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Structural analysis of sphingophospholipids derived from *Sphingobacterium spiritivorum*, the type species of genus *Sphingobacterium*

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Abstract

The unique feature of the genus *Sphingobacterium* is the presence of sphingophospholipids and ceramides, besides diacylglycerophospholipids. As major cellular lipid components, five kinds of sphingophospholipids were purified from *Sphingobacterium spiritivorum* ATCC 33861^T, the type species of genus *Sphingobacterium*. They were identified as ceramide phosphorylethanolamines (CerPE-1 and CerPE-2), ceramide phosphoryl-*myo*-inositols (CerPI-1 and CerPI-2), and ceramide phosphorylmannose (CerPM-1). The ceramide of CerPE-1, CerPI-1, and CerPM-1 was composed of 15-methylhexadecasphinganine (isoheptadeca sphinganine, *iso*-C17:0) and 13-methyltetradecanoic acid (isopentadecanoic acid, *iso*-C15:0), whereas that of CerPE-2 and CerPI-2 was composed of isoheptadeca sphinganine and 2-hydroxy-13-methyltetradecanoic acid (2-hydroxy isopentadecanoic acid, 2-OH *iso*-C15:0). These sphingophospholipids were also found in cellular lipids of *Sphingobacterium multivorum* ATCC 33613^T, *Sphingobacterium mizutaii* ATCC 33299^T, *Sphingobacterium faecium* IFO 15299^T, *Sphingobacterium thalpophilum* ATCC 43320^T, and *Sphingobacterium antarcticum* ATCC 51969^T. To our knowledge, the existence of CerPM-1 is a novel sphingophospholipid through eukaryotic and prokaryotic cells.

Keywords: *Sphingobacterium*; Sphingophospholipid; Ceramide; Bacteria; Taxonomy

1. Introduction

Sphingophospholipids (SPLs) are ubiquitous components of eukaryotic cell membranes, although most bacteria do not possess them. Certain bacteria that have SPLs as cellular lipids are *Bdellovibrio bacteriovorus* [1], *Flectobacillus major* [2], *Pedobacter* spp. [3], *Prevotella melaninogenica* (formerly *Bacteroides melaninogenicus*) [4–6], *Prevotella ruminicola* (formerly *Bacteroides ruminicola*) [6,7], *Bacteroides fragilis* [8–10], *Porphyromonas gingivalis* (formerly *Bacteroides gingivalis*) [8], and *Sphingobacterium* spp. [11–13]. However, the detailed structures and biosynthetic pathways of sphingolipids in bacteria remain

Abbreviations: SPLs, sphingophospholipids; SSPLs, sphingobacterial sphingophospholipids; TMS, trimethylsilyl; GC, gas chromatography; GC/MS, gas chromatography-mass spectrometry; FAB/MS, fast atom bombardment-mass spectrometry; NMR, nuclear magnetic resonance; FID, free induction decay; INEPT, insensitive nuclei enhanced by polarization transfer; 2-D, two-dimensional; COSY, correlated spectroscopy; ROESY, rotating frame nuclear overhauser effect spectroscopy; HMQC, heteronuclear multiple-quantum coherence; HMBC, heteronuclear multiple-bond correlation

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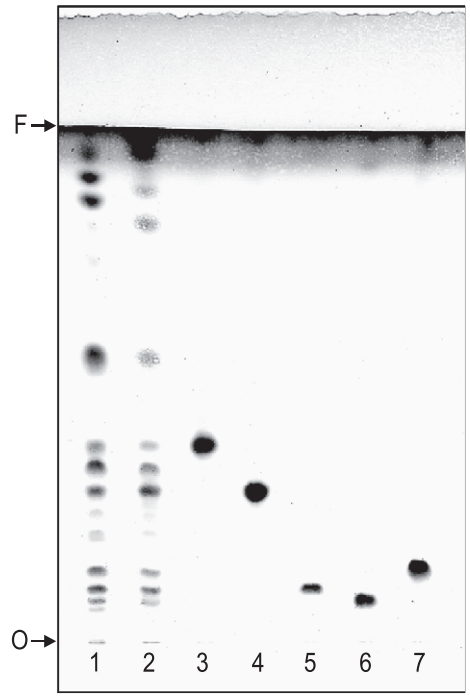

Fig. 1. TLC of lipids from *S. spiritivorum*. The developing solvent was chloroform–methanol–acetic acid–water (100:20:12:5, vol/vol/vol/vol). After spraying with 50% sulfuric acid solution, the spots were visualized by charring at 200 °C. O and F indicate origin and front of solvent, respectively. Lane 1, crude lipids; lane 2, alkali-stable lipids; lane 3, SSPL1, ceramide phosphorylethanolamine-1 (CerPE-1) containing non-hydroxy fatty acids; lane 4, SSPL2, ceramide phosphorylethanolamine-2 (CerPE-2) containing 2-hydroxy fatty acids; lane 5, SSPL3, ceramide phosphorylinositol-1 (CerPI-1) containing non-hydroxy fatty acids; lane 6, SSPL4, ceramide phosphorylinositol-2 (CerPI-2) containing 2-hydroxy fatty acids; lane 7, SSPL5, ceramide phosphorylmannose-1 (CerPM-1) containing non-hydroxy fatty acids.

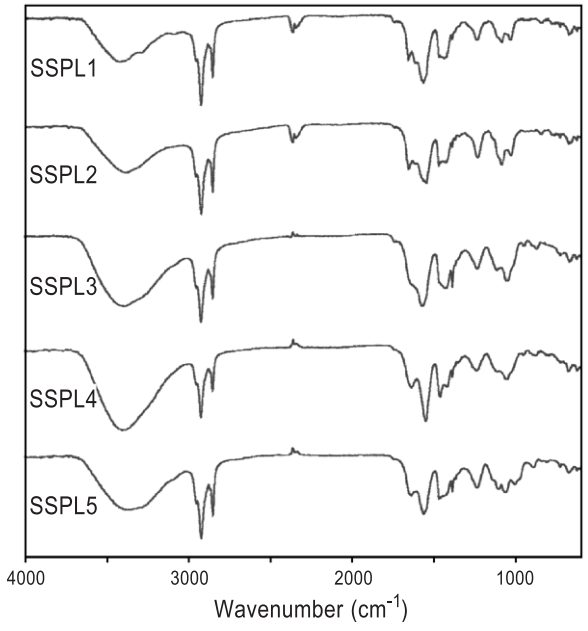

Fig. 2. Infrared absorption spectra of five classes of SSPLs. Infrared spectra were analyzed by a Fourier transform infrared spectrometer using potassium bromide.

Table 1
Distribution of SSPLs in *Sphingobacterium* spp.

Species	Strain no.	Sphingophospholipids				
		SSPL1	SSPL2	SSPL3	SSPL4	SSPL5
S. spiritivorum	ATCC 33861^T	+[a]	+	+	+	+
S. multivorum	ATCC 33613^T	+	+	+	+	+
S. mizutaii	ATCC 33299^T	+	+	tr[b]	tr	+
S. thalpophilum	ATCC 43320^T	+	+	+	+	+
S. faecium	IFO 15299^T	+	+	+	tr	+
S. antarcticum	ATCC 51969^T	n.d.[c]	n.d.	+	+	n.d.

[a] (+) Presence.
[b] (tr) Trace.
[c] (n.d.) Not detected.

unknown. We have previously reported the unique branched long-chain bases and ceramides in *Sphingobacterium* species [12,13]. The major molecular species of ceramides in sphingobacteria have been identified as 2-*N*-2′ -hydroxy-13′ -methyltetradecanoyl-15-methylhexadecasphinganine, 2-*N*-13′ -methyltetradecanoyl-15-methylhexadecasphinganine, and 2-*N*-13′ -methyltetradecanoyl-hexadecasphinganine [12,13], although the precise chemical structure of SPLs derived from the genus *Sphingobacterium* has not been clarified.

Sphingobacterium spiritivorum, the type species of genus *Sphingobacterium*, is a rod-shaped, aerobic, gram-negative, and oxidizes glucose in oxidation-fermentation media. Sphingobacteria are isolated from environments [11,14] and clinical specimens of blood, urine, and the uterus [15] from human patients with opportunistic infection. The important feature of sphingobacteria is the presence of high concentrations of SPLs as cellular lipid components. Based on genetic analysis by DNA–DNA hybridization and the existence of SPLs, it has been established as the genus *Sphingobacterium*, and is distinguished from the genus *Flavobacterium* [11]. Nowadays, the genus *Sphingobacterium* consists of six species, including *S. spiritivorum*, *Sphingobacterium multivorum*, *Sphingobacterium mizutaii*, *Sphingobacterium antarcticum*,

Table 2
The composition of fatty acids and long-chain bases in SSPLs

Ceramide component	Chain length	Sphingophospholipids				
		SSPL1	SSPL2	SSPL3	SSPL4	SSPL5
Fatty acid (%)	14:0			6.8		18.6
	iso-15:0	91.9		88.6		74.1
	16:0	8.1		4.6		7.3
	2-OH 14:0		3.5		6.0	
	2-OH *iso*-15:0		94.8		92.0	
	2-OH 16:0		1.7		2.0	
Long-chain base (%)	d[a]-16:0	5.8	5.9	7.8	6.2	16.3
	d-*iso*-17:0	83.6	84.6	81.1	89.0	79.0
	d-18:0	10.6	9.5	11.1	4.8	4.7

[a] (d) Dihydroxy sphingoid.

Fig. 3. TLC of water-soluble components of SSPL1 and SSPL2. Water-soluble components of SSPL1 and SSPL2 were obtained by hydrolysis with hydrofluoric acid at 0 °C for 50 h. The developing solvent was ethanol–water (1:9, vol/vol), and spots were visualized by spraying with ninhydrin reagent. Lane 1, authentic ethanolamine; lane 2, SSPL1; lane 3, SSPL2.

Sphingobacterium faecium, and *Sphingobacterium thalpophilum* [11,14,16].

In the present study, we describe the chemical structure of five classes of sphingobacterial sphingophospholipids (SSPLs), including a novel mannose-possessing SPL derived from *S. spiritivorum*.

2. Materials and methods

2.1. Bacterial strains and purification of SSPL

S. spiritivorum ATCC 33861, *S. multivorum* ATCC 33613, *S. mizutaii* ATCC 33299, *S. faecium* IFO 15299, *S. thalpophilum* ATCC 43320, and *S. antarcticum* ATCC 51969 were grown aerobically in culture media containing 1.0% polypeptone, 0.5% yeast extract, and 1.0% glucose with pH 7.0. After 30 h incubation with shaking at 30 °C, bacteria were harvested, and then crude lipids were extracted with chloroform–methanol (2:1, vol/vol). Crude lipids were hydrolyzed with 0.5 N sodium hydroxide in chloroform–methanol (1:1, vol/vol) at 37 °C for 1 h. After neutralization, alkali-stable lipids were extracted with chloroform–methanol (2:1, vol/vol) [12,13], and then were separated by thin-layer chromatography (TLC) of silica gel G (Analtech, Newark, DE) with the acidic solvent system, chloroform–methanol–acetic acid–water (100:20:12:5, vol/vol/vol/vol).

2.2. TLC and infrared spectrometric analyses of SSPLs

SSPLs were developed on TLC with the acidic solvent system. Lipids were visualized by spraying Dittmer–Lester reagent for phosphorus, ninhydrin reagent for amino group, and anthrone reagent for glycolipids. SSPLs were identified

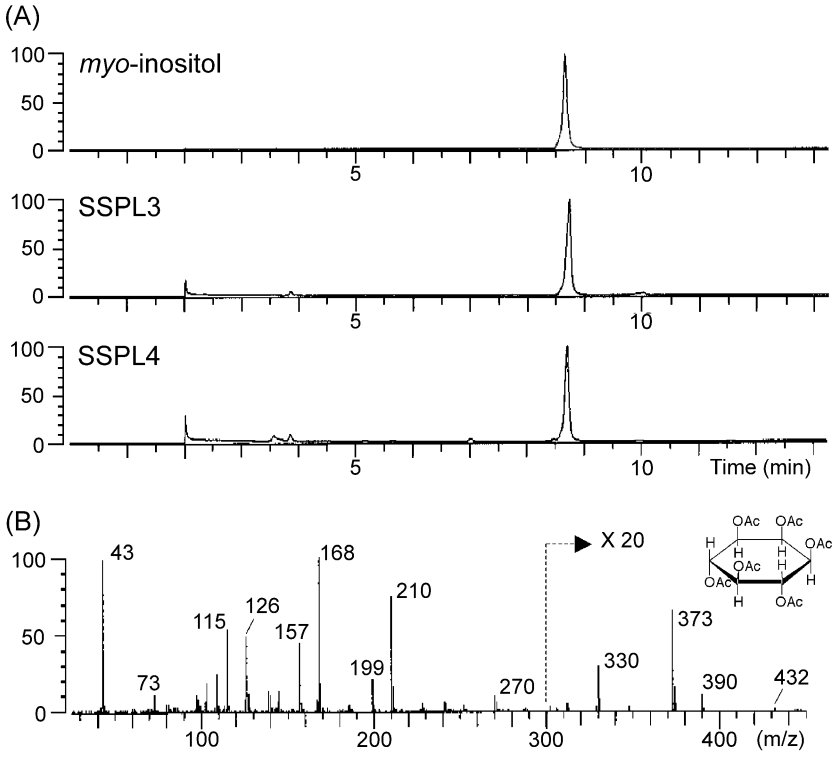

Fig. 4. Total ion gas chromatograms (A) and mass spectrum (B) of acetylated derivatives of water-soluble components of SSPL3 and SSPL4. The fused silica capillary column SP-2380 (15 m × 0.25 mm in internal diameter, Supelco) was used. GC oven was operated at 185 °C for 2 min, increasing to 230 °C at a rate of 4 °C/min, and flow rate of helium carrier gas was 1 ml/min. Ac; acetyl group (CH_3CO^-).

by R_f value on TLC. Infrared spectra were analyzed by a Fourier transform infrared spectrometer using potassium bromide (PARAGON 1000, Perkin-Elmer Ltd., Beaconsfield Bucks, England).

2.3. Analysis of fatty acids and long-chain bases

To analyze fatty acids and long-chain bases, SSPL1-5 were hydrolyzed with 2 N hydrochloric acid in methanol at 100 °C for 3 h. After cooling, an equal volume of water was added, and then fatty acid methyl esters were extracted with *n*-hexane. The aqueous phase was adjusted to pH 12 with potassium hydroxide, and then the long-chain base was extracted with *n*-hexane-diethyl ether (1:1, vol/vol). Long-chain bases were trimethylsilylated with pyridine-bis trimethylsilyl (TMS) trifluoroacetamide (1:2, vol/vol) at 70 °C for 30 min. Fatty acid methyl esters and TMS derivatives of long-chain bases were analyzed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC/MS) [12,13]. GC/MS was performed with a double focusing mass spectrometer (SX102A, JEOL, Tokyo, Japan) equipped with a GC system (HP5890 Series II, Hewlett Packard, Avondale, PA). SPB-1 (30 m × 0.25 mm in internal diameter, fused silica capillary column, Supelco Inc., Bellefonte, PA) was used as a column in the system.

2.4. Analysis of water-soluble components of SSPLs

SSPL1 and SSPL2 were hydrolyzed in 46% hydrofluoric acid at 0 °C for 50 h. After the procedure, ethanolamine was developed on TLC by using the solvent system, ethanol–water (1:9, vol/vol). Water-soluble components of SSPL3 and SSPL4 were obtained by hydrolysis with 6 N hydrochloric acid at 120 °C for 50 h. Inositol was acetylated with pyridine–acetic anhydride (1:1, vol/vol) at 100 °C for 1 h, and its acetylated derivative was analyzed by GC/MS [17]. Mannose of SSPL5 was identified as alditol acetate and partially methylated alditol acetate derivatives by GC/MS. Alditol acetate derivatives were obtained by hydrolysis with 2 N trifluoroacetic acid (120 °C, 2 h), reduction with sodium borohydride (room temperature, 2 h), and acetylation. Partially methylated alditol acetate derivative of SSPL5 was obtained by methylation with dimethyl iodide before formation of alditol acetate [18,19].

2.5. Fast atom bombardment-mass spectrometry (FAB/MS) spectra

FAB/MS of purified SSPLs was performed in the negative-ion mode using a double focusing mass spectrometer equipped with a MS-MD 7000 computer system (JEOL). The spectrum was recorded at an accelerating voltage of 8.0 kV. Xenon gas was used as the primary beam. Each sample was dissolved in chloroform–methanol (2:1, vol/vol) and adjusted to 5 μg/μl, and then 1 μl was applied to the target with a matrix solution of triethanolamine [20].

2.6. Nuclear magnetic resonance (NMR) spectrometry

Purified SSPL5 was deuterium exchanged by dissolving in $CDCl_3/CD_3OD$ (2:1, vol/vol), evaporating thoroughly under dry nitrogen, and then dissolved in $CDCl_3/CD_3OD/D_2O$ (3:1:0.1, vol/vol/vol) for NMR analysis [20–23]. Spectra of ^1H- and ^{13}C-NMR were recorded by an LA-400 spectrometer (JEOL) at 50 °C. Chemical shifts were referenced to internal chloroform (δ_H 7.24 ppm, δ_C 77.0 ppm). In the one-dimensional measurement (^1H- and ^{13}C-NMR), 64,000 data points were used for the free induction decay (FID). Heteronuclear coupling constants ($^1J_{H,C}$) were obtained using the standard pulse sequence of the insensitive nuclei enhanced by polarization transfer (INEPT)-nondecoupling by a LA-400 spectrometer. Two-dimensional (2-D) NMR, ^1H-^1H-gradient correlated spectroscopy (COSY), rotating frame nuclear overhauser effect spectroscopy (ROESY), heteronuclear multiple-quantum coherence (HMQC), and heteronuclear multiple-bond correlation (HMBC) experiments were performed on an AVANCE-600 spectrometer (Bruker, Rheinstetten, Germany) at 50 °C. The pulse programs of 2-D NMR experiments were supplied by the Bruker software library in the Bruker Avance-version [24,25].

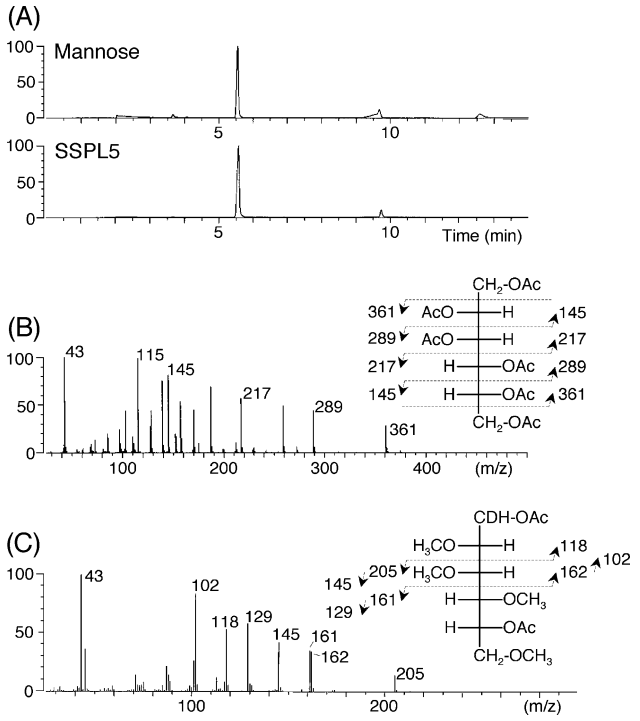

Fig. 5. Total ion gas chromatogram (A), mass spectrum (B) of alditol acetate derivative, and partially methylated alditol acetate derivative (C) of water-soluble component of SSPL5. The fused silica capillary column SP-2380 was used, and GC oven was operated to increase linearly 5 °C/min from 180 to 250 °C (A), and at 140 °C for 3 min, increasing to 220 °C at a rate of 5 °C/min (C). Flow rate of helium carrier gas was 1 ml/min. Ac; acetyl group (CH_3CO^-). The fragment pattern of partially methylated alditol acetate derivative of water-soluble component of SSPL5 indicated 2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetyl mannitol.

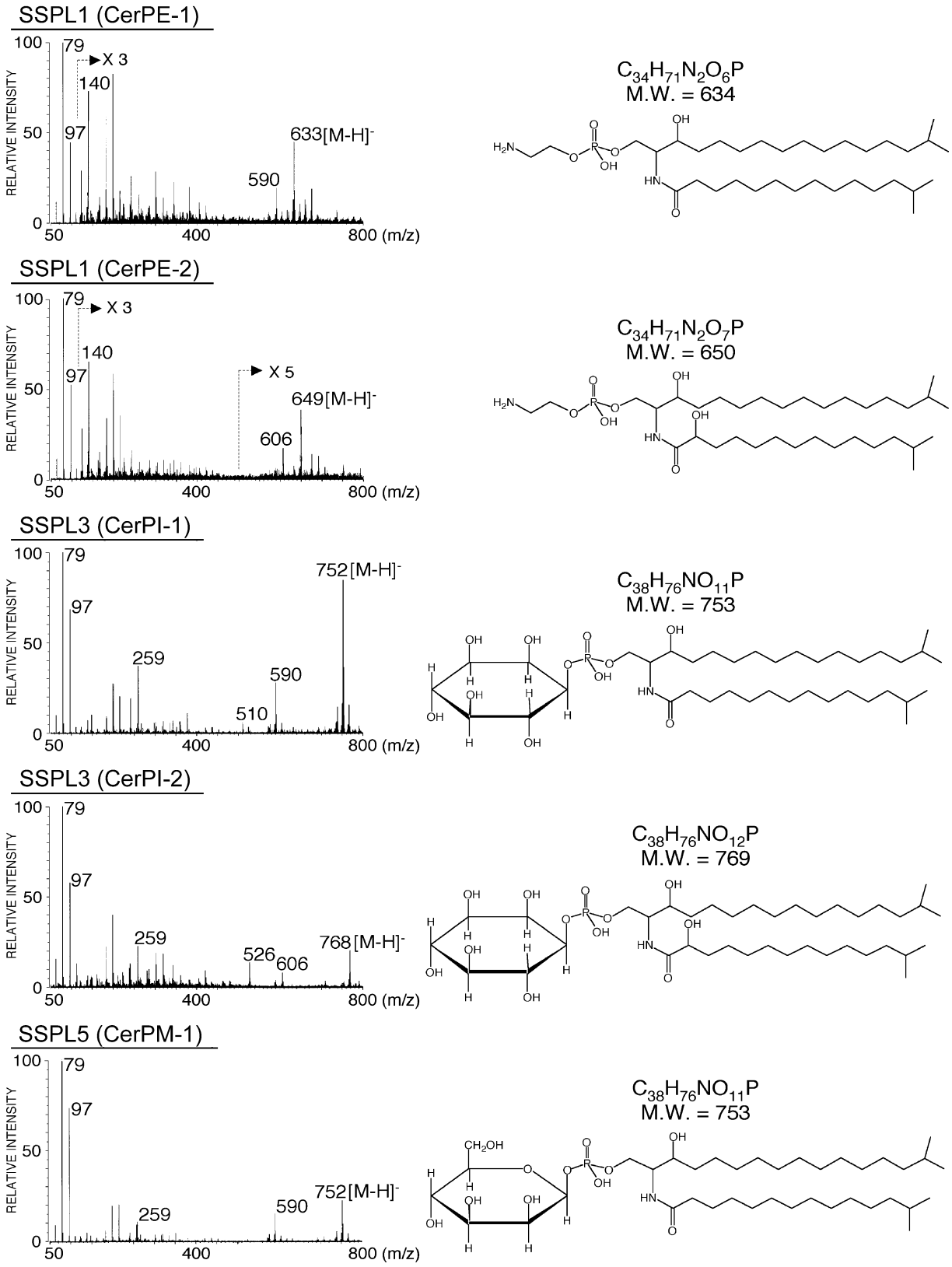

Fig. 6. Negative-ion mode FAB/MS spectra and chemical structures of five classes of SSPLs derived from *S. spiritivorum*. FAB/MS spectra of SSPL1, SSPL2, SSPL3, SSPL4, and SSPL5 showed quasimolecular ion $[M-H]^-$, *m/z* 633, 649, 752, 768, and 752, respectively. Proposed chemical structures of SSPLs were ceramide phosphorylethanolamines (CerPE-1 and CerPE-2), ceramide phosphoryl-*myo*-inositols (CerPI-1 and CerPI-2), and ceramide phosphoryl-1-β-mannose (CerPM-1).

3. Results

3.1. TLC and infrared spectrometric analyses of SSPLs

The yield of crude lipid was 31.6 mg per g wet weight of *S. spiritivorum* ATCC 33861. We calculated that the approximate amount of sphingolipids in crude lipid was 23% based on the colorimetric analysis of free sphingoid bases [26]. Five classes of alkali-stable lipids from *S. spiritivorum* ATCC 33861, SSPL1-5, were purified by using TLC developed with acidic solvent system, until a single spot was obtained (Fig. 1). The R_f values of SSPL1, SSPL2, SSPL3, SSPL4, and SSPL5 showed 0.38, 0.30, 0.10, 0.08, and 0.14 on the TLC, respectively. These lipids were resistant against mild alkali hydrolysis and showed positive reaction to Dittmer–Lester reagent. Both SSPL1 and SSPL2, but not SSPL3–5, exhibited positive reaction to ninhydrin reagent. SSPL3–5, but not SSPL1 and SSPL2, showed positive reaction to anthrone reagent. The infrared spectra of five classes of SSPLs showed no prominent absorption at 1720–1740 cm^{-1} due to an ester carbonyl group, but showed absorption at 1650–1635 cm^{-1} (C=O stretching) and 1560 cm^{-1} (N–H deformation) due to an acid amide linkage (Fig. 2). In addition, these lipids had absorptions at 1230 cm^{-1} (P=O stretching) and 1050–1020 cm^{-1} (P–O–C deformation) due to phosphate ester, suggesting that all SSPLs are SPLs [4,27]. Distribution of SSPLs from six species of the genus *Sphingobacterium* was summarized (Table 1). Alkali-stable lipids of *S. multivorum*, *S. mizutaii*, *S. faecium*, and *S. thalpophilum* possessed five classes of SSPLs analyzed by TLC. However, SSPL1, SSPL2, and SSPL5 were not detected in alkali-stable lipids of *S. antarcticum*.

3.2. Composition of fatty acids and long-chain bases

Based on the quasimolecular ion $[M]^+$ (*m/z* 256, *iso*-C15:0; *m/z* 272, 2-OH *iso*-C15:0), both isopentadecanoic acid and 2-hydroxy isopentadecanoic acid were identified. They had characteristic fragment ions of non-hydroxy fatty acid ($[M-43]^+$ and $[M-31]^+$) and 2-hydroxy fatty acid ($[M-59]^+$, *m/z* 90 and *m/z* 103). Thus, the major fatty acid was isopentadecanoic acid *(iso*-C15:0) in SSPL1, SSPL3, and SSPL5, and was 2-hydroxy isopentadecanoic acid (2-OH *iso*-C15:0) in SSPL2 and SSPL4. The mass spectra of TMS derivatives of long-chain bases from SSPLs showed a typical mass fragmentation pattern. An intense peak at *m/z* 132 was due to C2–C3 cleavage of TMS long-chain bases. The fragment ions of *m/z* 416 $[M-15]^+$, *m/z* 328 $[M-103]^+$, and *m/z* 299 $[M-132]^+$ indicated each molecule ion $[M]^+$ (*m/z* 431), although the molecular ion peak $[M]^+$ was very weak. Long-chain bases from five classes of SSPLs were dihydrosphingosines, and most of them were an isoheptadeca sphinganine. The composition of fatty acid and long-chain base was summarized in Table 2.

3.3. Water-soluble components of SSPLs

Water-soluble components of SSPL1 and SSPL2 obtained by hydrofluoric acid hydrolysis were reactive with ninhydrin reagent, and showed the identical R_f value with the authentic ethanolamine developed on TLC (Fig. 3). Acetylated derivatives of water-soluble components from SSPL3 and SSPL4 showed the similar retention time when compared to those of authentic *myo*-inositol (Fig. 4A). GC/MS spectra of acetylated derivatives from SSPL3 and SSPL4 showed the same fragment pattern, similar to authentic *myo*-inositol (Fig. 4B). The molecular ion $[M]^+$ (*m/z* 432) and fragment ion $[M-42]^+$ (*m/z* 390) were detected in the spectra of acetylated derivatives from SSPL3 and SSPL4. The fragment ion $[M-42]^+$ was due to loss of the element of ketene from the molecular ion $[M]^+$. These results showed a typical pattern of mass fragmentation of *myo*-inositolhexaacetates. The alditol acetate derivative of water-soluble component of SSPL5 exhibited the similar retention time to that of authentic D-(+)-mannose (Fig. 5A). The mass spectrum showed characteristic fragment ions at *m/z* 361, 289, 217, and 145 (Fig. 5B). The GC/MS spectrum of partially *O*-methylated alditol acetate derivative revealed characteristic fragment ions of 2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetyl mannitol at *m/z* 205, 162, 161, 145, 129, 118, and 102 (Fig. 5C). The result implies that the phosphoric acid is bound to C-1 position of mannose.

3.4. Negative-ion mode FAB/MS spectra of SSPLs

FAB/MS spectra of all lipids tested here showed common fragment ions of *m/z* 79 and 97 due to PO_3 and $H_2PO_4^-$, respectively. FAB/MS spectra of SSPL1, SSPL2, SSPL3, SSPL4, and SSPL5 showed quasimolecular ion $[M-H]^-$, *m/z* 633, 649, 752, 768, and 752, respectively. The characteristic fragment ion $[ceramide\text{-}HPO_4]^-$ due to phosphorylceramide moiety was *m/z* 590 (fatty acid composition, *iso*-C15:0) of SSPL1, SSPL3, and SSPL5, and *m/z* 606 (fatty acid composition, 2-OH *iso*-C15:0) of SSPL2 and SSPL4 (Fig. 6). FAB/MS spectra of all lipids showed the characteristic fragment ion $[M-ceramide]^-$ due to elim-

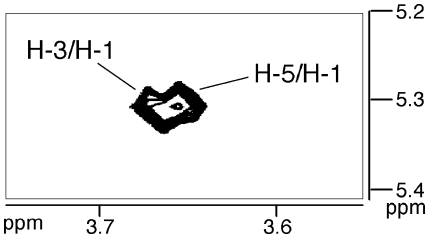

Fig. 7. Part of a 2-D 600-MHz ROESY spectrum of SSPL5. Shown are the ROE cross-peaks of the anomeric proton. The two ROE contacts between the H-3/H-1 and the H-5/H-1 protons of the mannose are diagnostic for the β-anomer.

Table 3
^{1}H and ^{13}C NMR assignments of SSPL5

Position in the structure[a]		Chemical sift[b]	
		δ_C (ppm) [(mult[c]), J (Hz)]	δ_H (ppm) [(mult), J (Hz)]
Mannose	1	95.85 [d, 5.0]	5.310 [d, 5.1]
	2	70.27	3.754
	3	73.94	3.674
	4	67.36	3.415
	5	70.36	3.651
	6	61.49	3.500/3.770
Ceramide	1′	65.05 [d, 4.0]	3.950 [br]/3.774 [br]
	2′	53.91	3.750
	3′	70.27	3.754
	4′	33.20	1.390
	5′	33.40	1.220
	6′	29.50	1.100
	7′	174.59	–
	8′	36.20	2.043 [t, 7.3]
	9′	27.29	1.413 [sept, 7.1]
	10′	29.50	1.100
	11′	38.80	0.969 [q, 6.8]
	12′	27.65	1.347 [sept, 6.7]
	13′	22.15	0.685 [d, 6.6]
	$(CH_2)_7$	29.00–29.65 [m]	1.090–1.150

[a] See Fig. 8 for number assignments.
[b] ^{1}H and ^{13}C chemical shifts at 50 °C in $CDCl_3/CD_3OD/D_2O$ (3:1:0.1, vol/vol/vol) relative to internal TMS.
[c] Multiplicity; d, doublet; br, broad; tr, triplet; sept, septet; q, quartet; m, multiplet.

ination of ceramide from SSPLs. The spectra of SSPL1 and SSPL2 showed fragment ions *m/z* 140 caused by phosphorylethanolamine, whereas spectra of SSPL3, SSPL4, and SSPL5 revealed fragment ions *m/z* 259 caused by phosphorylhexose (SSPL3 and SSPL4, phosphorylinositol; SSPL5, phosphorylmannose). Existence of [ceramide-HPO_4]$^-$ (phosphorylceramide ion) and [M-ceramide]$^-$ (phosphorylethanolamine ion and phosphorylhexose ion) suggests that phosphate ester is located between a ceramide and a water-soluble component (ethanolamine and hexose). Additionally, FAB/MS spectra of SSPL4 and SSPL5 showed fragment ions *m/z* 510 (fatty acid composition, *iso*-C15:0) and 526 (fatty acid composition, 2-OH *iso*-C15:0) due to ceramide moiety, respectively.

3.5. NMR spectrometry of SSPL5

The protons of C-methyl groups manifested themselves as doublet at δ_H 0.685 ppm (J = 6.6 Hz). It is, therefore, concluded that hydrocarbon chains of both fatty acid and long-chain base of ceramide terminate are present in *iso*-propyl residues. This fact was also supported by the presence of a multiplet at δ_H 1.347 ppm from two methine protons (H-12′) coupled with protons of both the methyl groups and adjacent methylene group of hydrocarbon chain as number assignments (Fig. 8).

The detailed assignments of the proton resonances of SSPL5 were obtained from homonuclear 2-D COSY. We next determined whether the sugar ring conformation of SSPL5 is pursued using through-space connectivity by a phase-sensitive homonuclear 2-D ^{1}H ROESY [24,28]. Part of a 2-D 600-MHz ROESY spectrum of SSPL5 was shown in Fig. 7. The coupling constant ($J_{1,2}$) of H-1 proton in mannose of SSPL5 was 5.1 Hz (d = 5.31 ppm) from ^{1}H NMR analysis. Heteronuclear coupling constant ($^{1}J_{H,C}$) was 169.7 Hz for SSPL5. In addition, ROE cross-peaks, H-1/H-3 and H-1/H-5, were observed by ROESY (Fig. 7). The data suggest a β-linked mannose present in SSPL5 [28]. The phosphorylation site was determined by assigning the ^{13}C NMR spectrum, and presence of ^{31}P-^{13}C scalar coupling. Usually, the carbon atoms of ^{1}H-^{13}C provide a singlet spectrum by coupling with proton atoms [29,30]. The carbon atoms in C−O−P elicit the split, as a doublet spectrum by scalar coupling with phosphorus atoms. In ^{13}C NMR spectrum of SSPL5, the doublet spectra based on ^{31}P-^{13}C scalar coupling were observed in C-1 ($^{2}J_{C,P}$, 5.0 Hz) and C-1′ ($^{2}J_{C,P}$, 4.0 Hz). These results suggest that phosphate ester is located between ceramide and mannose, and support the result of FAB/MS (Fig. 6). Additional information about the configuration of SSPL5 was summarized in Table 3. The long-range coupling was obtained from ^{1}H-^{13}C HMBC experiment (Fig. 8). We found strong $^{3}J_{C,H}$ and $^{2}J_{C,H}$ multibond cross-peaks from the H-2′ and H-8′ proton signals to C-7′ carbonyl signal, and also weak $^{4}J_{C,H}$ multibond cross-peaks from the H-1′ proton signal to C-7′ carbonyl signal. Multibond connectivities were seen from the H-1 proton signal to the C-2 and C-3 carbon.

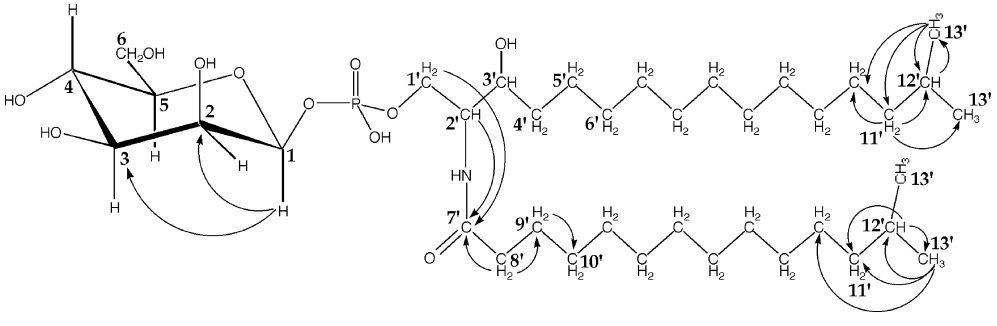

Fig. 8. Interaction of ^{1}H-^{13}C HMBC. Arrows indicate the long-range coupling in ^{1}H-^{13}C HMBC.

Similar results were obtained by using dimethyl sulfoxide-d_6 served as the solvent, instead of $CDCl_3/CD_3OD/D_2O$ (3:1:0.1, vol/vol/vol).

4. Discussion

Sphingobacterium spp. are oxidase-positive, indole-negative, gram-negative rods that form yellow-pigmented colonies. They have no flagella but may exhibit sliding motility. The currently described species of *Sphingobacterium* are *S. multivorum* (formerly *Flavobacterium multivorum*, CDC group IIk-2), *S. spiritivorum* (includes species formerly designated *Flavobacterium spiritivorum* and *Flavobacterium yabuuchiae*, and CDC group IIk-3), *S. mizutaii*, *S. thalpophilum*, *S. faecium*, *S. antarcticum*, and unnamed species *Sphingobacterium* genomospecies 1 and 2 [11,14,16]. *S. multivorum* and *S. spiritivorum* are the two species that have been most frequently recovered from human clinical specimens. *S. multivorum* has been isolated from various clinical specimens but has only rarely been associated with serious infections (peritonitis and sepsis) [31,32]. Blood and urine have been the most common sources for the isolation of *S. spiritivorum* [15].

Sphingolipids usually exist in eukaryotic cell membrane, but not in prokaryotes. Previously, we have isolated unique sphingoglycolipids possessing C21 cyclopropanoic long-chain base, 2-hydroxy myristic acid and anionic sugar such as glucuronic acid and galacturonic acid in the genus *Sphingomonas*, a gram-negative, glucose non-fermentative motile rod [20,33]. However, systematic investigation on the sphingophospholipids has not yet been performed in bacteria. In the present study, we have isolated and purified five classes of SSPLs from alkali-stable lipids of *S. spiritivorum*. The most abundant species of ceramide moiety were 2-*N*-13′ -methyltetradecanoyl-15-methylhexadecasphinganine in SSPL1, SSPL3, and SSPL5 and 2-*N*-2[1]-hydroxy-13′ -methyltetradecanoyl-15-methylhexadecasphinganine in SSPL2 and SSPL4 (Table 2). Analyses of water-soluble components of SSPLs, suggest the presence of phosphorylmonoethanolamine in SSPL1 and SSPL2, phophoryl-*myo*-inositol in SSPL3 and SSPL4, and phosphoryl-1-mannose in SSPL5. To identify the anomeric configuration of mannose in SSPL5, we examined the effect of α-mannosidase (ICN Biomedicals, Costa Mesa, CA) and β-mannosidase (Sigma-Aldrich, St. Louis, MO) [28]. However, these enzymes could not hydrolyze SSPL5. Because mannose is linked to phosphate ester, but not to oligosaccharides, α- and β-mannosidase may not digest SSPL5 as substrate. By contrast, ROE cross-peaks, H-1/H-3 and H-1/H-5 of a β-linked mannose, were observed by ROESY.

Based on these results, we propose the chemical structure of five classes of SSPLs derived from *S. spiritivorum* (Fig. 6). Chemically, they were identified as ceramide phosphorylethanolamine, SSPL1 (CerPE-1) and SSPL2 (CerPE-2); ceramide phosphoryl-*myo*-inositol, SSPL3 (CerPI-1) and SSPL4 (CerPI-2); and ceramide phosphoryl-1-β-mannose, SSPL5 (CerPM-1). Ceramide constituents of SSPLs were similar to ceramides of *Sphingobacterium* species [13]. The similar SSPLs were also seen in cellular lipids of *S. multivorum*, *S. mizutaii*, *S. faecium*, *S. thalpophilum*, and *S. antarcticum* assessed by TLC (Table 1).

CerPE has been demonstrated in *P. melaninogenica* [4–6], *B. fragilis* [8–10], *P. gingivalis* [8], and *F. major* [2] in prokaryotic cells, and oomycetes [34], rumen protozoan [35], adult housefly [36], blowfly [37], pupae of the green-bottle fly [38], chicken and rat liver [39], marine bivalve [40], and aquatic animals [41] in eukaryotic cells. CerPE-1 of *S. spiritivorum* was identical to CerPE isolated from *P. melaninogenica*. But CerPE-2 of *S. spiritivorum* differed in molecular species of CerPE in the previous reports. *P. melaninogenica* is gram-negative, non-spore forming, anaerobic bacteria. Clinical disease usually abscess formation with mixed anaerobic and facultative bacteria [6]. Microbial species now classified in *Provotella* and *Porphyromonas* genera were previously classified mainly in the genus *Bacteroides*. These anaerobic bacilli contain CerPE and CerPG (ceramide phosphorylglycerol) in common [8]. However, CerPG was not found in the alkali-stable lipids of *Sphingobacterium* spp.

CerPI has been found in *Phytophthora capsici* [42], *Phytophthora parasitica* [43], *Candida albicans* [44], *Cryptococcus neoformans* [45], and *Saccharomyces cerevisiae* [46,47] of fungi, and *F. major* [2] of bacteria. However, both CerPI-1 and CerPI-2 of *S. spiritivorum* differ in major ceramide moieties from CerPI found in the abovementioned microorganisms.

The presence of SPL containing mannose has been reported in eukaryotic cells, including fungi [28,44,45,48–57], protozoa [53], and plants [53,58]. Mannose of such SPLs derived from eukaryotic cells binds to inositol, but not to phosphate moieties. In contrast, mannose of CerPM-1 from *S. spiritivorum* binds directly to ceramide phosphate. To our knowledge, this study is the first report to demonstrate ceramide phosphoryl-1-β-mannose (CerPM-1) in prokaryotes and eukaryotes.

Our present study describes the distribution of unique SPLs in the genus *Sphingobacterium*, and demonstrates a novel SPL, CerPM-1, in eukaryotic and prokaryotic cells. Obviously, further studies are needed to clarify the biologic feature of SSPLs.

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