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Dihydroceramide was Highly Elevated by the Fumonisin B_1 and Desipramine in *Sphingomonas chungbukensis*

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Abstract – The sphingolipid metabolites act as lipid mediator for cell proliferation and apoptosis in mammalian cells. In bacteria, sphingolipid metabolism remains unknown. The purpose of this study was to investigate whether sphingolipid metabolism is potential target for fumonisin B_1(FB_1) and desipramine in *Sphingomonas chungbukensis*, Gram-negative bacteria, by comparing the intracellular contents of bacterial sphingolipids with ones of HIT-T15 β-cells, hamster pancreatic cells. The concentrations of ceramide and dihydroceramide were 18.0 ± 12.0 and 0.025 ± 0.018 nmol/mg protein, respectively, in HIT-T15 cells. However, the concentrations of ceramide and dihydroceramide in the bacterial culture were 2.0 ± 1.2 and 10.6 ± 5.5 nmol/mg protein, respectively. FB_1 decreased the level of ceramide from 18.0 to 3.8 nmol/mg protein in HIT-T15 β-cells. However, dihydroceramide content in FB_1-treated HIT-T15 cells was slightly decreased compared with the control culture. When *S. chungbukensis* was treated with either FB_1 or desipramine, dihydroceramide level was increased by 5- and 4-fold, respectively, compared with the control bacteria. These results indicate that FB_1 and desipramine may act as an activator in bacterial sphingolipid biosynthetic pathway, and bacterial sphingolipid metabolism pathway appears to be different from the pathway of mammalian cells.

Keywords: Dihydroceramide, fumonisin B_1, desipramine, sphingolipid, *Sphingomonas chungbukensis*

INTRODUCTION

Sphingolipids were found in cell membranes of animals, plants, yeasts, and some bacteria and fungi. The biological significance of sphingolipids in bacteria is not fully understood. In a group of Gram-negative bacteria called as *Sphingomonas*, glycosphingolipids occur (Kawasaki *et al.*, 1994). The function of glycosphingolipids has been suggested for bacterial attachment on mammalian cells (Hakomori and Igarashi, 1993).

Sphingomonas chungbukensis DJ77 was isolated from contaminated sediment of an industrial complex (Kim *et al.*, 1986). *S. chungbukensis* is a Gram- negative, aerobic, asporogenous, single polar flagellated bacterium. Some *Sphingomonas* strains contain ceramide glycolipids (C18-C21) composed of dihydrosphingosines and amide-linked 2-hydroxy straight chain saturated fatty acids (White *et al.*, 1996).

HIT-T 15 cells were originated from islet β-cells of Syrian golden hamster pancreas. The enzyme activity of membrane-bound sphingomyelinase was localized in isolated rat islets, mouse islets and clonal β cells (Kwon *et al.*, 1996). The regulation of ceramides for β cell function was reported. The exposure of pancreatic β cells to synthetic ceramides or purified sphingomyelinase markedly reduced the insulin production and mitogenesis (Sjoholm, 1995). Long-term exposure (24-96 h) of β cells to C2- or C6-ceramides significantly reduced glucose- and carbachol-induced insulin secretion from the β cells (Major *et al.*, 1999).

Ceramide is involved in the regulation of cell death and acts as a lipid mediator of cellular stress responses in mammalian cells (Hannun and Luberto, 2000). Ceramide level in cells is up-regulated by various types of stress conditions including ionizing radiation (Lu and Wong, 2004), serum deprivation (Colombaioni *et al.*, 2002; Yu *et*

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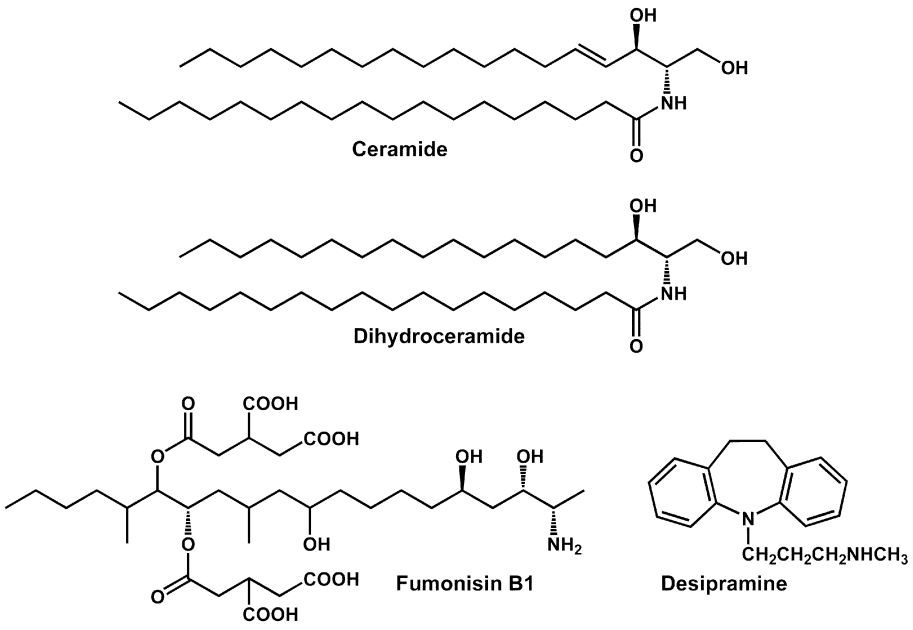

Fig. 1. Chemical structures of ceramide, dihydroceramide, fumonisin B_1 and desipramine. Dihydroceramide, unsaturated form of ceramide, occurs in *de novo* sphingolipid biosynthesis in mammalian cells. Fumonisin B_1 and desipramine are inhibitors of ceramide synthase and acidic sphingomyelinase, respectively, in sphingolipid metabolism pathway.

al., 2004) and anti-cancer drugs (Kok and Sietsma, 2004). There are two potential pathways for intracellular ceramide formation: *de novo* biosynthesis via the condensation of serine and palmitoyl-CoA, and the breakdown of sphingomyelin via sphingomyelinase. Fumonisin B_1 produced by *Fusarium* species, including *Fusarium verticilloides* Sheldon, is a specific inhibitor of ceramide synthase (Wang *et al.*, 1991) and desipramine, a tricyclic antidepressant, is an inhibitor of acidic sphingomyelinase in mammalian cells (Fig. 1).

In this study, *S. chungbukensis* was used for studying bacterial sphingolipid metabolism and was compared with HIT-T15 β-cells for confirming the difference in sphingolipid metabolism between bacterial and mammalian cells. The purpose of this study was to investigate whether sphingolipid metabolism in *S. chungbukensis* can be modulated by of FB_1 and desipramine, and sphingolipids can be useful biomolecules as promising source for cosmetic industry, atopic dermatitis therapy and the manufacture of stent coating material for coronary angioplasty.

MATERIALS AND METHODS

Materials

D-*erythro*-sphingosine was purchased from Biomol Research, Inc. (Plymouth Meeting, PA, USA). C_{17} sphingosine and C_{17} sphingosine-based ceramide were from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Pyridine, diisopropylether and heptane were from Sigma (St. Louis, MO, USA). Fetal bovine serum and culture medium for cell culture were obtained from Life Technologies, Inc. (Gaithersburg, MD, USA). HPLC-grade methanol was purchased from Merck KBaA (Darmstadt, Germany). o-Phthalaldehyde (OPA) was obtained from Molecular Probes, Inc. (Eugene, OR, USA). Other reagents were of the highest purity available.

Bacterial culture

Sphingomonas chungbukensis was grown in culture media containing 500 ml water with bactotrypton (1%), NaCl (0.5%) and yeast extract (0.5%) and streptomycin (0.01%) at 30°C for 24 h in the J-SWB2 shaker (Jisco Co., Seoul, Korea). In the main culture, 1 ml seed culture was inoculated into 19 ml media. *S. chungbukensis* pellets were harvested by centrifugation and kept at -20°C for sphingolipid analysis.

Cell culture

HIT-T15 cells originated from hamster's pancreas were obtained from KCLB (Korea Cell Line Bank, Seoul, Korea). HIT-T15 cells were grown in F-12 culture media containing 10% horse serum and 2.5% fetal bovine serum with sodium bicarbonate for 2 days at 37°C in a

humidified 5% CO_2 incubator. Cells were seeded on either each well of 6-well plate (10 cm^2/well) or 100 mm dish at a density of 4×10^5 cells/well. When cells reach confluency, the cultured cells were scraped with rubber policeman, harvested by centrifugation, and kept at -20°C for sphingolipid analysis.

Lipid extraction

Concentrations of ceramide and dihydroceramide in bacterial lysate and HIT-T15 cells were determined (Lee *et al.*, 2007). Bacteria and cell pellets were lysed with 0.2 *N* NaOH for the determination of protein content. Total lipid was extracted from the cell lysates of 100 mg protein content with 1 ml ethanol at 37°C for 1 h following the addition of C_{17} ceramide as an internal standard. The extract was centrifuged at 15,000×g for 10 min. The supernatant was dried in a Speed-Vac concentrator (Vision Scientific Co., Daejeon, Korea).

Thin layer chromatography (TLC)

The dry residue of the lipid extract was dissolved in 30 μl of chloroform/ methanol (1:2, v/v) and spotted on a high-performance thin-layer chromatography silica-gel plate (Merck, Darmstadt, Germany). The plate was developed in diisopropylether/methanol/29% NH_4OH (40:10:1, v/v/v). Ceramide standard lanes were cut from the sample lanes of the TLC plate and visualized by dipping the plate in 10% sulfuric acid and drying at 150°C. The areas in the sample lane with the same Rf values as the visualized band of C_{17} ceramide standard were scraped off, and both ceramide and dihydroceramide were eluted with 1 ml methanol. The eluates were transferred to polypropylene 1.5-ml tubes and dried in a Speed-Vac concentrator.

Enzymatic deacylation

The ceramide residue was mixed with reaction buffer containing 25 mM Tris-HCl buffer, pH 7.5, 1% sodium cholate, 15% fatty-acid-free BSA, and 150 μU SCDase (sphingolipid ceramide *N*-deacylase). Ceramide and dihydroceramide were deacylated into sphingosine and sphinganine, respectively, by SCDase at 37°C for 1 h. BSA in the reaction buffer was precipitated by adding ethanol and removed by centrifugation, and the supernatant was dried.

HPLC analysis

The sphingolipid extract was dissolved in 120 μl methanol, mixed with 15 μl OPA reagent (50 mg OPA, 1 ml ethanol, 200 μl β-mercaptoethanol and 50 ml 3% (w/v) boric acid buffer, pH 10.5), and incubated at room temperature for 30 min for derivatization. The HPLC analysis was performed using a Shimadzu (Tokyo, Japan) Model LC-10AT pump, SIL-$10A_{XL}$ auto sampler system and analytical Radial-Pak cartridge (Waters Associates, Inc., Milford, MA, USA) packed with Nova-Pak C_{18} reversed-phase column (4 μm, 100 mm×8 mm). The isocratic mobile phase composition of methanol/distilled water/triethylamine (92:8:0.1, v/v/v) and a flow rate of 1 ml/min were accurately controlled by HPLC system controller (Shimadzu SCL-10A). Shimadzu RF-10_{XL} fluorescence detector was set at an excitation wavelength of 340 nm and an emission wavelength of 455 nm. The resulting data and chromatographic profiles were evaluated using the Borwin system manager software (JMBS, France).

Protein assay

The total protein content in samples was determined in order to normalize the results. The lysate was solubilized with 0.2 *N* NaOH from the cell pellets or tissues, mixed with the PIERCE BCA reagents (Rockford, IL., USA) and incubated for 30 min. The protein content was quantified with a Molecular Devices ELISA reader (Sunnyvale, CA., USA) at 562 nm based on the BSA standard curve.

Statistics

All values were expressed as means±SD. Differences between untreated and treated samples were analyzed statistically by unpaired Student's *t*-test for single comparisons. Differences with $^{**}p<0.01$ were defined as statistically significant.

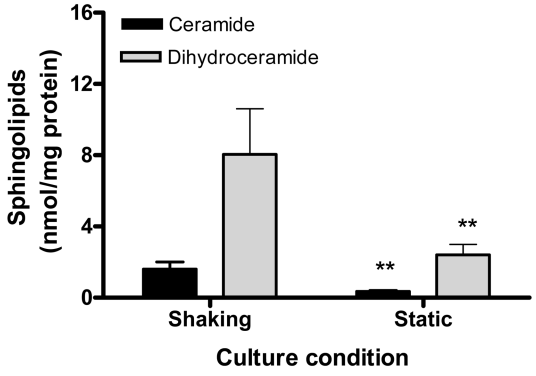

Fig. 2. Aeration effect of sphingolipid biosynthesis in *Sphingomonas chungbukensis*. *S. chungbukensis* was grown for 24 h. Ceramide and dihydroceramide were extracted from the bacterial pellets and analyzed by HPLC. Values are expressed as mean±S.D. Differences with $^{**}p<0.01$ were defined as statistically significant between counterparts of shaking culture condition.

RESULTS

Effects of shaking aeration for sphingolipid biosynthesis in *Sphingomonas chungbukensis*

Sphingolipids act as lipid mediators for cell growth in mammalian cells. However, bacterial sphingolipid metabolism pathway has not revealed yet. *S. chungbukensis* was reported to contain sphingoid bases (sphingosine and sphinganine) and their 1-phosphate (sphingosine 1-phosphate and sphinganine 1-phosphate) (Burenjargal *et al.*, 2007). The contents of bacterial sphingoid bases and their 1-phosphate were known to be related to the growth of *S. chungbukensis*. In this study, *S. chungbukensis* also contains ceramide and dihydroceramide in its composition. When *S. chungbukensis* was cultured under either shaking or static condition for 24 h (Fig. 2), the bacterial levels of ceramide and dihydroceramide under shaking culture condition were higher than that of static condition. The concentrations of bacterial ceramide under aeration and static culture conditions were 2.0 ± 1.2 and 0.4 ± 0.3 nmol/mg protein, respectively. The dihydroceramide concentrations of bacteria grown under the shaking and static conditions were 10.6 ± 5.5 and 3.0 ± 1.8 nmol/mg protein, respectively. It is surprising that bacterial dihydroceramide levels in both shaking and static culture conditions are much higher than ceramide levels by 5-8 folds. These results indicate that dihydroceramide rather than ceramide in *S. chungbukensis* may play major roles in bacterial physiology.

Effects of fumonisin B_1 on sphingolipid concentration in *Sphingomonas chungbukensis* and HIT-T15 cells

Fumonisin B_1 (FB_1) is a specific inhibitor of ceramide synthase in *de novo* sphingolipid biosynthesis pathway in mammalian cells (Fig. 1). However, there is no report regarding FB_1 inhinition of bacterial ceramide synthase. Thus, the levels of ceramide and dihydroceramide between *S. chungbukensis* and HIT-T15 cells, hamster pancreatic β-cells, were compared each other after were treated with FB_1 (Fig. 3). When HIT-T15 cells were treated with FB_1 at 10 μM for 24 h (Fig. 3A), dihydroceramide level was elevated to 0.19 ± 0.01 nmol/mg protein from 0.025 ± 0.018 nmol/mg protein in control culture, and ceramide content was decreased to 18 ± 12 nmol/mg protein from 3.8 ± 3.3 nmol/mg protein in control culture. These results demonstrated that *de novo* sphingolipid pathway in HIT-T15 cells was inhibited by FB_1. However, FB_1 elevated ceramide levels by 2.8-folds and dihydroceramide levels by 4.2-folds in *S. chungbukensis* culture

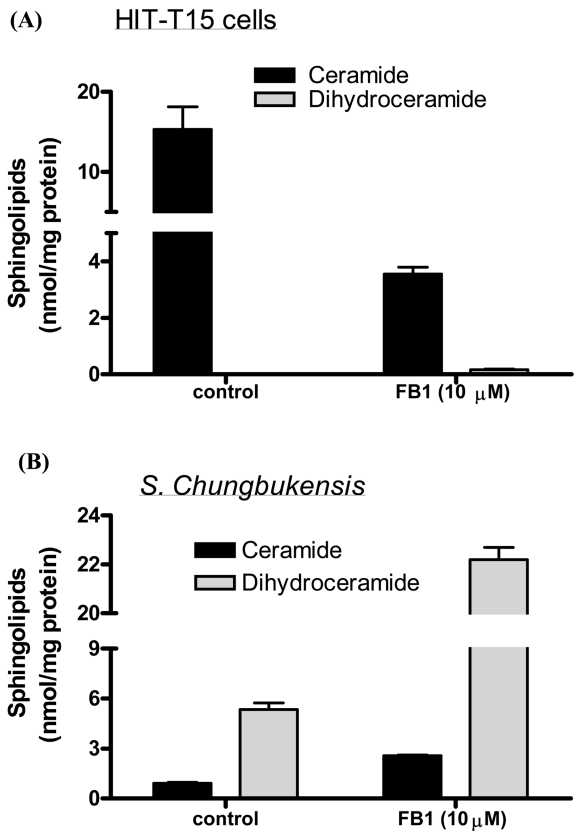

Fig. 3. Effect of fumonisin B_1 on the levels of ceramide and dihydroceramide in HIT-T15 cells and *Sphingomonas chungbukensis*. (A) HIT-T15 cells were cultured until 90% confluence and (B) *S. chungbukensis* was grown under aerobic shaking condition for 24 h in the presence of 10 μM fumonisin B_1, a specific inhibitor of ceramide synthase in mammalian cells. The concentrations ceramide and dihydroceramide were analyzed by HPLC. Fumonisin B_1 was abbreviated by FB_1. Values are expressed as mean ± S.D. Differences with $^{**}p<0.01$ were defined as statistically significant with ceramide value of control culture in *S. chungbukensis*.

(Fig. 3B). These results suggested that FB_1 is not an inhibitor of ceramide synthase-like enzyme in *S. chungbukensis*. It was surprising that FB_1 appeared to be an activator of ceramide biosynthesis, primarily dihydrceramide, for the unknown reason in *S. chungbukensis*.

Effects of desipramine on sphingolipid concentration in *Sphingomonas chungbukensis* and HIT-T15 cells

Desipramine is an inhibitor of acidic sphingomyelinase, which converts sphingomyelin into ceramide, in mammalian cells. Therefore, ceramide elevation-induced cell death was reduced by the desipramine treatment as a result of the decreased ceramide level (Hurwitz *et al.*,

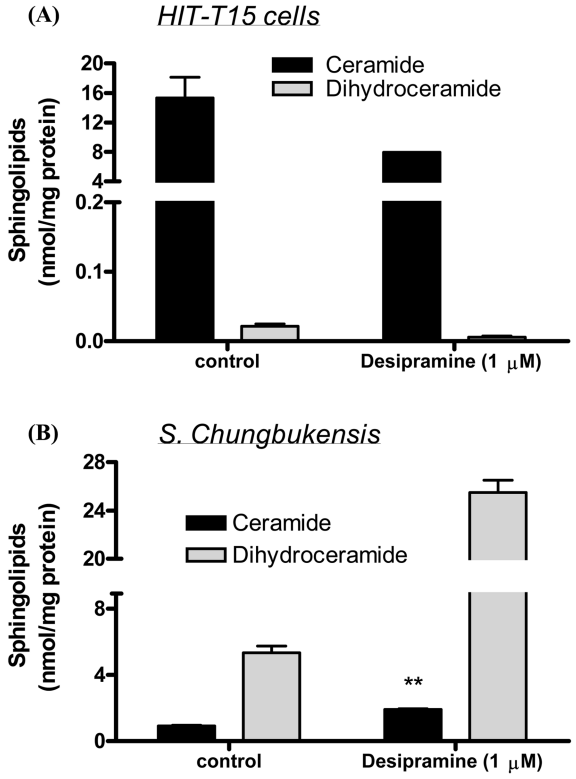

Fig. 4. Effect of desipramine on the levels of ceramide and dihydroceramide in HIT-T15 cells and *Sphingomonas chungbukensis*. (A) HIT-T15 cells were cultured until 90% confluence and (B) *S. chungbukensis* was grown under aerobic shaking condition for 24 h in the presence of 1 μM desipramine, a specific inhibitor of acidic sphingomyelinase. The concentrations of ceramide and dihydroceramide were analyzed by HPLC. Differences with $^{**}p<0.01$ were defined as statistically significant with ceramide value of control culture in *S. chungbukensis*.

1994). However, there is no report about the modulation of bacterial sphingomyelinase. In this study, the response of ceramide biosynthesis to desipramine was observed by measuring the concentrations of ceramide and dihydroceramide in *S. chungbukensis.* Sphingolipid intermediates between *S. chungbukensis* and HIT-T15 cells were compared after cells and the bacteria were treated with desipramine at 1 μM for 24 h (Fig. 4). When HIT-T15 cells were treated with deispramine (Fig. 3A), ceramide and dihydroceramide were decreased by 48% and 73%, respectively. This result showed that ceramide-sphingomyelin cycle in mammalian cells was inhibited by desipramine. In *S. chungbukensis* culture, desipramine increased the levels of ceramide and dihydroceramide by approximately 5-folds compared to control (Fig. 4B). These results suggested that desipramine is not an inhibitor of sphingomyelinase-like enzyme in *S. chungbukensis*. Oppositely, desipramine is an activator of dihydroceramide synthesis for the unknown mechanism in the bacteria.

DISCUSSION

Sphingolipids are important regulators of cellular physiology such as cell proliferation, apoptosis, differentiation, and angiogenesis as well as structural components of the cellular membranes in mammalian cells. However, the occurrence of ceramide and dihydroceramide, mammalian sphingolipid intermediates, in bacteria has been considered to be extremely rare (Minamino *et al.*, 2003). The sphingolipids of mammalian tissues, plants, fungi and yeasts have been characterized. Other than serine palmitoyltransferase (Ikushiro *et al.*, 2003), little is known about the biosynthetic pathway of sphingolipids in bacteria. The *Sphingomonas* species, however, is a relatively well-known strain as regards to sphingolipid metabolism in bacteria (Kawahara *et al.*, 2000; Burenjargal *et al.*, 2007). The optimal culture conditions for bacterial growth were found to be aeration and shaking (Burenjargal *et al.*, 2007). Aeration and shaking increased bacterial growth by 8-folds compared to the growth under the static culture condition in *S. chungbukensis*. The sphingolipid intermediates in *S. chungbukensis* have been known to include sphingosine, sphinganine and their 1-phosphate as well as total complex sphingolipids with the backbones of sphingoid bases (Burenjargal *et al.*, 2007). Ceramide and dihydroceramide were also determined at the amount of moles per mg protein in *S. chungbukensis* (Fig. 2). Thus, the composition of sphingolipids in *S. chungbukensis* appeared to be similar to one in mammalian cells, and the bacterial growth was also related to the levels of sphingolipid metabolites.

Sphingolipid metabolism pathway was modulated by several known inhibitors of sphingolipid metabolism enzymes in mammalian cells. FB_1 is a specific inhibitor for ceramide synthase of *de novo* sphingolipid biosynthesis in mammalian cells. In HIT-T15 cells, hamster β-cells, treated with FB_1, the levels of ceramide and dihydroceramide (Fig. 3B) were decreased. However, FB_1 elevated the concentrations of ceramide and dihydroceramide by several folds in *S. chungbukensis* (Fig. 3A). Therefore, FB_1 is not an inhibitor of ceramide synthase and appears to be an activator of ceramide biosynthetic pathway in *S. chungbukensis*. The major target for sphingolipid accumulation in *S. chungbukensis* is dihydroceramide rather than ceramide. Desipramine, a tricyclic antidepressant, inhibits acidic sphingomyelinase in mammalian cells and

reduced the intracellular levels of ceramide and dihydroceramide (Fig. 4B), endogenously occurring apoptosis inducer. However, desipramine increased ceramide and dihydroceramide in *S. chungbukensis* (Fig. 3A), indicating that desipramine seems to activate the ceramide biosynthetic pathway. Although sphingolipid metabolites including sphingoid bases, ceramide and dihydroceramide between mammalian cells and bacteria are common, *de novo* biosynthetic pathway in bacteria appeared to be much more active than that in mammalian cells because the rapid rate of bacterial cell division may require high amount of sphingolipids.

Few reports regarding bacterial sphingolipid metabolism have been published. *Sphingomonas paucimobilis* $EY2395^T$ has been shown to have serine palmitoyltransferase (SPT) activity (Ikushiro *et al.*, 2003). SPT appeared to be a common enzyme between bacteria and mammalian cells, but other enzymes in sphingolipid biosynthetic pathway were not elucidated in bacteria. Bacteria appeared to have different sphingolipid biosynthetic pathway from mammalian cells. The bacterial sphingolipids, primarily dihydroceramide, activated by the FB_1 and desipramine, may be a promising source for the industrial applications.

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