A facile enzymatic synthesis of sphingosine-1-phosphate and dihydrosphingosine-1-phosphate

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Summary A procedure is described to prepare sphingosine-1-phosphate by treatment of sphingosylphosphocholine with phospholipase D, isolated from *Streptomyces chromofuscus*. The phosphorylated long chain bases were purified by selective precipitation and differential extraction. Milligram quantities can be obtained in a yield of about 70%. Application of the procedure to dihydrosphingosylphosphocholine results in the synthesis of dihydrosphingosine-1-phosphate.—**Van Veldhoven, P. P., R. J. Foglesong, and R. M. Bell.** A facile enzymatic synthesis of sphingosine-1-phosphate and dihydrosphingosine-1-phosphate. *J. Lipid Res.* 1989. **30:** 611–616.

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A renewed interest in sphingolipids has developed since the discovery of sphingosine and lysosphingolipids as protein kinase C inhibitors in vitro (1, 2) and in vivo (1–4). A limited structure–activity study showed that the presence of a positive charge and a hydrophobic moiety was essential for inhibition (1–3). Sphingoid long chain bases are one of the few biological molecules that possess these characteristics and were therefore proposed as biological regulators of protein kinase C (1, 2).

According to this hypothesis, sphingosine would be produced in response to external stimuli and function to regulate protein kinase C activity negatively. Consequently, mechanisms must also exist to attenuate the signal. A major attenuation pathway for sphingosine (and probably lysosphingolipids) occurs through acylation of the free amino group (3–5). The N-acylated sphingolipids are inactive as protein kinase C modulators (2, 4).

For sphingosine, another attenuation pathway is possible: phosphorylation of the 1-OH-group, followed by cleavage to ethanolamine phosphate and palmitaldehyde (6–8). Upon

Abbreviations: SPC, sphingosylphosphocholine; (dihydro)sphingosine phosphate, (dihydro)sphingosine-1-phosphate.

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addition of labeled sphingosine to platelets, most of the label is found in sphingosine-1-phosphate (sphingosine phosphate) (9, 10). In contrast, acylation to ceramide is predominant in HL-60 cells (3) and human neutrophils (4, 5). Whether sphingosine phosphate acts as a protein kinase C inhibitor has not been established. This lipid is not commercially available and its chemical synthesis has never been reported.

Although Weiss (11) attempted to make sphingosine phosphate, those efforts were unsuccessful, probably due to the presence of the allylic OH-group in sphingosine. Synthesis of dihydrosphingosine phosphate was achieved, however, in four steps involving hydrogenation of sphingosine, carbobenzoxylation of the amino group, phosphorylation with diphenylphosphorylchloride, and removal of the protecting group. Other reports on the chemical synthesis of phosphorylated long chain bases are not known to us, but biochemical ways to make these lipids do exist. Cytosolic fractions from rat liver (12) or microsomal fractions of *Tetrahymena pyriformis* (13) have been used as a crude source of sphingosine kinase. Isolated platelets, which are apparently not able to metabolize sphingosine phosphate (9, 10), can also be used to obtain phosphorylated labeled long chain bases. Further isolation and purification of the produced phosphate esters is tedious, however, and hampered by their sparing solubility in polar and nonpolar solvents. Another possible approach consisted of the acidic hydrolysis of ceramide phosphate, which can be obtained by the action of *E. coli* diacylglycerol kinase on ceramide (14–16). Further isolation of the product turned out to be difficult (Bishop, W. R., and R. M. Bell, personal communication).

The procedure we report here to prepare milligram quantities of sphingosine phosphate relies on the action of a phospholipase D type enzyme on sphingosylphosphocholine (lysosphingomyelin; SPC). This enzyme, isolated from *Streptomyces chromofuscus* (17) and *Corynebacterium ovis* (18), removes the choline headgroup from sphingomyelin and phosphatidylcholine. Because this phospholipase acts also on lysophosphatidylcholine (17, 18), we reasoned that SPC could possibly be a substrate. Due to the limited solubility of the sphingosine phosphate product in aqueous and organic solvents, further purification turned out to be rather straightforward and did not require sophisticated equipment. The commercial availability of SPC and the phospholipase D enzyme increases the general applicability of this method.

By applying the method to dihydroSPC, dihydrosphingosine phosphate can be prepared in the same way.

MATERIALS AND METHODS

Phospholipase D (*Streptomyces chromofuscus,* type VI, 3000 U/mg solid),[2] choline oxidase (*Alcaligenes* sp., 15 U/mg solid), sphingosylphosphocholine (free base, batch 84F-4011), 4-aminoantipyrine, methyl-^{12}C-d_3 alcohol-*d* and acetic-d_3 acid-*d* were obtained from Sigma, St. Louis, MO; sphingomyelin (egg yolk) from Avanti Polar Lipids, Inc., Birmingham, AL; peroxidase (horse radish, grade I, 250 U/mg lyophilizate) from Boehringer Mannheim Biochemicals, Indianapolis, IN; phenol from Baker Chemical Co., Phillipsburg, NJ; rhodium on aluminium catalyst from Aldrich Chemical Company, Inc., Milwaukee, WI; Silica 60 G plates (20 × 20 cm; 0.25 mm) from EM Scienes, Cherry Hill, NJ; Silica 60 Å K6F plates (20 × 20 cm; 0.25 mm) from Whatman Chemical Separation Inc., Clifton, NJ, and Pyrex disposable screw-cap tubes from Corning Glass Works, New York, NY.

Phosphate determinations were performed as described (16) or modified as follows when analyzing lipids separated by TLC. The silica regions containing the compound of interest were scraped and collected into Pyrex glass tubes. Inorganic phosphate standards received the same amount of silica. After wetting the silica with 0.4 ml water, 0.2 ml 70% (w/v) $HClO_4$ was added and tubes were put in a heating block. The temperature was slowly raised to 165°C and kept at 165°C for 24 hr. After cooling, 1 ml water was added and samples were further processed as described (16). After development of the color, the silica was pelleted by centrifugation and the absorbance of the supernatant was measured at 820 nm.

Choline measurements were based on a coupled choline oxidase/peroxidase assay (17, 19), modified as follows. Samples, containing up to 100 nmol choline, were dried under N_2 and dissolved in 1 ml reaction mixture, containing 50 mM HEPES buffer, pH 7.5, 1 mM 4-aminoantipyrine, 2 mM phenol, 2 U choline oxidase, and 5 U of peroxidase. The choline oxidase was added from a concentrated stock solution (200 U/ml) made 4 M NaCl in 10 mM EDTA, pH 7.4, which was stored at 4°C. After a 20-min incubation at 37°C, absorbances were read at 500 nm (ϵ=12,200 $mol^{-1} \cdot min^{-1}$). Solutions were routinely centrifuged before reading the absorbance to remove some cloudiness, which was observed when the SPC concentration was 50 μM or higher and disturbed the readings at this wavelength.

Dihydrosphingosylphosphocholine was prepared by acidic hydrolysis dihydrosphingomyelin (20) which was obtained by catalytic hydrogenation of sphingomyelin under H_2 atmosphere in the presence of rhodium/aluminium catalyst.

TLC was performed on either Merck Silica 60 G or Whatman Silica 60 Å K6F plates developed in one of the following systems: (A) n-butanol–acetic acid–water 6:2:2

[2]The activity of the commercial phospholipase D preparation was 3000 units/mg solid lyophilized powder (containing approximately 50% protein). One unit is defined as the amount of enzyme hydrolyzing 1 μmol phosphatidylcholine/hr in 1 M Na-acetate buffer, pH 8.0, at 30°C. Therefore, the specific activity of this preparation for phosphatidylcholine is about 100 μmol/min per mg protein.

(v/v); (B) chloroform–methanol–water 65:25:4 (v/v); (C) chloroform–methanol–water 60:35:8 (v/v). Lipid spots were detected by iodine, while spraying with ninhydrin, molybdenum blue reagent, and Dragendorf reagent (21) was used to reveal the presence of amino, phosphate, and choline groups, respectively. Purity of SPC and dihydroSPC as checked by TLC in system A was higher than 95%.

FAB mass spectra were obtained with a VG Analytical 70S mass spectrometer (Manchester, United Kingdom) using a thioglycerol matrix and xenon as bombardment gas. NMR spectra were obtained with a General Electric GN-300 300 MHz NMR spectrometer.

RESULTS AND DISCUSSION

When SPC was treated with phospholipase D from *Streptomyces chromofuscus,* thin-layer chromatography showed the generation of a new compound (see Fig. 1). Based upon several criteria this compound was identical to sphingosine phosphate. Selective spraying revealed the presence of a free amino group and a phosphate group in this compound, but no choline, which is consistent with the known activity of the phospholipase used. Simultaneously measuring the generation of choline, via a coupled choline oxidase/peroxidase assay, and of the new compound, by measuring its phosphate content, resulted in a 1:1 stoichiometry (**Fig. 1**). The formation of these two products was also inversely proportional to the decrease in SPC (Fig. 1). In addition, the mobilities upon chromatography in different solvent systems are comparable to published values obtained with labeled sphingosine phosphate (9, 10). R_f values on Merck Silica G plates were 0.45, 0.02, and 0.19 with solvent systems A, B, and C, respectively. When chromatographed on Whatman Silica K6F plates, these values were 0.48, 0.02, and 0.23. Finally, the ^1H and ^{13}C NMR spectra (see below) and mass spectrum[3] were all consistent with the proposed structure.

Under the assay conditions described in the legend for Fig. 1, the reaction mixture starts to become cloudy after about 60 min. At the same time, SPC is not further cleaved by the enzyme. This seemed not to be due to inactivation of the enzyme since addition of an excess of enzyme at that time failed to produce more sphingosine phosphate (data not shown). On different occasions we noticed that, when using less substrate or more initial enzyme than the experiment shown in Fig. 1, the reaction slowed down when the choline[4] concentration reached 4 mM, suggesting product inhibition (22).

Using the choline oxidase/peroxidase assay to measure the initial rates of choline formation, the K_m and V_{max} values were estimated at 8.16 mM and 140 μmol/min per mg protein (present in the phospholipase D preparation). Compared to phosphatidylcholine,[2] the phospholipase D is thus slightly more active on SPC (assuming no influence

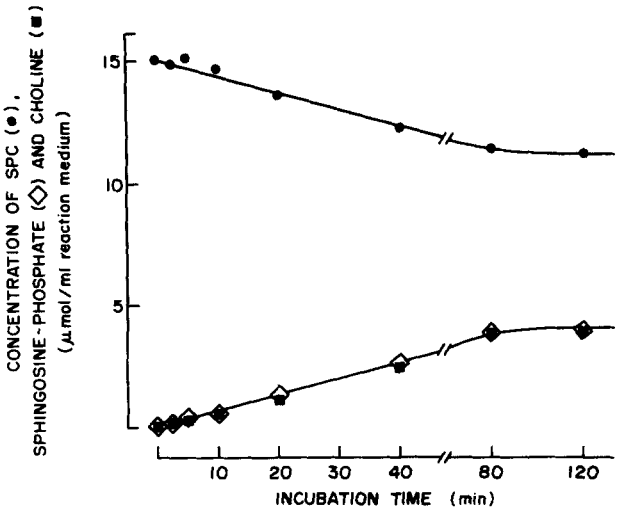

Fig. 1. Sphingosylphosphocholine hydrolysis by phospholipase D. SPC (15 μmol) was dissolved in 0.9 ml 50 mM ammonium acetate buffer, pH 8.0, mixed with 1.7 μg phospholipase D preparation (dissolved in 0.1 ml buffer) and incubated at 30°C. At the indicated time points, 25 μl reaction medium was removed and diluted into 150 μl chloroform–methanol 1:2 (v/v). Aliquots (25 μl) of this solution were spotted in duplicate on Whatman K6F plates and chromatograms were developed in system A. One plate was sprayed with ninhydrin to reveal lipids containing a free amino group. These spots were also phosphate-positive (data not shown). The duplicate plate was exposed to iodine and spots corresponding to SPC and reaction product were scraped and their phosphate content was determined. The sum of substrate and product equalled 90.8–105.4% of the initial amount of SPC. For the liberation of choline, 50 μl of the chloroform–methanol solutions were dried under N_2 and analyzed for choline; SPC, sphingosylphosphocholine.

of the assay buffer composition on the enzyme activity).

For the preparative purification of sphingosine phosphate, 10 μmol SPC was treated with 65 μg phospholipase D preparation in 2 ml 50 mM ammonium acetate buffer, pH 8.0, at 30°C. After 1 hr the insoluble reaction product was removed by centrifugation. The supernatant was allowed to react for another 1 hr, cooled to 4°C, and again centrifuged. The pelleted materials were combined and dissolved in water (1 ml) by sonication and cooled to 4°C. The precipitated material was sedimented by centrifugation. This step was repeated once and the final pellet, enriched in sphingosine phosphate, was dried under vacuum and dispersed in acetone (1 ml) by sonication. After cooling to 4°C, the solution was centrifuged and the supernatant was removed. This acetone precipitation step was repeated once. The acetone phase contained a neutral lipid which moved at the solvent front on TLC in system A. Apparently, it must be a contaminant present in the enzyme preparation

[3]Mass spectrum (FAB, Ar, thioglycerol), *m/z* (relative intensity) 488 (8.5), 380 (45.3, M^+ + H), 264 (100.0), 184 (10.7), 140 (22.6), 121 (4.2).

[4]Although the total concentration of sphingosine phosphate, measured as described in the legend to Fig. 1, equalled the concentrations of the produced choline, the actual concentration of the phosphorylated base in a soluble form is not known under these assay conditions.

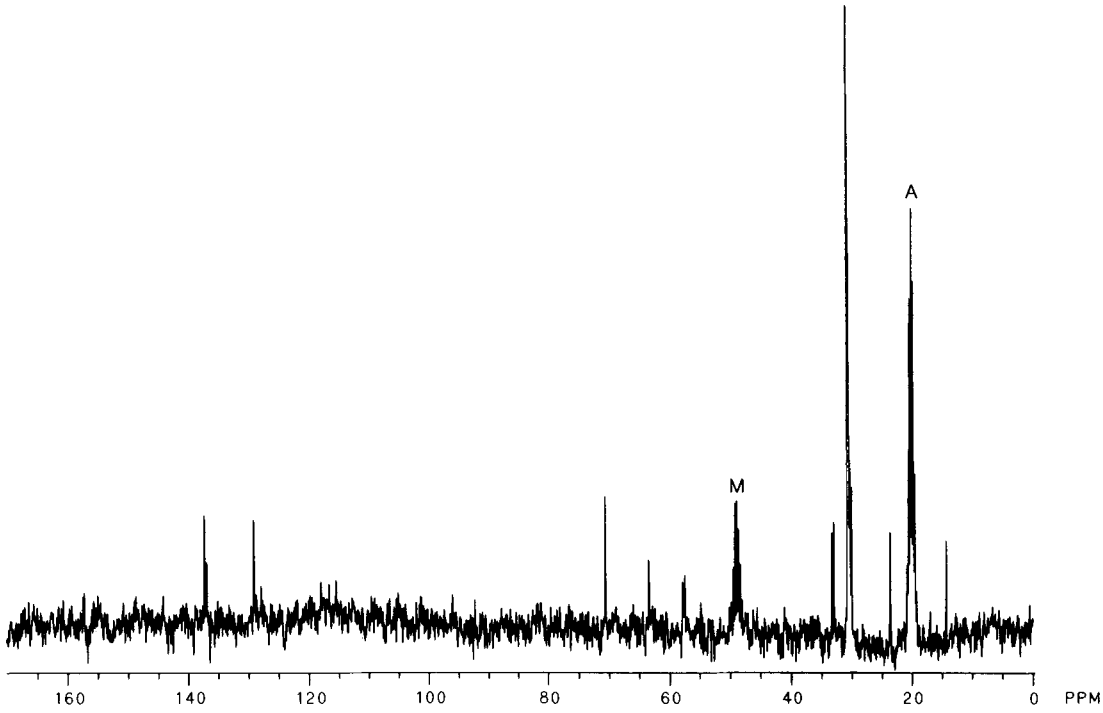

Fig. 2. Carbon-13 NMR proton-decoupled spectrum of sphingosine phosphate. Sphingosine phosphate was dissolved in methyl-^{12}C-d_3 alcohol-*d*-acetic-d_3 acid-*d* 9:1 (v/v) and NMR spectrum was obtained by power-gated decoupling at 75.0 MHz after 2400 scans. Chemical shifts for the carbons of sphingosine, expressed in parts per million (ppm) relative to the internal standard acetic-d_3 acid-*d* (20.0 ppm), are as follows: C1 (63.5), C2 (57.8 and 57.4), C3 (70.6), C4 (137.3 and 136.9), C5 (129.1 and 127.9), C6 (33.2), C16 (32.9), C7–C15 (30.6), C17 (23.6), and C18 (14.3); M, methanol resonance; A, acetic acid resonance.

which, indeed, was not pure.[2] Sphingosine, a small contaminant of commercially obtained SPC, is also removed by the acetone extraction. The acetone-insoluble material was dried under N_2 and dissolved in chloroform–methanol 1:1 (v/v) (1 ml) by sonication, cooled to 4°C, centrifuged, and the supernatant was removed. This step was repeated once and served to remove the last traces of substrate. Some sphingosine phosphate was also present in the chloroform–methanol solution. The final pellet consisted of apparently pure sphingosine phosphate, which could be dissolved in methanol, but to a limited extent. Upon analysis of the methanol solution by TLC, only one spot could be detected, being iodine-, ninhydrin-, and phosphate-positive and with an R_f value of 0.48 in butanol–acetic acid–water 6:2:2 (v/v). A yield of 66% was calculated.

The solubility of phosphorylated long chain bases is very peculiar as already noticed by others (10, 23, 24). Sphingosine phosphate was not soluble or very poorly soluble in petroleum ether, chloroform, acetone, butanol, ethanol, ethyl acetate, dimethylsulfoxide, and propyleneglycol, even upon warming, and sparingly soluble in methanol and water. Acidified organic solvents, such as methanol–3 N HCl 3:1 (v/v), methanol–acetic acid 9:1 (v/v), and methanol–chloroform–6 N HCl 7:4:1 (v/v) were more effective. As described by Weiss (11) for dihydrosphingosine phosphate, warm acetic acid was also a good solvent. Upon addition of one volume water to the acetic acid solution, the phosphate ester precipitated. We did not choose this purification step since some hydrolysis to sphingosine occurred (data not shown). Although the solubility in methanol is limited (the maximal concentration is about 3 mM) in our hands it was the best solvent to store and analyze this lipid by TLC.

Interestingly, capnine,[5] a sulfonated long chain base found in *Capnocytophaga* sp., a genus of gram-negative sliding bacteria, shows a similar low solubility in most solvents (25). Presumably this is due to intermolecular interactions between the oppositely charged ionic groups of these compounds, combined with hydrophobic interactions of the hydrocarbon chains.

Stoffel, LeKim, and Sticht (23) reported that the free acid and alkali salt of dihydrosphingosine phosphate were barely soluble in water, but the addition of Triton X-100 or the use of the triethanolamine salt resulted in a clear aqueous solution. The concentration range was not stated, however. Sphingosine phosphate as a triethanolamine salt could be dissolved in water, up to 3 mM, but these solutions had an opalescent appearance although without any sign of precipitation.

[5]Capnine is the trivial name assigned to 2-amino-3-hydroxy-15-methylhexadecane-1-sulfonic acid.

We also attempted to determine the stereoisomeric composition of the produced sphingosine phosphate. Presumably it is a mixture of L-*threo* and D-*erythro* forms, because the SPC[6] used in these studies was obtained by acidic hydrolysis of sphingomyelin (20, 26). These procedures are known to cause isomerization at C-3 of the sphingosine moiety (27–29). Although we think that the incomplete hydrolysis of SPC under the conditions described in Fig. 1 is due to product inhibition (see above), these data could also be interpreted that only (or preferentially) one of these isomers is recognized by the enzyme. We tried to use proton NMR to determine the isomeric ratios, but due to solubility problems the spectra were not useful for accurate ratio estimations. The proton NMR, combined with mass spectrum did, however, conclusively show that the material is sphingosine phosphate. Carbon-13 NMR proved to be more useful. Resonances of the C3 (28) and C4–C5 carbons (30) have been reported to be sensitive to their stereochemical environments. Indeed, ^{13}C NMR of our material showed the presence of two isomers in approximately the ratios expected based on the starting material (**Fig. 2**), indicating that the enzyme does not seem to express a preference of one isomer above another.[7] Such preference would also be surprising in view of the fact that this phospholipase works on phosphatidylcholine and lysophosphatidylcholine as well.

Attempts to obtain pure isomers of sphingosine phosphate were not successful, since we did not succeed in separating the SPC isomers either by silicic acid column chromatography or TLC. TLC in chloroform–methanol–water 60:35:8 (v/v) has been reported to separate these isomers, the *threo*-compound being slightly faster (27). In our hands, however, sufficient separation of the isomers could not be obtained with this system, in agreement with others (31). Acylation with an easily removable group and separation of the isomers might be useful and is under consideration at this moment.

Pure D-*erythro*-dihydrosphingosine phosphate could be prepared, however, by treating dihydroSPC with phospholipase D. Isomerization does not occur during acidic hydrolysis of sphingomyelin when the double bond of the long chain base is first reduced (32).

Sphingosine phosphate (as a triethanolamine salt) did not inhibit purified rat brain protein kinase C when assayed using mixed micellar methods (33) using 10 mol % phosphatidylserine, 2 mol % *sn*-1,2-dioctanoylglycerol, and 100 μM $CaCl^2$ (Bloomenthal, J., and R. M. Bell, unpublished data).

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[6]Proton-NMR of the starting SPC showed approximately a 72:28 mixture of D-*erythro* and L-*threo* forms, in agreement with the data of Sripada et al. (29) for SPC obtained by acidic hydrolysis of sphingomyelin according to the method of Gaver and Sweeley (26).

[7]We noticed that after TLC in system A and spraying for phosphate, the SPC spot was an unresolved doublet, probably reflecting the presence of these isomers, similar to the chromatogram in chloroform–methanol–water 60:35:8 (v/v) (27). Both components of this spot seemed to disappear at the same rate during phospholipase D treatment, another indication that the enzyme recognizes both forms.

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