Enzymatic Preparation of Sphingosine 1-Phosphate†

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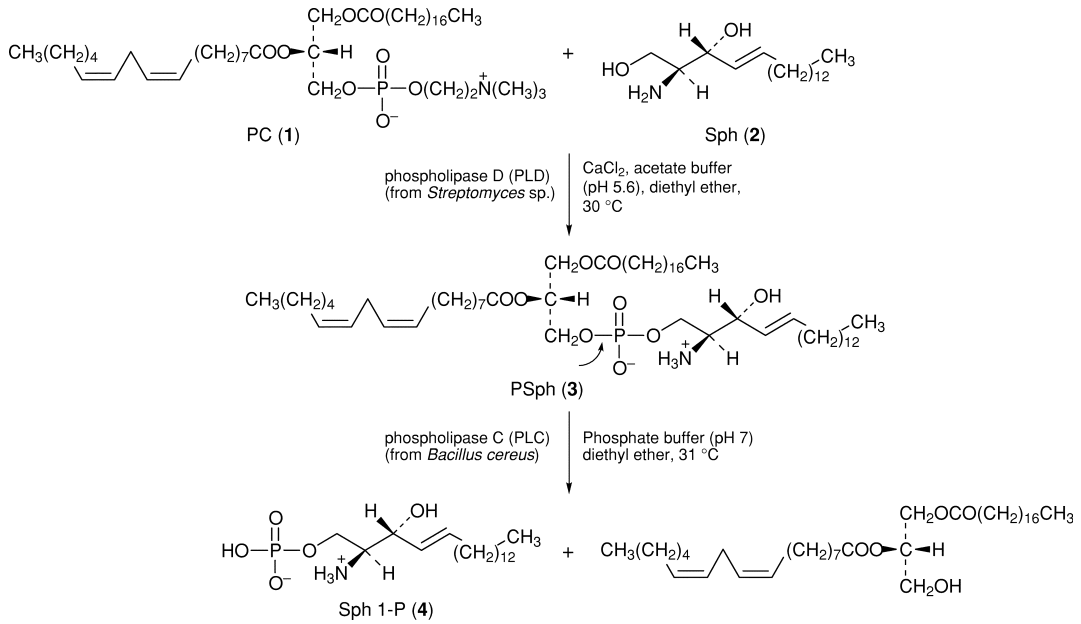
Sphingosine 1-phosphate was prepared from sphingosine by two steps involving conversion of sphingosine to phosphatidylsphingosine catalyzed by phospholipase D and enzymatic hydrolysis of the intermediate to a diacyglycerol and sphingosine 1-phosphate with phospholipase C.

In the sphingolipid metabolic cycle, sphingosine 1-phosphate (Sph 1-P) is produced from sphingosine (Sph) by sphingosine kinase and converted back to Sph catalyzed by sphingosine 1-phosphate phosphatase. Sph 1-P has been identified as a second messenger for various biochemical events. Zhang *et al.* suggested[1] that, in their study using Swiss 3T3 fibroblasts, Sph 1-P was involved in calcium release and the regulation of cell growth induced by sphingosine. By the same research group,[2] it was also demonstrated using the Swiss 3T3 cells that Sph 1-P induced a rapid increase in phosphatidic acid level which might be correlated with its effect on DNA synthesis in addition to calcium immobilization. This group also reported very recently[3] that Sph 1-P activated G protein-coupled orphan receptor EDG-1, over expression of which induced exaggerated cell–cell aggregation, enhanced expression of cadherins and formation of well-developed adherens junctions. Thus, Sph 1-P has been implicated as an important second messenger in proliferation and survival of cells. However, its receptors on the cell surface have not been identified yet. To promote studies of this field it is essential to supply Sph 1-P in sufficient quantities. A one step synthesis from sphingosine by utilizing sphingosine kinase seems to be impracticable since the enzyme is not readily accessible. The most recent report on the chemical synthesis of Sph 1-P was done by Kratzer and Schmidt.[4] Their method is based on the use of azidosphingosine in the phosphite amide method. This method seems to be the most efficient and versatile so far. However, it still requires several steps. The present report describes a very simple and convenient two-step enzymatic preparation of Sph 1-P using phospholinase D (PLD) and phospholipase C (PLC) *via* phosphatidylsphingosine (PSph) as an intermediate (Scheme 1). This strategy is basically the same as that reported by D'Arrigo *et al.*[5] In the present case, however, a problem arose in the PLC-catalyzed phosphate ester cleavage step.

PSph could be prepared very easily from phosphatidylcholine (PC) **1** and commercially available sphingosine (Sph) **2** by phospholipase D-catalyzed transphosphatidylation. In fact, as described in the experimental section, commercially available PLD from *Streptomyces* sp. catalyzed the reaction to afford PSph. Here, PC **1** bearing a linoleoyl group at the *sn*-2 position was employed to utilize the olefinic protons as a marker for structural analyses by NMR. As a key and last step, the PSph **3** was submitted to PLC-catalyzed phosphate ester cleavage. The enzymic reaction was conducted using PSph and PLC from *Streptomyces chromofuscus* in the presence of sodium deoxycholate, calcium chloride and lecitin in tris–HCl, pH 7.5. However, no reaction was observed. As another candidate, PLC from *Bacillus cereus* was examined under the same conditions. This enzyme is known to be inactive to phosphate cleavage at the sphingomyelin site suggesting that such a cleavage occurs at the phosphatidyl site of **3**[6] which is marked with a curved arrow in the reaction scheme. Here, the addition of lecitin broad-



Scheme 1 Enzymic synthesis of sphingosine 1-phosphate

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ens the specificity of the enzyme. Unexpectedly however, only a trace of Sph 1-P product was detected on TLC by molybdenum-blue coloration for phosphorus and ninhydrin-violet coloration for the amino group. Very recently, Kamata *et al.* found[7] that, in the phospholipase C-catalyzed hydrolysis of PC, calcium ion was not essential and it rather retarded the reaction. Thus, we used the same PLC from *Bacillus cereus* as described above for cleavage of the phosphate ester bond of **3** without Ca^{2+} under the reaction conditions described in the experimental section. TLC analysis indeed showed the formation of Sph 1-P. After purification by silica gel preparative TLC, the isolated yield was 30%. Although, the yields are unsatisfactory and should be improved further by alteration of enzyme source and/or reaction conditions, the present synthetic route with only two steps may be usable to obtain Sph 1-P in a preparative scale in a much shorter time.

Experimental

^{1}H NMR spectra (δ_H) were recorded on a Varian VXR 200 or 500 spectrophotometer. Ion spray mass spectra were taken with an API III triple quadrupole mass spectrometer (PE-Sciex) equipped with an ion spray interface.

Preparation of Phosphatidylsphingosine **3**.—To a solution of 1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (13.1 mg, 17 μmol) was added acetate buffer [0.26 ml, containing calcium chloride (0.1 M), excess of sphingosine (25 mg, 84 μmol), phospholipase D from *Streptomyces* sp. (*ca.* 50 units) and a trace of butylated hydroxytoluene (an antioxidant), and the solution was stirred at 30 °C for 4 h in a nitrogen atmosphere in the dark. Ethylenediaminetetraacetic acid (EDTA) (0.52 ml, 0.1 M) was added to stop the reaction and the product was extracted with chloroform five times. After evaporation of the solvent under reduced pressure, the residue was chromatographed on silica gel using a solvent system ($CHCl_3$–CH_3OH, from 60:25 to 65:10) giving the title compound (**3**) as a sticky liquid. Yield 25%. TLC ($CHCl_3$–CH_3OH, 60:25): $R_f = 0.69$). δ_H 0.88 (br, 9H, $3 \times CH_3$), 124 (m, 64H, $32 \times CH_2$), 1.58 (m, 4H, $2 \times OCOCH_2CH_2$), 2.04 (m, 6H, $3 \times C{=}CCH_2CH_2$), 2.28 (m, 4H, $2 \times OCOCH_2$), 2.76 (m, 2H, $C{=}CCH_2C{=}C$), 3.72 (m, 1H, $CH_2CHNH_3^+$), 3.96 (m, 2H, CH_2OP), 4.12–4.36 [m, 4H, $POCH_2CH(OCOR)CH_2OCO$, $POCH_2CHCN_3^+$], 4.47 [m, 1H, $CH(NH_3^+)CHOH$], 5.20 (m, 1H, $CHOCO$), 5.34 (m, 4H, olefin protons in the linoleoyl group), 5.42 [m, 1H, $CH(OH)CH{=}CH$)], 5.83 [m, 1H, $CH(OH)CH{=}CH$)]. Ion spray MS: m/z 982.8 $(M + H)^+$. Calc. for $C_{57}H_{104}NO_9P$, 981.8.

Preparation of Sphingosine 1-*phosphate*.—To a solution of PSph **3** (4 mg, 4 μmol) in diethyl ether (80 μl) was added phosphate buffer (0.48 ml, pH 7.0, 1/15 M), phospholipase C from *Bacillus cereus* (500 units, Sigma) and a trace of butylated hydroxytoluene, and the solution was stirred at 30 °C for 4 h in a nitrogen atmosphere in the dark. The reaction mixture was then made slightly acidic with 0.1 M HCl and the product was extracted with chloroform ten times. The solvent was evaporated under reduced pressure and the residue was chromatographed on preparative TLC affording the title compound. Yield 30%. TLC (silica gel G, $CHCl_3$–CH_3OH–H_2O, 50:40:10), $R_f = 0.36$. δ_H 0.88 (br, 3H, CH_3), 1.27 (m, 22H, $11 \times CH_2$), 2.08 [m, 2H, $C{=}C{-}CH_2{-}(CH_2)_{11}CH_3$], 3.60–4.42 [m, 4H, $P{-}O{-}CH_2{-}CH(NH_3^+){-}CH(OH)$], 5.44–5.53 [m, 1H, $CH(OH){-}CH{=}CH$], 5.70–5.81 [m, 1H, $CH(OH){-}CH{=}CH$]. This NMR spectrum coincided with that reported.[8] Ion spray MS: m/z 380.3 $(M + H)^+$. Calc. for $C_{18}H_{38}NO_5P$, 379.2.

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