Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 5, No. 23, pp. 2859-2862, 1995

0960-894X/95 $9.50+0.00

0960-894X(95)00514-5

A CARBENE-GENERATING BIOTINYLATED LACTOSYLCERAMIDE ANALOG AS NOVEL PHOTOREACTIVE SUBSTRATE FOR GM3 SYNTHASE

Yasumaru Hatanaka[a]*, Makoto Hashimoto[a], Kazuya I.-P. Jwa Hidari[b, 1], Yutaka Sanai[b], Yoshitaka Nagai[c], and Yuichi Kanaoka [d]

[a] Research Institute for Wakan-Yaku, Toyama Medicinal and Pharmaceutical University, Sugitani 3630, Toyama, 930-01 Japan, [b] Departments of Biochemical Cell Research, The Tokyo Metropolitan Institute of Medical Science, 18-22, Honkomagome 3-chome, Bunkyo-ku, Tokyo, 113 Japan, [c] Mitsubishi Kagaku Institute of Life Science, 11 Minami-ooya, Machida, Tokyo, 194 Japan, [d] Toyama Womens College, 444 Gankaiji, Toyama, 930-01 Japan

Abstract: A new biotinylated lactose derivative bearing a ^{14}C-labeled phenyldiazirine was synthesized. A convenient approach based on avidin-biotin technology was successfully applied for GM3 synthase assay and the *Km* value of this biotinylated photoprobe was determined as 180 μM using rat liver Golgi as the enzyme source. Further characterization revealed that this reagent could be a useful photoprobe for GM3 synthase.

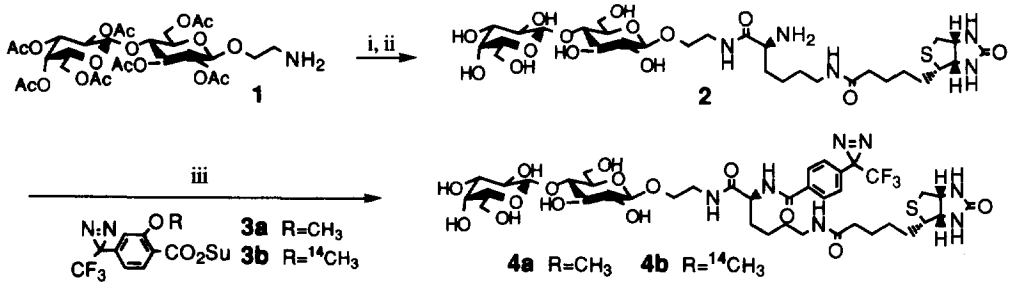
CMP-*N*-acetylneuraminic acid (CMP-NANA) : lactosylceramide α-2,3-sialyltransferase (GM3 synthase) catalyzes the transfer of sialic acid from CMP-sialic acid to the nonreducing terminal of lactosylceramide to form a ganglioside GM3 (NeuAcα2-3Galβ1-4Glc-ceramide).[2] The GM3 synthase is a potential candidate as a key regulatory enzyme, because it catalyzes the common step in the biosynthesis of nearly all gangliosides[3] and the cDNA cloning of this enzyme is recognized to be one of the major subject in the field of glycobiology. Photoaffinity labeling is an important chemical method for the analysis of biological macromolecules,[4] however, no useful photoprobe for GM3 synthase has been developed yet. We have already reported that the diazirine based photoaffinity labeling is more promising than the use of conventional aryl azide counterparts.[5] We also demonstrated that the combined use of diazirinyl probes with avidin-biotin technology is an effective approach for the specific manipulation of photolabeled products.[6] Sialyltransferases have the ability to utilize CMP-NANA as the common donor substrate, but differ in the specificity of acceptor sugar residue and the type of linkage formed. Recent study of site-specific mutagenesis suggested that the conserved region between sialyltransferases, the sialyl-motif, appears to participate in binding of the common donor substrate CMP-sialic acid.[7] Therefore, the development of photoreactive acceptor analogs may have several advantages to donor derivatives on the identification of respective sialyltransferase proteins as well as the structural analysis of unsolved acceptor binding regions. Here we wish to report the synthesis and characterization of lactosylceramide analog having a phenyldiazirine and a biotin as the first example of photoreactive acceptor analog for GM3 synthase.

Results and discussion

Synthesis. Although the Km value of GM3 synthase to lactosylceramide (Galβ1-4Glc-ceramide; CDH) is affected by its structure of ceramide moiety,[8] GM3 synthase is known to bind to an affinity column with lactosylceramide aldehyde which is missing one of two lipid tails in the original structure of CDH.[9] To mimic

this truncated form of CDH, β-lactoside derivative carrying a long chain alcohol with a carbene-generating phenyldiazirine and a biotin unit was designed for the ease of synthesis (Scheme 1). Biocytin was used instead of biotin itself because long-chain derivatives of biotin are usually requied to obtain optimal biotin-binding capabilities.[10] The α-amino group of lysine part of biocytin is also useful to introduce the photoreactive phenyldiazirin by simple acylation reaction. Thus, the aminoethyl glycoside of lactose **1** was coupled with α-Fmoc-biocytin *N*-hydroxysuccinimide ester followed by deprotection to give **2** which was treated with the active ester of diazirinyl-benzoic acid **3a**[11] to afford **4a**. After HPLC purification, the structure of compound **4a** was confirmed from its analytical and spectroscopic data.[12] The radioisotope labeled **4b** was analogously prepared using ^{14}C-diazirine (**3b**, specific activity 57 mCi / mmol).[11]

Scheme 1



i) Fmoc-biocytin-OSu, (iso-Pr)2NEt, DMF (40%), ii) NH3-MeOH (quant.), iii) Py, DMF (**4a**; 74%, **4b**; 17%)

Affinity isolation of 4 with immobilized avidin. The 44 μM solution of **4b** in 0.1 M phosphate buffered saline pH 7.2 (PBS) was loaded on an immobilized streptavidin (GIBCO) or a monomeric avidin (PIERCE) at room temperature. After washing the gel with PBS and distilled water, the elution of adsorbed materials was performed under the several conditions as described in Table 1. The yields of recovery from these avidin columns were comparable to that of the conventional assay using C_{18} column (Bond Elute, Analytichem International). Although the acidic elution condition required for streptavidin may disrupt the glycosidic linkages of oligosaccharide product in the assay mixture, the condition was satisfactory for the convenient assay of GM3 synthase as described below. Biotinylated compounds can be eluted from monomeric avidin under milder condition than from streptavidin and this advantage was successfully applied for the product analysis.

Table 1. Recovery of **4b** on different conditions

Column	Eluent	Recovery (%)
streptavidin	70% formic acid	89
monomeric avidin	2 mM *d*-biotin-PBS	84
C_{18}	$CHCl_3$-CH_3OH (1:1)	85

Enzyme assay of 4. Taking the advantage of biotin possessing substrate **4**, a new method of the enzyme assay was examined as an useful alternative to the conventional assay method.[13] The enzyme reaction was performed according to the literature with rat liver Golgi GM3 synthase. The assay mixture of 50 μl volume consisted of 3.2 mM CMP-[^{3}H]-NANA (4.2 mCi/mmol, DuPont NEN), 0.4% Triton CF-54, 10 mM $MnCl_2$, 10 mM

$MgCl_2$, 1 mM 2-mercaptoethanol, 150 mM cacodylate pH 6.5, 60 μg rat liver Golgi, and various concentration of **4a** was incubated at 37 °C for 2 h. After the addition of 450 μl of distilled water, the mixture was treated with the immobilized streptavidin by the similar manner described in the preceding experiment. From the Lineweaver-Burk plot analysis in Figure 1, the *Km* value of compound **4a** was estimated as 180 μM and this value seems sufficient to the reported *Km* value to the natural substrate CDH (50 - 130 μM).[8, 14]

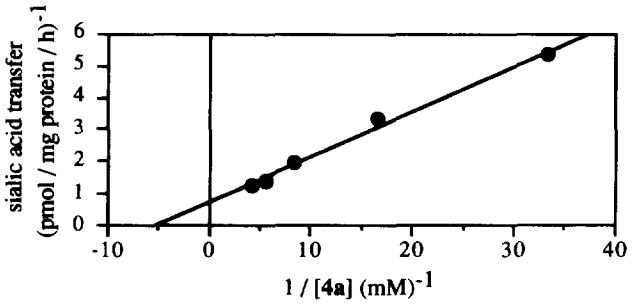


Figure 1. Lineweaver-Burk plot for compound **4a**

Competitive sialylation between lactosylceramide and compound 4. The inhibition of sialyltransfer to the natural substrate CDH by **4a** was examined changing the ratio of CDH to **4a**. The sialylation of **4a** was measured by the immobilized streptavidin method and the pass-through fraction from the streptavidin column was loaded on a C18 column to measure the formation of GM3.[13] This method provide a convenient approach to analyze biotinyl substrate as well as natural substrate when both are present in the same assay mixture. The sialylation of CDH was competitively inhibited on the increment of the **4a** concentration as shown in Table 2.

Table 2. Competition between CDH and **4a** in the sialyltransferase assay

CDH : **4a** (mM)	[^{3}H]-NANA incorporation (dpm)		Inhibition of CDH sialylation (%)
	CDH	**4a**	
0.18 : 0	10,810	-	0
0.18 : 0.18	7,710	3,850	33
0.18 : 0.90	4,940	8,610	64
0 : 0.18	-	9,480	100

TLC Analysis of enzyme products. The mixture of 25 μl volume consisted of 3.2 mM CMP-[^{14}C]-NANA (4 mCi/mmol, DuPont NEN), 0.4 % Triton CF-54, 10 mM $MnCl_2$, 10 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 150 mM cacodylate, pH 6.5, 60 μg rat liver Golgi, and the photoprobe **4a** (0.18 mM) was incubated at 37 °C for 2 h without or with the equimolar amount of competitive substrate CDH (0.18 mM). To prevent the acidic treatment which will cleave the glycosidic linkages, the enzyme products were recovered on the monomeric avidin instead of streptavidin. The TLC analysis of recovered products was performed on HPTLC plate (Merck, silica gel 60, No 5641) with a solvent system $CHCl_3$:CH_3OH:0.5 % $CaCl_2$ (55:45:2) according to the reported method.[13] After the plate was dried and wrapped with a plastic sheet, radioactive materials were visualized with BAS 2000 radio image analyzer (Fuji film) by exposing the plate for 24 h and the result is in Figure 2. The

starting material in lane 1 had been consumed in the lane 2 and the product was appeared at less mobile position than **4b**. Because of the introduction of charged sialic acid moiety, sialylated products are known to be poorly developed on TLC analysis compared to the starting substrates.[15] The sialylated product was depressed in the presence of CDH as shown in lane 3 and this observation is in consistent with the results from the competition assay described above. Although the spectrometric analyses have to be done to confirm the structure of the sialylated product in lane 2, the compound **4** was effectively sialylated by rat liver Golgi GM_3 synthase.

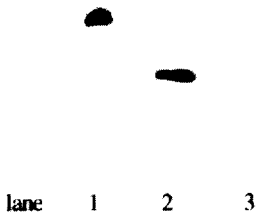


Figure 2. Autoradiogram of TLC separated biotinyl components from the enzyme reaction mixture. Following samples were used; **4b** (lane 1) and enzyme products from **4a** in the absence (lane 2) or presence (lane 3) of CDH.

In conclusion, our results demonstrate that the compound **4** could be a good acceptor substrate for GM_3 synthase. The purification of GM_3 synthase from rat liver Golgi was reported recently[9] and the structural analysis of its binding sites become increasingly important. Because of the relative ease of experiments, the biotinylated photoprobe **4** would be a useful tool to further explore the detailed molecular aspects of this enzyme.

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

References and Notes

1) Present address : Department of medicine, University of Oklahoma, 825 NE 13th Street, OKC, OK 73104, USA
2) Basu, M.; De, T.; Das, K. K.; Kyle, J. W.; Chon, H. -C.; Schaeper, R. J.; Basu, S. *Methods Enzymol.* **1987**, *138*, 575.
3) van Echten, G.; Sandhoff, K. *J. Biol. Chem.* **1993**, *268*, 5341.
4) Bayley, H. *Laboratory Techniques in Biochemistry and Molecular Biology* pp 25-44, Work, T. S.; Burdon, R. H., Eds.;, Elsevier; Amsterdam, 1983.
5) Yoshida, E.; Nakayama, H.; Hatanaka, Y.; Kanaoka, Y. *Chem. Pharm. Bull.* **1990**, *38*, 982.
6) Hatanaka, Y.; Hashimoto, M.; Kanaoka, Y. *BioMed. Chem.* **1994**, *2*, 1367-1373.
7) Datta, A. D.; Paulson, J. C. *J. Biol. Chem.* **1995**, *270* , 1497.
8) Kadowaki, H.; Grant, M. A. *J. Biol. Chem.* **1994**, *269* , 14931.
9) Melkerson-Watson, L. J.; Sweeley, C. C. *J. Biol. Chem.* **1991**, *266*, 4448-4457.
10) Wilchek, M.; Bayer, E. A. *Methods Enzymol.* **1990**, *184*, 123-138.
11) Hatanaka, Y.; Hashimoto, M.; Kurihara, H.; Nakayama, H.; Kanaoka, Y. *J. Org. Chem.* **1994**, *59*, 383.
12) UV(CH_3OH) λmax(ε) 295 (3300), 350 (470), FAB-MS m/z 982 $(MH)^+$, ^{1}H-NMR (CD_3OD) 7.80 (d, 1H, J=8.3), 7.01 (d, 1H, J=8.3), 6.85 (s, 1H), 4.49 (dd, 1H, J=4.9, 7.8), 4.35(d, 1H, J=7.8), 4.33 (d, 1H, J=7.3), 4.29 (dd, 1H, J=4.4, 7.8), 3.9-3.0 (m, 17H), 2.92 (dd, 1H, J=4.9, 12.7), 2.70 (d, 1H, J=12.7), 1.9-1.3(m, 16H)
13) Jwa Hidari, K. I. -P.; Kawashina, I.; Tai, T.; Inagaki, F.; Nagai, Y.; Sanai, Y. *Eur. J. Biochem.* **1994**, *221*, 603.
14) a) Richardson, C. L.; Keenan, T. W.; Morre, D. J. *Biochim. Biophys. Acta* **1977**, *488*, 88. b) Busam, K.; Decker, K. *Eur. J. Biochem.* **1986**, *160*, 23. c) Iber, H.; van Echten, G.; Sandhoff, K. *Eur. J. Biochem.* **1991**, *195*, 115.
15) Ando, S.; Chang, N. -C; Yu, R. K. *Anal. Biochim.* **1978**, *89*, 437.

(*Received in Japan* 18 *October* 1995; *accepted* 6 *November* 1995)