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Immunogens from a synthetic hexasaccharide fragment of the O-SP of *Vibrio cholerae* O:1, serotype Ogawa

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Abstract—The known 5-(methoxycarbonyl)pentyl α-glycoside of the hexasaccharide of *Vibrio cholerae* O:1, serotype Ogawa **24** was newly prepared. The efficiency of construction of the hexasaccharide from the disaccharide glycosyl acceptor **6** and each of the two tetrasaccharide glycosyl donors **16**, and **18**, as an alternative to the iterative coupling of the disaccharide glycosyl donor **7** with **6**, was evaluated. Compound **24** was treated with each of hydrazine hydrate, ethylenediamine, and hexamethylenediamine to give ligands **25**, **27**, and **29**, respectively, equipped with different linkers. Reaction of the foregoing compounds with squaric acid diethyl ester, and the reactions of the thus formed monoesters **26**, **28**, and **30** with BSA were evaluated. The rate of formation of the corresponding monoester was higher with hydrazide **25** than with amines **27** and **29**, whose reaction rates were virtually the same. Reactions of squaric acid derivatives **26**, **28**, and **30** with BSA were conducted under the same conditions (reaction temperature, ligand–BSA ratio, and concentration with respect to the hapten) and targeted for neoglycoconjugates with **24**–BSA ratio of ~5. Monitoring the conversions by SELDI-TOF mass spectrometry in conjunction with the ProteinChip® system made it possible to conduct the conjugations in controlled fashion, and to terminate the reactions when the desired carbohydrate–protein ratios were reached. Products from **26**, **28**, and **30**, neoglycoconjugates **31**–**33**, containing 4.9, 5.0, and 5.1 moles of **24**/BSA, respectively, were obtained in 87–93% yields. When the conjugation started with the initial molar carbohydrate–protein ratio of 15:1, the final molar carbohydrate–BSA ratio reached after 14 days with ligands **26**, **28**, and **30** was 9.0, 11.0, and 9.1, respectively.

1. Introduction

During the past few years, this laboratory has been working toward developing a conjugate vaccine for cholera from synthetic fragments of *O*-specific polysaccharides (O-SP) of *Vibrio cholerae* O:1. We have already been able to show[1] that a potent immunogen for protective anti *V. cholerae* O:1 antibodies can be obtained from the upstream[2] terminal hexasaccharide of the O-SP and bovine serum albumin (BSA). Development of conjugate vaccines from synthetic oligosaccharides is a very young field and structural requirements for a potent, medically acceptable synthetic vaccine are not known. Within our efforts to develop a clinically useful vaccine, we not only want to make conjugate immunogens following established protocols, but we also want to look at the effect of some fundamental variables upon conjugation and the immunogenicity. Because of its high efficiency[3,4] and experimental simplicity, our method of choice for conjugation of synthetic oligosaccharides to proteins is that developed by Tietze et al.,[5,6] which utilizes the unique reactivity of squaric acid diesters. When treated with an amine at pH 7, a substance of this class selectively forms a monoamide ester. The latter can be then converted to a diamide by reaction with another amine at pH 9. When the two amines are an oligosaccharide and a protein, the product will be a neoglycoconjugate. To make synthetic oligosaccharides suitable for conjugation to proteins by squaric acid chemistry, they are usually prepared in the form of glycosides, such as **24**, whose linker can be transformed to contain a primary amino group. This has been most frequently accomplished by aminolysis with ethylenediamine.[7] Squaric acid diesters have also been derivatized with hydrazides[8,9] but, to our knowledge, a direct comparison of the effect of the nature of the linker upon conjugation using squaric acid chemistry has not been made. The nature of the linker is an important variable in conjugate vaccine development. The linker (spacer) separates the specific antigen (the B-cell epitope) from the protein carrier (the T-cell epitope), thereby minimizing the steric influence of the protein, which can affect both

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chemistry of conjugation and immune response. Here we describe conjugation to BSA of the upstream, terminal hexasaccharide of the O-SP of *V. cholerae* O:1, serotype Ogawa equipped with three different linkers, and evaluate the effect of the nature of the linker upon the chemistry of conjugation.

To increase the potential of an Ogawa hexasaccharide–protein construct as a clinically useful vaccine for cholera,[1] we are continuously striving to improve the synthesis of the linker-equipped hexasaccharide. Here, we are comparing the block-wise assembly of the hexasaccharide intermediate **12** using different glycosyl donors.

Results of serological evaluation of the immune responses in small laboratory animals, challenged with products of conjugations here described, will be published in due time.

2. Results and discussion

2.1. Synthesis of the linker-equipped hexasaccharides

The synthesis of hapten **24** was first carried out in this laboratory almost 10 years ago.[10] The finding that a neoglycoconjugate based on that hexasaccharide has potential as a vaccine for cholera[1] prompted us to revise the original synthesis. Several improvements have been implemented in the newer variants,[11–14] but each has its advantages and drawbacks. The linker-equipped oligosaccharide **24** was now newly synthesized. The purpose of this new approach was to compare construction of hexasaccharide intermediate **12** from the disaccharide glycosyl acceptor **6** and tetrasaccharide glycosyl donors **16** and **18**, with the assembly from **6** by iterative extension of the oligosaccharide chain with the disaccharide glycosyl donor **7**.[15] Once assembled, compound **12** was further transformed to the known hexasaccharide **24**. Treatment of the latter with each of hydrazine hydrate, ethylenediamine, and hexamethylenediamine produced the upstream, terminal fragment of the O-SP of *V. cholerae* O:1, serotype Ogawa, equipped with different linkers. We expect that comparison of conjugations of these materials to BSA, and future evaluation of sera resulting from immunization of small laboratory animals with the resulting immunogens, will identify the most suitable derivative to be conjugated to a medically acceptable carrier.

The assembly of **12** (Scheme 1) started with the hitherto unknown glycosyl acceptor **3**, whose precursor **2** was now obtained from **1** in a higher yield (83% using $BF_3 \cdot Et_2O$ as a catalyst) than previously reported[14] (75%, with $SnCl_4$ as a catalyst). Reaction of **3** with thioglycoside **4**[16] (→**5**), followed by Zemplén deacetylation gave the key, linker-equipped disaccharide glycosyl acceptor **6**. The construction of the hexasaccharide **12** from **6** was achieved in three different ways. Following the route that led to the analogous methyl glycoside,[11,16] condensation of **6** with **7** gave the fully protected tetrasaccharide **8**, which was deacetylated, and the alcohol **9** thus formed was treated (Scheme 2) with the same donor **7**, to give the hexasaccharide intermediate **10**. Deacetylation (Zemplén) of **10**, followed by methylation of the formed **11** with the MeI/Ag_2O/Me_2S reagent[17] then gave **12**. In the other two approaches, alcohol **6** was condensed with each of tetrasaccharide glycosyl donors **16** and **18**, to give **10** and **12**, respectively.

Synthesis of the foregoing tetrasaccharide glycosyl donors (Scheme 2) utilized the known[18] hemiacetal **13**, which was now obtained in a more straightforward way by hydrolysis of thioglycoside **7**. Thus, all three approaches employ common intermediates **6** and **7**. Compound **13** was converted to trichloroacetimidate **14**, and treated with alcohol **15**, obtained by deacetylation of **7**, to give 2^{IV}-*O*-acetate **16**, which was readily transformed into the 2^{IV}-methyl ether **18**, through intermediate **17**.

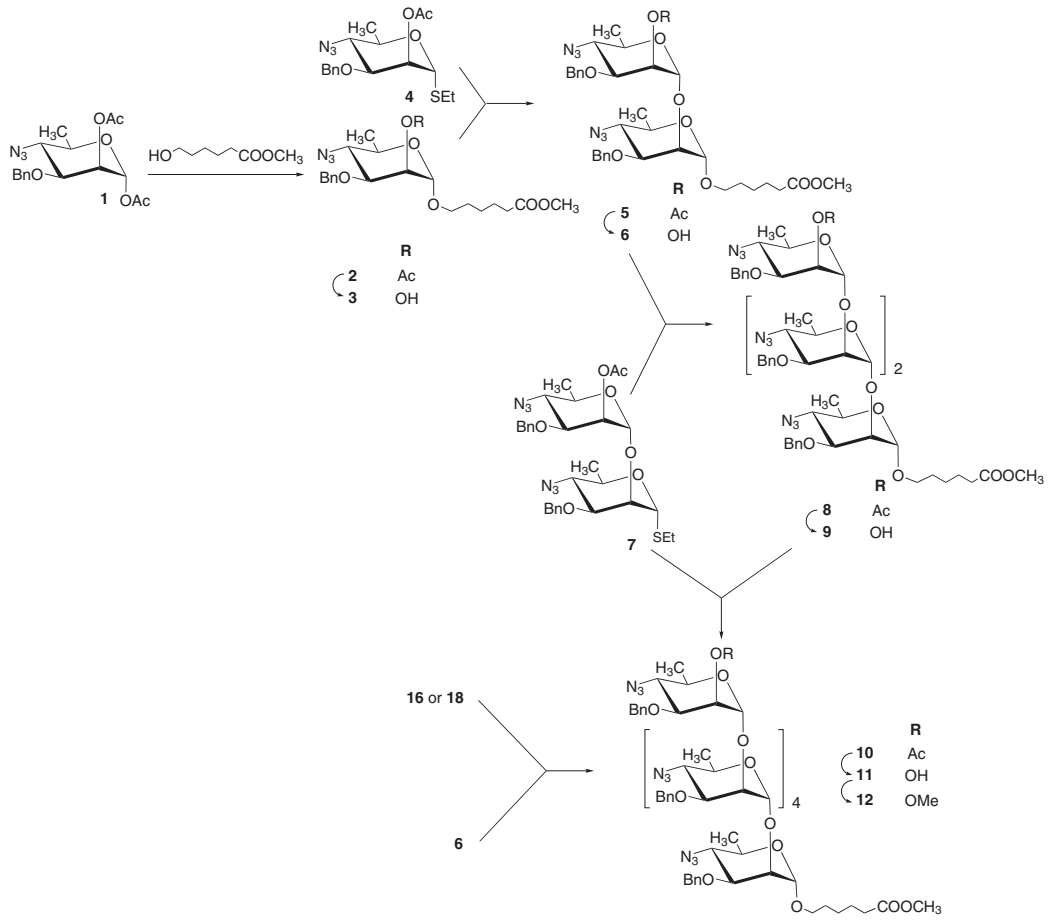
When starting with the glycosyl acceptor **6**, hexasaccharide **12** was obtained in 59% (**6** + **16** → **10** → **11** → **12**) and 55% yield (**6** + **18** → **12**) when tetrasaccharide glycosyl donors were used, and in 78.5% yield (**6** + **7** → **8** → **9** → **12**) with the disaccharide glycosyl donor (see Experimental). Thus, there is no advantage in using the tetrasaccharide synthons **16** and **18** to assemble the hexasaccharide **12**. It appears that, in this series, larger oligosaccharides are not as efficient glycosyl donors as their less bulky counterparts.

To introduce the 3-deoxy-L-*glycero*-tetronic acid side chain amine **19** (Scheme 3), prepared by selective reduction[15,19] of the azido functions in **12**, was treated with acid **21**, obtained by simple acetylation of **20**[20] with acetic anhydride in pyridine (Scheme 4), to give the fully protected hexasaccharide **22**. Preliminary experiments (not described in Experimental) showed that the product of amidation with the fully protected acid **21**, was easier to purify than the one resulting from amidation with **20**, or from acetylation of the crude product resulting from the reaction of **20** + **19**.

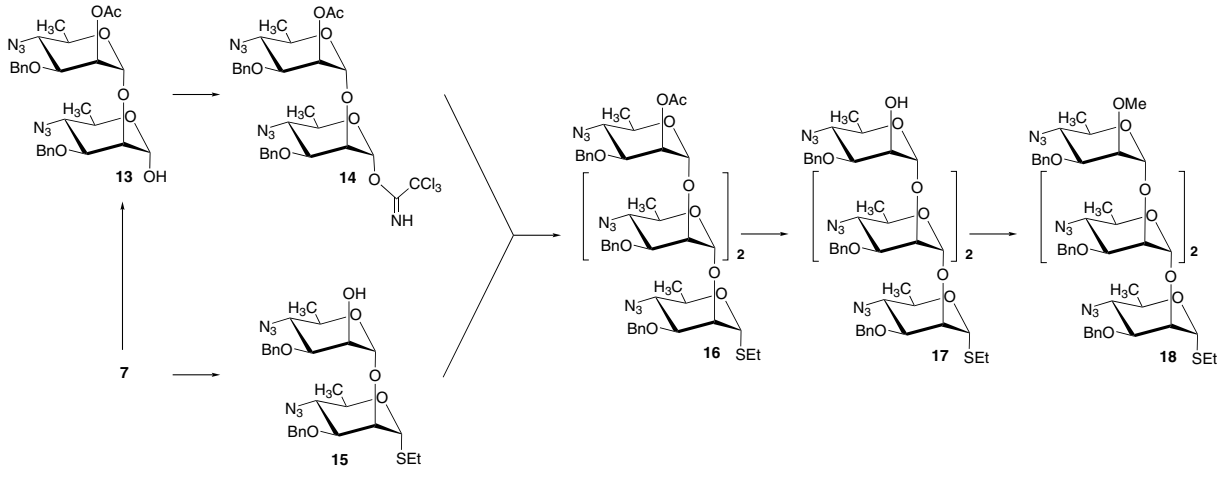
The aforementioned, fully protected hexasaccharide **22** was deprotected (**22** → **23** → **24**), and ester **24**, thus obtained, was treated with each of hydrazine hydrate, ethylenediamine, and hexamethylenediamine, to give synthons **25**, **27**, and **29**. Conversion of these materials to the corresponding monoethyl esters **26**, **28**, and **30**, respectively, was uneventful, but the reaction of the hydrazide derivative **25** with squaric acid diethyl ester was considerably faster. Contrary to the observations by others,[9] we have not observed violet color development during formation of squaric acid monoesters from hydrazides.

2.2. Conjugation

Serological evaluation[1] of neoglycoconjugates from BSA and the hexasaccharide determinant of *V. cholerae* O:1, serotype Ogawa, having carbohydrate–BSA ratio ~5, ~10, and ~15 has shown that the construct with the lowest degree of substitution was most useful as an experimental vaccine. To examine the effect of the nature of linker upon the conjugation by squaric acid chemistry and, perhaps, immunogenicity, the purpose of this

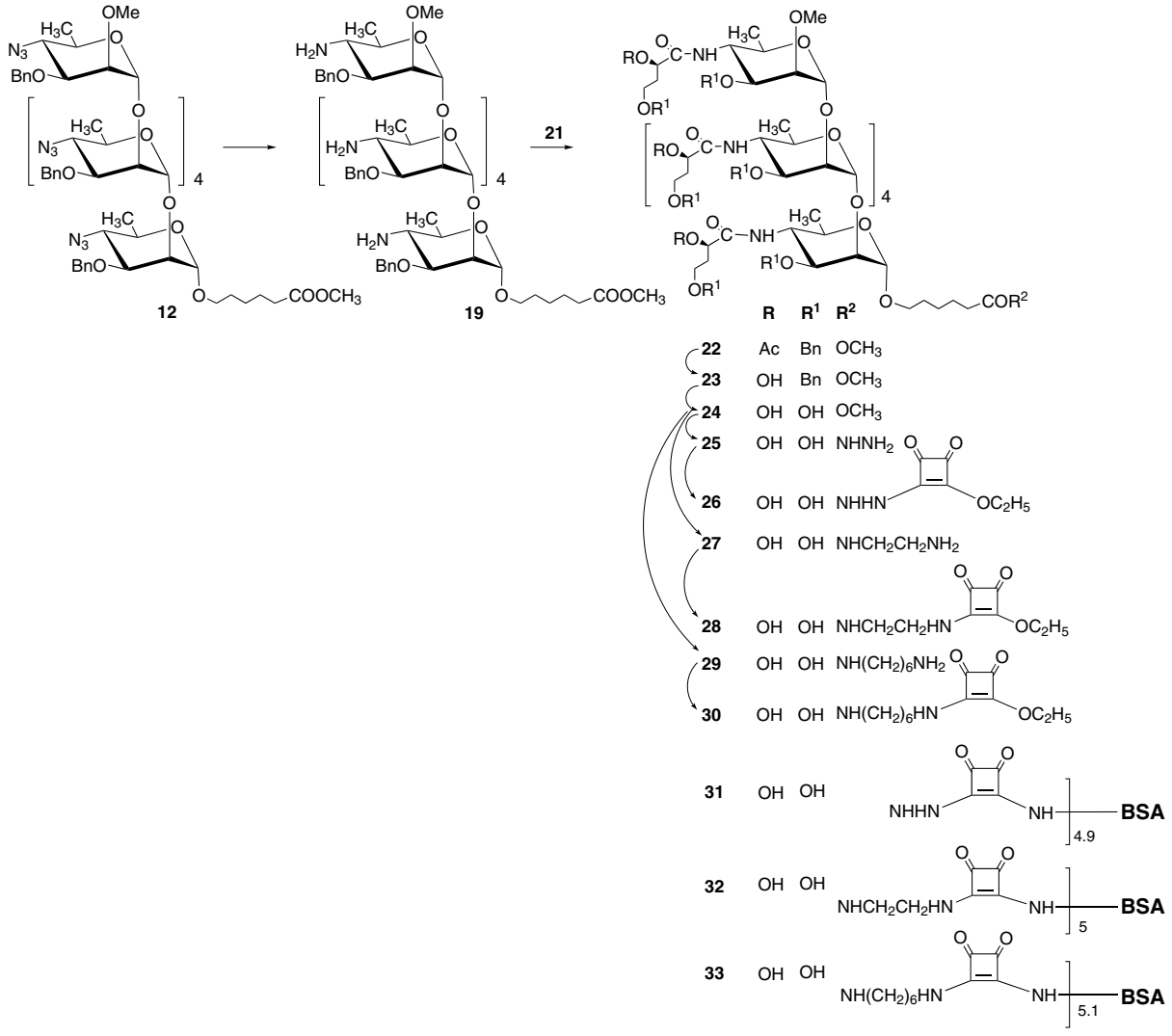


Scheme 1.

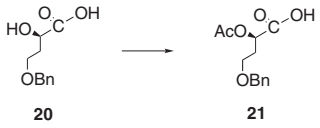


Scheme 2.

work was to prepare constructs similar to the one previously examined[1] and showing the best immunogenicity (the same hexasaccharide, carbohydrate–BSA ratio ~5) but involving different linkers. Previous work[13] where the conjugation was conducted with an initial molar carbohydrate–BSA ratio of 75 indicated that the desired carbohydrate density (carbohydrate–BSA ratio ~5) should be possible to achieve starting with much smaller excess of the carbohydrate, while maintaining an acceptable reaction rate. Conjugation under such conditions was expected to be slower and, thus, easier to control, and also more economical, in view of the labor-intensive hexasaccharide involved. Accordingly, conjugations with the three ligands **26**, **28**, and **30** were carried out at the initial molar hapten–BSA ratio of 15:1, and the increasing molecular mass of the product was monitored by SELDI-TOF mass spectrometry in conjunction with the ProteinChip® system.[8] When the



Scheme 3.



Scheme 4.

targeted carbohydrate–BSA ratio in each of the reactions was reached (Table 1), 90% of the reacting material was withdrawn, and the high molecular mass material was isolated, and freeze-dried, to give conjugates **31**–**33**. It was of interest to compare the highest carbohydrate–BSA ratio attainable with haptens containing different spacers. Therefore, the rest of the material was allowed to react further and periodical monitoring of the reaction was continued. After 14 days of reaction time, virtually no additional increase in the carbohydrate–protein ratio was noted with either of the conjugates **31**–**33**. This agreed with our previous observation[4] that after that period of time virtually all active monoester was consumed, due to conjugation or hydrolysis under the basic reaction conditions. The results in Table 1 show that the fastest and also most efficient conjugation was that with the monoethyl ester prepared from the pentanoic acid 2-(aminoethyl)amide.

Table 1. Effect of the nature of the linker upon conjugation of ligands **26**, **28**, and **30** to BSA[a]

Ligand	Ligand–BSA ratio/time required	Final molar ligand–BSA ratio after 14 days	Conjugation efficiency [%][b]
26	4.9/4 days	9	60
28	5/24 h	11	73
30	5.1/39 h	8.9	59

[a] All reactions have been carried out at an initial, molar ligand–BSA ratio of 15:1, at 15 mmol concentration of the ligand.

[b] After 14 days, based on the ratio of ligand linked to BSA over the initial amount of ligand used.

It is noteworthy that the molar carbohydrate–protein ratios found for all three conjugates prepared are virtually the same as those targeted (hapten–BSA = 5). This confirms the utility[8] of SELDI-TOF mass spectrometry in conjunction with the ProteinChip® system as an important tool in glycoconjugate chemistry. Monitoring

conjugation of synthetic oligosaccharides to carriers by this technique allows the reaction to be carried out in a predictable way, and match the desired and achieved carbohydrate–carrier ratios in neoglycoconjugates with unprecedented accuracy. Also, when squaric acid chemistry is applied, the reaction rate can be controlled by adjusting the initial molar hapten–carrier ratio.[3] Due to the efficiency of the conjugation observed with squarate **26**, ethylenediamine appears to be the reagent of choice for converting ester groups in spacers into an amide/amine, to make the parent ligand amenable to conjugation by squaric acid chemistry, unless future serological studies with constructs **31**–**33** would dictate otherwise.

3. Experimental

3.1. General methods

Unless stated otherwise, optical rotations were measured at ambient temperature for solutions in chloroform ($c \sim 1$), with a Perkin–Elmer automatic polarimeter, Model 341. All reactions were monitored by thin-layer chromatography (TLC) on Silica gel 60 coated glass slides. Column chromatography was performed by gradient elution from columns of silica gel with the high performance rapid flash chromatography system (RT Scientific). Solvent mixtures less polar than those used for TLC were used at the onset of separation. Nuclear magnetic resonance (NMR) spectra were measured at 300 MHz (^{1}H) and 75 MHz (^{13}C) with a Varian Gemini or Varian Mercury spectrometer. Assignments of NMR signals were made by first-order analysis of the spectra, and by comparison with spectra of related substances.[11,15,16,21] When the latter approach was used, advantage was taken of variations of line intensity expected for oligosaccharides belonging to the same homologous series to aid in the ^{13}C NMR signal-nuclei assignments.[22,23] 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 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. When reporting assignments of NMR signals, 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 tetrasaccharide, a resonance denoted H-3 (as opposed to, for example, H-3^{II}), can be that of H-3 of either sugar residue. Of two geminal protons showing distinctly different chemical shifts the one resonating at a lower field is designated Ha, and the one resonating at a higher field is designated Hb. Some signals in the ^{13}C NMR spectra of squaric acid derivatives appeared as doublets, the splitting of these signals being due to the vinylogous amide group, characteristic of squaric acid amide esters.[6] Liquid chromatography–electron spray-ionization mass spectrometry (LCESI MS) was performed with a Hewlett–Packard 1100 MSD spectrometer. Matrix assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed with a Shimadzu AXIMA/CFR spectrometer. Surface-enhanced laser desorption-ionization time-of-flight mass spectrometry (SELDI-TOF MS) was done by PBS-II Mass Reader® (Ciphergen Biosystems, Inc), calibrated with All in One Protein Standards (Ciphergen Biosystems, Inc). The H4 or NP20 Protein Chip Arrays® were used as sample carriers. The accuracy of molecular mass determination was increased by using the carrier protein (BSA, *m*/*z* 66,433 Da) as an internal standard.[13] 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%. Structures of these compounds follow unequivocally from the mode of synthesis, NMR data, and *m*/*z* values found in their low- and high-resolution mass spectra. TLC and NMR spectroscopy verified the purity of all compounds. BSA (Fraction V, Prod. No. A-4503) was purchased from Sigma Chemical Company, and purified as described by Chen.[24] Ethylenediamine (Aldrich Chemical Co.) was freshly distilled, bp 115–116 °C. Solid hexamethylenediamine, a product of DuPont, was freshly distilled, bp 92 °C/2 kPa. Palladium-on-charcoal catalyst (5%, ESCAT 103) was a product of Engelhard Industries. {*N*-[(Dimethylamino)-1*H*-1,2,3-triazolo-[4,5-*b*]-pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide} HATU was purchased from Applied Biosystems. 3,4-Diethoxy-3-cyclobutene-1,2-dione (squaric acid diethyl ester) was purchased from Aldrich Chemical Co., and used as supplied. Solutions in organic solvents were dried with anhydrous Na_2SO_4, and concentrated at 40 °C/2 kPa.

3.2. General method for glycosylation with *N*-iodosuccinimide/silver trifluoromethanesulfonate (NIS/AgOTf)

A mixture of the thioglycoside glycosyl donor (1.3 mmol), the glycosyl acceptor (1 mmol) and finely powdered 4 Å molecular sieves (0.5 g) in CH_2Cl_2 (10–15 mL) was stirred under argon for 15 min. Solid NIS (1.4 mmol) was added at 0 °C, followed by a solution of AgOTf (0.4 mmol) in dry toluene (4 mL), and the mixture was stirred at the same temperature for 3 min. Cooling was terminated and, when TLC showed that the reaction was complete (~15 min), the mixture was neutralized with Et_3N, washed successively with aq $NaHCO_3$ and water, dried, and concentrated. Chromatography gave the desired product.

3.2.1. Methoxycarbonylpentyl 4-azido-3-*O*-benzyl-4,6-dideoxy-α-D-mannopyranoside 3. Boron trifluoride etherate (8 mL, 62 mmol) was added to a stirred solution of **1**[16] (15 g, 41.3 mmol) and methoxycarbonylpentanol (9 g, 61.95 mmol) in CH_2Cl_2 (400 mL) and the stirring was continued for 24 h at room temperature, when TLC (4:1 toluene–EtOAc) showed that the reaction was complete. After neutralization with aqueous $NaHCO_3$

solution, the mixture was partitioned between water and CH_2Cl_2, the organic phase was dried, and concentrated. Chromatography of the residue gave methoxycarbonylpentyl 2-*O*-acetyl-4-azido-3-*O*-benzyl-4,6-dideoxy-α-D-mannopyranoside (**2**, 83%), which was indistinguishable (TLC, NMR) from the substance obtained previously in 75% yield, when $SnCl_4$ was used as a Lewis acid catalyst.

Deacetylation (Zemplén) of the foregoing acetate **2** gave the title alcohol **3** in virtually theoretical yield. A small amount of material was eluted from a silica gel column to afford the analytical sample, $[\alpha]_D = +66$ (*c* 0.4, $CHCl_3$). ^1H NMR ($CDCl_3$): δ 4.79 (d, 1H, $J_{1,2}$ 1.6 Hz, H-1), 4.71, 4.66 (2 d, 4H, 2 CH_2Ph), 3.96 (m, 1H, H-2), 3.72 (dd, 1H, $J_{2,3}$ 3.2, $J_{3,4}$ 9.4 Hz, H-3), 3.67 (s, partially overlapped, $COOCH_3$), 3.64, 3.61 (2 t, partially overlapped, *J* 6.7 Hz, H-1′a), 3.56–3.47 (m, 1H, H-5), 3.41 (t, partially overlapped, H-4), 3.41, 3.36 (2 t, partially overlapped, H-1′b), 2.49 (br d, 1H, OH), 2.32 (t, 2H, *J* 7.3 Hz, H-5′a,b), 1.73–1.52 (m, 4H, H-2′a,b,4′a,b), 1.40–1.30 (m, 5H, H-3′a,b, incl. d, 1.31, $J_{5,6}$ 6.0 Hz, H-6); ^{13}C NMR ($CDCl_3$): δ 98.87 (C-1), 78.38 (C-3), 71.94 (*C*H_2Ph), 67.45 (C-1′), 67.21 (C-2), 66.48 (C-5), 63.92 (C-4), 51.46 (COO*C*H_3), 33.88 (C-5′), 28.96 (C-2′), 25.64 (C-3′), 24.60 (C-4′), 18.38 (C-6). LCESI MS: *m*/*z* 430 ($[M+Na]^+$). Anal. Calcd for $C_{20}H_{29}N_3O_6$: C, 58.95; H, 7.17; N, 10.31. Found: C, 58.74; H, 7.22; N, 10.31.

3.2.2. Methoxycarbonylpentyl 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-D-mannopyranoside 5. Following the general procedure, the title disaccharide was obtained in 95% yield, when starting from **3** (12.9 g, 31.6 mmol), $[\alpha]_D = +48$ (*c* 0.9, $CHCl_3$). ^1H NMR ($CDCl_3$): δ 5.44 (dd, 1H, $J_{1,2}$ 1.9, $J_{2,3}$ 3.2 Hz, H-2^{II}), 4.84 (d, 1H, H-1^{II}), 4.73, 4.72, 4.64, 4.55 (4 d, partially overlapped, ^2J 11.1 Hz, 2 CH_2Ph), 4.68 (d, partially overlapped, $J_{1,2}$ 1.9 Hz, H-1^{I}), 3.85 (dd, partially overlapped, $J_{2,3}$ 3.1 Hz, H-2^{I}), 3.82 (dd, partially overlapped, $J_{3,4}$ 9.9 Hz, H-3^{II}), 3.76 (dd, 1H, $J_{3,4}$ 9.8 Hz, H-3^{I}), 3.68 (s, 3H, $COOCH_3$), 3.65–3.57 (m, 2H, H-5^{II}, 1′a), 3.52–3.29 (m, 4H, H-5^{I}, 1′b, incl. 3.41, 3.35, 2 t, $J \sim 9.8$ Hz, H-4^{I}, H-4^{II}, respectively), 2.34 (t, 2H, *J* 7.5 Hz, H-5′a,b), 2.09 (s, 3H, $COCH_3$), 1.71–1.53 (m, 4H, H-2′a,b,4′a,b), 1.41–1.30 (m, 8H, H-3′a,b, incl. 1.32, 1.31, 2 d, $J_{5,6}$ 6.2 Hz, H-6^{II}, H-6^{I}, respectively); ^{13}C NMR ($CDCl_3$): δ 99.02 (C-1^{II}), 98.38 (C-1^{I}), 77.58 (C-3^{I}), 75.18 (C-3^{II}), 73.82 (C-2^{I}), 71.81, 71.34 (2 *C*H_2Ph), 67.36 (C-5^{II}), 67.32 (C-1′), 67.00 (C-2^{II}), 66.86 (C-5^{I}), 63.94 (C-4^{I}), 63.63 (C-4^{II}), 51.38 (COO*C*H_3), 33.85 (C-5′), 28.80 (C-2′), 25.36 (C-3′), 24.40 (C-4′), 20.75 (CO*C*$_3$), 18.35, 18.25 (C-$6^{I,II}$). LCESI MS: *m*/*z* 733 ($[M+Na]^+$). Anal. Calcd for $C_{35}H_{46}N_6O_{10}$: C, 59.14; H, 6.52; N, 11.82. Found: C, 59.27; H, 6.66; N, 11.387.

3.2.3. Methoxycarbonylpentyl 4-azido-3-*O*-benzyl-4,6-dideoxy-α-D-mannopyranosyl-(1→2)-4-azido-3-*O*-benzyl-4,6-dideoxy-α-D-mannopyranoside 6. Zemplén deacetylation of **5** gave **6** in virtually theoretical yield, $[\alpha]_D = +96$ (*c* 1.3, $CHCl_3$). ^1H NMR ($CDCl_3$): δ 4.92 (d, 1H,$J_{1,2}$ 1.7 Hz, H-1^{II}), 4.70–4.58 (m, 5H, H-1^{I}, 2 CH_2Ph), 3.96 (m, 1H, H-2^{II}), 3.87 (br t, 1H, H-2^{I}), 3.72 (dd, partially overlapped, $J_{2,3}$ 3.0, $J_{3,4}$ 9.6 Hz, H-3^{I}), 3.70 (dd, partially overlapped, $J_{2,3}$ 3.2, $J_{3,4}$ 9.4 Hz, H-3^{II}), 3.65 (s, 3H, $COOCH_3$), 3.62–3.54 (m, 2H, H-5^{II}, 1′a), 3.49–3.25 (m, 4H, H-5^{I}, 1′b, incl. 3.43, 3.28, 2 t, $J \sim 10.1$ Hz, H-4^{I}, H-4^{II}, respectively), 2.56 (br d, 1H, OH), 2.30 (t, 2H, *J* 7.5 Hz, H-5′a,b), 1.68–1.50 (m, 4H, H-2′a,b,4′a,b), 1.38–1.26 (m, 8H, H-3′a,b, H-$6^{I,II}$); ^{13}C NMR ($CDCl_3$): δ 100.70 (C-1^{II}), 98.41 (C-1^{I}), 77.49 (C-3^{I}), 77.23 (C-3^{II}), 73.56 (C-2^{I}), 71.71, 71.57 (2 *C*H_2Ph), 67.17 (C-1′), 67.03 (C-5^{II}), 66.78 (C-2^{II}), 66.75 (C-5^{I}), 63.99 (C-4^{II}), 63.46 (C-4^{I}), 51.19 (COO*C*H_3), 33.58 (C-5′), 28.72 (C-2′), 25.38 (C-3′), 24.34 (C-4′), 18.32, 18.13 (C-$6^{I,II}$). LCESI MS: *m*/*z* 691 ($[M+Na]^+$). Anal. Calcd for $C_{33}H_{44}N_6O_9$: C, 59.27; H, 6.63; N, 12.57. Found: C, 59.11; H, 6.36; N, 12.55.

3.2.4. Methoxycarbonylpentyl 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-mannopyranoside 9. Reaction of the foregoing glycosyl acceptor **6** (4.0 g, 6.0 mmol) with donor **7**,[11,15] gave methoxycarbonylpentyl 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-mannopyranoside **8** (6.9 g, 95.8%). ^1H NMR ($CDCl_3$): δ 5.40 (dd, 1H, $J_{1,2}$ 1.9, $J_{2,3}$ 3.2 Hz, H-2^{IV}), 4.93 (d, 1H, $J_{1,2}$ 1.9 Hz, H-1^{III}), 4.87 (d, 1H, $J_{1,2}$ 1.9 Hz, H-1^{II}), 4.84 (d, 1H, $J_{1,2}$ 1.9 Hz, H-1^{IV}), 4.73–4.51 (m, 9H, 4 CH_2Ph, incl. d, ~4.60, H-1^{I}), 3.87 (br t, 1H, H-2^{III}), 3.83 (br t, 1H, H-2^{II}), 3.79–3.75 (m, 2H, H-2^{I}, 3^{IV}), 3.71–3.65 (m, 6H, H-$3^{I–III}$, incl. s, 3.67, $COOCH_3$), 3.65–3.15 (m, 10H, H-$4^{I–IV}$, $5^{I–IV}$, 1′a,b), 2.32 (t, 2H, *J* 7.7 Hz, H-5′a,b), 2.10 (s, 3H, $COCH_3$), 1.69–1.50 (m, 4H, H-4′a,b,2′a,b, in that order), 1.37–1.28 (m, 2H, H-3′a,b), 1.26, 1.20, 1.16 (3 d, partially overlapped, 12H, H-$6^{I–IV}$); ^{13}C NMR ($CDCl_3$): δ 100.37 (C-1^{II}), 100.02 (C-1^{III}), 99.05 (C-1^{IV}), 98.55 (C-1^{I}), 77.42, 76.77, 76.58 (C-$3^{I–III}$), 75.41 (C-3^{IV}), 73.93 (C-2^{I}), 73.40, 73.35 (C-$2^{II,III}$), 72.16, 72.09, 71.99, 71.51 (4 *C* H_2Ph), 67.75, 67.70, 67.60, 67.06 (C-$5^{I–IV}$), 67.46 (C-1′), 67.02 (C-2^{IV}), 64.29, 64.17, 63.97, 63.75 (C-$4^{I–IV}$), 51.50 (COO*C*$_3$), 33.87 (C-5′), 28.98 (C-2′), 25.63 (C-3′), 24.61 (C-4′), 20.96 (CO*C*H_3), 18.56, 18.45, 18.34 (2C, C, C; C-$6^{I–IV}$). LCESI MS: *m*/*z* 1255 ($[M+Na]^+$).

The foregoing acetate **8** (6.9 g) was deacetylated conventionally, to give the title alcohol **9** (5.65 g, 91%), $[\alpha]_D = +102$ (*c* 2.7, $CHCl_3$). ^1H NMR ($CDCl_3$): δ 4.97 (d, 1H, $J_{1,2}$ 1.8 Hz, H-1^{IV}), 4.94 (d, 1H, $J_{1,2}$ 1.8 Hz, H-1^{III}), 4.87 (d, 1H, $J_{1,2}$ 1.8 Hz, H-1^{II}), 4.75–4.55 (m, 9H, 4 CH_2Ph, incl. d, ~4.59, H-1^{I}), 3.99 (m, 1H, H-2^{IV}), 3.93 (br t, 1H, H-2^{III}), 3.82 (br t, 1H, H-2^{II}), 3.77 (br t, 1H, H-2^{I}), 3.73–3.68 (m, 7H, H-$3^{I–IV}$, incl. s, 3.67, $COOCH_3$), 3.60–3.15 (m, 10H, H-$4^{I–IV}$, $5^{I–IV}$, 1′a,b), 2.34–2.29 (m, 3H, OH, incl. t, 2.31, H-5′a,b), 1.68–1.49 (m, 4H, H-4′a,b,2′a,b, in that order), 1.37–1.13 (m, 14H, H-$6^{I–IV}$, 3′a,b); ^{13}C NMR ($CDCl_3$): δ 100.40 (C-1^{IV}), 100.34 (C-1^{III}), 100.16 (C-1^{II}), 98.53 (C-1^{I}), 77.61, 77.39, 76.90, 76.51 (C-$3^{I–IV}$), 73.90 (C-2^{I}), 73.46 (C-2^{II}), 73.13 (C-2^{III}), 72.14, 72.08, 72.05, 71.99 (4 *C*H_2Ph), 67.72, 67.70, 67.29, 67.00 (C-$5^{I–IV}$), 67.43 (C-1′), 67.05

(C-2^{IV}), 64.27, 64.12, 63.75 (C, 2C, C; C-$4^{I–IV}$), 51.48 (COO*C*H_3), 33.85 (C-5′), 28.95 (C-2′), 25.60 (C-3′), 24.59 (C-4′), 18.55, 18.45, 18.25 (2C, C, C; C-$6^{I–IV}$); LCESI MS: *m*/*z* 1191.7 ($[M+H]^+$). Anal. Calcd for $C_{59}H_{74}N_{12}O_{15}$: C, 59.48; H, 6.26; N, 14.11. Found: C, 59.60; H, 6.36; N, 14.10.

3.2.5. Methoxycarbonylpentyl 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-mannopyranoside 10. (a) Condensation of **7** with **9** (5.1 g, 4.2 mmol), as described in the general procedure for glycosylation, gave the title hexasaccharide **10** (6.4 g, 85%), $[\alpha]_D$ = +50 (*c* 7.6, $CHCl_3$). Structurally significant signals in the ^{1}H NMR ($CDCl_3$) spectrum were at: δ 5.41 (dd, 1H, $J_{1,2}$ 1.9, $J_{2,3}$ 3.2 Hz, H-2^{VI}), 4.95 (d, 1H, $J_{1,2}$ 1.6 Hz, H-1^{V}), 4.87 (d, 1H, $J_{1,2}$ 1.6 Hz, H-1^{II}), 4.86–4.84 (m, 3H, H-$1^{III,IV,VI}$), 4.75–4.52 (m, 13H, 6 C*H*$_2$Ph, incl. d, ∼4.59, H-1^{I}), 3.88 (br dd, 1H, H-2^{V}), 3.84 (br dd, 1H, H-2^{II}), 3.82–3.72 (m, 4H, H-$2^{I,III,IV}$, 3^{VI}), 3.67 (s, partially overlapped, $COOCH_3$), 2.31 (t, 2H, *J* 7.7 Hz, H-5′a,b), 2.11 (s, 3H, $COCH_3$), 1.69–1.49 (m, 4H, H-4′a,b,2′a,b, in that order), 1.37–1.12 (m, 20H, H-$6^{I–VI}$, 3′a,b,); ^{13}C NMR ($CDCl_3$): δ 100.32 (C-1^{II}), 100.09 (2C, C-$1^{III,IV}$), 100.01 (C-1^{V}), 99.05 (C-1^{VI}), 98.55 (C-1^{I}), 77.42, 76.80, 76.62, 76.51 (C, C, C, 2C; C-$3^{I–V}$), 75.41 (C-3^{VI}), 73.96 (C-2^{I}), 73.53 (C-2^{II}), 73.38 (2C; C-$2^{III,IV}$), 73.29 (C-2^{V}), 72.13, 72.03, 72.00, 71.52 (3C, C, C, C; 6 *C*H$_2$Ph), 67.78, 67.70, 67.61, 67.02 (3C, C, C, C; C-$5^{I–VI}$), 67.46 (C-1′), 67.08 (C-2^{IV}), 64.30, 64.19, 64.14, 63.99, 63.76 (C, C, 2C, C, C; C-$4^{I–VI}$), 51.50 (COO*C*H_3), 33.87 (C-5′), 28.98 (C-2′), 25.63 (C-3′), 24.61 (C-4′), 18.56, 18.46, 18.35 (2C, 3C, C; C-$6^{I–IV}$). Anal. Calcd for $C_{87}H_{106}N_{18}O_{22}$: C, 59.51; H, 6.08; N, 14.36. Found: C, 59.75; H, 6.14; N, 14.25.

(b) Condensation of alcohol **6** (4.7 g, 7 mmol) with the tetrasaccharide glycosyl donor **16**, as described in the general procedure for glycosylation gave, after chromatography, material (8.0 g, 65%), which was indistinguishable (TLC, NMR) from the above described substance.

3.2.6. Methoxycarbonylpentyl 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-mannopyranoside 11. Conventional deacetylation (Zemplén) of **10** gave alcohol **11** in 84% yield, $[\alpha]_D$ = +108 (*c* 1.7, $CHCl_3$). Structurally significant signals in the ^{1}H NMR ($CDCl_3$) spectrum were at: δ 4.99 (d, 1H, $J_{1,2}$ 1.7 Hz, H-1^{VI}), 4.97 (d, 1H, $J_{1,2}$ 2.0 Hz, H-1^{V}), 4.89 (d, 1H, $J_{1,2}$ 2.0 Hz, H-1^{II}), 4.85 (br d, 2H, H-$1^{III,IV}$), 4.75–4.59 (m, 13H, 6 C*H*$_2$Ph, incl. d, ∼4.59, H-1^{I}), 4.00 (m, 1H, H-2^{VI}), 3.95 (br dd, 1H, H-2^{V}), 3.84 (br t, 1H, H-2^{II}), 3.81, 3.79 (2 br t, 2H, H-$2^{III,IV}$), 3.77 (br dd, 1H, H-2^{I}), 3.67 (s, partially overlapped, $COOCH_3$), 2.36 (d, 1H, $J_{2,OH}$ 2.0 Hz, OH), 2.32 (t, 2H, *J* 7.3 Hz, H-5′a,b), 1.69–1.50 (m, 4H, H-4′a,b,2′a,b, in that order), 1.38–1.13 (m, 20H, H-3′a,b,$6^{I–VI}$); ^{13}C NMR ($CDCl_3$): δ 100.41 (C-1^{VI}), 100.31, 100.15, 100.06 (C, C, 2C; C-$1^{II–V}$), 98.52 (C-1^{I}), 77.61, 77.37, 76.91, 76.52, 76.46 (C, C, C, C, 2C; C-$3^{I–VI}$), 73.95 (C-2^{I}), 73.53 (C-2^{II}), 73.35 (2C; C-$2^{III,IV}$), 73.14 (C-2^{V}), 72.10, 71.99 (5C, C; 6 *C*H$_2$Ph), 67.75, 67.65, 67.29, 66.99 (3C, C, C, C; C-$5^{I–VI}$), 67.43 (C-1′), 67.05 (C-2^{IV}), 64.27, 64.11, 63.75 (C, 4C, C; C-$4^{I–VI}$), 51.47 (COO*C*H_3), 33.84 (C-5′), 28.95 (C-2′), 25.60 (C-3′), 24.58 (C-4′), 18.54, 18.45, 18.26 (2C, 3C, C; C-$6^{I–IV}$). Anal. Calcd for $C_{85}H_{104}N_{18}O_{21}$: C, 59.57; H, 6.12; N, 14.71. Found: C, 59.28; H, 6.11; N, 14.53.

3.2.7. Methoxycarbonylpentyl 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-mannopyranoside 12. (a) Me_2S (catalytic amount, ∼0.1 mL) was added to a suspension of alcohol **11** (0.5 g), Ag_2O (1 g) and MeI (2.5 mL) in 1,2-dimethoxyethane (10 mL), and the mixture was stirred overnight at room temperature. TLC (4:1 hexane–EtOAc) then showed that the starting material virtually completely disappeared. After filtration, the solids were washed with CH_2Cl_2, the combined filtrates were concentrated, and chromatography of the residue gave **12** (0.45 g, 90%), $[\alpha]_D$ = +103 (*c* 2.2, $CHCl_3$). Structurally significant signals in the ^{1}H NMR ($CDCl_3$) spectrum were at: δ 4.93–4.90 (m, 3H, H-$1^{II,V,VI}$), 4.85, 4.84 (2 d, partially overlapped, 2H, H-$1^{III,IV}$), 4.76–4.59 (m, 13H, 6 C*H*$_2$Ph, incl. d, ∼4,59, H-1^{I}), 3.96, 3.85, 3.82, 3.79 (4 br t, 4H, H-$2^{II–V}$), 3.76 (br t, 1H, H-2^{I}), 3.67 (s, partially overlapped, $COOCH_3$), 3.33, 3.30 (2 t, 2H, *J* 6.5 Hz, H-1′a,b), 3.21 (s, partially overlapped, OCH_3-2), 2.32 (t, 2H, *J* 7.3 Hz, H-5″a,b), 1.68–1.49 (m, 4H, H-4″a,b,2″a,b, in that order), 1.37–1.12 (m, 20H, H-3″a,b, $6^{I–VI}$); ^{13}C NMR ($CDCl_3$): δ 100.33, 100.19, 100.10, 100.07 (C-$1^{II–V}$), 98.73 (C-1^{VI}), 98.54 (C-1^{I}), 77.42, 77.12, 76.57, 76.50 (2C, C, C, 2C; C-$3^{I–VI}$), 76.32 (C-2^{VI}), 74.00 (C-2^{I}), 73.53, 73.38, 73.17, 73.10 (C-$2^{II–V}$), 72.34, 72.11, 72.08, 72.00 (C, 3C, C, C; 6 *C*H$_2$Ph), 67.80, 67.71, 67.02 (3C, 2C, C; C-$5^{I–VI}$), 67.43 (C-1′), 64.23, 64.14, 64.05 (2C, 3C, C; C-$4^{I–VI}$), 58.87 (OCH_3-2), 51.45 (COO*C*H_3), 33.85 (C-5′), 28.96 (C-2′), 25.61 (C-3′), 24.59 (C-4′), 18.54, 18.45, 18.37 (2C, 3C, C; C-$6^{I–IV}$). LCESI MS: *m*/*z* 1749 ($[M+Na]^+$). Anal. Calcd for $C_{86}H_{106}N_{18}O_{21}$: C, 59.78; H, 6.18; N, 14.59. Found: C, 59.86; H, 6.28; N, 14.59.

(b) Condensation of glycosyl acceptor **6** (0.7 g, 1.08 mmol) with donor **18**, following the general protocol for glycosylation gave, after chromatography (4:1 hexane–acetone)material (0.96 g, 55%), which was indistinguishable from the above-described substance.

3.2.8. 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,β-D-mannopyranose 13. Water (0.1 mL) was added at 0 °C to a stirred solution of ethyl 2-*O*-acetyl-4-azido-3-*O*-benzyl-4,6-dideoxy-α-D-mannopyranosyl-(1→2)-4-azido-3-*O*-benzyl-4,6-dideoxy-1-thio-α-D-mannopyranoside **7**[11,15] (313 mg, 0.5 mmol) in acetone (10 mL), followed by NIS (135 mg, 0.6 mmol). The cooling was removed after 5 min, and the solution was stirred at room temperature until TLC (hexane–EtOAc) showed that the reaction was virtually complete (2 h). One major product, showing slower mobility than the starting material, was formed. The mixture was neutralized with solid

$NaHCO_3$, acetone was evaporated, and the residue was extracted with CH_2Cl_2. After concentration, chromatography gave material (232 mg, 83%), which was indistinguishable from the previously, independently synthesized substance.[18]

3.2.9. Ethyl 4-azido-3-*O*-benzyl-4,6-dideoxy-α-D-mannopyranosyl-(1→2)-4-azido-3-*O*-benzyl-4,6-dideoxy-α-D-mannopyranoside 15. Zemplén deacetylation of ethyl 2-*O*-acetyl-4-azido-3-*O*-benzyl-4,6-dideoxy-α-D-mannopyranosyl-(1→2)-4-azido-3-*O*-benzyl-4,6-dideoxy-1-thio-α-D-mannopyranoside **7**[11,15] gave **15** in ~90% yield, $[\alpha]_D = +191$. ^1H NMR ($CDCl_3$): δ 5.17 (d, 1H, $J_{1,2}$ 1.5 Hz, H-1^I), 4.90 (d, 1H, $J_{1,2}$ 1.7 Hz, H-1^{II}), 4.75–4.58 (4 d, 4H, $J \sim 11.5$ Hz, 2 CH_2Ph), 3.98 (ddd, 1H, $J_{2,3}$ 3.3, $J_{2,OH}$ 1.8 Hz, H-2^{II}), 3.95 (dd, 1H, $J_{2,3}$ 2.8 Hz, H-2^I), 3.87–3.77 (m, 1H, H-5^I), 3.72 (dd, partially overlapped, $J_{3,4}$ 9.8 Hz, H-3^{II}), 3.65 (dd, partially overlapped, $J_{3,4} \sim 9.9$ Hz, H-3^I), 3.69–3.59 (m, partially overlapped, H-5^{II}), 3.43 (t, 1H, J 9.9 Hz, H-4^{II}), 3.32 (t, 1H, J 10.0 Hz, H-4^I), 2.66–2.48 (m, 2H, CH_2CH$_3$), 2.36 (d, 1H, OH), 1.30 (d, partially overlapped, H-$6^{I,II}$), 1.26 (t, partially overlapped, J 7.5 Hz, CH_2CH_3); ^{13}C NMR ($CDCl_3$): δ 100.89 (C-1^{II}), 83.39 (C-1^I), 78.13 (C-3^I), 71.60 (C-3^{II}), 75.86 (C-2^I), 72.14, 72.06 (2 *C*H$_2$Ph), 67.52 (C-5^I), 67.33 (C-5^{II}), 64.47 (C-4^I), 63.76 (C-4^{II}), 25.60 (*C*$_2$CH$_3$), 18.52, 18.45 (C-$6^{I,II}$), 14.92 (CH_2C_3); CIMS: *m*/*z* 602 ($[M+NH_4]^+$). Anal. Calcd for $C_{28}H_{36}N_6O_6S$: C, 57.52; H, 6.21; N, 14.37; S, 5.48. Found: C, 57.38; H, 6.14; N, 14.28; S, 5.53.

3.2.10. Ethyl 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)-4-azido-3-*O*-benzyl-4,6-dideoxy-1-thio-α-D-mannopyranoside 6. To a stirred solution of compound **13** (8.7 g, 14.95 mmol) and trichloroacetonitrile (6 mL, 60 mmol) in CH_2Cl_2 (200 mL) was added diazabicyclo[5.4.0]undec-7-ene (0.9 mL, 6 mmol) and the mixture was stirred for 10 min, when TLC showed that the reaction was complete. The mixture was concentrated and chromatography gave amorphous 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-D-mannopyranosyl trichloroacetimidate **14** (10.6 g, 95%). ^1H NMR ($CDCl_3$): δ 6.11 (d, 1H, $J_{1,2}$ 1.9 Hz, H-1^I), 5.44 (dd, 1H, $J_{1,2}$ 1.7, $J_{2,3}$ 3.0 Hz, H-2^{II}), 4.89 (d, 1H, H-1^{II}), 4.77–4.55 (4 d, partially overlapped, 2 CH_2Ph), 3.89 (br t, 1H, H-2^I), 3.80 (dd, partially overlapped, $J_{3,4}$ 9.9 Hz, H-3^{II}), 3.76 (dd, partially overlapped, $J_{2,3}$ 3.0, $J_{3,4}$ 9.9 Hz, H-3^I), 3.72–3.58 (m, 2H, H-$5^{I,II}$), 3.45, 3.42 (2 t, 2H, $J \sim 10$ Hz, H-$4^{I,II}$), 2.12 (s, 3H, COCH$_3$), 1.33, 1.32 (2 d, partially overlapped, 6H, $J_{5,6} \sim 6$ Hz, H-$6^{I,II}$); ^{13}C NMR ($CDCl_3$): δ 99.43 (C-I^{II}), 96.22 (C-I^I), 90. 70 (CN), 76.72 (C-3^I), 75.35 (C-3^{II}), 72.37, 71.61 (2 *C*H$_2$Ph), 72.10 (C-2^I), 70.05 (C-5^I), 67.81 (C-5^{II}), 67.08 (C-2^{II}), 63.63, 63.39 (C-$4^{I,II}$), 20.84 (CO*C*H$_3$), 18.52, 18.26 (C-$6^{I,II}$); CIMS: *m*/*z* 743 ($[M+NH_4]^+$).

A mixture of the foregoing imidate (10.6 g, 14.6 mmol), alcohol **15** (7.9 g, 13.5 mmol), and powdered 4 Å molecular sieves (7.5 g) in CH_2Cl_2 (250 mL) was stirred under argon for 15 min. Trimethylsilyl trifluoromethanesulfonate (catalytic amount, ~0.1 mL) was added, and stirring at room temperature was continued for 15 min, when TLC showed almost complete disappearance of the starting materials. After neutralization with triethylamine, the mixture was filtered, the filtrate was concentrated, and the residue was chromatographed to give **16** (13.1 g, 84%), $[\alpha]_D = +119$ (*c* 0.7, $CHCl_3$). ^1H NMR ($CDCl_3$): δ 5.41 (dd, 1H, $J_{1,2}$ 2.0, $J_{2,3}$ 3.1 Hz, H-2^{IV}), 5.09 (d, 1H, $J_{1,2}$ 1.2 Hz, H-1^I), 4.94 (d, 1H, $J_{1,2}$ 1.6 Hz, H-1^{III}), 4.85 (d, 1H, $J_{1,2}$ 2.0, H-1^{IV}), 4.83 (d, 1H, $J_{1,2}$ 1.6 Hz, H-1^{II}), 4.74–4.51 (4 d, 8H, 4 CH_2Ph), 3.87 (br t, 1H, H-2^{III}), 3.84–3.81 (m, 2H, H-$2^{I,II}$), 3.79–3.73 (m, 2H, H-3^{IV}, 5^I), 3.70 (dd, partially overlapped, $J_{2,3}$ 3.2, $J_{3,4} \sim 9.5$ Hz, H-3^{III}), 3.67 (dd, partially overlapped, $J_{2,3}$ 3.2, $J_{3,4}$ 9.8 Hz, H-3^{II}), 3.60 (dd, 1H, $J_{2,3}$ 2.8, $J_{3,4}$ 9.8 Hz, H-3^I), 3.56–3.18 (m, 7H, H-$4^{I–IV}$, $5^{II–IV}$), 2.64–2.46 (m, 2H, *C*H_2CH$_3$), 2.10 (s, 3H, COCH$_3$), 1.27–1.18 (m, 15H, H-$6^{I–IV}$, incl. t, 1.24, J 7.8 Hz, CH_2CH_3); ^{13}C NMR ($CDCl_3$): δ 100.55 (C-1^{II}), 100.02 (C-1^{III}), 99.07 (C-1^{IV}), 83.33 (C-1^I), 77.75 (C-3^I), 76.79 (C-3^{III}), 76.59 (C-3^{II}), 75.02 (C-2^I), 75.42 (C-3^{IV}), 73.37 (2C, C-$2^{II,III}$), 72.17, 72.02, 71.53 (2C, C, C; 4 *C*H$_2$Ph), 67.87, 67.80, 67.63 (C-$5^{II–IV}$), 67.51 (C-5^I), 67.10 (C-2^{IV}), 64.53 (C-4^I), 64.21 (C-4^{II}), 64.00 (C-4^{III}), 63.80 (C-4^{IV}), 25.60 (*C*H$_2$CH$_3$), 18.63, 18.58, 18.50, 18.43 (C-$6^{I–IV}$), 14.93 (CH_2CH_3); CIMS: *m*/*z* 1149 ($[M+H]^+$), 1171 ($[M+Na]^+$). Anal. Calcd for $C_{56}H_{68}N_{12}O_{13}S$: C, 58.52; H, 5.96; N, 14.62; S, 2.79. Found: C, 58.67; H, 6.08; N, 14.36; S, 2.68.

3.2.11. Ethyl 4-azido-3-*O*-benzyl-4,6-dideoxy-α-D-mannopyranosyl-bis(1→2)-(-4-azido-3-*O*-benzyl-4,6-dideoxy-α-D-mannopyranosyl)-4-azido-3-*O*-benzyl-4,6-dideoxy-1-thio-α-D-mannopyranoside 17. Deacetylation (Zemplén) of the foregoing acetylated tetrasaccharide gave alcohol **17** in virtually theoretical yield, $[\alpha]_D = +121$. ^1H NMR ($CDCl_3$): δ 5.09 (d, 1H, $J_{1,2}$ 1.2 Hz, H-1^I), 4.97 (d, 1H, $J_{1,2}$ 1.7 Hz, H-1^{IV}), 4.95 (d, 1H, $J_{1,2}$ 1.7 Hz, H-1^{III}), 4.84 (d, 1H, $J_{1,2}$ 1.7 Hz, H-1^{II}), 4.74–4.57 (m, 8H, 4 CH_2Ph), 4.00–3.97 (m, 1H, H-2^{IV}), 3.93 (br t, 1H, H-2^{III}), 3.83 (br t, 1H, H-2^I), 3.80 (br t, 1H, H-2^{II}), 3.80–3.74 (m, partially overlapped, H-5^I), 3.72–3.67 (m, 3H, H-$3^{II–IV}$), 3.60 (dd, 1H, $J_{2,3}$ 2.9, $J_{3,4}$ 9.9 Hz, H-3^I), 3.56–3.38 (m, 4H, H-$4,5^{II–IV}$), 3.34–3.18 (m, 3H, 2 H-4, incl. t, partially overlapped, J 9.9 Hz, H-4^I), 2.61–2.47 (m, 2H, CH_2CH$_3$), 2.39 (d, 1H, $J_{2,OH}$ 2.1 Hz, OH), 1.28–1.18 (m, 15H, 4 H-6, CH_2CH_3); ^{13}C NMR ($CDCl_3$): δ 100.48 (C-1^{II}), 100.39 (C-1^{IV}), 100.12 (C-1^{III}), 83.26 (C-1^I), 77.71 (C-3^I), 77.56, 76.85, 76.45 (C-$3^{II–IV}$), 75.95 (C-2^I), 73.39 (C-2^{II}), 73.12 (C-2^{III}), 72.05, 71.95 (3C, C; 4 *C*H$_2$Ph), 67.82, 67.72, 67.28 (C-$5^{II–IV}$), 67.45 (C-5^I), 67.03 (C-2^{IV}), 64.45 (C-4^I), 64.10, 63.72 (2C, C; C-$4^{II–IV}$), 25.54 (*C*$_2$CH$_3$), 18.58, 18.54, 18.44, 18.28 (C-$6^{I–IV}$), 14.88 (CH_2CH_3); CIMS: *m*/*z* 1129 ($[M+Na]^+$). Anal. Calcd for $C_{54}H_{66}N_{12}O_{12}S$: C, 58.58; H, 6.01; N, 15.18; S, 2.90. Found: C, 58.36; H, 5.99; N, 14.98; S, 2.86.

3.2.12. Ethyl 4-azido-3-*O*-benzyl-4,6-dideoxy-2-*O*-methyl-α-D-mannopyranosyl-(1→2)-bis(4-azido-3-*O*-benzyl-4,6-dideoxy-α-D-mannopyranosyl)-4-azido-3-*O*-benzyl-4,6-dideoxy-1-thio-α-D-mannopyranoside 18. Methyl iodide (0.225 mL, 36 mmol) was added to a stirred mix-

ture of **17** (2.67 g, 24 mmol) and powdered KOH (4.1 g, 72 mmol) in Me_2SO (30 mL). After 1 h TLC showed that the reaction was complete and that one product was formed. Water was added, to dissolve the solids, and the mixture was neutralized with aqueous AcOH (~10%, v/v). The mixture was partitioned between CH_2Cl_2 and brine, the organic phase was concentrated, and chromatography of the residue gave **18** (2.4 g, 89%), mp 79–80 °C (from EtOH), $[\alpha]_D = +140$ (*c* 0.8, $CHCl_3$). ^1H NMR ($CDCl_3$): δ 5.10 (d, 1H, $J_{1,2}$ 1.3 Hz, H-1^{I}), 4.91 (2 d, overlapped, 2H, $J_{1,2}$ 1.7 Hz, H-$1^{III,IV}$), 4.86 (d, 1H, $J_{1,2}$ 1.7 Hz, H-1^{II}), 4.75–4.56 (m, 8H, 4 CH_2Ph), 3.95 (br t, 1H, H-2^{III}), 3.85 (br t, 1H, H-2^{I}), 3.82 (br t, partially overlapped, H-2^{II}), 3.82–3.73 (m, partially overlapped, H-5^{I}), 3.72–3.66 (m, 3H, H-$3^{II–IV}$), 3.61 (dd, 1H, $J_{2,3}$ 2.7 Hz, $J_{3,4}$ 9.9 Hz, H-3^{I}), 3.59–3.48 (m, partially overlapped, H-5^{IV}), 3.48–3.41 (m, 3H, H-4^{IV}, $5^{II,III}$), 3.38 (br t, 1H, 2^{IV}), 3.34–3.20 (m, partially overlapped, H-$4^{I–III}$), 3.20 (s, partially overlapped, OCH_3), 2.64–2.44 (m, 2H, C$H_2$$CH_3$), 1.28–1.17 (m, 15H, H-$6^{I–IV}$, CH_2CH_3). ^{13}C NMR ($CDCl_3$): δ 100.47 (C-1^{II}), 100.10 (C-1^{III}), 98.63 (C-1^{IV}), 83.23 (C-1^{I}), 77.68 (C-3^{I}), 77.28, 77.00 (C-$3^{III,IV}$), 76.48 (C-3^{II}), 76.21 (C-2^{IV}), 75.92 (C-2^{I}), 73.17 (C-2^{II}), 73.01 (C-2^{III}), 72.22, 72.02, 71.88 (2C, C, C; 4 *C*H_2Ph), 67.77, 67.69, 67.60 (C-$5^{II–IV}$), 67.39 (C-5^{I}), 64.40 (C-4^{I}), 64.15, 64.01 (C-$4^{II,III}$), 63.95 (C-4^{IV}), 58.76 (CO*C*$_3$), 25.43 (*C*$_2$$CH_3$), 18.49, 18.42, 18.36, 18.31 (C-$6^{I–IV}$), 14.78 (CH_2*C*$_3$); FAB MS: *m*/*z* 1253 ($[M+Cs]^+$). Anal. Calcd for $C_{56}H_{68}N_{12}O_{13}S$: C, 58.91; H, 6.11; N, 14.99; S, 2.86. Found: C, 58.63; H, 6.08; N, 14.90; S, 2.80.

3.2.13. Methoxycarbonylpentyl 2-*O*-methyl-4-amino-3-*O*-benzyl-4,6-dideoxy-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-mannopyranoside 19. Hydrogen sulfide gas was passed through a solution of the azido derivative **18** (0.7 g) in pyridine–water [2:1 (v/v), 36 mL], for 30 min at 40 °C. The mixture was kept overnight at 40 °C in a loosely closed flask, when TLC (CH_2Cl_2–MeOH) showed that the starting material was no longer present, and that one, largely predominating product was formed. After concentration, the mixture was chromatographed to give **19** (0.6 g, 94%), $[\alpha]_D = 0$ (*c* 1.0, MeOH). Structurally significant signals in the ^1H NMR ($CDCl_3$) spectrum were at: δ 5.10–5.06 (3 br d, 4H, H-$1^{II–V}$), 5.00 (br d, 1H, H-1^{VI}), 4.75 (br d, 1H, H-1^{I}), 4.72–4.41 (m, 12H, 6 CH_2Ph), 4.09, 4.05–4.03 (br t, 1H, m, 3H, H-$2^{II–V}$), 3.93 (br t, 1H, H-2^{I}), 3.67 (s, partially overlapped, $COOCH_3$), 3.38, 3.34 (dt, 1H, *J* 6.5 Hz, H-1′b), 3.29 (s, 3H, OCH_3-2^{VI}), 2.93–2.80 (m, 6H, H-$4^{I–VI}$), 2.33 (t, 2H, *J* 7.5 Hz, H-5′a,b), 1.70–1.53 (m, 4H, H-4′a,b,2′a,b, in that order), 1.41–1.14 (m, 32H, 6 NH_2, H-3′a,b,$6^{I–VI}$); ^{13}C NMR ($CDCl_3$): δ 100.89, 100.81 (C, 3C, C-$1^{II–V}$), 99.02, 98.99 (C-$1^{I,VI}$), 79.46, 78.97, 78.60, 78.52 (C, 2C, C, 2C; C-$3^{I–VI}$), 75.68 (C-2^{VI}), 72.90, 72.46 (3C, C; C-$2^{II–V}$), 72.81 (C-2^{I}), 71.47, 71.12, 70.99, 70.95 (C, C, 2C, 2C; 6 *C*H_2Ph), 70.13, 70.05, 69.50 (4C, C, C; C-$5^{I–VI}$), 66.99 (C-1′), 58.72 (OCH_3-2^{VI}), 53.67, 53.50, 53.46, 53,43 (C, 3C, C, C; C-$4^{I–VI}$), 51.30 ($COOCH_3$), 33.75 (C-5′), 29.93 (C-2′), 25.58 (C-3′), 24.52 (C-4′), 18.10, 18.07, 18.03, 17.92 (3C, C, C, C; C-$6^{I–VI}$). Anal. Calcd for $C_{86}H_{118}N_6O_{21}$: C, 65.71; H, 7.57; N, 5.35. Found: C, 65.43; H, 7.56; N, 5.19.

3.2.14. Methoxycarbonylpentyl 3-*O*-benzyl-2-*O*-methyl-4-(2-*O*-acetyl-4-*O*-benzyl-3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-α-D-mannopyranosyl-(1→2)-tetrakis[4-(2-*O*-acetyl-4-*O*-benzyl-3-deoxy-L-*glycero*-tetronamido)-3-*O*-benzyl-4,6-dideoxy-α-D-mannopyranosyl]-(1→2)-4-(2-*O*-acetyl-4-*O*-benzyl-3-deoxy-L-*glycero*-tetronamido)-3-*O*-benzyl-4,6-dideoxy-α-D-mannopyranoside 22. A solution of crystalline 4-*O*-benzyl-L-*glycero*-tetronic acid[20] **20** (3.0 g) in 2:1 Ac_2O–pyridine [2:1, (v/v), 3.0 mL] was kept overnight at room temperature. TLC (15:1:0.1 CH_2Cl_2–MeOH–AcOH) showed the reaction to be complete. After concentration, and evaporation of several portions of water from the residue, to remove volatiles, the residue (3.3 g, 92%) contained pure (NMR, TLC) 2-*O*-acetyl-3-*O*-benzyl-L-*glycero*-tetronic acid **21**, $[\alpha]_D = -34$ (*c* 2.4, $CHCl_3$). ^1H NMR ($CDCl_3$): δ 9.87 (br s, 1H, COOH), 7.35–7.24 (m, 5H, aromatic), 5.22 (dd, 1H, $J_{2,3a}$ 5.0, $J_{2,3b}$ 8.5 Hz, H-2), 4.53, 4.45 (2 d, 2H, *J* 11.7 Hz, CH_2Ph), 3.65–3.53 (m, 2H, H-4a,b), 2.31–2.06 (m, 5H, H-3a,b, incl. s, 2.09, CO*C*H_3). ^{13}C NMR ($CDCl_3$): δ 72.97 (*C*H_2Ph), 69.04 (br, C-2), 65.15 (C-4), 31.17 (C-3), 20.50 (CO*C*$_3$). LCESI MS: *m*/*z* 275 ($[M+Na]^+$).

To a solution of **19** (100 mg, 0.064 mmol) and **21** (161 mg, 0.64 mmol) in CH_2Cl_2 (3 mL) was added HATU (243 mg, 0.64 mmol) followed by *N*-ethyldiisopropylamine (0.08 mL, 0.64 mmol), and the mixture was stirred overnight at room temperature. TLC (20:1 CH_2Cl_2–MeOH) showed that the reaction was complete and that one major product was formed. The mixture was partitioned between CH_2Cl_2 and water, which was kept slightly acidic (pH ~6) by addition of 1 M HCL. The organic phase was washed with a mixture (1:1, v/v) of brine and $NaHCO_3$ solution, dried, and concentrated. Chromatography of the residue gave **22** (122 mg, 80%), $[\alpha]_D = -37$. Structurally significant signals in the ^1H NMR ($CDCl_3$) spectrum were at: δ 6.40–5.83 (m, 6 NH), 5.30–5.21 (m, 6H, H-$2'^{I–VI}$), 5.03, 4.98 (br d, br s, 4H total, H-$1^{II–V}$), 4.87 (br s, 1H, H-1^{VI}), 4.68 (br s, 1H, H-1^{I}), 3.61 (s, overlapped, $COOCH_3$), 3.54–3.47 (m, 12H, $4'^{I–VI}$a,b), 3.19 (s, overlapped, OCH_3-2), 2.29 (t, 2H, *J* 7.4 Hz, H-5″a,b), 1.99, 1.95, 1.94, 1.92, 1.91 (5 s, one of them double intensity, partially overlapped with H-3b′$^{I–VI}$ signals, 6 $COCH_3$), 1.65–1.25 (2 m, H-2″a,b,3″a,b,4″a,b), 1.18–1.07 (6 d, partially overlapped, H-$6^{I–VI}$). Structurally significant signals in the ^{13}C NMR ($CDCl_3$) spectrum were at: δ 100.62, 99.74 (C, 3C, br, C-$1^{II–V}$), 98.93 (C-1^{VI}), 98.61 (C-1^{I}), 72.94, 72.87, 72.79, 72.72 (C, C, 3C, C; 6 *C*H_2Ph-$3^{I–VI}$), 71.47, 71.45, 71.42, 71.36 (C, C, C, 3C; C-$2'^{I–VI}$), 71.34, 71.12, 71.04, 70.87, 70.63, 70.46 (6 *C*H_2Ph-$4'^{I–VI}$), 69.24, 68.93, 68.82, 68.72, 67.75 (C, C, 2C, C, C; C-$5^{I–VI}$), 67.05 (C-1″), 65.93, 65.75, 65.70 (C, 3C, 2C; C-$4'^{I–VI}$), 58.94 (OCH_3-2^{VI}), 51.99, 51.79, 51.73 (C, 2C, 3C; C-$4^{I–VI}$), 51.52 ($COOCH_3$), 33.74 (C-5″), 31.99 (6C; C-$3'^{I–VI}$), 28.57 (C-2″), 25.65 (C-3″), 24.22 (C-4″), 20.78, 20.75, 20.70, 20.62 (C, C, 3C, C; 6 CO*C*H_3),

18.04, 17.96, 17.91 (4C, C, C; C-6$^{I–VI}$). LCESI MS: *m*/*z* 2998 ([M+Na]$^+$). Anal. Calcd for $C_{164}H_{202}N_6O_{45}$: C, 66.16; H, 6.84; N, 2.82. Found: C, 66.05; H, 6.87; N, 2.82.

3.2.15. Methoxycarbonylpentyl 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-mannopyranoside 24. Compound **22** (2.6 g) was deacetylated (Zemplén), to give methoxycarbonylpentyl 3-*O*-benzyl-4-(4-*O*-benzyl-3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-2-*O*-methyl-α-D-mannopyranosyl-(1→2)-tetrakis[4-(4-*O*-benzyl-3-deoxy-L-*glycero*-tetronamido)-3-*O*-benzyl-4,6-dideoxy-α-D-mannopyranosyl]-(1→2)-4-(4-*O*-benzyl-3-deoxy-L-*glycero*-tetronamido)-3-*O*-benzyl-4,6-dideoxy-α-D-mannopyranoside **23** (1.95 g, 81%). Structurally significant signals in the ^{1}H NMR ($CDCl_3$) spectrum were at: δ 6.95–5.60 (m, 6 NH), 5.06, 5.03, 5.01, 4.99 (4 br d, partially overlapped, 4H, H-1$^{II–V}$), 4.90 (br d, 1H, H-1^{VI}), 4.69 (br d, 1H, H-1^{I}), 4.66–4.39 (m, 24H, 12 *C*H$_2$Ph), 3.91 (br t, 1H, H-2^{I}), 3.62 (s, $COOCH_3$), 3.33, 3.36 (2 t, 1H, *J* 6.4 Hz, H-1″ b), 3.23 (s, overlapped, OCH_3-2^{VI}), 2.31 (t, 2H, *J* 7.5 Hz, H-5″a,b), 2.23–2.08, 1.96–1.80 (2 m, 12H, H-3′a,b$^{I–VI}$), 1.69–1.53 (m, 4H, H-2″a,b,4′a,b), 1.42–1.29 (m, 2H, H-3″a,b), 1.20–1.07 (6 d, 18H, partially overlapped, H-6$^{I–VI}$). Structurally significant signals in the ^{13}C NMR ($CDCl_3$) spectrum were at: δ 100.52, 98.87, 99.60 (C, C, br, 2C, br; C-1$^{II–V}$), 98.70 (C-1^{I}), 98.56 (C-1^{VI}), 73.72 (C-2^{I}), 73.41, 73.36, 73.30, 73.26, 73.20, 73.14 (6 *C*H$_2$Ph-3), 71.39, 71.14, 71.10, 70.95, 70.92, 70.60 (6 *C*H$_2$Ph-4′$^{I–VI}$), 69.63, 69.50, 69.30, 69.18, 69.02, 68.93 (C-4″$^{I–VI}$), 68.88, 68.80, 67.91 (2C, 3C, C; C-5$^{I–VI}$), 67.21 (C-1″), 58.97 (OCH_3-2^{VI}), 51.77, 51.70, 51.59, 51.49 (2C, C, C, 2C; C-4$^{I–VI}$), 51.40 ($COOCH_3$), 33.79, 33.60, 33.45 (4C, 2C, C; C-3′$^{I–VI}$, 5″), 28.85 (C-2″), 25.65 (C-3″), 24.45 (C-4″), 18.14, 18.09, 18.04, 17.97 (2C, 2C, C, C; C-6$^{I–VI}$). LCESI MS: *m*/*z* 2748 ([M+Na]$^+$).

Treatment of a solution of the foregoing compound **23** (1 g) in methanol (50 mL) with hydrogen in the presence of 5% palladium-on-charcoal catalyst (~200 mg) gave the title compound **24** (0.9 g, 88%), which was identical (TLC, NMR) with the independently described[10] substance.

3.2.16. Hydrazidocarbonylpentyl 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-mannopyranoside 25. This compound was prepared from ester **24** as described by Ogawa et al.[10] and was identical (TLC, NMR) with the independently described[10] substance.

3.2.17. (2-Aminoethylamido)carbonylpentyl 4-(3-deoxy-L-*glycero*-tetronamido)-2-*O*-methyl-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 27. This compound was prepared from ester **24** as described by Chernyak et al.[8] Structurally significant signals in the ^{1}H NMR (CD_3OD) spectrum were at δ 5.14, 5.12 (2 m, 5H, H-1$^{II–VI}$), 4.81 (d, 1H, $J_{1,2}$ 1.5 Hz, H-1^{I}), 4.22–4.16 (m, 6H, H-2′$^{I–VI}$), 3.48 (s, partially overlapped, OCH_3), 3.44, 3.41 (2, t, partially overlapped, H-1″b), 3.26 (t, 2H, *J* 6.4 Hz, H-6″), 2.74 (t, 2H, *J* 6.4 Hz, H-7″a,b), 2.22 (t, 2H, *J* 7.0 Hz, H-5″a,b), 2.08–1.77 (2 m, 12H, H-3′a,b), 1.70–1.58 (m, 4H, H-2″a,b,4″a,b), 1.47–1.36 (m, 2H, H-3″a,b), 1.18–1.13 m,, 18H, H-6$^{I–VI}$; ^{13}C NMR (CD_3OD): δ 102.70, 102.44 (C, 3C, C-1$^{II°V}$), 100.49 (C-1^{VI}), 100.19 (C-1^{I}), 80.68 (C-2^{VI}), 79.81 (C-2^{I}), 79.17, 79.00 (2C, 2C, C-2$^{II–V}$), 70.63 (C-2′), 69.69, 69.59, 69.46, 69. 39, 68.75 (C, 2C, 3C, 6C; C-3$^{I–V}$, 5$^{I–VI}$), 68.47 (C-1″), 59.42 (C-2′$^{I–VI}$), 59.15 (OCH_3), 54.74, 54.56, 54.50 (C, 3C, 2C, C-4$^{I–VI}$), 42.58 (C-6″), 41.91 (C-7″), 38.23 (C-3′$^{I–VI}$), 36.95 (C-5″), 30.11 (C-2″), 26.84 (C-3″), 26.53 (C-4″), 18.33, 18.28, 18.19 (2C, 3C, C; C-6$^{I–VI}$).

3.2.18. (6-Aminohexylamido)carbonylpentyl 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-mannopyranoside 29. A mixture of ester **24** (0.05 g) and hexamethylenediamine (0.5 mL) was heated overnight under argon at 65 °C. Excess of the reagent was removed by co-evaporation of water, and the residue was chromatographed. Lyophylization gave **29** (0.54 mg, 83%) as a white, amorphous solid.

Characteristic signals in the ^{1}H NMR (CD_3OD) spectrum were at δ 5.14, 5.11 (2 m, 5H, H-1$^{II–VI}$), 4.81 (d, 1H, $J_{1,2}$ 1.5 Hz, H-1^{I}), 4.22–4.16 (m, 6H, H-2′$^{I–VI}$), 3.67 (dd, 1H, $J_{1,2}$ 1.5, $J_{2,3}$ 3.3 Hz, H-2), 3.48 (s, partially overlapped, OCH_3-2), 3.44, 3.41 (2, t, partially overlapped, H-1″b), 3.17 (t, 2H, *J* 6.4 Hz, H-6″a,b), 2.70 (br t, 2H, H-11″a,b), 2.22 (t, 2H, *J* 7.0 Hz, H-5″a,b), 2.08–1.77 (2 m, 12H, H-3′a,b), 1.68–1.32 (m, 14H, H-2″a,b,3″a,b,4″a,b,7″a,b,8″a,b,9″a,b,10″a,b), 1.18–1.13 (m, 18H, H-6$^{I–VI}$). ^{13}C NMR (CD_3OD): δ 102.69, 102.42 (C, 3C; C-1$^{II–V}$), 100.45 (C-1^{VI}), 100.17 (C-1^{I}), 80.68 (C-2^{VI}), 79.76 (C-2^{I}), 79.13, 78.98 (2C, 2C; C-2$^{II–V}$), 70.65 (C-2′), 69.68, 69.59, 69. 41, 68.74 (C, 2C, 3C, 6C; C-3$^{I–VI}$, 5$^{I–VI}$), 68.50 (C-1″), 59.42 (6C, C-4′$^{I–VI}$), 59.14 (OCH_3-2), 54.77, 54.56, 54.50 (C, 3C, 2C; C-4$^{I–VI}$), 41.92 (C-6″), 40.19 (C-11″), 38.22 (6C; C-3′$^{I–VI}$), 36.97 (C-5″), 32.27 (C-7″), 30.29 (C-10″), 30.11 (C-2″), 27.65, 27.44 (C-8″, 9″), 26.83 (C-3″), 26.71 (C-4″), 18.26, 18.21 (5C, C; C-6$^{I–VI}$). LCESI MS: *m*/*z* 1749 ([M+Na]$^+$).

3.2.19. 1-{(5-Hydrazidocarbonylpentyl)carbonylpentyl 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-mannopyranoside}-2-ethoxycyclobutene-3,4 dione 26. Diethyl squarate (8 μL, 55 μmol) was added to a solution of the hydrazide **25** (50 mg, 30 μmol) as described,[8] to give squaric acid monoethyl ester **26** (40 mg, 83%). The NMR spectra (D_2O) were very similar to those of the analogous 2-methoxycyclobutene deriva-

tive, prepared from the same hexasaccharide.[8] Characteristic of the structure of **26** was the cross-peak between the multiplets at δ 1.50 and ~4.80 for the methyl and methylene group of the squaric acid ethyl ester group, respectively, present in the ^1H–^1H NMR COSY spectrum. The ^{13}C NMR spectrum showed signals characteristic of the corresponding carbon nuclei at 15.17 (d) and 70.12 (d) ppm, respectively. Similar resonances were present in the spectrum of the squaric acid monoethyl ester derived from the Ogawa monosaccharide.[3] LCESI MS: *m/2z* 884.387 ($[M/2+H]^+$).

3.2.20. 1-{(2-Aminoethylamido)carbonylpentyl 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-mannopyranoside}-2-ethoxycyclobutene-3,4-dione 28. This compound was prepared (245 mg, 82%), from **27** (280 mg, 0.16 mmol), following the protocol described in the preparation of **26**. The NMR spectra (D_2O) were very similar to those of the analogous 2-methoxycyclobutene derivatives, prepared from the same hexasaccharide.[8] Characteristic of the structure of **28** was the cross-peak between the multiplets at δ 1.55 and ~4.84 for the methyl and methylene groups belonging to the squaric acid ethyl ester group, respectively, present in the ^1H–^1H NMR COSY spectrum. The ^{13}C NMR spectrum showed signals characteristic of the corresponding carbon nuclei at 15.20 (d) and 70.75 (d) ppm, respectively. Similar resonances were present in the spectrum of the squaric acid monoethyl ester derived from the Ogawa monosaccharide.[3] LCESI MS: *m/2z* 898.4127 ($2[M/2+1]^+$).

3.2.21. 1-{(6-Aminohexylamido)carbonylpentyl 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-mannopyranoside}-2-ethoxycyclobutene-3,4-dione 30. This compound was prepared (52 mg, 80%), from **29** (61 mg, 0.035 mmol), following the protocol described in the preparation of **26**. The NMR spectra (D_2O) were very similar to those of the above 2-aminoethyl analog **28**. Characteristic of the structure **30** was the cross-peak between the multiplets at δ 1.40 and ~4.84 for the methyl and methylene groups belonging to the squaric acid ethyl ester group, respectively, present in the ^1H–^1H NMR COSY spectrum. The ^{13}C NMR spectrum showed signals characteristic of the corresponding carbon nuclei at 15.20 (d) and 70.75 (d) ppm, respectively. LCESI MS: *m/2z* 926 ($2[M/2+H]^+$).

3.2.22. Neoglycoconjugates from the hexasaccharide of *V. cholerae* O:1 and BSA 31–33. Each of the squaric acid derivatives **26** (4 mg, 2.25 μmol), **28** (4.05 mg, 2.25 μmol), and **30** (4.16 mg, 2.25 μmol) was added to a solution of BSA (10 mg, 0.15 μmol) in a pH 9 borate buffer (112 μL). The amount of buffer used assured the initial concentration of the hapten to be 20 mM. The mixture was stirred at room temperature and monitored periodically by SELDI-TOF MS. When a molar carbohydrate–BSA ratio of 5 had been reached, 90% of the solution was withdrawn and subjected to ultrafiltration using Millipore cells (Amicon, Stirred Ultrafiltration Cell, Model No. 8050), and filters (Ultrafiltration Membrane, Amicon, Milipore) with a molecular mass cutoff of 10,000 Da. The solution of the high molecular mass material was freeze-dried, to give the title conjugates **31** (9.6 mg, 93%), **32** (9.0 mg, 87%), and **33** (9.3 mg, 90%) as white, fluffy solids. For details, see Table 1.

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