Acknowledgment. We thank Professor W. A. Remers for suggestions and useful discussions. We gratefully acknowledge the support of the National Cancer Institute (Grant CA-25644) in this research. The use of the facilities of the UCSF Computer Graphics Laboratory (R. Langridge, Director, and T. Ferrin, Facility Manager), supported by NIH Grant RR-1081, is also gratefully acknowledged.

Appendix I

The chemical charges on mitomycin A and mitomycin C in the monovalent and covalent complexes with the decamer GC10 are listed in Table III.

Appendix II

The bond length, bond angle, and dihedral angle parameters corresponding to the additional atomic species types defined for mitomycin A and mitomycin C in their covalent and noncovalent complexes with the polynucleotide GC10, investigated in this study are listed in Table IV.

The species types used for the atoms of mitomycin are indicated within parentheses along with the names of the atoms as follows: C1 (CH), C2 (CH), N2 (N3), C3 (C2), N4 (N*), C4A (CQ), C5 (CY), O5 (OY), C6 (CQ), CM6 (C3), C7 (CQ), N7 (N), C8 (CY), O8 (OY), C8A (CQ), C9 (CZ), C9A (CZ), C10 (C2), O10 (OZ), C10A (CY), O10A (OY), N10A (N). The hydrogens in the amino groups at the C7 and C10A atoms were assigned the species type H, and those at C2 were assigned H3. In the case of the noncovalent complexes, N2 was assigned NW and the corresponding hydrogen HW. The parameters corresponding to HW are the same as those of H3. In the case of mitomycin A, the oxygen and the methyl atoms at C7 were respectively assigned OS and C3 species types.

Combined Chemical and Enzymatic Synthesis of Sialyloligosaccharides and Characterization by 500-MHz ^{1}H and ^{13}C NMR Spectroscopy

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Abstract: Sialyloligosaccharides that occur as terminal sequences in glycoproteins and glycolipids were synthesized by using combined chemical and enzymatic methodologies. Neutral oligosaccharides containing βDGal(1,3)βDGlcNAc (type 1), βDGal(1,4)βDGlc(NAc) (type 2), and βDGal(1,3)βDGalNAc (type 3) sequences were sialylated enzymatically by using three purified mammalian sialyltransferases each of which uses one of these above three sequences as a substrate. In each case a single oligosaccharide was produced that could be quantitatively purified by simple isolation procedures. In all, ten sialyloligosaccharides, including six which are novel, were prepared in 10–20-μmol scale. In addition, three more sialyloligosaccharides were isolated from a mixture of human or bovine milk oligosaccharides. All these compounds have been characterized by 500-MHz ^{1}H and ^{13}C NMR spectroscopy with complete assignments of ^{13}C chemical shifts. A comparison of the proton and ^{13}C chemical shifts in these linear sialosides with those published for branched structures found in gangliosides GM_1 and GM_2 indicates significant differences, especially for the atoms around the sialoside linkages, and a rationale for these differences based on the steric environment around these atoms in the linear sialosides and branched structures is discussed.

Sialyloligosaccharides of glycoproteins and glycolipids are known to mediate a variety of biological processes.[1] For example, sialyloligosaccharides serve as cell surface receptor determinants for influenza virus and other viruses,[2] for mycoplasma,[3,4] for blood group and tumor specific antibodies,[1,5] for interferon,[6] for recirculating lymphocytes seeking capillary sites of entry to the lymph system,[7] for bacterial toxins,[1] and for a variety of plant and animal lectins.[8,9] The diversity of sialyloligosaccharide sequences which occur naturally are evident in the most common carbohydrate groups of glycoproteins and glycolipids.[10,11] Thus, sialic acid[12] is frequently attached in 2,3 or 2,6-linkage to galactose, *N*-acetylglucosamine, or *N*-acetylgalactosamine and in the 2,8 linkage to another sialic acid in the terminal sequences (I–V) of glycoprotein oligosaccharides N-linked to asparagine or O-linked to threonine or serine.[13] Similar sequences and additional variation in structures are seen in the carbohydrate groups of glycolipids[14] as illustrated by ganglioside GT_{1b} (VI). Such diversity in structure

αDNeuAc(2,6)βDGal(1,4)βDGlcNAc-R (I)

αDNeuAc(2,3)βDGal(1,4/3)βDGlcNAc-R (II)

αDNeuAc(2,3)βDGal(1,3)
βDGlcNAc-R (III)
αDNeuAc(2,6)

αDNeuAc(2,8)αDNeuAc(2,3)βDGal-R (IV)

αDNeuAc(2,3)βDGal(1,3)
αDGalNAc-*O*-Thr/Ser (V)
αDNeuAc(2,6)

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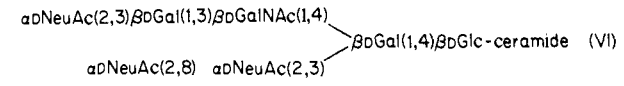

allows for biological specificity. Indeed, many of the sialic acid binding proteins which mediate the processes listed above preferentially bind only one or several of the naturally occurring sialyloligosaccharides sequences of glycoproteins and/or glycolipids.

In recent years the biological functions of many related cell surface oligosaccharides have become better understood due to the advances in chemical synthesis[15-17] and the wealth of information available regarding their conformational properties.[18] In particular, chemical synthesis of oligosaccharides with a suitable linking arm at the reducing end have made possible certain practical applications such as the preparation of artificial antigens and immunoadsorbents,[19,20] neoglycoproteins,[21] and affinity chromatographic columns.[22] Furthermore, synthesis allows such biologically relevant oligosaccharides to be prepared in sufficient amounts for investigations by high field nuclear magnetic resonance spectroscopy to obtain molecular parameters that relate to the secondary structures of these compounds in aqueous solution.[18,23-25] For example, with the aid of computer assisted molecular modeling procedures it has been possible to describe the detailed molecular topographies of oligosaccharides which are important in their binding specifities with antibodies and lectins.[26]

Similar investigations for sialyloligosaccharides of glycoproteins and glycolipids have not been possible due to the lack of availability of sufficient quantities of these compounds from either natural or chemical sources. In contrast to the advances in the synthesis of neutral sugars, there are few reports concerning the chemical synthesis of sialic acid containing oligosaccharides.[17,27-29] This is largely due to the additional problems in synthesis imposed by the properties of the glycosidic linkage involving sialic acid. The axial orientation of the bulky carboxyl group at the anomeric center and the presence of a deoxy carbon adjacent to this render the glycosidic linkage very labile, and consequently make its contruction difficult by the conventional glycosylation methods.[30] The best yields achieved in chemical synthesis range from 34% in the case of 2,6 sialosides[17] to 6% for 2,3 sialosides.[27] Furthermore, the synthesis of the natural α sialosides is complicated due to the formation of an equimolar amount of the unnatural β isomer which must be separated from the desired compound.

To facilitate the synthesis of a variety of biologically relevant sialyloligosaccharides, we have explored enzymatic synthesis as an alternative approach to chemical synthesis. The purpose was to synthesize these compounds in amounts sufficient for NMR spectroscopic investigations, with an ultimate aim of establishing their solution conformations. The enzymes of choice are highly purified mammalian glycosyltransferases which allow the synthesis of oligosaccharides of defined sequence because of their strict specificity for both nucleotide–sugar donor and oligosaccharide–acceptor substrates and their fidelity of transfer yielding a single anomeric linkage.[31] Indeed, Rosevear et al.[32] demonstrated the enzymatic synthesis of oligosaccharides related to human blood group determinants, and Wong et al.[33] showed that the neutral disaccharide *N*-acetyllactosamine could be synthesized in gram quantities using enzymes immobilized on a solid matrix. A number of sialyltransferases responsible for the synthesis of terminal sialyloligosaccharides of N-linked and O-linked oligosaccharides of glycoproteins have been purified to homogeniety.[31,34-36] To date, however, their use for synthesis of sialyloligosaccharides has been largely limited to characterization of the anomeric linkage of the product formed with neutral oligosaccharide acceptor substrates.[35-37]

In this report, we describe a combined approach using both chemical and enzymatic synthesis for the preparation of a variety of sialyloligosaccharides representing the terminal sequences in oligosaccharides of glycoproteins and glycolipids. Neutral oligosaccharides prepared by chemical synthesis were sialylated enzymatically with purified sialyltransferases to form the αDNeuAc(2,6)βDGal or αDNeuAc(2,3)βDGal linkages. Several additional compounds were isolated from human milk. In all, 13 sialyloligosaccharides ranging from a simple disaccharide to a hexasaccharide have been prepared in amounts sufficient to allow a detailed analysis by NMR spectroscopy. Each of these oligosaccharides including six which are novel has been characterized by 500-MHz ^{1}H- and ^{13}C NMR. For the ^{13}C spectra, the availability of a series of sialyloligosaccharides of increasing complexity has allowed the complete assignment of all the carbons for each compound reported here. Results have been analyzed for information they provide regarding the primary and secondary structures of sialyloligosaccharides.

Experimental Section[38]

Materials. All solvents and reagents were purified according to standard procedures.[39] UDP-glucose, UDP-galactose, CMP-NeuAc, UDP-glucose 4-epimerase (E.C. 5.1.3.2), bovine galactosyltransferase (E.C. 2.4.1.22), galactose oxidase (E.C. 1.1.3.9), nicotinamide adenine dinucleotide (NAD^+), and bovine serum albumin were purchased from Sigma. The Galβ1,4GlcNAc α2,6 sialyltransferase (E.C. 2.4.99.1), the Galβ1,3(4)GlcNAc α2,3 sialyltransferase (E.C. 2.4.99.5), and the Galβ1,3GalNAc α2,3 sialyltransferase (E.C. 2.4.99.4) were purified as previously described.[34,40] β-Galactosidase (E.C. 3.2.1.23) from bovine testes was provided by Dr. George Jourdian, University of Michigen.[41] Newcastle disease virus was prepared according to the published procedure.[42] Crude human milk oligosaccharides were a gift of Dr. Jean-Paul

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Prieels. A mixture of lacto-*N*-tetraose and lacto-*N*-neo-tetraose were isolated according to Kobata.[43]

Carbohydrate Analysis. Free and glycosidically linked sialic acid was quantitated by the periodate resorcinol procedure[44] and galactose by the galactose oxidase procedure.[45] Total hexose assays were done by the phenol–sulfuric acid method.[46] Thin-layer chromatography (TLC) was performed on precoated silica gel plates (60-F254; E. Merck. Darmstadt) and visualized, in the case of neutral sugars by charring after spraying with 5% sulfuric acid in ethanol, or for sialyloligosaccharides, by heating at 110 °C after spraying with resorcinol–HCl.

^{1}H NMR spectra were recorded on a Bruker WM-500 (500-MHz) spectrometer with 32 K points (Aspect 2000 computer). Unless specified, all the measurements were made at 296 K. A relaxation delay of 2 s was introduced between 90° pulses. The proton chemical shifts[38] in organic solvents are expressed relative to internal 1% tetramethylsilane, while solutions of compounds in deuterium oxide (D_2O) are expressed relative to the HOD signal (4.81 ppm at 296 K). ^{13}C Spectra were measured at 304 K with the above instrument operating at 125.76 MHz. A relaxation delay of 1 s was introduced between 90° pulses, and a line broadening factor of 2 Hz was included prior to the fourier transformation of the free induction decay. The chemical shifts are expressed relative to external tetramethylsilane with use of the deuterium lock of the spectrometer, which set the ^{13}C chemical shift of 1,4-dioxane in D_2O at 66.88 ppm at 304 K.[47,48]

Preparation of Neutral Oligosaccharides. Methyl β-D-galactopyranoside (**1**) was purchased from Sigma Chemial Co. Methyl β-D-lactoside **2b** was prepared from acetobromolactose[49] by condensation with methanol in the presence of silver carbonate followed by de-O-acetylation. Methyl 2-acetamido-2-deoxy-β-D-glycopyranoside (MeβDGlcNAc, **3**) was prepared in the same way by condensation of 2-acetamido-2-deoxy-α,β-D-glucopyranosyl chloride[50,51] with methanol followed by de-O-acetylation. De-O-acetylations were effected by using a specified volume of 0.5 M solution of sodium methoxide in dry methanol. δ-Caprolactone and D-galactosamine hydrochloride were purchased from Aldrich Chemical Co. For column chromatography, silica gel H (type 60, E. Merck. Darmstadt) and distilled solvents were used, and the columns were loaded in the range of 1:30–1:50.

Methyl β-D-Galactopyranosyl-(1–4)-2-acetamido-2-deoxy-β-D-glucopyranoside (4). The title compound was prepared enzymatically by a modification of the procedure described for the synthesis of *N*-acetyllactosamine.[32,37] UDP-glucose (274 mg, 0.53 mmol), MeβDglcNAc (**3**) (188 mg, 0.8 mmol), and bovine serum albumin (BSA, 36 mg) were dissolved in 36 mL of a solution containing 0.1 M sodium cacodylate (pH 6.5) and 0.1 M manganese chloride. UDP-glucose 4-epimerase (10 units) and galactosyltransferase (5 units) were added, and the reaction mixture was incubated at 37 °C for 24 h. The mixture was then applied to a column (4 × 18 cm) of Dowex 1-X2 (Cl^- form, 200–400 mesh), and the product was eluted unretarded with water (300 mL). The eluate was evaporated to dryness, dissolved in a minimum volume of water, and applied to a column (5 × 90 cm) of Bio Gel P2 (200–400 mesh). Fractions (5 mL) were collected and assayed for hexose content by the phenol–sulfuric acid method. The fractions containing the title compound **4** were identified by TLC (ethyl acetate–ethanol–water, 8:4:2), pooled, and evaporated to a dry residue (126 mg). The ^{13}C chemical shifts of **4** (Table III) were in accordance with those published.[52]

Methyl β-D-Galactopyranosyl-(1–3)-2-acetamido-2-deoxy-β-D-glucopyranoside (7). The title compound was prepared essentially as described by Lemieux et al.[53] for the corresponding 8-(methoxycarbonyl)octyl glycoside. A solution of MeβDGlcNAc (**3**) (1.07 g) in a mixture of acetonitrile–dimethylformamide (3:1, 25 mL) containing α,α-dimethoxytoluene (3 mL) and *p*-toluene sulfonic acid monohydrate (50 mg) was stirred at 70 °C for 3 h. Examination by TLC (ethyl acetate–ethanol–water, 14:4:1) indicated the complete disappearance of the starting material. The precipitate that appeared on cooling to 0 °C was filtered and washed with ice-cold acetonitrile, and this material (795 mg) was homogeneous on TLC. The filtrate was neutralized with triethylamine and evaporated to dry residue which on recrystallization from ethanol afforded 370 mg of a product having the same mobility on TLC as the precipitate. The NMR parameters of the product were found to be in agreement with the 4,6-O-benzylidene derivative **5** of MeβDGlcNAc.

The glycosylation of **5** with acetobromogalactose according to reported procedure[53] afforded after crystallization methyl 2-acetamido-2-deoxy-3-*O*-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-β-D-glucopyranoside (**6**). The conversion of **6** to the title compound **7** was done in the same way as reported.[53] The structure was confirmed on the basis of comparison of its ^{1}H and ^{13}C NMR (Table III) chemical shifts with those reported.[52]

β-D-Galactopyranosyl-(1–3)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1–3)-β-D-galactopyranosyl-(1–4)-α,β-D-glucopyranose (LNT, 8). The title compound, lacto-*N*-tetraose (LNT, **8**), was prepared from human milk oligosaccharides according to Kobata[43] with traces of lacto-*N*-neotetraose removed by digestion with Streptoccocus pneumoniae β-galactosidase prior to paper chromatography.[55] The ^{1}H NMR spectrum of the product was identical with that published.[56]

β-D-Galactopyranosyl-(1–4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1–3)-β-D-galactopyranosyl-(1–4)-α,β-D-glucopyranose (LNNT, 10). The title compound, lacto-*N*-neotetraose (LNNT, **10**), was prepared enzymatically from a mixture of LNT and LNNT. This entailed the removal of terminal galactose from both compounds with bovine testes β-galactosidase and replacement of galactose in the 1.4 linkage with bovine galactosyltransferase. The mixture of LNT and LNNT was prepared as follows. Crude human milk oligosaccharides (25 g) were dissolved in water (100 mL) and passed through a column (2.5 × 25 cm) of Dowex-1X2 (PO_4^{3-} form, 200–400 mesh) to remove most of the sialyloligosaccharides. The column was eluted with water, and fractions containing neutral oligosaccharides, detected by the phenol–sulfuric acid assay, were pooled and evaporated to a dry residue (21.0 g). The column was further eluted to recovery sialyloligosaccharides as described below for preparation of DSL (**25**). The mixture of LNT and LNNT was prepared from the neutral oligosaccharides essentially as described by Kobata.[43] Final purification following the initial separation on Bio Gel P4 (yield 3.3 g) was by chromatography (0.8 g at a time) on a column (5 × 90 cm) of Bio Gel P2 (200–400 mesh) equilibrated and eluted with water. Final yield was 160 mg. Examination of the sample by ^{1}H NMR and by comparison with the spectra of pure LNT and LNNT indicated the former material to be a 9:1 mixture of LNT and LNNT. The lyophilized sample was 50% oligosaccharide by weight as judged by quantitation of galactose.[45]

To prepare 2-acetamido--deoxy-β-D-glucopyranosyl-(1–3)-β-D-galactopyranosyl-(1–4)-α,β-D-glucopyranose (**9**), the mixture of LNT and LNNT (58.5 mg by weight) was dissolved in 2 mL of 0.1 M sodium acetate (pH 4.5) containing bovine serum albumin (2 mg) and 1 unit of bovine testes β-galactosidase and was incubated at 37 °C for 16 h. Estimation of the liberated galactose[45] indicated the presence of 29 μmoles of the product, and examination by TLC (ethyl acetate–acetonitrile–ethanol–water, 2:2:2:1) indicated the complete disappearance of the starting material. Purification on a column (5 × 90 cm) of Bio Gel P2 (200–400 mesh) in water afforded **9** as a colorless solid (17 mg): ^{13}C NMR (D_2O) see Table III.

To prepare LNNT (**10**), compound 9 (25.5 mg, 47 μmol) and UDP-galactose (50 mg, 88 μmol) were dissolved in a buffer (5 mL) containing 0.1 M sodium cacodylate (pH 7.0), 0.1 M manganese chloride, and bovine serum albumin (5 mg). Galactosyltransferase (2 units) was added to this, and the solution was incubated at 37 °C for 6 h, by which time examination by TLC (ethyl acetate–acetonitrile–ethanol–water, 2:2:2:1) indicated the disappearance of all the starting material. The reaction mixture was passed through a column 1.5 × 10 cm) of Dowex-1X2 (Cl^- form, 200–400 mesh) and eluted with 200 mL of water. The eluate containing the product was then evaporated to a dry residue, redissolved in water (2 mL), and desalted on a column (5 × 90 cm) of Bio Gel P2 (200–400 mesh). Lyophilization of the fractions afforded a colorless solid (27.6 mg, 39.1 μmol): ^{1}H NMR (D_2O) (Table II) δ 5.210 (d, H-1α, $J_{1\alpha,2}$ = 3.7 Hz), 4.694 (br d, 1 H, H-1″, $J_{1'',2''}$ = 8.5 Hz), 4.656 (d, 1 H, H-1, $J_{1,2}$ = 7.9 Hz), 4.471 (d, 1 H, H-1‴, $J_{1''',2'''}$ = 7.9 Hz), 4.430 (d, 1 H, H-1′, $J_{1',2'}$ = 7.6 Hz), 4.144 (d, 1 H, H-4′, $J_{4',3'}$ = 2.8 Hz), 3.914 (d, H-4‴, $J_{4''',3'''}$ = 2.8 Hz), 3.79 (H-2″), 3.5 (H-2′), 3.269 (t, H-2β, $J_{2\beta,3\beta}$ = 8. 5 Hz), 2.025 (3 H, $NHCOCH_3$); ^{13}C NMR (D_2O) see Table III.

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5-(Methoxycarbonyl)pentyl β-D-Galactopyranosyl-(1–3)-2-acetamido-2-deoxy-β-D-galactopyranoside (14). δ-Caprolactone (75.0 g) was dissolved in 300 mL of anhydrous methanol. Sodium methoxide solution (8 mL) was added and stirred at room temperature for 5 h. After neutralization with AG 50 W-X16 resin (20–50 mesh, hydrogen form), methanol was evaporated under reduced pressure (20 mmHg). Subsequent fractional distillation under vacuum afforded a constant boiling fraction (bp 82 °C at 0.1 mmHg) that showed infrared bands for hydroxyl group (3500–3000 cm^{-1}) and $COOCH_3$ (1750 cm^{-1}). Yield was 74 g: ^1H NMR ($CDCl_3$) δ 3.672 (s, 3 H, $COOCH_3$), 3.638 (t, 2 H, OCH_2), 2.335 (t, 2 H, CH_2COO), 1.661 (m, 4 H), and 1.398 (m, 2 H).

Galactosamine hydrochloride (5.0 g) was converted to 2-acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-α-,β-D-galactopyranosyl chloride (8.5 g, crude) according to the procedure described for the glucosamine hydrochloride.[57] Condensation of the crude chloride with 5-(methoxycarbonyl)pentanol was carried out as described for the corresponding glucose analogue.[53] Purification on a column 3.5 × 30 cm) of silica gel (ethyl acetate–hexane–acetonitrile, 4:5:1) afforded 5-(methoxycarbonyl)pentyl 2-acetamido-2-deoxy-3,4,6-tri-O-acetyl-β-D-galctopyranoside as a syrup homogeneous on TLC (3.5 g). The ^1H NMR spectrum was in accordance with that expected for a β glycoside.

The acetylated β glycoside was de-O-acetylated to get 5-(methoxycarbonyl)pentyl 2-acetamido-2-deoxy-β-D-galactopyranoside (**11**), which upon treatment with α,α-dimethoxytoluene afforded the corresponding 4,6-O-benzylidene derivative **12**. Condensation of **12** (1.5 g) with acetobromogalactose in the presence of mercuric cyanide and purification on a column (3.5 × 30 cm) of silica gel afforded 5-(methoxycarbonyl)-pentyl 2-acetamido-4,6-O-benzylidene-2-deoxy-3-O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-β-D-galactopyranoside (**13**) as a colorless solid (1.0 g, 38% yield): ^1H NMR ($CDCl_3$) δ 7.52 and 7.33 (m, 5 H, Ar), 6.08 (d, 1 H, NH, $J_{NH,2}$ = 6.1 Hz), 5.51 (s, 1 H, benzylic), 5.36 (d, 1 H, H-4′, $J_{4',3'}$ = 3 Hz), 5.23 (dd, 1 H, H-2′, $J_{2',1'}$ = 7.7 Hz, $J_{2',3'}$ = 9.2 Hz), 5.10 (d, 1 H, H-1, $J_{1,2}$ = 7.7 Hz), 4.99 (dd, 1 H, H-3′, $J_{3',2'}$ = 9.2 Hz, $J_{3',4'}$ = 3 Hz), 4.78 (dd, 1 H, H-3, $J_{3,2}$ = 9.5 Hz, $J_{3,4}$ = 3 Hz), 4.77 (d, 1 H, H-1′, $J_{1',2'}$ = 7.7 Hz), 4.32 (br d, 1 H, H-6_a, $J_{6a,b}$ = 11.5 Hz), 4.29 (d, 1 H, H-4, $J_{4,3}$ = 3 Hz), 4.14 (m, 1 H, H-2), 4.06 (br d, 1 H, H-6), 3.95–3.37 (9 H, H-5′, H-6′$_a$, H-6′$_b$, OCH_3, H-5, OCH_2 of the aglycon), 2.33 –1.36 (5 × $COCH_3$, and the remaining hydrogens in aglycon).

The O-benzylidene group of **13** was removed with 90% aqueous trifluoroacetic acid[58] and then de-O-acetylated with sodium methoxide in methanol. The residue was dissolved in 2 mL of water and then passed through a column (5 × 90 cm) of Bio Gel P-2 (200–400 mesh) in deionized water which afforded the title compound **14** as a colorless solid (290 mg, 43.5% yield): ^1H NMR (D_2O) δ 4.484 (d, 1 H, H-1, $J_{1,2}$ = 8.8 Hz), 4.430 (d, 1 H, H-1′, $J_{1',2'}$ = 7.6 Hz), 4.165 (d, 1 H, H-4, $J_{4,3}$ = 2.3 Hz), 3.976 (dd, 1 H, H-2, $J_{2,3}$ = 10.7 Hz), 3.893 (d, 1 H, H-4′, $J_{4',3'}$ = 3.7 Hz), 3.849 (dd, 1 H, H-3), 3.678 (s, 3 H, OCH_3), 3.511 (dd, 1 H, H-2′, $J_{2',3'}$ = 10.4 Hz), 2.387 (t, 2 H, CH_2COO), 2.006 (s, 3 H, $NHCOCH_3$), 1.576 (m, 4 H), 1.330 (m, 2 H); ^{13}C NMR (D_2O) see Table III.

Preparation of Sialyloligosaccharides. General Methods. All enzyme reactions were carried out in a 15-mL plastic tube, and the product was purified, unless otherwise specified, in the following way. After completion, the reaction mixture was diluted with distilled water to 13 mL and applied on a column (1.5 × 9 cm) of Dowex 1-X2 PO_4^{3-} form, 200–400 mesh). The column was washed with distilled water (175 mL) and then eluted with 5 mM of sodium phosphate buffer (pH 6.8). Fractions (5 mL) were collected and assayed for sialic acid by the periodate-resorcinol procedure.[44] The product, which eluted before free sialic acid, was pooled, evaporated to a dry residue, dissolved in 2 mL of water, and applied to a column (1.6 × 24 cm) of Sephadex G-15 (Sigma) equilibrated and eluted with water. The fractions (1.5 mL) containing the sialyloligosaccharides, as evidenced from the periodate–resorcinol procedure, were measured for conductivity to exclude the contamination by salts, pooled, and lyophilized.

Methyl (5-Acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonic acid)-(2–6)-β-D-galactopyranoside (15). Methyl β-D-galactopyranoside (**1**) (155 mg, 800 μmol) and CMP-NeuAc (13.5 mg, 22 μmol) were dissolved in 2 mL of 0.1 M sodium cacodylate buffer (pH 6.5). To this was added 226 milliunits of the β-galactoside α2,6 sialyltransferase, and the solution was incubated at 37 °C for 20 h. Yield was 3.6 mg (7.2 μmol): ^1H NMR (D_2O) δ 4.311 (d, 1 H, H-1, $J_{1,2}$ = 7.6 Hz), 3.944 (dd, 1 H, H-6_a, $J_{6a,5a}$ = 7.5 Hz, $J_{6a,6b}$ = 10.5 Hz), 3.929 (dd, 1 H, H-4, $J_{4,5}$ = 1.1 Hz, $J_{4,3}$ = 3.6 Hz), 3.874 (d, 1 H, H-6′, $J_{6',5'}$ = 10.5 Hz), 3.830 (t, 1 H, H-5′, $J_{5',4'} = J_{5',6'}$ = 9.5 Hz), 3.780 (m, 1 H, H-5), 3.716 (dd, 1 H, H-9′$_a$, $J_{9'a,8}$ = 1.2, $J_{9'a,9'b}$ = 10 Hz), 3.673 (m, 1 H, H-4′), 3.630 (dd, 1H, H-6_a, $J_{6b,5}$ = 4.6 Hz, $J_{6b,6a}$ = 10.5 Hz), 3.574 (s, 3 H, OCH_3), 3.488 (dd, 1 H, H-2, $J_{2,3}$ = 9.8 Hz), 2.731 (dd, 1 H, H-3′$_{eq}$, $J_{3'eq,4'}$ = 4.9, $J_{3'eq,3'ax}$ = 12.2 Hz), 2.034 (s, 3 H, $NHCOCH_3$), 1.697 (t, 1 H, H-3_{ax}, $J_{3'ax,3'eq} = J_{3'ax,4'}$ = 12.2 Hz).

Methyl (5-Acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonic acid)-(2–6)-β-D-galactopyranosyl-(1–4)-β-D-glucopyranoside (16). The same set of experimental conditions as described above was employed except methyl β-D-lactoside (**2b**) (800 μmol) was used as the acceptor substrate. Yield was 6.4 mg (9.3 μmol): ^1H NMR (D_2O) δ 4.427 (d, 1 H, H-1, $J_{1,2}$ = 8.0 Hz), 4.411 (d, 1 H, H-1′, $J_{1',2'}$ = 8.0 Hz), 4.01–3.94 (m, 2 H, H-6_a, H-6′$_a$), 3.939 (d, 1 H, H-4′, $J_{4',3'}$ = 3.5 Hz), 3.857 (t, 1 H, H-5″, $J_{5'',6''} = J_{5'',4''}$ = 9.7 Hz), 3.532 (dd, 1 H, H-2′, $J_{2',3'}$ = 10.0 Hz), 3.306 (t, 1 H, H-2, $J_{2,3}$ = 10.4 Hz), 2.706 (dd, 1 H, H-3″$_{eq}$, $J_{3''eq,4''}$ = 4.5 Hz, $J_{3''eq,3''ax}$ = 12.5 Hz), 2.034 (s, 3 H, $NHCOCH_3$), 1.747 (t, 1 H, H-3″$_{ax}$).

Methyl (5-Acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonic acid)-(2–6)-β-D-galactopyranosyl-(1–4)-2-acetamido-2-deoxy-β-D-glycopyranoside (17). The *N*-acetyllactosamine derivative **4** (16.1 mg, 40.55 μmol) and CMP-NeuAc (12.5 mg, 20.3 μmol) were dissolved in 2 mL of 0.1 M sodium cacodylate buffer (pH 6.5) containing 226 milliunits of the β-galactoside α2,6 sialyltransferase and incubated at 37 °C for 20 h. After workup, the title compound was obtained as a colorless powder. Yield was 13.8 mg, 19.4 μmol: ^1H NMR (D_2O) δ 4.486 (m, 1 H, H-1), 4.436 (d, 1 H, H-1′, $J_{1',2'}$ = 7.3 Hz), 2.657 (dd, 1 H, H-3″$_{eq}$, $J_{3'',4''}$ = 4.9 Hz, $J_{3''eq,3''ax}$ = 12.5 Hz), 2.050, 2.019 (2 s, 6 H, 2× $NHCOCH_3$), 1.705 (t, 1 H, H-3″$_{ax}$).

(5-Acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonic acid)-(2–6)-β-D-galactopyranosyl-(1–4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1–3)-β-D-galactopyranosyl-(1–4)-α,β-D-glucopyranoside (LSTc, 18). LNNT (**10**) (13.5 mg, 19 μmol) and CMP-NeuAc (25 mg, 40 μmol) were dissolved in 0.1 M sodium cacodylate solution (pH 6.5, 2 mL) containing bovine serum albumin (2 mg), and the reaction was conducted with 300 milliunits of the Galβ1,4GlcNAcα2,6 sialyltransferase for 22 h at 37 °C. The product was purified as described in the general methods except for the use of Sephadex G-25 (Sigma) instead of Sephadex G-15. Yield was 14.0 μmol: ^1H NMR (D_2O) δ 5.210 (d, H-1α, J = 3.7 Hz), 4.717 (br d, 1 H, H-1″, $J_{1'',2''}$ = 7.5 Hz), 4.653 (d, 1 H, H-1$_\beta$, J = 7.9 Hz), 4.446 and 4.429 (t, 2 H, H-1‴, H-1′, J = 7.5 Hz), 4.148 (d, 1 H, H-4′, $J_{4',3'}$ = 3.4 Hz), 3.98 (t, 1 H, H-6‴$_a$, J = 10 Hz), 3.269 (t, H-2$_\beta$, J = 8.2 Hz), 2.660 (dd, 1 H, H-3⁗$_{eq}$, $J_{3''''eq,4''''}$ = 4.9, $J_{3''''eq,3''''ax}$ = 12.5 Hz), 2.043 and 2.017 (2 s, 6 H, 2× $NHCOCH_3$), 1.715 (t, 1 H, H-3⁗$_{ax}$).

(5-Acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonic acid)-(2–3)-β-D-galactopyranosyl-(1–4)-α,β-D-glucopyranose (2,3 Sialyllactose, 19). Commercial sialyllactose from bovine milk (Sigma Chemical Co.) contained about 85% of the title compound and 15% of its 2,6 isomer. These were separated from each other by high-performance liquid chromatography on an amino column as described.[59] The ^1H NMR spectrum (Figure 1a) was identical with that published by Vliegenthart et al.[60]

Methyl (5-Acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonic acid)-(2–3)-β-D-galactopyranosyl(1–4)-β-D-glucopyranoside (20). Methyl β-D-lactoside **2b** (285 mg, 800 μmol) and CMP-NeuAc (12.5 mg, 20 μmol) were dissolved in 0.1 M sodium cacodylate (pH 6.5, 2 mL). The Galβ1,3(4)GlcNAc α2,3 sialyltransferase (48 milliunits) was added, and the solution was incubated at 37 °C for 24 h. The workup of the reaction mixture afforded the title compound as a colorless powder (3.8 mg, 5.7 μmol): ^1H NMR (D_2O) (Figure 1b) δ 4.512 (d, 1 H, H-1′, $J_{1',2'}$ = 7.9 Hz), 4.395 (d, 1 H, H-1, $J_{1,2}$ = 8.2 Hz), 4.101 (dd, 1 H, H-3′, $J_{3',2'}$ = 10.0 Hz), 3.995 (dd, 1 H, H-6_a, $J_{6a,5}$ = 2.45 Hz, $J_{6a,6b}$ = 12.5 Hz), 3.943 (d, 1 H, H-4′, $J_{4',3'}$ = 3.3 Hz), 3.288 (br t, 1 H, H-2), 2.745 (dd, 1 H, H-3″$_{eq}$, $J_{3''eq,4''}$ = 4.9 Hz, $J_{3''eq,3''ax}$ = 12.5 Hz), 2.017 (s, 3 H, $NHCOCH_3$), 1.786 (t, 1 H, H-3″$_{ax}$).

Methyl (5-Acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonic acid)-(2–3)-β-D-galactopyranosyl-(1–4)-2-acetamido-2-deoxy-β-D-glucopyranoside (21). Compound **4** (16 mg, 41 μmol) was converted to the title compound under the same conditions described for **20**. Yield was 6.5 mg (9.2 μmol): ^1H NMR (D_2O) (Figure 1c) δ 4.538 (d, 1 H, H-1′, $J_{1',2'}$ = 7.6 Hz), 4.445 (d, 1 H, H-1, $J_{1,2}$ = 7.9 Hz), 4.106 (dd, 1 H, H-3′, $J_{3',4'}$ = 2.7 Hz, $J_{3',2'}$ = 8.9 Hz), 4.003 (br d, 1 H, H-6_a, $J_{6a,6b}$ = 12.8 Hz), 2.746 (dd, 1 H, H-3″$_{eq}$, $J_{3''eq,4''}$ = 4.3 Hz, $J_{3''eq,3''ax}$ = 12.2 Hz), 2.020 (s, 6 H, 2 × $NHCOCH_3$), 1.788 (t, 1 H, H-3″$_{ax}$).

Methyl (5-Acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonic acid)-(2–3)-β-D-galactopyranosyl-(1–3)-2-acetamido-2-

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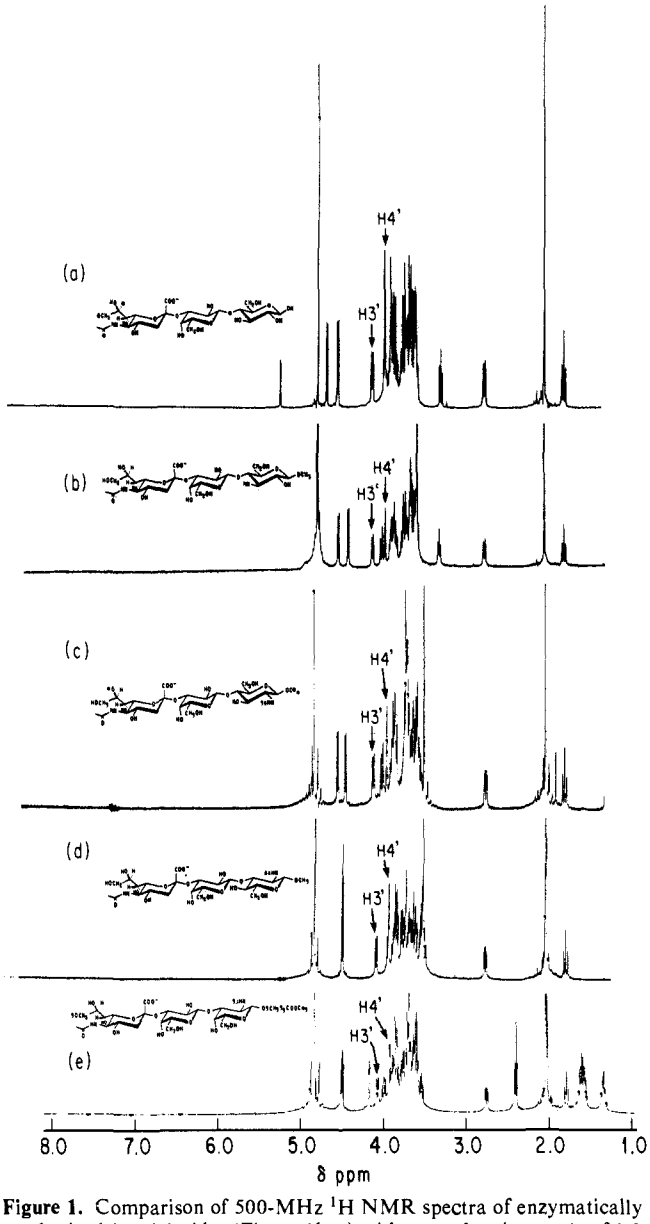


Figure 1. Comparison of 500-MHz ^{1}H NMR spectra of enzymatically synthesized 2,3 sialosides (Figure 1b–e) with an authentic sample of 2,3 sialyllactose (Figure 1a). Spectra are shown for αDNeuAc(2–3)-βDGal(1–4)βDGlc (**19**, a), αDNeuAc(2–3)βDGal(1–4)βDGlc–OCH_3 (**20**, b), αDNeuAc(2–3)βDGal(1–4)βDGlcNAc–OCH_3 (**21**, c), αDNeuAc(2–3)βDGal(1–3)βDGlcNAc–OCH_3 (**22**, d), and αDNeuAc(2–3)βDGal(1–3)βDGalNAc–$O(CH_2)_5COOCH_3$. The indicated signals for galactose hydrogens[38] (H3′ and H4′) are characteristic of linear α2–3 sialosides.

deoxy-β-D-glucopyranoside (22). βDGal(1,3)βDGlcNAc-OCH_3 (**7**) (8 mg, 20 μmol) and CMP-NeuAc (25 mg, 40 μmol) were dissolved in 0.1 M sodium cacodylate solution (pH 6.5, 2 mL) containing Triton-CF 54 (0.1%) and bovine serum albumin (2 mg). The Galβ1,3(4)GlcNAc α2,3 sialyltransferase (50 milliunits) was then added, and the reaction mixture was incubated at 37 °C. After 16 h, another portion of the disaccharide **7** (8 mg) was added, and the incubation was continued for an additional 8 h. Purification of the product afforded the sialyl glycoside **22** as a colorless powder (7.4 μmol): ^{1}H NMR (D_2O) (Figure 1d) δ 4.483 (d, 2 H, H-1, H-1′, J = 7.6 Hz), 4.074 (dd, 1 H, H-3′, $J_{3',4'}$ = 3.0 Hz, $J_{3',2'}$ = 9.8 Hz), 3.934 (dd, 1 H, H-6_a), 3.924 (d, 1 H, H-4′), 2.752 (dd, 1 H, H-3″$_{eq}$, $J_{3''eq,4''}$ = 4.6 Hz, $J_{3''eq,3''ax}$ = 12.5 Hz), 2.021 2.016 (2 s, 6 H, 2 × $NHCOCH_3$), 1.777 (t, 1 H, H-3″$_{ax}$).

(5-Acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonic acid)-(2–3)-β-D-galactopyranosyl-(1–4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1–3)-β-D-galactopyranosyl-(1–4)-α,β-D-glucopyranose (LSTa, 23). LNT (**8**) (20 mg, 20 μmol as estimated by galactose assay) and CMP-NeuAc (25 mg, 40 μmol) were dissolved in 0.1 M sodium cacodylate buffer (pH 6.5, 2 mL) containing 0.1% Triton-CF 54 and bovine serum albumin (2 mg). The Galβ1,3(4)-GlcNAcα2,3 sialyltransferase (50 milliunits) was added, and the reaction mixture was incubated at 37 °C for 48 h. The workup of the reaction mixture was carried out as described in general methods except for the use of Sephadex G-25 (Sigma) instead of Sephadex G-15. Yield was 6.9 μmol: ^{1}H NMr (D_2O) δ 5.208 (d, H-1α, J = 4.3 Hz), 4.723 (br d, 1 H, H-1″, $J_{1'',2''}$ = 8.5 Hz), 4.653 (d, H-1β, J = 7.9 Hz), 4.500 (d, 1 H, H-1‴, $J_{1''',2'''}$ = 7.9 Hz), 4.432 (d, 1 H, H-1′, $J_{1',2'}$ = 7.6 Hz), 4.140 (d, 1 H, H-4′, $J_{4',3'}$ = 3.7 Hz), 4.078 (dd, 1 H, H-3‴, $J_{3''',4'''}$ = 3.3 Hz, $J_{3''',2'''}$ = 10.0 Hz), 3.926 (d, H-4‴), 3.521 (dd, 1 H, H-2‴), 3.473 (m, 1 H, H-5″), 3.269 (br t, H-2β), 2.751 (dd, 1 H, H-3‴″$_{eq}$, $J_{3''''eq,4''''}$ = 4.9 Hz, $J_{3''''eq,3''''ax}$ = 12.5 Hz), 2.032 (s, 6 H, 2 × $NHCOCH_3$), 1.775 (t, 1 H, H-3‴″$_{ax}$).

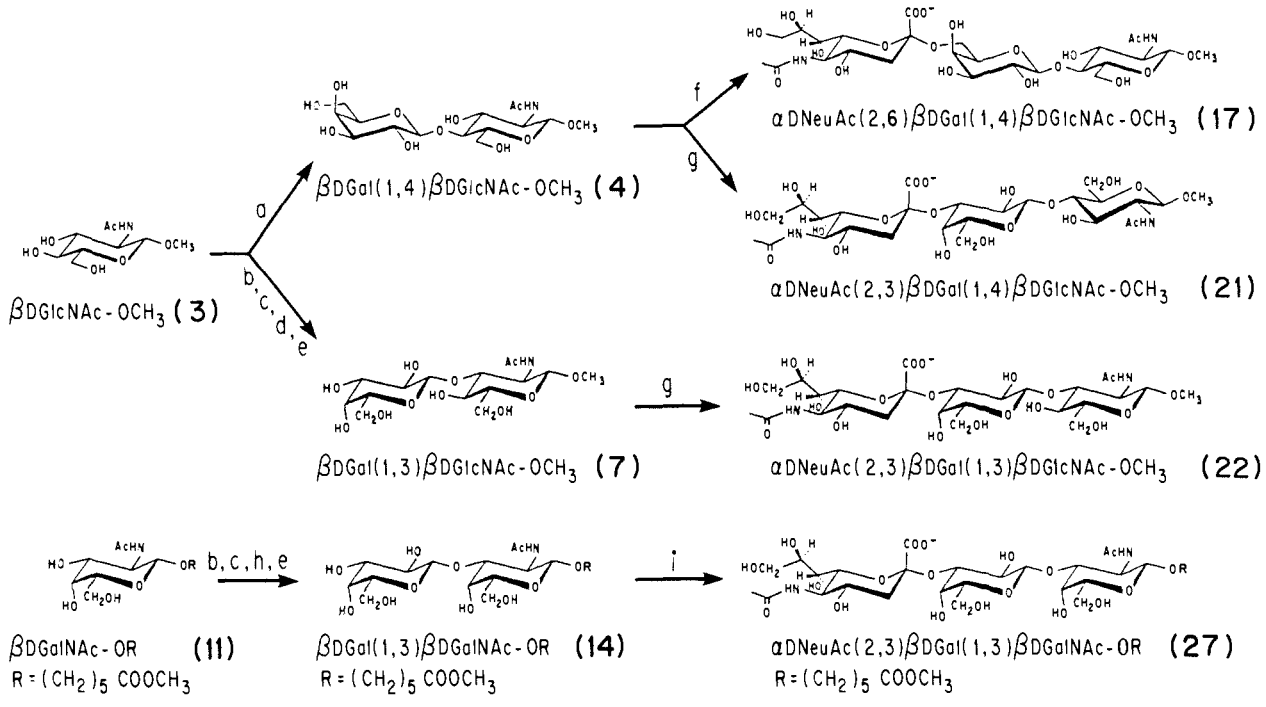
(5-Acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonic acid)-(2–3)-β-D-galactopyranosyl-(1–3)}(5-acetamido-3,5-dideoxy-α-D-*galacto*-2-nonulopyranosylonic acid)-(2–6)}-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1–3)-β-D-galactopyranosyl-(1–4)-α,β-D-glucopyranose (DSL, 25). As mentioned earlier (see preparation of LNNT, **10**), when a solution of a mixture of human milk oligosaccharides was passed through a column of Dowex 1-X2 (PO_4^{3-} form), the neutral oligosaccharides were eluted unretarded, while the sialyloligosaccharides were adsorbed on the column. Monosialyloligosaccharides were then eluted with 5 mM of sodium phosphate buffer (pH 6.8) till no more sialic acid could be detected in the eluant (periodate–resorcinol procedure). After washing the column with 10 mM of sodium phosphate buffer (pH 6.8, 100 mL), elution was continued with 50 mM of sodium phosphate buffer (pH 6.8). The fractions containing the sialic acid, as evidenced by the periodate–resorcinol method, were pooled, evaporated to a dry residue, dissolved in deionized water (2 mL), and applied to a column (1.6 × 19 cm) of Sephadex G-25 (Sigma) equilibrated and eluted with water. After lyophilization, the title compound was obtained as a colorless powder (104 mg): ^{1}H NMR (D_2O) δ 5.207 (d, H-1α, J = 3.7 Hz), 4.690 (br d, 1 H, H-1″, $J_{1'',2''}$ = 7.7 Hz), 4.651 (d, H-1β, J = 8.2 Hz), 4.490 (d, 1 H, H-1‴, $J_{1''',2'''}$ = 7.6 Hz), 4.427 (d, 1 H, H-1′, $J_{1',2'}$ = 7.9 Hz), 4.161 (d, 1 H, H-4′, $J_{4',3'}$ = 3.4 Hz), 4.069 (dd, 1 H, H-3‴, $J_{3''',4'''}$ = 3.1 Hz, $J_{3''',2'''}$ = 9.8 Hz), 3.917 (d, H-4‴), 3.520 (dd, 1 H, H-2‴, $J_{2''',1'''}$ = 7.6 Hz, $J_{2''',3'''}$ = 9.8 Hz), 3.269 (m, H-2β), 2.742 and 2.732 (m,[61] 2 H, H-3‴″$_{eq(2,3)}$, H-3‴″$_{eq(2,6)}$), 2.018, 2.014, and 2.010 (3 s, 9 H, 3 × $NHCOCH_3$), 1.772 (t, 1 H, H-3‴″$_{ax(2,3)}$, J = 12.0 Hz), 1.679 (t, 1 H, H-3‴″$_{ax(2,6)}$, J = 12.0 Hz).

β-D-Galactopyranosyl-(1–3)}(5-acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonic acid)-(2–6)}-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1–3)-β-D-galactopyranosyl-(1–4)-α,β-D-glucopyranose (LSTb, 26). To a solution of **25** (22 μmol) in 10 mL of 0.1 M sodium cacodylate (pH 6.5) Newcastle disease virus sialidase (150 milliunits) was added and incubated at 37 °C for 24 h. Analysis of the reaction mixture by the periodate–resorcinol procedure indicated a 1:1 ratio of free and glycosidically bound sialic acids. The reaction mixture was then purified as described under general methods except for the use of Sephadex G-25 (Sigma) instead of Sephadex G-15. Yield was 21.6 μmol: ^{1}H NMR (D_2O) δ 5.208 (d, H-1α, J = 4.0 Hz), 4.684 (br d, 1 H, H-1″, $J_{1'',2''}$ = 8.7 Hz), 4.652 (d, H-1β, J = 7.9 Hz), 4.427 (d, 2 H, H-1′ and H-1‴, J = 7.6 Hz), 4.165 (d, 1 H, H-4′, $J_{4',3'}$ = 3.4 Hz), 3.505 (dd, 1 H, H-2‴, $J_{2''',1'''}$ = 7.9 Hz, $J_{2''',3'''}$ = 9.8 Hz), 2.737 (dd, 1 H, H-3‴″$_{eq(2,6)}$, $J_{3''''eq,4''''}$ = 4.6 Hz, $J_{3''''eq,3''''ax}$ = 12.8 Hz), 2.021, 2.010 (2 s, 6 H, 2 × $NHCOCH_3$), 1.681 (t, 1 H, H-3‴″$_{ax(2,6)}$).

5-(Methoxycarbonyl)pentyl (5-Acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonic acid)-(2–3)-β-D-galactopyranosyl-(1–3)-2-acetamido-2-deoxy-β-D-galactopyranoside (27). Compound **14** (16 mg, 40 μmol) and CMP-NeuAc (25 mg, 40 μmol) were dissolved in 0.1 M sodium cacodylate buffer (pH 6.5, 2 mL) containing Triton-CF 54 (0.1%) and bovine serum albumin (2 mg). The Galβ1,3GalNAc α2,3 sialyltransferase (13 milliunits) was added, and the reaction mixture was

(61) The sialic acid hydrogens are accompanied by numbers in parentheses to indicate the sialoside linkage.

Scheme I[a]



[a]Shown are the steps in the synthesis of several sialyloligosaccharides using combined chemical (b, c, d, e, and h) and enzymatic (a, f, g, and i) methods. For each step is listed either the chemical reagents or the glycosyltransferase and the corresponding nucleotide sugar donor substrate used to carry out the transformations: (a) galactosyltransferase, UDP-galactose; (b) $C_6H_5(OCH_3)_2$, *p*-TsOH, CH_3CN; (c) acetobromogalactose, Hg(CN)$_2$, benzene, nitromethane; (d) 50% aqueous CH_3COOH, 100 °C; (e) $NaOCH_3$, CH_3OH; (f) Galβ1,4GlcNAc α2,6 sialyltransferase, CMP-NeuAc; (g) Galβ1,3(4)GlcNAc α2,3 sialyltransferase, CMP-NeuAc; (h) 90% CF_3COOH, 0 °C; (i) Galβ1,3GalNAc α2,3 sialyltransferase, CMP-NeuAc.

incubated for 48 h at 37 °C. Yield was 12.7 μmol: ^{1}H NMR (D_2O) (Figure 1e) δ 4.503 (d, 1 H, H-1′, $J_{1',2'}$ = 7.6 Hz), 4.486 (d, 1 H, H-1, $J_{1,2}$ = 8.2 Hz), 4.163 (d, 1 H, H-4, $J_{4,3}$ = 2.8 Hz), 4.036 (dd, 1 H, H-3′, $J_{3',2'}$ = 10 Hz), 3.986 (dd, 1 H, H-2, $J_{2,3}$ = 10 Hz), 3.925 (d, 1 H, H-4′, $J_{4',3'}$ = 3.0 Hz), 3.533 (dd, 1 H, H-2′, $J_{2',1'}$ = 7.6, $H_{2',3'}$ = 10 Hz), 2.746 (dd, 1 H, H-3″$_{eq}$, $J_{3''eq,4''}$ = 4.9 Hz, $J_{3''eq,3''ax}$ = 12.5 Hz), 2.390 (t, 2 H, CH_2COOCH_3), 2.021, 2.008 (2 s, 6 H, 2 × $NHCOCH_3$), 1.779 (t, 1 H, H-3″$_{ax}$), 1.586, 1.33 (m, 6 H, remaining hydrogens in aglycon).

Results

General Approach. Sialyloligosaccharides and the corresponding neutral oligosaccharide precursors described in this report are listed in Table I. The general approach of combined chemical and enzymatic synthesis is illustrated in Scheme I for four representative sialyloligosaccharides.

Preparation of Neutral Oligosaccharides. The choice of the neutral oligosaccharides listed in Table I was based on the frequent occurrence of their sialylated forms as terminal sequences in glycoproteins[10,11] and glycolipids.[14] All the disaccharides except **14** were synthesized as their methyl glycosides rather than the reducing sugar with the exception that these could be prepared readily based on the well-established chemical methodologies and that the ^{1}H and ^{13}C spectra would be much simpler than if the reducing terminal residue is present as a mixture of α,β anomers. The deliberate inclusion of a linking arm, namely the aglycon $(CH_2)_5COOCH_3$ as seen in the disaccharide **14**, is to demonstrate that the combination of chemical and enzymatic methodologies could be extended to prepare sialyloligosaccharide ligands that are suitable for making artificial antigens, immunoadsorbents,[19] and neoglycoproteins.[21] In addition, the two human milk tetrasaccharides LNT (**8**) and LNNT (**10**) were of interest since several of their sialylated forms also occur in human milk oligosaccharides[62] and as the oligosaccharide moieties of sialoglycolipids.[14]

Chemical synthesis was used to prepare the disaccharides **7** and **14** which started from their corresponding methyl and the 5-(methoxycarbonyl)pentyl glycosides **3** and **11**, respectively. The reactions involved the conversion of **3** and **11** to their corresponding 4,6-O-benzylidene derivatives followed by glycosylation with acetobromogalactose according to ther reported procedures[53,64] to obtain the disaccharides **6** and **13**, respectively (see Experimental Section). Removal of the protecting groups then afforded the disaccharides **7** and **14** as colorless solids, the ^{1}H and the ^{13}C chemical shifts of which were found to be in excellent agreement with those reported.[52,48]

The *N*-acetyllactosamine derivative **4** and the tetrasaccharide LNNT (**10**) were conveniently prepared by the enzymatic procedures using MeβD-GlcNAc (**3**) and the trisaccharide **9**, resepctively, as the acceptor substrates and UDP-galactose as the nucleotide sugar donor for the enzyme galactosyltransferase.[42,63] The trisaccharide **9** in turn was obtained from the 9:1 mixture of LNT (**8**) and LNNT (**10**) which was isolated from human milk, by treatment with the bovine testes β-galactosidase. The identity of **4** and LNNT (**10**) were firmly established on the basis of their ^{13}C chemical shifts (Table III).

Finally, LNT (**8**) was isolated from human milk according to Kobata.[43] This procedure afforded the desired product **8** which was contaminated by its isomer LNNT (**10**) by about 10%. However, digestion of this mixture with streptococous pneumoniae β-galactosidase, specific for cleavage of βDgal(1,4) linkages, selectively degraded LNNT to provide the trisaccharide **9**, which then could be easily removed by paper chromatography. The ^{1}H NMR spectrum of the LNT obtained by this procedure was identical with that reported[56] and also indicated a high degree of purity.

Preparation of Sialyloligosaccharides. Of the 13 sialyloligosaccharides listed in Table I, 10 were prepared by using three mammalian sialyltransferases which form the following sequences:

αDNeuAc(2,6)βDGalβ(1,4)βDGlcNAc- (a)
αDNeuAc(2,3)βDGalβ(1,3/4)βDGlcNAc- (b)
αDNeuAc(2,3)βDGalβ(1,3)βDGalNAc- (c)

(62) Kobata, A. ref 11, p 423.

(63) Nunez, H. A.; Barker, R. *Biochemistry* **1980**, *19*, 489.
(64) Ratcliffe, R. M.; Baker, D. A.; Lemieux, R. U. *Carbohydr. Res.* **1981**, *93*, 35.

Table I. List of Neutral Oligosaccharides and the Corresponding Sialyloligosaccharides (**15–18**, **20–24**, **27**) Prepared Enzymatically by Using Purified Mammalian Sialyltransferases[34,40,a]

neutral oligosaccharides	αDNeuAc(2–6)βDGal glycosides	αDNeuAc(2–3)βDGal glycosides	αDNeuAc(2–6)βDGlcNAc glycosides
βDGal–OCH_3 (**1**)	αDNeuAc(2–6)βDGal–OCH_3 (**15**)		
βDGal(1–4)βDGlc–OH (**2a**)		αDNeuAc(2–3)βDGal(1–4)βDGlc–OH (**19**)	
βDGal(1–4)βDGlc–OCH_3 (**2b**)	αDNeuAc(2–6)βDGal(1–4)βDGlc–OCH_3 (**16**)	αDNeuAc(2–3)βDGal(1–4)βDGlc–OCH_3 (**20**)	
βDGal(1–4)βDGlcNAc–OCH_3 (**4**)	αDNeuAc(2–6)βDGal(1–4)βDGlcNAc–OCH_3 (**17**)	αDNeuAc(2–3)βDGal(1–4)βDGlcNAc–OCH_3 (**21**)	
βDGal(1–3)βDGlcNAc–OCH_3 (**7**)		αDNeuAc(2–3)βDGal(1–3)βDGlcNAc–OCH_3 (**22**)	
βDGal(1–3)βDGlcNAc(1–3)-βDGal(1–4)DGlc, LNT (**8**)		αDNeuAc(2–3)βDGal(1–3)βDGlcNAc(1–3)-βDGal(1–4)DGlc, LSTa (**23**)	αDNeuAc(2–3)βDGal(1–3)-[αDNeuAc(2–6)]βDGlcNAc(1–3)-βDGal(1–4)DGlc, DSL (**25**) βDGal(1–3)[αDNeuAc(2–6)]-βDGlcNAc(1–3)βDGal(1–4)DGlc, LSTb (**26**)
βDGlcNAc(1–3)βDGal(1–4)DGlc (**9**)			
βDGal(1–4)βDGlcNAc(1–3)-βDGal(1–4)DGlc, LNNT (**10**)	αDNeuAc(2–6)βDGal(1–4)βDGlcNAc(1–3)-βDGal(1–4)DGlc, LSTc (**18**)	αDNeuAc(2–3)βDGal(1–4)βDGlcNAc(1–3)-βDGal(1–4)DGlc, LSTd (**24**)	
βDGla(1–3)βDGalNAc–$O(CH_2)_5$-$COOCH_3$ (**14**)		αDNeuAc(2–3)βDGal(1–3)βDGalNAc–$O(CH_2)_5$-$COOCH_3$ (**27**)	

[a] Compound **19** was isolated from bovine milk, while **25** and **26** were isolated from human milk (see Experimental Section).

Table II. ^1H Chemical Shifts[a] of LNT (**8**), LNNT (**10**), and Those Observed for the Sialyloligosaccharides **15–27**

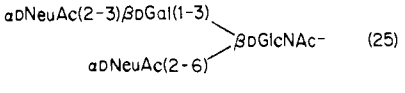
		chemical shifts, ppm														
sugar unit	hydrogen atoms	**8**	**10**	**15**	**16**	**17**	LSTc **18**	LSTb **26**	DSL **25**	**19**	**20**	**21**	**22**	**27**[b]	LSTa **23**	LSTd **24**
DGlc	1α	5.208	5.210				5.210	5.208	5.207	5.206					5.028	5.210
	1β	4.650	4.656		4.427		4.653	4.652	4.651	4.653	4.395				4.653	4.654
	2	3.262	3.269		3.306		3.269	3.270	3.269	3.270	3.288				3.269	3.271
βDGal	1	4.429	4.430				4.429	4.427	4.427						4.432	4.427
	2	~3.55	~3.59				~3.58								~3.55	~3.56
	4	4.143	4.144				4.148	4.165	4.161						4.140	4.150
βDGlcNAc	1	4.715	4.694			4.486[c]	4.717	4.684	4.690			4.445	4.483	4.503	4.723	4.687
or	2	~3.9	3.789			3.727[c]						~3.73	3.828	3.986	3.891	~3.79
βDGalNAc[b]	4													4.163		
	N–Ac	2.017	2.025			2.050	2.043	2.010	2.010			2.020	2.016	2.008	2.031	2.023
βDGal	1	4.429	4.471	4.311	4.411	4.436	4.446	4.427	4.490	4.521	4.512	4.538	4.483	4.486	4.500	4.551
	2	3.512	3.528	3.488	3.532	3.537	3.524	3.505	3.520	3.548	3.588	3.563	3.542	3.533	3.521	3.555
	3			3.61					4.069	4.103	4.101	4.106	4.074	4.036	4.078	4.110
	4	3.900	3.914	3.929	3.939	3.913		3.890	3.917	3.943	3.943	3.945	3.924	3.925	3.926	3.947
	6A			3.944			3.987									
αDNeuAc 2–3	3_{ax}								1.772	1.789	1.786	1.788	1.777	1.779	1.775	1.793
	3_{eq}								2.742	2.747	2.745	2.746	2.752	2.746	2.751	2.751
	–4									3.651	~3.675	~3.67	~3.67	~3.63	~3.678	~3.67
	–5								3.836[b]						3.852[b]	
	N–AC								2.014	2.017	2.017	2.020	2.021	2.021	2.032	2.023
αDNeuAc 2–6	3_{ax}			1.697	1.747	1.705	1.715	1.681	1.679							
	3_{eq}			2.731	2.706	2.657	2.660	2.737	2.732							
	4	~3.67		~3.67			~3.66									
	5			3.830	3.857	3.791			3.815[b]							
	N–Ac			2.034	2.034	2.019	2.017	2.021	2.018							

[a] See experimental for details. [b] Located by NOE. [c] Multiplets due to virtual coupling.[82]

commonly found as terminal sequences of glycoprotein[10,11] and glycolipid[14] oligosaccharides. These enzymes exhibit a high degree of specificity for the oligosaccharide sequence of the acceptor substrate, for CMP-βDNeuAc as the donor substrate, and for making a single anomeric linkage in the transfer reaction. Thus, the βDGal(1,4)βDGlcNAc α2,6 sialyltransferase (E.C. 2.4.99.1)[34] which forms sequence (a) was used to convert neutral oligosaccharides **4** and **10** containing the terminal sequence βDGal-(1,4)βDGlcNAc to the corresponding α2,6 sialosides **17** and **18**. Although methyl β-D-lactoside **2b** and methyl β-D-galactoside **1** are poor acceptor substrates of this enzyme,[33] it was also possible to prepare their 2,6 sialosides, compounds **15** and **16**. Similarly, the βDGal(1,3/4)βDGlcNAc α2,3 sialyltransferase (E.C. 2.4.99.5) which forms sequence (b) and preferentially uses oligosaccharides with the terminal βDGal(1,3)βDGlcNAc and βDGal(1,4)-βDGlcNAc sequences was used to convert oligosaccharides **2b**, **4**, **7**, **8**, and **10** to the α2,3 sialosides **20–24**. Finally, the βDGal(1,3)βDGalNAc α2,3 sialyltransferase (E.C. 2.2.99.4) was used to convert oligosaccharide **14** containing the preferred acceptor sequence βDGal(1,3)βDGalNAc to the α2,3 sialoside **27**.

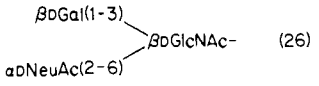
The general strategy in preparing the sialyltransferase reactions was as follows. In all reactions 20–40 μmol CMP-βDNeuAc was used, and the concentration was fixed at 10–20 mM. For abundant acceptor substrates and/or those known to react inefficiently, the acceptor substrate was either in large excess of or in equal molar concentration to CMP-βDNeuAc. The best acceptor substrates were used as the limiting substrate, with CMP-βDNeuAc in a two-fold molar excess. Sufficient enzyme was used to give a calculated 1 000–13 000-fold excess based on a reaction velocity of V_{max} over an incubation period of 24–48 h. Following the reaction, all products were purified by a simple protocol[42] involving adsorption to Dowex-1 (PO_4^{3-}), elution with 5 mM sodium phosphate, pH 6.8, removal of buffer salts by desalting on a column of Sephadex G-15 or G-25 equilibrated in water, and, finally, concentration by lyophylization. Overall yields when CMP-βDNeuAc was the limiting substrate ranged from 14 to 47% with an average of 27%, and when the best acceptor substrates were the limiting substrate, yields were 35–96 with an average of 63%. In all cases, sialyloligosaccharides prepared in this manner were found to be of high purity suitable for characterization by NMR (for examples see Figure 1b–e).

Three other sialyloligosaccharides examined in this report were isolated from mixed bovine or human milk oligosaccharides. The α2,3 isomer of sialyllactose was purified[59] from a mixture of the α2,3 and 2,6 isomers (Sigma) by liquid chromatography on a column of amino bonded silica (see Experimental Section). A number of sialyloligosaccharides bearing terminal sequences analogous to those on glycoprotein and glycolipid carbohydrate groups have been purified from human milk.[43] Several of these were conveniently prepared by sialylation of the neutral oligosaccharide precursors (LSTa (**23**) and LSTc (**18**). The disialylated hexasaccharide, DSL, containing the terminal sequence



(25)

was easily purified in a single step from the mixed oligosaccharides by ion exchange chromatography,[42] as described in the Experimental Section, using a modified procedure employing Dowex-1 ×2 (PO_4^{3-} form). To obtain the related monosialylated derivative LSTb



(26)

advantage was taken of the fact that Newcastle disease virus sialidase cleaves only the terminal sialic acid of DSL.[65,66] Indeed, DSL was quantitatively converted to LSTb, and the product was purified as for the other sialosides.

Characterization of Sialyloligosaccharides by ^{1}H NMR. In Figure 1b–e, the ^{1}H NMR spectra of the synthetic 2,3 sialosides are compared with that of the 2,3 sialyllactose (Figure 1a) isolated from bovine milk to indicate the high purity as well as the identity of the products obtained in the enzymatic reactions. The same high purity was seen from the NMR spectra of all the 2,6 sialosides. The chemical shifts for a number of hydrogens that could be determined directly or indirectly by double irradiation and NOE experiments for both 2,6 and 2,3 sialosides are compiled in Table II along with those of two asialo precursors LNT (**8**) and LNNT (**10**) for comparison. The chemical shift changes that accompany the sialylation of either the 6- or the 3-hydroxyls of a galactose are in general accord with those reported by Vliegenthart and co-workers[67] for the asparagine linked oligosaccharides of glycoproteins. For example, the conversion of LNNT (**10**) to the 2,6 sialoside LST_c (**18**) results in shielding of the anomeric hydrogen of the terminal galactose by 0.035 ppm, while the opposite is observed for the corresponding hydrogen in the 2,3 sialoside LST_d (24) (0.08 ppm deshielding, see Table II). Also, the signals for H-3_{ax} of the sialic acid residues in all the 2,6 sialosides appear at a higher field as compared to the 2,3 sialosides. As evident from Table II, the chemical shift of many additional hydrogens could be established with the high resolution of signals at 500 MHz confirming the structures of the enzymatic products. Of particular interest for the 2,3 sialosides, the sialylation is accompanied by a large deshielding (≈0.6 ppm) for the corresponding aglyconic hydrogen (H-3 of terminal galactose). Thus, the doublet of a doublet (J = 3.5, 9.0 Hz) seen around 4.1 ppm (Figure 1a–e) could be assigned to H-3 of the sialylated galactose[60] in view of its synclinal and antiperiplanar orientation to its adjacent H-4. In contrast, for the 2,6 sialosides, only one of the methylene hydrogens at C-6 is deshielded (see Experimental Section for complete assignments of chemical shifts for the disaccharide **15**).

It is noteworthy that the proton chemical shifts of the sialic acid residues in the trisaccharide **17** and the pentasaccharide **18** (Table II), are nearly the same as those reported by Vliegenthart and co-workers[67] for mono-, di-, and trianntennary structures of asparagine linked glycoproteins that bear the sequences of **17** and **18**. It is, therefore, likely that the hydrogens in the terminal sugar residues in these glycoproteins are in the same environment as the synthetic compounds.

Assignments of ^{13}C Chemical Shifts. Neutral Oligosaccharides. In Table III, the ^{13}C chemical shifts of all the neutral oligosaccharides are reported which were used for the assignments of those observed for the corresponding sialyloligosaccharides (Table IV and V). The ^{13}C chemical shift assignments for βDGal-OCH_3 (**1**), βDGal(1,4)βDGlc (**2a**), βDGal(1,4)βDGal-OCH_3 (**2b**), βDGal(1,4)βDGlcNAc-OCH_3 (**4**), and βDGal(1,3)βDGalNAc-O-$(CH_2)_5COOCH_3$ (**14**) were made (Table III) on the basis of the published report.[68] The chemical shift assignments for the carbon atoms in the trisaccharide **9** were made by comparison with those found for lactose (**2a**). The glycosylation of **3**-hydroxyl of galactose in **2a** is expected[69] to deshield the corresponding aglyconic carbon and shield the carbons vicinal to C-3. Indeed, in compound **9** (Table III), the C-3 of the galactose residue is deshielded by 9.52 ppm, while the adjacent C-2 is shielded by 0.77 ppm. However, practically no deshielding was observed for the other vicinal carbon, namely the C-4 of the galactose, and this seems to be typical of C-3 substitution of a galactose by a β-D-hexopyranoside.[70] Since both lacto-*N*-tetraose (**8**) and lacto-*N*-neotetraose (**10**) were derived by substitution with a βDGal unit respectively at the C-3 and C-4 hydroxyl groups of the terminal βDGlcNAc unit of **9**, following the general principle of glycosy-

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(66) Paulson, J. C.; Weinstein, J.; Dorland, L.; Van Halbeek, H.; Vliegenthart, J. F. G. *J. Biol. Chem.* **1982**, *257*, 12734.
(67) Vliegenthart, J. F. G.; Dorland, L.; Van Halbeek, H. *Adv. Carbohydr. Chem. Biochem.* **1983**, *41*, 209.
(68) For a review on ^{13}C chemical shifts of oligosaccharides see: Bock, K.; Pedersen, C.; Pedersen, H. *Adv. Carbohydr. Chem. Biochem.* **1984**, *42*, 193.
(69) Dorman, D. E.; Roberts, J. D. *J. Am. Chem. Soc.* **1971**, *93*, 4463.
(70) The same effect was seen in the trisaccharide βDGal(1,3)βDGal-(1,4)DGlc as well. Collins, J. G.; Bradbury, J. H.; Trifonoff, E.; Messer, M. *Carbohydr. Res.* **1981**, *92*, 136.

Table III. Assignments of ^{13}C Chemical Shifts[a] for Neutral Oligosaccharide Precursors which Served as a Basis for Assignment of the Chemical Shifts for Sialyloligosaccharides in Tables IV and V

sugar unit	carbon atom	chemical shifts							
		1	**2a**[b]	**4**[b]	**7**[b]	**14**	**9**	**8**	LNNT **10**
βDGlc	1		96.15				96.33	96.21	96.24
	2		74.24				74.45	74.33	74.34
	3		74.78				74.94	74.85	74.88
	4		78.83				79.13	7..09	78.96
	5		75.19				75.30	75.26	75.32
	6		60.58				60.81	60.69	60.69
βDGal	1	104.18	103.29				103.43	103.38	103.40
	2	71.00	71.38				70.61	70.51	70.50
	3	73.05	72.96				82.48	82.44	82.51
	4	68.94	68.97				68.91	68.79	68.84
	5	75.36	75.75				75.44	75.35	75.37
	6	61.23	61.43				61.50	61.40	61.48
βDGlcNAc	1			102.40	102.20	101.56	103.20	102.86	103.11
	2			55.54	54.91	52.42	56.32	55.20	55.75
	3			73.08	83.22	80.25	74.22	82.73	72.71
	4			79.18	69.33	68.36	70.42	69.00	78.93
	5			75.30	75.93	75.04	76.28	75.73	75.08
	6			60.71	61.33	61.24	61.21	61.10	60.51
	N—C=O			175.30	175.21	174.84		175.35	175.31
	CH_3			22.84	22.78	22.58	22.79	22.77	22.75
βDGal	1			103.42	104.05	105.14		103.90	103.40
	2			71.54	71.22	70.92		71.20	71.49
	3			73.08	73.07	72.80		73.01	73.08
	4			69.15	69.06	68.90		69.03	69.08
	5			75.89	75.79	75.30		75.73	75.85
	6			61.57	61.50	61.33		61.47	61.48

[a] See experimental for details. [b] Based on published report.[68]

lation, the ^{13}C assignments in these compounds were made. As expected, in the case of LNT, the chemical shift value for the C-3, C-4, and C-2 of the βDGlcNAc unit is altered by 8.51 (deshielding), −1.39 (shielding), and −1.12 ppm (shielding), respectively, while in LNNT, the chemical shifts of C-4, C-3, and C-5 of the corresponding unit are changed by 8.51 (deshielding), −1.51 (shielding), and −1.20 ppm (shielding), respectively, as compared to the trisaccharide **9** (Table III).

αDNeuAc(2,6)βDGal Glycosides. Table IV shows the ^{13}C chemical shift assignments of the 2,6 sialosides and the differences in the chemical shift values observed between the sialyloligosaccharides and the corresponding asialo-compounds (Table III). Significant changes due to sialylation are highlighted with bold typeface. Compound **15** represents the simplest of all of the sialyloligosaccharides. Its chemical shift assignments could be readily made on the basis of comparison with βDGal-OCH_3 (**1**) and MeαDNeuAc (**28**). As a result of glycosylation at 6-hydroxyl of **1** by an αDNeuAc residue, the chemical shifts of only C-6 and C-5 of compound **1** are expected to be significantly altered. As seen from Table IV, the observed values for C-6 and C-5 in compound **15** are 63.83 and 73.91 ppm, respectively. As evident from column 9 of Table IV, this corresponds to a deshielding of the aglyconic carbon, namely the C-6 of galactose by 2.60 ppm and a large shielding of about 1.45 ppm for the carbon (C-5 of βDGal) vicinal to the site of sialylation. In general, an overall correspondence in chemical shifts is observed for the remainder of the carbons as compared to **1** and **28** except for the C-2 of the NeuAc unit (Table IV, column 8) which is shielded in **15** by about 0.52 ppm as compared to the methyl sialoside. However, this is to be expected due to the difference in the nature of the aglycon. The effects of sialylation oberved for the disaccharide **15** are in fact seen in all of the remaining 2,6 sialosides as well.

For the trisaccharide **17**, the chemical shift assignments were made from comparison with **15** and the asialo compound **4**. An excellent correspondence is observed between **15** and **17** for the chemical shifts of αDNeuAc and most of the carbon atoms of the the βDGal residue. However, as noted by Berman,[71] sialylation caused the unusual deshielding of the chemical shifts of most of the carbon atoms of the βDGlcNAc residue as compared to the asialo precursor **4**. In the case of the pentasaccharide LSTc (**18**) (Table IV, also see ref 71), the assignment of ^{13}C chemical shifts for the terminal trisaccharide was made by comparison with **17**, and the chemical shifts of the inner lactose moiety were assigned on the basis of those found for LNNT (**10**). Again the unusual deshielding of the C-4 of the βDGlcNAc residue was observed.

αDNeuAc(2,3)βDGal Glycosides. The assignments of ^{13}C chemical shifts for (2,3) sialyllactose (**19**) were made on the basis of comparison with those found for lactose and methyl sialoside **28**. First, the chemical shift assignments were made to carbons atoms of α and β glucose units, as these signals had reduced intensity as compared to that of βDGal and αDNeuAc units. In compound **19**, there was only one signal seen in the region expected for an anomeric carbon involved in β-glycosidic linkage, which, therefore, could be unambiguously assigned to C-1 of galactose. The assignments of the two signals at 76.03 and 75.65 ppm were made in view of the following consideration. As a result of glycosylation at C-3 of galactose, the chemical shifts of only C-3 and the carbons vicinal to this should be altered, while the chemical shifts of C-1, C-5, and C-6 of the galactose in **19** should be essentially the same as observed in lactose (Table III). Therefore, the signal at 75.65 in **19** was first assigned to C-5 (C-5 of βDGal in lactose was 75.75 ppm) of the galactose, while the remaining signal at 76.03 ppm was assigned to C-3. It is to be noted that our assignments are opposite to that of the published report.[71] In the same way, the assignments for the remainder of the carbons atoms in **19** are made. Once the ^{13}C chemical shifts were established for (2,3) sialyllactose, the assignments for the αDNeuAc and the βDGal residues in the remaining trisaccharides could be readily made (Table V) on the basis of comparison with **19**, while for those residues at the reducing end from comparison with the corresponding asialo compounds.

Finally, the ^{13}C chemical shifts in the two pentasaccharides, namely LST_a (**23**) (Table V) and LST_d (**24**), were established on the basis of those found for the corresponding asialo compounds LNT (**8**) and LNNT (**10**) and the trisaccharides **22** and **21**. For example, in LST_d, the chemical shifts of the α-DNeuAc and the therminal βDGal residues are nearly the same as in **21** (Table V), while the chemical shifts for the remainder of the carbon atoms

(71) Berman, E. *Biochemistry* **1984**, *23*, 3754.

Table IV. Assignments of ^{13}C Chemical Shifts for (2–6) Sialyloligosaccharides and the Differences with Those Observed for the Corresponding Asialo Compounds (Table III)[a]

		chemical shifts, ppm										
sugar unit	carbon atom	**15**	**17**	LSTc **18**	LSTb **26**	DSL **25**	MeαDNeuAc **28**	**δ(1) − δ(15)**	**δ(4) − δ(17)**	**δ(10) − δ(18)**	**δ(8) − δ(26)**	**δ(8) − δ(25)**
βDGlc	1			96.17	96.14	96.21				0.07	0.07	0.00
	2			74.25	74.13	74.31				0.09	0.20	0.02
	3			74.76	74.78	74.86				0.12	0.07	0.01
	4			78.88	78.92	79.09				0.08	0.17	0.08
	5			75.23	75.21	75.27				0.09	0.05	0.01
	6			60.61	60.41	60.70				0.08	0.28	−0.01
βDGal	1			103.36	103.31	103.37				0.04	0.07	0.01
	2			70.43	70.38	70.45				0.07	0.13	0.06
	3			82.41	82.13	82.46				0.10	0.31	0.02
	4			68.89	68.67	68.76				0.05	−0.12	0.03
	5			75.32	75.41	75.46				0.05	0.06	−0.11
	6			61.40	61.44	61.48				0.08	0.04	−0.08
βDGlcNAc	1		102.08	102.93	102.99	102.86			0.32	0.18	−0.13	0.00
	2		55.12	55.45	55.14	55.11			0.42	0.30	0.06	0.09
	3		72.83	72.67	82.72	82.76			0.25	0.04	0.01	−0.03
	4		81.19	80.88	68.83	69.11			**−2.01**	**−1.95**	0.17	−0.11
	5		74.88	74.76	74.13	74.26			0.42	0.32	**1.60**	**1.47**
	6		60.79	60.67	63.29	63.48			−0.08	−0.16	**−2.19**	**−2.38**
	N—C=O		175.30	175.27[b]	175.25	175.23			0.00	0.04	0.10	0.12
	CH_3		22.69	22.76	22.68	22.86			0.15	0.01	0.09	−0.09
βDGal	1	104.24	103.86	103.83	103.80	103.83		−0.06	−0.44	−0.43	0.10	0.07
	2	71.02	71.12	71.20	71.11	69.61		−0.02	0.42	0.29	0.09	**1.59**
	3	73.01	72.95	72.90	72.92	76.13		0.04	0.13	0.18	0.09	**−3.12**
	4	69.03	68.77	68.89[b]	68.99	67.84		−0.09	0.38	0.19	0.04	**1.19**
	5	73.81	74.07	74.14	75.68	75.54		**1.55**	**1.82**	**1.71**	0.05	0.19
	6	63.77	63.72	63.79	61.45	61.58		**−2.54**	**−2.15**	**−2.31**	0.02	−0.11
								δ(28) − δ(15)	**δ(28) − δ(26)**	**δ(28) − δ(18)**	**δ(28) − δ(26)**	**δ(28) − δ(25)**
αDNeuAc (2–6)	1	173.74	173.80	173.86	173.76	173.65	173.67	−0.07	−0.06	−0.17	−0.09	−0.03
	2	100.84	100.54	100.61	100.62	100.70	100.99	0.52	0.45	0.38	0.37	0.29
	3	40.57	40.49	40.55	40.51	40.54	40.38	−0.19	−0.11	−0.17	−0.13	−0.16
	4	68.60	68.59	68.76	68.71	68.76	68.56	−0.04	−0.03	−0.20	−0.15	−0.20
	5	52.23	52.30	52.36	52.29	52.40	52.21	−0.02	−0.09	−0.15	−0.08	−0.19
	6	73.01	72.95	72.99	72.92	73.00	72.86	−0.15	−0.09	−0.13	−0.06	−0.14
	7	68.60	68.59	68.64[b]	68.71	68.63	68.49	−0.11	−0.10	−0.15	−0.22	−0.14
	8	72.12	72.09	72.16	72.11	72.15	71.94	−0.18	−0.15	−0.22	−0.17	−0.21
	9	63.02	63.06	63.15	63.04	63.18	62.94	−0.08	−0.12	−0.21	−0.10	−0.24
	N—C=O	175.41	175.61	175.33	175.41	175.38	175.39	0.02	0.20	−0.06	−0.02	0.01
	CH_3	22.35	22.42	22.50	22.46	22.57	22.30	−0.05	−0.12	−0.20	−0.16	−0.27

[a] Chemical shift differences of over 1 ppm are indicated in bold numbers. [b] Assignments that are different from the published report.[71]

Table V. Assignments of ^{13}C Chemical Shifts for (2–3) Sialyloligosaccharides and the Differences with Those Observed for the Corresponding Asialo Compounds (Table III)[a]

sugar unit	carbon atom	chemical shifts, ppm													
		19	**20**	**21**	**22**	**27**	LSTa **23**	LSTd **24**	DSL **25**	δ(**2a**) − δ(**19**)	δ(**4**) − δ(**21**)	δ(**7**) − δ(**22**)	δ(**14**) − δ(**27**)	δ(**8**) − δ(**23**)	δ(**10**) − δ(**24**)	δ(**8**) − δ(**25**)
βDGlc	1	96.26	103.58				96.20	96.18	96.21	−0.11				0.01	0.06	0.00
	2	74.33	73.30				74.27	74.26	74.31	−0.09				0.06	0.08	0.02
	3	74.84	74.89				74.84	74.82	74.86	−0.06				0.01	0.06	0.01
	4	78.86	78.94				78.93	78.89	79.09	−0.03				0.16	0.07	0.08
	5	75.29	75.28				75.27	75.24	75.27	−0.10				−0.01	0.08	0.01
	6	60.65	60.65				60.63	60.60	60.70	−0.07				0.06	0.09	−0.01
βDGal	1						103.39	103.37	103.37					−0.01	0.03	0.01
	2						70.51	70.44	70.45					0.00	0.06	0.06
	3						82.38	82.47	82.46					0.06	0.04	0.02
	4						75.26	68.76	68.76					0.10	0.08	0.03
	5						68.69	75.34	75.46					0.09	0.03	−0.11
	6						61.41	61.41	61.48					0.01	0.07	−0.08
βDGlcNAc	1			102.48	102.21	101.82	102.88	103.04	102.86		−0.08	−0.01	−0.19	0.03	0.07	0.00
or	2			55.61	54.83	52.60	55.07	55.67	55.11		−0.07	0.08	−0.18	0.13	0.08	0.09
βDGalNAc[b]	3			73.10	83.32	80.49	82.69	72.61	82.76		−0.02	−0.10	−0.24	0.04	0.10	−0.03
	4			79.35	69.41	68.44	68.99	78.65	69.11		−0.17	−0.08	−0.08	0.01	0.28	−0.11
	5			75.42	75.99	75.24	75.73	75.03	74.26		−0.12	−0.06	−0.20	0.00	0.05	**1.47**
	6			60.86	61.38	61.48	61.08	60.40	63.48		−0.15	−0.05	−0.24	0.02	0.11	**−2.38**
	N—C=O			175.16	175.55	175.00	175.27	175.26	175.23		0.14	−0.34	−0.16	0.08	0.05	0.12
	CH_3			22.81	22.91	22.86	22.83	22.66	22.86		0.03	−0.13	−0.28	−0.06	0.09	−0.09
βDGal	1	103.17	103.20	103.30	104.00	105.02	103.82	103.14	103.83	0.12	0.12	0.05	0.12	0.08	0.26	0.07
	2	69.86	69.87	70.00	69.65	69.56	69.56	69.83	69.61	**1.52**	**1.54**	**1.57**	**1.36**	**1.65**	**1.66**	**1.59**
	3	76.03[b]	76.04	76.19	76.24	76.15	76.10	75.98	76.13	**−3.07**	**−3.11**	**−3.17**	**−3.35**	**−3.09**	**−2.97**	**−3.12**
	4	68.00	68.01	68.18	67.87	67.96	67.76	67.96	67.84	**0.97**	**0.97**	**1.19**	**0.94**	**1.27**	**1.12**	**1.19**
	5	75.65[b]	75.66	75.78	75.63	75.30	75.56	75.62	75.54	0.10	0.11	0.16	0.00	0.18	0.23	0.19
	6	61.50	61.51	61.60	61.52	61.54	61.49	61.46	61.58	−0.07	−0.03	−0.02	−0.21	0.02	0.02	−0.11
										δ(**28**) − δ(**19**)	δ(**28**) − δ(**21**)	δ(**28**) − δ(**22**)	δ(**28**) − δ(**27**)	δ(**28**) − δ(**23**)	δ(**28**) − δ(**24**)	δ(**28**) − δ(**25**)
αDNeuAc (2–3)	1	174.23	174.23	174.27	174.38	174.31	174.25	174.18	174.17	−0.56	−0.60	−0.71	−0.64	−0.58	−0.51	−0.50
	2	100.32	100.34	100.28	100.28	100.26	100.17	100.29	100.10	0.67	0.71	0.71	0.73	0.82	0.70	0.69
	3	40.19	40.21	40.37	40.39	40.34	40.30	40.14	40.31	0.19	0.01	−0.01	0.04	0.08	0.24	0.07
	4	68.79	68.80	68.88	68.85	68.86	68.79	68.76	68.84	−0.23	−0.32	−0.29	−0.30	−0.23	−0.20	−0.28
	5	52.22	52.24	52.41	52.28	52.27	52.18	52.18	52.22	−0.01	−0.20	−0.07	−0.06	0.03	−0.03	−0.01
	6	73.39	73.41	73.55	73.37	73.35	73.30	73.36	73.30	−0.53	−0.69	−0.51	−0.49	−0.44	−0.50	−0.44
	7	68.67	68.67	68.88	68.70	68.68	68.59	68.60	68.63	−0.18	−0.39	−0.21	−0.19	−0.10	−0.11	−0.14
	8	72.25	72.26	72.37	72.34	72.31	72.28	72.21	72.25	−0.32	−0.43	−0.40	−0.38	−0.34	−0.27	−0.31
	9	63.14	63.17	63.36	63.12	63.12	63.03	63.09	63.05	−0.20	−0.42	−0.18	−0.18	−0.09	−0.15	−0.11
	N—C=O	175.48	175.51	175.60	175.55	175.51	175.44	175.45	175.43	−0.09	−0.21	−0.16	−0.12	−0.05	−0.06	−0.04
	CH_3	22.55	22.56	22.68	22.61	22.60	22.52	22.51	22.57	−0.25	−0.31	−0.30	−0.30	−0.32	−0.21	−0.27

[a] Chemical shift differences of 0.9 ppm or more are indicated in bold numbers. [b] Assignments that are different from those published.[71]

in the GDGlcNAc, inner βDGal, and the DGlc residues are nearly the same as in LNNT (**10**).

αDNeuAc(2,6)βDGlcNAc Glycosides. The assignments of chemical shifts in LST_b (**25**) are made by comparison with LNT (**10**). As seen from Table IV, the effect of branching at C-6 of GlcNAc of LNT to afford LST_b has very little effect on the chemical shifts of the terminal and inner βDGal units as well as the DGlc unit. In fact, the chemical shifts of all the carbons in these units fall within a range of ±0.2 ppm of those observed for LNT. As expected, the major changes observed as compared to LNT (Table IV, column 12) are the deshielding of 2.19 ppm for C-6 and the shielding of 1.6 ppm for the C-5 of the GlcNAc unit. The changes in chemical shifts of the carbon atoms in DSL **25**, which has the component oligosaccharides of LST_a (**23**) and LST_b (**26**), are essentially the sum of those observed in the latter two compounds which indicates that in DSL there is very little interaction between the two NeuAc residues.

Discussion

Sialyloligosaccharides prepared and characterized in this report appear as terminal sequences of glycoprotein and glycolipid carbohydrate groups.[10,11,14] These compounds were conveniently prepared in highly purified form by enzymatic sialylation of neutral sugar precursors using purified mammalian sialyltransferases, demonstrating the utility of these enzymes as reagents for synthesis of biologically relevant sialosides.[31] A natural limitation on the types of sialyloligosaccharides which can be prepared in this way is the substrate specificity of the sialyltransferases. Indeed, while the α2,3 sialosides of βDGal(1,3)βDGlcNAc-O-CH_3 and βDGal(1,3)βDGalNAc-O-$(CH_2)_5COOCH_3$ could be readily prepared by using two sialyltransferases which utilize these sequences as preferred acceptor substrates, the corresponding α2,6 sialosides could not, since these two saccharides are not acceptor substrates of the sialyltransferases which form the α2,6 linkage. Nonetheless, sialosides not found in nature may also be prepared within this specificity limitation as evidenced by the synthesis of several novel sialosides (**16**, **17**, **20**, **21**, **22**, **24**, and **27**).

The amount of each sialoside prepared was generally in the range of 10–20 μmol. This is on a par with the amount obtained currently by chemical synthesis and was sufficient for characterization by ^1H and ^{13}C NMR spectroscopy. However, the advantage of the enzymatic procedure is demonstrated by the preparation of a variety of 2,3 sialosides that have not been made so far by chemical synthesis. The feasibility of larger scale synthesis depends primarily on the availability of the sialyltransferases and the expensive donor substrate CMP-NeuAc, commercially available at a cost of $1600/mmol (Sigma). By using the amount of sialyltransferase obtained from a single purification,[34] the reactions for all of the sialosides described here could be scaled up 10–1000-fold. It may be useful in the future to consider immobilizing these enzymes[72] and developing an efficient synthesis of CMP-NeuAc to improve the economy of producing sialosides enzymatically.

In view of the interest in biological roles of sialyloligosaccharides, other potential applications involving their synthesis are apparent. As shown by the synthesis of αDNeuAc(2,3)-βDGal(1,3)βDGalNAc-O-$(CH_2)_5COOCH_3$, sialyloligosaccharides may be prepared with linker arms terminated with functional groups for attachment to proteins or insoluble supports for use as immunogens or affinity adsorbants. The potential of combined chemical and enzymatic synthesis of sialyloligosaccharides containing chemically modified sialic acid derivatives has been illustrated by Brossmer et al.[73] and Conradt et al.[74] Thus, the 9-azido-9-deoxy and 9-fluoro-9-deoxy derivatives of *N*-acetylneuraminic acid were activated to their respective CMP-sialic acid conjugates and subsquently transfered to glycoproteins by crude microsomal sialyltransferase preparations. In the case of the 9-azido derivative, it should be possible to synthesize sialyloligosaccharides which would permit photoactivated coupling to the binding site of sialic acid binding proteins.

The availability of all the sialyloligosaccharides mentioned in Table I in sufficient amounts has made possible a detailed investigation by both ^1H and ^{13}C NMR spectroscopy. As seen from Figure 1, although the proton spectra of even the trisaccharides were very complex, the resolution obtained at 500 MHz was sufficient to firmly establish their structural identity. Especially for the 2,3 sialosides, the resolved signals for the H-3 of the sialylated galactose (H-3′) and the characteristic signals for the H-3 hydrogens of the NeuAc residues provided direct proof to the identity of the compounds. Furthermore, since these hydrogens are located around the sialoside linkages, the above mentioned hydrogens' signals could be used for selective saturation in nuclear Overhauser enhancement studies in order to ascertain their internuclear distances. These studies together with the ^{13}C chemical shift assignments reported in Tables III, IV, and V are expected to provide information concerning the secondary structures of these compounds in aqueous solution.[75]

As expected, the ^{13}C signals of the sialyloligosaccharides were well resolved at 125 MHz to enable a complete assignment even for the hexasaccharide DSL **25**. These ^{13}C chemical shifts were then compared with their asialo compounds, and the differences were reported (Tables IV and V) to indicate the changes that accompany the sialylation. The chemical shift differences highlight the unique properties of the sialoside linkages as compared to the patterns observed with neutral sugars.[52] For example, sialylation of a primary and a secondary hydroxyl group by a α-DNeuAc residue deshielded the corresponding aglycon only by 2–3.5 ppm, which were about half or less than half of the changes observed upon glycosylation in neutral sugars. In contrast, the shielding chemical shifts observed for the carbons vicinal to the site of sialylation was either equal or more than that normally measured for glycosylations with neutral sugars. This is seen both for 2,3 and 2,6 sialosides as shown in Tables IV and V. These characteristic effects are probably a reflection of the influence of the carboxylate moiety on the atoms around the sialoside linkage. Since the polar carboxylate group is expected to be extensively hydrated in water, it should be in close proximity to the carbons near the glycosidic linkage and likely influence their chemical shifts through steric hindrance or electrical charge.[76,77] For the 2,3 sialosides the effect of the carboxyl group is restricted to the carbon atoms near the sialoside linkage, while in 2,6 sialosides this effect is more remarkable causing unusual changes in chemical shifts of carbons in βDGlcNAc residues of compounds **17** and **18**, which are separated from the carboxy group by more than eight bonds. It has been proposed[71] that these interunit deshieldings mentioned for the above compounds are due to the "fold over" of the NeuAc residue toward the βDGal and the βDGlcNAc residues so as to bring some parts of the NeuAc residue in close proximity to the glycosidic linkage between the βGal and the βGlcNAc residue. However, such interactions should alter the ^{13}C chemical shifts of the interacting atoms of the NeuAc unit as well and, therefore, should be different from that seen for the disaccharide **15**, where such interunit interactions are not possible. Yet relative to **15**, no changes are seen for the carbon atoms of the NeuAc residues of **17** and **18**. It is interesting that the unusual effects of sialic acid are not seen for a C-6 substitution at a βDGlcNAc unit, rather than a βDGal residue, as in compounds **25** and **26**. Our recent studies on the conformation of 2,6 sialosides suggest that the unusual effects on the carbon atoms of the GlcNAc units are due to the carboxylate group alone.[75] Similar effects of the carboxylate group have been observed by Prohaska et al.[78] in the sialoglyco-

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Table VI. Comparison of ^{13}C Chemical Shifts of αDNeuAc Residues in αDNeuAc(2–3)βDGal(1–4)βDGlc–OCH_3 (**20**), αDNeuAc(2–3)βDGal(1–4)βDGlcNAc–OCH_3 (**21**), αDNeuAc(2–3)βDGal(1–3)βDGlcNAc–OCH_3 (**22**), αDNeuAc(2–3)βDGal(1–3)βDGalNAc–$O(CH_2)_5COOH_3$ (**27**), LSTa (**23**), DSL (**25**), and LSTd (**24**) with Those Observed[a] for GM_2OS, GM_1OS, and GM_1

carbon atom	chemical shifts, ppm									
	20	**21**	**22**	**27**	**23**	**25**	**24**	GM_2OS	GM_1OS	GM_1
1	174.23	174.27	174.38	174.31	174.25	174.17	174.18	174.40	174.40	174.46
2	100.34	100.28	100.28	100.26	100.17	100.30	100.29	101.90	101.95	102.24
3	40.20	40.37	40.39	40.34	40.30	40.31	40.14	37.36	37.38	37.20
4	68.80	68.88	68.85	68.86	68.79	68.84	68.76	69.00	69.01	69.03
5	52.24	52.41	52.28	52.27	52.18	52.22	52.18	51.96	51.98	52.21
6	73.41	73.55	73.37	73.35	73.30	73.30	73.36	73.40	73.42	73.18
7	68.67	68.88	68.70	68.68	68.54	68.63	68.60	68.32	68.39	68.52
8	72.26	72.37	72.34	72.31	72.28	72.25	72.21	72.58	72.58	72.55
9	63.17	63.35	63.12	63.12	63.03	63.05	63.09	63.18	63.19	63.64
N—C=O	175.51	175.56	175.57	175.50	175.44	175.43	175.46	175.39	175.40	175.37
CH_3	22.56	22.68	22.60	22.60	22.52	22.57	22.51	22.39	22.42	22.65

[a] Reproduced from the published report.[48]

peptides from human glycophorin A that have the oligosaccharide sequence αDNeuAc(2,3)βDGal(1,3)[αDNeuAc(2,6)]αDGalNAc which is linked to serine and threonine of an octapeptide. The C^γ carbon of a valine residue, separated from the carboxyl group of sialic acid at C_6 of GalNAc by more than 16 bonds, experienced unusual deshielding as compared to the asialo compound. This was explained on the basis of a secondary structure, where the C^γ methyl group of the valine residue was in the proximity and in the plane of the carboxylate moiety.

Finally, it is of interest to compare the chemical shift of the atoms around the 2,3 sialoside linkages of the linear structures vs. those reported for the branched sialosides such as in gangliosides GM_1 and GM_2.[48,77,79,80] This is especially important in view of the fact that the orientations of the αDNeuAc residues in GM_1 (βDGal(1,3)βDGalNAc(1,4)[αDNeuAc(2,3)]βDGal-(1,4)βDGlc–O–ceramide) and GM_2 (βDGalNAc(1,4)-[αDNeuAc(2,3)βDGal(1,4)βDGlc–O–ceramide) have been shown[48] to be different from those normally seen in aldopyranosides. Therefore, it is important to understand whether this unusual orientation is due to the effect of branching or it is characteristic of all the sialosides. The hard sphere exoanomeric calculations for the branched structures of GM_1 and GM_2 indicated[48] two energy minima (−165, −15, conformer 1, and −75, 10, conformer 2) that were separated from each other by over 4.2 kcals in favor of conformer 1. This was found to be largely due to a greater number of attractive interactions between the sialic acid residue and the adjacent βDGalNAc unit in conformer 1, as compared to conformer 2 to the extent of about 2.9 k cals. However, in the absence of such interactions as is the case for the linear 2,3 sialosides reported in Table I, the energy difference between these two conformers should become smaller. Consequently, the population of each conformer should also be different, and this should, therefore, reflect in the proton and C-13 chemical shifts of atoms around the sialoside linkage. In fact, changes in both ^1H and ^{13}C chemical shifts are observed. In Table VI, the ^{13}C chemical shifts for the sialic acid residues in the linear 2,3 sialosides **20**, **21**, **22**, **23**, **24**, **25**, and **27** are compared with those found for the corresponding residues in GM_2OS, GM_1OS, and GM_1.[48] As seen in Columns 2–8, for all the linear 2,3 sialosides, the carbon atom C-2 is shielded by about 1.7 ppm, while C-3 is deshielded by about 3 ppm as compared to that found in gangliosides (columns 9–11). In contrast, the chemical shift of $H3_{ax}$ of sialic acid and H-4 of the terminal galactose (H-4′, Figure 1a–e) in the linear sialosides is shielded (not shown) by about 0.35 and 0.1 ppm, respectively, as compared to gangliosides. Veluraja and Rao proposed[81] on the basis of theoretical calculations that the above mentioned differences in chemical shifts are due to the difference in the conformations around the sialoside linkages of the linear and branched sialosides. However, the NOE studies on the synthetic sialyloligosaccharides (Table I)[75] provide no support to the above proposal. It now appears on the basis of HSEA models[75] that in the linear sialosides there is a greater conformational flexibility around the sialoside linkage as opposed to the rigid branched structures. This is especially important, since in the proposed conformation of the gangliosides, there is a severe interaction between atoms at C-3 of sialic acid and at C-3 of the adjacent galactose which cannot be relieved by a change in the torsion angles around the sialoside linkage or by a change in the valance angle of the glycosidic oxygen (as the NeuAc unit would bump into the adjacent βDGalNAc unit). However, these are possible in the linear sialosides as they appear to be more flexible and, consequently, the average conformer in solution might be devoid of such interactions. Experimental evidence in support of the above proposals has been obtained and this will be reported separately.[75]

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