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A fluorometric assay of ceramide glycanase with 4-methylumbelliferyl β-D-lactoside derivatives

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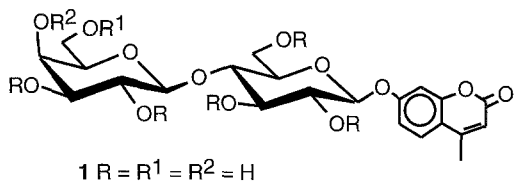
4-Methylumbelliferyl 6′-*O*-benzyl-β-D-lactoside (6′Bn-MU-Lac) and some related compounds were synthesized *via* different selective reactions including phase-transfer glycosylation. Their suitability as substrates for a fluorometric assay of ceramide glycanase (CGase) was evaluated. Among others, the 6′Bn-MU-Lac, which is resistant to exo-galactosidase, was found to be a suitable substrate for routine assay of the CGase activity. For American leech CGase, the K_m value is 0.232 mM at pH 5.

Keywords: ceramide glycanase, 4-methylumbelliferyl 6′-*O*-benzyl-β-D-lactoside derivative, fluorometric assay

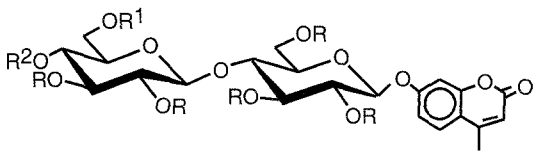
Abbreviations: CGase, ceramide glycanase; Gal, galactose; Glc, Glucose; Lac, lactose; MU, 4-methylumbelliferone; MU-Lac, 4-methylumbelliferyl β-D-lactoside; 6′Bn-Lac, 6′-*O*-benzyl-lactose; 6′Bn-MU-Lac, 4-methylumbelliferyl 6′-*O*-benzyl-β-D-lactoside; 4′6′Bd-MU-Lac, 4-methylumbelliferyl 4′,6′-*O*-benzylidene-β-D-lactoside; MU-Cel, 4-methylumbelliferyl β-D-cellobioside; 4′6′Bd-MU-Cel, 4-methylumbelliferyl 4′,6′-*O*-benzylidene-β-D-cellobioside; TLC, thin layer chromatography; ^{1}H-NMR, proton nuclear magnetic resonance; GSL, glycosphingolipids; CSA, 10-camphorsulfonic acid. See Scheme 1 for chemical structures.

Introduction

Ceramide glycanase (CGase) is an enzyme that releases the intact glycan chain from various glycosphingolipids by cleaving the linkage between the ceramide and the glycan chain [1]. This enzyme has been very useful for the structural analysis of glycosphingolipids (GSLs). In addition to hydrolysis, CGase also catalyses the oligosaccharide transferring reaction which can be used to synthesize neoglycoconjugates [2]. In the animal kingdom, CGase has been found in leech [3, 4], earthworm [5, 6], rabbit mammary gland [7], oyster [8] and hard-shell clam [9]. Similar enzymes have also been isolated from the microorganism, *Rhodococcus sp.* [10] and *Corynebacterium sp.* [11]. However, the biological functions of CGase remain to be elucidated.



1 R = R^1 = R^2 = H
2 R = H, R^1, R^2 = benzylidene
3 R = R^2 = H, R^1 = benzyl
4 R = R^1 = R^2 = Ac
5 R = Ac, R^1, R^2 = benzylidene
6 R = R^2, = Ac, R^1 = benzyl



7 R = R^1 = R^2 = H
8 R = H, R^1,R^2 = benzylidene
9 R = R^1 = R^2 = Ac

Scheme 1.

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To assay the CGase activity, Li *et al.* [4] employed the TLC-analysis of both the oligosaccharide and the ceramide released from GM1, while Ito *et al.* [10] monitored the reducing power generated from GM1. These methods are laborious and relatively insensitive. If a simple and sensitive method for the routine assay of CGase activity becomes available, it will facilitate the purification of this enzyme and also the studies of distribution and function of the enzyme. While studying the substrate specificity of leech CGase [4], we found that the enzyme also hydrolysed MU-Lac. Since most biological samples contain exoglycosidases such as β-galactosidase and β-glucosidase which can eventually release MU from Mu-Lac, MU-Lac is not suitable for the routine assay of CGase activity. In order to circumvent this problem, we have attached a benzyl group at the C-6 position of Gal in MU-Lac and found that the resulting 6′Bn-MU-Lac could be hydrolysed by CGase but not by β-galactosidase. In this paper, we describe the synthesis of 6′-modified 4-methylumbelliferyl β-D-lactoside and related substrates and their use for the assay of ceramide glycanase activity.

Materials and methods

SYNTHESES OF SUBSTRATES

General methods

^{1}H-NMR spectra were recorded at 25 °C with a Bruker AMX-300 spectrometer (300 MHz) in $CDCl_3$ (internal standard: tetramethylsilane, 0 ppm), D_2O (HDO, 4.78 ppm), or DMSO-*d*6 (dimethylsulfoxide, 2.50 ppm). Assignment of the sugar ring-protons was made by first-order analysis of the spectra and confirmed by homo-nuclear decoupling technique. Thin layer chromatography was carried out on precoated plates of silica gel (60F254, layer thickness, 0.25 mm; E. Merck), and the carbohydrate components were visualized by spraying the plates with 15% H_2SO_4 in 50% EtOH, followed by heating at 140 °C. Column chromatography was performed on Silica gel 60 (E. Merck). Evaporation was carried out below 40 °C under reduced pressure. Melting points were determined with a Fisher-Johns apparatus and not corrected. The ratios of solvents used for TLC and column chromatography are expressed in vol/vol.

4-Methylumbelliferyl hepta-O-acetyl-β-D-lactoside (4)

A solution of hepta-*O*-acetyl-α-D-lactosyl bromide (aceto-bromolactose) [12] (3.50 g, 5.0 mmol) in chloroform (30 ml) was added to a solution of the sodium salt of 4-methylumbelliferone (1.98 g, 10 mmol) and tetrabutylammonium bisulfate (1.70 g, 5.0 mmol) in water (30 ml). The mixture was shaken vigorously at room temperature using a wrist-action shaker. TLC (1:1 toluene:EtOAc) showed that the reaction was over after 10 h. The organic layer was then separated from the reaction mixture, washed with brine and water, dried ($MgSO_4$), filtered, and concentrated. The residue was subjected to column chromatography with 2:1:2 toluene:EtOAc:diethyl ether as eluant to give, first, 2-acetoxy-3,6-di-*O*-acetyl-4-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-1,5-anhydro-D-arabino-hex-1-enitol (2-acetoxy-hexa-*O*-acetyl-D-lactal) (1.51 g, 49%), then the 4-methylumbelliferyl lactoside derivative **4** (1.51 g, 38%).

2-Acetoxy-hexa-O-acetyl-D-lactal

M.p. 153–155 °C; R_F 0.34 (1:1 toluene:EtOAc); ^{1}H-NMR ($CDCl_3$) δ: 6.58 (s, 1 H, H-1), 5.68 (d, 1 H, *J*4.6 Hz, H-3), 5.36 (bd, 1 H, *J*3.4 Hz, H-4′), 5.19 (dd, 1 H, *J*7.9, 10.5 Hz, H-2′), 5.00 (dd, 1 H, *J*3.4, 10.4 Hz, H-3′), 4.63 (d, 1 H, *J*7.9 Hz, H-1′), 4.43–3.88 (m, 7 H, sugar protons), 2.16, 2.12, 2.09, 2.08, 2.05, 2.04, 1.98 (each s, each 3 H, 7 Ac).

Compound 4

M.p. 106.5–107 °C; R_F 0.29 (1:1 toluene:EtOAc); ^{1}H-NMR ($CDCl_3$) δ: 7.55 (d, 1 H, *J*8.8 Hz, H-5 in coumarin), 6.95 (d, 1 H, *J*2.5 Hz, H-8 in coumarin), 6.89 (dd, 1 H, *J*2.5, 8.8 Hz, H-6 in coumarin), 6.19 (d, 1 H, *J*1.1 Hz, H-3 in coumarin), 5.36 (bd, 1 H, *J*3.3 Hz, H-4′), 5.30 (t, 1 H, *J*8.9 Hz, H-3), 5.22 (t, 1 H, *J*8.3 Hz, H-2), 5.14 (dd, 1 H, *J*7.8, 10.4 Hz, H-2′), 5.13 (d, 1 H, *J*7.8 Hz, H-1), 4.97 (dd, 1 H, *J*3.4, 10.4 Hz, H-3′), 4.52 (dd, 1 H, *J*2.9, 11.2 Hz, H-6a), 4.50 (d, 1 H, *J*7.8 Hz, H-1′), 4.15 (dd, 1 H, *J*6.2, 11.3 Hz, H-6b), 4.16 (dd, 1 H, *J*6.2, 11.3 Hz, H-6′a), 4.09 (dd, 1 H, *J*7.2, 11.1 Hz, H-6′b), 3.92–3.81 (m, 3 H, H-4,5,5′), 2.40 (d, 3 H, *J*1.1 Hz, CH_3 in coumarin), 2.16, 2.13, 2.08 (2), 2.07, 2.06, and 1.98 (each s, each 3 H, 7 Ac).

4-Methylumbelliferyl β-D-lactoside (1)

A solution of **4** (1.46 g, 1.83 mmol) in 10 mM MeONa:-MeOH (50 ml) was stirred at room temperature for 5 h. The white precipitate was collected, washed with cold MeOH (2 × 10 ml), and dried to give **1** (800 mg, 87%), which was crystallized from 95% EtOH: m.p. 198–199.5 °C; R_F 0.22 (65:25:4 $CHCl_3$:MeOH:H_2O); ^{1}H-NMR (DMSO-*d*6) δ: 7.71 (d, 1 H, *J*8.5 Hz, H-5 in coumarin), 7.06 (d, 1 H, *J*2.4 Hz, H-8 in coumarin), 7.04 (dd, 1 H, *J*2.4, 8.5 Hz, H-6 in coumarin), 6.25 (bs, 1 H, H-3 in coumarin), 5.15 (d, 1 H, *J*7.6 Hz, H-1), 4.23 (d, 1 H, *J*7.4 Hz, H-1′), 3.78–3.40 (m, 12 H, sugar protons), 2.41 (bs, 3 H, CH_3 in coumarin).

4-Methylumbelliferyl 4′,6′-O-benzylidene-β-D-lactoside (2)

A mixture of **1** (501 mg, 1.0 mmol), α,α-dimethoxytoluene (0.188 ml, 1.25 mmol), and 10-camphorsulfonic acid (70 mg, 0.3 mmol) in *N,N*-dimethylformamide (10 ml) was stirred at 50 °C while being evacuated to remove the methanol generated. After 3 h, further α,α-dimethoxytoluene (0.15 ml, 0.1 mmol) was added and the reaction

was continued for another 3 h. TLC (65:25:4 $CHCl_3$:MeOH:H_2O) showed the complete disappearance of starting material **1**. The reaction mixture was then neutralized with sat. aq. sodium bicarbonate and evaporated *in vacuo*. The solid residue was heated in hot 95% EtOH, and the hot mixture was filtered to remove insoluble material. The filtrate was kept at 4 °C overnight. The white crystals formed were identified as **2** (145 mg). The mother liquor was concentrated and purified by a column (2.0 × 190 cm) of Sephadex LH-20 eluted with 95% EtOH, giving an additional product (301 mg). The total yield of **2**: 445 mg (76%). M.p. 179–180.5 °C, R_F 0.41 (5:1 $CHCl_3$:MeOH); ^{1}H-NMR (DMSO-*d*6) δ: 7.72 (d, 1 H, *J*8.5 Hz, H-5 in coumarin), 7.48–7.36 (m, 5 H, Ph), 7.08 (d, 1 H, *J*2.4 Hz, H-8 in coumarin), 7.05 (dd, 1 H, *J*2.4, 8.5 Hz, H-6 in coumarin), 6.26 (d, 1 H, *J*1.1 Hz, H-3 in coumarin), 5.58 (s, 1 H, benzylidene), 5.16 (d, 1 H, *J*7.7 Hz, H-1), 4.44 (d, 1 H, *J*7.3 Hz, H-1′), 4.11 (bd, 1 H, *J*3.2 Hz, H-4′), 4.04 (t, 2 H, *J*12 Hz, H-6′a,b), 3.78–3.41 (m, 9 H, sugar protons), 2.41 (d, 3 H, *J*1.1 Hz, CH_3 in coumarin).

4-Methylumbelliferyl 2,2′,3,3′,6-penta-O-acetyl-4′,6′-O-benzylidene-β-D-lactoside (5)

Acetylation of **2** (260 mg, 0.44 mmol) was carried out in a mixture of acetic anhydride (4 ml) and pyridine (4 ml) for 5 h. The reaction mixture was poured into ice-water and the solution was stirred at room temperature for 3 h. After evaporation, the residue was subjected to column chromatography using 3:2 toluene:EtOAc as eluant, giving **5** (311 mg, 88%). M.p. 227–228 °C; R_F 0.30 (1:1 toluene:EtOAc); ^{1}H-NMR ($CDCl_3$) δ: 7.52 (d, 1 H, *J*8.8 Hz, H-5 in coumarin), 7.47–7.38 (m, 5 H, Ph), 6.96 (d, 1 H, *J*2.4 Hz, H-8 in coumarin), 6.93 (dd, 1 H, *J*2.4, 8.8 Hz, H-6 in coumarin), 6.20 (d, 1 H, H-3 in coumarin), 5.49 (s, 1 H, benzylidene), 5.34–5.22 (m, 3 H, H-2,2′,3), 5.13 (d, 1 H, *J*7.6 Hz, H-1), 4.90 (dd, 1 H, *J*3.5, 10.3 Hz, H-3′), 4.56 (dd, 1 H, *J*1.3, 11.3 Hz, H-6a), 4.50 (d, 1 H, *J*7.9 Hz, H-1′), 4.36 (bd, 1 H, *J*3.5 Hz, H-4′), 4.32 (dd, 1 H, *J*1.1, 11.3 Hz, H-6′a), 4.18 (dd, 1 H, *J*5.2, 11.3 Hz, H-6b), 4.07 (dd, 1 H, *J*1.0, 11.3 Hz, H-6′b), 3.92–3.83 (m, 2 H, H-4,5), 3.50 (m, 1 H, H-5′), 2.41 (d, 3 H, *J*1.1 Hz, CH_3 in coumarin), 2.15, 2.08, 2.07, 2.06, and 2.05 (each s, each 3 H, 5 Ac).

4-Methylumbelliferyl 2,2′,3,3′,4′,6-hexa-O-acetyl-6′-O-benzyl-β-D-lactoside (6)

A mixture of the benzylidene derivative **5** (250 mg, 0.313 mmol) and molecular sieves 4A (1.5 g) in tetrahydrofuran (12 ml) was stirred at room temperature for 15 min, then sodium cyanoborohydride (196.7 mg, 3.13 mmol) was added. The mixture was cooled to 0 °C, followed by dropwise addition of 4 M HCl in dioxane until gas evolution ceased (pH was *ca.* 3). The mixture was then stirred at room temperature. After 14 h, TLC (1:1 toluene:EtOAc) showed the formation of a less mobile compound, but about half of the starting material still remained. After another portion of sodium cyanoborohydride (196 mg) and 4 M HCl were added dropwise until gas evolution ceased, and allowing the reaction to proceed 48 h in total, TLC showed that **5** was completely reacted. The reaction mixture was diluted with dichloromethane (60 ml) and filtered through a layer of Celite. The filtrate was washed successively with saturated sodium bicarbonate solution, brine, and water. The organic layer was dried ($MgSO_4$), filtered, and evaporated. To the white solid residue were added pyridine (4 ml) and acetic anhydride (4 ml). The mixture was stirred at room temperature overnight, and evaporated *in vacuo* and co-evaporated with toluene. The residue was chromatographed on silica gel with 3:2 toluene:EtOAc as eluant to give **6** (181 mg, 72%). R_F 0.44 (1:1 toluene:EtOAc); ^{1}H-NMR ($CDCl_3$) δ: 7.51 (d, 1 H, *J*8.6 Hz, H-5 in coumarin), 7.36–7.27 (m, 5 H, Ph), 6.93 (d, 1 H, *J*2.3 Hz, H-8 in coumarin), 6.90 (dd, 1 H, *J*2.3, 8.6 Hz, H-6 in coumarin), 6.19 (d, 1 H, *J*1.1 Hz, H-3 in coumarin), 5.45 (bd, 1 H, *J*2.6 Hz, H-4′), 5.30 (t, 1 H, *J*9.0 Hz, H-3), 5.21 (dd, 1 H, *J*7.5, 9.0 Hz, H-2), 5.12 (d, 1 H, *J*7.4 Hz, H-1), 5.11 (dd, 1 H, *J*7.8, 10.4 Hz, H-2′), 4.97 (dd, 1 H, *J*3.4, 10.4 Hz, H-3′), 4.54 and 4.42 (ABq, 2 H, *J*12 Hz, CH_2Ph), 4.50 (dd, 1 H, *J*1.8, 12.1 Hz, H-6a), 4.48 (d, 1 H, *J*7.8 Hz, H-1′), 4.13 (dd, 1 H, *J*5.9, 12.1 Hz, H-6b), 3.92–3.80 (m, 3 H, H-4,5,5′), 3.53 (dd, 1 H, *J*5.7, 9.2 Hz, H-6′a), 3.43 (dd, 1 H, *J*7.2, 9.2 Hz, H-6′b), 2.41 (d, 3 H, *J*1.1 Hz, CH_3 in coumarin), 2.11, 2.07 (2), 2.06, 2.00, and 1.97 (each s, each 3 H, 6 Ac).

4-Methylumbelliferyl 6′-benzyl-O-β-D-lactoside (3)

Compound **6** (161 mg, 0.2 mmol) was subjected to de-*O*-acetylation with 10 mM MeONa-MeOH solution (10 ml) for 5 h at room temperature. After neutralization with Dowex 50W-X8 (H^+ form), the resin was filtered and washed with methanol (2 × 10 ml). The filtrate and washings were combined and evaporated. The residue was crystallized from 95% EtOH to afford white crystalline **3** (98 mg, 83%). M.p. 186–187.5 °C; R_F 0.30 (5:1 $CHCl_3$:MeOH); ^{1}H-NMR (DMSO-*d*6): δ7.68 (d, 1 H, *J*8.8 Hz, H-5 in coumarin), 7.31–7.25 (m, 5 H, Ph), 7.04 (dd, 1 H, *J*2.3, 8.8 Hz, H-6 in coumarin), 7.00 (d, 1 H, *J*2.3 Hz, H-8 in coumarin), 6.18 (s, 1 H, H-3 in coumarin), 5.04 (d, 1 H, *J*7.8 Hz, H-1), 4.47 (s, 2 H, CH_2Ph), 4.26 (d, 1 H, *J*7.8 Hz, H-1′), 3.78–3.32 (m, 12 H, sugar protons), 2.34 (s, 3 H, CH_3 in coumarin).

4-Methylumbelliferyl hepta-O-acetyl-β-D-cellobioside (9)

A mixture of acetobromocellobiose (1.40 g, 2.0 mmol), 4-methylumbelliferone sodium salt (793 mg, 4 mmol), and tetrabutylammonium bisulfate (679 mg, 2.0 mmol) in $CHCl_3$ (20 ml) and 0.5 M aqueous sodium carbonate (20 ml) was shaken vigorously at room temperature for 6 h. The two phases were separated, and the aqueous layer

was extracted with chloroform (30 ml). The organic layer and the extract were combined, washed with brine and water, dried (Na_2SO_4), and filtered. The filtrate was evaporated and the residue was subjected to column chromatography with 2:1:2 toluene:EtOAc:diethyl ether as eluant to give **9** (668 mg, 42%), along with 2-acetoxy-3,6-di-*O*-acetyl-4-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-1,5-anhydro-D-arabino-hex-1-enitol (557 mg, 45%).

The 2-acetoxy-glycal

R_F 0.39 (1:1 toluene:EtOAc); ^{1}H-NMR ($CDCl_3$): δ6.59 (s, 1 H, H-1), 5.69 (d, 1 H, *J*4.7 Hz, H-3), 5.19 (t, 1 H, *J*9.3 Hz, H-3′), 5.09 (t, 1 H, *J*9.3 Hz, H-4′), 4.98 (dd, 1 H, *J*8.0, 9.2 Hz, H-2′), 4.68 (d, 1 H, *J*7.89 Hz, H-1′), 4.45–4.04 (m, 6 H, H-4,5,6a,b, and 6′a,b), 3.72–3.67 (m, 1 H, H-5′), 2.12, 2.09, 2.08, 2.055, 2.04, 2.02, and 1.97 (each s, each 3 H, 7 Ac).

Compound 9

R_F 0.36 (1:1 toluene:EtOAc); ^{1}H-NMR ($CDCl_3$): δ7.51 (d, 1 H, J8.7 Hz, H-5 in coumarin), 6.95 (d, 1 H, *J*2.4 Hz, H-8 in coumarin), 6.91 (dd, 1 H, *J*2.4, 8.8 Hz, H-6 in coumarin), 6.19 (d, 1 H, *J*1.2 Hz, H-3 in coumarin), 5.29 (t, 1 H, *J*8.6 Hz, H-3), 5.22 (t, 1 H, *J*7.4 Hz, H-2′), 5.17 (t, 1 H, *J*8.3 Hz, H-4′), 5.12 (d, 1 H, *J*7.1 Hz, H-1), 5.08 (t, 1 H, *J*8.2 Hz, H-2), 4.96 (t, 1 H, *J*8.0 Hz, H-3′), 4.56 (m, 1 H, H-6a), 4.55 (d, 1 H, *J*8.0 Hz, H-1′), 4.38 (dd, 1 H, *J*4.5, 12.5 Hz, H-6b), 4.16 (dd, 1 H, *J*5.5, 12.1 Hz, H-6′a), 4.08 (m, 1 H, H-6′b), 3.87–3.69 (m, 3 H, H-4,5,5′), 2.41 (d, 3 H, *J*1.2 Hz, CH_3 in coumarin), 2.15, 2.11, 2.07, 2.065, 2.06, 2.02, and 2.00 (each s, each 3 H, 7 Ac).

4-Methylumbelliferyl β-D-cellobioside (7)

Compound **9** (794 mg, 1.0 mmol) was dissolved in 10 mM MeONa:MeOH (30 ml), and the solution was stirred at room temperature for 5 h. After neutralization with Dowex 50W-X8 (H^+ form), the mixture was filtered, and the filtrate was concentrated. The white residue was crystallized from 95% EtOH to give **7** (400 mg, 80%). M.p. 101–102 °C; R_F 0.27 (65:25:4 $CHCl_3$:MeOH:H_2O); ^{1}H-NMR (DMSO-*d6*): δ 7.69 (d, 1 H, *J*8.6 Hz, H-5 in coumarin), 7.05–7.02 (m, 2 H, H-6,8 in coumarin), 6.21 (s, 1 H, H-3 in coumarin), 5.06 (d, 1 H, *J*7.9 Hz, H-1), 4.28 (d, 1 H, *J*7.7 Hz, H-1′), 3.72–3.01 (m, 12 H, sugar protons), 2.37 (s, 3 H, CH_3 in coumarin).

4-Methylumbelliferyl 4′,6′-O-benzylidene-β-D-cellobioside (8)

A mixture of **7** (352 mg, 0.7 mmol), α,α-dimethoxytoluene (0.157 ml, 1.05 mmol), and 10-camphorsulfonic acid (50 mg) was stirred at 50 °C for 6 h with evacuation to remove methanol generated. Then the solution was neutralized with aqueous sodium bicarbonate and concentrated to dryness. The residue was dissolved in hot 95% EtOH (100 ml) and insoluble materials were filtered out. The filtrate was concentrated and the solid residue was crystallized from 95% EtOH, giving **8** (298 mg, 72%). M.p. 195–196 °C; R_F 0.56 (5:1 $CHCl_3$:MeOH); ^{1}H-NMR (DMSO-*d6*): δ7.69 (d, 1 H, *J*8.6 Hz, H-5 in coumarin), 7.39–7.33 (m, 5 H, Ph), 7.05–7.02 (m, 2 H, H-6,8 in coumarin), 5.54 (s, 1 H, benzylidene), 5.05 (d, 1 H, *J*7.6 Hz, H-1), 4.48 (d, 1 H, *J*7.8 Hz, H-1′), 3.74–3.16 (m, 12 H, sugar protons), 2.36 (s, 3 H, CH_3 in coumarin).

CGASE ASSAY

For the fluorometric assay of CGase using the synthetic substrates, a 50 µl of the reaction mixture containing 1.35 mM of the substrate in 50 mM sodium acetate buffer, pH 5.0, was incubated with an appropriate amount of the enzyme. After incubation at 37 °C for a predetermined time, the reaction was stopped by the addition of 1.5 ml of 0.2 M sodium borate buffer, pH 9.8. The amount of MU released was determined fluorometrically using a Sequoia-Turner model 450 fluorometer with an NB360 excitation filter and an SC415 emission filter (excitation: 360 nm; emission, 440 nm). One unit of enzyme is defined as the amount that liberates 1 nmol of MU per min at 37 °C.

The initial rates of enzymatic reaction were evaluated from kinetic curves of the product accumulation as described by Boecker [13]. The parameters of the Michaelis-Menten type reaction were then evaluated by nonlinear regression using the Enzfitter program (Elsevier-Biosoft).

For TLC analysis of the products released by CGase, the reaction mixture contained 50 nmol of substrate and an appropriate amount of the enzyme in 30 µl of 50 mM sodium acetate buffer, pH 5.0. After incubation at 37 °C for a predetermined time, the reaction was stopped by addition of 30 µl of 100% ethanol, followed by removal of the insoluble material by centrifugation. A 10 µl aliquot of the incubation mixture was subsequently analysed by TLC using 2:1:1 n-butanol:acetic acid:H_2O as the developing solvent. To reveal glycoconjugates, the plate was sprayed with diphenylamine reagent [14] and heated at 115 °C for 15 min.

CERAMIDE GLYCANASE

Crude and purified CGase were prepared from *Macrobdella* leech as described previously [4]. *Rhodococcus* endoglycoceramidase [10] was purchased from Genzyme and Corynebacterium endoglycoceramidase [11] was a gift of Dr Kenji Yamamoto, Department of Food Science and Technology, Kyoto University, Japan.

Results and discussion

Synthesis of MU-glycosides

Initial experiments on the reaction of hepta-*O*-acetyl-α-D-lactosyl bromide (acetobromolactose) [12] with the

sodium salt of 4-methylumbelliferone in anhydrous *N,N*-dimethylformamide or acetone [15] failed to produce the desired 4-methylumbelliferyl lactoside derivative. The major product isolated from the reaction mixture was 2-acetoxy-3,6-di-*O*-acetyl-4-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-1,5-anhydro-D-arabino-hex-1-enitol (2-acetoxy-hexa-*O*-acetyl-D-lactal). In addition, the reaction of acetobromolactose with 4-methylumbelliferone under the catalysis of silver carbonate or triflate in toluene-nitromethane also did not yield the desired product. Finally, the desired product was obtained via a phase-transfer reaction. Thus, treatment of acetobromolactose with 4-methylumbelliferone (sodium salt) in a two-phase mixture (1 M aqueous sodium carbonate, chloroform) using tetrabutylammonium bisulfate as the phase-transfer catalyst gave 4-methylumbelliferyl hepta-*O*-acetyl-β-D-lactoside (**4**) in a 38% yield, together with the elimination product, 2-acetoxy-hexa-*O*-acetyl-D-lactal (50%). Attempts to improve the yield of **4** by using different molar equivalents of phase-transfer catalyst (0.5–1.0 mol. equiv.), or by using different aqueous solution (e.g. 0.5–1.0 M aq. sodium bicarbonate) were not successful. A considerable amount of the elimination product was formed in all cases. The Zemplén de-*O*-acetylation of **4** afforded 4-methylumbelliferyl β-D-lactoside (**1**) as a white solid. The relatively large coupling constant between H-1,2 in **1** ($J_{1,2}$ 7.6 Hz) indicated that the newly formed glycosidic linkage was of β-D-configuration.

Cyclic acetal formation and its selective opening

The 4′,6′-*O*-benzylidene derivative (**2**) was prepared *via* acetal exchange according to the procedure of Evans [16]. Compound **1** was reacted with α,α-dimethoxytoluene in *N,N*-dimethylformamide in the presence of 10-camphorsulfonic acid to provide 4-methylumbelliferyl 4′,6′-*O*-benzylidene-β-D-lactoside (**2**) (76%). To synthesize the 6′-*O*-benzyl derivative (**3**), compound **2** was first converted into the peracetylated derivative (**5**), and the reductive opening of the benzylidene ring in **5** was carried out through a reaction with sodium cyanoborohydride, hydrogen chloride in tetrahydrofuran [17] to afford 4-methylumbelliferyl 6′-*O*-benzyl-hexa-*O*-acetyl-β-D-lactoside (**6**) (72%) after *O*-acetylation. The location of the benzyl group was confirmed by comparing the ^{1}H-NMR spectra of **4** and **6**. The H-4′ in **4** and **6** resonated at almost the same position in a relatively low field (δ5.4), whereas the H-6′a,b in the two compounds appeared at different positions, with the H-6′a,b in **4** resonating in a relatively low field (δ4.1–4.2) and the H-6′a,b in **6** in a high field (δ3.4–3.5). These results clearly indicated that the benzyloxy group in **6** was attached to the 6′-position. The Zemplén de-*O*-acetylation of **6** provided 4-methylumbelliferyl 6′-*O*-benzyl-β-D-lactoside (**3**) (83%).

The 4′-epimer of 4-methylumbelliferyl β-D-lactoside, i.e. 4-methylumbelliferyl β-D-cellobioside (**7**), and its 4′,6′-*O*-benzylidene derivative (**8**) were also synthesized to provide opportunities to examine structural requirement of the substrate by the enzyme. The reaction of acetobromocellobiose [12] with the sodium salt of 4-methylumbelliferone under the phase-transfer condition provided a 42% yield of the per-*O*-acetylated 4-methylumbelliferyl cellobioside (**9**). Again, a by-product of the 2-acetoxy-glycal, attributable to 1,2-elimination of HBr from acetobromocellobiose, was isolated in a 45% yield. The Zemplén de-*O*-acetylation of **9** gave 4-methylumbelliferyl β-D-cellobioside (**7**) (80%). Finally, 7 was converted into 4-methylumbelliferyl 4′,6′-*O*-benzylidene-β-D-cellobioside (**8**) in a 72% yield through a reaction with α,α-dimethoxytoluene in *N,N*-dimethylformamide under the catalysis of CSA.

It should be noted that the 4-methylumbelliferyl β-D-lactoside derivatives **1**, **2**, and **3**, as well as 4-methylumbelliferyl 4′,6′-*O*-benzylidene-β-D-cellobioside (**8**) have lower solubilities in water than 4-methylumbelliferyl-β-D-cellobioside (**7**).

Hydrolysis of the synthetic substrates by the crude and purified CGases from the leech

The purified CGase released lactose and MU from MU-Lac (Fig. 1). However, the crude CGase obtained after ammonium sulfate fractionation [4] releases Gal and Glc from MU-Lac in addition to lactose. Under our TLC condition, Glc moved slightly ahead of Gal and the two sugars moved as a broad band as shown in Fig. 1, lane 7. These results indicate that MU-Lac is not suitable for

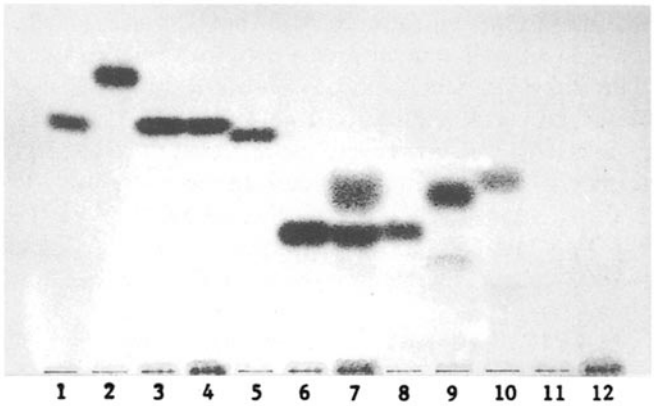

Figure 1. Thin-layer chromatogram showing the hydrolysis of 6′Bn-MU-Lac and MU-Lac by the crude and the purified leech CGase. 1, 6′Bn-Lac; 2, 6′Bn-MU-Lac; 3, 6′Bn-MU-Lac + purified CGase; 4, 6′Bn-MU-Lac + crude CGase; 5, MU-Lac; 6, MU-Lac + purified CGase; 7, MU-Lac + crude CGase; 8, Lac; 9, Gal; 10, Glc; 11, purified CGase; 12, crude CGase. CGase (0.1 unit) was incubated with 50 nmol of the substrates in 30 μl of 50 mM sodium acetate buffer, pH 5.0, at 37 °C for 6 h. The detailed assay conditions are described in the text.

routine fluorometric assay of CGase activity in crude enzyme preparations. Both the purified and the crude CGase released only 6′Bn-Lac from 6′Bn-MU-Lac (Fig. 1, lanes 3 and 4), indicating that the attachment of a benzyl group to the C-6 of Gal in lactose prevented the release of Gal by β-galactosidase. Both enzyme preparations could convert GM1 into $II^3NeuAcGgOse_4$ and ceramide. The crude CGase preparation also produced $II^3NeuAcGgOse_3$ in addition to $II^3NeuAcGgOse_4$, however, due to the presence of β-galactosidase (results not shown). Thus 6′-Bn-MU-Lac is superior to the natural substrates at this point. 4′6′Bd-MU-Lac was similarly cleaved by CGase and was resistant to β-galactosidase. However, the solubility of this substrate in water is lower than that of 6′Bn-MU-Lac and it is not useful for high-K_m enzymes. In addition, the K_m for this substrate was found to be higher than that for 6′Bn-MU-Lac (Table 1). Thus, 6′Bn-MU-Lac is a more useful substrate than 4′6′Bd-MU-Lac for routine fluorometric assay of CGase activity.

We found that CGase also cleaved MU-Cel. The purified CGase released cellobiose from MU-Cel while the crude CGase released Glc and cellobiose from this substrate due to the presence of β-glucosidase (results not shown). Thus as in the case of MU-Lac, Mu-Cel is not a suitable substrate for routine assay of CGase activity. Interestingly, 4′6′Bd-MU-Cel was found to be refractory to CGase.

Kinetic properties of leech CGase using 6′Bn-MU-Lac as substrate

The purified CGase isolated from *Macrobdella* leech exhibited a pH optimum between pH 4.8 and 5.2 with 6′Bn-MU-Lac as substrate (Fig. 2). This range is similar to that using GM1 as substrate [4]. The K_m values for the hydrolysis of synthetic substrates by CGase are summarized in Table 1. Among those substrates, 6′Bn-MU-Lac gave the lowest K_m, followed by 4′,6′Bd-MU-Lac, MU-Lac, and MU-Cel. However, as reflected in V_{max}, the efficiency of the enzyme to hydrolyse these substrates is in the following order: 4′,6′Bd-MU-Lac > MU-Cel > 6′Bn-MU-Lac > MU-Lac. As shown in Fig. 3, the apparent K_m for the hydrolysis of 6′Bn-MU-Lac was 0.232 mM, which is considerably higher than the K_m (0.0154 mM) for the hydrolysis of GM1 [4]. This indicates that the affinity of 6′Bn-MU-Lac to CGase is significantly lower than that of GM1. This may be due to the shorter sugar chain of the MU-substrate, or the bulky aglycon of methylumbelliferone. Previously, we have reported that both the sugar chain and the hydrophobic tail can influence the rate of hydrolysis of glycolipids by CGase

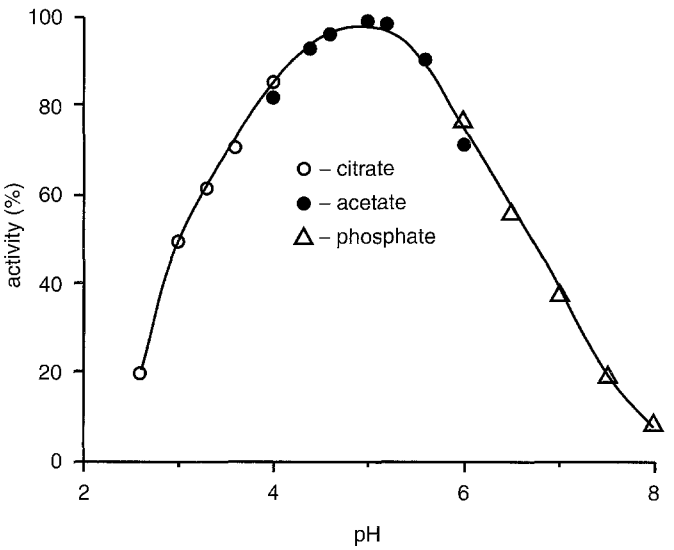

Figure 2. Effect of pH on the hydrolysis of 6′Bn-MU-Lac by the purified leech CGase. ○, 50 mM sodium citrate buffer; ●, 50 mM sodium acetate buffer; △, 50 mM sodium phosphate buffer. The detailed assay conditions are described in the text.

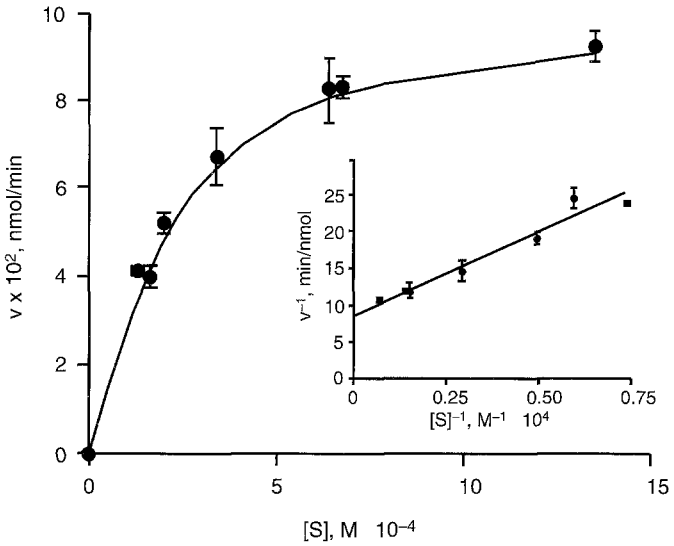

Figure 3. Kinetics of the hydrolysis of 6′Bn-MU-Lac by the purified leech CGase. The detailed assay conditions are described in the text.

Table 1. Michaelis-Menten constants for synthetic substrates with leech CGase.

Substrate	K_m *(mM)*	V_{max} *(nmol min^{-1} mg^{-1})*
Mu-Lac	0.77	29.5
6′Bn-MU-Lac	0.23	37
4′6′Bd-MU-Lac	0.60	54.5
MU-Cel	1.40	44.5
4′6′Bd-MU-Cel	(Not hydrolysed)	

[4]. It should be noted that a bifluorescence-labelled lactoside having a -O(CH2)$_5$-chain in the aglycon gave a K_m value of 7.7 μM [18]. Because of the lower affinity, the assay using 6′Bn-MU-Lac requires a higher concentration of the substrate than that of GM1. However, the 6′Bn-MU-Lac is much easier to prepare than the bi-fluorescence-labelled lactoside or GM1.

We also found that although the CGases from leech [4] and earthworm [6] were able to hydrolyse 6′Bn-MU-Lac, this synthetic substrate was not hydrolyzed by the endoglycoceramidase prepared from *Rhodococcus sp.* [10] and *Corynebacterium sp.* [11]. By using 6′Bn-MU-Lac, we have detected two CGase activities in the oyster hepatopancreas; one had a higher specificity toward GM1 while the other preferred the synthetic substrate 6′Bn-MU-Lac.

The specificities of leech and earthworm CGase toward different natural substrates have been compared [6]. In general, the ganglio-series glycosphingolipids are better substrates than the globo-series. The presence or absence of sialic acids and the length of the sugar chains also influenced the rate of hydrolyses. We found that the leech CGase hydrolysed GM1 much faster than 6′Bn-MU-Lac. The relative rates of the hydrolysis of GM1 and 6′Bn-MU-Lac by leech CGase was found to be 7:1. However, the reverse was true for the earthworm CGase, which hydrolysed 6′Bn-MU-Lac four times faster than GM1. The two CGase activities found in the oyster hepatopancreas differed greatly in their specificities toward GM1 and 6′Bn-MU-Lac. One hydrolysed GM1 twenty times faster than 6′Bn-MU-Lac while the other hydrolysed 6′Bn-MU-Lac 150 times faster than GM1. It should be emphasized that the activity toward 6′Bn-MU-Lac does not always reflect the activity toward the natural substrates.

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