Isolation and structural elucidation of biologically active phospholipids from *Scytonema julianum* (cyanobacteria)

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The role of platelet-activating factor (PAF) as a mediator appeared in rather primitive organisms like protozoans and was maintained in more evolved organisms. No reports exist for the presence of PAF or PAF analogues – or even compounds that exhibit PAF-like activity – in cyanobacteria, even though they belong to a group of organisms at a low evolutionary level where the content of alkylacyl forms of ether lipids is expected to be high. In addition, cyanobacteria serve as a rich source of novel bioactive metabolites. In the present study the total lipids of a strain of *Scytonema julianum*, a filamentous cyanobacterium isolated from a Greek cave, were separated into neutral lipids and phospholipids, the latter being further fractionated by HPLC. Each phospholipid fraction was tested *in vitro* for its ability to inhibit PAF-, arachidonic acid- and ADP-induced washed-rabbit-platelet aggregation and/or to cause platelet aggregation. Two types of phospholipids causing platelet aggregation

were detected and shown to be an acetylsphingomyelin and an acylacetylgllycerol phosphoacetylated glycolipid. The existence of the sphingomyelin analogues is very important, since ceramides, cerebroside and related lipids are intracellular second messengers. The identification of the phosphoglycoglycerolipid demonstrates a new type of lipid in cyanobacteria, namely one that exhibits a biological activity very similar to that of PAF. Its presence reinforces the concept that PAF is a member of a large family of lipid mediators, apparently having different physiological roles in prokaryotic and eukaryotic organisms. In addition, *Scytonema julianum* contains a phosphatidylcholine (C_{16:0}/C_{18:2}), even though bacteria in general seldom contain choline-containing phosphoacylglycerols.

Key words: acetylsphingomyelin, lipid analysis, phosphoglycolipid, platelet-activating factor.

INTRODUCTION

Platelet-activating factor (PAF), chemically characterized as 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine [1], is a highly active biological mediator in inflammation, allergy, immune disorders and ischaemic diseases [2,2a]. The role of PAF as a mediator appeared in rather primitive organisms like protozoans and was maintained during subsequent evolution [3]. The presence of PAF has been well established in various protozoans, such as *Tetrahymena pyriformis* [4], the cellular slime mould *Dictyostelium discoideum* [5], in yeast (*Saccharomyces cerevisiae*) [6], in higher plants (*Pinus halepensis* and *Urtica dioica*) [7,8], in earthworm (*Eisenia foetida*) [9], in lower vertebrates (*Salmo gairdneri*) [10] and in mammals (humans, rabbits, rats, guinea-pigs, baboons etc.) [11]. It is noteworthy that various synthetic as well as naturally occurring compounds have been found to exhibit PAF-like activity [12–15], reinforcing the notion that PAF is a member of a large family of lipid mediators. Research should therefore be focused not just on PAF, but also on PAF-like activity induced by different compounds apparently having different physiological roles in prokaryotic and eukaryotic organisms.

Prokaryotic organisms and, more specifically, eubacterial cells, were found to synthesize PAF after the addition of the appropriate lyso-PAF into the medium [16,17]. The synthesis of PAF in bacteria without the addition of a PAF precursor seems to be unlikely, since bacterial cells very seldom contain choline-containing phosphoacylglycerols.

The cyanobacteria (or blue-green algae) represent a large group within the prokaryotic Kingdom. They are the oldest oxygenic photosynthetic organisms known so far, and they also serve as a rich source of novel bioactive metabolites, including many cytotoxic, antifungal and antiviral compounds [18]. Analyses carried out on unpurified membrane fractions and whole cells of cyanobacteria have shown the presence of glycolipids and phospholipids. The major lipids of cyanobacteria are monogalactosyldiacylglycerols, digalactosyldiacylglycerols, sulphoquinovosyldiacylglycerols and, to a minor extent, phosphatidylglycerol [19]. The three glycolipids are also present in the chloroplast of higher plants, but are absent from intracytoplasmic membranes of photosynthetic bacteria, which contain predominantly phospholipids. There are no reports on the existence of PAF or PAF analogues – or even compounds that exhibit PAF-like activity – in cyanobacteria, even though they belong to a low evolutionary level where the content of alkylacyl forms of ether lipids is expected to be high [3]. In addition, the association between allergic skin irritation and allergic asthma – well-known actions of PAF [20,21] – with cyanobacterial exposure is well recorded [22]. However, these allergenic reactions may be quite unrelated to cyanobacterial toxins [23].

The first goal of the present study was therefore to test for the presence of PAF, PAF analogues or lipid compounds that exhibit PAF-like activity in cyanobacteria. A filamentous, nitrogen-fixing cyanobacterium isolated from a Greek cave, a strain of *Scytonema julianum*, was selected, since on one hand it exhibits a worldwide distribution and occupies a rather wide

Abbreviations used: AA, arachidonic acid; ESI, electrospray ionization; IC₅₀, concentration giving half-maximal inhibition; MS/MS, tandem MS; PAF, platelet-activating factor; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipids and phosphoglycolipids; SM, sphingomyelin.

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range of illuminated niches in terrestrial environments [24] and, on the other hand, few reports exist concerning its lipid composition [19,25]. The second goal of the present study was to test the biological activity of the phospholipids of *Scytonema julianum* and to make an effort to identify these compounds.

EXPERIMENTAL

Reagents

All reagents and chemicals were of analytical grade and were supplied by Merck (Darmstadt, Germany). HPLC solvents were from Rathburn (Walkerburn, Peeblesshire, Scotland, U.K.). Lipid standards, BSA and the ginkgolide BN 52021 (a receptor antagonist for PAF) were obtained from Sigma (St. Louis, MO, U.S.A.). Semisynthetic PAF (80% C₁₆ PAF/20% C₁₈ PAF) was synthesized in our laboratory as previously described [1]. Chromatographic material used for TLC was silica-gel H-60 (Merck). PAF acetylhydrolase was from human serum, purified by the method of Stafforini [26]. Arachidonic acid (AA) and ADP were obtained from Chrono-Par (Havertown, PA, U.S.A.).

Instrumentation

The separation of lipids was performed at room temperature on an HP HPLC Series 1100 liquid chromatograph (Hewlett-Packard, Waldbronn, Germany) equipped with a 100 µl loop Rheodyne (7725 i) loop valve injector, a G1322A degasser, a G1311A quat gradient pump and a G1314A HP UV spectrophotometer as a detection system. The spectrophotometer was connected to a Hewlett-Packard model HP-3395 integrator/plotter. The separation of phospholipids was performed on a Partisil 10 µm SCX column (250 mm long × 4.6 mm internal diameter) from Analysentechnik (Mainz, Germany) with an SCX (20 mm long × 4.0 mm internal diameter) pre-column cartridge and the detection was performed at 208 nm. A Unicam Helios Beta spectrophotometer (Spectronic Unicam, Cambridge, U.K.) was used to obtain the UV spectra. Samples were dissolved in methanol and the spectrum from 190 to 900 nm was obtained using quartz cuvettes. PAF-induced aggregation was measured in a Chrono-Log model 400-VS aggregometer coupled to a Chrono-Log recorder. The electrospray-ionization (ESI) MS experiments were performed on an API 100 PerkinElmer SCIEX single quadrupole mass spectrometer. Samples were dissolved at a concentration of 10 ng/µl in 1:1 (v/v) aq. methanol. Electrospray samples were typically introduced into the mass analyser at a rate of 4.0 µl/min. The positive and negative ions, generated by charged droplet evaporation, enter the analyser through an interface plate and a 100 mm orifice, while the declustering potential is maintained between 60 and 100 V to control the collisional energy of the ions entering the mass analyser. The emitter voltage is typically maintained at 4000 V. The ESI tandem MS (MS/MS) experiments were performed on a Finnigan LCQ quadrupole-ion-trap mass spectrometer.

Sampling, isolation and culture conditions of *Scytonema julianum*

The cyanobacterium *Scytonema julianum* was collected from the Koutouki cave in Paiania (Attika, Greece), located on the slopes of Mount Hymettus. Epilithic as well as edaphic samples ranging from the entrance to the inner parts of the cave were collected under sterile conditions during the years 2000–2001. In order to separate the various species from the cave specimens, both direct

isolation and enrichment cultures were applied. In direct isolation the material collected from specific sampling sites was treated by manual techniques under a dissection microscope. In enrichment cultures a common inorganic medium for cyanobacteria, BG11, was used [27]. It should be noted that, in the case of *Scytonema julianum*, the direct isolation of filaments was a satisfactory technique to obtain monospecific and even axenic cultures [28]. In the laboratory the cultures were kept under room conditions. Fresh (natural and cultured) cyanobacteria were studied under a Zeiss optical microscope.

The aerobic, nitrogen-fixing cyanobacterium, from natural as well as cultured material, was classified under the Nostocales and Scytonemataceae using the uniseriate, of even width, heterocystous, false branched trichomes as the main taxonomic criteria [29]. This cyanobacterium, which grows well in monospecific cultures, was identified as *Scytonema julianum*, on account of its morphology and ecology, which do not differ from those originally described [30].

Lipid extraction

Lipids were extracted using the method of Bligh and Dyer [31].

Recovery of pigment-free phospholipids

Pigment contamination was removed from total lipids by two successive preparative TLC [32] runs. Briefly, the chloroform phase from the extraction procedure, which contains total lipids, was evaporated to dryness and the extracts were redissolved in a small volume of chloroform/methanol (1:1, v/v) and applied to TLC plates. The chromatograms were developed in light petroleum (b.p. 40–60 °C)/benzene/acetic acid (30:70:2, by vol.). Phospholipids and phosphoglycolipids (PL), along with the pigments, remained at the origin, whereas neutral lipids migrated along the plates. The band of PL with pigments was scraped off separately and extracted as described by Bligh and Dyer [31], centrifuged, and the organic solvents were phased by adding appropriate volumes of chloroform and water to arrive at a final chloroform/methanol/water ratio of 1:1:0.9 (by vol.). The extracts, which contained PL and pigments, were evaporated to dryness and redissolved in a small volume as described above. The above extracts were rechromatographed on TLC plates, using acetone/methanol/water (40:20:1, by vol.) as the developing system. Pigments migrated near the solvent front, whereas PL migrated along the plate. The PL fraction was recovered.

Separation of phospholipids into classes

The PL fraction was further fractionated by HPLC [33]. Briefly, the lipids were separated on a 10 µm-particle-size cation-exchange HPLC column using an isocratic elution system consisting of acetonitrile/[methanol/water (4:1, v/v)] (6:4, v/v).

Biological assay

PAF and the samples examined were dissolved in saline containing 2.5 mg of BSA/ml. AA was dissolved in saline containing 100 mg of BSA/ml, whereas ADP was dissolved in saline alone. Various concentrations of the samples examined were added to the aggregometer cuvette and the aggregation induced by the sample was studied in a Chrono-log aggregometer. Experiments with the specific inhibitor BN 52021 (0.1 mM in 0.3% DMSO in water), were also performed. This inhibitor was added to the aggregometer cuvette containing washed rabbit platelets 1 min

prior to the addition of the sample examined. This experiment was carried out according to Lazanas et al. [34]. In desensitization and cross-desensitization experiments, platelets were desensitized by the addition of the test lipid to the platelet suspension at a concentration that caused reversible aggregation. Secondly, stimulation with PAF or ADP or AA was performed immediately after complete disaggregation.

The platelet aggregation induced by PAF (1.25×10^{-10} M final concn.) was measured as PAF-induced aggregation in washed rabbit platelets before (considered as 0% inhibition) and after the addition of various concentrations of the sample examined [34]. Consequently the plot of percentage inhibition (ranging from 20 to 80%) versus different concentrations of the sample is linear. From this curve, the concentration of the sample that inhibited 50% of PAF-induced aggregation was calculated. This value is defined as concentration for 50% inhibition, IC_{50} . This experiment was also performed with AA (0.02×10^{-4} M final concn.) and ADP (0.35×10^{-5} M final concn.) in order to assess the inhibition to AA- and ADP-induced aggregation respectively.

Treatment with acetylhydrolase

The effect of PAF acetylhydrolase from human serum, an enzyme specific to short or intermediate-length *sn*-2 chains, on the ability of fractions to induce platelet aggregation, was examined. Briefly 50 mM Tris buffer, pH 7.4, human serum acetylhydrolase and the examined sample in BSA at 2.5 mg/ml in saline were added to a prewarmed (37 °C) test tube. The enzymic system was incubated at 37 °C, and, at different time intervals, aliquots were taken to test their ability to induce washed rabbit platelet aggregation.

Phosphorus determination

This determination was performed as described by Bartlett [35].

RESULTS AND DISCUSSION

Scytonema julianum is known from a number of Greek caves [28]. Total lipids extracted were about 10 mg, while the fraction comprising PL was approx. 1 mg.

A typical profile of the separation of the PL fraction along with the retention times of standard phospholipids is shown in Figure 1. This HPLC method, even though it does not separate all phospholipid classes sufficiently, was selected, since it permits purification of PAF and PAF analogues from the other phospholipids.

Lipids eluted from the HPLC system, represented by fractions PL1–PL12 (Figure 1), were manually collected and each one was rechromatographed under the same HPLC conditions for its further purification from the adjacent peaks. Consequently all the purified fractions were tested for their ability to induce washed-rabbit-platelet aggregation and/or to inhibit PAF-induced as well as AA- and ADP-induced washed-rabbit-platelet aggregation. The PL1 fraction, which contains some phospholipids along with some polar glycolipids [36], was kept for further purification.

Three fractions, namely PL2, PL8 and PL11, were detected that inhibited PAF-induced washed-rabbit-platelet aggregation as well as AA-induced aggregation in a dose-dependent manner. Fraction PL11 slightly inhibited ADP-induced aggregation too (Table 1). The UV spectra of the above three fractions showed an absorption maximum at 202 nm with a small absorption band at 400 nm. Standards of phosphatidylcholine (PC), phosphatidylethanolamine (PE) and sphingomyelin (SM) gave a strong

Table 1 Biological activities of each PL fraction

Washed-rabbit-platelet aggregation induced by PAF (1.25×10^{-10} M final concn.), by AA (0.02×10^{-4} M final concn.) and by ADP (0.35×10^{-5} M final concn.). *, Fraction that inhibits PAF- and AA-induced aggregation; No, fraction with no biological activity; +, fraction that induces platelet aggregation; ‡, fraction that inhibits PAF-, AA- and ADP-induced aggregation.

Fraction	Inhibition	Aggregation
PL2	*	No
PL3	No	No
PL4	*	+
PL5	No	No
PL6	No	+
PL7	No	No
PL8	*	No
PL9	No	No
PL10	No	No
PL11	‡	No
PL12	No	No

Figure 1 Representative HPLC separation of phospholipids from *Scytonema julianum* on a cation-exchange column with acetonitrile/[methanol/water, 4:1 (v/v)] (6:4, v/v)

Abbreviations: PI, phosphatidylinositol; PS, phosphatidylserine; LPC, lysophosphatidylcholine. a.u.f.s., absorbance units full scale.

absorbance band at 202 nm when exposed to identical test conditions. Phosphorus was not detected in any of the aforementioned fractions, suggesting that PL2 is definitely a non-phospholipid. On the other hand, since it appears from both

Figure 2 MS/MS of the negative ion at m/z 742

Figure 3 Positive ESI-MS of fraction PL4 from *Scytonema julianum* phospholipids

The m/z ranges from 700 to 1000 (A) and from 100 to 400 (B).

the HPLC purification and the UV spectrum that fractions PL8 and PL11 contain a only small amount of lipid, the negative phosphorus determination could be due to there being an insufficient amount of lipid, so evidence for these being non-phospholipids is not strong.

Fractions PL3, PL5, PL7, PL9, PL10 and PL12 did not exert any biological activity. Fraction PL3 gave a positive phosphorus determination [4.3 μg of elemental phosphorus (P); total amount] and co-migrated with standard PC on the TLC plate and on HPLC separation. Its UV spectrum showed a strong absorbance band at 202 nm, along with a much smaller one at 400 nm.

The positive ESI mass spectrum gave a quite abundant $[M + \text{Na}]^+$ ion at m/z 780 corresponding to PC ($\text{C}_{34:2}$) and the ion corresponding to phosphocholine itself at m/z 184. The ESI mass spectrum of fraction PL3 produced abundant $[M - 15]^-$ ion at m/z 742, in the negative mode, resulting from demethylation reaction [37]. Collisional activation of the $[M - 15]^-$ ion

Figure 4 MS/MS of the positive ion at m/z 809 $[M + \text{Na}]^+$ from Figure 3(A) along with the proposed structure of the phospholipid with a molecular mass of 786 Da

Identified fragmentations are also represented.

(Figure 2) yielded abundant carboxylate ions at m/z 255 ($\text{C}_{16:0}$) and 279 ($\text{C}_{18:2}$), corresponding to the substituents at the *sn*-1 and *sn*-2 positions, respectively, since the most abundant carboxylate anion formed corresponds to the substituent at the *sn*-2 position [38]. Also, the fragment ion at m/z 168, the dimethylaminophosphate anion and the common fragment for all diacylglycerophospholipids at m/z 153, [glycerophosphate- $\text{H}_2\text{O}]^-$, were observed along with the low-abundance ions at m/z 504 and 480, corresponding to release of neutral ketene analogue structure from the *sn*-1 and *sn*-2 position respectively. The fragment at m/z 224 is likely to correspond to the release of the free fatty acid along with the release of the neutral ketene analogue structure from the *sn*-1 and *sn*-2 positions respectively.

The above data prove, for the first time, the existence of PC in cyanobacteria.

Fraction PL4, in low concentrations, induced washed-rabbit-platelet aggregation with a pattern different from that of PAF. The aggregation induced by this fraction was slow at the beginning and irreversible at all the tested concentrations. The maximum aggregation was observed at concentrations of the order of 10^{-6} M final concentration, while the EC_{50} value was at 0.7×10^{-6} M final concentration (both on the basis of phosphorus determination). It seems that this active phospholipid acts through the PAF receptor, since (1) platelets desensitized to PAF were not aggregated by this lipid and (2) BN 52021, a PAF-receptor-specific inhibitor, inhibited the aggregation induced by this active lipid. Incubation of this phospholipid with serum acetylhydrolase resulted in its inactivation, but the observed rate of hydrolysis is lower than that of PAF, suggesting that an acetyl group is not present at the *sn*-2 position of a glyceryl backbone.

This active phospholipid inhibited PAF-induced aggregation in a dose-dependent manner when tested at higher concentrations. More specifically, its IC_{50} value against PAF-induced

Figure 5 MS/MS of the positive ion at m/z 779 $[M+Na]^+$ from Figure 3(A) along with the proposed structure of the phospholipid with a molecular mass of 756 Da

Figure 6 Positive ESI-MS of fraction PL6 from *Scytonema julianum* phospholipids, m/z ranging from 1000 to 1300

aggregation was at 3.2×10^{-4} M final concentration (on the basis of phosphorus determination). Its UV pattern was similar to that of PC and SM, with an absorption maximum at 202 nm and a smaller peak at 400 nm. This phospholipid co-migrated with synthetic acetyl-SM on HPLC.

The positive ESI spectrum of fraction PL4 (Figure 3A) showed intense $[M+H]^+$ and/or $[M+Na]^+$ ions of SM species, since they all appeared at odd m/z values ranging from 721 to 849, along with the ions at m/z 184, 147, 125 and 104, which correspond to fragments of the phosphocholine moiety (Figure 3B).

The ESI-MS/MS spectrum of the fragment ion at m/z 809 is shown in Figure 4. The most abundant positive fragment ion at m/z 626 corresponded to the loss of polar choline phosphate head group as a neutral fragment of 183 Da from the sodiated SM molecule, as has been previously reported [39,40], while the ion at m/z 604 may correspond to the loss of the Na atom from the fragment at m/z 626. The fragment ions at m/z 147 and 184 are characteristic ions for the phosphocholine moiety. In addition, the observed ions at m/z 750 and 722 should correspond to the loss of $[N(CH_3)_3]$ and $[(CH_2)_2N(CH_3)_3]$ moieties from the

Figure 7 Proposed structure of fraction PL6 from *Scytonema julianum* phospholipids, along with the identified fragmentations

choline phosphate head group of the sodiated SM molecule respectively. The fragmentation resulted in an ion at m/z 750, which has recently been reported for sodiated, lithiated and potassiumated SM molecules [40]. Ions at m/z 264 and 304 reflect the long-chain base and the fatty acid in the molecule (Figure 4). The fragments corresponding to ions at m/z 502, 524 and 685, along with the proposed structure, are represented in Figure 4. It seems that this SM molecule has two esterified fatty acids: one at the free hydroxy group of the sphingoid chain and the other one at a free hydroxy group in the fatty acid chain. These fatty acids should be the acetic ones, since on the one hand the fragment ion at m/z 685 is produced (Figure 4) and on the other hand the chromatographic behaviour, along with the biological activity of this lipid, are identical with those of acetyl-SM [12,15].

Identical fragmentations were observed in the ESI-MS/MS spectra of the other SM molecules corresponding to fragments at m/z 779 (Figure 5), 805 and 829. Additionally, the fragment ion at m/z 721 (Figure 3A) corresponds to the loss of the acetyl group from the most abundant SM molecule at m/z 779.

The negative ESI spectrum of fraction PL4 showed fragment ions at m/z 767 and 791, which correspond to the expected $[M-15]^-$ fragment of the SM molecules with relative molecular masses 782 and 806 respectively.

Fraction PL6 induced washed-rabbit-platelet aggregation in a dose-dependent manner with a pattern similar to that of PAF. This active lipid seems to act through PAF receptors since (1) platelets desensitized to PAF were not aggregated by this lipid and vice versa, (2) platelets desensitized to AA or ADP were aggregated after the subsequent addition of this lipid and (3) BN 52021, a PAF-receptor-specific inhibitor, fully inhibited the aggregation induced by fraction PL6.

Incubation of this lipid with human serum acetylhydrolase resulted in its time-dependent inactivation, with a rate identical with that produced by PAF, suggesting the presence of an acetyl group at the *sn*-2 position of a glycerol backbone. Also, this lipid co-migrated with synthetic PAF on HPLC. Its UV pattern was similar to those of standard PC and PE, with an absorption maximum at 202 nm and a shoulder at 400 nm.

Consequently, an effort was made to elucidate its structure by ESI-MS, since its limited quantity did not permit any

Figure 8 Positive (A) and negative (B) ESI-MS of fraction PL6 from *Scytonema julianum* phospholipids

The positive ion at m/z 973 is also represented in the enlargement of the corresponding area.

chemical determinations. The ESI mass spectrum of fraction PL6 (Figure 6) gave positive ions at m/z 1251 $[M+H]^+$, at m/z 1191 $[M+H-AcOH]^+$, at m/z 1131 $[M+H-2AcOH]^+$ and at m/z 1235 $[1191-2H+2Na]^+$, supporting on the one hand the structure of a lipid with a molecular mass of 1250 Da and, on the other hand, the existence of at least two acetyl groups. It is noteworthy that the neutral loss of 60 units or 120 units is common for acetylated glycolipids. The absence of the characteristic positive fragment ion of phosphocholine moiety at m/z 184 is strong evidence that this lipid is not a PAF molecule.

Its chromatographic behaviour, its mass spectra, which share many similarities with those reported for glycolipids [25,41], along with the fact that galactosyldiacylglycerols are major membrane lipids in cyanobacteria [42], led to the proposed structure of the acylacetylgllycerol phosphoglycolipid represented in Figure 7, along with the identified fragmentations. The presence of the acetyl group at the *sn*-2 position of the glycerol backbone is also supported by the results of the acetylhydrolase experiments. In addition, we have previously reported that acyl-acetylgllycerol phosphoacetylated glycolipids can induce washed platelet aggregation [12,13].

The existence of the terminal sugar moiety is confirmed by the abundant negative ion at m/z 235 (Figure 8B) and by the positive ion at m/z 973 (Figure 8A), corresponding to $[M-sugar C-R_2CO]^+$. The other abundant negative ion at m/z 277 (Figure 8B) and the positive ion at m/z 713 (Figure 8A), $[M-sugar C-R_2CO-R_1CO]^+$, generated by the loss of the neutral ketene analogue structure from the *sn*-1 position of fragment 973, along with the fact that polyunsaturated fatty acids, represented by linoleic ($C_{18:2}$) and γ -linolenic ($C_{18:3}$) acids are localized mainly at the *sn*-1 position of diacylglycerolipids in cyanobacteria [41], provide strong evidence for the existence of a $C_{18:3}$ fatty acid in the structure of fraction PL6. In addition the MS/MS analysis of fragment 713 that is represented in Figure 9, along with its identified fragmentations, verified the aforementioned structure.

The presence of the sugar moiety B was determined by the positive ions at m/z 493, 381 and 365 (Figure 8A). Their

Figure 9 MS/MS of the positive ion at m/z 713 from Figure 8(A) along with the identified fragmentations

fragmentation pathways are represented in Figure 7. The MS/MS analysis of the ion at m/z 493 revealed ions at m/z 461 $[493-CH_3OH]^+$, at m/z 423 $[493-OC(CH_2)_2CH_3]^+$, at m/z 381, at m/z 259 (sugar moiety B), at m/z 171 $[259-HOOC-(CH_2)_2CH_3]^+$, at m/z 241 corresponding to the sodiated sugar moiety C and at m/z 183 $[241-OCOCH_3]^+$, all supporting the above structure. The neutral loss of methanol is a common fragmentation in MS/MS analysis of methoxy-sugar derivatives [43].

Finally, the negative ion at m/z 269 and the positive ions at m/z 625 and 413 (Figure 8), which support the presence of the sugar moiety A, are represented in Figure 7. The MS/MS analysis of fragment 413 gave predominant positive ions at m/z 81, 99 and 143 corresponding to $[HSO_3]^+$ (or $[H_2PO_3]^+$), to $[H_3SO_4]^+$ (or $[H_3PO_4+H]^+$) and to $[HSO_4Na_2]^+$ (or $[H_2PO_4Na_2]^+$), respectively, and another ion at m/z 189 which should represent the sugar moiety A after the loss of the $-SO_3$ group.

In conclusion, our results show that the studied cyanobacterium, *Scytonema julianum*, contains a PC ($C_{16:0}/_{18:2}$), even though bacteria very seldom contain choline-containing phosphoacylglycerols. In addition, two types of biologically active phospholipids have been detected and have been shown to be an acetyl-SM and an acylacetylgllycerol phosphoacetylated glycolipid. The existence of the sphingomyelin analogues was expected, since sphingoid-containing compounds seem to be ubiquitous and also very important. It is well known that ceramides, cerebrosides and related lipids are intracellular second messengers and mediate diverse biological processes, including growth and programmed cell death. The identification of the phosphoglycolipid demonstrates a new type of lipid in cyanobacteria that, in addition, exhibits a biological activity very similar to that of PAF. This active lipid may contribute to the allergic skin irritation and allergic asthma that appear after cyanobacterial exposure, since it is reported that these allergenic reactions may be quite unrelated to cyanobacterial toxins. Moreover, the presence of this phosphoglycolipid, which exhibits a biological activity very similar to that of PAF, reinforces the notion that PAF is a member of a large family of lipid mediators apparently having different physiological roles in unicellular and multicellular organisms.

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