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Linking Carbohydrates to Proteins Using *N*-(2,2-Dimethoxyethyl)-6-hydroxy Hexanamide

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Abstract: The title dimethyl acetal **4** and related compounds can be efficiently synthesized by treatment of 6-caprolactone with commercially available dialkyl acetals. Conventional glucosylation using **4** as a glycosyl acceptor gave mainly β-glycosides which were deprotected to give **13**. The latter was converted to the corresponding aldehyde **16**, which was used as a hapten in conjugation, by reductive amination, to chicken serum albumin (CSA). Effect of reaction time, concentration and molar ratio of hapten **16** to the number of L-lysine residue in CSA upon incorporation of the hapten was studied using MALDI-TOF spectrometry. High loading of CSA with hapten, up to 22 moles of **16**/CSA, could be achieved with 32% efficiency of utilization of the hapten. A glycoconjugate from a derivative, analogous to **16**, of the monosaccharide determinant of the O-PS of *Vibrio cholerae* O:1, serotype Ogawa and CSA was also prepared. The achieved incorporation of the *Vibrio cholerae* hapten was in remarkable agreement with the value expected, based on the study with the D-glucose derivative.

Keywords: Neoglycoconjugate, Reductive Amination, Antigen, Vibrio cholerae

The immunity of vertebrae to many diseases is related to the response of elements of their immune system to polysaccharide antigens, *e.g.* capsular polysaccharides (CP) or the O-specific polysaccharides (O-PS) that are part of the lipopolysaccharides (LPS) located on the surface of pathogenic bacteria. Carbohydrates themselves are often poorly immunogenic but glycoconjugates resulting from chemical linking of CP, O-PS, LPS, or fragments thereof to proteins show markedly improved immunogenicity. This was first demonstrated by Avery and Goebel[1-3] who prepared conjugates of the disaccharide, repeating unit of pneumococcus type 3 CP. While the disaccharide itself was not immunogenic, these conjugates induced type-specific antibodies that were protective against challenge with type 3 pneumococci. Use of the conjugate vaccine against *Hemophylus influenzae* b,[4] which is based on this principle, resulted in virtual elimination of meningitis in countries where the vaccine is routinely used. Similar materials prepared from other antigenic poly- or oligosaccharides are hoped to replace traditional vaccines prepared from killed or attenuated bacteria. One of the projects ongoing in this laboratory is aimed at preparation of glycoconjugates from fragments of the O-polysaccharide of *Vibrio cholerae* O:1. The study reported here was undertaken to explore conditions in which such conjugates could be efficiently prepared.

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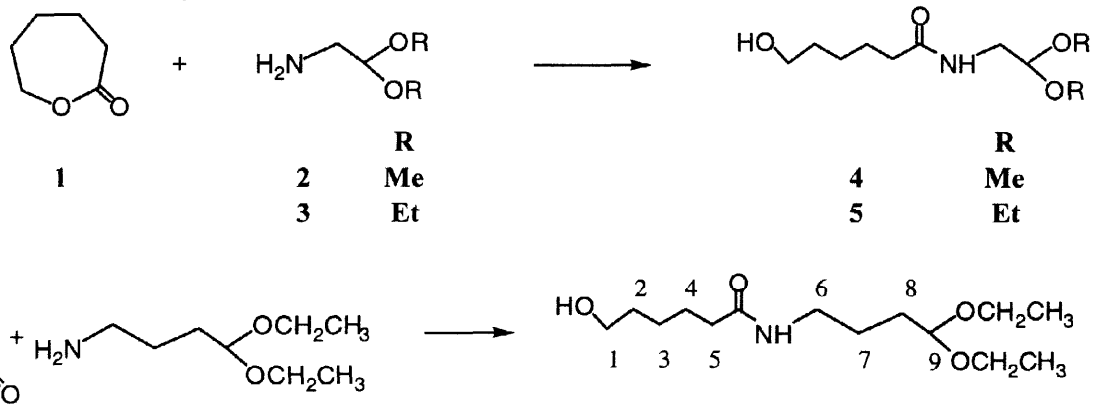
Reductive amination[5,6] has been extensively used for conjugation of carbohydrates to proteins. It involves the reaction of amino groups in proteins with various forms of aldehydes present or generated in carbohydrates, followed by reduction of the Shiff base initially formed into a stable product. In the case of polysaccharides, the aldehydo groups required can be generated, for example, by treatment of vicinal diols in the polymer with a limited amount of periodate. Reducing mono- and oligosaccharides are amenable to direct conjugation by reductive amination but the process is usually very slow, because the concentration of the acyclic aldehydo form of sugars is very low. Activation of mono- or oligosaccharides by periodate oxidation or many other means[7] is impractical, as it might change the structure of the determinant. The use of functionalized linkers in these situations is far more preferable. Thus, the requisite sugar is often prepared in the form of a glycoside whose aglycon contains functional groups suitable for linking to proteins. Here we describe the preparation of glycosides of the title, linking agent containing a latent aldehydo function, and their conjugation to chicken serum albumin (CSA) by reductive amination.

Many spacer-arm linkers suitable for conjugation of carbohydrates to proteins by reductive amination have been described.[8-13] Preparation of such molecules usually requires multi-step syntheses and tedious purification by chromatography of the intermediates involved, the target products, or both. We have recently reported briefly[14] on three new agents (**4**, **5** and **7**) suitable for linking carbohydrates and proteins by reductive amination and which do not suffer from such shortcomings. Details of the syntheses, and full characterization of the aforementioned dialkyl acetals is reported here.



Compounds **4**, **5** and **7** are prepared readily by treatment of commercially available amines **2**, **3**, and **6** with excess (~5-10 molar equivalents) of 6-caprolactone (**1**). Preliminary experiments (not described in the Experimental) showed the presence of base[15,16] to be detrimental to the outcome of the reaction: Byproducts were formed, showing faster chromatographic mobility than the desired addition product. The extent of formation of these byproducts was negligible when the reaction was carried out in the absence of base at room temperature (optimum conditions), albeit it took 5-7 days for all the amine to be consumed. We have isolated, by column chromatography, the byproduct (~3-6%) in one of the initial preparations of the dimethylacetal **4** (performed at 100 °C, not described in the Experimental). The ^{1}H NMR spectrum indicated that the compound was a product resulting from the addition reaction of the dimer of **1** with the dimethylacetal **2**. When the reaction was conducted under optimum conditions, the pure, desired compounds **4**, **5** and **7** were obtained without chromatography, by fractional distillation. Excess of ε-caprolactone was collected in the forerun, and it could be reused. The much higher-boiling byproduct, if present, remained in the distillation flask, after collection of the desired product. Relatively greatest amount of byproduct (still less than 10%, TLC) was formed from the reaction involving 4-aminobutyraldehyde dimethylacetal. This notwithstanding, vacuum distillation yielded analytically pure material (~90%). Because of its low boiling point, it is the dimethyl acetal **4** that is most conveniently prepared.

To test the potential of the above dialkyl acetals as glycosyl acceptors, compound **4** was treated with each of 2,3,4,6-tetra-*O*-benzyl- (**8**[17]), 2,3,4,6-tetra-*O*-benzoyl- (**9**[18]) α-D-glucopyranosyl bromides, as well as

with 2,3,4,6-tetra-*O*-benzyl-α- (**10**[19]) and -β-D-glucopyranosyl trichloroacetimidate (**11**[19]) under various conditions. The results are summarized in Table 1.

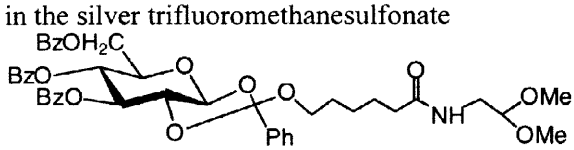
Table. 1 Glycosylation of **4** with various glycosyl donors[a]

Glycosyl donor	Reaction conditions[b]	Yield (%)[c]	Stereoselectivity of glycosylation (α:β)[d]
8	AgOTf (1.05 equiv[e]), DTMP[f] (1.10 equiv[e]), -78°C, 16 h	62	1 : 9
9	AgOTf (1.10 equiv[c]), TMU[g], (1.10 equiv[c]), r.t., 16 h	64[h]	0 : 1[h]
10	AgOTf (cat.[e]), -15°C → 5°C, 6 h	95	1 : 9
11	AgOTf (cat.[e]), -15°C → r.t., 18 h	93	3 : 2
10 + **11** (~1:1)	AgOTf (cat.[e]), r.t., 4 h	91	1 : 1
10 + **11** (~1:1)	LiOTf (cat.[e]), 50°C[i], 16 h	92	1 : 1

[a]All glycosylations were run at a molar ratio of glycosyl donor : **4** = 1:1.5; [b]The amount of AgOTf relative to the glycosyl donor; [c]After isolation by column chromatography; [d]Determined by ^{1}H NMR spectroscopy; [e]Relative to the amount of glycosyl donor; [f]Di-*t*-butyl-4-methylpyridine; [g]1,1,3,3-Tetramethylurea; [h]Glycoside **12** and orthoester **17** were formed (~1 : 1) in a combined yield of 57%, when Ag_2CO_3 was used as a promoter; [i]No reaction was observed (TLC) at room temperature.

	R	R^1	R^2		R
8	Bn	Br	H	**12**	Bz
9	Bz	Br	H	**14**	Bn
10	Bn	$OC(NH)CCl_3$	H		
11	Bn	H	$OC(NH)CCl_3$		

When performing these reactions with **9**, we could not apply the base deficient conditions[20] favorable for formation of glycosides *vs.* orthoesters, because of the acid-labile nature of the dialkyl acetals used. Thus, variable amount of orthoester **17** was present among the products of the conversions. Its structure followed from its NMR spectra showing features characteristic[21] of orthobenzoates. Notably, orthoester **17** and glycoside **12** were formed in a ratio of 1:1 when the reaction of **9** and **4** was mediated with Ag_2CO_3. An acceptable yield (64%) of the desired glycoside **12** was obtained from **9** only when 1,1,1,1- tetramethylurea was used as the base in the silver trifluoromethanesulfonate (triflate) mediated glycosylation. NMR spectroscopy indicated this reaction to be stereospecific. A similar yield of unseparable mixture (TLC) of glycoside **14** and the α-anomer **15** (9:1, NMR) was obtained from **8**.



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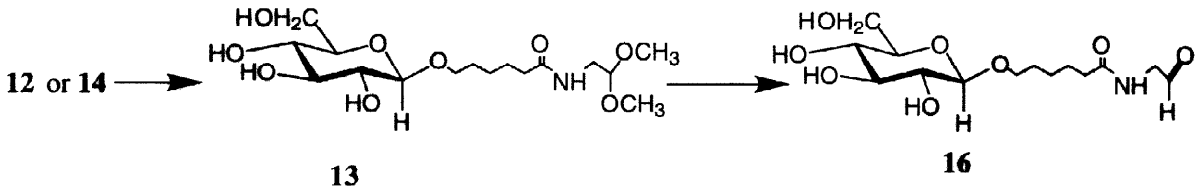
More efficient glycosylations were effected with imidates **10** and **11**, mediated with silver or lithium[22] triflate. Here, excellent yield (>90%) of glycosylations was observed, but only the glycosylation with the α-imidate **10** was highly stereoselective.



13 16

The internal part of O-polysaccharides (O-PS) of *Vibrio cholerae* O:1, serotype Ogawa and Inaba consists of the same repeating unit, α-(1-2)-linked 4-amino-4,6-dideoxy-D-mannose (D-perosamine) whose amino groups are acylated with 3-deoxy-L-*glycero*-tetronic acid. The two serotypes differ in that the upstream perosamine moiety terminating the O-PS of the Ogawa strain is methylated at *O*-2. Binding studies with methyl α-glycosides of mono- through hexasaccharide fragments of the O-PS of *Vibrio cholerae* O:1, serotype Ogawa revealed[23] that the terminal monosaccharide is the immunologically dominant determinant. Therefore, we felt it important to prepare a neoglycoconjugate based on that epitope, to be used in our future immunological studies. Oligosaccharides composed of perosamine or derivatives thereof have been successfully synthesized using thioglycosides as glycosyl donors.[24-27] Thus, we have also examined glycosylations of **4** with the thioglycoside **19**.

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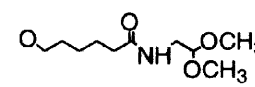
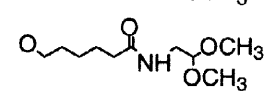
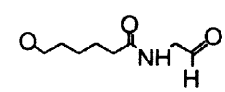
	R	R^1	R^2
19	Ac	H	SEt
20	Ac	SEt	H
21	Ac	H	
22	H	H	
23	H	H	

Before linking the precious aldehyde **23** to CSA, we used a more readily available derivative of D-glucose (**16**) to examine the effect of variables, such as the reaction time, concentration, and the molar ratio of the carbohydrate used to the number of lysine moieties present in CSA (46[28]), upon the outcome of the conjugation. Incorporation of the carbohydrate in the protein and the efficiency of the conjugation was determined from the molecular mass of the neoglycoconjugates obtained, as determined by MALDI–TOF mass spectrometry. The results showed (Tables 2-5) that the conjugation is very concentration dependent. At a reasonably high concentration of the hapten, its maximal incorporation into protein is achieved within about 16 h (Table 5), and prolonging reaction has little effect upon the final incorporation. At low concentration of hapten, its incorporation in the carrier protein can not be increased simply by increasing the excess of hapten used (Table 2). Since carbohydrate antigens to be conjugated are often very labor-intensive commodities, it was important to obtain data on the efficiency of coupling. Data show (Tables 2-5) that the efficiency is inversely proportional to the excess of the hapten used. This should not be surprising, as only a fraction of amino groups present in CSA is surface-exposed and readily available for the reaction. Reactivity of those buried within the three-dimensional structure appears limited, and some might be completely unavailable for the reaction.

Finally, conjugation of the monosaccharide determinant[23] of the O-PS of *Vibrio cholerae* O:1, serotype Ogawa to CSA was performed. The linker-equipped hapten **23** was prepared from the known[29] acetate **18**, here obtained by acetolysis of the parent methyl glycoside.[29] Compound **18** was converted to the ethyl α-1-thioglycoside **19**, which was treated with the dimethyl acetal **4** to give, stereospecifically, the fully protected glycoside **21**. Subsequent two-step deprotection gave aldehyde **23**.

Table 2. Effect of the amount of hapten used upon its incorporation in CSA at low concentration of hapten[a]

Hapten : L-lysine used [equiv]	m/z	Number of hapten residues incorporated/CSA	Efficiency [%]
1 : 2	69,063	6.5	28.2
1 : 1	69,988	9.4	20.4
2 : 1	70,014	9.5	10.3
3 : 1	70,047	9.6	6.9

[a] Concentration of hapten, 5.7 mM; Reaction time, 40 h.

Table 3. Effect of the amount of hapten used upon its incorporation in CSA at high concentration of hapten[a]

Hapten : L-lysine used [equiv]	*m/z*	Number of hapten residues incorporated/CSA	Efficiency [%]
1 : 2	70,592	11.3	49.1
1 : 1	71,341	13.5	29.3
3 : 2	72,317	16.7	24.2
2 : 1	72.895	18.5	20.1
3 : 1	73,284	19.7	14.2

[a] Concentration of hapten, 18.9 mM; Reaction time, 40 h.

Table 4. Effect of the concentration of hapten used upon its incorporation in CSA[a]

Concentration of hapten [mM]	m/z	Number of hapten residues incorporated/CSA	Efficiency [%]
5	70,015	9.5	13.7
10	70,721	11.8	19.1
15	72,850	18.4	26.6
20	73,474	20.3	29.4
25	74,070	22.2	32.1

[a] Reaction time, 40 h; Hapten : L-lysine used [equiv], 3 : 2.

Conjugation by reductive amination of **23** with CSA was carried out at room temperature for 40 h at a hapten concentration of 19.4 mM and a 3:1 molar ratio of the hapten to the number of L-lysine residues. Under these conditions, the expected incorporation of **23** in CSA (Table 3) is ~ 20 residues of **22**/CSA. MALDI-TOF spectrometry of the material obtained produced a peak centered at *m/z* 75,540 showing that, on average, the material contained 20.4 residues of **23**/CSA. This remarkably close agreement of the found degree of incorpora-

Table 5. Effect of the reaction time upon incorporation of hapten in CSA[a]

Time [h]	m/z	Number of hapten residues incorporated/CSA[b]
16	73,140	19.3
40	73,175	19.4
64	73,245	19.6
112	73,066	19.0
160	72,950	19.0
208	73,080	19.0

[a] Concentration of hapten, 15.7 mM; Reaction time, 40 h; Hapten : L-lysine used [equiv], 2 : 1.
[b] Efficiency, ~21%.

tion of **23** into CSA with that expected indicates that the results of the above study may be applicable in similar systems.

Experimental Section

Instruments and laboratory techniques used were the same as those described previously.[30] Mass spectra were obtained using PerSeptive BioSystems Voyager Elite DE-STR (PE-Biosystems, Framingham, MA) MALDI-TOF instrument. The instrument was operated in the linear mode with 25 kV accelerating voltage and a 300 nsec ion-extraction delay time. Samples for analysis (~0.1 mg) were dissolved in deionized water (50 μL), and applied as 1 μL droplets to separate positions in the center of the multiple-sample plate. An equal volume of matrix (saturated solution of sinnapinic acid in 1:1 acetonitrile–0.5% trifluoroacetic acid) was applied over each dried sample and redried before being inserted into the mass spectrometer. CSA, purchased from Sigma Chemical Company, was purified as described previously,[31] freeze-dried and used also as a mass standard. Results from all analyses yielded a molecular mass for this protein of about 66.9 kDa. Molecular masses of the glycoconjugates were calculated using the molecular mass of CSA of 66,973 Da, predicted from gene sequence.[28] Optical rotations were measured at ambient temperature with a Perkin-Elmer automatic polarimeter, Model 341. All reactions were monitored by thin-layer chromatography (TLC) on Silica gel 60 coated glass slides (Whatman or Analtech). During TLC of carbohydrate derivatives, detection was effected by charring with 5% (v/v) sulfuric acid in ethanol. When monitoring reactions leading to dialkylacetals **4**, **5**, and **7**, detection was effected by spraying with 5% (w/v) phosphomolybdic acid in ethanol, and heating until permanent spots were formed. Preparative 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 were made by first-order analysis of the spectra, supported by homonuclear decoupling experiments or homonuclear and heteronuclear 2-dimensional correlation spectroscopy. When reporting NMR data of glycosides prepared from **4**, the nuclei of the 3-deoxy-L-*glycero*-teronamide side-chain are noted as primed and those of the aglycon are noted as double-primed. Dialkylacetals **2**, **3**, and **6** were purchased from Aldrich Chemical Co. or from Fischer Scientific, and 6-caprolactone (monomer) was a product of Fluka Chemical Company. 2,3,4,6-Tetra-*O*-benzyl-D-glucose was purchased from Pfanstiehl Chemical Company, and used as supplied.

***N*-(2,2-Dimethoxyethyl)-6-hydroxy hexanamide (4).** A mixture of 2-aminoacetaldehyde dimethylacetal (5 g, 47.6 mmol) and 6-caprolactone (**1**, 50 g, 438 mmol) was kept at room temperature until TLC (hexane–acetone–conc NH_4OH 1:1:0.01) showed that all amine was consumed (~6-8 d). In addition to the major product **4** a trace of the by-product was formed (TLC). Fractional distillation gave first (~60°/~30 Pa) most of the unchanged lactone **1** which could be reused for the preparation of **4**. The main fraction collected next (~153-157 °C/8 Pa) contained pure **4** (9.35 g, ~90%, based on the amount of starting dimethylacetal). ^{1}H NMR ($CDCl_3$): δ 5.99 (m, 1 H, NH), 4.38 (t, 1 H, J = 5.2 Hz, H-7), 3.62 (t, 2 H, J = 6.5 Hz, H-1a,b), 3.39 (m, 8 H, incl 2 s, overlapped, 2 OCH_3, and t, H-6a,b), 2.52 (s, 1 H, OH), 2.21 (t, 2 H, J = 7.15, H-5a,b), 1.72–1.61 (m, partially overlapped, H-4a,b), 1.63–1.54 (m, partially overlapped, H-2a,b), 1.45–1.34 (m, 2 H, H-3a,b); ^{13}C NMR ($CDCl_3$): δ 173.4 (CO), 102.6 (C-7), 62.2 (C-1), 54.3 (2 C, 2 OCH_3), 40.8 (C-6), 36.3 (C-4), 32.2 (C-2), 25.2 (C-3), 25.2 (C-4); CIMS: *m/z* 220 ($[M + 1]^+$), 237 ($[M + 18]^+$). Anal. Calcd for $C_{10}H_{21}NO_4$: C, 54.79; H, 9.59; N, 6.39. Found: C, 54.54; H, 9.66; N, 6.30.

***N*-(2,2-Diethoxyethyl)-6-hydroxy hexanamide (5).** A mixture of 2-aminoacetaldehyde diethylacetal (5 g, 37.6 mmol) and 6-caprolactone (50 g, 438 mmol) was treated as described above for the preparation of **4**, to give **5** (8.4 g, ~90%, based on the amount of starting diethylacetal), bp 155–160 °C/~8Pa; ^{1}H NMR ($CDCl_3$): 5.84 (m, 1 H, NH), 4.51 (t, 1 H, J 5.1 Hz, H-7), 3.76–3.50 (m, 6 H, H-1a,b, 2 CH_2CH_3), 3.39 (t, 2 H, J = 5.4, H-6), 2.21 (t, 2 H, J = 7.3 Hz, H-5), 1.72–1.54 (m, 4 H, H-2a,b, H-4a,b), 1.46–1.38 (m, 2 H, H-3a,b), 1.22 (t, 6 H, J = 7.1 Hz, 2 CH_3); ^{13}C NMR ($CDCl_3$): δ 100.7 (C-7), 62.8 (2 C, 2 CH_2CH_3), 62.3 (C-1), 41.7 (C-6), 36.4 (C-5), 32.1 (C-2), 25.2 (C-3), 25.1 (C-4), 15.1 (2 C, 2 CH_3); CIMS: *m/z* 248 ($[M + 1]^+$); Anal. Calcd for $C_{12}H_{25}NO_4$: C, 58.30; H, 10.12; N, 5.67. Found: C, 58.31; H, 10.11; N, 5.60.

***N*-(2,2-Diethoxybutyl)-6-hydroxy hexanamide (7).** A mixture of 4-aminobutyraldehyde diethylacetal (5 g, 31 mmol) and 6-caprolactone (36 g, 315 mmol) was treated as described above for the preparation of **4**, to give **7** (8.0 g, 94%, based on the amount of starting diethylacetal), bp 185-193 °C/~10 Pa; ^{1}H NMR ($CDCl_3$): δ 5.96 (m, 1 H, NH), 4.48 (t, 1 H, J = 5.1 Hz, H-9), 3.71–3.61 (m, 4 H, H-1a,b, H-10a,b), 3.55–3.44 (m, 2 H, H-10a,b),

3.30–3.23 (m, 2 H, H-6a,b), 2.27 (bs, 1 H, OH), 2.18 (t, 2 H, J 7.3 Hz, H-5a,b), 1.71–1.54 (m, 8 H, H-2a,b, H-4a,b, H-7a,b, H-8a,b), 1.45–1.35 (m, 2 H, H-3a,b), 1.20 (t, 6 H, J = 7.0 Hz, 2 CH_3); ^{13}C NMR ($CDCl_3$): δ 102.7 (C-9), 62.3 (C-1), 61.4 (2 C, 2 C-10), 39.1 (C-6), 36.6 (C-5), 32.2 (C-2), 31.0 (C-8), 25.3 (C-3), 25.3 (C-4), 24.58 (C-7), 15.3 (2C, 2 CH_3); CIMS: *m/z* 276 ([M + 1]$^+$), 293 ([M + 18]$^+$). Anal. Calcd for $C_{14}H_{29}NO_4$: C, 61.09; H, 10.55; N, 5.09. Found: C, 61.18; H, 10.58; N, 5.00.

2,3,4,6-Tetra-*O*-benzoyl-β-D-glucopyranosyl *N*-(2,2-dimethoxyethyl)-5-hydroxy hexanamide (12). a. A mixture of **9** (132 mg, 0.2 mmol), **4** (44 mg, 0.2 mmol), Drierite (300 mg), and Ag_2CO_3 (55 mg, 0.2 mmol) in CH_2Cl_2 (10 mL) was stirred in the dark for 18 h. TLC (3:2 hexane–acetone) then showed that all glycosyl bromide was consumed. The two major products formed were isolated by chromatography. Eluted first was 3,4,6-tri-*O*-benzoyl-1,2-*O*-{[*N*-(2,2-dimethoxyethyl)-6-hydroxy hexanamide]-6-yl-orthobenzoyl}-α-D-glucopyranose (**17**). ^1H NMR ($CDCl_3$): δ 6.04 (d, 1 H, $J_{1,2}$ = 5.3 Hz, H-1), 5.76 (bdd, 1 H, $J_{2,3}$ = ~3.1, $J_{3,4}$ = ~1.2 Hz, H-3), 5.67 (m, 1 H, NH), 5.50 (bd, 1 H, $J_{4,5}$ = ~8.9 Hz, H-4), 4.77 (bddd, 1 H, $J_{2,4}$ = ~1 Hz, H-2), 4.52 (dd, 1 H, $J_{5,6a}$ = 3.0, $J_{6a,6b}$ = 12.1 Hz, H-6a), 4.37 (dd, partially overlapped, $J_{5,6b}$ = 4.6 Hz, H-6b), 4.34 (t, partially overlapped, J = 5.1, H-7''), 4.13 (ddd, 1 H, H-5), 3.40-3.22 (m, 10 H, H-1''a,b,6''a,b, incl s at 3.36 for 2 OCH_3), 2.14 (t, 2 H, J = 8.0 Hz, H-5''a,b), 1.63–1.48 (m, 4 H, H-2''ab,4''a,b), 1.36–1.24 (m, 2 H, H-3''a,b); ^{13}C NMR ($CDCl_3$): δ 121.29 (quaternary orthobenzoyl[21]), 102.6 (C-7''), 97.5 (C-1), 72.0 (C-2), 69.3 (C-3), 68.5 (C-4), 67.4 (C-5), 63.9 (C-1''), 63.9 (C-6), 54.3 ((2 C, 2 OCH_3), 40.8 (C-6''), 36.5 (C-5''), 29.1 (C-2''), 25.7 (C-3''), 25.2 (C-4''); CIMS: *m/z* 798 ([M + 1]$^+$, 815 ([M + 18]$^+$.

Eluted next was the desired, amorphous glycoside **12** ([α]$_D$ +15° (*c* 1.1, $CDCl_3$), ^1H NMR ($CDCl_3$): δ 5.91 (t, 1 H, J = 9.7 Hz, H-3), 5.68 (t, 1 H, J = 9.7, H-4), 5.61–5.48 (m, 2 H, NH, incl dd, at 5.52, $J_{1,2}$ = 7.8, $J_{2,3}$ = 9.9 Hz, H-2), 4.82 (d, 1 H, H-1), 4.65 (dd, 1 H, $J_{5,6a}$ = 3.4, $J_{6a,6b}$ = 12.1 Hz, H-6a), 4.50 (dd, 1 H, $J_{5,6b}$ = 5.0 Hz, H-6b), 4.35 (t, 1 H, J = 5.2 Hz, H-7''), 4.16 (m, 1 H, H-5), 3,95–3.88 (m, 1 H, H-1''a), 3.58–3.50 (m, 1 H, H-1''b), 3.40–3.55 (m, 8 H, H-6''a,b, incl s at 3.39, 2 OCH_3), 1.94–1.88 (m, 2 H, H-5''a,b), 1.59–1.44 (m, 4 H, H-2''a,b,4''a,b), 1.27–1.19 (m, 2 H, H-3''a,b); ^{13}C NMR ($CDCl_3$): δ 102.7 (C-7''), 101.3 (C-1), 72.9 (C-3), 72.2 (C-5), 72.0 (C-2), 70.0 (C-1''), 69.8 (C-4), 63.2 (C-6), 54.46, 54.3 (2 OCH_3), 40.8 (C-6''), 36.3 (C-5''), 29.1 (C-2''), 25.4 (C-3''), 25.1 (C-4''); CIMS: *m/z* 798 ([M + 1]$^+$), 815 ([M + 18]$^+$). *Anal.* Calc. for $C_{44}H_{47}NO_{13}$: C, 66.25; H, 5.90; N 1.76. Found: C, 66.00; H, 5.92; N, 1.69.

An intermediate, mixed fraction was also obtained. Combined yield, 92 mg (57%).

b. A mixture of **9** (330 mg, 0.5 mmol), Drierite (500 mg), **4** (100 mg, 0.45 mmol) and TMU (64 mg, 0.55 mmol) was stirred at room temperature and with the exclusion of moisture for 30 min. A solution of silver triflate (141 mg, 0.55 mmol) in a minimum amount of toluene was added dropwise, and stirring was continued for 18 h. TLC showed that, in addition to some product of hydrolysis of **9**, one major product was formed. Chromatography gave **12** (234 mg, 64%).

2,3,4,6-Tetra-*O*-benzyl-β-D-glucopyranosyl *N*-(2,2-dimethoxyethyl)-5-hydroxy hexanamide (14). A solution of AgOTf (280 mg, 1.1 mmol) in toluene (5 mL) was added dropwise at -78 °C to a mixture of **8** (prepared[17] from 2,3,4,6-tetra-*O*-benzyl-D-glucose, 540 mg, 1 mmol), **4** (315 mg, 3 mmol), 4 Å molecular sieves (0.4 g) and di-*t*-butyl-4-methylpyridine (DTBMP, 225 mg, 1.1 mmol) in CH_2Cl_2 (15 mL) which had been stirred at room temperature for 20 min. The stirring at -78 °C was continued for 3 h when the cooling was removed, and the stirring was continued overnight. Triethylamine (2 mL) was added, the mixture was filtered, the filtrate was concentrated, and the residue was chromatographed (3:1 hexane–EtOAc) to give a mixture of **14** and the α-anomer **15** (~10:1, as shown by ^1H NMR spectroscopy, 460 mg, 62%). ^1H NMR ($CDCl_3$) for the predominating β-anomer: δ 5.65 (m, 1 H, NH), 5.96–4.44 (m, 8 H, 4 CH_2Ph), 4.38 (d, partially overlapped, $J_{1,2}$ = 7.9 Hz, H-1), 4.34 (t, partially overlapped, J = 5.2 Hz, H-7''), 4.01-3.92 (m, 1 H, H-1''a), 3.76–3.35 (m, 15 H, H-2,3,4,5,6a,b,1''b,6''a,b, incl s at 3.33 for 2 OCH_3), 2.17–2.08 (m, 2 H, H-5''a,b), 1.71–1.60 (m, 4 H, H-2''a,b,4''a,b), 1.46–1.37 (m, 2 H, H-3''a,b); ^{13}C NMR ($CDCl_3$): δ 103.5 (C-1, $J_{C,H}$ 158.5 Hz), 102.5 (C-7''), 84.6 (C-3), 82.1 (C-2), 77.8 (C-4), 75.5, 74.8 (2 CH_2Ph), 74.7 (C-5), 74.6, 73.3 (2 CH_2Ph), 69.7 (C-1''), 68.8 (C-6), 54.2 (2 C, 2 OCH_3), 40.7 (C-6''), 36.3 (C-5''), 29.4 (C-2''), 25.7 (C-3''), 25.2 (C-4''); CIMS: *m/z* 759 ([M + 18]$^+$). *Anal.* Calc. for $C_{44}H_{55}N\,O_9$: C, 71.26; H, 7.42; N 1.89. Found: C, 71.32; H, 7.27; N, 1.79.

b. Sodium hydride (~5 mg) was added to a mixture of 2,3,4,6-tetra-O-benzyl- D-glucose (2.4 g, 4.4 mmol) trichloroacetonitrile (2 mL), and 4 Å molecular sieves (1.0 g) in CH_2Cl_2 (70 mL) which had been stirred at room temperature for 15 min. After 15 min TLC (5:1 hexane–EtOAc) showed that the reaction was complete and that two products were formed. After filtration through a Celite pad and concentration of the filtrate, the residue was chromatographed to give first the α-imidate **10**, ^{1}H NMR ($CDCl_3$): δ 8.58 (s, 1 H, NH), 6.53 (d, 1 H, $J_{1,2}$ = 3.6 Hz, H-1); ^{13}C NMR ($CDCl_3$): δ 94.4 (C-1);

Eluted last was the β-imidate **11**, ^{1}H NMR ($CDCl_3$): δ 8.71 (s. 1 H, NH), 5.81 (d, 1 H, $J_{1,2}$ = 7.7 Hz, H-1); ^{13}C NMR ($CDCl_3$): δ 98.2 (C-1).

An intermediate, mixed fraction was also obtained. Combined yield, 850 mg (92%).

A solution of silver triflate or a suspension of lithium triflate in the minimum of toluene was added dropwise with stirring to a mixture of the respective imidate, 4 Å molecular sieves and **4** in CH_2Cl_2 which had been stirred for 20 min. The reaction was allowed to proceed under conditions listed in Table 1, and the mixture of **14** and the α-anomer **15** formed was isolated by chromatography.

β-D-Glucopyranosyl *N*-(2,2-dimethoxyethyl)-5-hydroxy hexanamide (13). a. Conventional debenzoylation (Zemplén) of **12** (180 mg) gave amorphous, hygroscopic **13** (75 mg, 87%). ^{1}H NMR (D_2O): δ 4.51 (t, 2 H, J = 5.4 Hz, H-7''), 4.42, (d, 1 H, $J_{1,2}$ = 8.0 Hz, H-1), 3.93–3.85 (m, 2 H, H-6a,1''a), 3.71–3.60 (m, 2 H, H-6b,1''b), 3.48–3.31 (m, 11 H, H-3,4,5,6''a,b, incl s, 3.40, 2 OCH_3), 3.22 (dd, 1 H, $J_{2,3}$ = 9.1 Hz, H-2), 2.25 (t, 2 H, J = 7.3 Hz, H-5''), 1.66–1.54 (m, 4 H, H-2'',4''), 1.39–1.29 (m, 2 H, H-3''); ^{13}C NMR (D_2O): δ 102.7 (C-7''), 102.3 (C-1), 76.0, 76.0 (C-3,5), 73.3 (C-2), 70.4 (C-1''), 69.8 (C-4), 60.9 (C-6''), 54.6 (2 C, 2 OCH_3), 40.8 (C-6''), 35.6 (C-5''), 28.5 (C-2''), 25.2 (C-4''), 24.7 (C-3''); CIMS: *m/z* 382 ($[M + 1]^+$), 399 ($[M + 18]^+$).

b. Catalytic hydrogenolysis of **14** gave **13** in virtually theoretical yield.

Ethyl 3-*O*-Acetyl-4-(2,4-di-*O*-acetyl-3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-2-*O*-methyl-α- (19) and β-D-mannopyranose (20). A solution of methyl 3-*O*-acetyl-4,6-dideoxy-4-(2,4-di-*O*-acetyl-3-deoxy-L-*glycero*-tetronamido)-2-*O*-methyl-α-D-mannopyranoside[29] (7 g) in 50 : 20 : 0.5 Ac_2O : AcOH : H_2SO_4 (70.5 mL) was kept at room temperature for 1 h when TLC (1 : 1 hexane–acetone) showed that the reaction was complete. After addition of NaOAc trihydrate, to neutralize sulfuric acid, the mixture was partitioned between water and CH_2Cl_2, the organic phase was washed with aq. $NaHCO_3$, dried concentrated, and the residue was chromatographed to give **18** (6.2 g, 84%, α:β~20:1), whose NMR data agreed with those reported.[29]

Boron trifluoride etherate (1.9 g, 14 mmol) was added to a solution of the foregoing anomeric mixture of acetates (6.2 g, 13.8 mmol) and EtSH (1.3 g, 17.5 mmol) in CH_2Cl_2 (115 mL) and the mixture was stirred for 20 min, when TLC (3 : 2 hexane–acetone) showed that the reaction was complete. The mixture was neutralized with aqueous $NaHCO_3$ and washed successively with aqueous NaClO and $Na_2S_2O_5$ solutions, the organic phase was dried, concentrated, and the residue was chromatographed to give first the α-anomer **19** (5.1 g, 85%), mp 107–107.5 °C, $[\alpha]_D$ +122° (*c* 0.8, $CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 6.09 (d, 1 H, $J_{4,NH}$ = 9.3 Hz, NH), 5.37 (d, 1 H, $J_{1,2}$ = 1.4 Hz, H-1), 5.14 (dd, 1 H, $J_{2,3}$ = 3.1, $J_{3,4}$ = 11.0 Hz, H-3), 5.10 (dd, 1 H, $J_{2',3'a}$ = 3.8, $J_{2',3'b}$ = 7.1 Hz, H-2'), 4.34–4.24 (m, 1 H, H-4), 4.20–4.05 (m, 2 H, H-4'a,b), 4.04–3.95 (m, 1 H, H-5), 3.61 (dd, 1 H, H-2), 3.50 (s, 3 H, OCH_3), 2.74–2.55 (m, 2 H, CH_2CH_3), 2.18, 2.13, 2.06 (3 s, 3 H each, 3 $COCH_3$), 1.31 (t, J = 7.4 Hz, CH_3CH_2), 1.22 (d, 3 H, H-6); ^{13}C NMR ($CDCl_3$): δ 81.5 (C-1, $J_{C,H}$ = 167.3 Hz), 79.3 (C-2), 71.2 (C-3), 70.9 (C-2'), 68.7 (C-5), 59.9 (C-4'), 58.8 (OCH_3), 51.8 (C-4), 30.6 (C-3'), 25.3 (CH_2CH_3), 20.9, 20.7, 20.7 (3 $COCH_3$), 17.7 (C-6), 14.8 (CH_3CH_2); CIMS: *m/z* 450 ($[M + 1]^+$), 467 ($[M + 18]^+$). Anal. Calcd for $C_{19}H_{31}NO_9S$: C, 50.78; H, 6.90; N, 3.12; S, 7.13. Found: C, 50.79; H, 6.90; N, 3.13; S, 7.10.

Eluted next was the β-anomer **20** (0.5 g, 8%), mp 168.5–169 °C, $[\alpha]_D$ -48° (*c* 0.8, $CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 6.00 (d, 1 H, $J_{4,NH}$ = 9.1 Hz, NH), 5.07 (dd, partially overlapped, $J_{2,3'a}$ = 4.8, $J_{2,3'b}$ = 7.6 Hz, H-2'), 5.02 (dd, partially overlapped, $J_{2,3}$ = 2.9, $J_{3,4}$ = 10.9 Hz, H-3), 4.61 (d, 1 H, $J_{1,2}$ = ~1.0 Hz, H-1), 4.24–4.06 (m, 3 H, H-4,4'a,b), 3.67 (bdd, 1 H, H-2), 3.58 (OCH_3), 3.52–3.38 (m, 1 H, H-5), 2.82 (q, 2 H, J = 7.4 Hz, CH_2), 2.16, 2.14, 2.15 (3 s, 3 H each, 3 $COCH_3$), 1.29 (t, partially overlapped, CH_3), 1.27 (d, partially overlapped, $J_{5,6}$ = 6.4 Hz, C-6); ^{13}C NMR ($CDCl_3$): δ 83.96 (C-1, $J_{C-1,H-1}$ = 152.0 Hz), 79.8 (C-2), 76.2 (C-5), 74.0 (C-3), 71.0 (C-2'), 62.0 (OCH_3), 59.9 (C-4'), 51.8 (C-4), 30.6 (C-3'), 25.5 (CH_2CH_3), 20.8 (2 C), 20.7 (3 $COCH_3$), 18.1 (C-6), 15.0 (CH_2CH_3); CIMS: *m/z* 450 ($[M + 1]^+$), 467 ($[M + 18]^+$). Found: C, 50.87; H, 6.86; N, 3.10; S, 7.13.

3-*O*-Acetyl-4-(2,4-di-*O*-acetyl-3-deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-2-*O*-methyl-α-D-manno-pyranosyl *N*-(2,2-dimethoxyethyl)-5-hydroxy hexanamide (21). *N*-Iodosuccinimide (1.04 g, 4.6 mmol), followed by a solution of silver triflate (100 mg, 0.48 mmol) in a minimum amount of toluene, was added to a mixture of thioglycoside **19** (1.725 g, 3.84 mmol), **4** (1.01 g, 4.6 mmol) and 4Å molecular sieves (1 g) in CH_2Cl_2 (30 mL) which had been stirred at room temperature for 15 min. Reaction was almost instantaneous, as indicated by discoloration of the mixture, and TLC (1 : 1 toluene–acetone), showing disappearance of the glycosyl donor. The mixture was filtered, concentrated, and the residue was chromatographed, to give the desired, amorphous glycoside **21** (2.14 g, 88.6%), $[\alpha]_D$ 29° (*c* 1, $CHCl_3$). ^1H NMR ($CDCl_3$): δ 6.85 (d, 1 H, $J_{4,NH}$ = 9.6 Hz, NH'), 6.01 (bt, 1 H, J = ~5.7 Hz, NH''), 5.16 (dd, 1 H, $J_{2,3}$ = 3.1, $J_{3,4}$ = 11.0 Hz, H-3), 5.06 (dd, 1 H, $J_{2',3'a}$ = 4.3, $J_{2',3'b}$ = 8.4 Hz, H-2'), 4.81 (d, 1 H, $J_{1,2}$ = 1.8 Hz, H-1), 4.42 (t, 1 H, H-7''), 4.31–4.20 (m, 1 H, H-4), 4.18–4.08 (m, 2 H, H-4'a,b), 3.80–3.63 (m, 2 H, H-5,1''a), 3.51–3.49 (m, 4 H, H-2, incl s, OCH_3-2), 3.45–3.33 (m, 9 H, H-1''b,6''a,b, incl s 2 OCH_3-7''), 2.35–2.04 (m, 13 H, H-3'a,b5''a,b, incl 3 s, 3 $COCH_3$), 1.80–1.53 (m, 4 H, H-2''a,b, 4''a,b), 1.51–1.28 (m, 2 H, H-3''), 1.21 (d, 3 H, $J_{5,6}$ = 6.3 Hz, H-6); ^{13}C NMR ($CDCl_3$): δ 173.1, 171.4, 170.8, 169.9, 169.6 (5 CO), 102.4 (C-7''), 97.3 (C-1, $J_{C\text{-}1,H\text{-}1}$ = 167.8 Hz), 77.8 (C-2), 71.2 (C-3), 71.00 (C-2'), 67.8 (C-5), 66.6 (C-1''), 60.0 (C-4'), 59.4 (OCH_3-2), 54.2, 54.1 (2 OCH_3-7), 51.1 (C-4), 40.8 (C-6''), 36.3 (C-5''), 30.6 (C-3'), 28.6 (C-2''), 25.4 (C-3''), 24.7 (C-4''), 20.9, 20.7, 20.6 (3 $COCH_3$), 17.8 (C-6); CIMS: *m/z* 607 ($[M + 1]^+$), 624 ($[M + 18]^+$). *Anal.* Calc. for $C_{27}H_{46}N_2O_{13}$: C, 53.47; H, 7.59; N 4.62. Found: C, 53.41; H, 7.64; N, 4.53.

4-(3-Deoxy-L-*glycero*-tetronamido)-4,6-dideoxy-2-*O*-methyl-α-D-mannopyranosyl *N*-(2,2-dimethoxy-ethyl)-5-hydroxy-hexanamide (22). Conventional deacetylation (Zemplén) of **21** gave amorphous dimethyl acetal **22**, $[\alpha]_D$ +10.4° (*c* 0.8, $CHCl_3$). ^1H NMR ($CDCl_3$): δ 7.47 (d, 1 H, $J_{4,NH}$ = 8.0 Hz, NH-4), 6.39 (t, 1 H, $J_{6'',NH}$ = 5.8 Hz, N''H); ^1H NMR (D_2O): δ 4.96 (d, 1 H, $J_{1,2}$ = 1.5 Hz, H-1), 4.51 (t, 1 H, J = 5.4 Hz, H-7''), 4.26 (dd, 1 H, $J_{2',3'a}$ = 3.9, $J_{2',3'b}$ = 8.7 Hz, H-2'), 3.94 (dd, 1 H, $J_{2,3}$ = 3.3, $J_{3,4}$ = 10.2 Hz, H-3), 3.86–3.64 (m, 5 H, H-4,5,4'a,b,1''a), 3.56–3.44 (m, 5 H, H-2,1''b, incl s at 3.46 for OCH_3-2), 3.40 (s, 6 H, 2 OCH_3-7), 3.32 (d, 2 H, $J_{6'',7''}$ = 5.8 Hz, H-6''a,b), 2.25 (t, 2 H, J = 7.3 Hz, H-5''a,b), 2.07–1.96, 1.88–1.76 (2 m, 1 H each, H-3'a,b), 1.65–1.54 (m, 4 H, H-2''a,b,4''a,b), 1.40–1.29 (m, 2 H, H-3''a,b), 1.14 (d, 3 H, $J_{5,6}$ = 5.8 Hz, H-6); ^{13}C NMR (D_2O): δ 102.6 (C-7''), 96.7 (C-1), 79.4 (C-2), 69.1 (C-2'), 68.0 (C-1''), 67.9 (C-3), 67.3 (C-5), 58.9 (OCH_3-2), 57.3 (C-4'), 54.6 (2 C, 2 OCH_3-7''), 53.5 (C-4), 40.8 (C-6''), 36.1 (C-3'), 35.6 (C-5''), 28.3 (C-2''), 25.2 (C-3''), 24.9 (C-4''), 16.9 (C-6); CIMS: *m/z* 481 ($[M + 1]^+$), 498 ($[M + 18]^+$). *Anal.* Calc. for $C_{21}H_{40}N_2O_{10}$: C, 52.50; H, 8.33; N 5.83. Found: C, 52.24; H, 8.28; N, 5.72.

General procedure for reductive amination. Dimethyl acetals **15** or **22** were converted to the corresponding aldehydes **16** or **23**, respectively, by heating their ~0.5% solution in 0.05 mM trifluoroacetic acid at 100 °C for 10 min. Complete conversions were evidenced by disappearance in the ^1H NMR spectra of a singlet and a triplet, respectively at δ ~3.38 (2 OCH_3-7'') and 4.50 (H-7''), and appearance of a singlet and a triplet, respectively at δ ~9.41 (CHO) and 4.85 (hydrated form of the aldehyde). After concentration, to remove TFA, a requisite amount of CSA, followed by borane-pyridine complex (10 equiv.) was added to a solution of the residue in 0.1 M phosphate buffer (pH 7.0), and the mixture was stirred at room temperature for 40 h. After dialysis (5 exchanges, 2 L each, of deionized water during 2 days), or removal of the low-molecular mass material by centrifugation using 10K Centricon (Amicon, Inc.), and freeze-drying, the white, solid product obtained was analyzed by MALDI-TOF mass spectrometry, *c.f.* Tables 2-5.

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