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Note

Synthesis of amphiphilic lactosides that possess a lactosylceramide-mimicking *N*-acyl structure: Alternative universal substrates for *endo*-type glycosylceramidases

Yoshiaki Miura [1], Tsutomu Arai, Tatsuya Yamagata *

Department of Biomolecular Engineering, Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology, Yokohama 226, Japan

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The *endo*-type glycosylceramidases, EGCase [1,2], and CGase [3,4], are both unique enzymes in action and are essential in the hydrolysis of glycosphingolipids (GSLs) into ceramide and oligosaccharide moieties. Although they are useful for GSL carbohydrate structural analyses [5,6] and for elucidating possible biological functions of GSLs [7], the determination of their activity has required the use of GSLs or radiolabelled compounds, followed by laborious and variable thin-layer chromatographic methods. The authors thus produced a chromogenic amphiphilic lactoside, 2-(*N*-hexadecanoylamino)-4-nitrophenyl β-lactoside (**1**), as a universally reliable substrate for *endo*-type glycosylceramidases. It should be noted that EGCase/CGase does not act on *p*-nitrophenyl β-lactoside, presumably owing to its nonlipophilic nature. Novel lipophilic lactosides whose *N*-acyl structures are introduced via ethanolamine or 2-mercaptoethylamine were also synthesized in this study. The aglycon, consisting of ethanolamine and an *N*-acyl chain in the lactosides, *N*-acylaminoethyl β-lactosides (**16–19**),

Abbreviations: EGCase, endoglycoceramidase; CGase, ceramide glycanase; GSL, glycosphingolipid; GM1, β-D-Gal-(1 → 3)-β-D-GalNAc-(1 → 4)-[α-Neu5Ac-(2 → 3)]-β-D-Gal-(1 → 4)-β-D-Glc-1,1′-Cer; TMSTf, trimethylsilyl trifluoromethanesulfonate; Fmoc-OSu, 9-fluorenylmethyl *N*-succinimidyl carbonate

* Corresponding author.

[1] Current address: The Burnham Institute, 10901 N. Torrey Pines Road, La Jolla, CA 92037, USA.

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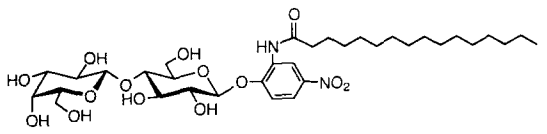
partially mimics the ceramide structure, and, consequently, the lactoside should prove far more susceptible to hydrolyzing enzymes than conventional synthetic glycolipids possessing a simple alkyl chain. In contrast to those lactosides, a thiolactoside, *N*-hexadecanoylaminoethyl β-thiolactoside (**20**), which also possesses a corresponding *N*-acyl chain, should provide a useful tool for the study on kinetics of the enzymes and for constructing affinity ligands.



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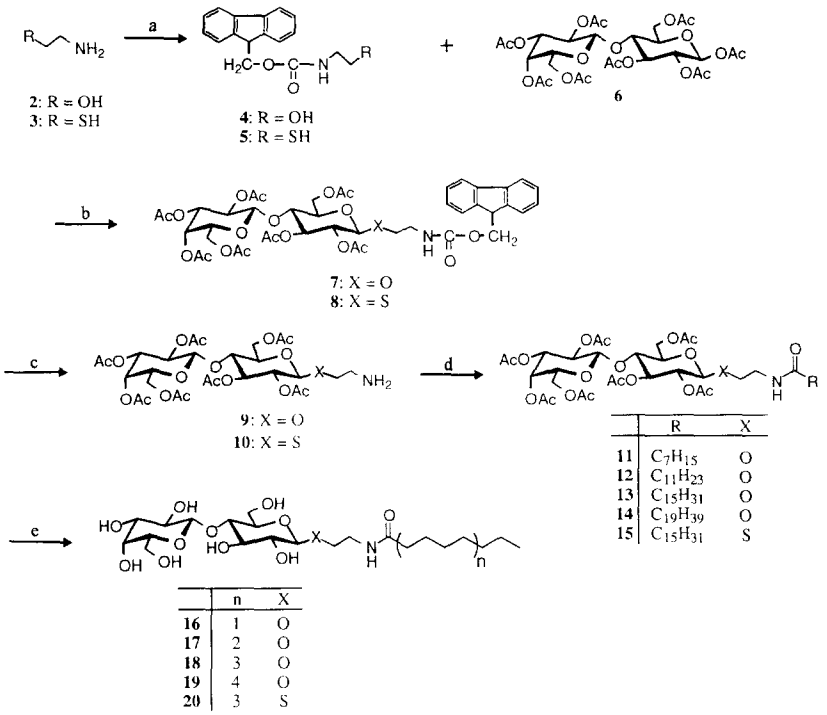
It has been found that lactosylceramide (LacCer) is the simplest structure among susceptible GSLs for EGCase/CGase and that a single alkyl chain could be the aglycon of the substrates for EGCase/CGase. In order to understand the effects of the amide bond present in ceramide and the hydrophobicity of the aglycon of the amphiphilic lactosides on the rate of hydrolysis, we prepared *N*-acylaminoethyl lactosides in which an octanoyl (**16**), dodecanoyl (**17**), hexadecanoyl (**18**), or eicosanoyl (**19**) chain has been introduced. Based on considerations of simplicity and cost reduction, lactose peracetate (**6**) was used in this study as the starting material for the syntheses discussed in the following (see Scheme 1). To provide an efficient and somewhat shorter route to *N*-acylaminoethyl β-lactosides, *N*-(9-fluorenylmethyloxycarbonyl, Fmoc)-aminoethanol (**4**), readily prepared from commercially available Fmoc-OSu and ethanolamine (**2**) in high yield, was first condensed with **6** in the presence of TMSTf to give the β-anomer of *N*-Fmoc-aminoethyl lactosides (**7**). The reaction mixture contained a major component in TLC (2:3 toluene–AcOEt) that moved distinctly slower (R_f 0.39) than **6** (R_f 0.5). The TLC of the mixture showed one additional minor spot (R_f 0.12). Since it seemed to be a lactoside lacking a 2-*O*-acetyl group, which was diminished after the acetylation of the mixture by pyridine–Ac_2O, purification was not carried out to increase the yield of the desired compound. The Fmoc group, which could be removed under mild conditions, was selectively removed from the compound **7** with stirring in morpholine at room temperature for 30 min. Crude aminoethyl hepta-*O*-acetyl β-lactoside (**9**) was used directly for *N*-acylation. Acylation of the amino group was conducted by reaction with an acyl chloride (C_8, C_{12}, C_{16}, or C_{20}), followed by deacetylation under standard conditions. Purification by silica-gel chromatography afforded the desired compounds **16**–**19** in overall 22–30% yield from **6**. The compounds obtained were identified as the pure corresponding *N*-acylaminoethyl β-lactoside, as evidenced by NMR data and elemental analyses. The anomeric configuration of the β-lactosides was confirmed by the chemical shift for H-1 and the $J_{1,2}$ coupling constant. Synthesis of an *N*-hexadecanoylaminoethyl β-thiolactoside **20** was accomplished in a similar manner using 2-mercaptoethylamine hydrochloride in place of ethanolamine. *N*-Fmoc-aminoethanethiol (**5**) was obtained in high yield as well as *N*-Fmoc-aminoethanol. The structure of **5** was fully proved by 270 MHz ^{1}H NMR spectroscopy. By essentially the



	R	X
11	C_7H_{15}	O
12	$C_{11}H_{23}$	O
13	$C_{15}H_{31}$	O
14	$C_{19}H_{39}$	O
15	$C_{15}H_{31}$	S

	n	X
16	1	O
17	2	O
18	3	O
19	4	O
20	3	S

Scheme 1. **Reagents and conditions:** (a) CH_2Cl_2, Fmoc-OSu, triethylamine, r.t., 2 h; (b) CH_2Cl_2, TMSTf, r.t., 2 h; (c) morpholine, r.t., 30 min; (d) pyridine, acyl chloride, r.t., 3 h; (e) sodium methoxide, methanol, r.t., 18 h.

same way as described for **16–19**, compound **20** was afforded in 26% yield from **6**. The structure was confirmed by ^{1}H NMR spectroscopy. H-1 of **20** resonated at δ 4.25 as a wide doublet with a relatively larger $J_{1,2}$ of 9.7 Hz, which is generally seen for β-thioglycosidically linked anomeric protons. Since the elemental analysis for some of the lactosides showed slight deviations, the lactosides were further analyzed by matrix-assisted laser-desorption ionization-mass spectroscopy (MALDIMS). All lactosides tested gave a peak corresponding to the sodium-adduct ion $(M + Na)^+$ because of the matrix DHB which is known to produce mainly $(M + Na)^+$ signals for oligosaccharides. The mass found for each lactoside agreed very closely with that calculated (see Experimental section).

Lipophilic *p*-nitrophenyl lactoside should prove to be a chromogenic and useful substrate for *endo*-type glycosylceramidase. The facile synthesis of **1** is presented in this paper for the first time. Per-*O*-acetyl lactosyl bromide and the sodium salt of 2-*N*-hexadecanoylamino-4-nitrophenol were refluxed in acetone to give the corresponding per-*O*-acetyl lactoside. Following deacetylation, recrystallization from EtOH of the crude compound afforded **1** in an overall yield of 44% from lactose peracetate. ^{1}H NMR spectroscopy and elemental analysis confirmed the structure of **1** (see Tables 1 and 2, respectively).

Table 1
Selected ^{1}H NMR chemical shifts [a] (ppm) and coupling constants (Hz, in parentheses) for synthesized amphiphilic lactosides

Compound	Glc H-1	Gal H-1	$NHCOCH_2$	$COCH_2CH_2$	$(CH_2)_nCH_3$	CH_3	Phenyl
1	5.08 (7.3)	4.32 (7.0)	2.48 (7.3)	1.66	1.22–1.44, $n \approx 12$	0.89 (6.6)	7.42 (9.2) 7.98 (9.2, 2.6) 9.09 (2.6)
16	4.25 (7.6)	4.26 (7.6)	2.11	1.52	1.22–1.33, $n = 4$	0.90 (6.6)	–
17	4.25 (7.6)	4.26 (7.6)	2.10 (7.8)	1.52	1.18–1.33, $n = 8$	0.89 (6.8)	–
18	4.25 (7.6)	4.25 (7.6)	2.10 (7.3)	1.52	1.22–1.33, $n = 12$	0.89 (6.2)	–
19	4.25 (7.8)	4.26 (7.6)	2.10 (7.6)	1.52	1.23–1.33, $n = 16$	0.89 (6.2)	–
20	4.37 (9.7)	4.27 (7.6)	2.09 (7.6)	1.52	1.28–1.34, $n = 12$	0.89 (6.8)	–

[a] Measured in 95:5 Me_2SO-d_6–D_2O at 60 °C and 270 MHz.

The compounds obtained in this study were assayed for susceptibility to CGase from leeches. A Lineweaver–Burk plot showed K_m for the hydrolysis of the chromogenic substrate **1** to be 28 μM, this value being consistent with that estimated for GM1 (15 μM) [3]. We have found that the chromogenic substrate **1** was digested by both EGCases from *Rhodococcus* sp. and *Corynebacterium* sp., and the CGase (Y. Miura, T. Arai, A. Ohtake, M. Ito, K. Yamamoto, and T. Yamagata, unpublished work), suggesting that the compound **1** would be an alternative universal substrate for *endo*-type glycosylceramidases. While hydrolysis of the compound **1** was monitored readily by a spectroscopic method with high sensitivity, susceptibilities for the EGCase/CGase of *N*-acylaminoethyl β-lactosides **16–19** were detected by a conventional high-performance TLC method. Although the lactoside **18** showed high susceptibility for the CGase-catalyzed hydrolysis, the corresponding thiolactoside **20** was found to undergo virtually no hydrolysis by CGase. The results of precise determinations of substrate specificity of *endo*-type glycosylceramidases to the above lactosides synthesized in this study will be published elsewhere (Y. Miura, T. Arai, A. Ohtake, M. Ito, K. Yamamoto, and T. Yamagata, unpublished work).

Table 2
Elemental analysis of synthesized amphiphilic lactosides [a]

Compound	Formula	Anal. Calcd			Found		
		C	H	N	C	H	N
1	$C_{34}H_{56}N_2O_{14}\cdot H_2O$	55.57	7.96	3.81	55.27	7.94	3.73
16	$C_{22}H_{41}NO_{12}\cdot H_2O$	49.90	8.18	2.65	49.31	7.70	2.70
17	$C_{26}H_{49}NO_{12}\cdot H_2O$	53.32	8.78	2.39	54.37	8.91	2.43
18	$C_{30}H_{57}NO_{12}\cdot H_2O$	56.14	9.27	2.18	56.77	9.41	2.18
19	$C_{34}H_{65}NO_{12}\cdot H_2O$	58.51	9.68	2.01	58.65	9.81	2.10
20	$C_{30}H_{57}NO_{11}S\cdot H_2O$	54.77	9.04	2.13	55.38	9.02	2.09

[a] Deviations outside the usual $\pm 0.4\%$ from the theoretical percent was sometimes unavoidable due to the nature of these compounds. For such compounds (e.g., **16**, **18**, and **20**), acceptable MALDIMS values for (M + Na) were obtained, and the NMR spectra showed no extraneous peaks.

1. Experimental

General methods.—^{1}H NMR spectra were recorded at 270 MHz with a JEOL EX270 spectrometer in $CDCl_3$ or 95:5 dimethyl sulfoxide-d_6–D_2O using tetramethylsilane (Me_4Si) or sodium 3-(trimethylsilyl)propanesulfonate (DSS), respectively, as an internal reference. All reactions were monitored by TLC on Silica Gel 60 F-254 or HPTLC Silica Gel 60 (Merck, Darmstadt), with detection by UV light or by visualizing with orcinol–H_2SO_4. Fmoc-OSu was purchased from Peptide Institute, Osaka. Column chromatography was performed on Silica Gel 60 (70–230 mesh, Merck, Darmstadt). Positive-ion MALDI mass spectra were recorded on a KOMPACT MALDI 1 mass spectrometer (Kratos, UK) with a 2,5-dihydroxybenzoic acid (DHB) matrix.

2-N-(Hexadecanoylamino)-4-nitrophenyl β-lactoside (**1**).—To a solution of the sodium salt of 2′-hydroxy-5′-nitrohexadecananilide (1.24 g, 3 mmol) [8] in 10 mL of acetone was added 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1 → 4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl bromide (3 mmol). The mixture was stirred for 2 h and refluxed for 12 h. The mixture was brought to room temperature and then acidified with 6 M HCl. The mixture was filtered and evaporated to a syrup. The residue was dissolved in dry MeOH, and to the solution was added NaOMe (1 mmol). The mixture was stirred for 18 h. After neutralization with Dowex 50W-X2 (200–400 mesh) and filtration, the mixture was evaporated to dryness. The crude product was purified by column chromatography on silica gel (60:25:4 $CHCl_3$–MeOH–water), followed by recrystallization from EtOH to yield 0.97 g (44% from per-*O*-acetyl-lactosyl bromide) of **1**. ^{1}H NMR (60 °C, 95:5 Me_2SO-d_6–D_2O): δ 0.89 (t, 3 H, *J* 6.6 Hz, CH_2CH_3), 1.22–1.40 (m, 24 H, $(CH_2)_{12}CH_3$), 1.66 (m, 2 H, CH_2CH_2CONH), 2.48 (t, 2 H, *J* 7.3 Hz, CH_2CH_2CONH), 7.42 (d, 1 H, *J* 9.2 Hz, phenyl), 7.98 (dd, 1 H, *J* 9.2 Hz, *J* 2.6 Hz, phenyl), 9.09 (d, 1 H, *J* 2.6 Hz, phenyl), 4.32 (d, 1 H, *J* 7.0 Hz, H-1′), 5.08 (d, 1 H, *J* 7.3 Hz, H-1); MALDIMS: Calcd for $C_{34}H_{56}N_2NaO_{14}$: $(M + Na)^+$, 739.4; found: $(M + Na)^+$, 740.1. For Anal., see Table 1.

N-(9-Fluorenylmethyloxycarbonyl)aminoethanol (**4**).—To a solution of 9-fluorenylmethyl succinimidyl carbonate (Fmoc-OSu) (10 g, 30 mmol) in 80 mL of CH_2Cl_2, was added 1.8 mL (30 mmol) of **2**, and the mixture was stirred at 4 °C. To the mixture was added 4.2 mL of triethylamine with stirring for 3 h. The mixture was washed with 0.1 M HCl and distilled water, the organic phase was dried, and the residue was recrystallized after removal of the solvent to yield **4** (6.9 g, 83%). ^{1}H NMR ($CDCl_3$): δ 7.26–7.78 (m, 8 H, Ar), 5.15 (br, 1 H, NH), 4.42–4.45 (m, 2 H, CH_2-Fmoc), 4.22 (t, 1 H, *J* 7.0 Hz, CH-Fmoc), 3.70–3.74 (m, 2 H, $NHCH_2$), 3.33–3.37 (m, 2 H, CH_2OH), 2.07 (t, 1 H, OH). Anal. Calcd for $C_{17}H_{17}NO_3$: C, 72.07; H, 6.05; N, 4.94. Found: C, 72.43; H, 6.05; N, 4.91.

N-(9-Fluorenylmethyloxycarbonyl)aminoethanethiol (**5**).—This compound was prepared from 1.14 g (10 mmol) of 2-mercaptoethylamine hydrochloride and 3.0 g (10 mmol) of Fmoc-OSu in CH_2Cl_2 in the presence of two mol-equiv of triethylamine as described for the synthesis of **4**, giving 2.16 g (72%) yield: ^{1}H NMR ($CDCl_3$): δ 7.29–7.78 (m, 8 H, Ar), 5.14 (br, 1 H, NH), 4.42–4.45 (m, 2 H, CH_2-Fmoc), 4.22 (t, 1 H, CH-Fmoc), 3.34–3.41 (m, 2 H, $NHCH_2$), 2.62–2.70 (m, 2 H, CH_2SH), 1.34 (t, 1 H,

SH). Anal. Calcd for $C_{17}H_{17}NO_2S$: C, 68.20; H, 5.72; N, 4.68. Found: C, 68.35; H, 5.85; N, 4.75.

General procedures for the synthesis of N-*acylaminoethyl β-lactosides* (**16–19**).—To a solution of *N*-Fmoc-aminoethanol (2 mmol) and lactose peracetate (2 mmol) in 40 mL of CH_2Cl_2 was added molecular sieves 4A, and the mixture was stirred for 3 h. To the solution was added an mol-equiv of TMSTf with stirring for 2 h. The mixture was washed with satd aq $NaHCO_3$ and water, dried over Na_2SO_4, and concentrated. The residue containing the β-anomer **7** was dissolved in 6 mL of morpholine, stirred for 30 min, then concentrated. The residue was dissolved in CH_2Cl_2 and washed with aq 5% NaCl, dried (Na_2SO_4), and concentrated. The amino-deblocked crude **9** was used directly for *N*-acylation.

To a solution of **9** in pyridine (10 mL) was added 2 mmol of acyl chloride, the mixture was stirred for 3 h and coevaporated with toluene. The heptaacetate **11**, **12**, **13**, or **14** was dissolved in dry MeOH, and if necessary, a few drops of tetrahydrofuran was added to give a clear solution. To the solution was added NaOMe in MeOH with stirring for 18 h. Purification by silica gel chromatography (60:25:4 $CHCl_3$–MeOH–water) afforded the desired compounds **16–19** in overall 22–30% yield from **6**.

N-*Octanoylaminoethyl β-lactoside* (**16**).—Yield 0.318 g (30%); ^{1}H NMR (60 °C, 95:5 Me_2SO-d_6–D_2O): δ 0.90 (t, 3 H, *J* 6.6 Hz, CH_2CH_3), 1.22–1.33 (m, 8 H, $(CH_2)_4CH_3$), 1.52 (m, 2 H, CH_2CH_2CONH), 2.11 (m, 2 H, CH_2CH_2CONH), 4.25 (d, 1 H, *J* 7.6 Hz, H-1), 4.26 (d, 1 H, *J* 7.6 Hz, H-1′); MALDIMS: Calcd for $C_{22}H_{41}NNaO_{12}$: $(M + Na)^+$, 534.25; found: $(M + Na)^+$, 534.97.

N-*Dodecanoylaminoethyl β-lactoside* (**17**).—Yield 0.328 g (28%); ^{1}H NMR (60 °C, 95:5 Me_2SO-d_6–D_2O): δ 0.89 (t, 3 H, *J* 6.8 Hz, CH_2CH_3), 1.18–1.33 (m, 16 H, $(CH_2)_8CH_3$), 1.52 (m, 2 H, CH_2CH_2CONH), 2.10 (t, 2 H, *J* 7.8 Hz, CH_2CH_2CONH) 4.25 (d, 1 H, *J* 7.6 Hz, H-1), 4.26 (d, 1 H, *J* 7.6 Hz, H-1′); MALDIMS: Calcd for $C_{26}H_{49}NO_{12}Na$: $(M + Na)^+$, 590.32; found: $(M + Na)^+$, 590.82.

N-*Hexadecanoylaminoethyl β-lactoside* (**18**).—Yield 0.385 g (30%); ^{1}H NMR (60 °C, 95:5 Me_2SO-d_6–D_2O): δ 0.89 (t, 3 H, *J* 6.2 Hz, CH_2CH_3), 1.22–1.33 (m, 24 H, $(CH_2)_{12}CH_3$), 1.52 (m, 2 H, CH_2CH_2CONH), 2.10 (t, 2 H, *J* 7.3 Hz, CH_2CH_2CONH), 4.25 (d, 1 H, *J* 7.6 Hz, H-1), 4.25 (d, 1 H, *J* 7.6 Hz, H-1′); MALDIMS: Calcd for $C_{30}H_{57}NNaO_{12}$: $(M + Na)^+$, 646.38; found: $(M + Na)^+$, 640.40.

N-*Eicosanoylaminoethyl β-lactoside* (**19**).—Yield 0.307 g (22%); ^{1}H NMR (60 °C, 95:5 Me_2SO-d_6–D_2O): δ 0.89 (t, 3 H, *J* 6.2 Hz, CH_2CH_3), 1.23–1.33 (m, 32 H, $(CH_2)_{16}CH_3$), 1.52 (m, 2 H, CH_2CH_2CONH), 2.10 (t, 2 H, *J* 7.6 Hz, CH_2CH_2CONH), 4.25 (d, 1 H, *J* 7.8 Hz, H-1), 4.26 (d, 1 H, *J* 7.6 Hz, H-1′); MALDIMS: Calcd for $C_{34}H_{65}NNaO_{12}$: $(M + Na)^+$, 702.44; found: $(M + Na)^+$, 702.45.

N-*Hexadecanoylaminoethyl β-thiolactoside* (**20**).—To a solution of 2 mmol of **5** and 2 mmol of **6** in 40 mL of CH_2Cl_2 was added molecular sieves 4A, and the mixture was stirred for 3 h. The reaction mixture was worked up as described for the general synthesis of **16–19**, followed by removal of the Fmoc group and direct *N*-acylation with hexadecanoyl chloride (2 mmol). After the deacetylation in NaOMe–MeOH and purification by silica gel chromatography, decolorization on charcoal was carried out in EtOH to afford **20** (0.342 g, 26% from **6**): ^{1}H NMR (60 °C, 95:5 Me_2SO-d_6–D_2O): δ 0.89 (t, 3 H, *J* 6.8 Hz, CH_2CH_3), 1.28–1.34 (m, 24 H, $(CH_2)_{12}CH_3$), 1.52 (m, 2 H,

CH_2CH_2CONH), 2.09 (t, 2 H, J 7.3 Hz, CH_2CH_2CONH), 4.37 (d, 1 H, J 9.7 Hz, H-1), 4.27 (d, 1 H, J 7.6 Hz, H-1′); MALDIMS: Calcd for $C_{30}H_{57}NNaO_{11}S$: $(M + Na)^-$, 662.36; found: $(M + Na)^+$, 662.18.

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