ELSEVIER

Carbohydrate Research 306 (1998) 341–348

CARBOHYDRATE RESEARCH

Synthesis of aryl 3′-sulfo-β-lactosides as fluorogenic and chromogenic substrates for ceramide glycanases

Lai-Xi Wang[a], Nadejda V. Pavlova[b], Michael Yang[a], Su-Chen Li[b], Yu-Teh Li[b], Yuan C. Lee[a]*

[a] *Department of Biology, The Johns Hopkins University, Baltimore, MD 21218-2685, USA*

[b] *Department of Biochemistry, Tulane University Medical Center, New Orleans, Louisiana 70112-2699, USA*

Received 12 September 1997; accepted in revised form 25 October 1997

Abstract

We have previously reported that 4-methylumbelliferyl 6′-*O*-benzyl-β-lactoside (**2**) is a useful substrate for a fluorometric assay of ceramide glycanase (CGase) (L.-X. Wang, N.V. Pavlova, S.-C. Li, Y.-T. Li and Y.C. Lee, *Glycoconjugate J.*, 13 (1996) 359–365). The introduction of a 6-*O*-benzyl group at the terminal Gal efficiently protected the substrate from its hydrolysis by exo-galactosidase, permitting the assay of CGase in crude biological materials. However, a drawback of this substrate is its low water-solubility and relatively high Km (at a mM level). Introduction of a sulfate group into 4-methylumbelliferyl β-lactoside (**1**) led to the formation of 4-methylumbelliferyl 3′-*O*-sulfo-β-lactoside (**3**), which was found to be a more effective substrate than **2**. Moreover, the presence of a 3′-*O*-sulfate group not only increases the water solubility tremendously, but also protects the substrate from cleavage by exo-β-galactosidase as the 6′-*O*-benzyl group in **2** does. In addition to the fluorogenic substrate (**3**), two sulfated chromogenic substrates, *N*-tetradecanoyl-4-*O*-(3′-sulfo-β-lactosyl)-3-nitro-L-tyrosine methyl ester (**9**) and 2-*N*-(tetradecanoylamino)-4-nitrophenyl 3′-sulfo-β-lactoside (**12**), were synthesized and their suitability for a photometric assay of CGase was evaluated. Substrates **9** and **12**, with a long fatty acid chain attached to the aglycon part, have a *K*m value close to that of the natural substrate GM1 (at a μM level).

Keywords: Ceramide glycanase; Sulfated lactoside; Fluorogenic substrate; Chromogenic substrate; Glycosphingolipid

1. Introduction

Glycosphingolipids are membrane constituents that are believed to play roles in many important biological events such as cell–cell recognition and cell growth regulation [1,2]. Ceramide glycanase (CGase) is specific for glycosphingolipid and cleaves the linkage between the ceramide and the glycan chain [3–6]. Therefore, CGase has become a very useful enzyme for structural as well as functional studies of glycosphingolipids. Although the enzyme has been found in some animals and microorganisms [3–10], its biological function

*Corresponding author. Tel: 410-516-7041; Fax: 410-516-8716; e-mail: yclee@jhu.edu

0008-6215/97/$19.00
PII S0008-6215(97)10082-9

remains to be elucidated. To facilitate the purification of CGase and also the studies of its distribution and physiological function, a simple and sensitive method suitable for routine assay is highly desirable. For this purpose, we have previously described a fluorometric assay of CGase using 4-methylumbelliferyl 6′-*O*-benzyl-β-lactoside [6′Bn-Lac-MU, (**2**)] as substrate [11]. The introduction of a 6-*O*-benzyl group at the terminal Gal efficiently protected the substrate from its hydrolysis by exo-galactosidase, permitting the assay of CGase in crude biological sources. However, a drawback of this substrate is its poor solubility in water. In addition, its *K*m value (0.23 mM) is relatively high as compared with the natural substrate GM1 (*K*m 15 μM). Combination of these properties substantially detracts its usefulness. We reasoned that introduction of a hydrophilic functional group, such as a sulfate group, at the non-reducing terminal would play dual roles of increasing the water solubility and blocking the cleavage of the substrate by exo-glycosidases. Moreover, the 3′-sulfate group may mimic the function of a sialyl group in various gangliosides. Therefore, we have introduced a sulfate group at the 3′-position of 4-methylumbelliferyl β-lactoside [Lac-MU, (**1**)] and found that the resulting 4-methylumbelliferyl 3′-sulfo-β-lactoside [3′SLac-MU, (**3**)] has an excellent water solubility and can serve as an efficient fluorogenic substrate for CGase. In addition, we also describe the preparation of two nitrophenyl 3′-sulfo-β-lactosides (**9** and **12**) with a fatty-acid chain attached to the aglycon and their usefulness as chromogenic substrates for CGases.

2. Results and discussion

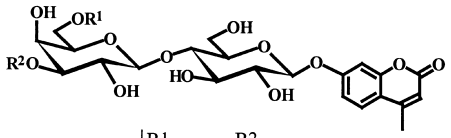
Synthesis of the substrates.—Selective sulfation at the 3′-hydroxyl group of 4-methylumbelliferyl lactoside (**1**) was achieved via selective activation through the formation of a cyclic stannylene complex at the cis-3′,4′-dihydroxyl groups [12,13]. Thus, **1** was first treated with dibutyltin oxide in DMF, and then treated with sulfur trioxide-trimethylamine complex to give the desired 3′-sulfated lactoside (**3**) in 68% yield, together with the formation of 3′,6′-disulfated lactoside derivative **4** (17%). We found that the commonly used solvents, such as methanol and toluene [12,13], are not suitable for the formation of the stannyl complex of 4-methylumbelliferyl lactoside (**1**), even under refluxing conditions for a long time, because of the poor solubility of **1** in these solvents. The structure of **3** was confirmed by ^{1}H NMR. The spectrum of **3** showed the expected downfield shift for the H-3′ and H-4′ at δ 4.363 (dd, $J = 3.5$ and 9.7 Hz) and 4.325 (br d, $J = 3.5$ Hz), respectively, clearly indicating sulfation at the 3′-hydroxyl group [14,15].

It has been previously reported that the shortest carbohydrate chain on a glycosphingolipid which could be efficiently hydrolyzed by CGase is lactose [5]. While simple alkyl β-lactosides with an alkyl chain longer than C_8 can serve as substrate for CGase, nitrophenyl β-lactoside (Lac-PNP) is a poor substrate (*K*m = 7.2 mM), probably because of the lack of enough hydrophobicity or chain length of its aglycon portion. Based on these facts, we speculate that introduction of a fatty acid chain on the nitrophenyl portion might lead to an efficient chromogenic substrate for the enzyme. Therefore, we designed two potential chromogenic substrates: *o*-nitrophenyl 3′-sulfo-β-lactoside derivative **9** and *p*-nitrophenyl 3′-sulfo-β-lactoside derivative **12**, both with a tetradecanoyl chain attached to the aglycon. In **9**, 3-nitrotyrosine was chosen as the aglycon because of its ready availability and its further manipulability.

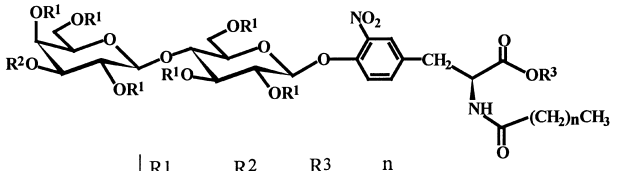
Phase transfer-catalyzed reaction [16] of acetobromolactose and 3-nitrotyrosine ethyl ester gave a high yield of the per-*O*-acetylated β-lactoside with a high stereoselectivity, despite the presence of a free amino group. *N*-Acylation of the coupling product with tetradecanoyl chloride and acetyl chloride and subsequent *O*-deacetylation provided, respectively, the *N*-tetradecanoyl derivative **7** and the *N*-acetyl derivative **8**. 3′-*O*-Sulfation of **7** was carried out in the same way as described for the preparation of 3′SLac-MU (**3**) to give the 3′-sulfate derivative **9** in 72% yield.

The *p*-nitrophenyl lactosides were prepared in a similar way. First, phase transfer-catalyzed coupling of acetobromolactose with 2-*N*-(tetradecanoylamino)-4-nitrophenol gave a 91% yield of the *p*-nitrophenyl lactoside derivative **10**. *O*-Deacetylation of **10** gave 2-*N*-(tetradecanoylamino)-4-nitrophenyl β-lactoside (**11**). Finally, 3′-*O*-sulfation of **11** provided 2-*N*-(tetradecanoylamino)-4-nitrophenyl 3′-sulfo-β-lactoside (**12**) in 58% yield.

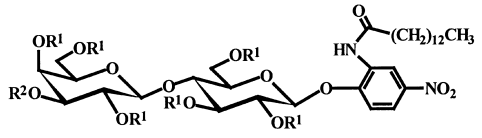
Substrate activities.—*Fluorogenic substrates*. Substitution of the terminal Gal at 6-position by *O*-benzyl group in Lac-MU efficiently protects the



	R1	R2
1	H	H
2	Bn	H
3	H	SO_3Na
4	SO_3Na	SO_3Na



	R1	R2	R3	n
5	Ac	Ac	Et	12
6	Ac	Ac	Et	0
7	H	H	Me	12
8	H	H	Me	0
9	H	SO_3Na	Me	12



	R1	R2
10	Ac	Ac
11	H	H
12	H	SO_3Na

Fig. 1. Structures of the synthetic substrates and reaction intermediates.

glycoside from hydrolysis by β-galactosidase [11]. Introduction of a sulfate group to Lac-MU to form 3′SLac-MU (**3**) has a similar protective effect, as shown in Fig. 2. The purified CGase from American leech *Macrobdella decora* can release lactose from Lac-MU (Fig. 2, lane 6). Crude CGase [5] that contains β-galactosidase released Gal and Glc, in addition to lactose (Fig. 2, lane 7). In contrast, both the purified and the crude CGase released only 3′-sulfo-lactose from 3′SLac-MU (**3**) (Fig. 2, lanes 3 and 4). Along with the protection of the substrate from the cleavage by β-galactosidase, introduction of the charged sulfate group to the substrate significantly increases the water solubility of 3′SLac-MU (**3**), which is a big advantage for assaying CGases. For example, the solubility of Lac-MU (**1**) in water is 8.6 mM at 25 °C, whereas the solubility of 3′SLac-MU (**3**) is 350 mM at the same temperature, which is about 40 times higher than that of the non-sulfated **1**. However, a potential problem for the sulfated substrate is that the sulfate group could be cleaved by non-specific sulfatases, which are sometimes present in crude biological samples. To overcome this drawback, we used sodium sulfite (Na_2SO_3) to inhibit sulfatase activity. Using a crude CGase from the oyster *Crassostrea virginica* [17], which contains a high sulfatase activity, we found that the presence of 10 mM Na_2SO_3 completely inhibited sulfatase activity. The presence of Na_2SO_3 in the incubation mixture also had some inhibitory effect toward the hydrolysis of the substrate by CGase (data not shown). Therefore, it is necessary to increase the incubation time for CGase assay when Na_2SO_3 is included.

The purified CGase from leech exhibits pH optimum 3.0–3.5 with both 3′SLac-MU (**3**) and 3′,6′SLac-MU (**4**) as substrates (Table 1). This optimal pH is lower than that for the hydrolysis of Lac-MU and 6′Bn-Lac-MU by the same enzyme [11], and it may be due to the influence of the sulfate group. The kinetic data for the hydrolysis of the fluorogenic substrates by CGase are summarized in Table 1. The values of Km for 3′SLac-MU (**3**) and 3′,6′SLac-MU (**4**) are very similar and slightly higher than that for 6′Bn-Lac-MU [11]. Nevertheless, the efficiency of the substrates, as reflected by the *V*max value, is significantly higher for 3′SLac-MU (**3**). The *V*max value for 3′,6′SLac-MU (**4**) is close to that for 6′Bn-Lac-MU. Thus, the introduction of the second sulfate group appears to reduce the efficiency of the substrate. The excellent water solubility makes the sulfated substrates very useful for CGase assay.

Chromogenic substrates. We have found that all the nitrophenyl lactoside derivatives with a long fatty acid chain (compounds **7**, **9**, **11**, and **12**) can be hydrolyzed efficiently by leech CGase to give a yellowish aglycon, although the nitrophenyl lactoside derivative (**8**) without a long fatty acid chain at the aglycon is actually not hydrolyzed by CGase under the same assay conditions. However, a practical problem with substrates **7**, **9**, **11**, and **12** is that, after hydrolysis, the chromogenic aglycon products are actually water insoluble, which makes the quantitative determination difficult. To overcome this problem, we have modified the traditional assay procedure by stopping the reaction with a mixture of methyl Cellosolve and 0.2 M borate buffer (4/1, v/v). This mixture dissolved the aglycon products completely and maintained the

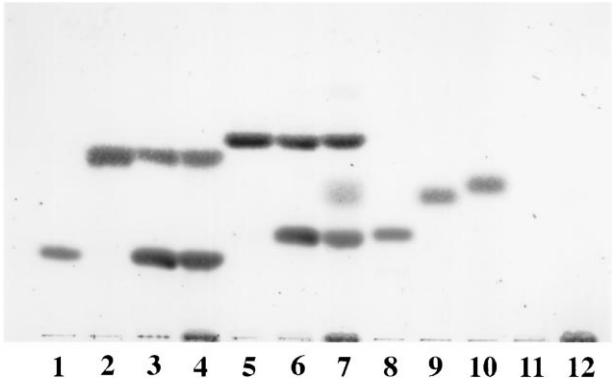


Fig. 2. Thin-layer chromatogram showing the hydrolysis of 3′SLac-MU and Lac-MU by the crude and the purified leech CGase. 1.3′SLac; 2.3′SLac-MU; 3.3′SLac-MU + the purified CGase; 4.3′SLac-MU + the crude CGase; 5. Lac-MU; 6. Lac-MU + the purified CGase; 7. Lac-MU + the crude CGase; 8. Lac; 9. Gal; 10. Glc; 11. The purified CGase; 12. The crude CGase.

Table 1
pH optima and Michaelis–Menten constants for the hydrolysis of the fluorogenic substrates by leech CGase

Substrates	pH optimum	*K*m (mM)	*V*max (nmol/min/mg)
Lac-MU (**1**)	4.8–5.2	0.77	29.5
6′Bn-Lac-MU (**2**)	4.8–5.2	0.23	37
3′SLac-MU (**3**)	3.0–3.5	0.37	132
3′,6′SLac-MU (**4**)	3.0–3.5	0.41	44.4

pH at 9.8, necessary for terminating the reaction. The pH optima for the hydrolysis of the chromogenic substrates by leech CGase were all close to pH 5. As shown in Table 2, the *K*m values for the sulfated substrates (**9** and **12**) are close to that of the natural substrate GM1 (at a μM level). However, the *K*m for **7** is significantly higher, and closer to the *K*m for Lac-PNP (at a mM level). The reason for this is not clear. Very recently, Miura et al. [18] reported that 2-(*N*-hexadecanoylamino)-4-nitrophenyl β-lactoside was a chromogenic substrate for several CGases. Comparison of the *K*m and *V*max in Table 2 among the chromogenic substrates reveals that the *o*-nitrophenyl substrates (**7** and **9**) are more efficient substrates than the *p*-nitrophenyl substrates (**11** and **12**) and, along with this, the sulfated substrates (**9** and **12**) are better substrates than the non-sulfated (**7** and **11**). Moreover, the introduction of sulfate group into **9** and **12** efficiently protects the substrates from β-galactosidase-catalyzed cleavage (data not shown). Therefore, **9** and **12** should be more useful chromogenic substrates for CGase assay.

Table 2
pH optima and Michaelis–Menten constants for the hydrolysis of chromogenic substrates by leech CGase

Substrates	pH optimum	*K*m (mM)	*V*max (nmol/min/mg)
Lac-PNP	5.0	7.2	12.5
7	5.0–5.5	1.46	10.9
9	4.5–5.0	0.026	23
11	4.8–5.0	0.027	2.7
12	4.5–5.0	0.039	4.2

3. Experimental

Synthesis of the substrates.—General methods. Melting points were determined with a Fisher–Johns apparatus and not corrected. ^{1}H NMR spectra were recorded at 25 °C with a Bruker AMX-300 NMR spectrometer (300 MHz) at 25 °C. The chemical shifts (δ) are expressed in parts per million (ppm) relative to internal standards: Me_4Si (0 ppm), residual Me_2SO (2.5 ppm), or residual MeOD (3.31 ppm). Multiplicities of signals are abbreviated as follows: s, singlet; d, doublet; t, triplet; and m, multiplet. Thin-layer chromatography (TLC) was carried out on precoated plates of silica gel 60 F_{254} (layer thickness, 0.25 mm; E. Merck, Darmstadt, Germany). Column chromatography was performed on silica gel 60 (E. Merck). The ratios of solvents used for TLC and column chromatography are expressed in vol/vol.

4-Methylumbelliferyl 3′-sulfo-β-*lactoside* (**3**) *and 3′,6′-disulfo*-β-*lactoside* (**4**). A mixture of 4-methylumbelliferyl β-lactoside **1** [11] (300 mg, 0.6 mmol) and dibutyltin oxide (179 mg, 0.72 mmol) in DMF (8 mL) and toluene (3 mL) was stirred for 6 h at 90 °C with azeotropic removal of water, then the clear solution was evaporated in vacuo to dryness. The residue was dissolved in DMF–THF (1:1, 6 mL), $Me_3N{\cdot}SO_3$ (104 mg, 0.75 mmol) was added, and the mixture was stirred at 25 °C for 20 h when TLC (65:25:4 $CHCl_3$–MeOH–water) indicated the disappearance of starting material **1** and the formation of two components of lower mobility corresponding to the mono- and di-sulfated derivatives. After adding MeOH (0.5 mL) to stop the reaction, the mixture was evaporated to dryness, and the two sulfated compounds were separated by column chromatography (65:25:4 $CHCl_3$–MeOH–water), which were then converted into the sodium salts by passing through a column of Dowex 50W-X8 (Na^+ form), giving the 3′-sulfo-β-

lactoside **3** (246 mg, 68%) and the 3′,6′-disulfo-β-lactoside **4** (72 mg, 17%) as white powders after lyophilization.

The 3′-sulfate derivative **3**. R_f 0.14 (65:25:4 $CHCl_3$–MeOH–water); ^1H NMR (Me_2SO-d_6 + 5% D_2O): δ 7.610 (d, 1 H, *J* 8.8 Hz, H-5 in coumarin), 7.060 (dd, 1 H, *J* 2.1, 8.8 Hz, H-6 in coumarin), 6.975 (d, 1 H, *J* 2.1 Hz, H-8 in coumarin), 6.110 (s, 1 H, H-3 in coumarin), 5.209 (d, 1 H, *J* 7.6 Hz, H-1), 4.629 (d, 1 H, *J* 7.8 Hz, H-1′), 4.363 (dd, 1 H, *J* 3.5, 9.7 Hz, H-3′), 4.325 (br d, 1 H, *J* 3.4 Hz, H-4′), 4.076–3.670 (m, 10 H, other sugar protons), 2.345 (s, 3 H, Me in coumarin). Anal. Calcd for $C_{22}H_{27}NaO_{16}S$: C, 43.85; H, 4.52; S, 5.32. Found: C, 43.70; H, 4.51; S, 5.20.

The 3′,6′-disulfate derivative **4**. R_f 0.08 (65:25:4 $CHCl_3$–MeOH–water); ^1H NMR (Me_2SO-d_6 + 5% D_2O): δ 7.700 (d, 1 H, *J* 8.8 Hz, H-5 in coumarin), 7.121 (dd, 1 H, *J* 2.2, 8.8 Hz, H-6 in coumarin), 7.071 (d, 1 H, *J* 2.2 Hz, H-8 in coumarin), 6.215 (s, 1 H, H-3 in coumarin), 5.264 (d, 1 H, *J* 7.8 Hz, H-1), 4.651 (d, 1 H, *J* 7.9 Hz, H-1′), 4.386 (dd, 1 H, *J* 3.2, 9.8 Hz, H-3′), 4.373 (br d, 1 H, *J* 3.2 Hz, H-4′), 4.257 (d, 2 H, *J* 6.0 Hz, H-6′a,b), 4.072 (t, 1 H, *J* 6.0 Hz, H-5′), 4.060–3.804 (m, 5 H, H-3,4,5,6a,b), 3.763–3.671 (m, 2 H, H-2,2′), 2.417 (s, 3 H, Me in coumarin). Anal. Calcd for $C_{22}H_{26}Na_2O_{19}S_2$: C, 37.50; H, 3.72; S, 9.10. Found: C, 37.21; H, 4.05; S, 8.82.

N-*Tetradecanoyl-4*-O-*(hepta*-O-*acetyl*-β-*lactosyl)-3-nitro*-L-*tyrosine ethyl ester* (**5**) *and* N-*acetyl-4*-O-*(hepta*-O-*acetyl*-β-*lactosyl)-3-nitro*-L-*tyrosine ethyl ester* (**6**). A two-phase solution of 3-nitro-L-tyrosine ethyl ester hydrochloride (291 mg, 1.0 mmol), acetobromolactose (1.05 g, 1.5 mmol), and tetra-*n*-butylammonium hydrogensulfate (339 mg, 1.0 mmol) in aq. Na_2CO_3 (10 mL, 1 M) and $CHCl_3$ (10 mL) was shaken for 5 h at 25 °C. The two layers were then separated and the aq. layer was extracted with $CHCl_3$ (2×8 mL). The organic layer and the extracts were combined and washed with sat. $NaHCO_3$ and water, dried (Na_2SO_4), and filtered. The filtrate was evaporated and the residue was subjected to column chromatography (1:2 to 1:3 toluene–EtOAc) to give 4-*O*-(hepta-*O*-acetyl-β-lactosyl)-3-nitro-L-tyrosine ethyl ester (707 mg, 81%) as a syrup, R_f 0.13 (1:3 toluene–EtOAc), which was used directly for the following *N*-acylation reaction without further characterization.

The free amino compound thus obtained was dissolved in CH_2Cl_2 (15 mL) containing Et_3N (0.282 mL, 2.0 mmol). The solution was cooled to 0 °C and tetradecanoyl chloride (0.44 mL, 1.62 mmol) was added dropwise. The resulted mixture was stirred for 3 h at 25 °C. Methanol (0.2 mL) was then added and the mixture was evaporated to dryness. The crude product was purified by column chromatography (1:1 toluene–EtOAc) to give *N*-tetradecanoyl-4-*O*-(hepta-*O*-acetyl-β-lactosyl)-3-nitro-L-tyrosine ethyl ester (**5**) (702 mg, 80%). Similarly, *N*-acetylation of 4-*O*-(hepta-*O*-acetyl-β-lactosyl)-3-nitro-L-tyrosine ethyl ester with acetyl chloride gave *N*-acetyl-4-*O*-(hepta-*O*-acetyl-β-lactosyl)-3-nitro-L-tyrosine ethyl ester (**6**).

Compound **5**. R_f 0.52 (1:2 toluene–EtOAc); ^1H NMR ($CDCl_3$): δ 7.536 (d, 1 H, *J* 2.0 Hz, aromatic H), 7.307 (dd, 1 H, *J* 2.0, 8.6 Hz, aromatic H), 7.211 (d, 1 H, *J* 8.6 Hz, aromatic H), 6.011 (d, 1 H, *J* 7.3 Hz, NH), 5.368 (br d, 1 H, *J* 3.3 Hz, H-4′), 5.282 (t, 1 H, *J* 8.2 Hz, H-3), 5.218–5.115 (m, 3 H, H-1,2,2′), 4.985 (dd, 1 H, *J* 3.3, 10.4 Hz, H-3′), 4.846 (m, 1 H, C*H*NH), 4.579 (dd, 1 H, *J* 1.8, 12 Hz, H-6a), 4.547 (d, 1 H *J* 7.9 Hz, H-1′), 4.212 (q, 2 H, *J* 7.1 Hz, OCH_2CH_3), 4.170–3.810 (m, 6 H, 4,5,5′,6b,6′a,6′b), 3.203 (dd, 1 H, *J* 5.9, 14 Hz, 1/2 $ArCH_2$), 3.109 (dd, 1 H, *J* 5.3, 14 Hz, 1/2 $ArCH_2$), 2.215 (t, 2 H, *J* 7.2 Hz, CH_2CO), 2.171, 2.125, 2.103, 2.081, 2.069, 2.064, and 1.983 (each s, each 3 H, 7 Ac), 1.308–1.257 (m, 25 H, 11 CH_2 and OCH_2CH_3), 0.884 (t, 3 H, *J* 6.6 Hz, CH_3 in alkyl).

Compound **6**. R_f 0.32 (1:3 toluene–EtOAc); ^1H NMR ($CDCl_3$): δ 7.540 (d, 1 H, *J* 2.1 Hz, aromatic H), 7.330 (dd, 1 H, *J* 2.1, 8.5 Hz, aromatic H), 7.219 (d, 1 H, *J* 8.5 Hz, aromatic H), 6.048 (d, 1 H, *J* 7.1 Hz, NH), 5.370 (br d, 1 H, *J* 3.3 Hz, H-4′), 5.283 (t, 1 H, *J* 8.2 Hz, H-3), 5.218–5.115 (m, 3 H, H-1,2,2′), 4.987 (dd, 1 H, *J* 3.3, 10.5 Hz, H-3′), 4.842 (m, 1 H, C*H*NH), 4.590 (dd, 1 H, *J* 2.2, 12 Hz, H-6a), 4.549 (d, 1 H *J* 7.8 Hz, H-1′), 4.220 (q, 2 H, *J* 7.1 Hz, OCH_2CH_3), 4.184–3.780 (m, 6 H, 4,5,5′,6b,6′a,6′b), 3.210 (dd, 1 H, *J* 5.8, 14 Hz, 1/2 $ArCH_2$), 3.120 (dd, 1 H, *J* 5.5, 14 Hz, 1/2 $ArCH_2$), 2.171, 2.126, 2.104, 2.082, 2.073, 2.062, 2.037, and 1.984 (each s, each 3 H, 8 Ac), 1.291 (t, 3 H, *J* 7.1 Hz, OCH_2CH_3).

N-*Tetradecanoyl-4*-O-*(*β-*lactosyl)-3-nitro*-L-*tyrosine methyl ester* (**7**) *and* N-*acetyl-4*-O-*(*β-*lactosyl)-3-nitro*-L-*tyrosine methyl ester* (**8**). *O*-Deacetylation of **5** (400 mg, 0.369 mmol) was carried out with NaOMe–MeOH (10 mL, 10 mM) for 10 h at 25 °C, and the white precipitate formed was collected by filtration to give the *N*-tetradecanoyl derivative **7** (250 mg, 87%). Similarly, de-*O*-acetylation

of **6** (720 mg, 0.787 mmol) with NaOMe–MeOH (25 mL, 10 mM) gave the *N*-acetyl derivative **8** (406 mg, 85%).

Compound **7**. R_f 0.60 (9:4:2 EtOAc–2-propanol-water); ^{1}H NMR (Me_2SO-d_6 + 5% D_2O): δ 7.644 (d, 1 H, *J* 2.1 Hz, aromatic H), 7.483 (dd, 1 H, *J* 2.1, 8.8 Hz, aromatic H), 7.303 (d, 1 H, *J* 8.8 Hz, aromatic H), 5.083 (d, 1 H, *J* 7.7 Hz, H-1), 4.446 (m, 1 H, C*H*NH), 4.223 (m, 1 H, H-1′), 3.889–3.272 (m, 12 H, sugar protons), 3.582 (s, 3 H, OCH_3), 3.051 (dd, 1 H, *J* 5.8, 14 Hz, 1/2 ArCH_2), 2.864 (dd, 1 H, *J* 9.8, 14 Hz, 1/2 ArCH_2), 2.013 (t, 2 H, *J* 7.1 Hz, CH_2CO), 1.354 (m, 2 H, CH_2CH_2CO), 1.189 (m, 20 H, 10 CH_2), 0.807 (t, 3 H, *J* 6.6 Hz, CH_3 in alkyl). Anal. Calcd for $C_{36}H_{58}N_2O_{16}$: C, 55.80; H, 7.54; N, 3.62. Found: C, 55.62; H, 7.90; N, 3.58.

Compound **8**. R_f 0.45 (65:25:4 $CHCl_3$–MeOH–water); ^{1}H NMR (Me_2SO-d_6 + 5% D_2O): δ 7.675 (d, 1 H, *J* 2.0 Hz, aromatic H), 7.458 (dd, 1 H, *J* 2.0, 8.6 Hz, aromatic H), 7.320 (d, 1 H, *J* 8.6 Hz, aromatic H), 5.115 (d, 1 H, *J* 7.7 Hz, H-1), 4.448 (m, 1 H, C*H*NH), 4.225 (m, 1 H, H-1′), 3.740–3.270 (m, 12 H, sugar protons), 3.591 (s, 3 H, OCH_3), 3.060 (dd, 1 H, *J* 5.8, 14 Hz, 1/2 ArCH_2), 2.871 (dd, 1 H, *J* 9.4, 14 Hz, 1/2 ArCH_2), 1.778 (s, 3 H, Ac). Anal. Calcd for $C_{24}H_{34}N_2O_{16}$: C, 47.52; H, 5.65; N, 4.62. Found: C, 47.43; H, 5.70; N, 4.53.

N-*Tetradecanoyl-4*-O-*(3′-sulfo*-β-*lactosyl)-3-nitro*-L-*tyrosine methyl ester* (**9**). A suspension of **7** (77.5 mg, 0.1 mmol) and dibutyltin oxide (30 mg, 0.12 mmol) in MeOH (6 mL) was refluxed with stirring. The suspension became a clear solution as the reaction proceeded, and the reaction was continued for 8 h. After evaporation, the residue was co-evaporated with toluene (2×5 mL). The syrupy residue was dissolved in THF (3 mL)–DMF (0.5 mL), $Me_3N{\cdot}SO_3$ (22.3 mg, 0.16 mmol) was added, and the mixture was stirred for 12 h at 25 °C when TLC (9:4:2 EtOAc–2-propanol–water) indicated the disappearance of starting material **7** and the formation of a less mobile component corresponding to the monosulfated product. After adding MeOH (0.1 mL), the mixture was evaporated to dryness, and the product was purified by column chromatography (65:25:4 $CHCl_3$–MeOH–water) and converted into its sodium salt by passing through a column of Dowex 50W-X8 (Na^+ form) to give the 3′-sulfo-lactoside derivative (**9**) (63 mg, 72%): R_f 0.47 (9:4:2 EtOAc–2-propanol–H_2O); ^{1}H NMR (CD_3OD + 10% D_2O): δ 7.668 (d, 1 H, *J* 2.1 Hz, aromatic H), 7.479 (dd, 1 H, *J* 2.1, 8.8 Hz, aromatic H), 7.380 (d, 1 H, *J* 8.8 Hz, aromatic H), 5.112 (d, 1 H, *J* 7.5 Hz, H-1), 4.672 (m, 1 H, C*H*NH), 4.520 (d, 1 H, *J* 7.7 Hz, H-1′), 4.257 (dd, 1 H, *J* 3.3, 9.1 Hz, H-3′), 4.243 (br d, 1 H, *J* 3.3 Hz, H-4′), 3.914– 3.545 (m, 10 H, sugar protons), 3.722 (s, 3 H, OCH_3), 3.205 (dd, 1 H, *J* 5.3, 14 Hz, 1/2 ArCH_2), 2.988 (dd, 1 H, *J* 9.2, 14 Hz, 1/2 ArCH_2), 2.171 (t, 2 H, *J* 7.4 Hz, CH_2CO), 1.520 (m, 2 H, CH_2CH_2CO), 1.275 (m, 20 H, 10 CH_2), 0.894 (t, 3 H, *J* 7.0 Hz, CH_3 in alkyl). Anal. Calcd for $C_{36}H_{57}N_2NaO_{19}S$: C, 49.30; H, 6.55; N, 3.19; S, 3.65. Found: C, 48.92; H, 6.58; N, 3.02; S, 3.48.

2-N-*(Tetradecanoylamino)-4-nitrophenyl hepta*-O-*acetyl*-β-*lactoside* (**10**). To a suspension of 2-amino-4-nitrophenol (3.1 g, 20 mmol) in 0.5 M $NaHCO_3$ (80 mL) containing EtOH (10 mL) was added dropwise a solution of tetradecanoyl chloride (6.8 mL, 25 mmol) in acetone (5 mL). The mixture was stirred for 2 h at 25 °C, and then the mixture was adjusted to pH 2–3 by adding 6 M HCl. The precipitate was collected by filtration, washed with 1 M HCl and water, and dried. Crystallization from ethyl acetate provided 2-*N*-(tetradecanoylamino)-4-nitrophenol (4.6 g, 63%) as a yellow solid. ^{1}H NMR (Me_2SO-d_6 + 5% D_2O) of 2-*N*-(tetradecanoylamino)-4-nitrophenol: δ 8.902 (d, 1 H, *J* 2.8 Hz, H-3 in the phenol), 7.878 (dd, 1 H, *J* 2.8, 8.9 Hz, H-5 in the phenol), 7.010 (d, 1 H, *J* 8.9 Hz, H-6 in the phenol), 2.406 (t, 2 H, *J* 7.3 Hz, CH_2CO), 1.581–1.222 (m, 22 H, 11 CH_2), 0.845 (t, 3 H, *J* 6.6 Hz, CH_3).

The phase transfer-catalyzed reaction of 2-*N*-(tetradecanoylamino)-4-nitrophenol (1.82 g, 5.0 mmol) with acetobromolactose (4.4 g, 6.25 mmol) in 0.5 M Na_2CO_3 (30 mL) and $CHCl_3$ (30 mL) containing tetra-*n*-butylammonium hydrogensulfate (1.7 g, 5 mmol) was performed as described for the preparation of **5**. The coupling product was then purified by column chromatography (2:1 toluene–EtOAc) to give **10** (4.5 g, 91%): R_f 0.54 (1:1 toluene–EtOAc); ^{1}H NMR ($CDCl_3$): δ 9.390 (d, 1 H, *J* 2.6 Hz, aromatic H), 7.907 (dd, 1 H, *J* 2.6, 9.0 Hz, aromatic H), 6.990 (d, 1 H, *J* 9.0 Hz, aromatic H), 5.413 (t, 1 H, *J* 9.4 Hz, H-3), 5.385 (br d, 1 H, *J* 3.3 Hz, H-4′), 5.240 (dd, 1 H, *J* 7.8, 9.6 Hz, H-2), 5.138 (dd, 1 H, *J* 7.8, 10.4 Hz, H- 2′), 5.104 (d, 1 H, *J* 7.8 Hz, H-1), 4.985 (dd, 1 H, *J* 3.3, 10.4 Hz, H-3′), 4.542 (dd, 1 H, *J* 1.8, 12 H, H-6a), 4.510 (d, 1 H, *J* 7.8 Hz, H-1′), 4.210–3.868 (m, 6 H, H-4,5,5′,6b,6′a,6′b), 2.455 (t, 2 H, *J* 7.3 Hz, CH_2CO), 2.178, 2.133, 2.112, 2.103, 2.087, 2.080,

and 1.986 (each s, each 3 H, 7 Ac), 1.722 (m, 2 H, CH_2CH_2CO), 1.350–1.259 (m, 20 H, 10 CH_2), 0.882 (t, 3 H, *J* 6.5 Hz, CH_3 in alkyl).

2-N-(Tetradecanoylamino)-4-nitrophenyl β-lactoside (**11**). *O*-Deacetylation of **10** (3.0 g, 3.05 mmol) with NaOMe–MeOH (35 mL, 10 mM) was performed as described for the preparation of **7** to provide **11** (1.93 g, 92%): R_f 0.53 (9:4:2 EtOAc–2-propanol–H_2O); ^{1}H NMR (Me_2SO-d_6 + 5% D_2O): δ 8.972 (d, 1 H, *J* 2.8 Hz, aromatic H), 7.944 (dd, 1 H, *J* 2.8, 9.3 Hz, aromatic H), 7.325 (d, 1 H, *J* 9.3 Hz, aromatic H), 5.069 (d, 1 H, *J* 7.3 Hz, H-1), 4.231 (m, 1 H, H-1′), 3.772–3.316 (m, 12 H, sugar protons), 2.399 (t, 2 H, *J* 7.1 Hz, CH_2CO), 1.557 (m, 2 H, CH_2CH_2CO), 1.228–1.161 (m, 20 H, 10 CH_2), 0.784 (t, 3 H, *J* 6.4 Hz, CH_3). Anal. Calcd for $C_{32}H_{52}N_2O_{14}$: C, 55.80; H, 7.61; N, 4.07. Found: C, 55.54; H, 7.82; N, 3.85.

2-N-(Tetradecanoylamino)-4-nitrophenyl 3′-sulfo-β-lactoside (**12**). A mixture of **11** (827 mg, 1.2 mmol) and dibutyltin oxide (358.5 mg, 1.44 mmol) in MeOH (15 mL) was refluxed for 8 h. MeOH was evaporated and the residue was co-evaporated with toluene (2×15 mL) to give a pale-yellow residue. The residue was dissolved in THF–DMF (1:1, 6 mL), $Me_3N{\cdot}SO_3$ (217 mg, 1.56 mmol) was added, and the mixture was stirred for 10 h at 25 °C. TLC (9:4:2 EtOAc–2-propanol–water) indicated the formation of a major, less-mobile product, together with several minor products. After adding MeOH (0.5 mL), the reaction mixture was evaporated to dryness, and the major product was purified by column chromatography (65:25:4 $CHCl_3$–MeOH–water) and converted into the form of sodium salt by passing through a column of Dowex 50W-X8 (Na^+ form), giving the 3′-sulfolactoside **12** (550 mg, 58%): R_f 0.39 (9:4:2 EtOAc–2-propanol–H_2O); ^{1}H NMR (CD_3OD + 10% D_2O): δ 9.085 (d, 1 H, *J* 2.7 Hz, aromatic H), 8.020 (dd, 1 H, *J* 2.7, 9.0 Hz, aromatic H), 7.394 (d, 1 H, *J* 9.0 Hz, aromatic H), 5.111 (d, 1 H, *J* 7.1 Hz, H-1), 4.531 (d, 1 H, *J* 7.7 Hz, H-1′), 4.266 (dd, 1 H, *J* 3.3, 9.4 Hz, H-3′), 4.252 (br d, 1 H, *J* 3.3 Hz, H-4′), 3.939–3.674 (m, 10 H, sugar protons), 2.504 (t, 2 H, *J* 7.4 Hz, CH_2CO), 1.720 (m, 2 H, CH_2CH_2CO), 1.368–1.274 (m, 20 H, 10 CH_2), 0.889 (t, 3 H, *J* 6.6 Hz, CH_3). Anal. Calcd for $C_{32}H_{51}N_2NaO_{17}S$: C, 48.60; H, 6.50; N, 3.54; S, 4.05. Found: C, 48.27; H, 6.63; N, 3.40; S, 3.91.

Substrate activity.—Ceramide glycanase (CGase). The crude and the purified CGase from leech *M. decora* [5] and the crude CGase from oyster *C. virginica* [17] were prepared as described previously.

CGase assay. For the fluorometric assay of CGase using 3′SLac-MU or 3′,6′SLac-MU as substrate, 50 μL of the reaction mixture containing 1.35 mM of the substrate in 50 mM sodium formate buffer, pH 3.0, was incubated with an appropriate amount of the enzyme. After incubation at 37 °C for a preset time, 1.5 mL of 0.2 M sodium borate buffer, pH 9.8, was added to the reaction mixture to stop the reaction. The released MU was determined using a Sequoia–Turner Model 450 fluorometer. One unit (U) of enzyme activity is defined as the amount that liberates 1 nmol of MU per min at 37 °C.

For the assay of CGase using the chromogenic substrates, 100 μL of the reaction mixture containing 1–2 mM of the substrate in 50 mM sodium acetate buffer, pH 5.0, was incubated with an appropriate amount of the enzyme for a preset time at 37 °C. To stop the reaction, 1.0 mL of methyl cellosolve in 0.2 M sodium borate buffer, pH 9.8, (4/1, v/v) was added. The liberated aglycons of compounds **7** and **9** were determined using Spectronic 1001 spectrophotometer at its absorption maximum, 430 nm ($\epsilon = 4.54\times10^3\ M^{-1}\,cm^{-1}$). The liberated aglycons of compounds **11** and **12** were determined at its absorption maximum, 422 nm ($\epsilon = 1.61\times10^4\ M^{-1}\,cm^{-1}$). One unit (U) of enzyme activity is defined as the amount that liberates 1 nmol of modified ONP or PNP per min at 37 °C.

The initial rates of enzymatic reaction were evaluated from kinetic curves of the product accumulation as described by Boecker [19]. The parameters of the Michaelis–Menten type kinetics were evaluated by nonlinear regression using the Enzfitter program (Elsevier–Biosoft).

For TLC analysis of the products released by CGase, the reaction mixture containing 30 nmol of substrate and an appropriate amount of the enzyme in 30 μL of 50 mM of sodium formate buffer, pH 3.0, or sodium acetate buffer, pH 5.0, was incubated at 37 °C for a predetermined time, and the reaction was stopped by adding 30 μL of EtOH. The mixture was dried in a Speed-Vac concentrator, redissolved in 12 μL of MeOH–water (1/2, v/v), and applied to a TLC plate. The tubes were rinsed twice with 10 μL $CHCl_3$–MeOH (2/1, v/v) to solubilize any remaining glycoconjugates, and the rinse was also applied to the same positions on the TLC plate. The plates were developed

with 1-butanol–AcOH–water (2/1/1, v/v/v), sprayed with the diphenylamine reagent [20], and heated for 20 min at 115 °C to visualize the glycoconjugates.

Acknowledgements

This work was supported by National Institutes of Health Grants DK 09970 (to Y.C.L.) and NS 09626 (to Y.-T.L).

References

[1] S. Hakomori, *Annu. Rev. Biochem.*, 50 (1981) 733–764.
[2] A. Makita and N. Taniguchi, in H. Wiegandt (Ed.), *Glycosphingolipid*, Elsevier Scientific, New York, 1985, pp 59–82.
[3] M. Ito and T. Yamagata, *J. Biol. Chem.*, 261 (1986) 14278–14282.
[4] Y.-T. Li, Y. Ishikawa, and S.-C. Li, *Biochem. Biophys. Res. Commun.*, 149 (1987) 167–172.
[5] B. Zhou, S.-C. Li, R.A. Laine, R.T.C. Huang, and Y.-T. Li, *J. Biol. Chem.*, 264 (1989) 12272–12277.
[6] H. Ashida, K. Yamamoto, H. Kumagai, and T. Tochikura, *Eur. J. Biochem.*, 205 (1993) 729–735.
[7] Y.-T. Li and S.-C. Li, (1989) Methods Enzymol, 179 (1989) 479–487.
[8] M. Ito and T. Yamagata, *Methods Enzymol.*, 179 (1989) 488–496.
[9] B.Z. Carter, S.-C. Li, and Y.-T. Li, *Biochem. J.*, 285 (1992) 619–623.
[10] Z, Li, T. Rambasek, B. Dastghei, S. Hosseini, and S. Basu, *FASEB J.*, 8 (1994) A 1439.
[11] L.-X. Wang, N.V. Pavlova, S.-C. Li, Y.-T. Li, and Y.C. Lee, *Glycoconjugate J.*, 13 (1996) 359–365.
[12] J. Alais, A. Maranduba, and A. Veyrières, *Tetrahedron Lett.*, 24 (1983) 2383–2386.
[13] B. Guilbert, N.J. Davis, and S.L. Flitsch, *Tetrahedron Lett.*, 35 (1994) 6563–6566.
[14] R.R. Contreras, J.P. Kamerling, and J.F.G. Vliegenthart, *Carbohydr. Res.*, 179 (1988) 411–418.
[15] H. Kogelberg and T. Rutherford, *Glycobiology*, 4 (1994) 49–57.
[16] R. Roy and F.D. Tropper, *Can. J. Chem.*, 69 (1991) 817–821.
[17] N.V. Pavlova, K. Noda, S.-C. Li, and Y.-T. Li, *Glycobiology*, 4 (1994) 734.
[18] Y. Miura, T. Arai, and T. Yamagata, *Carbohydr. Res.*, 289 (1996) 193–199.
[19] E.A. Boecker, *Biochem. J.*, 203 (1982) 117–123.
[20] G. Harris and I.C. MacWilliams, *Chem. Ind. (London)*, (1954) 249.