J. Biochem. 125, 746-749 (1999)

Specific and Sensitive Assay for Alkaline and Neutral Ceramidases Involving C12-NBD-Ceramide[1]

Motohiro Tani, Nozomu Okino, Susumu Mitsutake, and Makoto Ito[2]

Laboratory of Marine Biochemistry, Faculty of Agriculture, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581

Received November 26, 1998; accepted December 25, 1998

A fluorescent analogue of ceramide, C12-NBD-ceramide, was found to be hydrolyzed much faster than ^{14}C-labeled ceramide by alkaline ceramidase from *Pseudomonas aeruginosa* and neutral ceramidase from mouse liver, while this substrate was relatively resistant to acid ceramidase from plasma of the horseshoe crab. The radioactive substrate was used more preferentially by the acid ceramidase. It should be noted that C6-NBD-ceramide, which is usually used for ceramidase assays, was hardly hydrolyzed by any of the enzymes examined, compared to C12-NBD-ceramide. For the alkaline and neutral enzymes, the V_{max} and k (V_{max}/K_m) with C12-NBD-ceramide were much higher than those with ^{14}C-ceramide. In contrast, for the acid enzyme these parameters with C12-NBD-ceramide were less than half those with the radioisotope-labeled substrate. It is noteworthy that the labeling of ceramide with NBD did not itself reduce the K_m of the alkaline enzyme, but did that of the neutral enzyme. It was also found that C12-NBD-ceramide was preferentially hydrolyzed by the alkaline and neutral enzymes, but not the acid one, in several mammalian cell lines. This study clearly shows that the attachment of NBD, but not dansyl, increases the susceptibility of ceramide to alkaline and neutral enzyme, and decreases that to acid enzymes. Thus the use of this substrate provides a specific and sensitive assay for alkaline and neutral ceramidases.

Key words: ceramidase, ceramide, C12-NBD-ceramide, sphingolipid ceramide *N*-deacylase (SCDase).

Ceramide (Cer) acts as a second messenger for cell growth, differentiation, and apoptosis (for reviews see Ref. *1*). Ceramidase (*N*-acylsphingosine amidohydrolase, EC 3.5.1.23) is an enzyme capable of cleaving the *N*-acyl linkage of Cer to produce a fatty acid and a sphingosine base (for reviews see Ref. *2*). The enzyme can be basically classified into two types—acid or alkaline—according to the catalytic pH optimum. Acid ceramidase is thought to primarily degrade Cer in lysosomes. Recently, the gene of an acid ceramidase was cloned (*3*). In contrast, the biological significance of alkaline ceramidase, which are mainly associated with the membrane fraction, is still unclear. Interestingly, the alkaline enzyme present in rat glomerular mesangial cells has been shown to be up-regulated by growth factors, and to be possibly involved in sphingosine- or sphingosine-1-phosphate-mediated signal transduction pathways (*4*). Very recently, we purified a novel type of alkaline ceramidase from *Pseudomonas aeruginosa* which had been isolated from a patient with atopic dermatitis (*5*). In addition, a ceramidase showing a neutral pH optimum has been found in rat hepatocytes (*6*), although the relationship between the alkaline and neutral enzymes remains unclear.

Sphingolipid ceramide *N*-deacylase (SCDase) is an enzyme capable of hydrolyzing the *N*-acyl linkage of ceramide in various glycosphingolipids and sphingomyelin (*7*). Recently, it was found that free fatty acids were condensed efficiently to sphingosine by the enzyme to produce Cer under specific conditions (*8*). By using the condensation reaction of SCDase, radioisotope- (*8*) and fluorescence-labeled Cer (*9*) were easily prepared in high yields. We found a significant difference between these two substrates in their susceptibility to ceramidase.

Here we report a sensitive and specific assay for alkaline and neutral ceramidases involving a fluorescence-labeled Cer. The method was based on the observation that alkaline and neutral ceramidases preferentially hydrolyze NBD-labeled *N*-dodecanoylsphingosine (C12-NBD-Cer), compared with a ^{14}C-labeled substrate, while the fluorescent substrate is relatively resistant to acid ceramidase.

MATERIALS AND METHODS

Materials—SCDase was purified from the culture fluid of *Pseudomonas* sp. TK4 as described in Ref. *7* or purchased from Takara Shuzo (Shiga). ω-Aminododecanoic acid and NBD-fluoride were obtained from Wako Chemical (Osaka)

[1] This work was supported in part by a Grant-in Aid for Scientific Research (B) (09460051) from the Ministry of Education, Science, Sports and Culture of Japan.

[2] To whom correspondence should be addressed. Tel: +81-92-642-2900, Fax: +81-92-642-2900 (or 2907), E-mail: makoto_i@agr.kyushu-u.ac.jp

Abbreviations: Cer, ceramide; DNS, dansyl; NBD, 4-nitrobenzo-2-oxa-1,3-diazole; SCDase, sphingolipid ceramide *N*-deacylase; TLC, thin-layer chromatography.

and Dojindo Laboratories (Kumamoto), respectively. D-Sphingosine was from Sigma (USA). C6-NBD-Cer was purchased from Molecular Probe (USA).

Preparation of Labeled Cer—Fluorescence-labeled *N*-dodecanoylsphingosine was prepared by the method described previously (*9*). Briefly, ω-aminododecanoic acid was condensed to sphingosine (d18:1) by SCDase after blocking of the ω-amino group with trifluoroacetate. After the reaction, the block was removed by mild alkaline treatment to produce ω-amino-Cer (ω-amino-C12:0, d18:1). C12-NBD-Cer (NBD-C12:0, d18:1) and C12-DNS-Cer (DNS-C12:0, d18:1) were easily prepared from the ω-amino-Cer by coupling with NBD-fluoride and DNS-chloride, respectively. ^{14}C-Cer was prepared by condensation of ^{14}C-fatty acids to sphingosine using SCDase as described previously (*8*).

Preparation of Ceramidases—The alkaline ceramidase was purified from the culture fluid of *P. aeruginosa* AN17 as described previously (*5*). The neutral ceramidase was partially purified from mouse liver. Briefly, livers were homogenized with 0.25 M sucrose containing 1 mM EDTA (sucrose-EDTA). Debris was removed by centrifugation at $600\times g$ for 10 min, and the supernatant was centrifuged at $27{,}000\times g$ for 30 min. The sediment (membrane fraction) was suspended in sucrose-EDTA, frozen at −80°C immediately, and then thawed under running tap water. The suspension was centrifuged at $105{,}000\times g$ for 90 min, and the supernatant was applied to a DEAE-Sepharose FF column, pre-equilibrated with 20 mM phosphate buffer, pH 7.0, containing 0.1% (w/v) Lubrol PX (buffer A), using a BPLC-600FC HPLC system (Yamazen, Japan). The enzyme was adsorbed onto the column and eluted with buffer A containing 1 M NaCl. The fractions containing CDase activity were pooled and used as a neutral ceramidase. For an acid ceramidase, the $150{,}000\times g$ supernatant of plasma of the Japanese horseshoe crab (*Tachypleus tridentatus*) was used. The optimum pH for the enzyme from bacteria is 8.5 (alkaline ceramidase), that from mouse liver 7.5 (neutral ceramidase), and that from horseshoe crab 4.5 (acid ceramidase). To prepare cell lysates of various mammalian cells, semi-confluent cells ($2\text{–}3\times10^7$) were washed with phosphate-buffered saline 3 times and then suspended in an appropriate amount of 10 mM phosphate buffer, pH 7.0.

Assay for Ceramidase—An appropriate amount of a fluorescence- or ^{14}C-labeled Cer in chloroform/methanol (1:2, v/v) was dried in an Eppendolf tube under N_2 gas. The substrate was dissolved in the appropriate buffer indicated below. To dissolve the substrate completely, the Eppendolf tube containing the substrate solution was kept in a boiling water bath for 10 s, followed by sonication for 5 min. For the standard assay, 100 pmol of C12-NBD-Cer or C12-^{14}C-Cer was incubated with an enzyme at 37°C for 30 min in 20 μl of 25 mM Tris-HCl buffer, pH 8.5, for the *Pseudomonas* enzyme, 25 mM Tris-HCl buffer, pH 7.5, for the mouse liver enzyme, and 25 mM sodium acetate buffer, pH 4.5, for the horseshoe crab enzyme. The buffers contained 0.25% (w/v) Triton X-100 for the enzymes from bacteria and horseshoe crab, and 1% (w/v) sodium cholate for the enzyme from mouse liver. After incubation, the reaction was terminated by the addition of 100 μl of chloroform/methanol (2:1, v/v), followed by drying in a Speed Vac concentrator, and dissolved in 15 μl of the same solvent. An appropriate amount of sample was applied to a TLC plate which was then developed with chloroform/methanol/25% ammonia (90:20:0.5, v/v). Fluorescence-labeled products were determined with a Shimadzu CS-9300 TLC chromatoscanner (Shimadzu, Kyoto). Excitation was performed at 470 and 350 nm, and emission was detected at 525 and 455 nm for NBD and DNS, respectively. For determination of radioactive substrates, an imaging analyzer BAS1000 (Fuji Film, Tokyo) was used. One enzyme unit was defined as the amount capable of catalyzing the hydrolysis of 1 μmol of substrate per min with an appropriate substrate (C12-NBD-Cer for the alkaline and neutral enzymes, and C12-^{14}C-Cer for the acid enzyme) under the standard conditions described above. A value of 10^{-6} units of enzyme was expressed as 1 μU in this study.

RESULTS AND DISCUSSION

TLC (Fig. 1) revealed the release of fluorescence-labeled dodecanoic acid from C12-NBD-Cer (A) or C12-DNS-Cer (B), and ^{14}C-labeled lauric acid from C12-^{14}C-Cer (C) through the action of ceramidase. The reaction products were clearly separated from the parent substrates on the TLC plates, and determined using a TLC-chromatoscanner with a fluorescence detector (for fluorescent substrates) and an image analyzer (for the radioisotope-labeled substrate), respectively. No less than 400 fmol of the released NBD-fatty acids was detectable when 100 pmol of C12-NBD-Cer was used for the assay. The sensitivity with C12-NBD-Cer as a substrate was found to be comparable to that with the radioactive substrate.

The time courses of Cer hydrolysis by the ceramidases were examined using three different labeled-substrates (Fig. 2). Interestingly, C12-NBD-Cer was hydrolyzed much

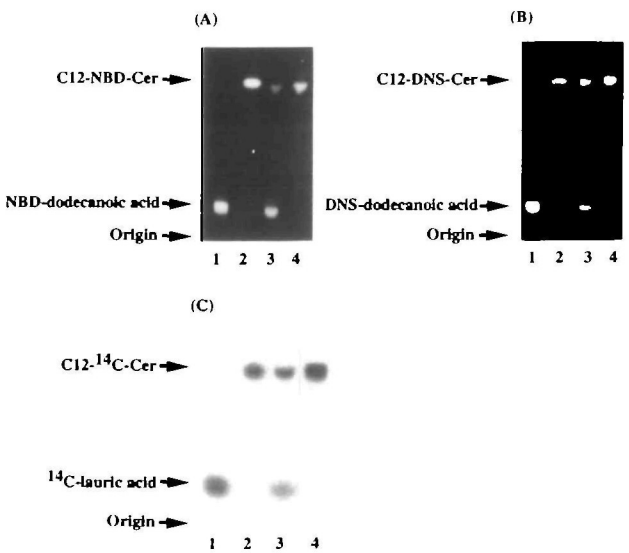

Fig. 1. **Detection of fluorescent or radioactive fatty acid released from labeled-ceramides by the action of a ceramidase.** The action of the ceramidase from *P. aeruginosa* on (A) C12-NBD-Cer, (B) C12-DNS-Cer, and (C) C12-^{14}C-Cer. Lane 1, labeled-fatty acid standard; lane 2, labeled-Cer standard; lane 3, labeled-Cer+enzyme; lane 4, labeled-Cer+boiled enzyme. Enzyme digestion was performed by the standard method described under "MATERIALS AND METHODS." The reaction was carried out at 37°C for 16 h.

faster than ^{14}C-Cer by the alkaline ceramidase from *P. aeruginosa* (Fig. 2A) and the neutral ceramidase from mouse liver (Fig. 2B), while this substrate was relatively resistant to the acid ceramidase from plasma of the horseshoe crab (Fig. 2C). The radioactive substrate was used more preferentially by the acid ceramidase (Fig. 2C). It should be noted that C6-NBD-Cer, which is usually used for ceramidase assays (*6*), was hardly hydrolyzed by any of the enzymes examined under the conditions used (Fig. 2, A-C). C12-DNS-Cer was also found to be somewhat resistant to hydrolysis by the alkaline and acid enzymes (Fig. 2, A and C). The kinetics of the three enzymes with C12-NBD-Cer and ^{14}C-Cer were examined (Fig. 2, D-F). For the alkaline and neutral enzymes, the V_{max} and k (V_{max}/K_m) with C12-NBD-Cer were much higher than those with ^{14}C-Cer (Table I). In contrast, for the acid enzyme these parameters with C12- NBD-Cer were less than half those with the radioisotope-labeled substrate. These results indicated that the attachment of NBD to the fatty acid moiety of Cer strongly affected the susceptibility of the substrate to ceramidase, *i.e.*, the susceptibility to the alkaline and neutral ceramidases was increased by attachment of NBD, whereas that to the acid enzyme was decreased. It is noteworthy that the labeling of Cer with NBD did not itself reduce the K_m of the alkaline enzyme, but did that of the neutral enzyme (Table I). Furthermore, it is also interesting that another fluorescent dye, DNS, did not enhance the apparent activity of the alkaline enzyme and strongly inhibited that of the acid enzyme (Fig. 2, A and C).

To confirm the superiority of C12-NBD-Cer for the assaying of alkaline and neutral ceramidases, we measured the ceramidase activities of several mammalian cultured cell lines using three different types of labeled-Cer at different pH values. As shown in Fig. 3, at pH 8.5 C12-NBD-Cer was hydrolyzed much more rapidly than C12-^{14}C-Cer for all the cell lines examined, whereas the latter appeared to be a better substrate at pH 4.0. In particular, for HL60 cells (C) and KB2 cells (D), both of which were

TABLE I. **Kinetic parameters of ceramidase for C12-NBD-Cer or ^{14}C-Cer.**

	K_m (μM)	V_{max} (pmol/min)	k (V_{max}/K_m)
Alkaline ceramidase			
C12-^{14}C-Cer	63.2	0.9	1.4×10^{-2}
C12-NBD-Cer	111	2.5	2.3×10^{-2}
Neutral ceramidase			
C16-^{14}C-Cer	71.4	0.5	0.7×10^{-2}
C12-NBD-Cer	22.3	4.7	21.1×10^{-2}
Acid ceramidase			
C12-^{14}C-Cer	40.4	1.8	4.3×10^{-2}
C12-NBD-Cer	59.0	0.8	1.4×10^{-2}

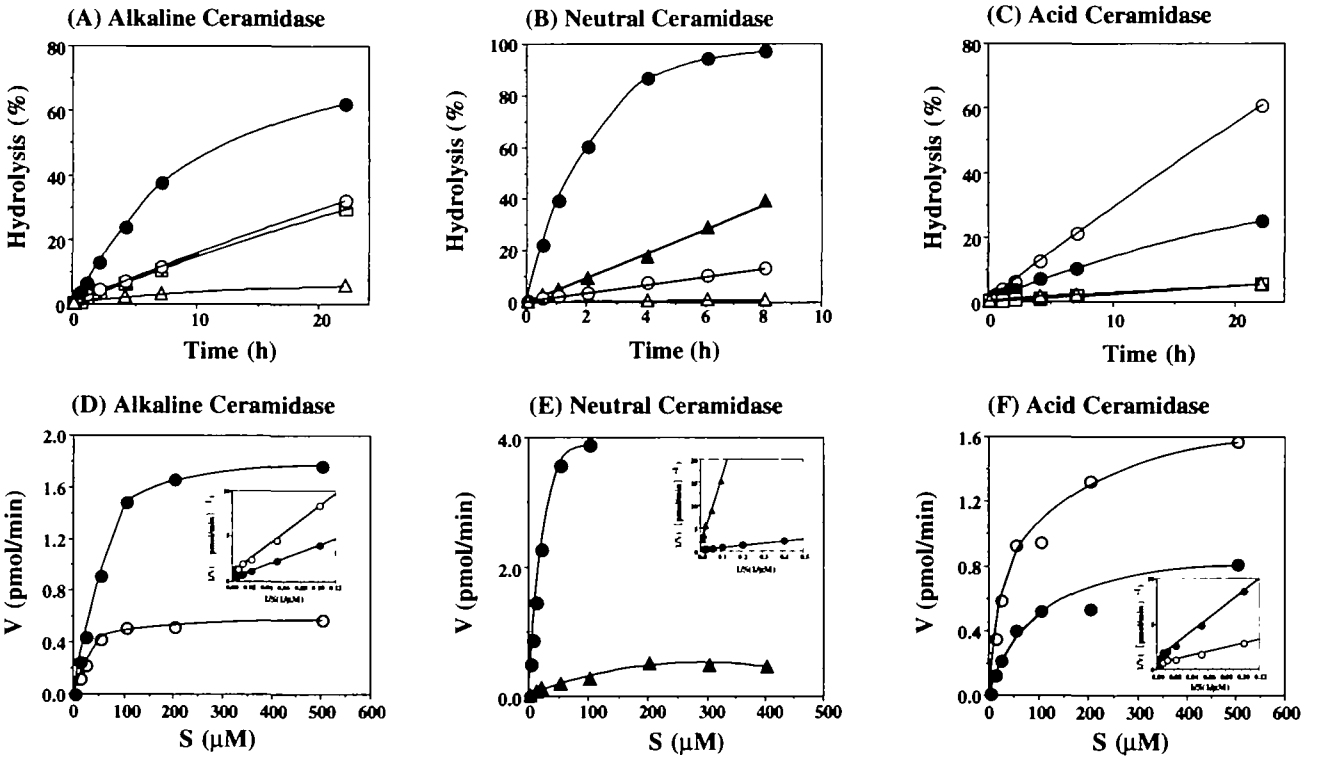

Fig. 2. **Kinetic analysis of the hydrolysis of fluorescence- or radioisotope-labeled Cer by ceramidases.** Time courses of the reactions with (A) the alkaline ceramidase from *P. aeruginosa*; (B) the neutral ceramidase from mouse liver; and (C) the acid ceramidase from the horseshoe crab. Substrate saturation curves and Lineweaver-Burk plots for (D) the alkaline ceramidase; (E) the neutral ceramidase; and (F) the acid ceramidase. The assay was performed by the standard method as described in "MATERIALS AND METHODS" with some modifications. The amount of enzyme used was 1 μU for (A), (C), (D), and (F), and 2 μU for (B) and (E). The concentration of the substrate was 5 μM for the neutral enzyme (B), and 50 μM for the alkaline and acid enzymes (A, C). For analysis of kinetics, the concentrations of substrates were varied as indicated, and the incubation times were 3 h for the *Pseudomonas* (D) and horseshoe crab (F) enzymes, and 30 min for the mouse enzyme with C12-NBD-Cer and 2 h with C16-^{14}C-Cer (E). ●, C12-NBD-Cer; △, C6-NBD-Cer; ▫, C12-DNS-Cer; ○, C12-^{14}C-Cer; ▲, C16-^{14}C-Cer. The values are the means for duplicate determinations.

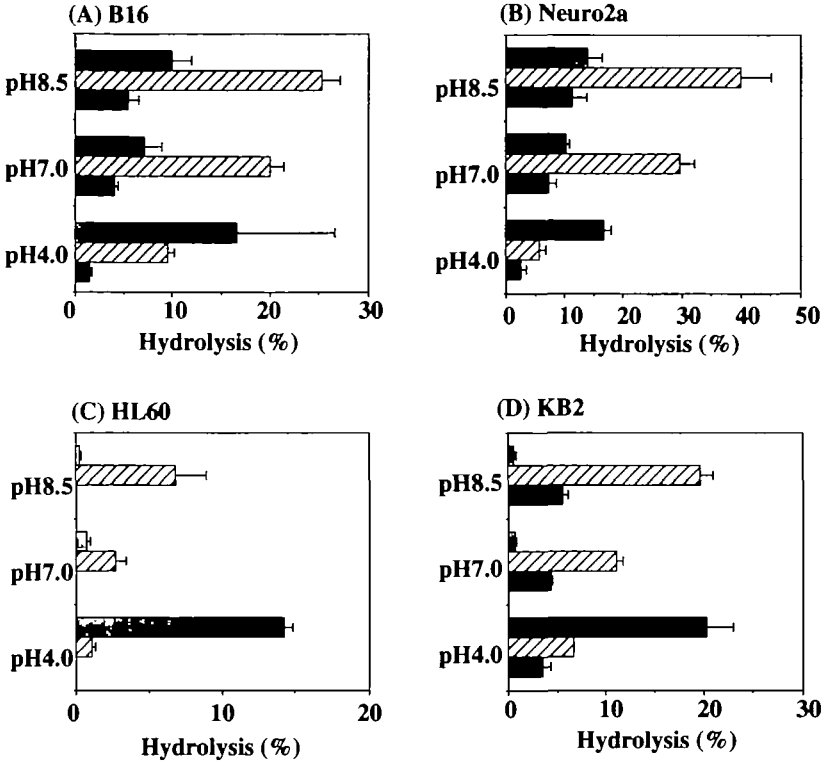

Fig. 3. **Assaying of ceramidase activities in various mammalian cells.** The assay was performed by the standard method with some modifications. For assaying of the neutral enzyme, 25 mM phosphate buffer, pH 7.0, containing 0.25% (w/v) Triton X-100 was used. The incubation times and amounts of protein used are shown in parentheses. (A) B16 mouse melanoma cells (3 h, 50 μg); (B) Neuro2a mouse neuroblastoma cells (18 h, 100 μg); (C) HL60 human myelogenous leukemia cells (18 h, 100 μg); and (D) KB2 human epidermoid carcinoma cells (6 h, 100 μg). The values are the means for triplicate determinations. ▩, C12-^{14}C-Cer; ▨, C12-NBD-Cer; ■, C12-DNS-Cer.

derived from human cancers, definite activity under alkaline conditions was detected only when C12-NBD-Cer was used as the substrate. Interestingly, at pH 7.0 C12-NBD-Cer also seemed to be a good substrate, but at present it is not certain whether or not the ceramidase having a neutral pH optimum is present in these cell lines. These results suggested that at least two different enzymes showing different pH optima and specificities were present in the cell lysates employed, and that C12-NBD-Cer was preferentially hydrolyzed by the alkaline enzyme in all the cell lines examined. Again, C12-DNS-Cer was shown to be a poor substrate under alkaline, neutral and acidic conditions, although the tendency for susceptibility to ceramidases at different pH values was similar to that for C12-NBD-Cer (Fig. 3).

One consideration that may arise is that NBD can only compensate for the shortage of the chain length of the fatty acids in Cer, since the Cer used most preferentially by the *Pseudomonas* ceramidase was one containing palmitic acid (C16:0) (*5*). However, it should be emphasized that C12-NBD-Cer was hydrolyzed by the alkaline and neutral enzymes much more rapidly than ^{14}C-Cer containing not only lauric acid (C12:0) (Fig. 2, A and B) but also palmitic acid (C16:0) (Fig. 2B).

The present findings clearly indicate that the attachment of NBD, but not DNS, increases the susceptibility of Cer to alkaline and neutral ceramidases and decreases that to acid ceramidase. Thus the use of this substrate provides a specific and sensitive assay for alkaline and neutral ceramidases. This assay involving C12-NBD-Cer will facilitate not only the purification and characterization of neutral and alkaline ceramidases, but also elucidation of their biological functions.

The authors wish to thank Drs. S. Iwanaga and S. Kawabata of Kyushu University for supplying the horseshoe crab plasma. They also thank Dr. T. Nakamura for the encouragement throughout the course of this work.

REFERENCES

1. Hannun, Y.A. and Bell, R.M. (1993) The sphingomyelin cycle: A prototypic sphingolipid signaling pathway. *Adv. Lipid Res.* **25**, 27-40
2. Hassler, D.F. and Bell, R.M. (1993) Ceramidase: Enzymology and metabolic roles. *Adv. Lipid Res.* **26**, 49-57
3. Koch, J., Gartner, S., Li, C.-M., Quintern, L.E., Bernardo, K., Levran, O., Schnabel, D., Desnick, R.J., Schuchman, E.H., and Sandhoff, K. (1996) Molecular cloning and characterization of a full-length complementary DNA encoding human acid ceramidase. *J. Biol. Chem.* **271**, 33110-33115
4. Coroneos, E., Martinez, M., McKenna, S., and Kester, M. (1995) Differential regulation of sphingomyelinase and ceramidase activities by growth factors and cytokines. *J. Biol. Chem.* **270**, 23305-23309
5. Okino, N., Tani, M., Imayama, S., and Ito, M. (1998) Purification and characterization of a novel ceramidase from *Pseudomonas aeruginosa*. *J. Biol. Chem.* **273**, 14368-14373
6. Karakashian-N., M., Morgan, E.T., Alexander, C., Liotta, D.C., and Merrill, A.H., Jr. (1997) Bimodal regulation of ceramidase by interleukin-1β. *J. Biol. Chem.* **272**, 18718-18724
7. Ito, M., Kurita, T., and Kita, K. (1995) A novel enzyme that cleaves the *N*-acyl linkage of ceramide in various glycosphingolipids as well as sphingomyelin to produce their lyso forms. *J. Biol. Chem.* **270**, 24370-24374
8. Mitsutake, S., Kita, K., Okino, N., and Ito, M. (1997) [^{14}C]-Ceramide synthesis by sphingolipid ceramide *N*-deacylase: New assay for ceramidase activity detection. *Anal. Biochem.* **247**, 52-57
9. Tani, M., Kita, K., Komori, H., Nakagawa, T., and Ito, M. (1998) Enzymatic synthesis of ω-amino-ceramide: Preparation of a sensitive fluorescent substrate for ceramidase. *Anal. Biochem.* **263**, 183-188