1→2)]-[3-*O*-acetyl-4-(2,4-di-*O*-acetyl-3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranosyl]oxy}hexanamide (**13**), $[\alpha]_D$ +31° (*c* 0.7). ^{1}H NMR ($CDCl_3$): 6.80–6.44 (m, 6 H, 6 NH′), 5.78 (t, 1 H, *J* 5.5, NH″), 5.28–4.90 (m, 16 H, 4 H-1, H-3$^{I–VI}$,2′$^{I–VI}$, incl d, 4.90, 1 H, *J* 2.1 Hz, H-1), 4.73 (bd, 1 H, H-1^{I}), 4.42 (t, 1 H, *J* 5.2, H-7″), 4.35–4.04 (m, 22 H, H-2$^{II–V}$,4$^{I–VI}$,4′$^{I–VI}$), 3.93 (bt, 1 H, H-2^{I}), 3.90–3.70 (m, 7 H, H-5$^{I–VI}$, 1″a), 3.64 (bdd, 1 H, H-2^{VI}), 3.54 (s, partially overlapped, OCH_3-2^{VI}), 3.52–3.30 (m, 9 H, H-1″b, 6″a,b, incl s, 3.40, 2 OCH_3-7″), 2.20–2.05 (m, 7 H, H-5″,3′a$^{I–VI}$ a, 6 $COCH_3$), 1.86–1.67 (m, 6 H, 3′b), 1.65–1.15 (m, H-6$^{I–VI}$,2″,3″,4″); ^{13}C NMR ($CDCl_3$): 102.30 (C-7″), 100.24 (3 C), 99.90 (C-1$^{II–V}$), 99.45 (C-1^{I}), 98.45 (C-1^{VI}), 77.77 (C-2^{VI}), 75.38 (2 C), 75.18 (2 C), 74.63 (C-2$^{I–V}$), 71.12, 70.90 (2 C), 70.81 (4 C), 70.73, 70.35, 70.01, 69.75, 69.70, 69.37 (2 C), 69.13, 68.92 (2 C), 68.61 (C-3$^{I–VI}$, 5$^{I–VI}$,2′$^{I–VI}$), 6948 (C-1″), 59.96, 59.89 (5 C, C-4′$^{I–VI}$), 59.60 (OCH_3-2^{VI}), 54.32, 54.10 (2-OCH_3-7″), 51.58 (4 C), 51.37, 51.05 (C-4$^{I–VI}$), 40.89 (C-6″), 36.42 (C-5″), 30.50 (C-3′$^{I–VI}$), 28.21 (C-2″), 25.01 (C-3″), 24.06 (C-4″), 17.83 (2 C), 17.77 (2 C), 17.71, 17.62 (C-6$^{I–VI}$); FABMS: *m*/*z* 2604 $[M + Cs]^+$. Anal. Calcd for $C_{107}H_{161}N_7O_{58}$: C, 51.96; H, 6.56; N, 3.96. Found: C, 51.73; H, 6.59; N, 3.95.

Single-point-attachment conjugation of the terminal, hexasaccharide determinant of the O-antigen of V. cholerae *O:1, serotype Ogawa to CSA*.—A solution of **12** (1.8 mg, 1.05 μmol) in 0.02 M TFA (1 mL) was kept at rt for 3 days. ^{1}H NMR then showed that the reaction was complete and that byproducts from cleavage of interglycosidic linkages were not formed. Compared to the spectrum of **12**, the ^{1}H NMR spectrum of **14** (D_2O) showed the disappearance of the singlet at δ 3.40 [$CH(OCH_3)_2$]. The triplet for H-7″ [$CH(OD)_2$], appearing in the spectrum of **12** [$CH(OCH_3)_2$] at δ 4.51, was shifted downfield to δ 5.04. The solution was freeze-dried, to give **14** as a white solid. To a solution of the foregoing material and CSA (3 mg, 0.045 μmol, corresponding to a 1:2 ligand–NH_2 ratio, based on 46 lysine residues/CSA) in phosphate buffer (50 μL, forming a solution concentration of the hapten, 21 mM, which is well above the minimum that is recommended [8]), was added borane–pyridine complex (8 M, 1 μL, 8 μM). The solution was kept at rt for 4 days. The mixture was filtered through a 30 K Centricon device, and the retained material was washed with deionized water (3 × 2 mL). The retained material was freeze-dried, to give a white, hygroscopic solid (3.2 mg). MALDI-TOF analysis showed the molecular mass of the neoglycoconjugate thus obtained to be 78,650 Da, corresponding to an incorporation of approximately seven residues of **14**/CSA.

Preparation of the glycocluster conjugate **20**.—Subcarrier **19** (1.98 mg, 1 μmol) was added to a solution of **14** [prepared from **12** (14 mg, 8.16 μmol)] in 0.1 M phosphate buffer (pH 7.0, 400 μL), followed by boron–pyridine complex (8.2 μL, 82 μmol). The mixture was stirred at rt for 40 h, filtered, washed (thrice)

using a 3 K Centricon centrifugal filtering device, and the retained material was freeze-dried, to give the HCS.

Diethyl squarate (14 μL, 82 μmol) was added to a solution of the above HCS in 0.1 M phosphate buffer (0.8 mL, pH 7.0), and the solution was stirred overnight at rt. The mixture was filtered, washed (thrice) using a 3 K Centricon centrifugal filtering device, and the retained material was freeze-dried to give the DHCS as a white solid (8 mg). On MALDI-TOF analysis, the material produced a broad peak centered at $\sim m/z$ 8600.

A solution of the foregoing DHCS (1 mg) in borate buffer (0.1 mL, pH 9.0) was treated with CSA (0.49 mg) for 2 days at rt. The mixture was processed as described for the preparation of DHCS, except that a 30 K Centricon centrifugal filtering device was used to remove the low-molecular-mass material. The retained material was freeze-dried to give the target product **20** as an amorphous solid (1.3 mg). The material, when analyzed by phenol–sulfuric acid assay, was shown to contain 51% of carbohydrate (expressed as the methyl glycoside analog of **14**, a synthetic sample of which [21] was used as a reference).

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