Synthesis and Characterization of a Carbene-Generating Biotinylated Lactosylceramide Analog as a Novel Chromogenic Photoprobe for GM_3 Synthase

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Received December 1, 1995; accepted January 23, 1996

A new biotinylated lactose derivative bearing a nitro-substituted chromogenic diazirine was synthesized. The biotinyl group within the structure enabled the performance of a convenient assay of GM_3 synthase based on avidin–biotin technology, and the K_m values of this biotinylated photoprobe were determined as 40 and 47 μM using bovine brain and rat liver Golgi as enzyme sources, respectively. Furthermore, the sialylation of lactosylceramide, a natural acceptor substrate for GM_3 synthase, was competitively inhibited by this synthetic analog. The reagent could be a useful chromogenic photoprobe for GM_3 synthase.

Key words photoaffinity labeling; ganglioside; sialyltransferase; biotinylated probe; chromogenic diazirine; avidin–biotin system

Cytidine 5′-monophospho-*N*-acetylneuraminic acid (CMP-NANA): lactosylceramide α-2,3-sialyltransferase (GM_3 synthase) has been recognized as an important key enzyme for the biological processing of glycolipids, because it catalyzes the common step in the biosynthesis of nearly all gangliosides.[1] The GM_3 synthase catalyzes the transfer of sialic acid from CMP–sialic acid to the terminal galactose of lactosylceramide (Galβ1-4Glc-ceramide; CDH) to form a ganglioside, GM_3 (NeuAcα2-3Galβ1-4Glc-ceramide).[2] The cDNA cloning of this enzyme is now one of the major research subjects in the field of glycobiology. In aiming for this goal, photoaffinity labeling would be a useful chemical method for the molecular identification of GM_3 synthase protein as well as the defined structural analysis of its active site.[3] However, no useful photoreactive analogs of these substrates have been developed yet. We have already reported that diazirine-based photoaffinity labeling is more promising than the use of conventional aryl azide photoprobes[4] and have thus developed a family of useful diazirines, including nitro-substituted phenyldiazirines.[5] A radioactive derivative of the nitrodiazirine was also prepared for tracer experiments and was applied for the photoaffinity labeling study of sodium channels.[6] As the nitrodiazirine was originally designed for the spectroscopic monitoring of labeled products during HPLC, the chromogenic property of this diazirine is potentially useful for isolating substantial amounts of labeled peptides for the sequence analysis at the final stage of a photolabeling study. We also demonstrated that the use of biotinylated photoprobes would be practical for efficient photoaffinity labeling enabling the specific manipulation of labeled products.[7] The development of biotinylated glycolipid analogs carrying the chromogenic diazirine could provide useful probes for the field of glycobiology. Here we wish to report the synthesis and characterization of a CDH analog having a chromogenic diazirine and biotin as a novel photoreactive substrate for GM_3 synthase.

Results and Discussion

Synthesis The sialyltransferase family has the ability to utilize CMP-NANA as the common donor substrate, but each enzyme differs in the specificity of acceptor sugar residue and the type of linkage formed. A recent study of site-specific mutagenesis suggested that the conserved region between sialyltransferases, the sialyl-motif, appears to participate in the binding of the common donor substrate, CMP–sialic acid.[8] Therefore, the development of photoreactive acceptor analogs may have several advantages over donor derivatives for the identification of respective sialyltransferase subtypes as well as for the structural analysis of unsolved acceptor binding regions. Although the K_m values of GM_3 synthase to naturally occurring CDHs are affected to some extent by the length of ceramide moiety,[9] there are only a few examples of the characterization of synthetic lactose derivatives. Recently, GM_3 synthase was shown to bind to the affinity column, having a CDH from which is deleted one of hydrophobic lipid tails by oxidative cleavage at the olefinic bond of the ceramide moiety.[10] For the ease of synthesis, we chosed biocytin (ε-*N*-biotinyl-L-lysine) to mimic the lipid tail of CDH as well as to allow for the use of avidin–biotin technology. The α-amino group on the lysine part of biocytin is also useful for introducing the photoreactive group by simple acylation reaction, and the long-chain of biotin derivatives is usually required to obtain optimal biotin-binding capabilities.[11] Thus, a β-lactoside derivative **1**, having a hydrophobic tail of nitrodiazirine-attached biocytin, was designed as a biotinylated and chromogenic photoprobe for GM_3 synthase (Fig. 1). The chromogenic nitrodiazirine **2** was introduced *via* its active ester to the α-amino group of biocytin yielding **3**, which was derivatized to its *N*-hydroxysuccinimide ester **4** (Chart 1). Heptaacetyl lactosyl

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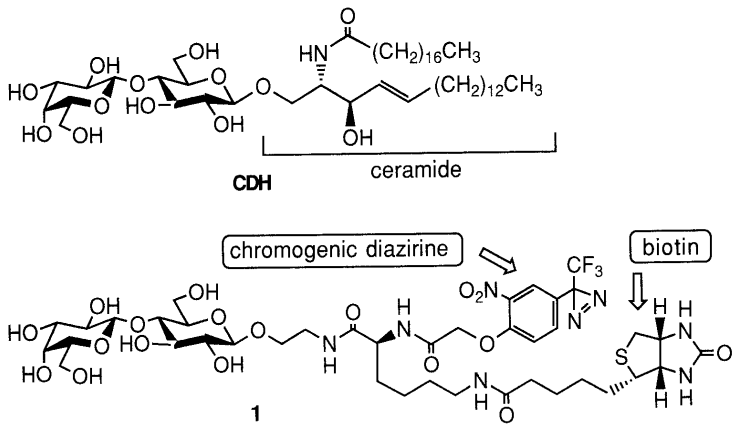

Fig. 1. Structural Feature of CDH and Photoprobe **1**

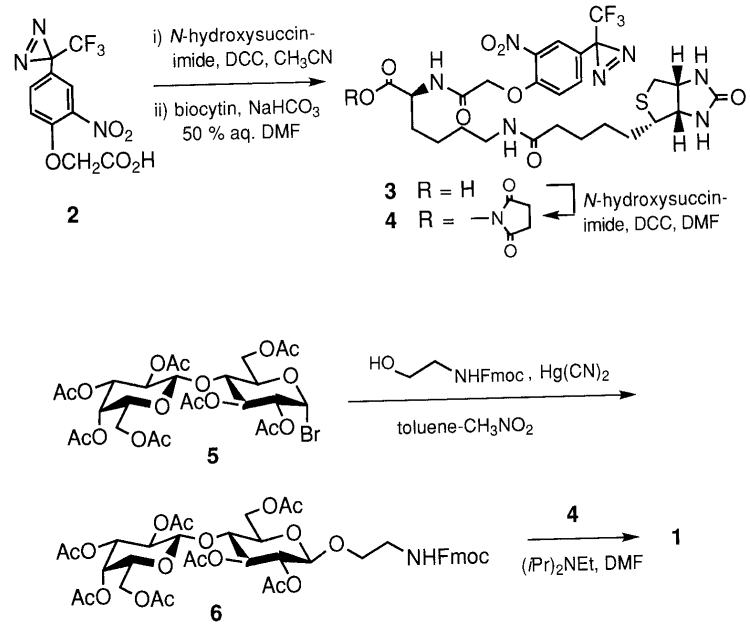

Chart 1

bromide **5** was converted to a β-lactoside derivative **6**. After deprotection of **6**, the photoreactive biocytin **4** was coupled to give the desired photoprobe **1**.

Biological Assay Taking advantage of biotin-possessing substrate **1**, an enzyme assay method based on avidin–biotin technology was examined as a useful alternative to the conventional method using a reversed phase C_{18}-silica gel column.[12] The biotinyl components were easily separated from the assay mixture by using an immobilized streptavidin column. Components trapped to the column can be eluted with 70% formic acid, and the efficiency of elution is similar (1.05 times effective) to the conventional C_{18}-silica gel column method. For the control experiment, an assay using CDH as the acceptor substrate was performed using the C_{18}-silica gel column method. From the analysis of Lineweaver–Burk plots in Fig. 2, the K_m values of compound **1** for the GM_3 synthase from bovine brain and rat liver Golgi were estimated as 40 and 47 μM, respectively. The V_{max} values were also determined to be 35.4 and 41.5 (pmol/mg protein/h), respectively. The observed K_m value for **1** using the bovine brain membrane was better than that of CDH (122 μM), whereas the V_{max} value was lower than that of CDH

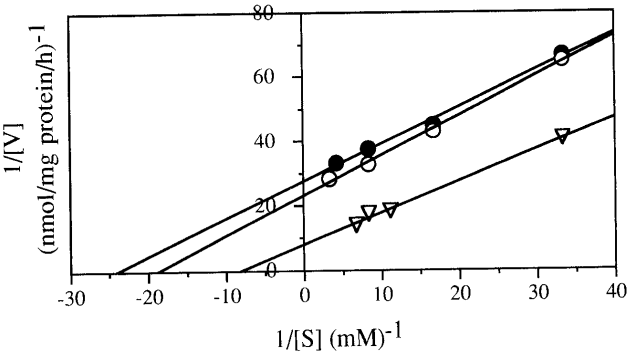

Fig. 2. Lineweaver–Burk Plots for Photoprobe **1** and CDH

The sialyltransferase assay of **1** was carried out at 37 °C using bovine brain total membrane fraction (○) or rat liver (●). The control assay of CDH was performed using the bovine brain membrane (▽).

(125 pmol/mg protein/h). The K_m value of **1** using rat liver Golgi was also better than the reported values for CDH (50—130 μM).[13] Since the photolabeling experiments primarily depend on the binding ability of the reagent to the target molecule, reagent **1** seems to have desirable features as the photoprobe for GM_3 synthase.

Competitive Inhibition of CDH Sialylation and Com-

Table 1. Inhibition of CDH Sialylation with **1**

1 (mM)	**1**:CDH	Rate of sialyltransfer (pmol/mg protein/h)	Inhibition (%)
0	—	116.3	0
0.3	1:1	32.7	72
0.6	1:2	21.1	82

pound 1 The inhibition of sialyltransfer to CDH by the photoprobe was examined in the presence of different concentrations of **1**. The sialylation of CDH was competitively inhibited by incremental increase in the concentration of **1**, as shown in Table 1. An equimolar amount of **1** inhibited more than 70% of sialyl transfer to CDH under the experimental conditions.

In conclusion, our results demonstrate that the photoprobe **1** showed about two to three times higher affinity for GM_3 synthase compared to the natural substrate CDH. The probe was also found to be an effective inhibitor of the sialylation of CDH. The radioisotope labeled analog of this probe can be analogously prepared using ^{14}C-labeled nitrodiazirine if needed.[5b] Because of the relative ease of these experiments, the biotinylated photoprobe **1** would be a useful tool for exploring the detailed molecular aspects of GM_3 synthase.

Experimental

CMP-[^{3}H]-NANA was obtained from DuPont NEN and diluted to the specific activity of 4.2 mCi/mmol with cold CMP-NANA. Immobilized streptavidin was obtained from GIBCO BRL Products. The reagent for the determination of protein concentration was micro BCA Protein Assay Reagent from Pierce. The cellulose for column chromatography was Avicel (Asahi Kasei Kogyo Co., Ltd.). Structural characterization of synthetic products was performed with a Shimadzu UV-160 spectrophotometer (UV spectra), a JEOL JNM GX-400 spectrometer (^{1}H- and ^{13}C-NMR spectra), and a JEOL JMS-HX110 spectrometer (FAB-MS).

***N*^{6}-Biotinyl-*N*^{2}-{2-nitro-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]phenoxy}acetyl-L-lysine (3)** 2-[Nitro-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]phenoxy] acetic acid (**2**, 0.153 g, 0.5 mmol) was converted to its succinimide ester in 0.5 ml of CH_3CN as described previously,[7] and the CH_3CN solution was added to a solution of biocytin (0.1 g, 0.26 mmol) in 4 ml of H_2O–*N*,*N*-dimethylformamide (DMF) (1:1) containing $NaHCO_3$ (0.42 g, 0.5 mmol) at room temperature. After being stirred at room temperature for 1 h, the reaction mixture was concentrated and the aqueous residue was made acidic with HCl. The precipitates were recrystallized from ethanol–ether to afford **3** as colorless needles (0.152 g, 89%). *Anal.* Calcd for $C_{26}H_{32}F_3N_7O_8S \cdot 1/2\ H_2O$: C, 46.70; H, 4.97; N, 14.66. Found: C, 46.71; H, 4.94; N, 14.26. FAB-MS *m/z*: 660 ($M^+ + H$). ^{1}H-NMR (acetone-d_6) δ: 8.25 (d, 1H, *J*=8.2), 7.88 (d, 1H, *J*=2.2), 7.74 (t, 1H, *J*=4.9), 7.64 (dd, 1H, *J*=2.2, 8.8), 7.35 (d, 1H, *J*=8.8), 6.41 (s, 1H), 6.35 (s, 1H), 4.89 (d, 1H, *J*=4.4), 4.83 (d, 1H, *J*=4.4), 4.30 (dd, 1H, *J*=4.9, 6.6), 4.19 (d, 1H, *J*=4.9), 4.15—4.10 (m, 2H), 3.12—3.09 (m, 1H), 2.99 (dd, 1H, *J*=6.6, 12.6), 2.82 (dd, 1H, *J*=4.9, 12.6), 2.55 (t, 2H, *J*=6.0), 2.04 (t, 2H, *J*=7.1), 1.71 (t, 2H, *J*=8.2), 1.60—1.24 (m, 12H).

***N*^{6}-Biotinyl-*N*^{2}-{2-nitro-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]phenoxy}acetyl-L-lysine Succiniimido Ester (4)** The acid **3** (0.132 g, 0.2 mmol) was dissolved in hot DMF (2 ml). To this solution, *N*-hydroxysuccinimide (25 mg, 0.22 mol) and dicyclohexylcarbodiimide (45 mg, 0.22 mol) were added at room temperature and the mixture was stirred at room temperature for 1 h. The mixture was filtered and the filtrate was diluted with ether to precipitate the product which was recrystallized from 2-propanol to afford **4** as a pale yellow solid (0.12 g, 79%). *Anal.* Calcd for $C_{30}H_{35}F_3N_8O_{10}S \cdot 1/2\ H_2O \cdot 1/2$ 2-propanol: C, 47.54; H, 5.07; N, 14.08. Found: C, 47.10; H, 4.93; N, 13.76. UV λ_{max}^{EtOH} nm (ε): 329 (2165). FAB-MS *m/z*: 757 ($M^+ + H$). ^{1}H-NMR (CD_3OD) δ: 7.88 (d, 1H, *J*=2.2), 7.62 (dd, 1H, *J*=2.2, 8.8), 7.42 (d, 1H, *J*=8.8), 4.32 (dd, 1H, *J*=4.9, 6.6), 3.85—3.80 (m, 2H), 3.22—3.20 (m, 3H), 2.99—2.82 (m, 2H), 2.68 (s, 4H), 2.21—2.18 (m, 2H), 1.20—2.00 (m, 12H).

9-Fluorenylmethoxycarbonylaminoethyl 2,3,6-Tri-*O*-acetyl-4-*O*-(2,3,-4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-β-D-glucopyranoside (6) The bromide **5**[14] (0.50 g, 0.7 mmol) and mercuric cyanide (0.18 g, 0.7 mmol) were suspended in distilled toluene (10 ml). To this, 1-*N*-(9-fluorenylmethoxycarbonyl)ethylamine (0.21 g, 0.7 mmol) in distilled nitromethane (20 ml) was added and the reaction mixture was heated at 50 °C for 1.5 h under an atmosphere of nitrogen. The insoluble materials were filtered off, the filtrate was washed with $NaHCO_3$ and brine, and the organic layer was dried over $MgSO_4$. After evaporation, the residual oil was purified by column chromatography on silica gel (ethyl acetate: CH_2Cl_2=1:2) to give **6** as a colorless glass (0.20 g, 31%). *Anal.* Calcd for $C_{43}H_{51}F_3NO_{20} \cdot 1/2\ H_2O$: C, 56.70; H, 5.75; N, 1.54. Found: C, 56.53; H, 5.65; N, 1.32. FAB-MS *m/z*: 902 ($M^+ + H$). ^{1}H-NMR ($CDCl_3$) δ: 7.78—7.30 (m, 8H), 5.35 (d, 1H, *J*=3.4), 5.20 (t, 1H, *J*=9.2), 5.19 (dd, 1H, *J*=7.8, 10.3), 4.96 (dd, 1H, *J*=3.4, 10.3), 4.87 (dd, 1H, *J*=7.8, 9.2), 4.56—4.40 (m, 4H), 4.49 (d, 1H, *J*=7.8), 4.35 (d, 1H, *J*=7.8), 4.21—4.06 (m, 4H), 4.01 (dd, 1H, *J*=5.4, 11.7), 3.87 (m, 1H), 3.75 (m, 2H), 3.67 (m, 1H), 3.35 (m, 2H), 2.16—1.97 (seven s, 21H).

***N*^{6}-Biotinyl-*N*-2-[4-*O*-(β-D-galactopyranosyl)-β-D-glucopyranosyl]-ethyl-*N*^{2}-{2-nitro-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]phenoxy}-acetyl-L-lysineamide (1)** A methanolic solution (4 ml) of compound **6** (23 mg, 0.06 mmol) was saturated with dry ammonia at 0 °C. After evaporation of the solvent, the residue was dissolved in DMF, followed by the addition of **4** (45 mg, 0.06 mmol) and diisopropylethyl-amine (25 μl). After being stirred at room temperature for 1 h, the reaction mixture was concentrated and the residue was purified by cellulose column chromatography (1-propanol: 2.5 M aq. NH_3=8:1) to afford **1** as a colorless glass (21 mg, 33%). UV $\lambda_{max}^{H_2O}$ nm (ε): 320 (1840). FAB-MS (negative) *m/z*: 1025 ($M^- - H$). ^{1}H-NMR (CD_3OD) δ: 7.88 (d, 1H, *J*=2.0), 7.61 (dd, 1H, *J*=2.0, 8.3), 7.41 (d, 1H, *J*=2.0), 4.49 (dd, 1H, *J*=4.9, 7.8), 4.40 (dd, 1H, *J*=5.9, 8.3), 4.35 (d, 1H, *J*=7.8), 4.33 (dd, 1H, *J*=4.9, 8.3), 4.32 (d, 1H, *J*=7.3), 3.94—3.88 (m, 2H), 3.85 (d, 1H, *J*=4.4), 3.81 (d, 1H, *J*=2.4), 3.78 (dd, 1H, *J*=4.4, 11.2), 3.70 (dd, 1H, *J*=4.4, 11.2), 3.68—3.60 (m, 2H), 3.57 (dd, 1H, *J*=4.9, 7.8), 3.54 (dd, 1H, *J*=7.8, 7.8), 3.48 (dd, 1H, *J*=2.9, 9.8), 3.40—3.20 (m, 12H), 2.92 (dd, 1H, *J*=4.9, 12.7), 2.70 (d, 1H, *J*=12.7), 2.19 (t, 2H, *J*=7.3), 1.84—1.40 (m, 12H). ^{13}C-NMR (CD_3OD) δ: 176.1, 173.9, 169.2, 166.1, 153.6, 140.8, 134.4, 129.4, 125.8, 123.1, 117.8, 105.2, 104.3, 80.7, 79.6, 79.3, 79.0, 77.1, 76.6, 76.4, 74.9, 72.6, 70.3, 69.4, 69.2, 63.4, 62.6, 62.0, 61.7, 57.0, 54.6, 41.1, 40.7, 40.1, 36.8, 33.2, 30.1, 29.8, 29.5, 29.1, 26.9, 26.8, 24.1.

Enzyme Assay Enzyme reactions with suspensions of bovine brain total membrane fraction or rat liver Golgi were performed using a method similar to that described previously.[12] The assay mixture, 50 μl in volume, consisting of 0.3 mM CMP-[^{3}H]-NANA (4.2 mCi/mmol), 0.4% Triton CF-54, 10 mM $MnCl_2$, 10 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 150 mM cacodylate pH 6.5, 60 mg bovine brain total membrane protein, and various concentrations of **1**, was incubated at 37 °C for 2 h. After the addition of distilled water (450 μl), the mixture was loaded on an immobilized streptavidin column (100 μl) and the column was successively washed with 0.1 M phosphate buffer, 0.15 M NaCl, pH 7.3, and distilled water. The elution of adsorbed materials was performed by incubating the gel with 70% formic acid at 80 °C for 3 min. The eluate was evaporated to dryness with a continuous stream of N_2 gas at 40 °C, and the radioactivity of the residue was measured by liquid scintillation counting. The assay with rat liver Golgi was carried out similarly except that 60 mg protein was used. The control assay with CDH was performed as described previously.[12]

Inhibition of CDH Sialylation with 1 The incubation was performed similarly to the enzyme assay of CDH using 200 μg of bovine brain protein in the presence of 0.3 or 0.6 mM of **1**. After incubation at 37 °C for 2 h, the reaction mixture was applied on the immobilized streptavidin as described above to remove the biotinyl components, and the pass-through fraction from the column was loaded on a pre-packed C_{18} column (Bond Elute C18, Analytichem) followed by the conventional assay procedures for CDH sialylation.[12]

Acknowledgments This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas (05274102) and an additional Grant-in-Aid (06453193) from the Ministry of Education, Science, Sports and Culture, Japan. Research Fellow of the Japan Society for the Promotion of Science (M. H.).

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