^{1}H NMR Studies of a Biosynthetic Lacto–Ganglio Hybrid Glycosphingolipid: Confirmation of Structure, Interpretation of "Anomalous" Chemical Shifts, and Evidence for Interresidue Amide–Amide Hydrogen Bonding†

Steven B. Levery,*,‡ Eric H. Holmes,§ Dwayne D. Harris,‡ and Sen-itiroh Hakomori‡

The Biomembrane Institute and University of Washington, 201 Elliott Avenue West, Seattle, Washington 98119, and Pacific Northwest Research Foundation, 720 Broadway, Seattle, Washington 98122

Received May 16, 1991; Revised Manuscript Received October 21, 1991

ABSTRACT: Glycosphingolipids bearing GlcNAcβ1→3 and GalNAcβ1→4 linked to β-Gal of lactosylceramide (lacto–ganglio hybrids), first isolated from a murine myelogenous leukemia cell line [Kannagi, R., Levery, S. B., & Hakomori, S. (1984) *J. Biol. Chem. 259*, 8444–8451], have since been found as normal components of mullet roe and English sole liver. In order to clarify the biosynthetic pathways responsible for its occurrence both as a product of normal tissues and as a possible mammalian cancer-associated antigen, the lacto–ganglio hybrid core structure $LcGg_4Cer$ was synthesized from Lc_3Cer using a GalNAcβ1→4 transferase preparation from English sole liver. A preliminary characterization of the enzyme, which may be identical to the GalNAc T-1 responsible for synthesis of GM_2 ganglioside, is presented. The enzymatically synthesized product was analyzed by 1- and 2-D ^{1}H NMR spectroscopy, confirming its primary structure as GalNAcβ1→4-(GlcNAcβ1→3)Galβ1→4Glcβ1→1Cer. In addition to assigning all nonexchangeable glycosyl proton resonances, measurements of several properties of the amide NH protons, including chemical shift, coupling constants, exchange rates, and temperature shift coefficients, were obtained and compared to those in the simpler constituent triglycosylceramides, Lc_3- and Gg_3Cer. An approximate three-dimensional structure for $LcGg_4Cer$ is proposed, consistent with all data obtained, which should be useful in discussing the results of ^{1}H NMR analysis of compounds containing this core tetrasaccharide. The structure is characterized by an unusual arrangement of terminal *N*-acetylhexosamine residues, resulting in a π–H hydrogen-bonding interaction between their acetamido groups.

Glycosphingolipids bearing GlcNAcβ1→3 and GalNAcβ1→4 linked to β-Gal of lactosylceramide (see Figure 1) are hybrids of the lacto- and ganglio series (*neo*-lactoganglio when extended by Galβ1→4 to β-GlcNAc). First isolated from a murine myelogenous leukemia cell line (M1) in its undifferentiated form (Kannagi et al., 1983, 1984), glycosphingolipids of this type were subsequently found in mullet roe (DeGasperi et al., 1987) and in the liver of English sole (Ostrander et al., 1988a) (see Table I). Lactoganggliotetraosylceramide ($LcGg_4Cer$)[1] has been synthesized both chemically (Shigeta et al., 1987) and enzymatically, using lactotriaosylceramide (Lc_3Cer) and a β-GalNAc transferase from guinea pig bone marrow (Das et al., 1987a). It is thus possible that, once the enzymatic pathways governing its biosynthesis are clarified, and the signs of its presence become more familiar to analytical biochemists using modern techniques such as high-resolution NMR, this glycosphingolipid core structure, which was at first considered a novelty confined to a unique in vitro murine system, may be found to be distributed in a wider variety of normal (and abnormal) tissues.

We report the results of studies in both of these areas (biosynthesis and NMR), which are closely related in that both are considerably illuminated by an appreciation of three-dimensional structure. These results include (1) a preliminary characterization of a GalNAcβ1→4 transferase from English sole liver which may produce both $LcGg_4Cer$ and GM_2 ($II^3NeuAcGg_3Cer$) in vivo; (2) use of this enzyme to synthesize in vitro enough $LcGg_4Cer$ to facilitate a thorough characterization using 1- and 2-D ^{1}H NMR techniques, including (3) complete assignment of all nonexchangeable glycosyl protons; (4) confirmation of saccharide linkage structure by detection of interglycosidic NOEs; and (5) assignment of amide NH protons and measurement of several of their properties, including exchange rates and chemical shift temperature dependencies. We propose an approximate three-dimensional structure for $LcGg_4Cer$, derived with aid of simplified molecular mechanics calculations, which is consistent with all available data and which can be used as a starting point in a discussion of the substrate specificity of the glycosyltransferase responsible for its synthesis. It is further hoped

† This study was supported by funds from National Institutes of Health Outstanding Investigator Grant CA42505 (to S.H.), National Institute of Environmental Health Sciences/NIH Research Grant ES04370 (to E.H.H.), and funds from The Biomembrane Institute, in part under a research contract from Otsuka Pharmaceutical Co. E.H.H. is supported by National Cancer Institute Research Career Development Award K04 CA01343.

* Author to whom correspondence should be addressed.

‡ The Biomembrane Institute.

§ Pacific Northwest Research Foundation.

[1] Abbreviations: Gg_3Cer, gangliotriaosylceramide (GalNAcβ1→4Galβ1→4Glcβ1→1Cer); Lc_3Cer, lactotriaosylceramide (GlcNAcβ1→3Galβ1→4Glcβ1→1Cer); $LcGg_4Cer$, lactoganggliotetraosylceramide (GalNAcβ1→4[GlcNAcβ1→3]Galβ1→4Glcβ1→1Cer); nLc_4Cer, lacto-*neo*-tetraosylceramide (Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer); GM_2, $II^3NeuAcGg_3Cer$ (GalNAcβ1→4[NeuAcα2→3]Galβ1→4Glcβ1→1Cer); GM_3, $II^3NeuAcLacCer$ (NeuAcα2→3Galβ1→4Glcβ1→1Cer); CM, chloroform–methanol; CMW, chloroform–methanol–water; IHW, isopropanol–hexane–water, EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PBS, phosphate-buffered saline (8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4); TLC, thin-layer chromatography; HPTLC, high-performance thin-layer chromatography; HSEA, hard sphere/exoanomeric effect; NOE, nuclear Overhauser effect; NOESY, 2-D nuclear Overhauser effect spectroscopy; PS, phase sensitive; DQF, double-quantum filtered; COSY, correlation spectroscopy; TOCSY, total correlation spectroscopy; TPPI, time-proportional phase increments.

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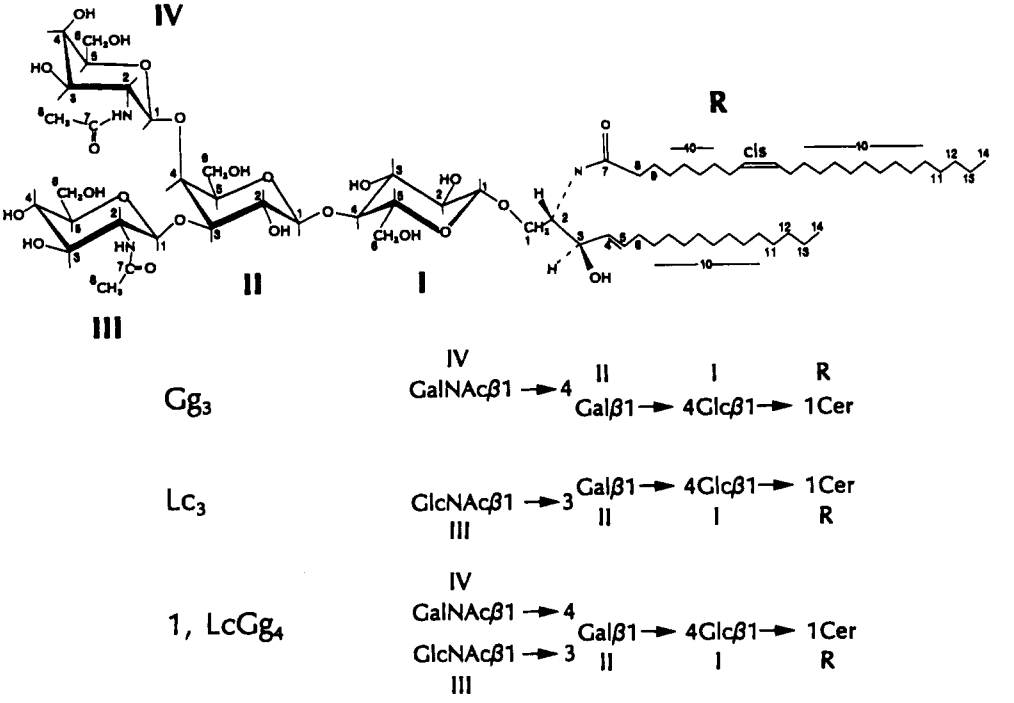


FIGURE 1: Structure and numbering for lacto-ganglio series core ($LcGg_4$) and constituent glycosphingolipids, Gg_3 and Lc_3.

Table I: Structures of Glycosphingolipids with (neo-)Lacto-Ganglio Series Core Structures

1[a,b]	GalNAcβ1→4 Galβ1→4Glcβ1→1Cer GlcNAcβ1→3
2[b]	GalNAcβ1→4 Galβ1→4Glcβ1→1Cer Galα1→3Galβ1→4GlcNAcβ1→3
3[c]	GalNAcβ1→4 Galβ1→4Glcβ1→1Cer Fucα1→3GalNAcβ1→3Galα1→3Galβ1→4GlcNAcβ1→3
4[d]	GalNAcβ1→4 Galβ1→4Glcβ1→1Cer NeuAcα2→3Galβ1→4GlcNAcβ1→3
5[d]	GalNAcβ1→4 GalNAcβ1→4 Galβ1→4Glcβ1→1Cer Galβ1→4GlcNAcβ1→3 NeuAcα2→3

[a] Kannagi et al. (1983). [b] Kannagi et al. (1984). [c] Ostrander et al. (1988a). [d] DeGasperi et al. (1987).

that by correlating the three-dimensional structure with the ^1H NMR data, it can be shown that these results, which include some unusual chemical shift effects, are consistent with ordinary knowledge of oligosaccharide stereochemistry, making the data more useful to workers who may encounter related structures and who may use 1- or 2-D ^1H NMR as a primary tool in their elucidation.

Experimental Procedures

Materials. English sole were obtained from local waters by otter trawl. UDP-*N*-acetylgalactosamine, deoxycholate, Triton X-100, Triton CF-54, and Brij-58 were obtained from Sigma Chemical Co., St. Louis, MO. UDP-[^{14}C]*N*-acetylgalactosamine (303 mCi/mmol) was obtained from Amersham (Arlington Heights, IL). *Neo*-lactotetraosylceramide (nLc_4Cer) was prepared by desialylation of sialosyl-*neo*-lactotetraosylceramide, which was prepared from bovine erythrocytes (Chien et al., 1978). Desialylation was performed in 1% acetic acid at 100 °C for 1 h. Lc_3Cer was prepared from nLc_4Cer by overnight hydrolysis with jackbean β-galactosidase in 0.1 M citrate buffer, pH 4.5, containing 0.1% taurodeoxycholate. A similar sequence of reactions was used to produce Gg_3Cer from GM_1 ganglioside (Sigma), except that β-galactosidase from bovine testis was employed. Released sialic acid and unreacted ganglioside were removed following acid treatment by passage through activated DEAE Sephadex in solvent A (CMW, 30:60:8). The products following β-galactosidase digestion were purified by a sequence of solid-phase extraction on a C_{18}-silica cartridge (Analytichem International, Harbor City, CA), passage through DEAE Sephadex (to remove deoxytaurocholate), and a second solid-phase C_{18}-silica extraction (to remove salts released by the DEAE Sephadex). Lactosylceramide, globotriaosylceramide, and globoside were obtained from human erythrocytes. GM_3 was obtained from canine erythrocytes. The detergent G-3634-A was a gift from Dr. Subhash Basu, Notre Dame University, Notre Dame, IN. DMSO-d_6 (Aldrich) and D_2O (Cambridge Isotope Laboratory) were both obtained at 99.96 atom % purity. All other reagents were of the highest purity commercially available.

Preparation of Golgi Membrane-Rich Fraction from English Sole Liver. All procedures were carried out at 0–4 °C using a modification of the procedure previously described (Senn et al., 1981). Frozen liver (8–10 g) was thawed, minced, and homogenized in two volumes of 50 mM HEPES–NaOH buffer, pH 7.0, containing 0.5 M sucrose and 1 mM EDTA with two strokes of a Potter–Elvehjem homogenizer. The crude homogenate was centrifuged at 1000*g* for 10 min. The resulting supernatant fraction was removed, and the 1000*g* pellet was washed twice with one volume of the homogenization buffer. The combined 1000*g* supernatant fractions were then centrifuged at 27000*g*, the supernatant was removed, and the pellet fraction was resuspended in 7 mL of the homogenization buffer by one stroke of the homogenizer. This

suspension was transferred to another centrifuge tube, applying it to the top of 5 mL of a solution containing 50 mM HEPES-NaOH buffer, pH 7.0, 1.2 M sucrose and 1 mM EDTA as a cushion. This was then centrifuged at 94000*g* for 90 min in an AH-627 swinging bucket rotor (Sorvall, Newton, CT). The Golgi membrane-rich fraction was isolated from the interface between the 0.5 and 1.2 M sucrose layers and was washed first with homogenization buffer containing 0.25 M sucrose and then with distilled water, with collection by centrifugation at 27000*g* for 30 min between each step. The Golgi membrane-rich fraction was finally suspended in homogenization buffer and utilized for subsequent studies.

β1→4-N-Acetylgalactosaminyltransferase Assay. The β1→4-*N*-acetylgalactosaminyltransferase activity of membrane fractions was determined in reaction mixtures containing 2.5 μmol HEPES buffer, pH 7.2, 30 μg of acceptor glycolipid, 100 μg of G-3634-A, 1 μmol of $MnCl_2$, 17 nmol of UDP-[^{14}C]*N*-acetylgalactosamine (15 000 cpm/nmol), and 0.3–0.5 mg of protein in a total volume of 0.1 mL. The reaction mixtures were incubated for 2 h at 25 °C and terminated by addition of 6 μmol of EDTA and 0.1 mL of 2:1 CM. The reaction mixtures were quantitatively streaked onto a 4 cm wide strip of Whatman 3 paper and developed with water overnight. The papers were dried, the origins were removed, and the labeled ganglioside product was extracted with 2–5-mL washes of 10:5:1 CMW. The combined eluates were concentrated to dryness by nitrogen stream and dissolved in 20 μL of 2:1 CM. A 10-μL aliquot of each was spotted onto a TLC plate and developed in a solvent system of 60:40:9 CMW, 0.02% $CaCl_2 \cdot 2H_2O$. The radioactive products were located by autoradiography, scraped from the plate, and quantitated by liquid scintillation counting.

One unit of activity is defined as 1 pmol of UDP-[^{14}C]*N*-acetylgalactosamine transferred per hour under assay conditions.

Protein Determination. Protein concentrations were determined by the method previously described (Lowry et al., 1951), using BSA standard.

Isolation of Reaction Products from Preparative Scale in Vitro Biosynthesis. Reaction products from scaled-up reaction mixtures were initially transferred to Spectra/Por-3 dialysis tubing and dialyzed extensively with water to remove salts and unreacted labeled sugar nucleotide. The dialyzed product was concentrated to dryness, dissolved in 2:1 CM, and subjected to preparative TLC on HPTLC plates (Merck) using a solvent system of 60:40:9 CMW with 0.02% $CaCl_2 \cdot 2H_2O$. The labeled product was located by autoradiography, scraped from the plate, and eluted from the silica gel with 55:25:20 IHW (upper phase removed). The reaction product was then concentrated to dryness, dissolved in chloroform, and chromatographed on a 2-mL resin bed volume Bio-Sil A column equilibrated in chloroform. The glycolipid bound to the Bio-Sil A column was eluted by increasing amounts of methanol in the elution solvent. The reaction product from transfer of *N*-acetylgalactosamine to Lc_3Cer eluted at a solvent composition of 7:3 CM.

Proton Nuclear Magnetic Resonance Spectroscopy. Samples (≈1 mg of $LcGg_4Cer$, 2 mg of Lc_3Cer, and 2 mg of Gg_3Cer) were deuterium-exchanged by repeated lyophilization from DMSO-d_6–D_2O (98:2) and dissolved in 0.4 mL of this solvent for ^{1}H NMR spectroscopy (Dabrowski et al., 1980). All spectra were recorded at 308 ± 2 K on a Bruker (W. Germany) AM-500 Fourier transform spectrometer/Aspect 3000 data system, using quadrature detection. The residual HOD resonance was suppressed using a presaturation pulse during the preparatory delay (PD) period. For the 1-D spectrum, the sweep width was 5000 Hz, collected over 16K data points, and PD was 2.0 s. A Lorentzian to Gaussian transformation was used for resolution enhancement.

One-dimensional transient NOE spectra (Gordon & Wüthrich, 1978) were obtained by selective inversion–recovery in the difference mode (SIR-ΔNOE) (Andersen et al., 1987). Eight mixing times (from 10 to 800 ms) were used; PD was 2.0 s, the sweep width was 5000 Hz, collected over 16K data points.

Pure absorption PS-COSY spectra were recorded using the method of time-proportional phase increments (TPPI) (Marion & Wüthrich, 1983), with double-quantum filtering (DQF) (Piantini et al., 1982; Rance et al., 1983). Time domain spectra were acquired with a sweep width of 3200 Hz over 4K data points in t_2 and 1K experiments in t_1. Sixteen transients were accumulated per t_1 with a PD of 2.0 s. The time domain data were transformed as a 2K (real) × 2K matrix with phase-shifted sine-bell apodization applied in both dimensions ($\pi/3$ in t_2 and t_1).

One-step relayed coherence transfer spectroscopy (RELAY) (Eich et al., 1982) was performed using a mixing time (τ) of 52 ms, which was chosen to be optimal for $^3J_{1,2} + {}^3J_{2,3} = 19$ Hz (Bax & Drobny, 1985), a compromise value for the expected β-GlcNAc and β-GalNAc couplings. A total of 512 t_1 experiments were collected with 64 transients per t_1 and a PD of 1.5 s. An increment to t_2 of 312 μs per experiment gave a $t_{2max} = 160$ ms. The data were transformed as a 2K × 1K matrix, with a non-phase-shifted sine-bell window function applied in both dimensions, as recommended (Bax & Drobny, 1985).

TOCSY spectra (Braunschweiler & Ernst, 1983) were recorded using the MLEV-17 pulse scheme described by Bax and Davis (1985), with a total mixing time of 100 ms. A total of 950 TPPI experiments were collected, with 48 transients per t_1 and a PD of 1.7 s. The data were transformed as a 2K × 1K matrix with phase-shifted sine-bell apodization applied in both dimensions ($\pi/3$ in t_2 and t_1).

Homonuclear PS-NOESY spectra were recorded in the TPPI mode (Bodenhausen et al., 1984), with a mixing time (τ) of 300 ms. A total of 512 t_1 experiments were collected with 96 transients per t_1 and a PD of 2.0 s. A random variation of τ of ±20% was introduced to cancel scalar correlation effects. The data were transformed as a 2K × 1K matrix with phase-shifted sine-bell apodization applied in both dimensions ($\pi/3$ in t_2 and t_1).

1-D and 2-D PS-DQF-COSY experiments were also performed in DMSO-d_6–H_2O (98:2), using a sweep width of 7000 Hz. Chemical shifts of NH protons were measured at 5-deg intervals from 303 to 328 K. The results were plotted and slopes calculated by single-parameter linear regression.

Deuterium exchange experiments were performed at 298 ± 2 K. Samples were dissolved in pure DMSO-d_6, and exchange was initiated by addition of a volume of D_2O equivalent to 2% of the total volume. "t_0" spectra were obtained at 4 min from exchange initiation, and at 1-h intervals thereafter (referenced to true t_0).

Conformational Analysis. Minimum energy conformational calculations were performed using the GESA version (Paulsen et al., 1984) of the hard sphere/exoanomeric effect (HSEA) program (Thøgersen et al., 1982; Lemieux et al., 1982), kindly provided by Dr. Bernd Meyer of the UGA Complex Carbohydrate Research Center, Athens, GA.

In defining glycosidic torsion angles ϕ and ψ, the common non-IUPAC system is used; that is, for neutral α- or β-D-

pyranosides, ϕ is the dihedral angle defined by H-1′–C-1′–O-1′–C-x and ψ is the dihedral angle defined by C-1′–O-1′–C-x–H-x.

Results

Enrichment of β1→4-N-Acetylgalactosaminyltransferase Activity in Membrane Fractions from English Sole Liver

Previous results (Ostrander et al., 1988b) indicated that GM_2 was the major ganglioside found in English sole liver, comprising 80% of the total ganglioside. For this reason, English sole liver was chosen at a convenient source for β1→4-*N*-acetylgalactosaminyltransferase activity. Golgi-rich membrane fractions, prepared as described under Experimental Procedures, contained 20% of the total activity but had a specific activity 4-fold higher than the crude liver homogenate at 476 pmol/(h·mg of protein). This membrane fraction was utilized for characterization of enzyme activity in all studies described.

Effects of Reaction Conditions on Activity

pH and Buffer. Transfer of GalNAc to GM_3 as a function of pH and buffer was tested. The enzyme was active over a pH range from about 6.5 to 8.0. The highest activity was obtained at pH 7.0–7.2 with HEPES–NaOH buffer.

Detergent. The effect of various detergents on transfer of GalNAc to GM_3 was also tested. High activity was observed in the absence of added detergent and was diminished 1.3–38-fold by a variety of nonionic and ionic detergents when present at a 0.1% final concentration. Weakly to moderately inhibiting were Triton X-100, Triton CF-54, octyl glucoside, and deoxycholate, while strong inhibition was observed with Empigen BB and Brij 58. In contrast, the cationic detergent G-3634-A provided a slight (≈25%) stimulation of the activity compared to control (no detergent). Variation of the amount of G-3634-A added indicated that 0.1% was the optimal final concentration for enzyme activation.

Reaction Requirements. The complete assay system was composed of HEPES–NaOH buffer, pH 7.2, 30 μg of GM_3, 0.1% G-3634-A, 10 μM Mn^{2+}, and 170 μM UDP-[^{14}C]GalNAc. Removal of detergent or exogenous GM_3 acceptor resulted in decreased transfer. Supplementation with CDP-choline, useful to protect the labeled sugar nucleotide from hydrolytic activity, caused marked reduction in activity, perhaps due to weak competitive binding with the sugar nucleotide. The enzyme requirement for divalent Mn^{2+} was near absolute, as addition of EDTA or substitution of a variety of alternate divalent metal ions, yielded activity which was 29–200-fold lower than that obtained in the presence of Mn^{2+}. Supplementation of the reaction mixture with a variety of purified phospholipids in each case resulted in decreased activity compared to that found in their absence. Under the optimal conditions determined, the reaction was linear with respect to both time and protein concentration.

Incubation Temperature. Analysis of the variation of the reaction rate with temperature was conducted in order to determine an optimal assay temperature. It was observed that the greatest transfer of GalNAc occurred at 25 °C and declined rapidly at temperatures that would be physiological for mammals. Enzyme assays were therefore routinely conducted at 25 °C.

Acceptor Specificity. Transfer of GalNAc to a variety of potential acceptors, catalyzed by the Golgi-rich membrane fraction from English sole liver, was studied, and the results of these experiments are summarized in Figure 2. The enzyme was found to transfer GalNAc effectively to Gal residues in β1→4 linkage, whether these were present as terminal sugars

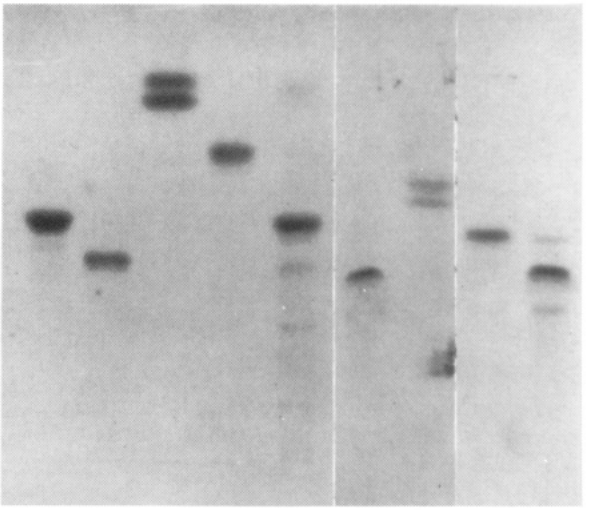


FIGURE 2: TLC analysis of reaction products with differing acceptors catalyzed by English sole β1→4-*N*-acetylgalactosaminyltransferase. Lane 1, standard GM_3; lane 2, standard GM_2; lane 3, standard lactosylceramide; lane 4, standard Lc_3; lane 5, standard nLc_4. Lanes 6–9 are autoradiographs of reaction products from transfer of [^{14}C]*N*-acetylgalactosaminyltransferase to the indicated acceptor. Lane 6, transfer to GM_3; lane 7, transfer to lactosylceramide; lane 8, transfer to Lc_3; lane 9, transfer to nLc_4. The plate was developed in a solvent system composed of 60:40:9 CMW, 0.02% $CaCl_2 \cdot 2H_2O$. Standard glycolipids were visualized by orcinol spray.

(as in lactosylceramide) or were already glycosylated by NeuAcα2→3 (GM_3) or by GlcNAcβ1→3 (Lc_3Cer or nLc_4Cer). The specific activity of the enzyme preparation with these acceptors was found to be 153, 249, 155, and 269 pmol/(h·mg of protein), respectively. No transfer to globo-series structures Gb_3- or Gb_4Cer (bearing a terminal β1→3GalNAc) was observed.

Kinetics. Saturation of the GalNAc β1→4 transferase with both donor and acceptor substrates was studied and indicated an apparent K_m for UDP-GalNAc of 40 μM when GM_3 was used as acceptor and apparent K_m values for acceptors GM_3, Lc_3Cer, and nLc_4Cer of 55, 50, and 75 μM, respectively.

Transfer of GalNAc to saturating concentrations of GM_3 and Lc_3Cer were not additive when both acceptors were present, indicating that the same enzyme is most probably responsible for transfer to the alternate acceptors.

Preparative Synthesis of the Lacto–Ganglio Hybrid Structure $LcGg_4Cer$

Preparative scale biosynthetic reaction mixtures were defined to prepare milligram quantities of the biosynthetic product from transfer of GalNAc to acceptor Lc_3Cer. The reaction mixtures contained 25 μmol of HEPES–NaOH buffer, pH 7.2, 1 mg of G-3634-A, 170 nmol of UDP-[^{14}C]GalNAc (500 cpm/nmol), 2 mg of Lc_3Cer, and enzyme in a total volume of 1 mL. The reaction was incubated at 25 °C for 48 h, and the labeled reaction product was isolated as described under Experimental Procedures. The total product from 20 individual reaction mixtures was obtained and pooled to yield 1 mg of $LcGg_4Cer$ for further chemical analysis.

Proton Nuclear Magnetic Resonance Spectroscopy

The biosynthetic product was initially characterized by ^{1}H NMR spectroscopy at 308 K in DMSO-d_6 containing 2% D_2O (Figure 3) or H_2O (for obtaining resonances of exchangeable amide protons). Expanded downfield regions of the spectrum are reproduced in Figures 4 and 5, along with those of Gg_3Cer and the precursor Lc_3Cer, presented for comparison as well

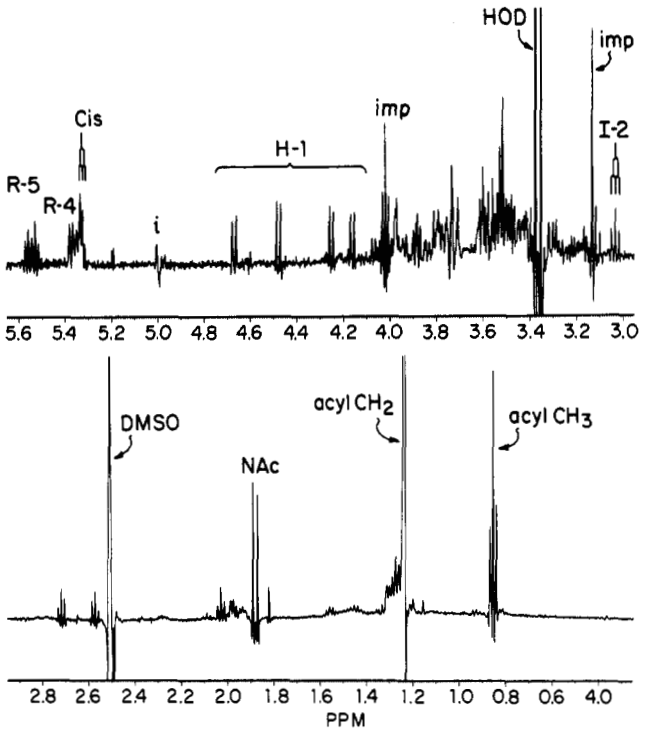


FIGURE 3: Resolution-enhanced ^1H NMR spectrum of biosynthetic $LcGg_4Cer$. The spectrum was obtained at 308 ● 2 K in DMSO-d_6 containing 2% D_2O. Signals marked "imp" are attributed to an unknown impurity; *i*, interference spike; DMSO and HOD, residual solvent proton signals.

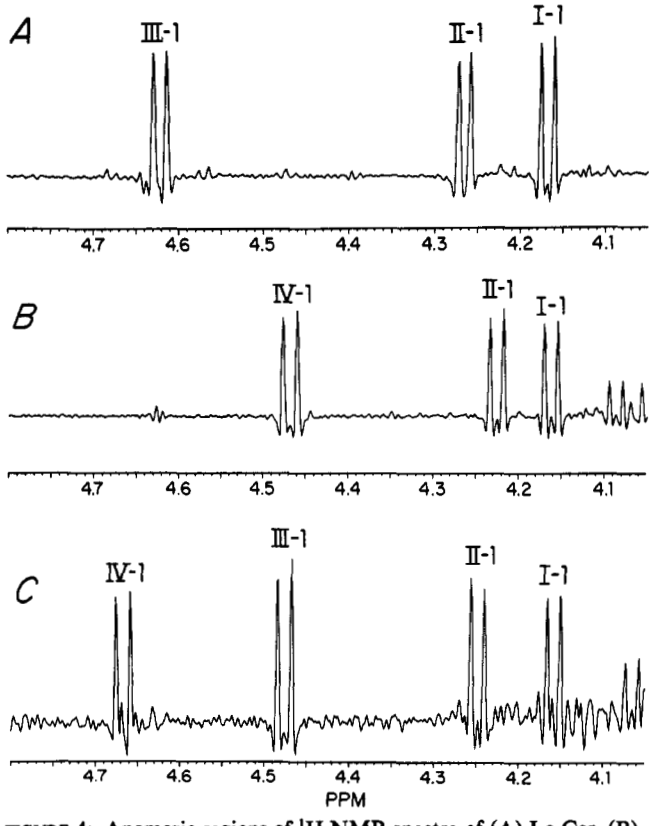


FIGURE 4: Anomeric regions of ^1H NMR spectra of (A) Lc_3Cer, (B) Gg_3Cer, and (C) biosynthetic $LcGg_4Cer$. Spectra were obtained at 308 ● 2 K in DMSO-d_6 containing 2% D_2O.

as verification of their identity and purity. Four β-anomeric resonances ($^3J_{1,2}$ = 7–9 Hz) can be observed for the product in the anomeric region (Figure 4C), at chemical shifts virtually identical to those found previously for natural $LcGg_4Cer$ from

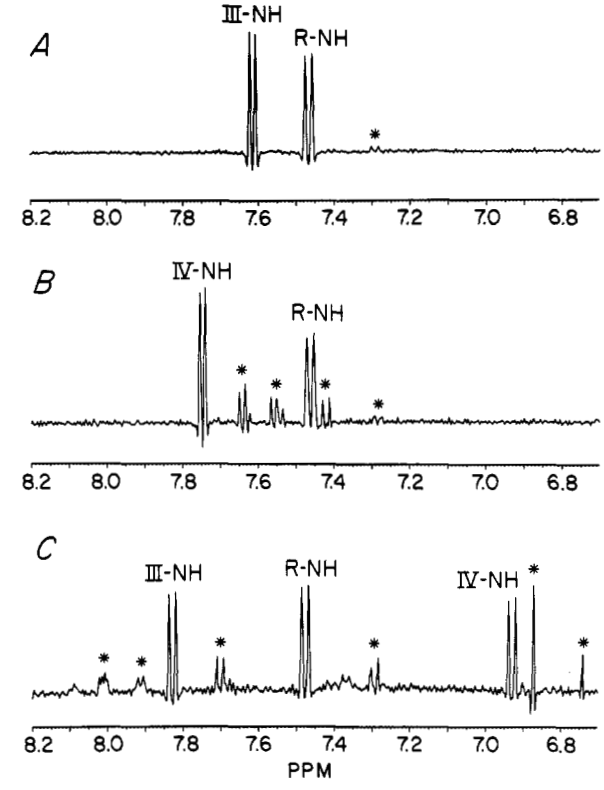


FIGURE 5: Amide proton regions of ^1H NMR spectra of (A) Lc_3Cer, (B) Gg_3Cer, and (C) biosynthetic $LcGg_4Cer$. Spectra were obtained at 308 ± 2 K in DMSO-d_6 containing 2% H_2O.

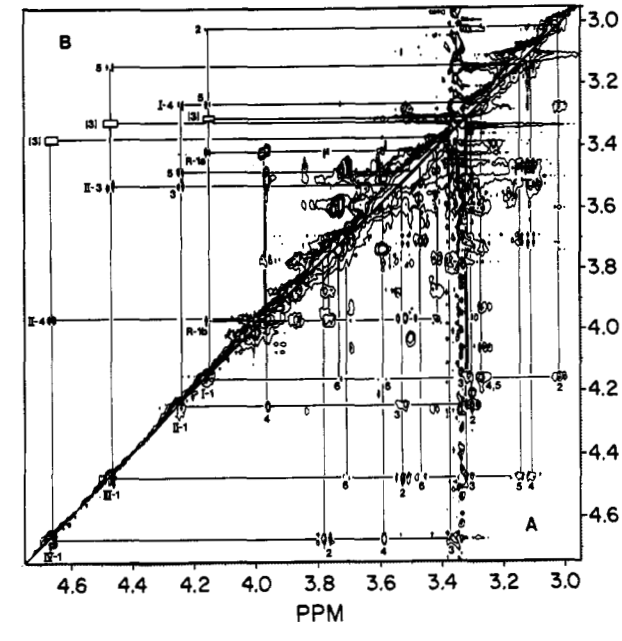


FIGURE 6: (A) Section (4.75–2.95 ppm) of the TOCSY spectrum of biosynthetic $LcGg_4Cer$. (B) Section of the TPPI mode PS-NOESY spectrum of biosynthetic $LcGg_4Cer$.

M1$^-$ cells (Kannagi et al., 1984), as well as for the chemically synthesized glycosphingolipid (Shigeta et al., 1987). The presence of an additional amino sugar is also indicated by the observation of a second 2-acetamido methyl resonance and a third amide NH signal in the spectrum (Figures 3 and 5C). In order to provide further confirmation of structure, as well as to clarify points of dispute concerning chemical shift assignments (DeGasperi et al., 1987), the product was subjected to a sequence of 2-D ^1H–^1H correlation experiments, including PS-DQF-COSY, RELAY, and TOCSY (see Figure 6A). Complete chemical shift assignments, along with a great

Table II: Proton Chemical Shifts [ppm from $(CH_3)_4Si$] for Gg_3Cer,[a] Lc_3Cer,[b,c] and $LcGg_4Cer$[b] in DMSO-d_6–D_2O (98:2)

GalNAcβ1⟶4 (IV); GlcNAcβ1⟶3 (III); Galβ1⟶4Glcβ1⟶1Cer (II, I, R)

	IV	III	II	I	R
H-1	4.462		4.222	4.161	3.987, 3.442
H-2	3.614		3.245	3.036	3.768
H-3	3.519		3.519	3.335	3.870
H-4	3.614		3.789	3.288	5.346
H-5	3.335		3.500	3.293	5.535
H-6	3.400		3.478	3.598	
H-6′	3.478		3.604	3.745	
NH[d]	7.762				7.475
NAc	1.884				
H-1		4.621	4.265	4.168	3.976, 3.420
H-2		3.350	3.421	3.045	3.775
H-3		3.335	3.451	3.325	3.880
H-4		3.095	3.839	3.300	5.353
H-5		3.105	3.475	3.289	5.542
H-6		3.447	3.527	3.606	
H-6′		3.656	3.527	3.745	
NH[d]		7.629			7.476
NAc		1.837			
H-1	4.666	4.475	4.247	4.158	3.974, 3.419
H-2	3.792	3.542	3.314	3.032	3.775
H-3	3.382	3.324	3.542	3.320	3.880
H-4	3.600	3.115	3.971	3.285	5.353
H-5	3.185	3.170	3.493	3.285	5.540
H-6	3.493	3.481	3.593	3.606	
H-6′	3.578	3.719	3.411	3.744	
NH[d]	6.929	7.828			7.476
NAc	1.887[e]	1.867[e]			

[a] Obtained from Koerner et al. (1983a) (303 K). [b] Obtained in the present study (308 K). [c] Partial assignment previously obtained by Dabrowski et al. (1980) at 338 K. [d] Obtained for the present study in DMSO-d_6–H_2O, 98:2, 308 K. [e] Connectivity established by 1-D SIR-ΔNOE experiment.

Table III: $^3J_{1,2}$ Coupling Constants (Hz) for Methine Protons of Oligosaccharide Residues I–IV and Amide NH Protons of Gg_3Cer,[a] Lc_3Cer,[b] and $LcGg_4Cer$[b] in DMSO-d_6–D_2O (98:2)

GalNAcβ1⟶4 (IV); GlcNAcβ1⟶3 (III); Galβ1⟶4Glcβ1⟶1Cer (II, I, R)

	IV	III	II	I	R
$J_{1,2}$	8.4		7.8	7.8	
$J_{2,3}$	10.0		8.9	8.2	
$J_{3,4}$	2.5		2.0	9.4	
$J_{4,5}$	<1.5		<1.5	10.3	
$J_{2,NH}$	6.9				9.5
$J_{1,2}$		7.9	7.3	7.9	
$J_{2,3}$		ND[c]	ND	8.1	
$J_{3,4}$		ND	2.4	ND	
$J_{4,5}$		ND	<1.5	ND	
$J_{2,NH}$		7.7			9.5
$J_{1,2}$	8.6	8.1	7.8	7.8	
$J_{2,3}$	10.4	9.8	10.1	7.8	
$J_{3,4}$	2.8	9.5	2.4	ND	
$J_{4,5}$	<1.5	9.2	<1.5	ND	
$J_{2,NH}$	9.5	8.6			9.5

[a] Obtained from Koerner et al. (1983a) (303 K). [b] Obtained in the present study (308 K). [c] ND, not determined.

number of two- and three-bond coupling constants, were thus obtained. These are presented in Tables II and III, compared with data for Lc_3Cer, obtained under identical conditions, and data for Gg_3Cer, previously published by Koerner et al. (1983a).

It is apparent from inspection of these data that the spin system originating from H-1 at 4.475 ppm is of a 2-acetamido-2-deoxyhexopyranose in the β-gluco configuration, while that arising from H-1 at 4.666 ppm has the β-galacto configuration. In the latter case, the equatorial orientation of H-4 yields a characteristic peak shape for this resonance, due to the small coupling with H-3 and almost negligible coupling with H-5 (both axial) (Dabrowski et al., 1980; De Bruyn & Anteunis, 1976). A further consequence of the lack of significant H-4/H-5 coupling is that coherence can not be efficiently transferred between them; therefore, while one can trace the coupling pathway of a β-Glc(NAc) residue from H-1 all the way to H-6 in a typical TOCSY experiment, it is difficult to follow the spin system of a β-Gal(NAc) residue beyond H-4 from H-1 (Lerner & Bax, 1987; Inagaki et al., 1987, 1989; Dabrowski et al., 1987, 1988a). These characteristics are evident in the TOCSY of Figure 6A. Clearly, therefore, the β-GlcNAc H-1 resonance has been shifted significantly upfield from its position in Lc_3Cer ($\Delta\delta = -0.146$ ppm) upon addition of the β-GalNAc residue, while the β-GalNAc H-1 resonates considerably downfield ($\Delta\delta = +0.204$ ppm) from its chemical shift in Gg_3Cer. Inspection of Table IV shows that other substantial shift changes occur. Of particular interest are the magnitudes of the shift changes for the β-GlcNAc and β-GalNAc NH proton resonances. Changes in the $^3J_{2,NH}$ coupling constants are also apparent (see Table III).

Table IV: Glycosylation-Induced Chemical Shift Effects for $LcGg_4Cer$ Tetraosylceramide Relative to the Constituent Triaosylceramides Gg_3Cer and Lc_3Cer (Derived from Data in Table II)

GalNAcβ1⟶4 (IV); GlcNAcβ1⟶3 (III); Galβ1⟶4 (II)

glycosylation	proton	IV	III	II
Lc_3Cer	H-1		−0.146	−0.018
+	H-2		+0.192	−0.107
GalNAc IV	H-3		−0.011	+0.091
↓	H-4		+0.020	+0.132
$LcGg_4Cer$	H-5		+0.065	+0.018
	NH		+0.199	
	CH_3		+0.030	
Gg_3Cer	H-1	+0.204		+0.025
+	H-2	+0.178		+0.069
GlcNAc III	H-3	−0.137		+0.023
↓	H-4	−0.014		+0.182
$LcGg_4Cer$	H-5	−0.150		−0.007
	NH	−0.833		
	CH_3	+0.003		

The increased splitting of the GalNAc H–N resonance in $LcGg_4$ vs Gg_3Cer indicates a conformational change of the GalNAc acetamido group. The value of 6.9 Hz measured for this proton coupling in Gg_3Cer corresponds either to a stable dihedral angle close to ±140° (Bystrov et al., 1973; Pardi et al., 1984) or to averaging over two or more low-energy rotamers (Acquotti et al., 1990). The PCILO calculations of Yadav and Luger (1983) on GalNAc indicated that the conformation of lowest energy was to be found with a HCNH torsion angle of 160° ($\Phi_{IUPAC} = -140°$),[2] stabilized by a hydrogen bond between the acetamido carbonyl and HO-3. This

[2] Φ_{IUPAC} values are given for dihedral angles H–N–C-2–H-2 of the GalNAc 2-acetamido group in analogy to the IUPAC-IUB conventional angle for amino acid residues in peptides. These relate to the dihedral angles θ by the formula $\theta = |\Phi - 60|$ and are quoted for the purpose of relating this work to studies in which Karplus-type functions have been derived primarily for peptide conformational analysis (Bystrov et al., 1973; Pardi et al., 1984).

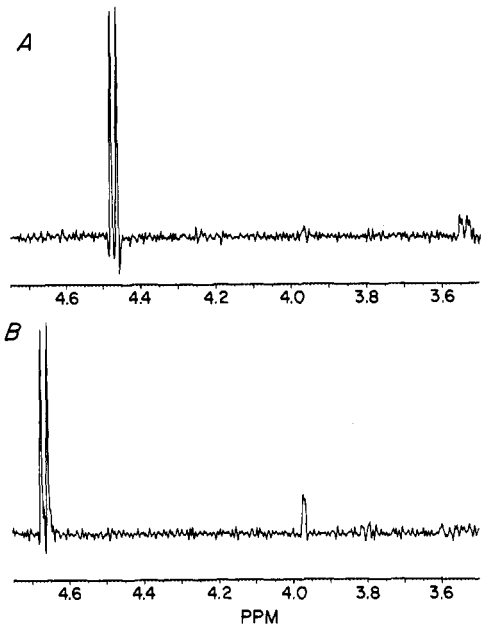


FIGURE 7: One-dimensional selective inversion–recovery NOE difference spectra with irradiation of anomeric resonances occurring at (A) 4.475 ppm (β-GlcNAc III) and (B) 4.666 ppm (β-GalNAc IV).

angle would still require $^3J_{2,NH}$ in the neighborhood of 9 Hz. In Gg_3Cer, there would be an opportunity for an additional interresidue hydrogen-bonding interaction between the acetamido H–N and O-3 of the aglyconic β-Gal residue. This could produce a further deviation from the antiperiplanar conformation for this proton, to approximately 140° (Φ_{IUPAC} = −160°), consistent with the smaller measured vicinal coupling constant. The increase in this coupling constant, due to the presence of the GlcNAcβ1→3 residue in $LcGg_4Cer$, is then consistent with a realignment of the acetamido group. This could be due to an attractive interaction between H–N of GalNAc and the GlcNAc acetamido carbonyl group. The value of 9.5 Hz for $^3J_{2,HN}$ in $LcGg_4Cer$ is close to the maximum reported $^3J_{HN,\alpha}$ for antiperiplanar conformations of amino acid residues in bovine pancreatic trypsin inhibitor (Pardi et al., 1984). On this basis, one could propose an enforced dihedral angle close to 180° (Φ_{IUPAC} = −120°) between H–N and H-2 of GalNAc in $LcGg_4Cer$.

Since H-1 chemical shift similarities alone might not be considered absolute proof of primary structure, further evidence was sought in the form of 1- and 2-D NOE experiments (Figures 6B and 7). Although the GlcNAcβ1→3Gal linkage is not in question (as it occurs in the precursor Lc_3Cer), it is worth noting that a substantial dipolar correlation can be observed between H-1 of β-GlcNAc and H-3 of β-Gal, as would be expected (Dabrowski et al., 1981, 1988b; Hanfland et al., 1981). Although the latter resonance occurs at the same chemical shift as that of H-2 of β-GlcNAc, close inspection of the higher resolution 1-D difference spectrum (Figure 7A) shows that the splitting of the enhanced resonance is that expected for β-Gal H-3, rather than for β-GlcNAc H-2 (see Table III).

Of clear significance for the primary structure is the dipolar correlation evident between H-1 of β-GalNAc and H-4 of β-Gal (Figures 6B and 7B). The close spatial proximity of these protons is consistent with a 1→4 linkage between the two sugars (Koerner et al., 1983b, 1987), although exceptions to the general pattern, attributable to unique conformational effects, are known [Lemieux et al., 1980; Bush et al., 1986; see also the comments on this analytical problem in Dabrowski et al. (1988b)].

Table V: Temperature Dependencies[a] of Amide Proton Chemical Shifts (in ppb/deg) for Glycosphingolipids in DMSO-d_6–H_2O (98:2) and Half-Lives (h)[b] for Deuterium Exchange of Amide Protons of Glycosphingolipids in DMSO-d_6–D_2O (98:2) at 298 K (Italicized)

compound	GlcNAc	GalNAc	Cer
Lc_3Cer	−5.49		−7.45
	8.5		*90.4*
Gg_3Cer		−4.15	−7.46
		14.7	*92.2*
$LcGg_4Cer$	−6.67	−2.30	−7.41
	13.6	*72.0*	*86.1*

[a] Calculated slope from linear least-squares analysis of six data points from 303 to 328 K. [b] Calculated inverse slope from decay of the NH resonance over 15 h, fitted to single exponential.

Amide Proton Temperature-Shift Coefficients

Chemical shifts of GlcNAc, GalNAc, and ceramide amide protons were measured in DMSO-d_6–H_2O (98:2 v/v) over a temperature range of 25 K. The temperature-shift coefficients, listed for comparison in Table V, are all negative and in the typical range for the conditions used. The coefficients of the ceramide amide protons are identical, within experimental error, and provide an indication of the reproducibility of the experimental conditions.

It is apparent that the temperature susceptibility of the GalNAc NH chemical shift in $LcGg_4Cer$ is about half the value found for the same proton in Gg_3Cer. A reduction in temperature susceptibility has been commonly accepted as an indicator of reduced interaction with solvent, due either to intramolecular hydrogen bonding, steric hindrance (crypticity), or both, in proteins and polypeptides (Llinâs & Klein, 1975; Kopple & Go, 1977; Kessler et al., 1983; Pope et al., 1984; Kartha et al., 1984; Gellman et al., 1990), as well as carbohydrates (Heatley et al., 1982; Scott et al., 1983, 1984; St. Jacques et al., 1976; Harvey & Symons, 1976), although Buffington et al. (1989) have cautioned that reliance solely on the conventional interpretation of temperature-shift coefficients can lead to erroneous results. A small increase in susceptibility is observed for the GlcNAc NH proton in $LcGg_4Cer$ vs Lc_3Cer, which would be consistent with a transfer of electron density from the N–H bond as a result of hydrogen bonding involving the acetamido carbonyl as electron donor. However, steric differences leading to increased exposure might also account for the increased temperature dependence.

Amide Proton Exchange Kinetics

In order to measure relative exchange rates of amide protons, samples were dissolved in pure DMSO-d_6 at 298 K, and a volume equivalent to 2% D_2O added to initiate the exchange reaction, which was then followed by monitoring the disappearance of the N–^{1}H resonances in the NMR spectra. The results with $LcGg_4Cer$ are illustrated in Figure 8, which shows the near disappearance of the GlcNAc NH signal within 17 h; the GalNAc and Cer NH signals clearly persist much longer. When plotted as a single term exponential decay (Table V), the half-life of the GalNAc NH was found to be ≈5× that of the GlcNAc NH. Since this result could reflect an intrinsic difference between the two residues, control experiments were performed with Lc_3Cer and Gg_3Cer under the same conditions. It was found (see Table V) that the GlcNAc NH in Lc_3Cer does exchange approximately twice as fast as the GalNAc NH in Gg_3Cer. However, it is apparent that the GalNAc NH exchange is slowed by a factor of 5 upon addition of the GlcNAc residue to Gg_3Cer to produce $LcGg_4Cer$. There appears to be a small increase in half-life of the Lc_3Cer GlcNAc NH upon addition of the GalNAc residue.

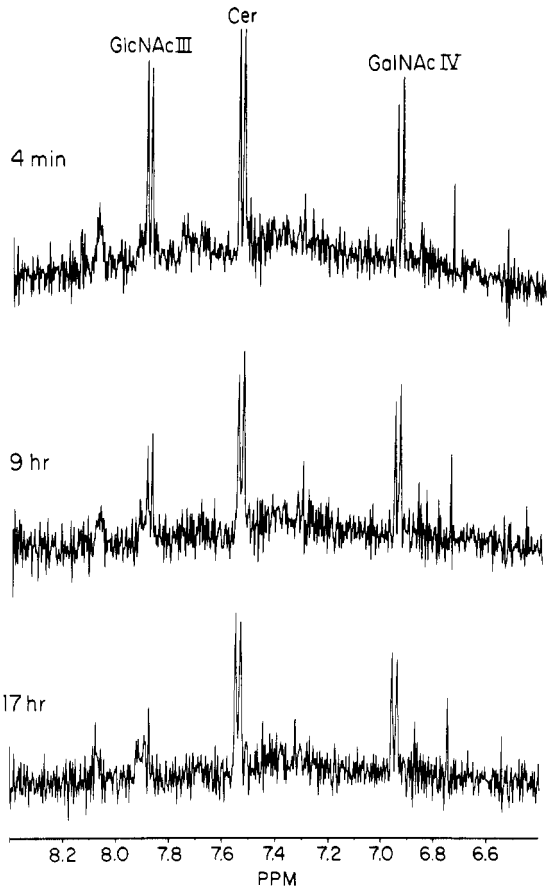


FIGURE 8: Decay of amide proton resonances of $LcGg_4Cer$ during deuterium exchange. 2% D_2O was added to $LcGg_4Cer$ in pure DMSO-d_6 at 298 ± 2 K.

Table VI: Linkage Conformations (ϕ, ψ) and Their Relative Energies (E) Obtained by GESA Calculations on Lc_3, Gg_3, and $LcGg_4$ Oligosaccharides[a]

	ϕ/ψ (deg)		
	GlcNAc-β1→3Gal	GalNAc-β1→4Gal	E_{rel} (kcal)
Lc_3	58/−8 (gl)[b]		−2.7
	160/10		+0.7
	40/160		+2.5
Gg_3		54/10 (gl)	−3.4
$LcGg_4$	63/−15 (gl)	38/16 (gl)	−6.7
two-parameter	65/−15	40[c]/16[c]	−6.7
	80/50	40[c]/16[c]	−2.5
	150/20	40[c]/16[c]	−0.7
	−20/−35	40[c]/16[c]	+0.5
	65[c]/−15[c]	40/16	−6.7
three-parameter	66/−14	40/16[c]	−6.6

[a] Galβ1→4Glc angles found to be 55 ± 1/4 ± 1 during all global minimum searches. These angles were held constant during all grid searches, since they appeared not to affect significantly the calculations of other angles. [b] gl = global minimum. [c] Angle held constant during grid search.

It should be pointed out that these kinetic results were not obtained under saturation conditions (the ratio of D_2O to total exchangeable H was calculated to be ≈200:1); nevertheless, the results are consistent with an inhibition of GalNAc NH/D exchange due either to sterically reduced solvent accessibility, hydrogen bonding, or both.

Conformational Analysis

The GESA potential energy minimization program (Paulsen et al., 1984) was applied to Lc_3, Gg_3, and $LcGg_4$. In addition

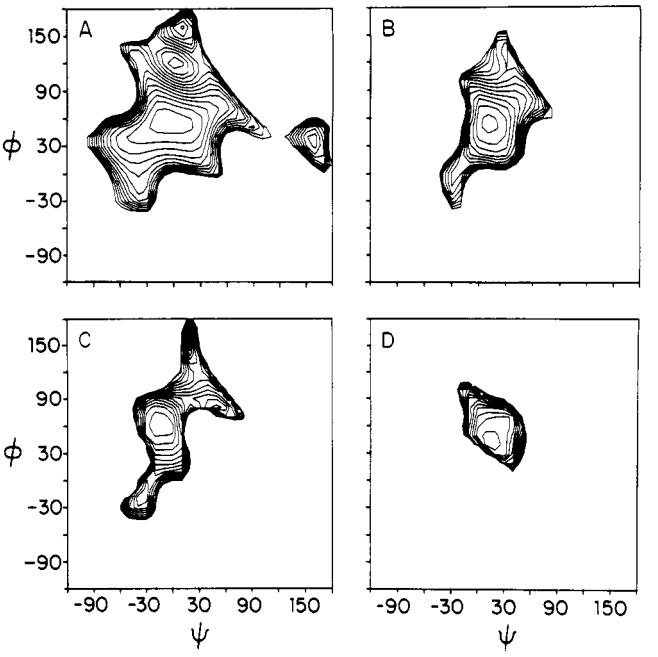


FIGURE 9: Two-parameter grid searches for ϕ and ψ of glycosidic bonds: (A) GlcNAcβ1→3Gal in Lc_3; (B) GalNAcβ1→4Gal in Gg_3; (C) GlcNAcβ1→3Gal in $LcGg_4$; (D) GalNAcβ1→4Gal in $LcGg_4$.

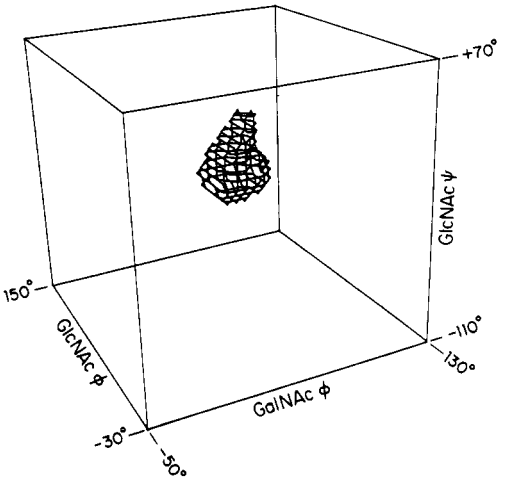


FIGURE 10: Three-parameter grid search of ϕ and ψ of GlcNAcβ1→3Gal and ϕ of GalNAcβ1→4Gal, in $LcGg_4$. ψ of GalNAcβ1→4Gal was held constant at 16°. Contour encloses $E \leq -5$ kcal.

to global energy minimization, two-parameter grid searches were done for ϕ and ψ angles vs internal energy for the GlcNAcβ1→3Gal linkage of Lc_3, for the GalNAcβ1→4Gal linkage of Gg_3, and for each of these linkages in $LcGg_4$ while holding the other constant at its minimum. Results are presented for comparison in Table VI and Figure 9.

The calculations for the $LcGg_4$ tetrasaccharide predict essentially a single minimum (Table VI), as illustrated both in Figure 9 panels C and D (two-parameter grid searches) and in Figure 10 (three-parameter grid search). The results are perhaps better visualized using the two-parameter grid searches in which one linkage is varied while the other is held rigid. The potential wells for each linkage in $LcGg_4$ are deeper than for their trisaccharide counterparts (Figure 9A,B) and conformationally more restricted, in particular the GalNAcβ1→4Gal linkage. Small shifts in minimum energy linkage positions, relative to those in the trisaccharides, are predicted. Since the addition of one sugar would be expected to have virtually no effect on the exoanomeric component of the other

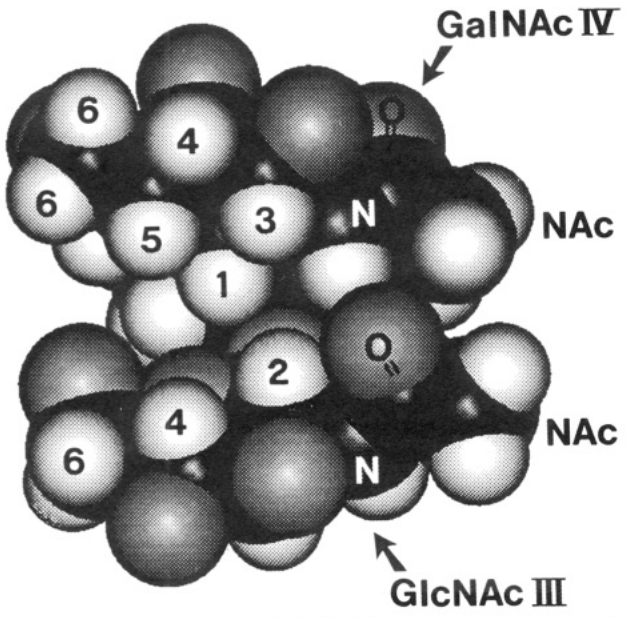


FIGURE 11: Space-filling model of minimum energy conformation of $LcGg_4$ calculated using the GESA program, viewed from the nonreducing end.

sugar's conformation-dependent potential energy, these differences are presumably the result of nonbonded (hard-sphere) interactions between the two vicinally linked terminal sugars. Figure 9 could be interpreted as representing an equilibrium position for each of the linkages, along with several allowable modes of motion in its vicinity, particularly concerted deviations of ϕ and ψ in the direction of the troughs. The precise conformational dynamics and equilibrium positions are difficult to verify experimentally. Overall, $LcGg_4$ could be expected to be somewhat less "floppy" than either of the constituent trisaccharides, a result which could perhaps be predicted intuitively. Figure 11 depicts the GESA-calculated minimum energy conformation of $LcGg_4$, viewed from the nonreducing end.

It remains to be established to what extent the global minimum predicted by GESA is consistent with available experimental data. The appearance of a number of pseudominima for the GlcNAcβ1→3Gal linkage (Table VI), although they are calculated to be of higher energy than that occurring at 65/−15, should call for some degree of attention, since these calculations are dependent on a set of torsional parameters whose relative weighting has been assigned on an empirical basis which is still the subject of controversy (Tvaroška, 1984). Unfortunately, the available set of interresidue dipolar correlations is a small one. At present, reconciliation of the proposed model with a limited number of chemical shift changes and with the slowed exchange of the GalNAc acetamido NH, may add to the confidence level of the conformational analysis, although alternative explanations for these phenomena need to be considered.

The substantial NOEs between GalNAc H-1 and Gal H-4 and between GlcNAc H-1 and Gal H-3 are both predicted. There are no indications from these results of correlations between GalNAc H-1 and Gal H-3 or between GlcNAc H-1 and Gal H-4, as suggested by DeGasperi et al. (1987). A measurement of the NOE buildup rate between GlcNAc H-1 and Gal H-3, relative to the GlcNAc H-1/H-5 correlation, gave an internuclear distance estimate of 2.26 ± 0.1 Å. Imposition of this single restraint excludes substantial contributions from glycosidic conformations in the vicinity of 80/50 and 150/20, while allowing those near 65/−15 and −20/−35. The latter is clearly disfavored on energetic grounds (Table VI). A nearly identical NOE buildup rate was observed between GalNAc H-1 and Gal H-4, which would lead to a similar estimate for the internuclear distance between them, provided one assumes similar correlation times for the GalNAc and GlcNAc residues. This assumption is necessary, since an intraresidue calibrating NOE is not available for the GalNAc residue as it is for the GlcNAc (H-1/H-5). A similar distance (2.2–2.3 Å) is compatible with a conformation close to the GESA-calculated minimum for the GalNAcβ1→4Gal linkage.

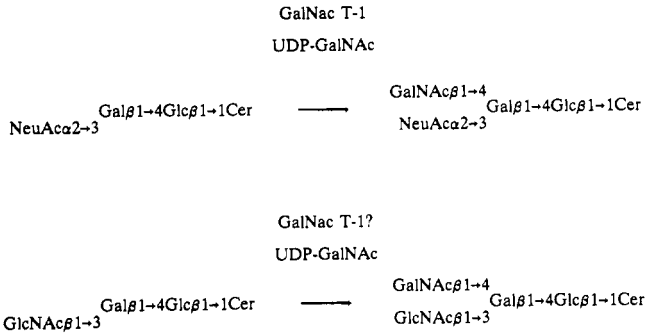
Only one further dipolar interaction is predicted at the global minimum, a small NOE between GalNAc H-1 and GlcNAc H-2, which was not observed in either the 1-D or 2-D dipole–dipole cross-relaxation experiments. This could be due to substantial uncorrelated motion and/or a shift in the equilibrium position of one or both linkages not predicted by GESA.

The substantial downfield shift of Gal H-4 on addition of the GlcNAc residue can most likely be ascribed to the proximity of the GlcNAc ring oxygen (O-5). That the effect is caused directly by the GlcNAc residue, rather than indirectly through a conformational adjustment of the GalNAc residue, is supported by the fact that a similar effect can be observed on Gal H-4 of lactosylceramide upon addition of GlcNAc to make Lc_3Cer. Such deshielding effects have been claimed to indicate an H–O internuclear distance less than the sum (2.7 Å) of their van der Waals radii (Lemieux & Bock, 1983). Such a distance appears consistent with a conformation near 65/−15 for the GlcNAcβ1→3Gal linkage, but with none of the other pseudominima. If one assumes that the deshielding of GalNAc H-1 in $LcGg_4$ is also caused by O-5 of GlcNAc, this distance is consistent with conformations near 65/−15 and −20/−35. The latter conformation can be rejected on the basis of one further argument: that it requires GlcNAc H-1 to be ca. 2.3 Å from Gal H-4, close enough to produce a strong NOE, which is clearly absent. The chemical shift data show that Gal H-4 is also deshielded by addition of the GalNAc residue. Ascribing this effect to O-5 of the GalNAc residue is compatible with the GESA minimum for the GalNAcβ1→4Gal linkage.

Discussion

Studies of the glycosphingolipids of English sole liver (Ostrander et al., 1988a,b) have produced a number of notable observations, including (1) the presence of ganglio-series structures with GM_2 comprising ca. 80% of the total ganglioside fraction and (2) the presence of a *neo*-lacto–ganglio hybrid core structure in a novel glycosphingolipid found abundantly in the neutral fraction. Similarly, studies of gangliosides from roe of striped mullet (Li et al., 1984; DeGasperi et al., 1987) showed the presence of GM_2 as the major ganglioside, accompanied by a number of novel structures possessing *neo*-lacto–ganglio hybrid core chains. It is notable also that the first observations of glycosphingolipids with hybrid lacto–ganglio and *neo*-lacto–ganglio saccharide structures were made on cells ($M1^-$) which are high expressors of asialo-GM_2 (Kannagi et al., 1983, 1984). Thus, the biosynthesis of *neo*-lacto–ganglio structures appears, at least superficially, to correlate with a high activity of GalNAc transferases known collectively as GM_2 synthase or GalNAc T-1 (Basu et al., 1987). This idea is supported by a recent communication claiming that a partially purified GalNAc T-1 from guinea pig bone marrow, previously reported to have catalyzed the transfer of GalNAcβ1→4 to both GM_3 and lactosylceramide to make GM_2 and Gg_3Cer, respectively (Das et al., 1987b), could also be used to biosynthesize $LcGg_4Cer$ from Lc_3Cer

Scheme I: Biosynthetic Reactions for Production of GM_2 Ganglioside or $LcGg_4Cer$



(Das et al., 1987a; see Scheme I). Although the present study was prompted by a desire to examine spectral and three-dimensional structural features of the lacto–ganglio core tetraglycosylceramide, it was also seen as an opportunity to study properties of a transferase responsible for its biosynthesis, with regard to both substrate specificities and species differences.

Previous studies of GM_2 synthases have been conducted using crude enzyme preparations from a number of mammalian sources, such as rat brain and liver (Cumar et al., 1971; DiCesare & Dain, 1971; Senn et al., 1981; Keenan et al., 1974), embryonic chicken brain (Chien et al., 1973; Steigerwald et al., 1975), mouse neuroblastoma cells (Kemp & Stoolmiller, 1976), bovine thyroid (Pacuszka et al., 1978), guinea pig bone marrow (Basu et al., 1974), fetal pig brain (Steigerwald et al., 1975), NIL hamster cells (Lockney & Sweeley, 1982), 3T3 cells (Fishman & Brady, 1976), and human melanoma (Thurin et al., 1986). The basic properties observed with the English sole liver enzyme are quite similar to those found previously for the mammalian transferases, particularly in the requirements for Mn^{2+} and detergent (Basu et al., 1987). On the other hand, it is interesting to note that the optimum temperature for the English sole liver enzyme is rather low at 25 °C. Similar results have been observed with $\beta1\rightarrow4$ galactosyl and $\alpha2\rightarrow3$ sialosyltransferases of rainbow trout liver (Jenner & Holmes, 1990; Ostrander & Holmes, 1991). In consideration of the physiological differences in poikilothermic animals, this result is quite reasonable. In addition, the results indicated rather low K_m values for acceptor glycosphingolipids compared with many of the previously studied mammalian enzymes. K_m values ranging from 50 to 75 μM for the English sole enzyme are between 5- and 10-fold lower than those found for mammalian enzymes. This observation is in accord with the ganglioside profile from English sole liver, in which a lower apparent steady-state concentration of the precursor GM_3 was found compared to many mammalian sources (Ostrander et al., 1988b). Combined with the unusually high proportion of GM_2, this finding attests to the presence of a highly efficient GalNAc T-1 enzyme.

The apparent acceptor specificity of this enzyme was determined to match that from mammalian sources, i.e., transfer to both $\alpha2\rightarrow3$ sialosyl and $\beta1\rightarrow3$ GlcNAc derivatives of lactosylceramide. None of these enzymes has ever been analyzed in a homogeneous state. Therefore, we cannot be certain at this time that the same enzyme catalyzes all reactions. Available evidence indicates that the activity observed with transfers to mixtures of alternate acceptors is not additive compared to that observed with the individual acceptors, consistent with the involvement of a single enzyme.

The broad specificity of the English sole liver GalNAc $\beta1\rightarrow4$ transferase for acceptor glycosphingolipids suggested that it would be useful for biosynthesis of lacto–ganglio hybrid structures. It should be pointed out that this property is not unique, having been noted previously in several of the systems mentioned. We believe that the present study is the first attempt to exploit the broad specificity of a putative GalNAc T-1 for the purpose of obtaining and analyzing macroscopic quantities of lacto–ganglio hybrid, although it is fair to say that the existence of such a structure as a realistic biosynthetic target was not suspected when most of the previous studies were done.

With regard to the analysis of the product, the results of ^1H NMR spectroscopic experiments clearly show that (1) the glycosphingolipid is identical in primary saccharide structure to that originally obtained from mouse leukemia $M1^-$ cells (Kannagi et al., 1984) and (2) both the structure originally proposed, as well as the anomeric proton assignments and interpretation of 1-D NOE data, were correct. Thus, the H-1 chemical shifts of β-GalNAc and β-GlcNAc in $LcGg_4Cer$ are anomalous, compared with those in the triaosylceramides, while the dipolar coupling data, insofar as they reflect the positions of interglycosidic linkages, fit the more general pattern (Koerner et al., 1987) despite suggestions to the contrary (DeGasperi et al., 1987). It was expected that a more detailed examination of the data, in connection with simplified molecular mechanics calculations of three-dimensional structure, would shed light not only on the source of the unusual chemical shifts noted herein and previously (Kannagi et al., 1984) but also on properties of the GalNAc T-1 related to its interactions with its substrates and on the potential for specific recognition of lacto–ganglio structures by monoclonal antibodies as possible cancer-associated antigens. Given the relatively small number of distance constraints available from interresidue dipolar couplings, the HSEA minimum energy conformational calculations must remain relatively unconfirmed, although there is little reason to consider it unsatisfactory as a first approximation.

The most interesting aspect of the predicted minimum energy structure is the juxtaposition of the two terminal HexNAc residues nearly parallel to each other, bringing their 2-acetamido groups into close proximity. The GalNAc 2-N–H is clearly in the shielding zone of the GlcNAc acetamido carbonyl group, a feature which is consistent with the substantial *upfield* shift of that proton on addition of the GlcNAc residue. Furthermore, distance and geometry appear well-suited to formation of a hydrogen bond of the π-acceptor type. As indicated by two additional criteria, temperature-shift coefficients and exchange kinetics, the GalNAc amide proton is at least cryptic, if not hydrogen-bonded. Some further evidence is provided by a change in the $^3J_{2,NH}$ coupling constant for the GalNAc residue on addition of the GlcNAc residue, indicating a conformational adjustment of the acetamido side chain, although steric interactions alone might be responsible. The formation of such an interresidue hydrogen bond could contribute to a less flexible structure for the $LcGg_4$ oligosaccharide in a manner which is not normally accounted for by the GESA program.

Although a number of studies have utilized NMR spectroscopy to examine the role of acetamido groups in the intra- and interresidue interactions of oligosaccharides (Scott et al., 1981, 1983, 1984; Heatley et al., 1982; Huang et al., 1987), the situation occurring in $LcGg_4$ appears to be unique. Even in peptide literature, the reporting of a *shielding* hydrogen-bonding interaction is rare. Nevertheless, strong evidence for hydrogen bonding with a carbonyl π-system has been presented, for example, by Joris and Schleyer (1968), in infrared

studies of hydroxyketones with restricted geometry. Although π-systems are generally considered to be weaker hydrogen-bond acceptors than oxygen lone pairs (Davis & Deb, 1970), it is possible that a stronger interaction may take place with an amide carbonyl than with a ketone, due to additional charge delocalization via the extended amide π-system (Llinás & Klein, 1975; Popov & Zheltova, 1971; Buffington et al., 1989). One would expect a slight weakening of the N–H bond in the GlcNAc residue to result from this interaction, due to transfer of electron density into the hydrogen bond. This could be used to rationalize the slight increase in temperature susceptibility of the GlcNAc amide proton (but not the slight increase in its exchange half-life). In addition, it could account in part for the downfield shift of that proton on addition of the GalNAc residue to Lc_3Cer without attributing it solely to the anisotropic effect of the GalNAc acetamido group.

Although a number of proton chemical shift changes are relatively easy to explain on the basis of the proposed model [for example, the downfield shifts of β-GalNAc H-1 (due to β-GlcNAc O-5) and β-Gal H-4 (β-GlcNAc and -GalNAc O-5) and the upfield shifts of β-GalNAc N–H (β-GlcNAc acetamido carbonyl) and β-Gal H-2 (β-GalNAc acetamido carbonyl)], a greater number of these can not be obviously attributed to any one factor [the downfield shifts of β-Gal H-3 (on addition of β-GalNAc), β-GlcNAc H-2 and N–H, and β-GalNAc H-2, and the upfield shifts of β-GlcNAc H-1, β-GalNAc H-3 and H-5]. We would argue that changes in orientation and hybridization of an acetamido group, induced by hydrogen bonding, could be responsible for some of the chemical shift changes of protons attached to the same ring, although a number of alternate mechanisms need to be considered.

At the time when lacto–ganglio structures were first described, it was proposed that a GalNAc T-1 with an unusual substrate specificity, enriched in undifferentiated leukemia cells, must be responsible for their synthesis (Kannagi et al., 1984). At present it appears more likely that no "aberrant" enzyme is required, at least judging by a number of cases now reported involving synthesis by normal animal tissues (DeGasperi et al., 1987; Ostrander et al., 1988a; Das et al., 1987). The GalNAcβ1→4 transferases found in the piscine systems studied may be more efficient, rather than simply having a looser substrate specificity than the corresponding mammalian enzymes, while the occurrence of lacto–ganglio hybrid structures in $M1^-$ cells may be influenced more by substrate availability. In comparing molecular models of GM_3 and Lc_3, there seems to be little physical resemblance between the NeuAcα2→3 of the former and the GlcNAcβ1→3 residue of the latter. Considering the data obtained for acceptor specificity, one may conclude that the identity or even the absence of the 3-linked Gal substituent has relatively little influence on the activity of English sole liver GalNAc T-1, within reasonable experimental error.

ACKNOWLEDGMENTS

We thank Dr. Stephen Anderson for his expert assistance in preparation of the manuscript.

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