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Structural analysis of sialic acid-containing carbohydrates by the reductive-cleavage method

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ABSTRACT

The applicability of the reductive-cleavage method to the structural analysis of sialic acid-containing carbohydrates was investigated using fully methylated methyl α- and β-*N*-acetylneuraminic acid (**1** and **2**, respectively). Both compounds were fully stable to reductive cleavage in the presence of borane dimethylsulfide and boron trifluoride etherate but were slowly degraded in the presence of triethylsilane and promoters such as trimethylsilyl trifluoromethanesulfonate, boron trifluoride etherate, or a mixture of trimethylsilyl methanesulfonate and boron trifluoride etherate. However, after selective reduction of the ester groups of **1** and **2** with sodium borohydride, the products (**3** and **4**, respectively) underwent rapid reductive cleavage to yield the expected anhydroalditols, which were characterized as their acetates (**8** and **11**) by GLC–MS. The major product was also characterized as its benzoate (**9**) by ^{1}H NMR spectroscopy. The usefulness of this analytical method was demonstrated using 3′-sialyllactose, i.e., α-Neu5Ac-(2 → 3)-β-D-Gal*p*-(1 → 4)-D-Glc. The methylated and ester-reduced trisaccharide (**13**) was reductively cleaved with $Me_2S \cdot BH_3$–$BF_3 \cdot Et_2O$ and the products were acetylated. Analysis by GLC–MS revealed the products expected from terminal (nonreducing) Neu5Ac (**8** and **11**), 3-linked Gal*p* (**16**), and 4-linked Glc*p* (**15**) residues in relative molar ratios of 1.2:1.0:1.0, respectively. However, direct reductive cleavage of the fully methylated trisaccharide (**12**) yielded the fully methylated disaccharide-anhydroalditol derivative α-Neu5Ac-(2 → 3)-1,5An-D-Gal (**14**) and the product (**15**) derived from 4-linked Glc*p* residues. These experiments therefore established both the composition and the sequence of the trisaccharide.

INTRODUCTION

The reductive-cleavage method[1] is being developed in an attempt to simplify the chemical methods used for the structural characterization of complex carbohydrates and to address the shortcomings of currently used chemical procedures. The method has already been shown to be applicable to the simultaneous analysis of positions of linkage and ring forms in glycans containing a wide variety of

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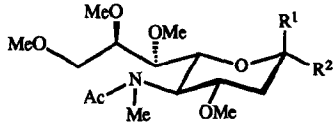
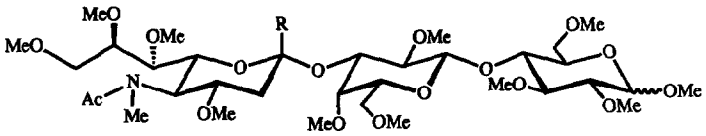
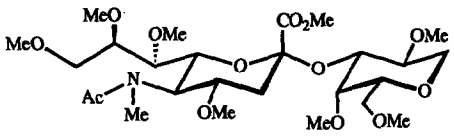
monosaccharide residues. Since the structural analysis of sialic acid-containing carbohydrates is an important problem in glycobiology, the present study was undertaken to establish whether the method was applicable to their analysis as well. Described herein are the results of a study using the fully methylated derivatives of α- and β-*N*-acetylneuraminic acid (**1** and **2**, respectively) and their ester-reduced derivatives (**3** and **4**, respectively) and fully methylated 3′-sialyllactose (**12**) and its ester-reduced derivative (**13**).



	R^1	R^2
1	CO_2Me	OMe
2	OMe	CO_2Me
3	CH_2OH	OMe
4	OMe	CH_2OH
5	OMe	CD_2OH
6	H	CO_2Me
7	CO_2Me	H
8	H	CH_2OAc
9	H	CH_2OBz
10	H	CD_2OBz
11	CH_2OAc	H



12 R=CO_2Me
13 R=CH_2OH



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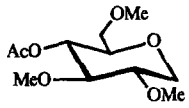

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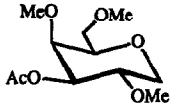

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RESULTS

*Reductive cleavage of methyl [methyl 5-(acetylmethylamino)-3,5-dideoxy-4,7,8,9-tetra-O-methyl-*D*-glycero-α-*D*-galacto-2-nonulopyranosid]onate* (**1**) *and methyl [methyl 5-(acetylmethylamino)-3,5-dideoxy-4,7,8,9-tetra-O-methyl-*D*-glycero-β-*D*-galacto-nonulopyranosid]onate* (**2**).—Compounds **1** and **2** were prepared by methylation[2] of the corresponding methyl glycosides[3,4]. Reductive cleavage of **1** and **2** was carried out under the usual conditions with triethylsilane (Et_3SiH) as the reducing agent and either trimethylsilyl trifluoromethanesulfonate ($Me_3SiOSO_2CF_3$)[5], boron trifluoride etherate ($BF_3 \cdot Et_2O$)[1], or a mixture[6] of trimethylsilyl methanesulfonate (Me_3SiOSO_2Me) and $BF_3 \cdot Et_2O$ as the promoter, and also with borane dimethylsulfide ($Me_2S \cdot BH_3$) as the reducing agent and $BF_3 \cdot Et_2O$ as the promoter (I.H. Oh and G.R. Gray, unpublished work). The reactions were monitored by GLC and GLC combined with chemical-ionization mass spectrometry (CIMS) using ammonia as the reagent gas. Starting materials and products were identified by characteristic molecular ions at M + 1 $(M + H)^+$ and M + 18 $(M + NH_4)^+$. The linear temperature programmed gas–liquid chromatography retention indices (LTGC-RI)[7,8] of starting materials and products were also determined as a further aid to characterization.

The reductive cleavage of **1** was conducted using a mixture comprised of 84% of **1** (LTGCRI 2305.6) and 16% of the corresponding β anomer (**2**; LTGCRI 2369.9) (see Table I). Both compounds were found to be fully stable to reductive cleavage for 24 h in the presence of $Me_2S \cdot BH_3$ and $BF_3 \cdot Et_2O$ (Table I). Compound **1** was unstable, however, when reductive cleavage was carried out in the presence of Et_3SiH as the reducing agent and $Me_3SiOSO_2CF_3$, $BF_3 \cdot Et_2O$ or Me_3SiOSO_2Me–$BF_3 \cdot Et_2O$ as the promoter (Table I). Small amounts (9–38%) of the expected product (either **6** or **7**; LTGCRI 2306.7) were observed in the latter reactions, but several unidentified products were also formed.

The reductive cleavage of **2** was conducted using a mixture consisting of 91% of **2** and 9% of **1** (see Table I). Again, both compounds were fully stable to reductive cleavage for 24 h in the presence of $Me_2S \cdot BH_3$ and $BF_3 \cdot Et_2O$. Compound **2** was also fairly stable to reductive cleavage with Et_3SiH and $BF_3 \cdot Et_2O$, but substantial degradation (41%) occurred when **2** was exposed to reductive cleavage with Et_3SiH and $Me_3SiOSO_2CF_3$ (Table I). In the latter reaction, the two major degradation products, which were formed in yields of 30% (LTGCRI 2289.0) and 5% (LTGCRI 2260.2) were both found by GLC–CIMS to have M_r 343, which corresponds to the elimination of two molecules of MeOH from the starting glycoside (**2**). Interestingly, reductive cleavage in the presence of Et_3SiH and Me_3SiOSO_2Me–$BF_3 \cdot Et_2O$ gave substantial amounts of two products (LTGCRI 2306.7, 33%; LTGCRI 2324.8, 14%) having the molecular weight (377) of the expected products (**6** and **7**).

Reductive cleavage of the ester-reduced derivatives (**3** *and* **4**) *of neuraminic acid derivatives* **1** *and* **2**.—Suspecting that the electron-withdrawing C-1 methoxycar-

TABLE I

Mole fractions of products derived by reductive cleavage of fully methylated methyl α- and β-*N*-acetyl neuraminic acid (**1** and **2**, respectively)

Compound	Reducing agent, promoter	Reaction time (h)	Mole fraction		
			1	**2**	**6** or **7**
1	None		0.84	0.16	
1	$Me_2S \cdot BH_3$ $BF_3 \cdot Et_2O$	24	0.84	0.16	
1	Et_3SiH $Me_3SiOSO_2CF_3$	20		0.17	0.09
1	Et_3SiH $BF_3 \cdot Et_2O$	22		0.16	0.38
1	Et_3SiH Me_3SiOSO_2Me $-BF_3 \cdot Et_2O$	2		0.04	0.26
2	None		0.09	0.91	
2	$Me_2S \cdot BH_3$ $BF_3 \cdot Et_2O$	24	0.09	0.91	
2	Et_3SiH $Me_3SiOSO_2CF_3$	24		0.59	
2	Et_3SiH $BF_3 \cdot Et_2O$	24	0.09	0.87	
2	Et_3SiH Me_3SiOSO_2Me $-BF_3 \cdot Et_2O$	2		0.47	0.47

bonyl group of **1** and **2** was preventing reductive cleavage of the glycoside, that group was selectively reduced to the primary alcohol by treatment of **1** and **2** with $NaBH_4$ in MeOH, and the products (**3** and **4**, respectively) were again subjected to reductive cleavage. The results are summarized in Table II. As expected, both the α-alcohol (**3**; LTGCRI of acetate 2396.9) and β-alcohol (**4**; LTGCRI of acetate 2437.2) were readily cleaved under all reductive-cleavage conditions, as determined by GLC and GLC–CIMS (NH_3) of *O*-acetyl derivatives. Treatment of **3** (a 91:9 mixture of **3** and **4**, respectively, was used) with $Me_2S \cdot BH_3$ and $BF_3 \cdot Et_2O$ for 1 h yielded anhydroalditols **8** (LTGCRI 2395.3) and **11** (LTGCRI 2380.1) in yields of 77 and 17%, respectively, and an additional product (LTGCRI 2300.00) in 6% yield. The latter product was found by CIMS to have M_r 359, corresponding to the loss of MeOH from one of the expected products (**8** or **11**; M_r 391). Treatment of **3** with Et_3SiH and $Me_3SiOSO_2CF_3$ for 15 min also gave compounds **8** and **11**, but in yields of only 32 and 20%, respectively. Two other products in yields of 39% (LTGCRI 2300.0) and 9% (LTGCRI 2316.8) were formed in this reaction and both were found to have M_r 359, corresponding to the elimination of MeOH from the expected product. The product formed in 39% yield had the same retention index (2300.0) and mass spectrum as the minor product (6% yield) derived from the reduction of **3** with $Me_2S \cdot BH_3$ and $BF_3 \cdot Et_2O$. Similar results were obtained when **3** was exposed to Et_3SiH and Me_3SiOSO_2Me–$BF_3 \cdot Et_2O$ for 15 min; i.e., compounds **8** and **11** were formed in yields of 27 and 31%, respectively, and the

TABLE II

Mole fractions of products derived by reductive cleavage of the ester-reduced derivatives (**3** and **4**) of fully methylated methyl α- and β-*N*-acetyl neuraminic acid (**1** and **2**, respectively).

Compound	Reducing agent, promoter	Reaction time (h)	Mole fraction			
			3 [a]	**4** [a]	**8**	**11**
3	None		0.91	0.09		
3	$Me_2S \cdot BH_3$ $BF_3 \cdot Et_2O$	1			0.77	0.17
3	Et_3SiH $Me_3SiOSO_2CF_3$	0.25			0.32	0.20
3	Et_3SiH $BF_3 \cdot Et_2O$	0.25		0.21	0.42	0.28
3	Et_3SiH Me_3SiOSO_2Me $-BF_3 \cdot Et_2O$	0.25			0.27	0.31
4	None		0.08	0.92		
4	$Me_2S \cdot BH_3$ $BF_3 \cdot Et_2O$	1 h [b]			0.85	0.15
4	Et_3SiH $Me_3SiOSO_2CF_3$	0.5			0.07	0.07
4	Et_3SiH $BF_3 \cdot Et_2O$	0.5			0.12	0.11
4	Et_3SiH Me_3SiOSO_2Me $-BF_3 \cdot Et_2O$	0.5			0.31	0.29

[a] Compounds **3** and **4** were analyzed as their acetates by GLC and GLC–CIMS. [b] Identical results were obtained after 24 h.

two products possessing a molecular weight of 359 were formed in yields of 36% (LTGCRI 2300.0) and 6% (LTGCRI 2316.8). Reductive cleavage of **3** for 15 min in the presence of Et_3SiH and $BF_3 \cdot Et_2O$ also gave **8** and **11** as major products (42 and 28%, respectively) and a single product (LTGCRI 2300.0) with M_r 359 in 8% yield; the remainder of the product was the unreacted β anomer (**4**), identified as its acetate.

Similar results were obtained in reductive-cleavage experiments with the β anomer of the alcohol (**4**) (a 92:8 mixture of **4** and **3**, respectively, was used). Treatment of **4** with $Me_2S \cdot BH_3$ and $BF_3 \cdot Et_2O$ for 1 h gave **8** and **11** in yields of 85 and 15%, respectively, and no other products were detected. In contrast, however, silane reductions employing $Me_3SiOSO_2CF_3$ or $BF_3 \cdot Et_2O$ as the promoter gave low yields of the expected products (**8** and **11**) and many other products were observed. Silane reductions employing Me_3SiOSO_2Me–$BF_3 \cdot Et_2O$ as the promoter gave better results; i.e., the expected products **8** and **11** were formed in yields of 31 and 29%, respectively, and the two products arising as a result of elimination of MeOH from the expected products were formed in yields of 35% (LTCGRI 2300.0) and 5% (LTGCRI 2316.8).

The structure of the major product (**8**) formed in these reactions was confirmed by isolation and characterization of its corresponding benzoate (**9**) as well as the

benzoate of the 1,1-dideuterio derivative (**10**). Compound **9** was prepared by subjecting the β anomer of the alcohol (**4**) to reductive cleavage for 1 h in the presence of 5 equiv each of $Me_2S \cdot BH_3$ and $BF_3 \cdot Et_2O$, followed by benzoylation in situ. The major product (**9**) was subsequently isolated by reversed-phase HPLC using a semi-preparative C_{18} column. The ^1H NMR spectrum of **9** clearly revealed an apparent quartet (J 12.0 Hz) at δ 1.42, attributable to H-3*ax*. The multiciplicity of this resonance arises because $J_{3eq,3ax}$ and the two *trans*-diaxial couplings ($J_{2,3ax}$ and $J_{3ax,4}$) are approximately equal in magnitude, as can only occur in the compound (**9**) having an equatorial $-CH_2OBz$ group at C-2. The spectrum of **9** also displayed a resonance for H-3e*q* at δ 2.31 (multiplet), and its assignment as H-3*eq* was confirmed by selective decoupling of H-3*ax*. The spectrum of **9** also clearly revealed two doublets of doublets at δ 4.35 (J 7.5, 12.0 Hz) and δ 4.41 (J 3.0, 12.0 Hz) attributable to the diastereotopic hydrogens at C-1. In an attempt to identify and assign the H-2 resonance of **9**, the corresponding 1,1-dideuterio derivative (**10**) was also prepared (via reductive cleavage of **5**). The spectrum of **10** was virtually identical to that of **9**, except for the absence of the C-1 hydrogen resonances, but the ring proton region was still too complex to permit assignment of H-2. However, analysis of the spectrum of **10** confirmed the aforementioned assignments of H-3*ax* and H-3*eq*.

Reductive cleavage of permethylated 3'-sialyllactose (**12**) *and its ester-reduced derivative* (**13**).—The foregoing experiments suggested that it should be possible to sequence sialic acid-containing carbohydrates and determine their glycosyl linkages by reductive cleavage of their fully methylated and ester-reduced forms, respectively. As a test of this assumption, fully methylated 3'-sialyllactose (**12**) and its ester-reduced derivative (**13**) were subjected to reductive cleavage for 24 h with $Me_2S \cdot BH_3$ in the presence of $BF_3 \cdot Et_2O$ and the products were acetylated. Analysis by GLC and GLC–CIMS revealed the presence of the fully methylated disaccharide-anhydroalditol α-Neu5Ac(2 → 3)-1,5An-D-Gal (**14**; $[M + H]^+ = 582$; $[M + NH_4]^+ = 599$) and the product (**15**) derived from the 4-linked D-glucopyranosyl residue in a relative molar ratio of 1.3 : 1.0, respectively. After selective reduction of the methyl ester of **12**, the reduced trisaccharide (**13**) was subjected to reductive cleavage under the same conditions. Analysis of the acetylated products by GLC and GLC–CIMS revealed the presence of products derived from terminal (nonreducing) Neu5Ac residues (**8** and **11**), 3-linked D-galactopyranosyl residues (**16**), and 4-linked D-glucopyranosyl residues (**15**) in relative molar ratios of 1.2 : 1.0 : 1.0, respectively. These experiments therefore established both the composition and the sequence of the trisaccharide.

DISCUSSION

The present study was undertaken in order to develop satisfactory procedures for the analysis of sialic acid-containing saccharides by the reductive-cleavage method. The α- and β-methyl glycosides of Neu5Ac were chosen as models for

this study because in past work methyl glycosides have been found to give results in good agreement with those ultimately obtained with the corresponding glycosidically linked sugars. The fully methylated Neu5Ac derivatives (**1** and **2**) were therefore subjected to reductive cleavage with the reducing agents and promoters commonly employed, and the products so obtained were identified and quantified (Table I).

Neither **1** nor **2** was very stable to reductive cleavage in the presence of triethylsilane as the reducing agent. Although the expected products (**6** and/or **7**) were observed in some of these reactions, other, uncharacterized products were also formed. Two of the major side-products, although not structurally characterized, were found by GLC–CIMS analysis to have molecular weights corresponding to the loss of 2 moles of MeOH from the starting materials. These results differ from those in a previous report[9] in that much lower yields of reductive cleavage products were observed in the present study. The former study[9], however, used a different promoter ($BF_3 \cdot Et_2O$ in trifluoroacetic acid[1]). Two artifacts were also observed in the former study, but they were not characterized.

Given the complexity of the product mixtures arising by direct reductive cleavage of the fully methylated Neu5Ac derivatives (**1** and **2**), other conditions for the analysis were sought. Treatment of **1** and **2** with our newly developed reductive-cleavage reagents, i.e., $Me_2S \cdot BH_3$ and $BF_3 \cdot Et_2O$ (I.H. Oh and G.R. Gray, unpublished work), failed to give any reductive-cleavage products but, indeed, also failed to give any degradation products. These results, together with the fact that the presence of the methoxycarbonyl groups of **1** and **2** would be expected[10] to hinder or prevent reductive cleavage of the glycoside, suggested an alternative strategy. The ester groups of **1** and **2** were therefore selectively reduced, and the products (**3** and **4**, respectively) so obtained were again subjected to reductive cleavage under various conditions (Table II). As expected, reductive cleavage of the glycoside now proceeded readily under all conditions (to give **8** and **11**). Although substantial amounts of degradation products were observed in silane reductions, reductions in the presence of $Me_2S \cdot BH_3$ and $BF_3 \cdot Et_2O$ gave the expected products (**8** and **11**) in either quantitative yield (for **4**) or nearly so (for **3**).

Finally, this newly-developed strategy for analysis of Neu5Ac residues was shown to be applicable to a sialic acid-containing trisaccharide, i.e., 3′-sialyllactose. Reductive cleavage of the fully methylated trisaccharide (**12**) gave disaccharide-anhydroalditol **14** and the product (**15**) arising from the reducing terminal D-glucopyranosyl residue, as expected, as a consequence of the stability of the α-Neu5Ac-(2 → 3)-D-Gal glycosidic linkage. In contrast, the ester-reduced trisaccharide derivative (**13**) was reductively-cleaved completely under the same conditions and only the products arising from terminal (nonreducing) Neu5Ac residues, 3-linked D-galactopyranosyl residues, and 4-linked D-glucopyranosyl residues were detected.

The strategy developed herein therefore provides a means to quantitatively analyze Neu5Ac residues and to establish the identity of the sugar to which they are glycosidically linked.

EXPERIMENTAL

General methods—^{1}H NMR spectra were recorded on a Varian VXR-500S NMR spectrometer in $CDCl_3$ as the solvent and were referenced to internal Me_4Si. HPLC was performed using a Beckman model 338 System Gold chromatograph. Chromatography was performed on a 5-μm particle size octadecylsilica gel column (0.46 × 25 cm; Kromasil KR100-5C18, Eka Nobel, Bohus, Sweden) at a flow rate of 1.5 mL/min. The column was eluted for 15 min with 30% MeCN in H_2O, followed by a linear gradient over 10 min to 95% MeCN in H_2O. Analytical GLC was performed with a Hewlett–Packard Model 5890A gas–liquid chromatograph equipped with a flame-ionization detector, a Hewlett–Packard Model 3392A integrator, and a J&W Scientific DB-5 fused-silica capillary column (0.25 mm × 30 m, 0.25-μm film thickness). The temperature of the column was programmed from 80°C to 300°C at 6°/min. LTGCRI values were determined on the same column as described by Elvebak et al.[7].

GLC–MS analyses were performed using a Finnegan MAT 95 high-resolution, double-focusing, reverse geometry mass spectrometer equipped with a Hewlett–Packard 5890A Series II gas–liquid chromatograph and a Digital Equipment Corporation model 2100 workstation. CI mass spectra were acquired with NH_3 as the reagent gas at a source temperature of 180°C, and NH_3 was introduced at a pressure of 4×10^{-4} torr as indicated on the source ionization gauge. For CI spectra, the instrument was scanned from m/z 60–650 at 1 s/decade. Spectra were acquired at a resolution of 1000 (10% valley definition). Chromatography was conducted on a DB-5 column under the previously indicated conditions. Fast-atom bombardment mass spectrometry (FABMS) was performed using a VG Analytical LTD Model 7070E-HF high-resolution, double-focusing mass spectrometer.

N-Acetylneuraminic acid (Neu5Ac) was obtained from Sigma Chemical Co., and 3′-sialyllactose was obtained from BioCarb Chemicals. Triethylsilane, trimethylsilyl trifluoromethanesulfonate, boron trifluoride etherate, trimethylsilyl methanesulfonate, borane dimethylsulfide (10% in CH_2Cl_2), methyl iodide, sodium borohydride, and sodium borodeuteride were obtained from Aldrich Chemical Co. Dowex-AG501X-8 (D) mixed bed ion-exchange resin was obtained from Bio-Rad laboratories. Samples for reductive cleavage were dried overnight under high vacuum over P_2O_5. Solution transfers requiring anhydrous conditions were performed under N_2 using standard syringe techniques. Unless otherwise stated, all reactions were performed at ambient temperature.

Reductive cleavages were performed in Wheaton V-vials equipped with Teflon-lined screw caps. The samples (100 μg–5 mg) to be analyzed and a small stirring bar were placed in the vial and sufficient dry CH_2Cl_2 was added to give a 0.1 M solution. Reductive cleavages were performed using 5 equiv of Et_3SiH in the presence of 5 equiv of $Me_3SiOSO_2CF_3$[5], 5 equiv of $BF_3 \cdot Et_2O$[1], or a mixture[6] of 5 equiv of Me_3SiOSO_2Me and 1 equiv of $BF_3 \cdot Et_2O$, as well as using 5 equiv of $Me_2S \cdot BH_3$ in the presence of 5 equiv of $BF_3 \cdot Et_2O$ (I.H. Oh and G.R. Gray,

unpublished work). Acetylations and benzoylations were performed as previously described[7].

Methyl [methyl 5-(acetylmethylamino)-3,5-dideoxy-4,7,8,9-tetra-O-*methyl*-D-glycero-*α*-D-galacto-*2-nonulopyranosid]onate* (**1**).—Methylation was carried out by a modification[2] of the Hakomori procedure[11]. Methyl 5-*N*-acetyl-*α*-D-neuraminate, sodium salt[3] (9.0 mg, 0.027 mmol) was dissolved in dry Me_2SO (1 mL) under N_2 and stirred for 30 min. Potassium dimsylate (0.5 mL, 3 M) was added and stirring was continued for 2 h, then the reaction was cooled in an ice bath and MeI (0.2 mL) was added. The reaction was allowed to warm to room temperature and after 2 h was diluted with water (2 mL) and extracted (3 ×) with 50-mL portions of $CHCl_3$. The organic layer was extracted (3 ×) with 20-mL portions of water, then dried over anhyd Na_2SO_4. Evaporation of the solvent left a syrup that was chromatographed on a column (1.5 × 25 cm) of silica gel. Elution with a linear gradient of 1 to 15% MeOH in CH_2Cl_2 provided **1** (7.2 mg, 68%) as a clear syrup. ^1H NMR: δ 1.69 (t, 1H, *J* 12.5 Hz, H-3*ax*), 2.22 (s, 3H, NAc), 2.75 (s, 3H, NMe), 2.83 (dd, 1 H, *J* 4.5, 12.5 Hz, H-3*eq*), 3.33, 3.38, 3.39, 3.44, 3.47 (5 s, 15 H, 5 MeO), 3.86 (s, 3H, CO_2Me), and 3.26–4.12 (complex, 7H, H-4,5,6,7,8,9,9′). CI (NH_3)-mass spectrum: *m/z* 408 [100%, $(M + H)^+$] and 425 [1%, $(M + NH_4)^+$]. LTGCRI (DB-5): 2305.6.

Methyl [methyl 5-(acetylmethylamino)-3,5-dideoxy-4,7,8,9-tetra-O-*methyl*-D-glycero-*β*-D-galacto-*2-nonulopyranosid]onate* (**2**). Methyl 5-*N*-acetyl-*β*-D-neuraminate, sodium salt[4] (61.5 mg, 0.178 mmol) was converted to **2** as described for **1** to provide **2** (58.5 mg, 81%) as a clear syrup. ^1H NMR: δ 1.62 (dd, 1 H, *J* 11.0, 13,5 Hz, H-3*ax*), 2.22 (s, 3 H, NAc), 2.64 (dd, 1 H, *J* 5.0, 13.5 Hz, H-3*eq*), 2.85 (s, 3 H, NMe), 3.27, 3.31, 3.38, 3.39, 3.46 (5 s, 15 H, 5 MeO), 3.81 (s, 3H, CO_2Me), and 3.20–4.10 (complex, 7 H, H-4,5,6,7,8,9,9′). CI (NH_3)-mass spectrum: *m/z* 408 [100% $(M + H)^+$], 425 [2%, $(M + NH_4)^+$]. LTGCRI (DB-5): 2369.9.

Methyl 5-(acetylmethylamino)-3,5-dideoxy-4,7,8,9-tetra-O-*methyl*-D-glycero-*α*-D-galacto-*2-nonulopyranoside* (**3**).—Compound **1** (7 mg) was dissolved in MeOH (2 mL) and added at 0°C to a solution of $NaBH_4$ (26 mg) in 2 mL of MeOH[12]. The reaction was stirred at ambient temperature for 3 h, then diluted with water and neutralized at −20°C with Dowex-50 (H^+) ion-exchange resin. After removal of the resin, the solvent was evaporated under vacuum and the residue was dissolved in MeOH and evaporated (3 ×) to remove methyl borate. The residue was chromatographed on a column (1.5 × 25 cm) of Silica Gel-60 (230–400 mesh; EM Science) to yield pure **3** (6 mg, 92%) upon elution with a linear gradient of 2–20% MeOH in CH_2Cl_2. ^1H NMR: δ 1.74 (dd, 1 H, *J* 10.0, 13.5 Hz, H-3*ax*), 2.24 (s, 3 H, NAc), 2.32 (dd, *J* 6.5, 13.5 Hz, H-3*eq*), 2.44 (m, 1 H, OH), 2.80 (s, 3 H, NMe), 3.33, 3.39, 3.42 (3 s, 9 H, 3 MeO), 3.41 (s, 6 H, 2 MeO), 3.36–4.3 (complex 9 H, H-1,1′4,5,6,7,8,9,9′). For acetate; CI (NH_3)-mass spectrum: *m/z* 358 [100%, $(M - 2MeOH + H)^+$], 390 [77%, $(M - MeOH + H)^+$], and 422 [44%, $(M + H)^+$]. LTGCRI (DB-5): 2396.9.

Methyl 5-(acetylmethylamino)-3,5-dideoxy-4,7,8,9-tetra-O-*methyl*-D-glycero-*β*-D-

galacto-*2-nonulopyranoside* (**4**).—Compound **2** (65 mg) was converted to **4** (55 mg, 92%) as described above for the conversion of **1** to **3**. For **4**; ^{1}H NMR: δ 1.53 (dd, 1 H, *J* 11.0, 13.5 Hz, H-3*ax*), 2.22 (s, 3 H, NAc), 2.48 (dd, 1 H, *J* 5.0, 13.5 Hz, H-3*eq*), 2.84 (s, 3 H, NMe), 3.33, 3.38, 3.44 (3 s, 9 H, 3 MeO), 3.39 (s, 6 H, 2 MeO), 3.26–4.08 (complex, 9 H, H-1,1′,4,5,6,7,8,9,9′). For acetate; CI (NH_3)-mass spectrum: *m/z* 358 [45%, $(M - 2\,MeOH + H)^+$], 390 [64%, $(M - MeOH + H)^+$], 422 [100%, $(M + H)^+$], and 439 [0.2%, $(M + NH_4)^+$]. LTGCRI (DB-5): 2437.2.

Methyl 5-(acetylmethylamino)-3,5-dideoxy-1,1-dideuterio-4,7,8,9-tetra-O-*methyl*-D-glycero-β-D-galacto-*2-nonulopyranoside* (**5**).—Compound **2** (15 mg) was converted to **5** (12 mg, 86%) as described above for the conversion of **1** to **3**, except that sodium borodeuteride was used. For **5**; ^{1}H NMR: δ 1.53 (dd, 1 H, *J* 11.0, 13.5 Hz, H-3*ax*), 2.22 (s, 3 H, NAc), 2.48 (dd, 1 H, *J* 5.0, 13.5 Hz, H-3*eq*), 2.84 (s, 3 H, NMe), 3.33, 3.38, 3.44 (3 s, 9 H, 3 MeO), 3.39 (s, 6 H, 2 MeO), 3.2–4.1 (complex, 7 H, H-4,5,6,7,8,9,9′). For acetate; CI(NH_3)-mass spectrum: *m/z* 360 [62%, $(M - 2MeOH + H)^+$], 392 [86%, $(M - MeOH + H)^+$] and 424 (100%, $(M + H)^+$]. LTGCRI(DB-5): 2436.7.

1-O-*Acetyl-5-(acetylmethylamino)-2,6-anhydro-3,5-dideoxy-4,7,8,9-tetra*-O-*methyl*-D-erythro-L-gluco-*nonitol* (**8**).—Compound **8** was identified by GLC and GLC–MS as the major product when both **3** and **4** were subjected to reductive cleavage ($Me_2S \cdot BH_3$, $BF_3 \cdot Et_2O$) followed by acetylation. For **8**: CI (NH_3)-mass spectrum: *m/z* 392 [100%, $(M + H)^+$] and 409 [0.5%, $(M + NH_4)^+$]. LTGCRI (DB-5): 2395.3.

5-(Acetylmethylamino)-2,6-anhydro-1-O-*benzoyl-3,5-dideoxy-4,7,8,9-tetra*-O-*methyl*-D-erythro-L-gluco-*nonitol* (**9**).—Compound **4** (5 mg) was subjected to reductive cleavage for 1 h with 5 equiv each of $Me_2S \cdot BH_3$ and $BF_3 \cdot Et_2O$, then the reaction was quenched by the addition of water (5 equiv). After stirring for 30 min, benzoic anhydride (300 mg) and *N*-methylimidazole (100 μL) were added and stirring was continued for 30 min. The reaction was processed in the usual way[7], and the major benzoate product (**9**) was isolated by reversed-phase HPLC. Compound **9** was found to elute at 16–17 min. ^{1}H NMR: δ 1.42 (q, 1 H, *J* 12.0 Hz, H-3*ax*), 2.21 (s, 3 H, NAc), 2.34 (ddd, 1 H, *J* 2.0, 5.0, 12.5 Hz, H-3*eq*), 2.78 (s, 3 H, NMe), 3.24, 3.32, 3.33, 3.38 (4 s, 12 H, 4 MeO), 3.2–3.9 (complex, 8 H, H-2,4,5,6,7,8,9,9′), 4.35 (dd, 1 H, *J* 7.5, 12.0 Hz, H-1), 4.41 (dd, 1 H, *J* 3.0, 12.0 Hz, H-1′), 7.43 (t, 2 H, *J* 7.5 Hz, *m*-Ar), 7.56 (t, 1 H, *J* 7.5 Hz, *p*-Ar), and 8.05 (d, 2 H, *J* 7.5 Hz, *o*-Ar). CI(NH_3)-mass spectrum: *m/z* 454 [100%, $(M + H)^+$].

5-(Acetylmethylamino)-2,6-anhydro-1-O-*benzoyl-3,5-dideoxy-1,1-dideuterio-4,7,8,9-tetra*-O-*methyl*-D-erythro-L-gluco-*nonitol* (**10**).—Compound **10** was prepared from **5** as described for the conversion of **4** to **9**. For **10**; ^{1}H NMR δ 1.44 (q, 1 H, *J* 12.0 Hz, H-3*ax*), 2.24 (s, 3 H, NAc), 2.34 (ddd, 1 H, *J* 2.0, 5.0, 12.5 Hz, H-3*eq*), 2.82 (s, 3 H, NMe), 3.27, 3.34, 3.35, 3.41 (4 s, 12 H, 4 MeO), 3.2–3.9 (complex, 8 H, H-2,4,5,6,7,8,9,9′), 7.46 (t, 2 H, *J* 7.5 Hz, *m*-Ar), 7.59 (t, 1 H, *J* 7.5 Hz, *p*-Ar), and 8.08 (d, 2 H, *J* 7.5 Hz, *o*-Ar).

1-O-*Acetyl-5-(acetylmethylamino)-2,6-anhydro-3,5-dideoxy-4,7,8,9-tetra*-O-

methyl-D-erythro-L-manno-*nonitol* (**11**).—Compounds **11** was identified by GLC and GLC–MS as the minor product when both **3** and **4** were subjected to reductive cleavage ($Me_2S \cdot BH_3$, $BF_3 \cdot Et_2O$), followed by acetylation. For **11**; CI (NH_3)-mass spectrum: *m/z* 392 [100%, $(M + H)^+$] and 409 [1.1%, $(M + NH_4)^+$]. LTGCRI (DB-5): 2380.1.

Permethylated 3'-sialyllactose (12) and its ester-reduced derivative (**13**).—3'-Sialyllactose, sodium salt (5 mg) was dissolved in water and passed through a small column (10 mL) of Dowex-50 (H^+), and the eluate was collected and neutralized to pH 8 by the addition of a dilute solution of tetrabutylammonium hydroxide. Lyophilization afforded the tetrabutylammonium salt as a powder. The latter was dissolved in dry Me_2SO (500 μL) and stirred under N_2 for 1 h, then freshly prepared potassium dimsylate (500 μL, 3 M) was added. After stirring for another hour, the mixture was cooled to 0°C and MeI (100 μL) was added. The solution was stirred at ambient temperature for 4 h, then processed as described for the synthesis of **1**. The product (**12**), which was contaminated with tetrabutylammonium salts, was used without further purification. For **12**; FABMS: *m/z* 816 [44%, $(M + H)^+$]. Compound **13** was prepared by selective reduction ($NaBH_4$) of the ester group of **12** as already described for the synthesis of **3**. For **13**; FABMS: *m/z* 810 [27%, $(M + Na)^+$].

Molar response factors of reductive-cleavage products in flame-ionization detection.—Integrated areas of GLC peaks were corrected for molar response by the effective carbon response (ECR) method[13,14], which has previously been shown[15] to be applicable to anhydroalditol acetates. The ECR values of amino sugar derivatives (**8**, **11**, and **14**) were calculated based upon the work of D'Ambra and Gray[16]. The following values were used in the present study: **8** and **11**, 0.955; **14**, 1.44; **15**, 0.545, and **16**, 0.545. Integrated areas were divided by the appropriate ECR value in order to correct for molar response.

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