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A bi-fluorescence-labeled substrate for ceramide glycanase based on fluorescence energy transfer ☆

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Abstract

An alkyl lactoside containing two different fluorescence probes as an energy donor and an energy acceptor was synthesized as a substrate for ceramide glycanase. *n*-Pentenyl β-lactoside was converted into its 4′,6′-*O*-(2-naphthylmethylidene) derivative with subsequent benzoylation of all remaining OH groups. The fully protected lactoside was treated with borane–trimethylamine complex and aluminum chloride in tetrahydrofuran [P.J. Garegg, *Pure Appl. Chem.*, 56 (1984) 845–858] for selective opening of the 4′,6′-acetal group to give the 6′-*O*-(2-naphthylmethyl) derivative in high yield. After *O*-debenzoylation, the ω-alkenyl group at the reducing end was extended by Michael addition with $HS(CH_2)_2NH_2 \cdot HCl$ to provide an amino group at the terminal position. The amino group was then dansylated to give the target lactoside, which has two different fluorescent probes at each end. Excitation at 290 nm (of the 2-naphthyl group) of the bi-fluorescence-labeled lactoside showed emissions at 335 nm (2-naphthyl) and at 540 nm (dansyl). The distance between the naphthyl group and the dansyl group was estimated to be 12 Å by the Förster relationship. Digestion of this lactoside with American leech (*Macrobdella decora*) ceramide glycanase [B. Zhou et al., *J. Biol. Chem.*, 264 (1989) 12,272–12,277] resulted in an increase in the naphthyl emission with a concomitant decrease in the dansyl emission. These changes can be used for continuous monitoring of the ceramide glycanase activity.

Keywords: Bi-fluorescence-labeled lactoside; Fluorescence energy transfer; Ceramide glycanase

☆ For a preliminary account of this work see ref. [1].

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1. Introduction

The value of resonance energy transfer using a suitable pair of fluorescent probes for measurement of distances in biomolecules was promoted by Stryer and Haugland [2]. The efficiency of intermolecular resonance energy-transfer is strongly dependent on both the distance between donor and acceptor, and the spectral overlap of the donor emission spectrum with the absorption of the acceptor. Fluorescence energy transfer, most suitable for the distance range of 10–60 Å, has been applied to distance measurements in proteins, oligonucleotides, and lipids [3,4]. Recently Rice and co-workers [5–7] have utilized fluorescence energy transfer to determine solution conformations of the complex-type glycopeptides.

Energy transfer can be also used for enzyme assays. Fluorescently labeled substrates for proteases have been used to study the kinetics of renin by some workers [8–10]. The substrates used were octa- to deca-peptide labeled, for example, with 5-[(2-aminoethyl)amino]naphthalenesulfonic acid group (EDANS) at the *C*-terminus and with 4-{[4-(dimethylamino)phenyl]azo}benzoyl group (DABCYL) at the *N*-terminus. When these groups co-exist on a peptide, the DABCYL group effectively quenches the fluorescence emission of the EDANS group. As the enzyme cleaves the substrate, this quenching is removed and the emission by the EDANS group is restored. These substrates have proved to be very useful for the assay of endo-peptidases.

Ceramide glycanase (CGase) is an enzyme which cleaves the glycosidic linkage between oligosaccharide and ceramide in ceramide glycosides (glycoshingolipids; GSLs). This enzyme has been useful in structural analysis of GSLs, modification of the cell surface of glycolipids, and construction of neoglycolipids [11]. A number of ceramide glycanases have been isolated from the European leech *Hirudo medicinalis* [12], the American leech *Macrobdella decora* [11,13], earthworn *Lumbricus terrestris* [14], *Rhodococcus* sp. [15,16], and *Corynebacterium* sp [17]. The American leech CGase can cleave a wide variety of GSLs as well as a number of synthetic β-lactosides [13]. Zhou et al. [13] found that CGase require the presence of a detergent such as sodium cholate for effective hydrolysis of GSL. The enzyme is stable between pH 4.5 and 8.5, and the optimum pH of the enzyme was 5.0 when G_{M1} was used as substrate, for which the K_M was estimated to be 15.4 μM. Unlike the assays for the exo-glycosidases, for which chromogenic or fluorogenic substrates are available, the assay for CGases (and other endo-type carbohydrases) is by means of separation of the products followed by quantification [13,16]. For example, the typical assay requires 0.2 unit of the enzyme and 30 nmol of GSL, and is not convenient for a large number of samples nor is it amenable to continuous monitoring of the enzyme activity.

Although the same principle of energy transfer used for endo-peptidases should be applicable to the assays of endo-glycosidases, glyco-amidases, or ceramide glycanases, to our knowledge only our substrate has been reported as a preliminary communication [1]. In this paper, we describe the synthesis of the bi-fluorescence-labeled lactoside and its use for kinetic studies of the leech ceramide glycanase. The lactoside **1** (Fig. 1) has a hydrophobic aglycon which is required for successful cleavage by CGase [13].

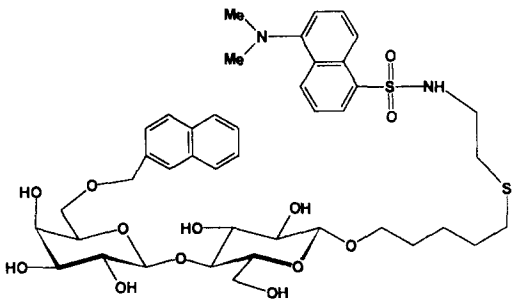

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Fig. 1. The structure of NLD, 5-[2-(5-dimethylamino-1-naphthalenesulfonamido)ethanethio]pentyl [6-*O*-(2-naphthylmethyl)-β-D-galactopyranosyl]-(1 → 4)-β-D-glucopyranoside (**1**).

2. Experimental

General methods.—Unless otherwise stated, all commercially available solvents and reagents were used without further purification. Chloroform, *N*,*N*-dimethylformamide (DMF), tetrahydrofuran (THF), and MeOH were stored over molecular sieves 3 Å (MS 3 Å) before use. Pyridine and Et_3N were stored over NaOH pellets. Pulverized MS 4 Å was dried in vacuo at ca. 100°C overnight before use.

Melting points (uncorrected) were determined with a Fisher–Johns apparatus. ^1H NMR and proton-decoupled carbon NMR spectra were recorded at 300 and 75.5 MHz respectively with a Bruker AMX-300 spectrometer in chloroform-*d* or methyl-d_3 alcohol-*d*, using Me_4Si or methanol (3.3 ppm for ^1H or 49.0 ppm for ^{13}C) as internal standards. Assignment of the ring-protons was made by first-order analysis of the spectra, and were confirmed by homonuclear decoupling experiments. Samples were dried (ca. 24 h) in vacuo (50°C, 0.1 Torr) over NaOH pellets before elemental analyses (Galbraith Lab., Inc., Knoxville, TN).

Synthetic and hydrolytic reactions were monitored by thin-layer chromatography (TLC) on precoated plates of silica gel 60 F_{254} (layer thickness, 0.25 mm; E. Merck, Darmstadt). The solvent systems used were (A) 5:1 (v/v) toluene–EtOAe, (B) 9:1, (C) 5:1 (v/v) $CHCl_3$–MeOH, (D) 65:25:4 (v/v/v) $CHCl_3$–MeOH–water, (E) 60:35:8 (v/v/v) $CHCl_3$–MeOH–0.02% aq $CaCl_2$. For detection of the components, TLC plates were sprayed with a solution of 85:10:5 (v/v/v) MeOH–concd H_2SO_4–*p*-anisaldehyde, and heated for a few minutes (for carbohydrate) or sprayed with an aqueous solution of 5% (w/w) $KMnO_4$ and heated similarly (for double bond). Visualization of aldehydic components was by heating the plate sprayed with a 2,4-dinitrophenylhydrazine (DNPH) solution which was made by dissolving 0.4 g of DNPH in 100 mL of 2 M HCl and 100 mL of 95% EtOH. Amino groups were visualized by heating the plates sprayed with 0.2% ninhydrin in 95% EtOH. Column chromatography was performed on silica gel (Silica Gel 60; 0.015–0.040 mm, E. Merck). A Cool White fluorescent lamp (A 4 W F4T5/CW) was used to promote in the Michael addition.

n-Pentenyl β-lactoside (**3**) was prepared by the method previously reported [18]. 2-Naphthaldehyde, borane–trimethylamine complex, sodium cholate, and dansyl chloride were purchased from Aldrich Chemical Co. (Milwaukee, WI). G_{M1} as a standard solution of 5 mg/mL in 2:1 (v/v) $CHCl_3$–MeOH was purchased from Matreya Inc. (Pleasant Gap, PA). Ceramide glycanase (CGase) from American leech (*Macrobdella decora*) was purchased from V-Labs, Inc. (Covington, LA). The CGase as supplied usually contains 0.29 units/10 mL and 21.8 mg protein/10 mL. One unit of CGase is defined as the amount of the enzyme which hydrolyzes 1 nmol of G_{M1} per min at pH 5.0, 37°C. The stock sodium cholate solution (1.0 g/10.0 mL) was prepared in deionized water. A 1 mM methanolic solution of the 2-naphthylmethylated lactoside with ω-dansyl aglycon (NLD) was prepared and stored at −15°C in the dark. Fluorescence measurements were carried out with a Perkin–Elmer luminescence spectrometer LS50B (Perkin–Elmer Corp., Rockville, MD), and the data were processed with an OBEY fluorescence data manager software (Perkin–Elmer).

2-Naphthylaldehyde di-(i-butyl) acetal (**2**).—To a solution of 2-naphthaldehyde (2.00 g, 12.8 mmol) in 2-methylpropanol (20 mL) containing powdered Drierite (3.0 g) was added 10-camphorsulfonic acid (CSA) (3.0 mg, 12.8 μmol) with stirring, and the mixture was continuously stirred for 2 days at room temperature. The mixture was diluted with $CHCl_3$ (50 mL) and filtered. The filtrate was washed with cold saturated $NaHCO_3$ and brine, and then dried (Na_2SO_4). The $CHCl_3$ solution was concentrated in vacuo to give syrupy **2** (3.29 g, 89.6%) containing a trace of starting material: R_f 0.81 (solvent toluene), and the compound was used for the next step without further purification.

n-*Pentenyl [2,3-di-*O*-benzoyl-4,6-*O*-(2-naphthylmethylidene)-β-*D*-galactopyranosyl]-(1 → 4)-2,3,6-tri-*O*-benzoyl-β-*D*-glucopyranoside* (**4**).—To a solution of *n*-pentenyl (β-D-galactopyranosyl)-(1 → 4)-β-D-glucopyranoside (**3**) (2.04 g, 4.97 mmol) and **2** (2.85 g, 9.94 mmol) in dry DMF (20 mL) at 0°C was added CSA (0.93 g, 4.00 mmol), and the mixture was heated for 2 h at 55°C while removing the liberated 2-methylpropanol with a water aspirator. The mixture was neutralized with Et_3N (0.56 mL, 4.02 mmol) and evaporated in vacuo to remove DMF. The residual syrup was fractionated on a column of Sephadex LH-20 (5 × 200 cm) using 95% EtOH as eluant. The fractions containing both carbohydrate and aldehyde as analyzed by TLC were concentrated. The syrup was dissolved in pyridine (30 mL), to which BzCl (5.83 mL, 49.7 mmol) was added with stirring at 0°C. After 3 h at room temperature, MeOH (0.5 mL) was added dropwise to the mixture, and the mixture was diluted with $CHCl_3$, and the solution was successively washed with cold 0.5 M H_2SO_4, cold satd $NaHCO_3$, and brine. The extract was dried (Na_2SO_4) and evaporated. The residue was chromatographed on a column of silica gel, eluted first with 20:1 then with 10:1 (v/v) toluene–EtOAc to give pure **4** (2.90 g, 54.6%): R_f 0.45 (solvent A); mp 243–245°C (from MeOH); ^{1}H NMR δ ($CDCl_3$) 1.53 (m, 2 H, CH_2CH=), 2.35 (m, 2 H, OCH_2CH_2), 3.02 (s, 1 H, H-5′), 3.6 (m, 2 H, OCH_2), 3.63 (dd, 1 H, $J_{6'a,6'b}$ 10.7 Hz, H-6′a), 3.8 (m, 2 H, H-5, 6′b), 4.23 (t, 1 H, $J_{4,5}$ 9.0 Hz, H-4), 4.37 (d, 1 H, $J_{4',5'}$ ~ 0 Hz, H-4′), 4.44 (dd, 1 H, $J_{5,6a}$ 4.2 Hz and $J_{6a,6b}$ 12.6 Hz, H-6a), 4.62 (dd, 1 H, $J_{5,6b}$ 2.0 Hz, H-6b), 4.67 (d, 1 H, $J_{1,2}$ 7.8 Hz, H-1), 4.76 (m, 2 H, CH_2=CH), 4.86 (d, 1 H, $J_{1',2'}$ 7.9 Hz, H-1′), 5.20 (dd, 1 H, $J_{3',4'}$ 3.6 Hz, H-3′), 5.33 (dd, 1 H, $J_{2,3}$ 9.5 Hz, H-2), 5.44 [s, 1 H, CH-(2-naphthyl)], 5.57 (m, 1 H,

C*H*=CH_2), 5.83 (dd, 1 H, $J_{2',3'}$ 10.4 Hz, H-2'), 5.86 (t, 1 H, $J_{3,4}$ 9.5 Hz, H-3), 7.59 (m, 32 H, 5 Bz, 2-naphthyl). Anal. Calcd for $C_{63}H_{56}O_{16} \cdot H_2O$: C, 69.61; H, 5.37. Found C, 69.74; H, 5.55.

A small amount of perbenzoylated *n*-pentenyl lactoside was also obtained as a byproduct: R_f 0.59 (solvent A); ^{1}H NMR δ ($CDCl_3$) 1.57 (m, 2 H, CH_2CH=), 1.93 (m, 2 H, OCH_2CH_2), 3.64 (m, 2 H, OCH_2), 3.69 (dd, 1 H, $J_{6'a,6'b}$ 11.3 Hz, H-6'a), 3.75 (dd, 1 H, 6'b), 3.90 (t, 1 H, $J_{5',6'a}$ 6.9, $J_{5',6'b}$ 6.5 Hz, H-5'), 3.9 (m, 1 H, H-5), 4.27 (t, 1 H, $J_{4,5}$ 9.6 Hz, H-4), 4.50 (dd, 1 H, $J_{5,6a}$ 4.2, $J_{6a,6b}$ 12.1 Hz, H-6a), 4.61 (dd, 1 H, $J_{5,6b} < 1$ Hz, H-6b), 4.69 (d, 1 H, $J_{1,2}$ 7.9 Hz, H-1), 4.77 (m, 2 H, CH_2=CH), 4.89 (d, 1 H, $J_{1',2'}$ 7.9 Hz, H-1'), 5.38 (dd, 1 H, $J_{3',4'}$ 3.4 Hz, H-3'), 5.48 (dd, 1 H, $J_{2,3}$ 9.8 Hz, H-2), 5.60 (m, 1 H, C*H*=CH_2), 5.74 (dd, 1 H, $J_{2',3'}$ 10.3 Hz, H-2'), 5.74 (m, 1 H, H-4'), 5.82 (t, 1 H, $J_{3,4}$ 9.3 Hz, H-3), 7.61 (m, 35 H, 7 Bz).

n-*Pentenyl [2,3-di*-O-*benzoyl-6*-O-*(2-naphthylmethyl)-β*-D-*galactopyranosyl](1 → 4)-2,3,6-tri*-O-*benzoyl-β*-D-*glucopyranoside* (**5**).—A mixture of **4** (703 mg, 0.66 mmol), powdered MS 4 Å (1.5 g), and borane–Me_3N complex (346 mg, 4.61 mmol) in 10 mL of dry THF was stirred for 40 min at room temperature. Anhydrous $AlCl_3$ (614 mg, 4.61 mmol) was added with stirring, and stirring was continued for 11 h at room temperature. When TLC showed complete disappearance of the acetal, the mixture was filtered, and the powdered MS 4 Å was thoroughly washed with $CHCl_3$. The filtrate was successively washed with cold 0.5 M H_2SO_4, cold satd $NaHCO_3$, and brine, dried (Na_2SO_4) and concentrated. The syrup was separated on a column of silica gel eluting with 10:1 (v/v) toluene–EtOAc to give pure **5** (668 mg, 94.7%): R_f 0.42 (solvent A); mp 171–173°C (from MeOH); ^{1}H NMR δ ($CDCl_3$) 1.52 (m, 2 H, CH_2CH=), 1.87 (m, 2 H, OCH_2CH_2), 2.26 (d, 1 H, OH-4'), 3.05 (m, 2 H, H-6'a, 6'b), 3.46 (t, 1 H, $J_{5,6'}$ 5.9 and 6.2 Hz, H-5'), 3.58 (m, 2 H, OCH_2), 3.78 (m, 1 H, H-5), 4.16 (t, 1 H, $J_{4,5}$ 9.2 Hz, H-4), 4.19 (br t, 1 H, $J_{4',OH}$ 2.3 Hz, H-4'), 4.38 [m, 2 H, OCH_2-(2-naphthyl)], 4.39 (dd, 1 H, $J_{5,6a}$ 4.6, $J_{6a,6b}$ 11.8 Hz, H-6a), 4.57 (dd, 1 H, $J_{5,6b} \sim 1$ Hz, H-6b), 4.62 (d, 1 H, $J_{1,2}$ 7.8 Hz, H-1), 4.74 (m, 2 H, CH_2=CH), 4.77 (d, 1 H, $J_{1',2'}$ 7.8 Hz, H-1'), 5.11 (dd, 1 H, $J_{3',4'}$ 3.1 Hz, H-3'), 5.37 (dd, 1 H, $J_{2,3}$ 9.5 Hz, H-2), 5.56 (m, 1 H, C*H*=CH_2), 5.69 (dd, 1 H, $J_{2',3'}$ 10.3 Hz, H-2'), 5.71 (t, 1 H, $J_{3,4}$ 9.0 Hz, H-3), 7.58 (m, 32 H, 5 Bz, 2-naphthyl). Anal. Calcd for $C_{63}H_{58}O_{16}$: C, 70.65; H, 5.45. Found C, 70.76; H, 5.65.

A small amount of **5** (22 mg, 0.2 mmol) was *O*-acetylated by the usual method with pyridine–Ac_2O to give the pure monoacetate **6**: R_f 0.57 (solvent A); ^{1}H NMR ($CDCl_3$) 1.52 (m, 2 H, CH_2CH=), 1.78 (s, 3 H, Ac), 1.87 (m, 2 H, OCH_2CH_2), 2.78 (t, 1 H, $J_{5',6'}$ 8.1, $J_{6'a,6'b}$ 9.1 Hz, H-6'a), 2.97 (dd, 1 H, $J_{5',6'b}$ 5.2 Hz, H-6'b), 3.51 (m, 2 H, OCH_2), 3.61 (dd, 1 H, H-5'), 3.77 (m, 1 H, H-5), 4.18 (t, 1 H, $J_{4,5}$ 9.6 Hz, H-4), 4.36 [m, 2 H, OCH_2-(2-naphthyl)], 4.38 (dd, 1 H, $J_{5,6a}$ 4.4, $J_{6a,6b}$ 12.1 Hz, H-6a), 4.56 (dd, 1 H, $J_{5,6b} < 1$ Hz, H-6b), 4.62 (d, 1 H, $J_{1,2}$ 7.8 Hz, H-1), 4.76 (d, 1 H, $J_{1',2'}$ 7.8 Hz, H-1'), 4.77 (m, 2 H, CH_2=CH), 5.22 (dd, 1 H, $J_{3',4'}$ 3.3 Hz, H-3'), 5.38 (dd, 1 H, $J_{2,3}$ 9.6 Hz, H-2), 5.55 (m, 1 H, C*H*=CH_2), 5.53 (d, 1 H, $J_{4',5'} \sim 0$ Hz, H-4'), 5.54 (dd, 1 H, $J_{2',3'}$ 10.4 Hz, H-2'), 5.69 (t, 1 H, $J_{3,4}$ 9.0 Hz, H-3), 7.56 (m, 32 H, 5 Bz, 2-naphthyl).

n-*Pentenyl [6*-O-*(2-naphthylmethyl)-β*-D-*galactopyranosyl]-(1 → 4)-β*-D-*glucopyranoside* (**7**).—To a solution of **5** (667.6 mg, 0.623 mmol) in dry methanol (5 mL) and dry THF (5 mL) was added NaOMe (20.2 mg, 0.374 mmol), and the solution was stirred for 64 h at room temperature. The mixture was neutralized by adding Dowex

50W X-8 (H^+) resin. The suspension was filtered, and the filtrate was evaporated in vacuo. The residue was purified by passing through a column of Sephadex LH-20 (2.5 × 50 cm), eluting with 95% EtOH, to give homogeneous **7** (260.8 mg, 76.0%): R_f 0.18 (solvent B); ^{1}H NMR δ (CD_3OD) 1.66 (m, 2 H, CH_2CH=), 2.10 (m, 2 H, OCH$_2$CH_2), 3.25 (m, 1 H, H-2′), 3.51 (m, 1 H, H-3′), 3.53 (m, 1 H, H-2), 4.24 (d, 1 H, $J_{1',2'}$ 7.8 Hz, H-1′), 4.32 (d, 1 H, $J_{1,2}$ 7.4 Hz, H-1), 4.68 [m, 2 H, CH_2-(2-naphthyl)], 4.95 (m, 2 H, CH_2=CH), 5.79 (m, 1 H, CH=CH_2), 7.62 (m, 10 H, 2-naphthyl); ^{13}C NMR δ (CD_3OD) 30.06 (OCH$_2$CH_2), 31.21 (CH_2CH=), 62,05 (C-6), 70.24, 70.37, 70.55, 72.38, 74.48, 74.68, 74.79, 75.30, 76.27, 76.45, 81.53 [C-2, 3, 4, 5, 2′, 3′, 4′, 5′, 6′, OCH_2CH$_2$, CH_2-(2-naphthyl)], 104.22 (C-1), 105.19 (C-1′), 115.19 (CH_2=CH), 126.92, 127.06, 127.10, 127.73, 128.64, 128.98, 129.11, 134.49, 134.74, 136.95 (10 C, attributable to 2-naphthyl), 139.47 (CH=CH_2).

*5-(2-Aminoethanethio)pentyl [6-O-(2-naphthylmethyl)-β-*D*-galactopyranosyl](1 → 4)-β-*D*-glucopyranoside hydrochloride salt* (**8**).—A solution of **7** (40.4 mg, 73.4 μmol) in dry MeOH (0.5 mL) was allowed to react with 2-aminoethanethiol hydrochloride (85.1 mg, 0.734 mmol) under UV illumination for 3 days at room temperature. The mixture was evaporated in vacuo, and the residue was dissolved in a small amount of water. The aqueous solution was fractionated on a column of Sephadex G-10 (1.5 × 14 cm), first eluted with water then with 0.5 M aq AcOH, to give homogeneous **8** (32.1 mg, 65.9%): R_f 0.13 (solvent D).

*5-[2-(5-Dimethylamino-1-naphthalenesulfonamido)ethanethio]pentyl [6-O-(2-naphthylmethyl)-β-*D*-galactopyranosyl]-(1 → 4)-β-*D*-glucopyranoside* (**1**; NLD).—To a solution of **8** (32.1 mg, 48.3 μmol) and dry Et_3N (13.4 μL, 96.7 μmol) in dry MeOH (2.0 mL) was added dansyl chloride (dansyl, 5-dimethylamino-1-naphthalenesulfonamido) (52.1 mg, 193 μmol) at room temperature. After 30 min, the mixture was directly applied to a column of Sephadex LH-20 (2.5 × 50 cm) eluted with 95% EtOH to give crude **1** (37.8 mg, 90.9%). An analytical sample was chromatographed on silica gel with 10:1 (v/v) $CHCl_3$–MeOH as eluent to give pure **1**: R_f 0.33 (solvent C); ^{1}H NMR δ (CD_3OD) 1.36 (m, 4 H, 2 CH_2), 1.55 (m, 2 H, OCH$_2$CH_2), 2.26 (m, 2 H, CH_2SCH$_2$CH$_2$NH), 2.39 (m, 2 H, SCH_2CH$_2$NH), 2.98 (m, 2 H, CH_2NH), 4.28 (d, 1 H, $J_{1',2'}$ 7.8 Hz, H-1′), 4.37 (d, 1 H, $J_{1,2}$ 7.3 Hz, H-1), 4.73 [m, 2 H, OCH$_2$-(2-naphthyl)], 7.25–8.57 (m, 13 H, dansyl, 2-naphthyl).

Hydrolysis of NLD (**1**) *with ceramide glycanase.*—The enzymatic reaction conditions were based on the published method reported in ref. [13]. A solution of NLD (30 nmol) and a solution of sodium cholate (2.0 mL, 200 mg) was placed in a 1.5-mL screw-capped vial (USA/Scientific Plastics, Ocala, FL), and the solution was evaporated to dryness in vacuo with a SpeedVac (Savant Instruments, Inc., NY). To the dried residue, 200 μL of 50 mM NaOAc buffer, pH 5.0, was added, and the mixture was ultrasonicated for 1 min. The enzyme (10 μL, 0.29 U) was added to the vial, and the vial was shaken in a water bath at 37°C in the dark. After incubation at 37°C for 17 h, a portion of the mixture was directly analyzed by TLC using solvents (C), (D), or (E). The components were detected either by UV illumination or by charring.

Continuous assay of ceramide glycanase with NLD by intramolecular fluorescence quenching.—Measurement of fluorescence emission spectra and excitation spectra was carried out at ambient temperature, in a Teflon-stoppered cuvette (12.5 mm wide × 45

mm high) containing a 3.0-mL sample. The slit width of the excitation and the emission was 5.0 nm, and the scan speed was 500 nm/min.

An enzymatic reaction is carried out at room temperature as follows: appropriate amounts of 1 mM NLD solution and the sodium cholate solution in a screw-capped vial was evaporated to dryness. To the vial was added 4.0 mL of 50 mM NaOAc buffer, pH 5.0, and the vial was sonicated for 1 min. A 3.0 mL portion was pipeted into a Teflon-stoppered cuvette, and the enzyme (0.57 U) was added and quickly mixed by inversion. The initial spectrum as 0 min was taken immediately after the enzyme was added. The quantification of NLD by measurement of dansyl emission was estimated by subtracting the dansyl emission (540 nm) at 420 min from the value at 0 min. The amount of NLD hydrolyzed during this period was regarded to be equal to the 6′-(2-naphthylmethyl)-lactose (NL) formed in the same reaction period. Linear proportionation of the decrease of the dansyl emission yielded the quantity of the hydrolyzed NLD. These emission spectra showed an isoemissive point at 437 nm.

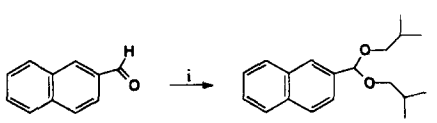
3. Results and discussion

Synthesis of bi-fluorescence-labeled lactoside.—*n*-Pentenyl glycosides are versatile intermediates in carbohydrate chemistry [19]. They can be used as glycosylating agents in different ways, and the ω-alkenyl group in their aglycon is amenable to modification such as oxidation (producing an aldehyde), gemhydroxylation (for improved water solubility), and Michael addition (for extension of the aglycon chain). In the present work, *n*-pentenyl β-lactoside was modified with 2-naphthaldehyde at the galactosyl residue, and the *n*-pentenyl group was extended by Michael addition followed by dansylation.

(A) n-*Pentyl β-4′,6′-O-(2-naphthylmethylidene)-lactoside.*—2-Naphthaldehyde was readily converted to the acetal derivative **2** in an exellent yield in the presence of CSA, 2-methylpropanol and powdered Drierite as dehydrating agent (Scheme 1).

When the *n*-pentenyl lactoside was treated with 2-naphthaldehyde di(isobutyl) acetal (**2**) with continual removal of 2-methylpropanol produced (Scheme 2) in the presence of CSA as an acid catalyst, the acetal exchange reaction proceeded smoothly. The cyclic acetal derived from the lactoside was purified on a Sephadex LH-20 column, and the acetal was benzoylated to give **4** in 55% yield.

(B) n-*Pentenyl 6′-O-(2-naphthylmethyl)-β-lactoside.*—The regioselective ring-opening reaction of a cyclic acetal reported by Garegg [20] requires that the substituent



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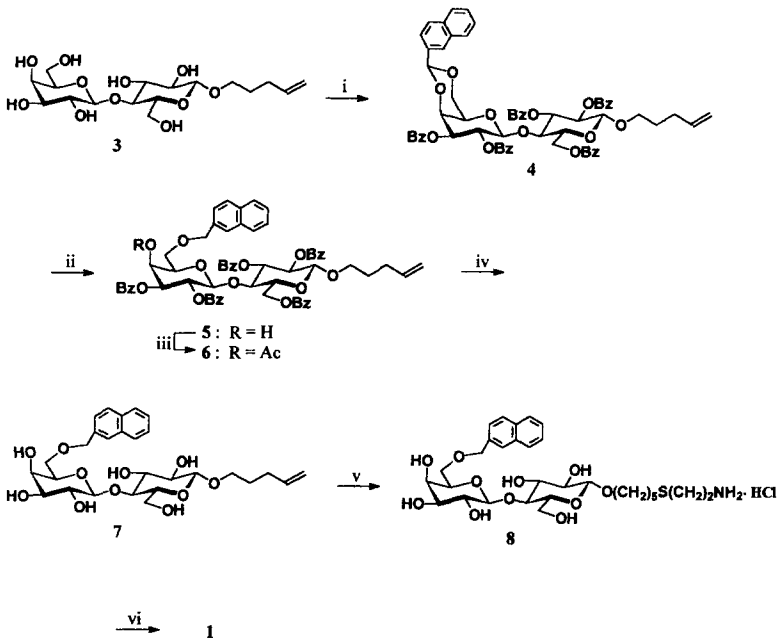
Scheme 1. Reagents and conditions: i, 2-methylpropanol, powdered Drierite, 10-camphorsulfonic acid, room temperature, 2 days.



Scheme 2. Reagents and conditions: ia, **2**, 10-camphorsulfonic acid, DMF, reduced pressure, 55°C, 2 h,; ib, benzoyl chloride, pyridine, 0°C to room temperature, 3 h; ii, borane–Et_3N complex, powdered MS 4 Å, aluminum chloride, THF, room temperature, 11 h; iii, Ac_2O, pyridine, room temperature; iv, sodium methoxide, MeOH; v, $HSCH_2CH_2NH_2 \cdot HCl$, MeOH, UV irradiation, room temperature, 3 days; vi, Et_3N, dansyl chloride, MeOH, room temperature, 30 min.

adjacent to the cyclic acetal to be a bulky group (e.g., benzyl, benzoyl, or a sugar). Since the 2-naphthylmethyl group is susceptible to hydrogenolysis, the benzyl group is excluded as the neighboring substituent. The benzoyl group, however, meets our requirement, and yielded the desired product successfully.

When the reductive opening of a cyclic acetal was carried out with only a small excess of the reagents, the reaction is sometimes incomplete. Therefore reductive cleavage of the cyclic acetal (**4**) was carried out with 7-mol equivalent each of borane–trimethylamine complex and $AlCl_3$ in the presence of dried powdered MS 4 Å in THF at room temperature. After 11 h, the reaction was complete as judged by TLC, and the 2-naphthylmethylated lactoside **5** was obtained in 95% yield. The structure was confirmed by the ^1H NMR spectrum of the acetylated **6** (only a single acetate was found). The lactosyl derivative was *O*-debenzoylated by the Zemplén method to provide **7**, which showed fluorescence emission at 335 nm for the 2-naphthyl group (excited at 290 nm) in 50 mM sodium acetate buffer, pH 5.0, containing 200 mg/3.0 mL of sodium cholate at ambient temperature.

(C) *Synthesis of the bi-fluorescence labeled lactoside.*—The thiol group of cysteamine has been successfully added to a C=C double bond previously [21,22], providing a thioether in an anti-Markovnikov manner. Using an analogous reaction, the lactosyl derivative **7** was extended with cysteamine by the Michael addition to the

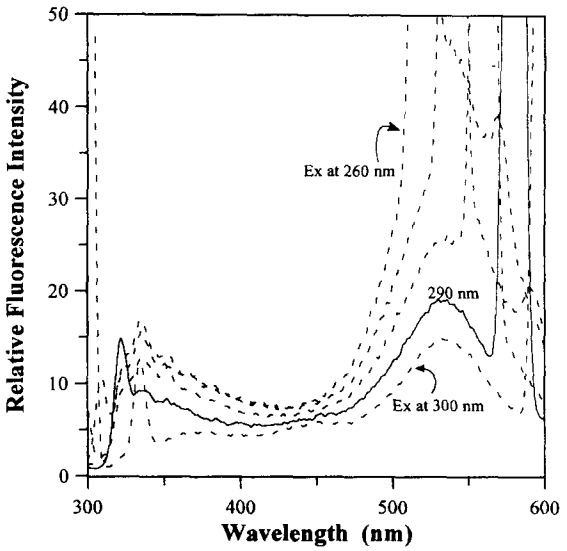


Fig. 2. Dependence of excitation wavelength of NLD on the emission spectra. The conditions were as follows: ambient temperature, 3.0 mL of 50 mM NaOAc, pH 5.0, containing 200 mg of sodium cholate. Slit size of 5.0 nm was used for both the excitation and emission. The spectra were scanned at 500 nm/min. The curves decreased most at ~ 540 nm as the excitation wavelength was increased from 260 to 300 nm stepwise by 10 nm.

ω-alkenyl group to afford **8** in 66% yield. Dansylation of the amino group was carried out in dry methanol at room temperature to give **1** in 91% yield.

Physical properties of NLD.—UV-absorption spectrum of NLD showed the presence of peaks at 260 and 335 nm, as expected from the presence of the 2-naphthyl group and the dansyl group. The NLD having bi-fluorescence probes at the each terminal was tested as a function of excitation wavelength from 260 to 300 nm and the emission spectra of 5 μM solution of NLD are shown in Fig. 2. These emission spectra included two peaks attributable to 2-naphthyl group at around 335 nm and dansyl group at 540 nm because the fluorescence energy transfer was observed from 2-naphthyl as the energy donor to dansyl as the acceptor. Although the relative fluorescence intensity of emission peaks excited at 260 nm was greater than all others (Fig. 2), a double scatterd peak of the excitation wavelength strongly (around 520 nm) obscured this emission peak. We tentatively selected 290 nm as the appropriate excitation wavelength for further enzymatic reactions.

The energy-transfer efficiency (E) was calculated from the integrated fluorescence intensity when the donor and acceptor are present in the same molecule (F^{DA}) and that obtained when the acceptor is absent (F^{D}):

$$E = 1 - (F^{DA}/F^{D}) \quad (1)$$

Fluorescence spectra showed that for the 2-naphthyl group the excitation maximum is at 260 nm, and the emission maximum at 335 nm. The integrated fluorescence emission

intensity for the 2-naphthyl group in NLD (Naphthyl-Lac-Dansyl; Lac, lactose), **1**, and NLP (2-Naphthylated lactosyl pentenyl glycoside or Naphthyl-Lac-Pent; Pent, $CH_2=CHCH_2CH_2CH_2$), **7**, were calculated from the peak area from 300 to 400 nm. The average distance (r) between the donor–acceptor can be directly related to the transfer efficiency (E) using the Förster equation [23]

$$r = R_0[(1-E)/E]^{1/6} \quad (2)$$

in which R_0 is the Förster distance for a given donor–acceptor pair, at which the efficiency of transfer is 50%. Using the value of 20.8 Å for the Förster distance (R_0) for the 2-naphthyl and dansyl pair [5], the average distance between the naphthyl and the dansyl groups in NLD was estimated to be 12 Å. This was shorter than the value calculated when the glycoside is fully extended.

Digestion of NLD by the CGase.—NLD and G_{M1} were separately incubated with 10 μL of CGase (0.29 U) in 200 μL of 50 mM NaOAc buffer, pH 5.0, at 37°C for 17 h as previously reported [13]. The TLC showed that both G_{M1} and NLD were completely hydrolyzed. The released 2-naphthylmethylated lactose (NL) had an R_f of 0.44, and the other product 5-(2-dansylamidoethanethio)pentanol had R_f 0.94 in the solvent (c). Zhou et al. [13] tested many ceramide glycoside substrates, including *Al*-Lac-Cer (the lactosyl-ceramide in which the primary alcohol (6′-OH) of the galactose residue was

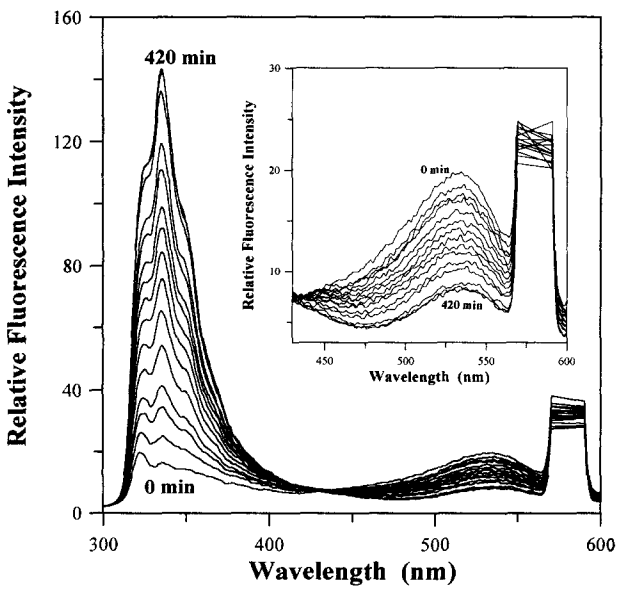


Fig. 3. The time course (0, 10, 20, 28, 45, 60, 75, 90, 105, 120, 150, 180, 270, 360, and 420 min) of the relative fluorescence emission of NLD during hydrolysis with CGase. The reaction conditions were as follows: ambient temperature, 3.0 mL of 50 mM NaOAc, pH 5.0, containing 200 μg of sodium cholate, 5 μM of the NLD (15 nmol/3.0 mL), 0.57 U of the CGase. Slit size of 5.0 nm was used for both the excitation and emission. The spectra were scanned at 500 nm/min exciting at 290 nm for the 2-naphthyl group. The inset shows the expand spectra around 540 nm attributable to dansyl emission.

converted into an aldehyde group). Hydrolysis of *Al*-Lac-Cer was only 5% of Lac-Cer, and they suggested that the galactose residue in the Lac-Cer was necessary to interact with the enzyme. Our results showed that NLD in which the 6′ position of the galactose is substituted by a hydrophobic and bulky 2-naphthylmethyl group could be cleaved by ceramide glycanase as readily as G_{M1}, showing the unimportance of the free 6′-OH group. The failure of *Al*-Lac-Cer to serve as substrate may be interpreted as the requirement of the $-CH_2O-$ group by the enzyme, or the side reactions attributable to the –CHO group (e.g., intramolecular hemiacetal formation) which somehow prevents the enzyme from acting.

Time course of enzymatic reaction using NLD as substrate.—Digestion of NLD with CGase was carried out in a cuvette at ambient temperature using the following conditions: To a freshly prepared solution (3.0 mL) of 10 μM in NLD containing 200 μg of sodium cholate placed in the cuvette was added CGase (20 μL, 0.57 U) and mixed quickly. An emission spectrum was taken immediately after the enzyme addition and this was regarded as the 0 min spectrum. Subsequently, spectra were taken at appropriate intervals (up to 420 min) (Fig. 3).

As expected, the enzymic digestion caused a decrease in the dansyl emission by diminishing the energy transfer from the 2-naphthyl group. It also caused an increase in the 2-naphthyl emission by removing the quenching by dansyl group. The 2-naphthyl emission at 335 nm gradually increased up to 360 min, after which, the reaction tapered off until it had almost stopped at 420 min. The decreases in dansyl emission at 540 nm as the reaction progressed were also measured. Using this time course of enzymatic reactions, enzyme kinetic constants were estimated to be $K_m = 7.7$ μM and $V_{max} = 0.16$ nmol/min, respectively [1].

In conclusion, we have synthesized a bi-fluorescence-labeled lactoside (NLD) and demonstrated its usefulness as a substrate for CGase. NLD provides a number of advantages such as sensitivity, speed, and feasibility of continuous assay.

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