Glycoconjugate Journal 16, 507–515 (1999)
© 2000 Kluwer Academic Publishers. Manufactured in The Netherlands

Allylmalonamide as a bivalent linker: Synthesis of biantennary GM_3-saccharide—Keyhole limpet hemocyanin glycoconjugate and the immune response in mice†

Wei Zou*, Mary Abraham, Michel Gilbert, Warren W. Wakarchuk and Harold J. Jennings

Institute for Biological Sciences, National Research Council of Canada, Ottawa, Canada K1A OR6

A biantennary GM_3-saccharide (sialyllactoside) derivative (4) was constructed using allylmalonic acid as a bivalent linker, both carboxylic acids of which were condensed with 3-aminopropyl lactoside (2) prior to enzymatic sialylation with a fusion enzyme. While ozonolysis of its allyl group generated a saccharide having a terminal aldehyde (6), we were unable to couple 6 directly to protein by reductive amination. However, extension of the spacer by means of introducing a maleimide group to 6 through its aldehyde group to give 7 enabled the latter to be successfully coupled to thiolated proteins. The average ratios of saccharide to protein were observed to be 35 in KLH conjugate (13) and 9–12 in HSA conjugates (14 and 15). The antisera obtained by immunizing mice with the biantennary sialyllactoside-KLH conjugate (13) together with MPL adjuvant were analyzed by ELISA. Using several structurally related saccharide-HSA conjugates as screening antigens, it was concluded that anti-sialyllactoside antibodies, both IgG and IgM, were effectively raised. This was further supported by competitive inhibition experiments using lactoside (1), sialyllactoside (8) and biantennary sialyllactoside (4) as inhibitors.

Keywords: **GM_3 antigen, sialyllactoside, biantennary, glycoconjugate, antibody**

Abbreviation: **CTP, Cytidine 5′-triphosphate; KLH, Keyhole Limpet Hemocyanin; M_2C_2H, 4-(4-N-maleimidomethyl) cyclohexane-1-carboxyl hydrazide; MPL, Monophosphoryl lipid A; Sulfo-GMBS, N-(γ-maleimidobutyryloxy) sulfosuccinimide ester; BSA, Bovine serum albumin; HSA, Human serum albumin; GBSPIa (GBSPIII), Type Ia (III) group B Streptococcus polysaccharide**

Introduction

Gangliosides including GM_3 are over-expressed on the surface of several human tumors, particularly melanomas [1–4], although GM_3 is not unique to melanomas as it is also found on normal human cells, but at much lower density. However, a murine IgM mAb (M2590) was reported to react with GM_3 antigen on B16 melanoma but not on normal cells. This was attributed to differences in the density and tertiary structure of GM_3-saccharides on the surface of these cells [5,6]. In addition a GM_3-enriched microdomain in B16 melanoma has been reported to be involved in cell adhesion and signal transduction [7], which could be important in promoting metastasis.

The humoral immune response to specific gangliosides on tumor cells plays an important role in host protective immunity and correlates with prolonged survival [8–11], however, GM_3, unlike other gangliosides, is incapable of inducing an antibody response [12] because of its immune suppressive role [13,14]. Considerable effort has been devoted in trying to raise anti-GM_3 antibodies, particularly IgG, by using various adjuvants, but this has met with limited success [15–17]. Only when GM_3 lactone was used were IgG antibodies raised, which cross-reacted with GM_3 and inhibited the melanoma cell growth [18].

Improvement in the immunogenicity of ganglioside conjugate vaccines was obtained using KLH as a carrier [19,20]. This was particularly true for GD_2 and GM_2 in terms of both antibody production and host protection [21,22]. Because of the high concentration of GM_3 on the tumor cell surface we hypothesized that synthetic glycoconjugates having a multivalent rather than monovalent presentation of GM_3 would be more successful in triggering immune response by raising tumor specific antibodies.

†NRCC publication No. 42399.

*To whom correspondence should be addressed. Tel.: 613-991-0855; Fax: 613-952-9092; E-mail: *Wei.Zou@nrc.ca*

Therefore, as the first step in the assembly of multiantennary GM_3-saccharide motifs coupled to KLH we describe the synthesis of a KLH glycoconjugate of a biatennary GM_3-saccharide, and preliminary studies on its immune response in mice.

Materials and Methods

General methods

^1H and ^{13}C NMR spectra were recorded at 500 MHz and 125 MHz, respectively, with INOVA-500 instrument at 293 K unless otherwise noted. Chemical shifts are given in ppm relative to the signal of internal acetone δ_H 2.225 in D_2O for ^1H NMR spectra, and to δ_C 31.07 for ^{13}C NMR spectra. The ^1H NMR chemical shifts of oligosaccharides were assigned on the basis of 2D ^1H-COSY and ^1H-^{13}C chemical-shift correlated experiments. ES-MS were performed with QUATTRO (MICROMASS). MALDI-MS spectra were recorded with Voyager-DE™STR (PerSeptive Biosystems). HPLC analysis was performed with an instrument of Hewlett Parkard Series 1100 using a Superose 12 10/30 column (Pharmacia), PBS buffer was used as eluent. Both UV and RI monitors were equipped.

KLH and maleimide crosslinkers (Sulfo-GMBS and M_2C_2H) were the products of PIERCE. HSA was a product of Sigma. All other chemicals were purchased from Aldrich without further purification. Two glycoconjugates (GBSPIa-HSA and GBSPIII-HSA) were prepared with the procedure previously reported [23].

Synthesis of biantennary sialyllactosyl-KLH conjugate

3-Aminopropyl lactoside (**2**). A solution of **1** (0.5 g) in water (5 ml) was added Pd/C (100 mg, 50% wet). The mixture was subjected to hydrogen pressure (35 p.s.i.) for 2 h, when TLC indicated the reaction was complete. The filtrate was passed through a Sephadex G-10 column, using water as eluent. The fractions were pooled and lyophilized to afford **2** (0.43 g, 90%) as an amorphous. Calcd for $C_{15}H_{29}NO_{11}$: 414.4 Found: 415.3 (M+H) (f.a.b positive). The NMR data are listed in Table 1.

N,N'-Di-lactosylpropyl allylmalonamide (3). To a solution of **2** (100 mg, 0.25 mmole) in DMF (0.3 ml) were added iPr_2NEt (40 µL) and a solution of pentafluorophenyl allylmalonate (50 mg, 0.11 mmole) in DMF (0.25 ml). The mixture was kept at room temperature for 4 h and precipitated by the addition of diethyl ether. The pellet was dissolved in water and purified on a Biogel P-2 column using water as eluent to obtain a white solid **3** (65 mg, 65%) after lyophilize as major product. Calcd for $C_{36}H_{62}N_2O_{24}$: 906.9. Found: 907.2 (M + H) (f.a.b. positive); 905.4 (M − H) (ES-MS negative). The NMR data are also listed in Table 1.

*N,N'-Di-(sialyllactosyl)propyl allylmalonamide (**4**) and N-lactosylpropyl-N'-(sialyllactosyl) propyl allylmalonamide (**5**).* A solution of **3** (27 mg) in water (20 ml) were added 0.1*M* $MgCl_2$ (2 ml), 0.1 *M* NeuAc (1 ml), and 0.1 *M* CTP (1 ml) and adjusted to pH 7.5 by the addition of N Tris base. To above solution were added inorganic pyrophosphatase (10 U), fusion enzyme (5 U, PEG pellet). Again the pH was adjusted to 7.5 and the mixture was incubated for another 5 h at 37 °C. The insoluble material was removed by centrifuge. The resulting solution was lyophilized and further purified by a Biogel P-2 column using water as eluent to afford **4** (26 mg, 63%) and **5** (5 mg, 11%).

For **4:** Calcd for $C_{58}H_{96}N_4O_{40}$: 1489.4. Found: 1489.1 (ES-MS negative). Both ^1H and ^{13}C NMR data are summarized in Table 1.

For **5:** Calcd for $C_{47}H_{79}N_3O_{32}$: 1198.1. Found: 1198.0 (ES-MS negative). ^1H NMR δ_H (D_2O): 5.78 (m, 1H, $CH_2-CH=CH_2$), 5.12 (2 d, 2H, $CH_2-CH=CH_2$), 4.53 (d, 1H, H-1, $J_{1,2}$ 7.7 Hz, NeuAc*Gal*), 4.47 (d, 2H, 2 × H-1, $J_{1,2}$ 7.7 Hz, 2 × Glc), 4.46 (d, 1H, H-1, $J_{1,2}$ 7.7 Hz, Gal), 2.57 (dd, 2H, $CH_2-CH=CH_2$, J 8.0 Hz, J 7.0 Hz), 1.83 (m, 4H, 2 × $OCH_2CH_2CH_2N$) ppm.

*N,N'-Di-(sialyllactosyl)propyl carbonylmethylenemalonamide (**6**).* A solution of **4** (18 mg) in dry MeOH (3 ml) was bubbled with ozone at −78 °C for 5 min, then with nitrogen for 5 min. Dimethyl sulfide (0.3 ml) was added and the mixture was stirred for 3 h. The solvent was evaporated to afford a white powder of **6** in a quantitative yield. ^1H NMR showed the disappearance of allyl group ($CH_2-CH=CH_2$) and the presence of $CH_2CH(OH)_2$ and the hydrated aldehyde proton at δ 5.23 and 5.10 ppm, Free aldehyde (c.a 10%) could be observed at δ 9.14 ppm. Calcd for $C_{57}H_{94}N_4O_{41}$:1491.4. Found: 1489.8 (M−H) and 1509.0 ($M+H_2O$) in ES-MS negative mode.

Preparation of M_2C_2H *derivative (**7**).* To a solution of **6** (10 mg) in 0.1 M sodium acetate buffer (1 ml, pH 5.5) M_2C_2H (15 mg) in DMSO (150 µl) was added. The mixture was stirred at room temperature for 20 min, and $NaCNBH_3$ (10 mg) was then added to the mixture. The mixture was incubated at room temperature overnight. Purification by a Sephadex G-10 column, with 0.1M PBS buffer (pH 6.0) containing 5 mM EDTA, afforded **7** (9.2 mg) based on the carbohydrate content assay.

Biantennary GM_3 *saccharide conjugates of HSA and KLH (**12** and **13**).* Compound **7** and thiolated HSA or KLH were mixed in PBS buffer and adjusted to pH 7.2. The mixture was incubated at room temperature for 16 h. The progress of the reaction was monitored by HPLC. Purification of the conjugates was performed on a Biogel A 0.5 column, eluted with 0.01 M PBS buffer (pH 7.3). The fractions containing conjugate were pooled, and the contents of sialic acid and protein were analyzed. HSA conjugate **12** after dialysis and lyophilize was also analyzed by MALDI-MS, and KLH conjugate **13** was subjected to quantitative sialic acid and protein contents assay. The results showed that the average molar ratios of biantennay sialyllactoside to HSA and KLH are ap-

Table 1. Chemical shift data for **2, 3, 4, 8,** and **9**[a,b]

		2		**3**		**4**		**8**		**9**	
Compound	*atom*	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
	3					1.79	40.5	1.79	40.4	1.80	40.7
						2.76		2.76		2.75	
	4					3.69	69.0	3.68	69.1	3.69	69.3
	5					3.85	52.5	3.84	52.7	3.85	52.8
α-NeuAc(2→	6					3.64	73.8	3.63	73.7	3.64	74.0
	7					3.89	72.7	3.88	72.5	3.88	72.7
	8					3.59	69.0	3.59	68.8	3.59	69.1
	9					3.65	63.4	3.64	63.4	3.64	63.6
						3.87		3.85		3.86	
	NAc					2.03	22.8	2.02	22.8	2.03	22.8
	1	4.46	103.9	4.46	103.8	4.53	103.6	4.53	103.5	4.53	103.6
	2	3.55	72.0	3.55	71.8	3.57	70.3	3.57	70.1	3.57	70.4
3)-β-D-Gal(1→	3	3.67	73.6	3.66	73.5	4.11	76.5	4.11	76.3	4.10	76.6
	4	3.93	69.6	3.93	69.4	3.96	68.5	3.96	68.2	3.95	68.4
	5	3.73	76.4	3.73	76.2	3.70	76.1	3.70	75.9	3.70	76.1
	6	3.74	61.9	3.75	61.9	3.71	61.9	3.71	61.9	3.72	62.1
		3.77		3.78		3.74		3.74		3.74	
	1	4.50	103.1	4.47	103.1	4.47	103.0	4.48	102.9	4.51	103.2
	2	3.32	73.8	3.33	73.8	3.32	73.8	3.31	73.7	3.34	73.9
4)-β-D-Glc(1→	3	3.65	75.5	3.64	75.2	3.64	75.3	3.64	75.1	3.66	75.4
	4	3.65	79.4	3.65	79.4	3.65	79.3	3.65	79.1	3.66	79.2
	5	3.60	75.8	3.60	75.6	3.59	75.7	3.59	75.5	3.61	75.7
	6	3.81	61.0	3.81	61.0	3.82	61.0	3.81	60.8	3.83	61.0
		3.98		3.98		3.99		3.98		3.99	
1 2 3	1	3.76	69.3	3.70	68.7	3.75	68.6	3.75	68.2	3.82	68.9
$OCH_2CH_2CH_2N$		4.00		3.94		4.00		3.99		4.05	
	2	1.82	31.6	1.82	29.3	1.82	29.2	1.91	28.9	2.01	27.8
	3	2.81	38.6	3.32	37.4	3.31	37.4	3.45	48.7	3.16	38.8
	1			3.29	54.2	3.29	54.2				
1 2 3 4	2			2.55	34.6	2.55	34.5				
>CHCH$_2$CH=CH$_2$	3			5.77		5.77					
	4			5.12	118.7	5.12	118.7				

[a]First-order data recorded in D_2O at 293 K.
[b]Internal acetone was used as references for both proton (2.225 ppm) and carbon-13 (31.07 ppm).

proximately 5 and 35, respectively. The analytical data are shown in Table 2.

Synthesis of HSA conjugates 14 and 15 as screening reagents for ELISA

*3-Azidopropyl sialyllactoside (**8**) and 3-aminopropyl sialyllactoside (**9**).* 3-Azidopropyl lactoside (**1**) (240 mg) was sialylated at the 3-*O* position of galactose with a fusion enzyme as described above in the preparation of **4** to afford, after purification on a Biogel P-2 column with water as eluent and lyophilize, 3-azidopropyl sialyllactoside (**8**) (320 mg, 78%). Calcd for $C_{26}H_{44}N_4O_{19}$: 716.7. Found: 715.1, 716.0 (ES-MS negative). The NMR data are presented in Table 1.

A solution of **8** (100 mg) in water (5 ml) was subjected to catalytic (Pd/C) hydrogenation (30 p.s.i.) for 2 h. After passage through a Saphdex G-10 column and lyophilize, compound **9** (84 mg, 90%) was obtained as an amorphous. Calcd for $C_{26}H_{46}N_2O_{19}$: 690.7. Found: 689.2 (M-H), 690.1 (M) (ES-MS negative). The NMR data are also presented in Table 1.

*Synthesis of lactosyl and sialyllactosyl-HSA conjugates (**14** and **15**).* A solution of compound **9** or **2** (8 mg) in 20 mM PBS buffer (pH 7.2) was mixed with Sulfo-GMBS (20 mg). The solution was kept at room temperature for 2 h, when TLC (n-BuOH-AcOH-H_2O 2:1:2) indicated the reaction was complete with the formation of a faster moving product. Purification on Sephadex G-10 column, eluted with water, gave **10** (9.5 mg, 94%) and **11** (15.0, 91%) as an

Table 2. Analysis of neoglycoconjugates (**12, 13, 14** and **15**) by MALDI-MS

Conjugate	*Saccharide*	*Protein*	*MS observed*	*Average molar ratio*
12	Biantennary sialyllactoside	HSA[a]	75655.6[b]	5
13	Biantennary sialyllactoside	KLH	n.d.	35[c]
14	Lactoside	HSA	74399.7[b]	12
15	Sialyllactoside	HSA	75581.4[b]	9

[a]The molecular weight of HSA was observed at 66423.5 by MALDI-MS.
[b]Average molecular weight, the typical half-peak width is about 3500 Dalton.
[c]Based on sialic acid content with resorcinol analysis.

amorphous after lyophilize, respectively. For **10**: ^{1}H NMR δ_H (D_2O) 1.70–1.74 (m, 3H, H-3a of NeuAc, CH_2CH$_2$CO), 1.82 (m, 2H, CH_2CH$_2$N), 1.95 (s, 3H, NAc of NeuAc), 2.17 (t, 2H, CH_2CO, J 7.0 Hz), 2.68 (dd, 1H, H-3e of NeuAc, $J_{3a,3e}$ 12.0 Hz, $J_{3a,4}$ 4.5 Hz), 4.03 (dd, 1H, H-3 of Gal, $J_{2,3}$ 10.0 Hz, $J_{3,4}$ 3.0 Hz), 4.40 (d, 1H, H-1 of Glc, J_{12} 8.0 Hz), 4.45 (d, 1H, H-1 of Gal, J_{12} 8.0 Hz), 6.76 (s, 2H, maleimide). Calcd for $C_{34}H_{53}N_3O_{22}$: 855.8. Found: 855.1 [M], 854.2 [M-H] (ES-MS negative), and 900.1 [M−H+2Na], 877.8 [M−H+Na] (MOLDI-MS).

For **11**: ^{1}H NMR δ_H (D_2O) 1.78–1.82 (m, 4 H, CH_2CH$_2$N, CH_2CH$_2$CO), 2.26 (t, 2H, CH_2CO, J 7.0 Hz), 3.22 (t, 2 H, NCH_2CH$_2$CH$_2$CO, J 7.0 Hz), 3.26 (t, 2 H, CH$_2$CH$_2$CH_2N, J 7.0 Hz), 4.42 (d, 1H, H-1 of Gal, $J_{1,2}$ 8.0 Hz), 4.45 (d, 1H, H-1 of Glc, $J_{1,2}$ 8.0 Hz), 6.82 (s, 2H, maleimide). Calcd for $C_{23}H_{36}N_2O_{14}$: 564.5. Found: 588.2 [M+Na], 587.3 [M−H+Na] (ES-MS positive).

A solution of thiolated HSA (5 mg) in 20 mM PBS buffer (pH 7.2) was mixed with **10** or **11** (5 mg) prepared above. The reaction mixture was incubated at room temperature for 4 h. Conjugation was indicated by shift of the protein peak in HPLC. Purification on a Biogel A 0.5 column, eluted with 0.01 M PBS buffer (pH 7.3), gave after dialysis and lyophilize lactoside and sialyllactoside conjugates **14** (4.5 mg) and **15** (4.1 mg), respectively. The average molar ratios of saccharide to protein were measured by MALDI-MS, and the data are shown in Table 2.

Immunization of mice

Groups of female BALB/c mice, 6 to 8 weeks of age, were immunized intraperitoneally with KLH glycoconjugate **13.** Each mouse in groups of four was injected with 2 µg of saccharide in 0.15 ml PBS buffer with MPL (2.0 µg). Two mice in a control group were injected with same volume of PBS buffer. The mice were boosted on day 7, 14, and 21. The mice were bled on day 0, 7, 14, 21 with a final bleeding on day 31.

ELISA. Antisera were assayed against HSA conjugates (1.0µg/100µl PBS/well) in 96-well EIA plates. Wells were coated at 37°C for 2 h then washed with PBS-T (0.05% Tween 20) three times and blocked with 200µl 1% BSA-PBS for 1h at room temperature. Mouse antiserum serially diluted in 1% BSA-PBS were added and incubated for 1 h at room temperature. Following washing with PBS-T, alkaline phosphatase labeled goat anti-mouse IgG or IgM (Caltag Laboratories, San Francisco, CA) diluted 1:2000 in 3% BSA-PBS was added for 1 h at room temperature. The plates were then washed and developed with p-NPP Phosphate Substrate System (Kirkegard and Perry Laboratories, Gaithersberg, MD). After 20 min at room temperature, the plates were scanned at 410 nm in a Dynatech MR 5000 microplate reader.

Competitive inhibition assay

The inhibition experiments were performed following the ELISA procedure as described above with the following modifications. After the blocking step, 50 µl of oligosaccharide (concentration 0.1 mg/ml) in 0.5%BSA/0.02% Tween/PBS buffer was added to the wells, which were serially diluted twofold with the same buffer. Then 50 µl of antiserum, which was diluted 200 times in the same buffer to give an OD of approximately 1 in the absence of inhibitors, was added, and the mixture was incubated at room temperature for 3 h. The remainder of the procedure was followed as described above. OD vs. log concentration curves was plotted for each inhibitor (see Fig. 3).

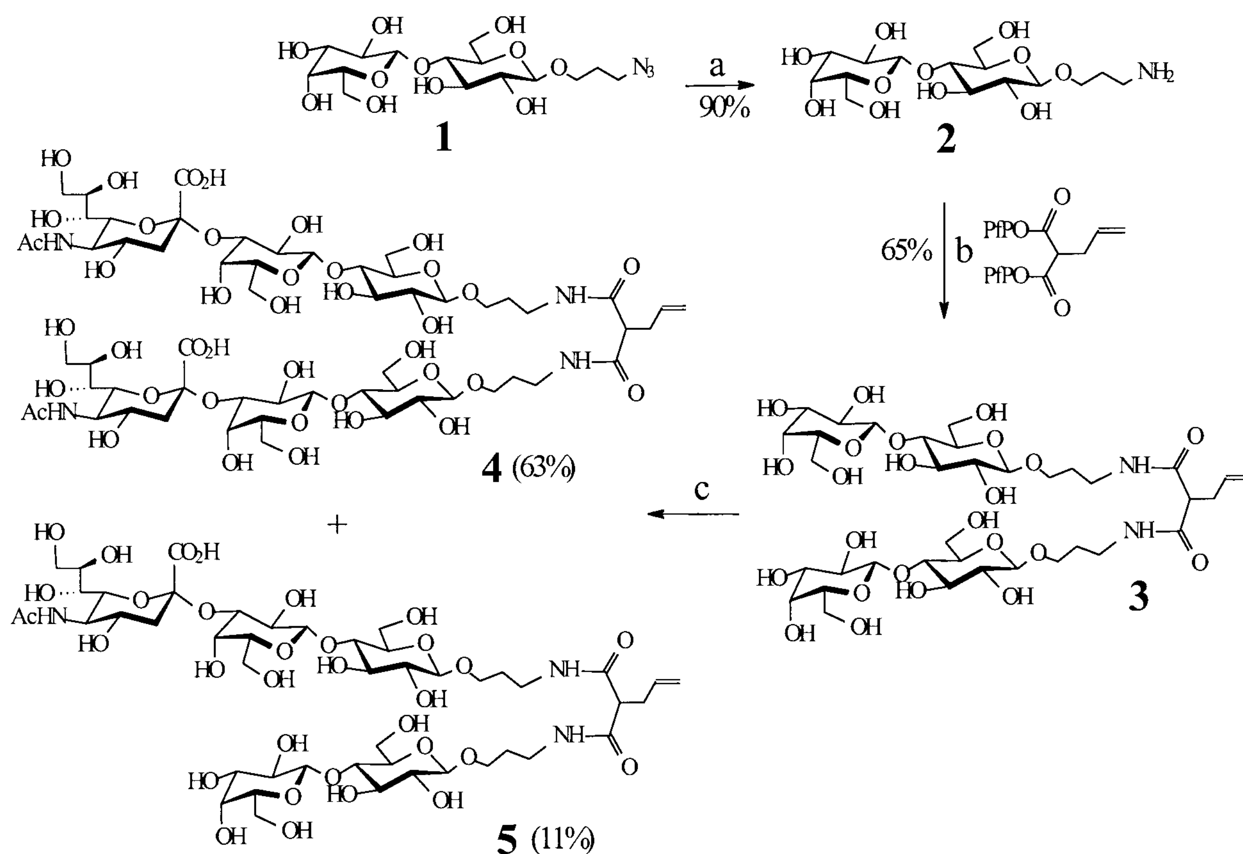
Results and discussion

Synthesis of glycoconjugates

Multivalent antigen presentation may create a three-dimensional epitope, which can mimic the topology of the cell surface carbohydrates to elicit a cancer-specific immune response. For this reason KLH has been widely used as a carrier because of its ability to be conjugated to high amounts of oligosaccharide antigens [20]. However, the presentation of antigens in such a cluster is not defined. By using allylmalonamide as a bivalent linker in the synthesis of glycoconjugates we can achieve a well-defined bivalent antigen presentation.

3-Aminopropyl lactoside 2 prepared from catalytic hydrogenation of its azido-derivative 1 [24], was reacted with an activated ester of allymalonic acid in DMF (Scheme 1) to afford 3 as major product (65%). The structure of the product was characterized according to NMR and ES-MS. Two lactoside moieties were identical in terms of their chemical shifts. Anomeric protons were observed at δ 4.46 ppm ($J_{1,2}$ 7.7 Hz) for galactosyl residues and 4.47 ($J_{1,2}$ 7.7 Hz) for glucosyl residues. Anomeric carbons signal at 103.8 ppm for Gals, and 103.1 for Glcs, respectively.

Enzymatic sialylation has been widely used in a setting



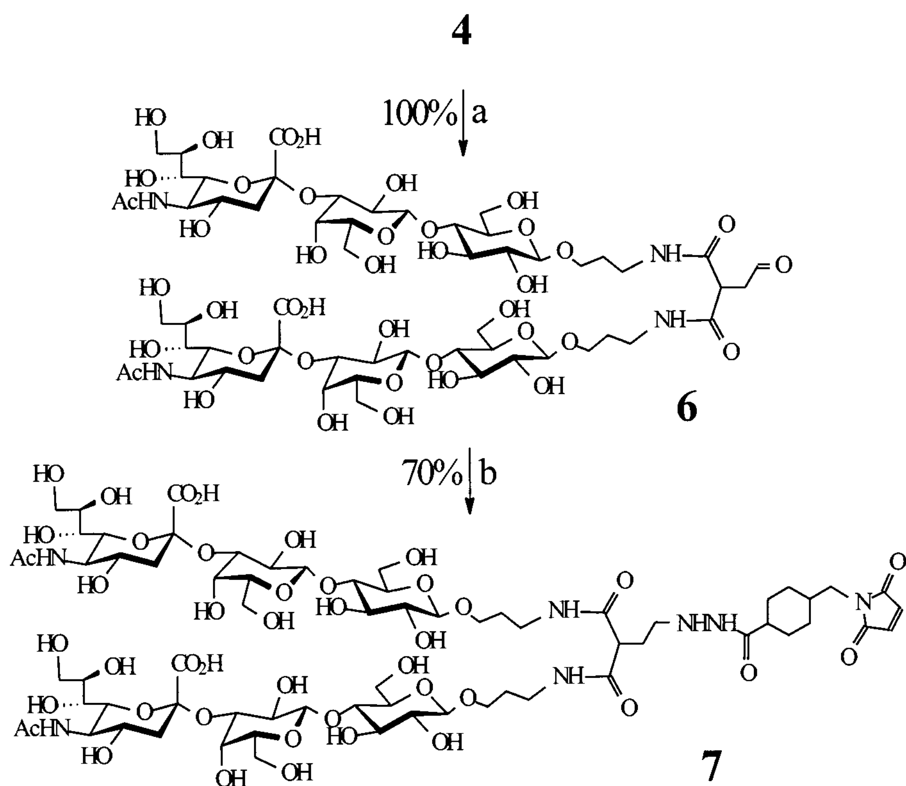
Scheme 1. Reagents and conditions: (a) Pd/C-H_2 in water, 2 h at rt; (b) iPr_2NEt/DMF, 4 h at rt; (c) NeuAc/CTP, fusion enzyme, pH 7.5, 5 h at 37 °C.

of one-pot, two-enzyme system [25]. Similarly, GM_3 has been synthesized using a chemo-enzymatic combination [26–29]. Recently, we developed a recombinant fusion enzyme that combines CMP-NeuAc synthetase and NeuAc transferase together [30]. This enzyme was efficiently applied to the introduction of sialic acids at 3-*O*-positions of the galactosyl residues of **3,** to afford bisialylated **4** as a major product and monosialylated **5** as a minor product in an overall yield 70–80%. The ratio of **4** to **5** was about 6:1 according to the measurement by ^1H NMR. Both compounds were fully characterized with ^1H and ^{13}C NMR (see Table 1) and ES-MS.

The allyl group of **4** was converted to an aldehyde by ozonolysis [31,32] to afford **6,** which was obtained mainly in its hydrated acetal form according to its ^1H NMR spectrum, where only about 10% aldehyde proton was observed at 9.14 ppm. ES-MS analysis further confirmed the hydrated **6** by the observation of a corresponding molecular peak (m/z 1509.0, M+H_2O). Conjugation of compound **6** to proteins (HSA and KLH) directly via reductive amination was attempted with sodium cyanoborohydride in $NaHCO_3$ buffer (pH 8.3), PBS buffer (pH 7.8), and NaOAc buffer (pH 5.5). However, we failed to achieve significant conjugation under above conditions, as determined by lack of significant change in HPLC profiles obtained as the reaction proceeded. When an ozonolized **3,** instead of **6,** was used, however, marginal success in conjugation to HSA resulted. MALDI-MS showed 2–3 moles of saccharide were attached per mole of HSA (data not shown). The above results are very similar to the observation reported by Ragupathi et al [33]. When a second spacer such as M_2C_2H was used, a higher ratio of carbohydrate to protein was achieved [33]. Compound **6** was therefore coupled to M_2C_2H by reductive amination to yield maleimide-containing, biantennary **7** (70%) (see Scheme 2), which was further coupled to thiolated proteins (HSA and KLH) resulting in glycoconjugates **12** (HSA) and **13** (KLH), respectively. MALDI-MS analysis indicated that five biantennary GM_3 saccharides were attached to HSA, while thirty-five were incorporated to KLH as determined by quantitative analysis of sialic acid and protein [34] (see Table 2).

With KLH glycoconjugate **13** in hand for immunization, we had to make other glycoconjugates for ELISA studies to avoid cross-reactivity to the protein and linkage structure of **13.** Lactoside and sialyllactoside-HSA conjugates (**14** and **15**), possessing a different crosslinker, were therefore synthesized (see Fig. 1).

3-Aminopropyl sialyllactoside (**9**) was prepared by sialylation of lactoside **1** to **8** (70–80%) with the same procedure used in the synthesis of **4,** followed by reduction of the



Scheme 2. Reagents and conditions: (a) O_3/MeOH, −78 °C, Me_2S, 4 h; (b) C_2M_2H/PBS-DMSO, $NaCNBH_3$, 16 h at rt.

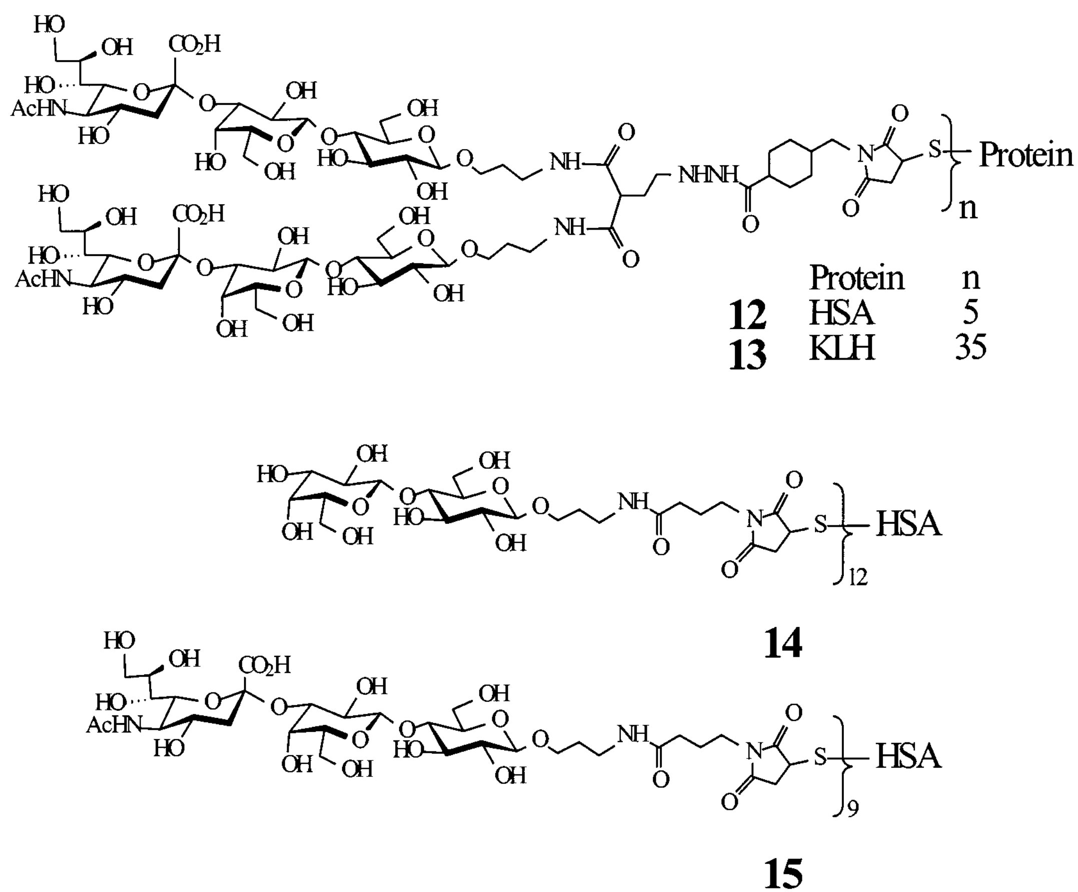
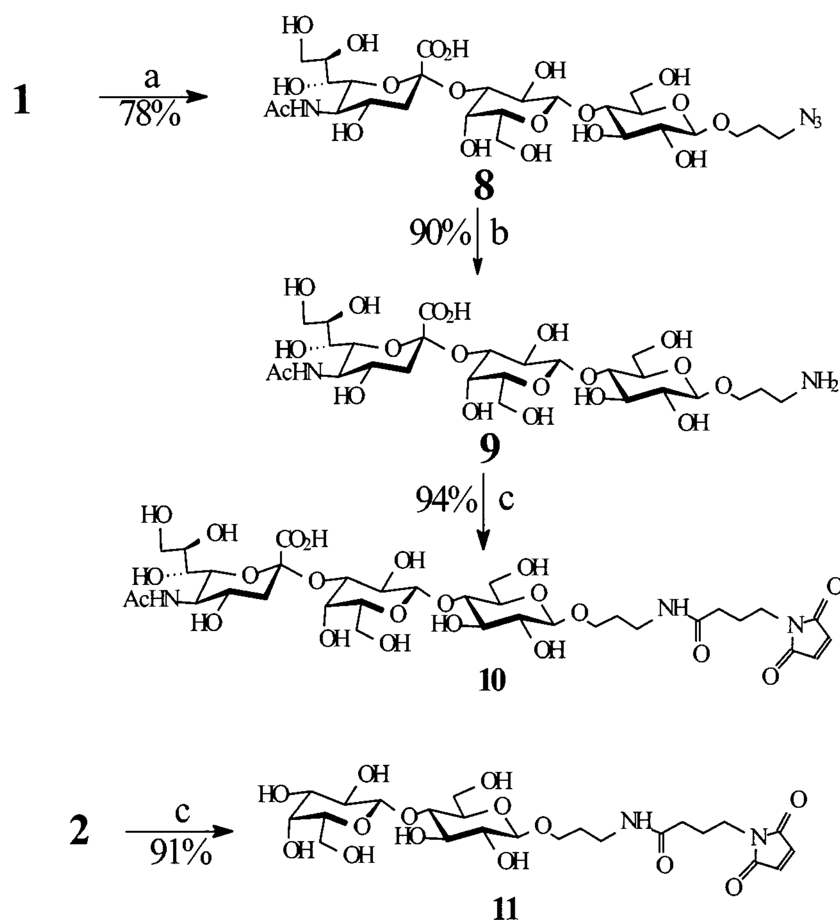


Figure 1. The structures of neoglycoconjugates synthesized in this study.



Scheme 3. Reagents and conditions: (a) NeuAc/CTP, fusion enzyme, pH 7.5, 16 h at 37 °C; (b) Pd/C-H_2 in water, 2 h at rt; (c) Sulfo-GMBS/PBS, 2 h at rt.

azidopropyl (H_2-Pd/C in water, 90%) (see Scheme 3). The amino group was designed for coupling purpose. The NMR data for both **8** and **9** are also summarized in Table 1.

To introduce a meileimide-containing spacer, 3-aminopropyl lactoside **2** or sialyllactoside **9** was reacted with a water-soluble activated ester crosslinker (Sulfo-GMBS) in PBS buffer, respectively. The completion of the reactions was indicated by TLC analysis with the disappearance of starting material and the formation of a faster moving product. Both products, **10** (94%) and **11** (91%), were purified by a Sephadex G-10 column and characterized by NMR and MS spectroscopic analysis. Further conjugation to thiolated HSA afforded products, **14** and **15,** respectively, after purification by a Biogel A 0.5 column. MALDI-MS analysis of the glycoconjugates shown that an average molar ratio of protein to saccharide 1:12 in **14** and 1:9 in **15** were achieved (see Table 2).

Immunological properties

In order to evaluate the specificity of the antibodies raised by immunization of mice with a biantennary sialyllactoside-KLH conjugate (**13**), four glycoconjugates, the structures of which are shown in Figure 1 and Figure 2, were used for ELISA. Glycoconjugates **14** and **15** were constructed with same crosslinker and were otherwise structurally similar except that the sialic acid residue in **15** is absent in **14.** The differences in antibody titer between **14** and **15** in screening should correlate to some extent to the specific antibodies against the sialyllactoside epitope. Glycoconjugates of GBS Ia and III capsular polysaccharide were included for the purposes of determining whether antibodies to terminal sialyl-galactose were raised, and if so, whether the multiple sialyl-galactose epitopes presented in side chains of the polysaccharide have a cluster effect in the antibody binding. All the screening glycoconjugates used had different protein carrier (HSA) and linkage constructs to the glycoconjugate immunogen (**13**) so that we could focus exclusively on the immune response of the saccharide moiety. The antibody titers (ELISA) of the binding of the HSA conjugates (**14** and **15**) to the biantennary sialyllactoside-KLH conjugate (**13**) antisera are shown in Table 3.

High titers of anti-sialyllactoside antibodies (both IgG and IgM) were obtained. While the IgG response to **13** was

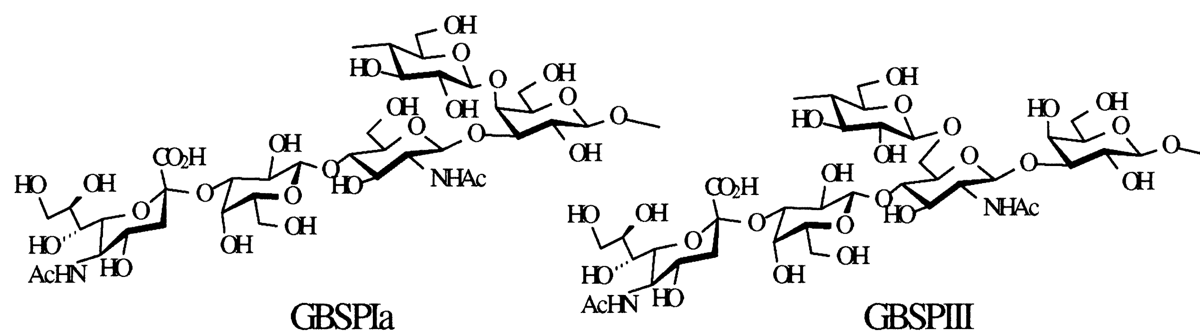


Figure 2. The structures of type Ia and III group B streptococcal capsular polysaccharides (GBSPIa and GBSPIII). Their HSA conjugates were prepared by partial oxidation of exo-cyclic chain of sialic acid, followed by reductive amination with amino groups of protein.

Table 3. Antibody titers by ELISA against HSA conjugates of lactoside (**14**), sialyllactoside (**15**), GBSPIa, and GBSPIII[a,b]

	14		**15**		**GBSPIa**		**GBSPIII**	
Mouse	*IgG*	*IgM*	*IgG*	*IgM*	*IgG*	*IgM*	*IgG*	*IgM*
1	1,300	3,200	6,000	6,000	125	70	200	80
2	1,600	1,600	64,000	1,000	80	–	500	–
3	1,400	3,500	80,000	12,800	200	–	400	100
4	6,000	6,500	250,000	25,600	75	70	700	150

[a]The mice were immunized with biantennary GM_3 saccharide—KLH glycoconjugate (**13**) with MPL adjuvant.
[b]The titers were the readings of last bleeding (day 31), and the highest dilution at OD $>$ 0.10.

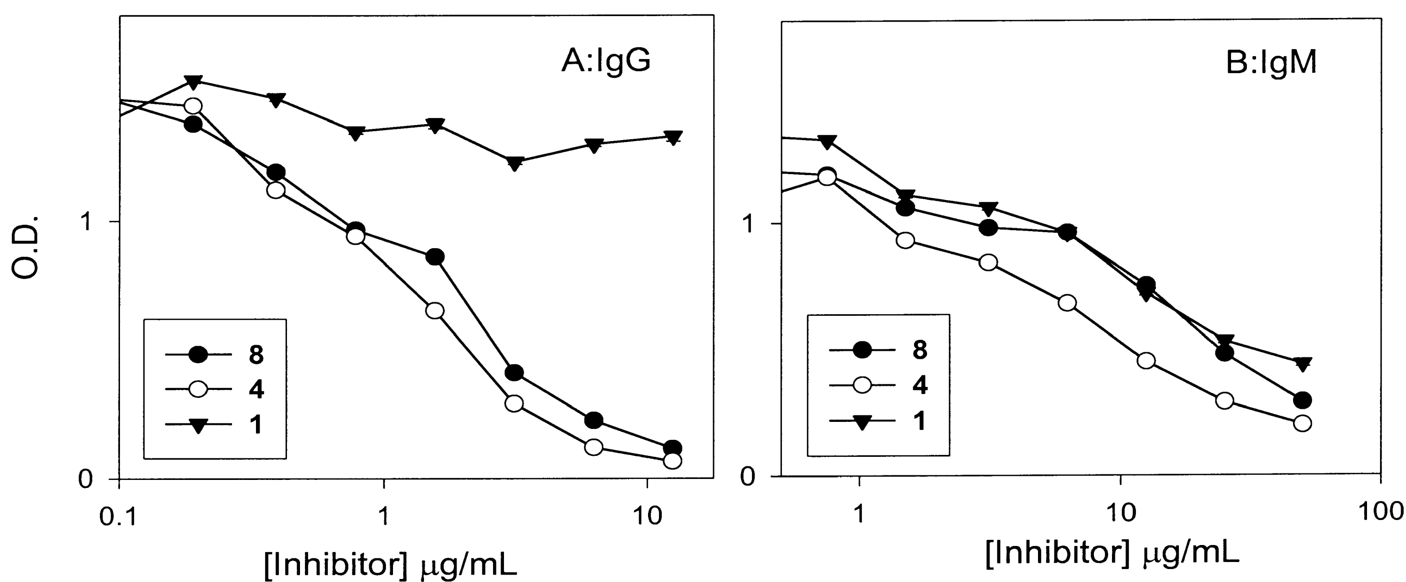


Figure 3. The competitive inhibition ELISA on the binding of glycoconjugate **15** to antisera using lactoside (**1**), biantennary sialyllactoside (**4**), and sialyllactoside (**8**) as inhibitors.

predominantly sialyllactoside-specific there was a small amount of lactose cross-reactive IgG antibodies detected. In contrast the IgM response to **13** was different because the amount of cross-reactive lactoside-binding antibodies was much larger. This basic difference between the IgG and IgM responses to **13** was also reflected in inhibition experiments shown in Figure 3. These experiments were performed using lactoside (**1**), sialyllactoside (**8**) and biantennary sialyllactoside (**4**) as inhibitors of mouse IgM and IgG induced by **13.** While lactoside failed to inhibit the IgG response to **13,** it was able to inhibit the IgM response as well as sialyllactoside. Thus the IgM response was much less dependent on the presence of terminal sialic acid. For both IgM and IgG response biantennary sialyllactoside was the best inhibitor although only marginally so in the case of the latter.

The binding of both GBSP Ia and GBSP III-HSA conjugates to the antisera induced by **13** (see Table 3) was very weak, indicating that only minimal amounts of (NeuAcGal)-specific antibodies were induced by **13.** Interestingly the IgG response in terms of binding to GBSP Ia and GBSP III was much higher than that the IgM response. This is consistent with our previous findings that terminal sialic acid plays a larger role in the IgG response to **13.**

In conclusion we have synthesized a biantennary GM_3 saccharide-KLH conjugate by a method that is adaptable to the synthesis of other biantennary glycoconjugates. We have demonstrated that it is effective in eliciting a predominant sialyllactoside-specific response but we were unable to detect any antibodies induced by an epitope unique to GM_3 in its biantennary form. This is consistent with NMR evidence where the chemical shifts of **2, 3, 4,** and **9** (see Table 1) are almost identical, which is indicative of the conformational equivalent of the sialyllactoside motifs in its monovalent and bivalent forms.

Acknowledgments

The authors thank Dr. P. Thibault and Mr. D. Krajcarsky for MS analysis.

References

1 Hakomori S-I (1984) *Ann Rev Immunol* **2:** 103–26.
2 Hakomori S-I (1996) *Cancer Res* **56:** 5309–18.
3 Tsuchida T, Saxton R, Irie RF (1987) *J Natl Cancer Inst* **78:** 55–60.
4 Lloyd KO, Furukawa K (1998) *Glycoconjugate J* **15:** 627–36.
5 Hirabayashi Y, Hamaoka A, Matsumoto A, Matsubara T, Tagawa M, Wakabayashi S, Taniguchi M (1985) *J Biol Chem* **260:** 13328–33.
6 Nores GA, Dohi T, Taniguchi M, Hakomori S-I (1987) *J Immunol* **139:** 3171–76.
7 Iwabuchi K, Yamamura S, Prinetti A, Handa K, Hakomori S-I (1998) *J Biol Chem* **273:** 9130–38.
8 Jones PC, Sze L, Liu PY, et al.(1981) *J Natl Cancer Inst* **66:** 249.
9 Livingston PO, Wong G, Adluri S, Tao Y, Padavan M, Parente R, Hanlon C, Calves MJ, Helling F, Ritter G, Oettgen HF, Old LJ (1994) *J Clin Oncol* **12:** 1036–44.
10 Livingston PO (1992) *Current Opinion in Immunology* **4:** 624–29.
11 Tai T, Cahan LD, Tsuchida T, Saxton R, Irie RF, Morton DL (1985) *Int J Cancer* **35:** 607–12.
12 Nakano J, Yasui H, Muto M (1998) *Pigment Cell Research* **11:** 213–15.
13 Li R, Gage D, McKallip R, Ladisch S (1996) *Glycoconjugate J* **13:** 385–89.
14 Li R, Villacreses N, Ladisch S (1995) *Cancer Res* **55:** 211–14.
15 Ravindranath MH, Kelly MC, Jones RC, Amiri AA, Bauer PM, Morton DL (1998) *Int J Cancer* **75:** 117–24.
16 Hoon DSB, Wang Y, Sze L, Kanda H, Watanabe T, Morrison SL, Morton DL, Irie RF (1993) *Cancer Res* **53:** 5244–50.
17 Wolchok JD, Livingston PO, Houghton AN (1998) *Melanoma* **12:** 835–48.
18 Dohi T, Nores GA, Hakomori S-I (1988) *Cancer Res* **48:** 5680–85.
19 Livingston PO (1995) *Cancer Biology* **6:** 357–66.
20 Livingston PO (1995) *Immunol Rev* **145:** 147–56.
21 Livingston PO (1998) *Seminars in Oncology* **25:** 636–45.
22 Zhang H, Zhang S, Cheung N-K, Ragupathi G, Livingston PO (1998) *Cancer Res* **58:** 2844–49.
23 Wessels MR, Paoletti LC, Kasper DL, DiFabio JL, Michon F, Holme K, Jennings HJ (1990) *J Clin Invest* **86:** 1428.
24 Zou W, Jennings HJ (1996) *J Carbohydr Chem* **15:** 279–96.
25 Wong C-H, Halcomb RL, Ichikawa Y, Kajimoto T (1995) *Angew Chem Int Ed Engl* **34:** 521–46.
26 Zehavi U, Tuchinsky A (1998) *Glycoconjugate J* **15:** 657–62.
27 Earle MA, Manku S, Hultin PG, Li H, Palcic M (1997) *Carbohydr Res* **301:** 1–4.
28 Ito Y, Paulson J (1993) *J Am Chem Soc* **115:** 1603–05.
29 Sabesan S, Paulson J (1986) *J Am Chem Soc* **108:** 2068–80.
30 Gilbert M, Bayer R, Cunningham A-M, DeFrees S, Gao Y, Watson D, Young NM, Wakarchuk WW (1998) *Nature Biotechnology* **16:** 769–72.
31 Bernstein MA, Hall DL (1980) *Carbohydr Res* **78:** C1–C3.
32 Danishefsky SJ, Behar V, Randolph JT, Lloyd KO (1995) *J Am Chem Soc* **117:** 5701–11.
33 Ragupathi G, Koganty RR, Qiu D, Lloyd KO, Livingston PO (1998) *Glycoconjugate J* **15:** 217–21.
34 Svennerholm L (1957) *Biochim Biophys Acta* **24:** 604–11.

Received 13 July 1999, revised 28 September 1999, accepted 4 October 1999