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BIOSYNTHESIS *in vitro* OF A GLOBOSIDE CONTAINING A 2-ACETAMIDO-2-DEOXY-β-D-GALACTOPYRANOSYL GROUP (1→3)-LINKED AND FORSSMAN GLYCOLIPID BY TWO *N*-ACETYLGALACTOSAMINYLTRANSFERASES FROM CHEMICALLY TRANSFORMED GUINEA PIG CELLS*

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ABSTRACT

Two *N*-acetylgalactosaminyltransferase activities (GalNAcT-2 and GalNAcT-3) have been characterized in chemically transformed, cultured guinea-pig cell lines (104C1 and 106B). Line 104C1 is a benz[*a*]pyrene-transformed tumorigenic variant, whereas line 106B is a 7,12-dimethylbenz[*a*]anthracene-transformed nontumorigenic variant obtained from fetal guinea-pig cells at 43 days of gestation. The GalNAcT-2 (UDP-GalNAc:$GbOse_3Cer$ β-*N*-acetylgalactosaminyltransferase) isolated from both 104C1 and 106B cells catalyzed the transfer of GalNAc from UDP-GalNAc to the ^3H-labeled terminal galactose group of Gb3 ([6-^3H]Galα1→4Galβ1→4Glc→Cer). The ^3H-labeled globoside was purified and then subjected to exhaustive methylation. After acetolysis, the partially methylated sugars were separated by two-dimensional, thin-layer chromatography. ^3H-Label was detected in two major areas, 2,4,6-tri-*O*-Me-Gal (40%) and 2,3,4,6-tetra-*O*-Me-Gal (46%). In a separate experiment, 80% of the GalNAc was released when labeled $GbOse_4Cer$ ([^3H]GalNAc→Galα1→4Galβ1→4Glc→Cer) was treated with purified clam β-hexosaminidase. The present results establish the formation of a β-D-Gal*p*NAc-(1→3) linkage in the terminal region of the biosynthesized globoside.

GalNAcT-3 activity (UDP-GalNAc:$GbOse_4Cer$ α-GalNAc-transferase), which catalyzes the transfer of GalNAc from UDP-[^{14}C]- or -[^3H]GalNAc to $GbOse_4Cer$ (GalNAcβ1→3Galα1→4Galβ1→4Glc→Cer), was three times higher

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in 106B cells than in 104C1 cells. The isolated, purified radioactive product formed an immunoprecipitin line against rabbit anti-Forssman antibody.

INTRODUCTION

Forssman glycolipid is a pentaglycosylceramide having a terminal 2-acetamido-2-deoxy-α-D-galactopyranosyl group present in erythrocytes of several Forssman-positive animals. The occurrence of Forssman antigen was first demonstrated[1] in 1911. The structure of the globoside (β-D-Gal*p*NAc-(1→3)-α-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-β-D-Glc-(1→1)-Cer; $GbOse_4Cer$; **2**), which is probably a precursor of Forssman antigen, was first reported by Yamakawa *et al.*[2,3] and Makita *et al.*[4,5]. The revised structures of globoside and Forssman glycosphingolipid (GalNAcα1→3$GbOse_4Cer$) were established by Hakomori and associates[6,7]. The structure of the Forssman antigen from equine spleen[7], ovine and caprine erythrocytes[8,9],, and canine erythrocytes[10] has also been reported. The structure of cytolipin R, which is closely related to globoside, except for the α-D-(1→3) internal linkage, has been established for murine tissues[11,12]. Although the guinea pig is a Forssman-positive animal, the major glycolipid present in guinea pig erythrocytes is a triglycosylceramide, $GgOse_3Cer$ (GalNAcβ1→4Galβ1→4Glc-Cer; **1**), as shown by Seyama and Yamakawa[13]. We have reported the biosynthesis, *in vitro*, of this glycosphingolipid (GSL) from lactosylceramide and UDP-[^{14}C]GalNAc by the use of an enzyme obtained from the bone-marrow membrane of guinea-pig, GalNAcT-1 [UDP-GalNAc:Lac→Cer(β1→4)GalNAc-transferase][14].

The stepwise biosynthesis, *in vitro*, of globoside was reported for an embryonic chicken-brain system[15]. The conversion of globoside to Forssman antigen has been achieved with guinea pig kidney membranes[16,17] and with cultured mouse adrenal Y-1-K tumor[18], as well as with guinea pig 104C1 cells[19]. A tandem mechanism of Forssman GSL biosynthesis has been proposed[20]. Makita and associates, using dog spleen microsomes, have solubilized and purified the enzyme catalyzing the synthesis[21] of Forssman GSL from globoside[22]. However, the exact chemical structures of the biosynthesized products, globoside and Forssman glycosphingolipids, are not yet known.

We report herein the biosynthesis *in vitro* of Forssman GSL (**3**) from globotriaosylceramide ($GbOse_3Cer$; **1**) *via* globoside ($GbOse_4Cer$; **2**) by two types of chemically transformed, guinea pig cells, 104C1 (tumorigenic) and 106B (non-tumorigenic)[23,24]. We have identified, in these cell lines, two distinct *N*-acetylgalactosaminyltransferases, βGalNAcT-2 and αGalNAcT-3 that catalyze reactions (*1*) and (*2*).

$$\text{Gal}\alpha1\rightarrow4\text{Gal}\beta1\rightarrow4\text{Glc}\rightarrow\text{Cer}\ (GbOse_3Cer)\ (\mathbf{1})\ +\ \text{UDP-[}^{14}\text{C]- or UDP-[}^{3}\text{H]-GalNAc}\xrightarrow[Mn^{2+}]{\beta\text{GalNAcT-2}}\text{[}^{14}\text{C]- or [}^{3}\text{H]-GalNAc}\beta1\rightarrow3\text{Gal}\alpha1\rightarrow4\text{Gal}\beta1\rightarrow4\text{Glc}\rightarrow\text{Cer}\ (GbOse_4Cer)\ (\mathbf{2})\ +\ \text{UDP} \qquad (1)$$

$$\text{GbOse}_4\text{Cer (2)} + \text{UDP-[}^{14}\text{C]- or UDP-[}^{3}\text{H]-GalNAc} \xrightarrow[\text{Mn}^{2+}]{\alpha\text{GalNAcT-3}} [^{14}\text{C}]\text{- or}$$

$$[^{3}\text{H}]\text{-GalNAc}\alpha1\rightarrow3\text{GalNAc}\beta1\rightarrow3\text{Gal}\alpha1\rightarrow4\text{Gal}\beta1\rightarrow4\text{Glc}\rightarrow\text{Cer}$$

(Forssman GSL) (3) (2)

EXPERIMENTAL

Materials. — The following materials were obtained from commercial sources: sodium taurocholate (Sigma Chemical Co.); Florisil (Fisher Scientific Products); Unisil (Clarkson Chemical Co.); Silica gel G (Brinkman Instruments); and Whatman 3MM paper (Scientific Products). Purified protein A from *Staphylococcus aureus* was purchased from Pharmacia Fine Chemicals and iodinated by the Chloramine-T method[25]. Tissue culture flasks were purchased from Corning Glass Works. RPMI-1640 medium, fetal calf serum, penicillin, and streptomycin were obtained from Gibco Laboratories. All the glycosphingolipids used as substrates were prepared in our laboratory according to previously published methods: Globoside ($GbOse_4Cer$) (**2**) and globotriaosylceramide ($GbOse_3Cer$) (**1**) were isolated from pig heart[15] and rabbit erythrocytes[26], respectively; Forssman glycolipid was isolated from sheep erythrocytes by the use of a newly developed, radial thin-layer chromatographic method[27]. Before use as substrates, the purified glycolipids were analyzed by g.l.c.–m.s. M.s. data were interpreted according to Björndal *et al.*[28] and Sung *et al.*[10]. Jack bean β-hexosaminidase and a mixture of clam α- and β-hexosaminidases[29] were prepared in our laboratory. Rabbit anti-Forssman antibody was prepared according to the method used for preparation of anti-globoside serum[30]. UDP-D-[^{14}C]GalNAc of high specific activity was purchased from New England Nuclear. Unlabeled UDP-D-GalNAc was prepared according to the method of Carlson *et al.*[31].

Cell culture. — Guinea pig clonal cells 104C1 and 106B were grown and maintained in culture routinely in our laboratory. Both cell lines are chemically transformed variants of fetal cells at 43 days of gestation[23]. 104C1 is a benz[*a*]-pyrene-transformed tumorigenic variant, whereas 106B is a 7,12-dimethylbenz[*a*]-anthracene-transformed nontumorigenic clone. The culture were grown in 250-mL plastic T-flasks containing RPMI-1640 medium (15 mL) supplemented with 10% fetal calf serum in the presence of penicillin (100 units/mL) and streptomycin (100 μg/mL). The cells were incubated in a humidified atmosphere of 95% air and 5% CO_2 (pH 7.0) at 37°. The medium was changed once before harvesting. The cells were subcultured, when the monolayer reached confluence, by use of 0.25% of trypsin and harvested with phosphate-buffered saline (PBS) containing 0.1% EDTA.

Preparation of enzyme. — The enzymes that catalyze the stepwise biosynthesis of Forssman glycolipid were detected in homogenates of both 104C1 and 106B cells. All steps of enzyme preparation were carried out at 0–4°. The packed

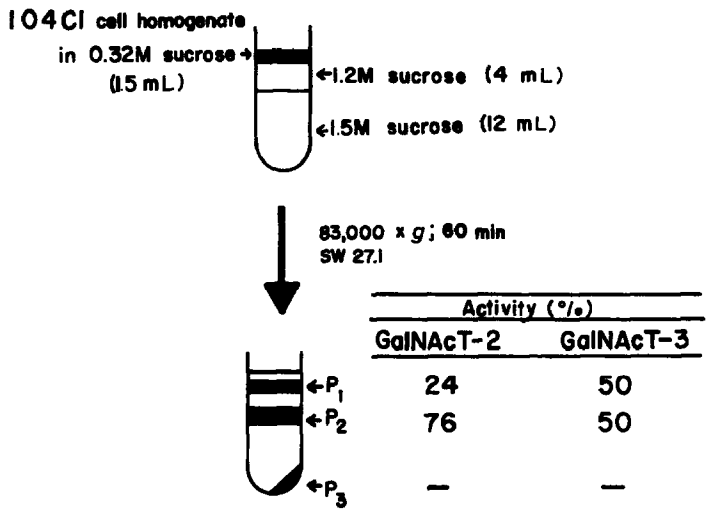


Fig. 1. Fractionation of 104C1 cell membranes.

cells (0.2–0.5 mL) were homogenized in 0.32M sucrose, mM EDTA, and 0.14mM mercaptoethanol (SME) (3 vol.) according to our previously published method[32] with a Polytron 10-ST homogenizer for 20–30 s. The homogenate was then layered on top of a discontinuous sucrose gradient (1.2 and 1.5M) and centrifuged for 1 h at 83 000 *g* in a swinging bucket rotor (SW 27.1) in a Beckman ultracentrifuge (Model L2-65B). Both β-GalNAcT-2 and α-GalNAcT-3 activities were isolated in membrane fractions P_1 (at the junction of 0.32 and 1.0M SME), P_2 (membrane fraction at the junction of 1.0 and 1.5M SME), and P_3 (pellet at the bottom of 1.5M SME). However, the relative activities of these two enzymes were different in these membrane fractions (Fig. 1), as discussed later.

Enzyme assay. — Complete incubation mixtures contained the following components in a final volume of 50 μL: acceptor glycosphingolipid ($GbOse_3Cer$ or $GbOse_4Cer$) (25–50 nmol); Triton DF-12 detergent (GalNAcT-2, P/D 1.5; GalNAcT-3, P/D 2.0); HEPES buffer (GalNAcT-2), cacodylate · HCl(GalNAcT-3), 10 μmol, pH 7.0; $MnCl_2$ (0.25 nmol); UDP-[^{3}H]- or [^{14}C]-GalNAc (10–15 nmol; 3–5 × 10^6 c.p.m./μmol); and enzyme fraction P_3 or homogenate (0.1–0.3 mg of protein estimated by the method of Lowry *et al.*[33]). The mixture was incubated for 1–2 h, and the reaction was stopped by addition of 0.25M EDTA (10 μL) and methanol (10 μL). Incubation mixtures containing [^{14}C]$GbOse_4Cer$ (**2**) and [^{14}C]$GbOse_5Cer$ (**3**) were then assayed by a double chromatographic technique in 1% $Na_2B_4O_7$, followed by reverse chromatography in 10:5:1 (v/v) chloroform–methanol–water on Whatman 3MM paper according to a previously published method[15]. Incubation mixtures containing [^{3}H]$GbOse_4Cer$ and [^{3}H]$GbOse_5Cer$ were spotted directly on SG-81 paper[34] and chromatographed in descending fashion in 1% $Na_2B_4O_7$ (pH 9.1). The radioactivity of the product was determined quantita-

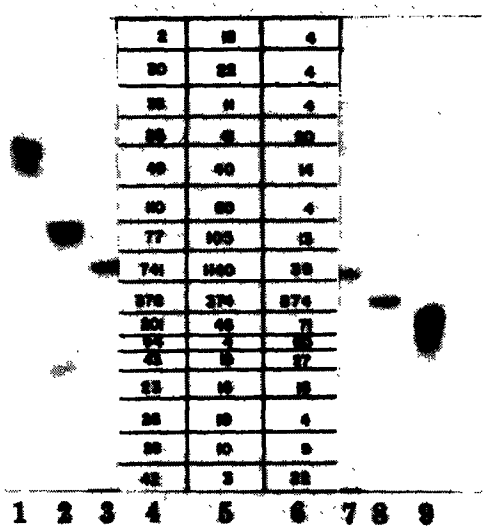


Fig. 2. Thin-layer chromatography of ^{14}C-labeled Forssman products: (1) LacCer (Galβ1→4Glc→Cer); (2) $GbOse_3Cer$ (**1**); (3) $GbOse_4Cer$ (**2**); (4) [^{14}C]$GbOse_4Cer$ (**2**) (1500 c.p.m. from the 104C1 GalNAcT-2-catalyzed reaction); (5) [^3H]$GbOse_4Cer$ (**2**) (2500 c.p.m. from the 106B GalNAcT-2-catalyzed reaction); (6) [^3H]$GbOse_5Cer$ (2500 c.p.m. from the 106B GalNAcT-3-catalyzed reaction); (7) $GbOse_4Cer$ (**2**) (porcine erythrocytes); (8) Forssman GSL **3**; ovine erythrocytes); and (9) $nLcOse_5Cer$ (Galα1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc-Cer), rabbit erythrocytes). The plate was developed in a solvent system of 11:9:2 (v/v) chloroform–methanol–0.2% $CaCl_2$ in water. Lanes 4, 5, and 6 were scraped and the radioactivity was counted in a liquid-scintillation system; the results are shown by numbers. The spots in the other lanes were detected by spraying with the diphenyl-amineaniline reagent.

tively by counting appropriate areas in a toluene scintillation system using a Beckman scintillation counter (Model 3133T).

Isolation and purification of radioactive products. —The radioactive products of both reactions (104C1 and 106B) were isolated from incubation mixtures the volume of which had been increased 30-fold (1.5 mL). After incubation for 12 h at 37°, the reactions were stopped by adding 0.25M EDTA (10 μL, pH 7.0). The content of each reaction tube was spotted on SG-81 paper and chromatographed in descending fashion in 1% $Na_2B_4O_7$. After the paper had been developed and dried, the origin including 2.5 cm on either side was cut from each sheet and the radioactive products were eluted from the strips with 60:35:8 (v/v) chloroform–methanol–water. Further purification of the radioactive products extracted from SG-81 was achieved by chromatography on a Bio-Sil column (0.7 × 9 cm) with increasing concentrations of methanol in chloroform. The final purity of the radioactive product was monitored by t.l.c. on Silica gel G plates with authentic substrates (Fig. 2).

α- and β-Hexosaminidase treatment of the radioactive products. — The purified radioactive products (**2** and **3**) were hydrolyzed with jack bean or clam hexosaminidase for 12 h at 37° as follows: ^{14}C- or ^3H-products (1000–2000 c.p.m.); detergent (sodium taurodeoxycholate, 50 μg); 0.25mM sodium citrate phosphate

buffer (pH 4.5); and enzyme protein (1–5 munits:1 unit of enzyme hydrolyzes 1.0 μmol of *p*-nitrophenyl 2-acetamido-2-deoxy-α- or -β-D-galactopyranoside per min) of either jack bean β-hexosaminidase[29] or α- and β-hexosaminidases from clam[35]. After reduction with $NaBH_4$, the cleaved radioactive 2-acetamido-2-deoxy-D-galactitol was recovered by p.c. in 1% $Na_2B_4O_7$. The appropriate areas of the chromatograms were analyzed quantitatively by a liquid scintillation technique.

Binding of [^{125}I]Protein A to Forssman antibody-bound cell surfaces. — Rabbit anti-Forssman antibody was produced according to a modification of our previously published method[15]. Newly harvested, guinea pig tumor cells (5×10^4) were placed on 96-hole microtiter plates (MTP) and incubated for 18 h at 37° with RPMI-1640 (0.1 mL/hole) containing 10% fetal bovine serum in a humidified atmosphere of 95% air and 5% CO_2. After the addition of anti-Forssman antibody (50 μL) in the dilutions indicated in Fig. 10, the MTP was kept at room temperature for 60 min. The excess antibody was washed three times with phosphate-buffered saline (PBS, Ix), and then [^{125}I]protein A (50 μL, 1 mg/mL) was added to each well and incubated at room temperature for 30 min[36]. The excess, radiolabeled protein A was washed three times with 0.1% ovalbumin-containing PBS (Ix), and 1.0M NaOH was added to each well (0.1 mL). The alkaline mixture from each well was removed into a plastic minivial with filter paper, and the radioactivity was quantitatively determined with a Beckman gamma counter.

RESULTS

Requirements. — The complete incubation mixture needed the presence of Mn^{2+} (Table I) for both the GalNAcT-2- and the GalNAcT-3-catalyzed reactions. Of many detergents tested (Table II), Triton DF-12 stimulated the highest activity at the indicated protein-to-detergent ratio, between 1.5 and 2.0 (Fig. 3). Both reaction rates remained constant up to 2 h over a range of protein concentrations from 0.1 to 0.3 mg/50 μL of incubation mixture.

When $GbOse_3Cer$ (**3**) was used as substrate, the GalNAcT enzymes isolated from 106B and 104C1 showed two different pH profiles (Fig. 4). For 104C1, only one pH optimum, at pH 7.1, was observed. However, two distinct pH optima (7.1 and 7.9) in HEPES buffer were observed with 106B cell-derived GalNAcT using $GbOse_3Cer$ (**3**) as substrate. The pH optimum for GalNAcT-3 activity in both 104C1 and 106B was pH 7.0 in cacodylate · HCl buffer.

Substrate specificity. — The effects of various concentrations of $GbOse_3Cer$ (**1**) on the rate of reaction are shown in Figs. 5A and 5B. The calculated K_m values were 160 and 110μM with GalNAcT-2 activities isolated from 104C1 and 106B, respectively. The apparent K_m value was 180μM (Fig. 6) with 106B GalNAcT-3 activity when $GbOse_4Cer$ (globoside, **2**) was used as an acceptor. The apparent K_m values for UDP-[^{14}C]GalNAc with GalNAcT-2 from 104C1 and 106B were 130–140μM (Figs. 7A and 7B).

TABLE I

REQUIREMENTS FOR GSL:*N*-ACETYLGALACTOSAMINYLTRANSFERASES FROM GUINEA PIG TRANSFORMED CELLS[a]

Incubation mixture	*[^{3}H]GalNAc incorporated (nmol/mg of protein/2 h)*			
	GalNAc-T-2		*GalNAcT-3*	
	104C1	*106B*	*104C1*	*106B*
Complete	1.82	3.1	0.9	1.5
Complete minus substrate	0.62	1.1	0.59	0.64
Complete minus detergent	0.99	2.1	0.67	0.84
Complete minus Mn^{2+}	0.17	0.19	0.21	0.33
Complete minus Mn^{2+} plus EDTA (1.25 μmol)	0.3	0.98	0.13	0.30
Complete plus EDTA	0.15	0.26	0.29	0.20
Complete heat-killed enzyme	0.15	0.22	0.15	0.58

[a]The complete incubation mixtures contained the following components in a final volume of 0.04 mL. *GalNAc-2 assay*: $GbOse_3Cer$ (**1**) (46 nmol), Triton DF-12 (150 μg; protein/detergent 1.5:1), HEPES buffer (10 mmol, pH 7.0), $MnCl_2$ (0.2 mmol), UDP-[^{3}H]GalNAc (35 000 c.p.m.; 4.6×10^6 c.p.m./μmol), and enzyme protein (200 μg for 104C1 and 170 μg for 106B cells). *GalNAcT-3 assay*: The conditions were the same as given for the GalNAcT-2 assay except for the following: $GbOse_4Cer$ (**4**) (40 nmol) Triton DF-12 (100 μg, protein/detergent 2.0:1.0), and cacodylate · HCl buffer (10 mmol, pH 7.0). After 2 h at 37°, the mixtures were analyzed by 1% $Na_2B_4O_7$ descending chromatography on SG-81 paper as described in the Experimental section. The radioactive product remained at the origin and was quantitatively determined in a liquid-scintillation system. The rates of the reactions remained constant until 2 h and were proportional to protein concentration up to 5 mg/mL.

TABLE II

EFFECT OF VARIOUS DETERGENTS ON GalNAcT ACTIVITIES[a]

Detergents[b]	*[^{3}H]GalNAc incorporated (pmol/mg of protein/h)*			
	GalNAcT-2		*GalNAcT-3*	
	104C1	*106B*	*104C1*	*106B*
Triton DF-12	1047	1177	94	1653
Triton CF-54	778	1234	99	432
Triton X-100	378	992	24	80
Nonidet P-40	450	986	44	164
Taurocholate	175	122	77	49
Octyl glucopyranoside	224	380	80	150
Cutscum	350	1062	0	226
Zwittergent	355	818	0	0

[a]The incubation conditions were the same as described in the footnote to Table I, except that various detergents were used. [b]The ratios of protein to detergent for GalNAcT-2 and GalNAcT-3 were 1.5 and 2.0, respectively.

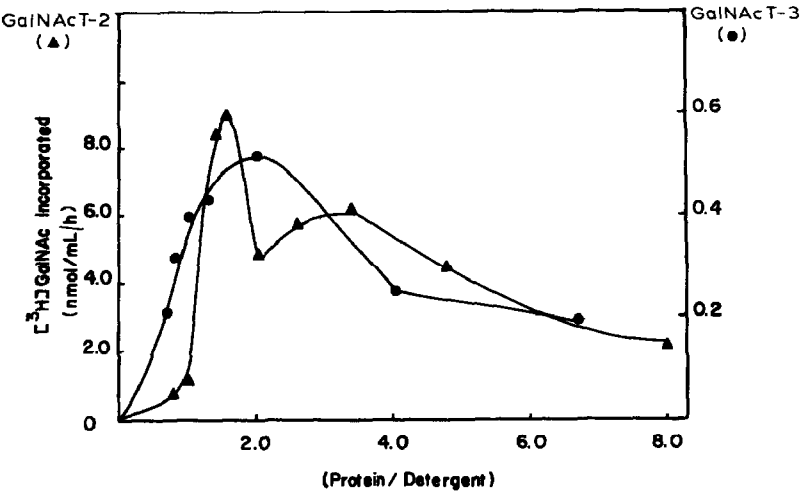


Fig. 3. Effect of detergent concentration on βGalNAcT-2 and αGalNAcT-3 activities. The complete incubation mixture contained the components as described in the footnote to Table I, except that various concentrations of detergent (Triton DF-12) and 104C1 membrane-bound enzyme (4.4 mg/mL of protein) were used. The protein-to-detergent ratio was varied as indicated: $GbOse_3Cer$ (▲) and $GbOse_4Cer$ (●) were used as substrates for GalNAcT-2 and GalNAcT-3 activities, respectively.

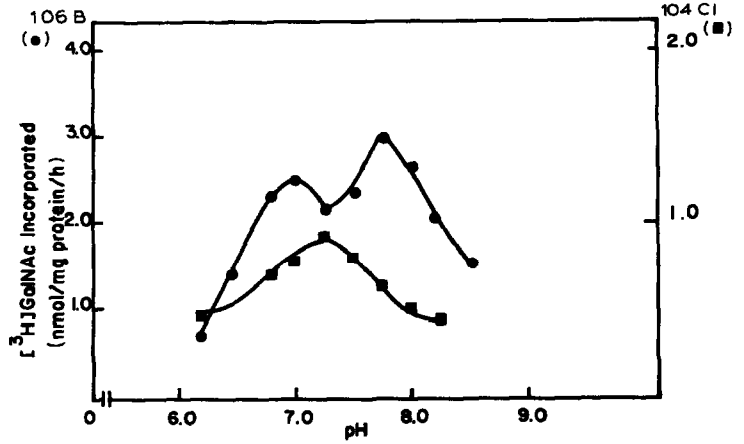


Fig. 4. Effect of pH on GalNAcT-2 activities, isolated from 104C1 and 106B cells with **1** as acceptor. The incubation conditions were the same as described in the footnote to Table I, except that HEPES buffer was used over the pH range 6.2–8.75. The incubation mixtures were incubated for 2 h at 37° and analyzed by SG-81 chromatography.

The incorporation of [^{14}C]GalNAc into various potential acceptors was tested with UDP-[^{14}C]GalNAc as nucleotide donor and GalNAcT-2 plus GalNAcT-3-enriched membrane fractions (Table III). Both lactosylceramide and GM3 were almost inactive as substrates, whereas $GbOse_3Cer$ (**1**) and $GbOse_4Cer$ (**2**) were the most active substrates with membrane preparations isolated from untransformed,

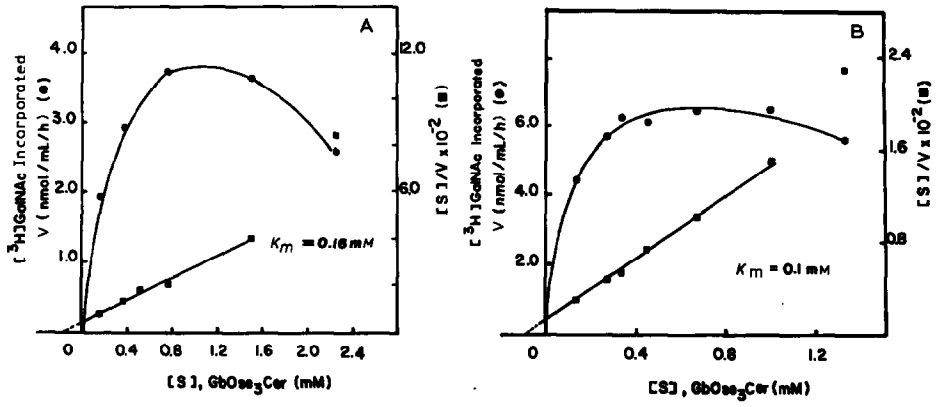


Fig. 5. Effect of $GbOse_3Cer$ (**1**) concentration on the rate of formation of radioactive globoside. The experimental conditions were the same as described in the footnote to Table I, except that various concentrations of **1** were used: (A) βGalNAcT-2 from 104C1 cells and (B) βGalNAcT-2 from 106B cells. All values have been corrected for endogenous compounds.

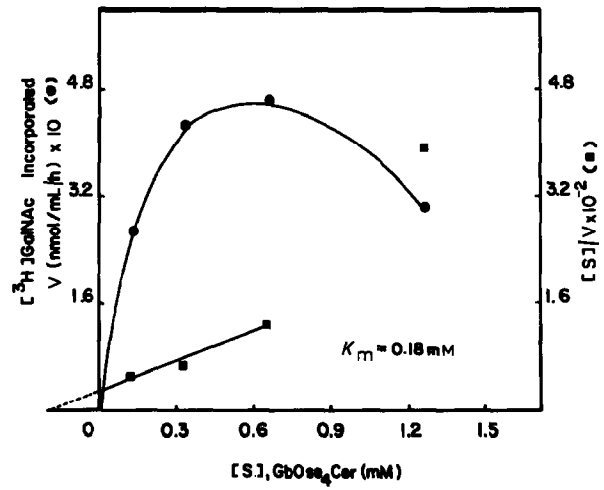


Fig. 6. Effect of globoside concentration on the rate of formation of radioactive Forssman GSL (**3**). The incubation conditions were the same as described in the footnote to Table I for GalNAcT-3, except that various concentrations of globoside **2** ($GbOse_4Cer$) were used. The values have been corrected for endogenous compounds.

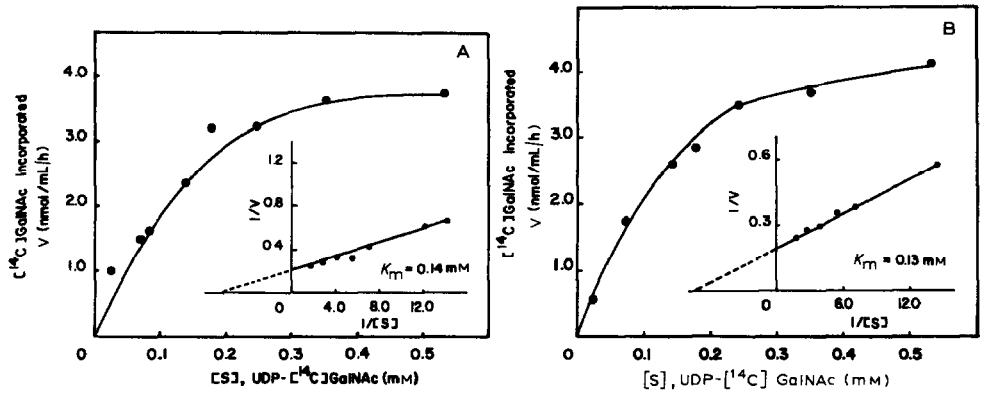


Fig. 7. Effect of donor concentration on the rate of formation of [^{14}C]GalNAc-GbOse$_3$Cer. The experimental conditions were the same as described in the footnote to Table I, except that various concentrations of UDP-[^{14}C]GalNAc (0.01 μmol; 4.6 × 10^6 c.p.m./μmol) were used: (A) βGalNAcT-2 from 104C1 cells and (B) βGalNAcT-2 from 106B cells. All values have been corrected for endogenous compounds.

TABLE III

GLYCOLIPID:*N*-ACETYLGALACTOSAMINYLTRANSFERASE ACTIVITIES IN MEMBRANES ISOLATED FROM GUINEA PIG TUMOR CELLS[a]

Glycolipid acceptor (0.5mM)	*Expected linkage in the product*	*[^{14}C]GalNAc incorporated (pmol/mg of protein/2 h)*		
		103	*104C1*	*106B*
None	(?)	470	360	470
LacCer	β-D-(1→4)	400	380	490
GM3	β-D-(1→4)	450	340	430
GbOse$_3$Cer (**1**)	β-D-(1→3)	1720	1250	1270
GbOse$_4$Cer (**2**)	α-D-(1→3)	1000	580	1600
nLcOse$_4$Cer	β-D-(1→3)(?)	1070	930	810

[a]The conditions were the same as described in the footnote to Table I except that various substrates and UDP-[^{14}C]GalNAc (10 nmol, 3.4 × 10^6 c.p.m./μmol) were used. After 2 h of incubation, the mixtures were analyzed by the chromatographic method described in the Experimental section.

guinea pig embryonic cells (103) and their chemically transformed variants (104C1 and 106B). The GalNAcT-2 activity in 104C1 and 106B remained almost the same, whereas the GalNAcT-3 activity in 106B was five times higher (after subtraction from endogenous values, Table III). However, the activity with nLcOse$_4$Cer was

TABLE IV

TREATMENT OF RADIOACTIVE PRODUCTS WITH PURIFIED HEXOSAMINIDASES

Glycosidase	*Radioactive GalNAc (%) released from*		
	GalNAcT-2 products		*GalNAcT-3 products*
	104C1	*106B*	*106B*
Jack bean β-D-hexosaminidase (5.0 mu)	40	22	0
Clam β-D-hexosaminidase (2.0 mu)	90	44	<9
Clam α-D-hexosaminidase (4.0 mu)			61

50% higher in 104C1 than in 106B, indicative of a different GalNAc-transferase.

Hydrolysis with hexosaminidases. — The radioactive products of GalNAcT-2 and GalNAcT-3 reactions from both 104C1 and 106B were treated separately with purified hexosaminidases. Jack bean β-hexosaminidase cleaved 48 and 22% of the radioactive 2-acetamido-2-deoxy-D-galactose from the GalNAcT-2-catalyzed products of 104C1 and 106B, respectively. When the same two radioactive products were treated with purified clam β-hexosaminidase, almost 90% (104C1) and 44% (106B) of the [^{14}C]GalNAc was cleaved (Table IV). These results indicated the presence of mostly β-D-linked GalNAc groups at the terminus of the 104C1 GalNAcT-2-derived product, whereas the 106B GalNAcT-2 product could be a mixture of $GbOse_4Cer$ and $GbOse_5Cer$ (see Discussion). On the other hand, treatment of the radioactive product ([^{3}H]GalNAc→GalNAcβ1→3Galα1→4Galβ1→4Glc→Cer) of the 106B GalNAcT-3-catalyzed reaction with purified clam β-hexosaminidase released less than 9% of the [^{3}H]GalNAc. This result proved the absence of any β-D-linkage in the GalNAcT-3 catalyzed product. However, purified clam α-hexosaminidase cleaved 61% of the terminal 2-acetamido-2-deoxy-D-galactopyranosyl group of the ^{14}C-Forssman product obtained from 106B GalNAcT-3-catalyzed reaction.

Identification of the terminal GalNAc→Gal linkage in the labeled-globoside product by permethylation studies. — ^{3}H-Labeled $GbOse_3Cer$ (**1**) ([6-^{3}H]Galα1→4Galβ1→4Glc→Cer) was prepared by oxidation of **1** with galactose oxidase, followed by reduction with NaB^3H_4. By use of unlabeled UDP-GalNAc and [6-^{3}H]$GbOse_3Cer$, the radioactive products (GalNAcβ→[6-^{3}H]Galα1→4Galβ1→4Glc→Cer) of the 104C1 and 106B GalNAcT-2-catalyzed reactions were isolated and purified on a Bio-Sil column and by t.l.c. The purified product (~20 000–30 000 c.p.m.) was subjected to Hakomori permethylation, followed by acetolysis and hydrolysis. The permethylated, hydrolyzed products were mixed with nonradioactive, partially methylated galactose standards and were subjected to two-dimensional t.l.c. (Fig. 8). The spots were revealed with the diphenylamine spray and scraped, and the radioactivity in each spot was determined in a liquid-scintillation system. Almost 80–85% of the radioactivity was recovered from only

Fig. 8. Separation of partially methylated sugars by two-dimensional t.l.c. Methylated galactose standards were chromatographed in two directions in two different solvent systems. Solvent *I*: acetone–5% NH_4OH (50:1). Solvent *II*: acetone–28% NH_4OH–water (50:5:1). The spots were detected by spraying with the diphenylamine reagent and heating the plate for 1 h at 110°: (a) 2,3,4,6-tetra-*O*-Me-Gal, (b) 2,3,6-tri-*O*-Me-Gal, (c) 2,4,6-tri-*O*-Me-Gal, (e) 2,3,4-tri-*O*-Me-Gal, (f) 3,4,6-tri-*O*-Me-Gal, and (g) 2,3-di-*O*-Me-Gal.

TABLE V

DISTRIBUTION OF RADIOACTIVITY BETWEEN AREAS CONTAINING PARTIALLY METHYLATED GALACTOSE AFTER 2D T.L.C.[a]

Area	*Permethylated sugar*	*GalNAc-[6-^{3}H]GbOse$_3$Cer (% of radioactivity) from*	
		104C1	*106B*
a	2,3,4,6-Tetra-*O*-Me-Gal	46	55
b	2,3,6-Tri-*O*-Me-Gal	2	5
c	2,4,6-Tri-*O*-Me-Gal	40	25
d	[b]	0	2
e	2,3,4-Tri-*O*-Me-Gal	2	3
f	3,4,6-Tri-*O*-Me-Gal	1	5
g	2,3-Di-*O*-Me-Gal	1	1

[a]Partially methylated galactose standards were mixed with radioactive, permethylated galactose (obtained from radioactive product, GalNAc→[6-^{3}H]GbOse$_3$Cer) and subjected to two-dimensional t.l.c. as illustrated in Fig. 8. After the spots had been detected with the diphenylamine spray, the area of each visible spot was scraped and the radioactivity quantitatively determined in a toluene-scintillation system. [b]Not identified.

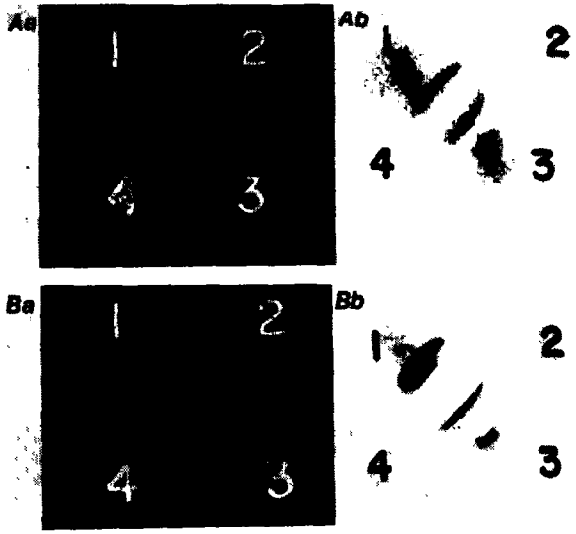


Fig. 9. Microimmunodiffusion pattern with ^{14}C-labeled globoside and Forssman products: (A) The center well contained rabbit anti-Forssman antiserum, and the outer wells contained the following: Well 1, ^{14}C-product (2000 c.p.m.) obtained from $GbOse_3Cer$ (**1**) in the 106B GalNAcT-catalyzed reaction; Well 3, the same ^{14}C-product (2000 c.p.m.) mixed with nonradioactive Forssman GSL (**3**) (in aqueous solution; Wells 2 and 4 contained aqueous solutions of $GbOse_4Cer$ (**2**) and Forssman GSL (**3**) both 10 μg/10 μL), respectively. The precipitin lines were developed with Amido Black as described in the Experimental section. 9Ab is an autoradiogram of plate 9Aa exposed to Kodak X-omat AR X-ray film for 3 months. (B) The conditions were the same except that Well 1 contained the ^{14}C-product (3000 c.p.m.) obtained from $GbOse_4Cer$ (**2**) in the 106B GalNAcT-catalyzed reaction, and Well 3 the ^{14}C-product (3000 c.p.m.) mixed with nonradioactive Forssman GSL (**3**) in aqueous solution. 9Bb is an autoradiogram of plate 9Ba exposed to Kodak X-omat AR X-ray film.

two areas, 2,3,4,6-tetra- and 2,4,6-tri-*O*-methylgalactose (Table V). Less than 5% of the radioactivity was detected in 2,3,4-tri- or 3,4,6-tri-*O*-methylgalactose with the radioactive globoside obtained from either the 104C1- or the 106B-derived GalNAcT-2 reaction. These results unambiguously proved the presence of a GalNAc1→3Gal linkage in the terminal region of these two biosynthetized globosides.

Microimmunodiffusion reaction. — A microimmunodiffusion plate was set up according to our previous procedure[15] using rabbit anti-Forssman antibody in the center well (Fig. 9A and 9B). In Fig. 9Aa, the purified ^{14}C-product obtained from the membrane-bound GalNAcT-catalyzed reaction (GSL, ^{14}C-**2** and ^{14}C-**3**; 2000 c.p.m.) was placed in Well 1. Well 3 contained the same product (2000 c.p.m.) mixed with nonradioactive **3** (10 μg). Nonradioactive **2** and **3** were placed in Wells 2 and 4, respectively. All samples were in 0.1% Triton X-100 solution. The plate was kept in a humidified chamber at 4° for 72 h. The precipitin lines were detected with 2% Amido Black (Fig. 9Aa). A radioautogram of the stained plate was obtained by exposure of the X-ray plate for 3 months (Fig. 9Ab); the ^{14}C-product formed a precipitin line with rabbit anti-Forssman antibody (Wells 1 and 3), and

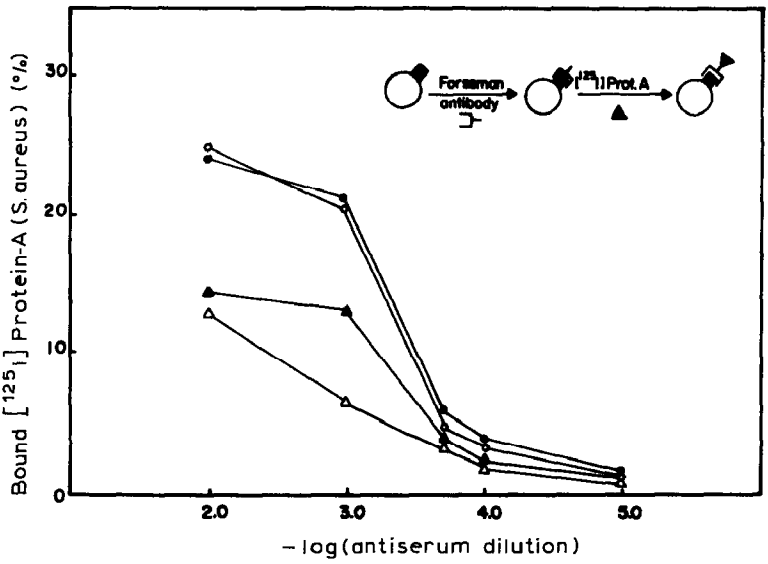


Fig. 10. Binding of [^{125}I]protein-A to Forssman antibody-bound cells: (—○—○—) Cells 103 (passage 50), (—●—●—) cells 106B (passage 120), (—△—△—) cells 107C3 (passage 24), and (—▲—▲—) cells 1046C (passage 30).

the line fused with that of nonradioactive Forssman GSL (**3**) (Well 4). This suggests that the product of the GalNAcT-2-catalyzed reaction may have been converted partly into Forssman GSL by a tandem reaction[20] in the presence of GalNAcT-3 in the membrane preparation.

A second set of microimmunodiffusion plates was set up with the ^{14}C-product from the GalNAcT-3-catalyzed reaction (Fig. 9B). Anti-Forssman antibody was placed in the center well. Well 1 contained the purified ^{14}C-product from **2** as substrate (3000 c.p.m.), and Well 3 had the same amount of radioactive product mixed with nonradioactive Forssman GSL (**3**) in aqueous solution. Wells 2 and 4 had the aqueous solutions of nonradioactive **2** and **3**, respectively. The reaction was continued for 72 h, and the plate was developed as described earlier. As seen in Fig. 9Bb, the radioactive band from the ^{14}C-product fused with that from nonradioactive Forssman antibody, indicating the presence of radioactive **3** in the GalNAcT-3-catalyzed reaction.

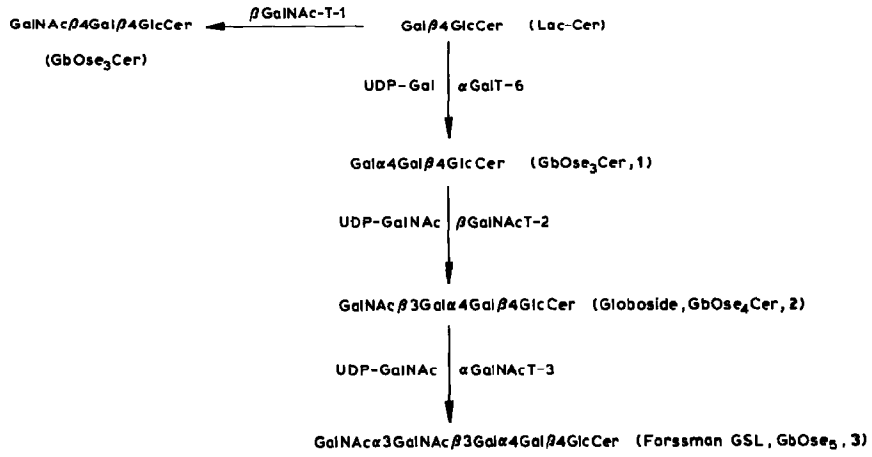
Anti-Forssman antibody binding to cell surfaces. — Polyclonal antibodies against GalNAcα1-3-R (Forssman glycoconjugates) have been isolated from rabbit serum and used as a probe to determine the carbohydrate profile of the guinea pig, tumor-cell surface and the tentative structures of the glycoconjugates. Binding of anti-Forssman antibody to embryonic, guinea pig cell line 103 and its chemically transformed variants (106B, 107C3, and 104C1) was quantitatively determined by further binding with [^{125}I]protein A (Fig. 10). Both cell lines 103 and 106B are

nontumorigenic and appeared to have 50% more Forssman glycoconjugate at the surfaces than the tumorigenic variants, 107C3 and 104C1. 107C3 cells had the fewest Forssman-type receptors on the surfaces.

DISCUSSION

The pattern of glycosphingolipids of the Forssman type[37] on tumor cell surfaces of primate and nonprimate origin is not well understood. Forssman-positive animals contain either Forssman GSL (**3**) or both **2** and **3** in their tissues and in erythrocytes. It is expected that the precursor globoside **2** is the major GSL in human erythrocytes, although normal human tissues do not contain any Forssman-positive GSL. However, expression of Forssman antigen in gastric and colonic mucosa of human cancer patients has been detected[38]. By use of a radioimmunoassay[39], a few more Forssman-positive human cancer cases have been reported. Anti-Forssman antibodies in sera from human cancer patients have also been detected[40].

If Forssman antigen **3** or its intermediate **2** is related to metastasis or any tumorigenic process, the gene for αGalNAcT-3 might be controlled during the oncogenic process. It also became important to understand the mechanism of stepwise biosynthesis of Forssman antigen in any tumor system (Scheme 1). Both 104C1 (tumorigenic) and 106B (nontumorigenic) cultured cells from guinea pig embryos represent appropriate model systems for studying (a) the specificity of the two different *N*-acetylgalactosaminyltransferases (βGalNAcT-2 and αGalNAcT-3) toward both glycolipid and glycoprotein potential acceptors and (b) the cell surface changes that may occur during the tumorigenic process under various growth conditions.



Scheme 1. *N*-Acetylgalactosaminyltransferases in guinea pig tumor cells.

We report herein results that establish the biosynthesis *in vitro* of globoside from globotriaosylceramide **1** in both 104C1 and 106B cells (Scheme 1). From pH optima and methylation studies, it became obvious that the 104C1 cell line contains only one βGalNAc transferase, βGalNAcT-2, and not the other βGalNAcT-1 enzyme (UDP-GalNAc:lactosylceramide or GM3:GalNAcT) previously reported to be present in guinea pig bone-marrow[14]. On the other hand, the presence of two pH optima (Fig. 4) for the enzyme from 106B-derived membranes suggested the presence of two enzyme activities (βGalNAcT-1 and βGalNAcT-2) in these cells. The radioactive product derived at pH 7.0 from 106B cell membranes showed the formation of only one type of linkage (Table V) after methylation. Both βGalNAcT-1 and βGalNAcT-2 activities (Scheme 1) are present in embronic chicken brains[41–43]. Permethylation studies of the radioactive products (produced at pH 7.0) from both 104C1 and 106B have also established, for the first time, the biosynthesis of a β-D-GalNAc-(1→3) linkage to the penultimate D-[^{3}H]galactosyl[44] residue of the globoside product (GalNAcβ1→3[^{3}H]Galα1→4Galβ1→4Glc→Cer). Conversion of **2** to **3**, the radioactive product of the GalNAcT-3-catalyzed reaction, also gave a sharp precipitin band with anti-Forssman antibody.

It will be interesting to determine whether the αGalNAcT-3 activity is controlled by various growth conditions in these tumorigenic and nontumorigenic cell lines. Turning "on" and "off" the gene for the αGalNAcT-3 enzyme might be crucial to the oncogenic process in cell lines where Forssman antigen is necessary as an active antigen on the cell surface. It is also important to elucidate the roles of these two enzymes (βGalNAcT-2 and αGalNAcT-3) in the expression of Forssman-related (*i.e.*, GalNAcα1→3GalNAcβ1→3Gal→R) glycoproteins on the surfaces of these cells. The last goal will be possibly reached only after the two enzymic activities have been solubilized and purified from these cell lines. Separation of these two enzyme activities with a detergent-solubilized preparation is in progress in our laboratory[45]. It has been shown that the radioactive glycolipid may coprecipitate in a glycolipid–antiglycolipid system, perhaps owing to mixed-micelle formation[46]. In order to prove the terminal GalNAc-linkage unambigously, further permethylation analysis of the Forssman-active enzymatic product is under way.

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