Detection and Identification of *Bacteriovorax stolpii* UKi2 Sphingophosphonolipid Molecular Species

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Bacteriovorax stolpii is a predator of larger gram-negative bacteria and lives as a parasite in the intraperiplasmic space of the host cell. This bacterium is unusual among prokaryotes in that sphingolipids comprise a large proportion of its lipids. We here report the presence of 18 molecular species of *B. stolpii* UKi2 sphingophosphonolipids (SPNLs). ^{31}P NMR spectroscopy and analysis of P_i released by a differential hydrolysis protocol confirmed the phosphonyl nature of these lipids. The SPNLs were dominated by those with 1-hydroxy-2-aminoethane phosphonate (hydroxy-aminoethylphosphonate) polar head groups; aminoethylphosphonate was also detected in minor SPNL components. The long-chain bases (LCBs) were dominated by C_{17} iso-branched phytosphingosine; C_{17} iso-branched dihydrosphingosine was also present in some SPNLs. The N-linked fatty acids were predominantly iso-branched and most contained an α-hydroxy group (C_{15} α-hydroxy fatty acid was the major fatty acid). Minor molecular species containing nonhydroxy fatty acids were also detected. The definitive iso-structures of the predominant fatty acids and LCBs present in the *B. stolpii* SPNLs were established using ^{13}C and ^{3}H nuclear magnetic resonance spectroscopy; less than 20% were unbranched. Detection and analyses of intact compounds by MS-MS were performed by a hybrid quadrupole time-of-flight (Q-TOF-II) MS equipped with an electrospray ionization source. Analyses of peracetylated derivatives verified the structural assignments of these lipids. (J Am Soc Mass Spectrom 2007, 18, 394–403) © 2007 American Society for Mass Spectrometry

Bacteriovorax stolpii, previously called *Bdellovibrio stolpii* [1] is a small predatory bacterium that attacks and parasitizes larger gram-negative bacteria. These organisms can scavenge host molecules [2, 3] but some strains have been placed in axenic cultures allowing for biochemical analyses without problems of contamination from host compounds [4]. It was earlier reported that three sphingophosphonolipids (SPNLs) were present in axenically cultured *B. stolpii* strain UKi2 as shown by ^{32}P-labeling, differential hydrolysis, susceptibility to digestion by enzymes, thin-layer chromatography (TLC), and gas chromatography (GC) techniques [4]. These workers reported that the two components that migrated slowest were sphingophosphonolipids and the one with the highest migration rate was a sphingophospholipid [4].

Sphingolipids are ubiquitous in eukaryotic fungal, protozoan, animal, and plant cells and can occur in membranes as glycosphingolipids, phosphosphingolipids, and inositolsphingolipids [5–15]. These lipids and their precursors and metabolic products such as sphingosine, sphingosine-1-phosphate, and ceramide can be highly bioactive agents in cell proliferation, differentiation, aging, and apoptosis, transmembrane signal transduction, and other cell functions [16–19]. In recent years, these compounds have also attracted the interest of cell biologists with the finding that there are microdomains in biomembranes known as lipid rafts that are enriched in sphingolipids and sterols [20–26].

Sphingolipids are not widely distributed among prokaryotes, and have been mainly studied among organisms in the genus *Sphingomonas* [27] but these compounds have been reported in *Bacteroides melaninogenicus* [28] and *Bacteriovorax stolpii* [4, 29]. Furthermore, naturally occurring phosphonolipids are rare, and most reports of these compounds have been

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from analyses of the ethananolamine glycerophosphonolipids and SPNLs of protozoa [8–12, 15, 30–32]. Thus, it is significant that these compounds are present in *B. stolpii* [4, 29]. As the finding of SPNLs in prokaryotes is novel and significant, and despite the elegant work performed previously on these *B. stolpii* compounds [4], state-of-the-art MS-MS technologies [33–35] available today can provide much more information and far greater sensitivity than was available when these lipids were first examined. Currently there is a lack of MS data and fragmentation patterns of sphingophosphonolipids in the literature. We here report on 18 SPNL molecular species identified in *B. stolpii* based mainly on MS techniques. Structural assignments were further provided by analyses using nuclear magnetic resonance (NMR) spectroscopy, TLC, susceptibility to phospholipases, and differential hydrolysis.

Materials and Methods

Bacterial Culture and Extraction of Lipids

Bacteriovorax stolpii UKi2 was obtained from the American Type Culture Collection (ATCC 27,052) and cultured axenically in 0.3% yeast extract and 1% bactopeptone as described previously [29].° Cell° density° as determined by $Abs_{660\ nm}$ indicated that cultures grown in the enriched medium yielded ~28% higher bacterial numbers than when grown with 0.3% yeast extract alone. Standing cultures were incubated at 30 °C until they reached stationary phase.

Cells were harvested by centrifugation at 10,000 × *g* for 5 min, washed in physiological saline, and recentrifuged into a packed pellet. The cell pellet was extracted for total lipids using chloroform ($CHCl_3$):methanol (MeOH)°(2:1,°vol/vol)°[29]°or°by°the°method°of°Bligh and°Dyer°[36].

Components of the culture medium (1.88 g yeast extract, 5.66 g bactopeptone) were extracted to determine whether the fatty acids found in *B. stolpii* could have been taken up from the medium. After acidic trans-esterification°[12]°fatty°acid°methyl°esters°were recovered and analyzed by GC-TOF/MS. The fatty acid methyl esters have a characteristic fragment ion at *m/z* 74 and the α-hydroxy fatty acid methyl esters have an ion at *m/z* 90 (McLafferty rearrangement). The number of carbon atoms in the fatty acid moiety can be determined from the molecular ion.

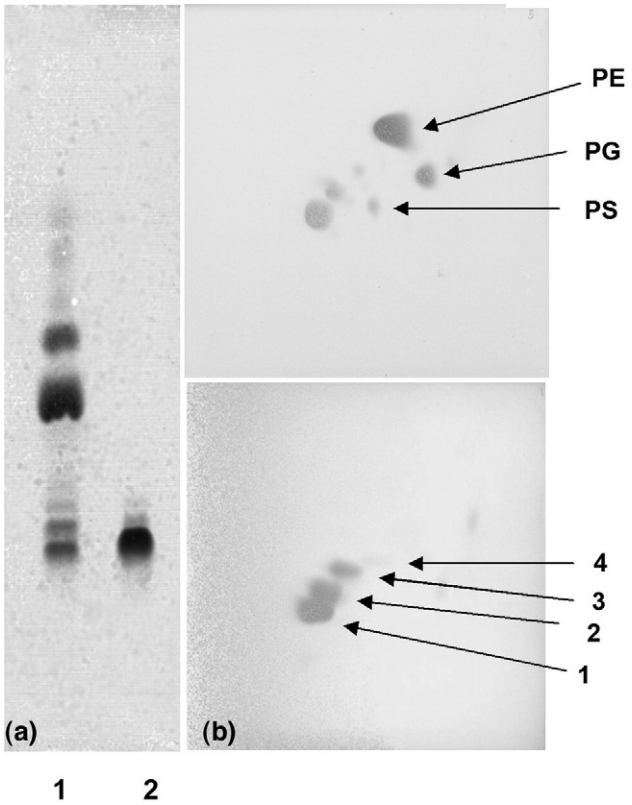


Figure 1. *B. stolpii* Uki2 phospholipids separated by 1-D and 2-D HPTLC and then stained for phosphorus. (**a**) 1-D HPTLC developed in SS#2. Lane 1, intact phospholipids; lane 2, phospholipids analyzed after alkaline hydrolysis. The presence of alkali-resistant lipids suggests that these are sphingolipids. (**b**) 2-D HPTLC. Top, total lipids. PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PS, phosphatidylserine. Bottom, lipids resistant to mild alkaline hydrolysis. Plates were developed in SS#1 and then SS#3 in the first dimension. SS#2 was used for separation in the second dimension. SPNL components are identified by the numbers 1, 2, 3, and 4.

Isolation of SPNLs, TLC, and Hydrolysis

Total lipids were fractionated by silicic acid column chromatography (Unisil, Clarkson Co., Williamsport, PA); the neutral lipid fraction was eluted using $CHCl_3$

Table 1. TLC analyses and differential hydrolysis of *Bacteriovorax stolpii* UKi2 sphingophosphonolipids[a]

Sample	R_f	% of Total SPNL[b]	% of Total SPNL[c]	P[d]	ninhydrin
Band 1	0.259	53.5 + 8.9	40.0 + 3.1	+	+
Band 2	0.319	25.5 + 4.1	29.7 + 2.6	+	+
Band 3	0.380	15.5 + 5.2	21.3 + 4.2	+	+
Band 4	0.434	5.6 + 1.1	2.8 + 1.5	+	ND[e]

TLC was performed on Silica Gel G, H, or 60 developed in propanol:n-propylamine:H_2(80:15:5,vol/vol/vol).
[a]Total SPNL was obtained by alkaline hydrolysis of the polar lipid fraction. Preparative 1-D
[b]Values represent weight of lipids + SEM recovered from each TLC band, n = 4.
[c]Values were obtained by densitometry of TLC plates stained with phosphomolybdate, n = 5.
[d]Phosphorus stain [37].
[e]Not detected.

Table 2. Total fatty acids of culture medium components. Hydroxy fatty acids were not detected

FAME[a]	$M^{+\cdot}$ (*m/z*)	$M^{+\cdot}$ peak intensity	% of total
14:0	242	860	4.0
15:0	256	1,870	8.8
16:0	270	10,880	50.6
16:1	268	1,270	6.0
17:0	284	1,440	6.8
18:0	298	3,840	18.0
18:1	296	1,250	5.9

[a]Fatty acid methyl esters.

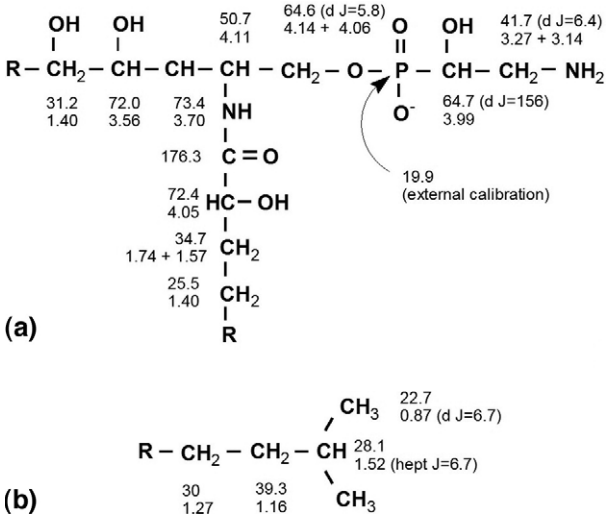


Figure 2. NMR data for the major sphingophosphonolipid fraction, TLC Band 1. ^{13}C NMR (ppm) top number, bottom number (coupling constant in Hz). (**a**) Structure of the head group found in Band 1 as determined by 2-D NMR (COSY, HSQC, HMBC). (**b**). Structure of the lipid tail found in Band 1 as determined by 2D NMR (COSY, HSQC, HMBC).

and the polar lipid fraction was eluted with MeOH. The polar lipid fraction was analyzed by TLC using Silica Gel G, H, or 60 glass-backed plates or by high-performance TLC (HPTLC). Development of plates for 1-D TLC was performed using $CHCl_3$:MeOH:H_2O (60:35:8, vol/vol/vol; SS#1) or *n*-propanol:*n*-propylamine:H_2O (80:15:5, vol/vol/vol; SS#2) as the solvent systems.

For 2-D TLC, plates were developed in SS#1, allowed to dry and then redeveloped in the first dimension using $CHCl_3$:MeOH:acetic acid (90:2:8, vol/vol/vol; SS#3). After plates were thoroughly dried, separation in the second dimension was performed using SS#2. Separated components were visualized by general lipid stains, ninhydrin, or a phosphorus-specific reagent [37].

In some cases, total lipids or the polar lipid fraction were first subjected to mild alkaline methanolysis to degrade the glycerolipids leaving the sphingolipids intact and then the individual SPNL bands were isolated by TLC. Lipid samples (up to 20 mg) were dried in shell vials, to which 0.6 mL of $CHCl_3$:MeOH (2:1, vol/vol) and 0.6 mL of freshly prepared solution of 1M NaOH in methanol were added. After 2 h at room

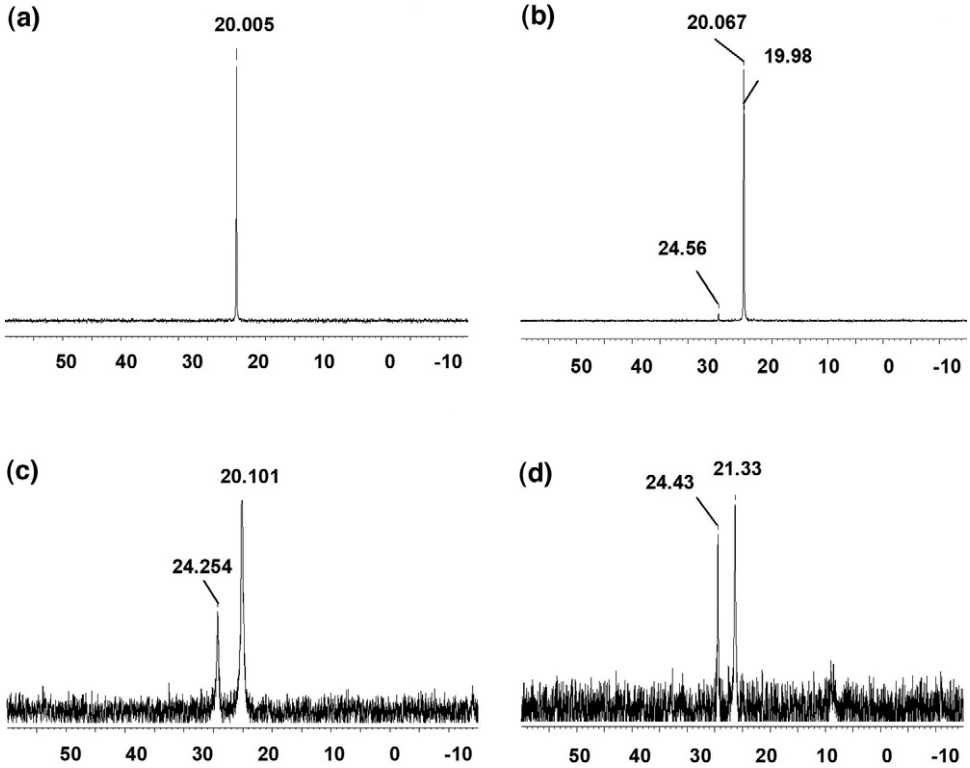


Figure 3. 243 MHz ^{31}P NMR analysis of TLC-isolated *B. stolpii* sphingolipid fractions. (**a**), Band 1; (**b**), Band 2; (**c**), Band 3; (**d**), Band 4.

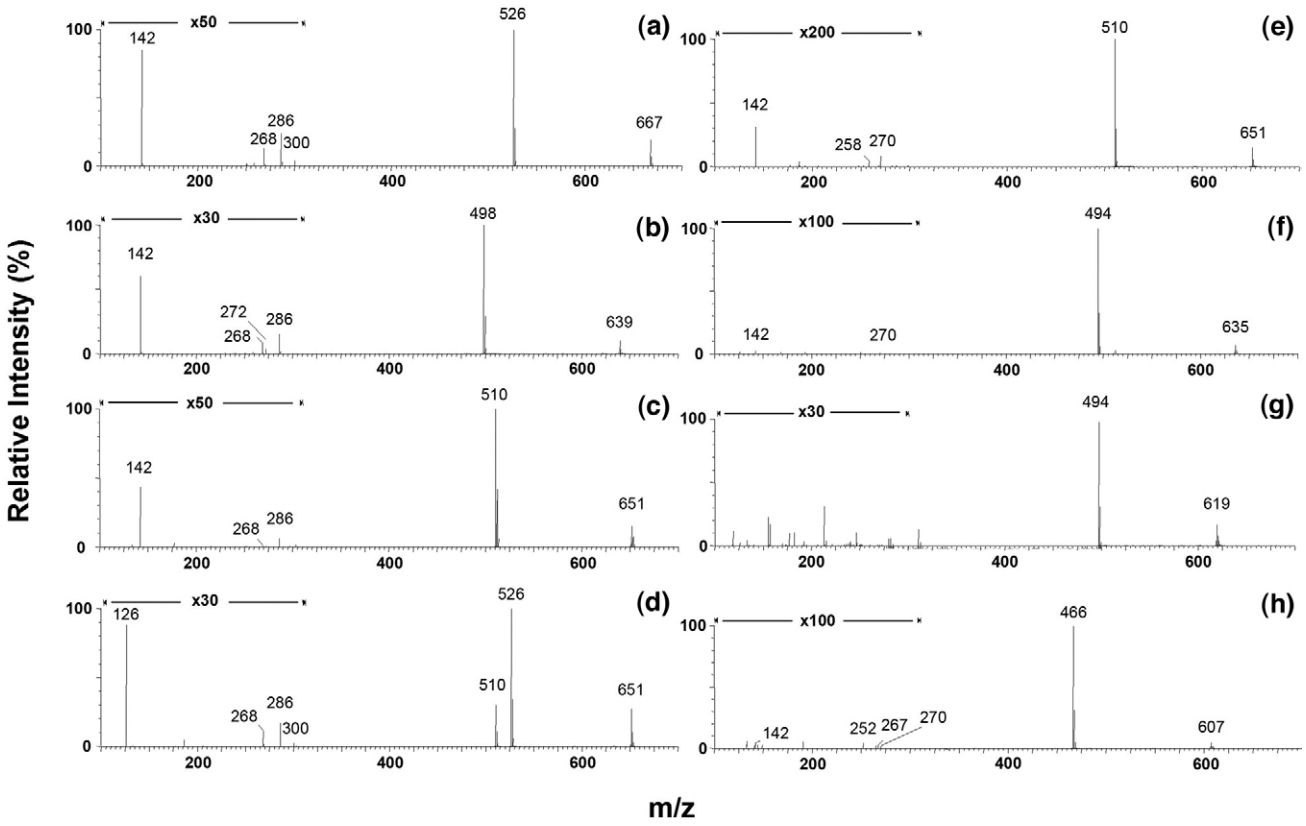


Figure 4. Product ion mass spectra obtained by collision-induced dissociation (CID) MS of *B. stolpii* Uki2 sphingophosphonolipids (SPNL) from the $[M + H]^+$ ions generated by ESI in the positive-ion mode. (**a**) The major SPNL N-2′-hydroxy-isopentadecanoyl-2-amino-3,4-dihydroxy-isoheptadecan-1-phosphono-(1-hydroxy-2-aminoethane). (**b**) The second most abundant SPNL N-2′-hydroxy-isotridecanoyl-2-amino-3,4-dihydroxy-isoheptadecan-1-phosphono-(1-hydroxy-2-aminoethane). (**c**) N-isopentadecanoyl-2-amino-3,4-dihydroxy-isoheptadecan-1-phosphono-(1-hydroxy-2-aminoethane). (**d**) N-2′-hydroxy-isopentadecanoyl-2-amino-3,4-dihydroxy-isoheptadecan-1-phosphono-(2-aminoethane). *m/z* 510 is a fragment due to the loss of the PHG from $[M + H]^+$ at *m/z* 651, an isomer contaminant in the sample from an adjacent TLC band. (**e**) N-2′-hydroxy-isopentadecanoyl-2-amino-3-hydroxy-isoheptadecan-1-phosphono-(1-hydroxy-2-aminoethane). (**f**) N-isopentadecanoyl-2-amino-3-hydroxy-isoheptadecan-1-phosphono-(1-hydroxy-2-aminoethane). (**g**) N-isopentadecanoyl-2-amino-3-hydroxy-isoheptadecan-1-phosphono-(2-aminoethane). (**h**) N-isotridecanoyl-2-amino-3-hydroxy-isoheptadecan-1-phosphono-(1-hydroxy-2-aminoethane).

temperature with intermittent shaking, 0.5 mL of 6N HCl was added. Biphasic partitioning was accomplished by adding 0.6 mL $CHCl_3$, 0.2 mL MeOH, and 0.4 mL H_20. The lower organic phase was dried and subjected to TLC and the separated lipid components were visualized by staining for phosphorus [37].

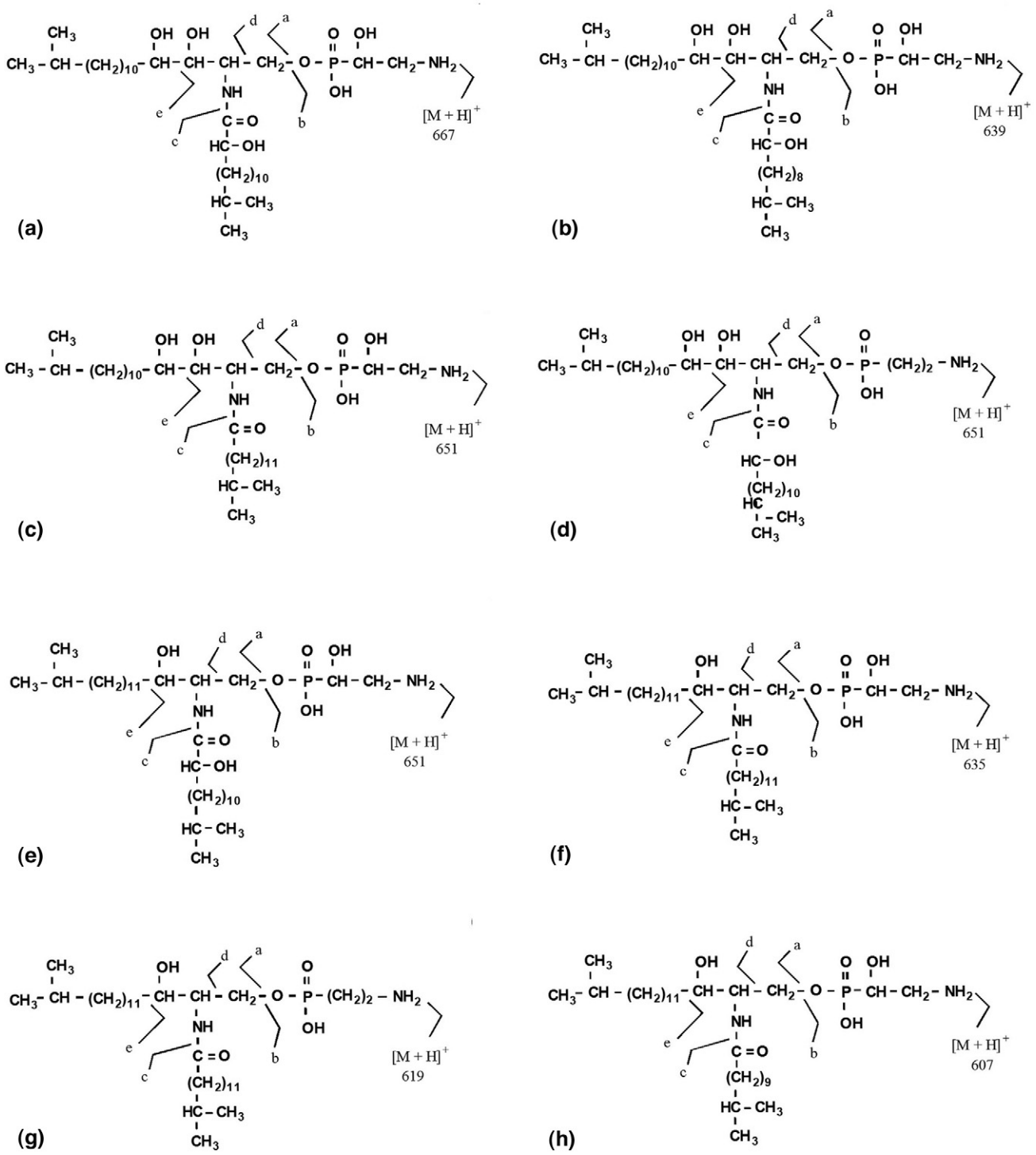
Water-soluble polar head groups (PHG) were obtained by acid hydrolysis using MeOH:H_2O:HCl (11: 2.6:1,vol/vol/vol) and incubated at 80 °C overnight. Phospholipase C (*Clostridium perfringens* E.C. 3.1.4.3, Sigma Chemical Co., St. Louis, MO) and phospholipase D (*Streptomyces chromofuscus*, EC. 3.1.4.4, Sigma) were tested for their ability to release the PHG of the SPNLs.

NMR Spectroscopy

Total TLC-purified sphingophosphonolipids and material in individual TLC-purified SPNL bands were analyzed by ^{31}P, ^{13}C, and ^{1}H NMR spectroscopy. NMR spectra were measured using a 600 MHz Bruker Avance instrument at 30 °C in a solvent consisting of the lower phase of a mixture of $CDCl_3/CD_3OD/D_2O$ 2:1:0.6 (by vol). Calibration of the ^{1}H and ^{13}C spectra was to internal TMS, ^{31}P spectra were calibrated to external 85% H_3PO_4. Connectivity assignments were made by heteronuclear multiple bond correlation (HMBC), heteronuclear single quantum coherence (HSQC), and correlated spectroscopy (COSY) 2D-NMR experiments.

Mass Spectrometry

Intact compounds isolated by TLC were analyzed by MS-MS techniques using a hybrid quadrupole time-of-flight (Q-TOF-II) (Waters Corp., Milford, MA)-mass spectrometer equipped with electrospray ionization (ESI) [38]. Samples were dissolved in $CHCl_3$:MeOH (3:1, vol/vol) at 10 pg/μL and were infused into the source by means of a syringe pump (Harvard Apparatus, Holliston, MA). Spectra were obtained in both the positive- and negative-ion modes. The data were col-



lected and processed with a Mass Lynx Data system (Waters). Like FAB-MS, ESI-MS is used to analyze intact molecules but this technique is more sensitive, does not require a matrix for the sample, and can be interfaced to high-performance liquid chromatographic instrumentation.

Calibration of the instrument before the analyses of the samples resulted with a mass accuracy of less than 10 ppm. Exact mass (to 4 decimal places) of a known peak was also used as lock mass to correct the masses of other peaks in the spectrum (normally $[M + Na]^+$ is used). The elemental composition was obtained from these exact masses.

The $[M + H]^+$ were subjected to collision-induced dissociation (CID) to achieve fragmentation. The initial collision energy was 20 eV, which was increased until

Table 3. Characterization of *Bacteriovorax stolpii* UKi2 sphingophosphonolipid molecular ion and fragments. Mass spectra are shown in Figure 4 and structures are shown in Figure 5

	Ion mass (*m/z*) Elemental composition[a]					
Mass spectrum[b]	$[M + H]^+$	$[a + 2H]^+$	$[b]^+$	$[M + H]^+$ - d - e	$[M + H]^+$ - a - c	$[M + H]^+$ - a - c - H_2O
A	667.5003	142.0235	526.4813	300.2539	286.2745	268.2654
	$C_{34}H_{72}N_2O_8P$	$C_2H_9NO_4P$	$C_{32}H_{64}NO_4$	$C_{17}H_{34}NO_3$	$C_{17}H_{36}NO_2$	$C_{17}H_{34}NO$
B	639	142	498	272	286	268
	$C_{32}H_{68}N_2O_8P$	$C_2H_9NO_4P$	$C_{30}H_{60}NO_4$	$C_{15}H_{30}NO_3$	$C_{17}H_{36}NO_2$	$C_{17}H_{34}NO$
C	651	142	510		286	268
	$C_{34}H_{72}N_2O_7P$	$C_2H_9NO_4P$	$C_{32}H_{64}NO_3$		$C_{17}H_{36}NO_2$	$C_{17}H_{34}NO$
D	651	126	526	300	286	268
	$C_{34}H_{72}N_2O_7P$	$C_2H_9NO_3P$	$C_{32}H_{64}NO_4$	$C_{17}H_{34}NO_3$	$C_{17}H_{36}NO_2$	$C_{17}H_{34}NO$
E	651	142	510		270	252
	$C_{34}H_{72}N_2O_7P$	$C_2H_9NO_4P$	$C_{32}H_{64}NO_3$		$C_{17}H_{36}NO$	$C_{17}H_{34}N$
F	635	142	494		270	
	$C_{34}H_{72}N_2O_6P$	$C_2H_9NO_4P$	$C_{32}H_{64}NO_2$		$C_{17}H_{36}NO$	
G	619	126	494			
	$C_{34}H_{72}N_2O_5P$	$C_2H_9NO_3P$	$C_{32}H_{64}NO_2$			
H	607	142	466		270	252
	$C_{32}H_{68}N_2O_6P$	$C_2H_9NO_4P$				

[a]Spectra are shown in Figure 4 and are designated by the same letters used in this table.
[b]Some elemental compositions were deduced from exact mass data obtained. Exact masses for fragments were obtained for some (e.g., SPNL A) but not all fragments. Hence, nominal masses are shown for those fragments that were deduced from fragmentation patterns of intact molecules and hydrolysis products.

Figure 5. Collision-induced dissociation (CID) patterns and product ions from the [M + H] positive ions obtained from ESI of the sphingophosphonolipids (SPNL) of *B. stolpii* Uki2 and the molecular structures deduced from them. The CID spectra are presented in Figure 4. (**a**) Product ion pattern of the $[M + H]^+$ of the major SPNL N-2′-hydroxy-isopentadecanoyl-2-amino-3,4-dihydroxy-isoheptadecan-1-phosphono-(1-hydroxy-2-aminoethane). This compound has a molecular weight (MW) of 666 Da. The $[M + H]^+$ is *m/z* 667. The fragment ion *m/z* 526, due to the loss of the head group 1-phosphono-(1-hydroxy-2-aminoethane,) is also the base peak. (**b**) Fragmentation pattern of the $[M + H]^+$ of the second most abundant SPNL N-2′-hydroxy-isotridecanoyl-2-amino-3,4-dihydroxy-isoheptadecan-1-phosphono-(1-hydroxy-2-aminoethane). The $[M + H]^+$ is at *m/z* 639. The fragmentation pattern is similar to the major SPNL except for the fragments containing the fatty acid (*m/z* 639, *m/z* 498, *m/z* 272), which are 28 Da less than those of the major SPNL. (**c**) The fragmentation pattern of the $[M + H]^+$ of the SPNL N-isopentadecanoyl-2-amino-3,4-isoheptadecan-1-phosphono-(1-hydroxy-2-aminoethane) whose $[M + H]^+$ is *m/z* 651. The base peak at *m/z* 510 is 16 Da less than that of the major SPNL. This indicates that the molecule contains one less oxygen atom. The fragmentation pattern is similar to that of the major SPNL except for the ion at *m/z* 510 and the absence of the ion at *m/z* 300. This indicates that the fatty acid moiety is isopentadecanoic acid instead of 2′-hydroxy-isopentadecanoic acid. The ions at *m/z* 286, *m/z* 268, and *m/z* 250 are derived from the LCB. (**d**) The CID spectrum of the $[M + H]^+$, *m/z* 651, of the SPNL N-2′-hydroxy-isopentacecanoyl-2-amino-3,4-dihydroxy-isoheptadecan-1-phosphono-(2-aminoethane). The fragmentation pattern is similar to that of the major SPNL except for the ion at *m/z* 126, which is 16 Da less than *m/z* 142. Since this ion is due to the PHG, this indicates that the PHG contains 2-aminoethane instead of 1-hydroxy-2-aminoethane. (**e**) The fragmentation pattern of the $[M + H]^+$ of N-2′-hydroxy-isopentadecanoyl-2-amino-3-hydroxy-isoheptadecan-1-phosphono-(1-hydroxy-2-aminoethane). The $[M + H]^+$ at *m/z* 651 and the base peak at *m/z* 510 are both 16 Da less than the respective ions of the major SPNL. The ions at *m/z* 270 and *m/z* 252 are again 16 Da less than the *m/z* 286 and *m/z* 268 ions of the major SPNL, which are from the LCB. This shows that the LCB is dihydrosphingosine instead of phytosphingosine. (**f**) Fragmentation pattern of the $[M + H]^+$ *m/z* 635 shows an ion at *m/z* 142, which is 32 Da less than the major SPNL. This is due to the presence of the same PHG in both compounds, which is phosphono-(1-hydroxy-2-aminoethane). The ion at *m/z* 270 is due to the LCB dihydrosphingosine. Hence this SPNL is N-isopentadecanoyl-2-amino-3-hydroxy-isoheptadeca-1-phosphono-(1-hydroxy-2-aminoethane). (**g**) The product ion pattern of the $[M + H]^+$ *m/z* 619 of the SPNL N-isopentadecanoyl-2-amino-3-hydroxy-isoheptadeca-1-phosphono-2-aminoethane shows the base peak at *m/z* 494, which is 125 Da less than the $[M + H]^+$ indicating that the PHG is phosphono-(2-aminoethane). The $[M + H]^+$ *m/z* 619 is 48 Da less than that of the major SPNL, which is *m/z* 667 indicating that this molecule has three oxygen atoms less in its elemental composition. The peak at *m/z* 126 is due to the PHG phosphono-(2-aminoethane). (**h**) The fragmentation pattern of the $[M + H]^+$ *m/z* 607 is similar to that exhibited by SPNL F, and the ions at *m/z* 607 and *m/z* 466 are 28 Da less than the corresponding ions of molecule F, namely *m/z* 635 and 494. The PHG is the same as indicated by *m/z* 142. The SPNL is therefore N-isotridecanoyl-2-amino-3-hydroxy-isopentadecan-1-phosphono-(1-hydroxy-2-aminoethane).

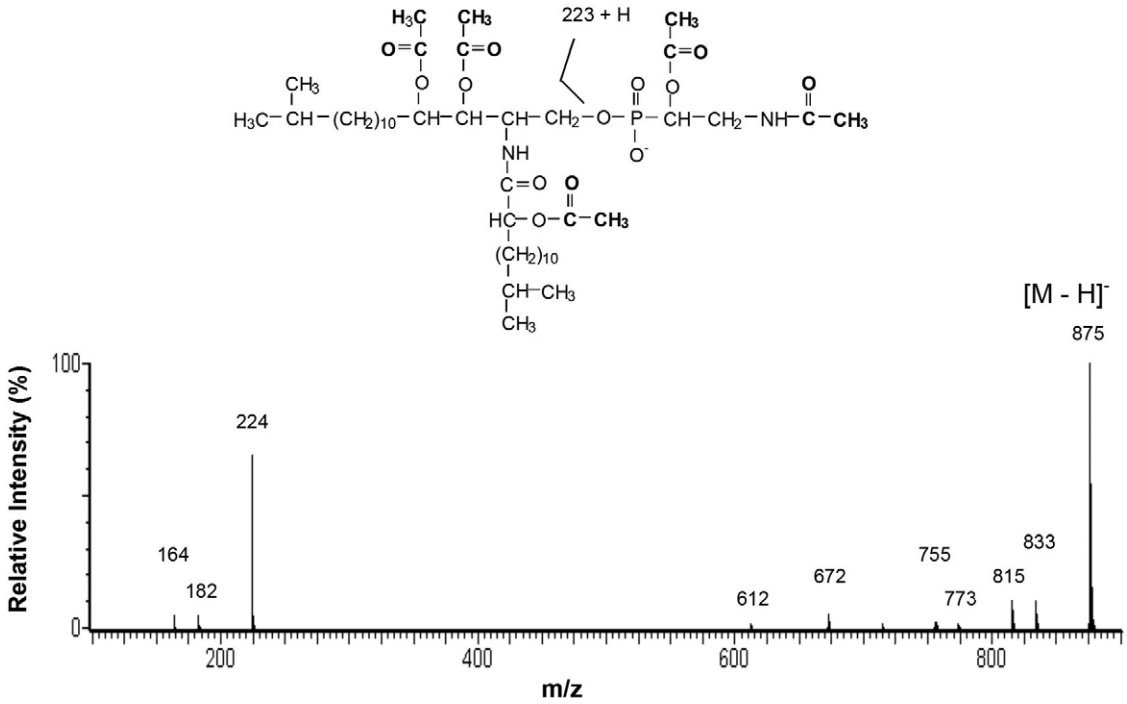


Figure 6. The peracetylated major SPNL N-2′-acetyloxyisopentadecanoyl-2-amino-3,4-diacetyloxy-isoheptadecan-1-phosphono-(1-acetyloxy-2-acetylamino-ethane). The MW of this compound is 876 Da. The pseudo molecular ion $[M - H]^-$ in this spectrum is at *m/z* 875. The ion at *m/z* 224 is due to the acetylated PHG. The molecular ion is 210 Da higher than the MW of the major SPNL. The increase in MW is due to the acetylation of all the available hydroxyl groups and the amino group in the PHG. Since one acetyl group contributes 42 Da there are a total of five sites available for acetylation (210/42 = 5). This also shows that the compound has a phosphono head group.

optimal fragmentation was attained; argon was used as the collision gas. The exact mass of the $[M + H]^+$ was used to correct the masses of the fragments and the elemental compositions and identity of the fragments were determined from the exact masses by information based on fragmentation patterns and by calculating mass differences. Identification of the SPNL hydrolysis products (PHG, LCB, fatty acid) aided in the structural assignments of the complex SPNLs. In these cases, the masses of fragments were not calculated as exact masses with precision to 4 decimal places. The structures of the intact SPNLs were also verified as trimethylsilyl (TMS) and acetylated derivatives by ESI-MS. TMS derivatives were prepared using the reagent BSTFA (Supelco, Bellefonte, PA). Acetylation was done by adding 0.1 mL of acetic anhydride and a drop of triethylamine to the TLC-isolated SPNL samples and the reaction was allowed to progress at room temperature overnight. The sample was dried under a stream of N_2 and the peracetylated SPNLs were dissolved in $CHCl_3$ and analyzed by ESI run in the negative ion mode on the Q-TOFF-II.

Other MS techniques were used for analyzing the *B. stolpii* SPNLs, these included ESI-MS in both the negative- and positive-ion modes, by FAB-MS and by secondary ion MS (SIMS). Shown here are mainly MS data by ESI-MS in the positive-ion mode.

Results and Discussion

Identification of Sphingophosphonolipids: TLC

The major glycerophospholipid in *B. stolpii* UKi2 was identified as phosphatidylethanolamine. In addition to the SPNLs, phosphatidylglycerol and phosphatidylserine were also detected. Following hydrolysis under alkaline conditions, the glycerophospholipids with ester-linked fatty acids were degraded and the slower-migrating bands on the TLC plates remained intact; four sphingolipid components were resolved by 1-D and 2-D TLC (Figure 1, Table 1). The fastest-migrating, smallest component was not reported in the earlier study of *B. stolpii* SPNLs [4].

Sphingophosphonolipid Fatty Acids

The *B. stolpii* SPNL fatty acids are dominated by those with an iso-structure and an α-hydroxyl group [4] (see below). The GC/MS analysis of yeast extract and bactopeptone total fatty acids showed that they were mainly unsaturated and even carbon-numbered (Table 2). Since α-hydroxy fatty acids were not detected in the culture medium components, it was concluded that the fatty acid moiety of SPNLs were synthesized by *B. stolpii*.

NMR Analyses

Total SPNLs and individual TLC-purified *B. stolpii* SPNL fractions were subjected to NMR analysis. The dominant structural features were the presence of phosphonyl P, fatty acids containing an α-hydroxy group, and iso-branched LCB and fatty acids (Figure 2). However, the presence of minor components (< 20%) without iso-branching groups could not be ruled out by NMR.

^{31}P NMR analysis of total SPNLs and all TLC-isolated fractions showed a major peak at 20.0 ppm and minor peaks at 24.5 ppm and 21.3 ppm (Figure 3). These results agree with the chemical shift of compounds with a P—C bond, which is far downfield from compounds with P—O—C bonds [39, 40]. Phosphoryl P was not detected in the total SPNL fraction or in lipids recovered from the four bands separated by TLC.

Phospholipase Digestion

The SPNLs were sensitive to phospholipase C digestion, which catalyzed the hydrolysis at the P—O—C bond releasing the ceramide and the water-soluble head groups comprised of both the phosphorus and the base. In lipids such as glycerophospholipids and sphingophospholipids that contain phosphoryl groups, phospholipase D acts at the other P—O—C bond releasing only the base. The *B. stolpii* SPNLs were resistant to phospholipase D action; thus these results are consistent with a P—O—C bond between the head group and the ceramide moiety and a P—C bond between the base and the phosphorus.

Identification of Sphingophosphonolipids: Mass Spectrometric Analyses

The spectrum obtained for the dominant SPNL in the largest TLC Band 1 by Q-TOF ESI-MS-MS operated in the positive-ion mode was consistent with N-2′-hydroxy-iso-pentadecanoyl-2-amino-3,4-dihydroxy-iso-heptadecan-1-phosphono-(1-hydroxy-2-aminoethane) as its structure (Figure 4a). The structure and fragmentation pattern of the intact compound is shown in Figure 5a, and the properties of fragments are presented in Table 3. The mass spectrum of the ESI positively charged product ions showed $[M + H]^+$ at *m/z* 667. The peak at *m/z* 142 (141 + H) corresponds to the PHG. The ion at *m/z* 526 represents the loss of the PHG $[M + H - 141]^+$, from $[M + H]^+$. The ion at *m/z* 286 is due to the loss of both the PHG and the fatty acid moieties from the $[M + H]^+$, and the ions at *m/z* 268 and *m/z* 250 represent the loss of one and two H_2O from *m/z* 286, respectively. The peak at *m/z* 300 has the elemental composition $C_{17}H_{34}NO_3$ [HC(OH) = CH-NH_2-CO-CH(OH)-$(CH_2)_{10}$-$CH(CH_3)_2$]$^+$, which identified the fatty acid. This major SPNL was estimated to comprise ~50% of the total SPNL of *B. stolpii*.

The structure of the intact major *B. stolpii* SPNL was also verified by ESI in the negative ion mode (not shown). Furthermore, analysis of its TMS derivative was performed indicating the $[M + H]^+$ ion at *m/z* 1027.6986 (calculated 1027.7003) with an elemental composition of $C_{49}H_{112}N_2O_8Si_5$ (not shown). A fragment ion at *m/z* 444.3341 (calculated mass 444.3329) with an elemental composition of $C_{23}H_{50}NO_3Si_2$ represented both the LCB and fatty acid. This confirmed the presence of two hydroxyl groups in the LCB, a hydroxyl group in the fatty acid and a hydroxyl group and the amine in the PHG that were available for silylation.

The second most abundant SPNL, which comigrated by TLC with the major SPNL in Band 1 (Table 1), differed only by having a C_{13} α-hydroxy fatty acid (Figure 4b; Figure 5b). This SPNL, N-2′-hydroxy-iso-tridecanoyl-2-amino-3,4-dihydroxy-iso-heptadecan-1-phosphono-(1-hydroxy-2-aminoethane), constituted ~18% of the total *B. stolpii* SPNL.

This large, broad TLC Band 1 contained minor components such as N-iso-pentadecanoyl-2-amino-3,4-dihydroxy-iso-heptadecan-1-phosphono-(1-hydroxy-2-aminoethane) (Figure 4c, Figure 5c), which contained a nonhydroxy fatty acid. Also present were minor SPNL molecular species. These included components with the hydroxy-aminoethylphosphonate (HAEP) head group and C_{17} phytosphingosine LCB. One contained a C_{13} nonhydroxy fatty acid ($[M + H]^+$ = 623); the others contained either C_{11}, C_{12}, or C_{14} α-hydroxy fatty acids ($[M + H]^+$ = 611, 625, and 653, respectively) (not shown).

The second largest Band 2 isolated from TLC plates (Table 1) contained an SPNL with dihydrosphingosine rather than phytosphingosine as the LCB moiety (Figure 4e; Figure 5e). The fragment ions at *m/z* 252 and *m/z* 270 indicated the LCB was C_{17} dihydrosphingosine. The product ion analysis of the $[M + H]^+$ ion *m/z* 651 detected by ESI in the positive ion mode identified the head group at *m/z* 142, which was HAEP. The fatty acid moiety was identified by the detection of the fragment at *m/z* 510. This TLC band also contained a similar SPNL but with a C_{13} α-hydroxy fatty acid ($[M + H]^+$ = 623) (not shown).

The third TLC Band 3 (Table 1) contained an SPNL with C_{17} phytosphingosine, C_{15} α-hydroxy fatty acid, and aminoethylphosphonate (AEP) as the head group (Figure 4d; Figure 5d); an SPNL with C_{17} dihydrosphingosine, C_{15} nonhydroxy fatty acid, and HAEP as its head group (Figure 4f; Figure 5f); and an SPNL containing C_{17} dihydrosphingosine, C_{13} nonhydroxy fatty acid, and a HAEP head group (Figure 4h; Figure 5h). Trace amounts of two other SPNLs were detected in this band. These both contained C_{17} phytosphingosine and the head group was AEP; one contained a C_{13} α-hydroxy fatty acid ($[M + H]^+$ = 623) and the other had a C_{11} α-hydroxy fatty acid ($[M + H]^+$ = 595) (not shown).

Band 4, the smallest and fastest-migrating SPNL component resolved by TLC (Table 1) contained the LCB C_{17} dihydrosphingosine, a C_{15} non-hydroxy fatty acid, and AEP as its head group with $[M + H]^+$ at *m/z* 619 (Figure 4g; Figure 5g). Minor components detected in Band 4 all contained C_{17} dihydrosphingosine and non-hydroxy fatty acids. One contained a C_{15} fatty acid and HAEP ($[M + H]^+$ = 635). The structure of this minor SPNL was also verified by FAB-MS product ion spectrum (not shown),

which showed $[M + H]^+$ at *m/z* 635. Loss of the head group was indicated by an ion at *m/z* 494 and the peak at *m/z* 142 was identified as the head group (HAEP) fragment. Another minor SPNL in Band 4 contained a C_{13} fatty acid and AEP ($[M + H]^+ = 591$); and another contained a C_{13} fatty acid and HAEP ($[M + H]^+ = 607$) (not shown).

Further confirmation of the phosphonate nature of these compounds came from mass spectral analysis of peracetylated derivatives. The spectra showed that all the available hydroxyl groups and the amino group in the PHG were acetylated (Figure 6). The major SPNL contains four hydroxyl groups; two in the LCB, one in the fatty acid side-chain, and one at the PHG. These were all acetylated in addition to the acetyl group on the amine. Thus, there were a total of five acetyl groups observed by analysis in the negative ion mode. The spectrum of the major SPNL indicated an ion at *m/z* 665 corresponding to $[M - H]^-$ (not shown). The spectrum of peracetylated major SPNL analyzed in the negative ion mode indicated the presence of an ion at *m/z* 875, which is 210 Da higher than the ion at *m/z* 665. This showed that all the five available sites were acetylated. Similarly, spectra of all the other SPNLs indicated the expected number of acetyl groups. These MS results together with the ^{31}P NMR data, sensitivity to phospholipase C but not D hydrolysis, and the differential hydrolysis assay results showed that all SPNLs in *B. stolpii* have phosphonyl bonds; no phosphoryl bonds were detected.

Fatty Acids in B. stolpii Culture Medium

Analysis of total fatty acids present in yeast extract and bactopeptone, components for the *B. stolpii* culture medium, showed that hydroxy fatty acids were not detectable (Table 2). Therefore, it was concluded that the α-hydroxy fatty acids found in *B. stolpii* were synthesized by the organism; they were not scavenged from the medium.

Unlike *B. stolpii*, the eukaryotic ciliated protozoans *Paramecium* and *Tetrahymena* synthesize both sphingophosphonolipids and glycerophosphonolipids with AEP head groups [10–12, 30–32]. *Paramecium* also synthesizes their serine counterparts, which suggests these are the direct precursors of its respective AEP compounds [31, 32]. The biosynthetic pathways involved in the synthesis of AEP head groups in ciliates are still poorly understood, but clearly they are not synthesized from ethanolamine [31, 32, 40–43]. The present study also showed that the *B. stolpii* SPNLs contained some minor components with AEP head groups but the dominant head group was HAEP. Further studies are needed to determine whether HAEP is derived from AEP and whether serine is a precursor of this novel head group in *B. stolpii*.

Conclusions

Bdellovibrio stolpii has at least 18 SPNL molecular species, dominated by those containing the unique head group HAEP; minor components with AEP were also detected by sensitive MS techniques. ^{31}P NMR, differential sensitivity to phospholipase enzymes, and MS of peracetylated derivatives demonstrated the absence of phosphoryl P in these SPNLs, hence they are all sphingophosphonolipids. The most prevalent LCB in the SPNL is C_{17} iso-phytosphingosine, which might be derived from C_{17} iso-dihydrosphingosine, which was detected in minor components. C_{15} iso-α-hydroxy fatty acid was the most abundant amide-linked fatty acid but α-hydroxy fatty acids with different chain lengths as well as nonhydroxy fatty acids were also found as minor components. The iso-branched nature of the LCB and fatty acid moieties was previously suggested by Steiner et al. [4] and we here confirmed these structural features of the dominant *B. stolpii* SPNL components by NMR. Establishing the definitive structures and diversity of *B. stolpii* UKi2 SPNL is important since it might lead to elucidating the role of sphingolipids in the predatory behavior of this bacterium.

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