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CHEMICAL SYNTHESIS OF D-*ERYTHRO*-SPHINGOSINE-1-PHOSPHATE, AND ITS INHIBITORY EFFECT ON CELL MOTILITY

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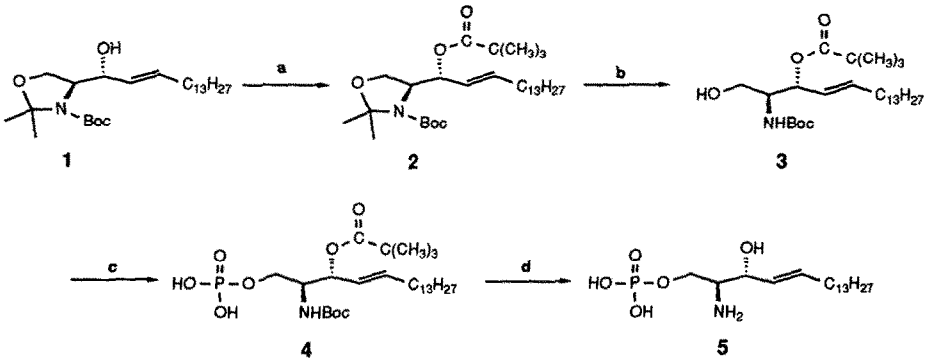
Abstract. The first chemical synthesis of D-*erythro*-sphingosine-1-phosphate (which occurs naturally) is described. This synthetic product had an inhibitory effect on motility of mouse melanoma B16/F1 cells in an *in vitro* assay system.

Sphingosine-1-phosphate (SPN-1-P) has been known for many years as an intermediate product during degradation of sphingosine (SPN) by SPN kinase to ethanolamine-1-phosphate and a long-chain aldehyde (e.g., palmital) by a pyridoxal phosphate-dependent lyase reaction[1]. The biological significance of SPN-1-P has been reported recently[2]. However, its physiological function in cells remains unclear, except for its Ca^{2+} mobilizing activity in some cells[2c]. Recently, we demonstrated that SPN-1-P inhibits motility of melanoma cells at a very low concentration (10 nM), at which SPN, N,N-dimethyl-SPN, and N,N,N-trimethyl-SPN have no inhibitory effect[3]. Furthermore, SPN-1-P is far less cytotoxic than these other three compounds, and does not inhibit protein kinase C. These biological findings suggest that SPN-1-P may act as an agent for prevention of tumor cell metastasis and inflammatory processes, both of which are highly dependent on cell motility. Unlike SPN and its N-methylated derivatives, the chemical synthesis of SPN-1-P has not been reported. The only known method for preparation of SPN-1-P is by treatment of sphingosylphosphorylcholine (SPC) with phospholipase D from *Streptomyces chromofuscus*[4], which gives a mixture of D-*erythro* and L-*threo* isomers. During design and synthesis of cell motility inhibitors derived from SPN-1-P, we developed a chemical synthesis of D-*erythro*-SPN-1-P, the naturally-occurring isomer.

The chemical synthesis of SPN-1-P is quite straightforward. In order to selectively phosphorylate the primary hydroxyl group of SPN, its amino and allylic hydroxyl groups must be protected. We chose the protected D-*erythro*-olefinic alcohol **1**

Scheme I



(a) pivaloyl chloride, pyridine; (b) p-TsOH, CH_3OH; (c) (i) $POCl_3$, Et_3N, CH_2Cl_2, (ii) H_2O, pyridine, dioxane; (d) (i) $nBu_4N^+OH^-$ (aq), dioxane; Amberlite IR-120 (H^+), (ii) 50% TFA/CH_2Cl_2.

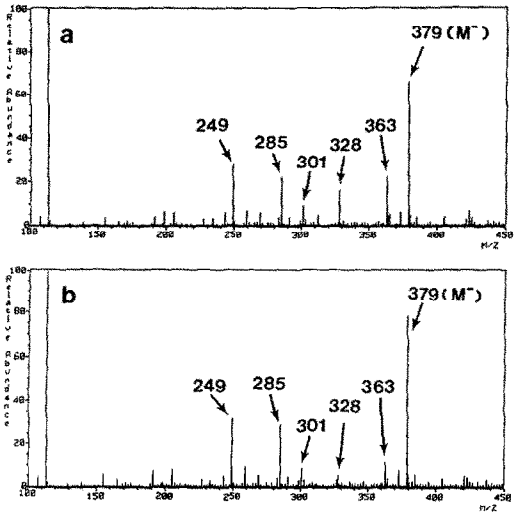


Figure 1. Negative-ion FAB mass spectra (DMIX as a matrix) of sphingosine-1-phosphate from (a) sphingosylphosphocholine with phospholipase D; (b) chemical synthesis.

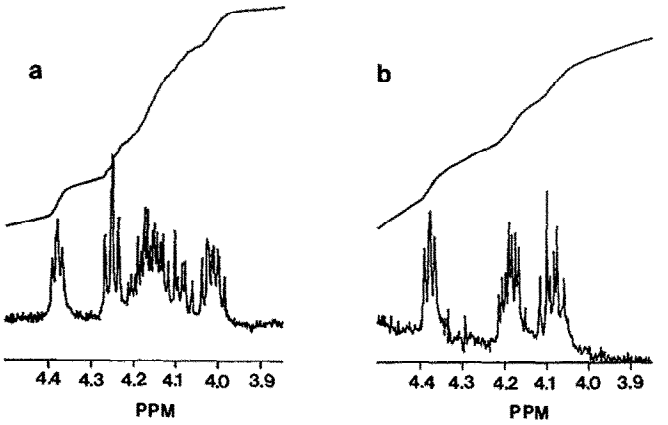


Figure 2. Portions of ^{1}H-NMR spectra (500 MHz) of sphingosine-1-phosphate from (a) sphingosylphosphocholine with phospholipase D, δ (ppm) 397-4.22 (4H, m, POC<u>H</u>$_2$), 4.25, 4.38 (2H, t, C<u>H</u>(OH)); (b) chemical synthesis, δ (ppm) 4.09, 4.18 (2H, m, POC<u>H</u>$_2$), 4.38 (1H, t, C<u>H</u>(OH)), taken in methyl-^{12}C-d_3-alcohol-d-acetic-d_3-acid-d 8:2 (v/v).

([α]^D -25.9° (c= 1.43, $CHCl_3$); lit.[5b] -25.2° (c= 0.215, $CHCl_3$)), readily available from L-serine by the known method[5], as the starting material. We also chose the acid-stable pivaloyl group for protection of the allylic-OH group, which could be left intact through modification of the 1-OH group, with no C-3 to C-1 migration[6]. Synthesis of SPN-1-P is summarized in Scheme 1. Esterification of **1** (6 eq. $(CH_3)_3CCOCl$ in dry pyridine, 0→25°C, 5 h) gave **2** (98%) as a colorless oil ($[\alpha]_D$ -25.7° (c= 1.22, $CHCl_3$)[7]. Selective deprotection of the 1-OH group (1.3 eq. TsOH·H_2O in methanol, 5 h) produced **3** (70%) as a colorless oil ($[\alpha]_D$ -12.9° (c= 1.20, $CHCl_3$). Phosphorylation of the primary hydroxyl group of **3** (4 eq. $POCl_3$, 4.5 eq. Et_3N in dry CH_2Cl_2, 0→25°C, 2 h), followed by hydrolysis (H_2O, pyridine in dioxane, 1.5 h), gave the precursor of SPN-1-P, **4** (53%). Finally, sequential deprotection of **4** (excess amount 40% wt. $nBu_4N^+OH^-$ (aq) in dioxane, 4 h, then Amberlite IR-120(H^+); 50% TFA/CH_2Cl_2, 0.5 h) yielded the naturally-occurring D-*erythro*-SPN-1-P, **5**, as a white solid in 78% yield after chromato-

graphy on silica gel (nBuOH/H_2O/AcOH, 5:1:1). Mobility of this synthetic product on silica-gel TLC (nBuOH/H_2O/AcOH, 6:1:1) and HPTLC ($CHCl_3$/MeOH/H_2O, 65:35:8) was indistinguishable from that of enzymatically-derived material prepared by the method of van Veldhoven et al.[8] Negative ion FAB-MS (Figure 1) revealed that the

TABLE I. Inhibitory effects of chemically and enzymatically synthesized SPN-1-P on F1 melanoma cell motility.

Compound	Concentration (μM)	% migration of F1 cells[a]
Control		100±32
SPN-1-P[b]	5	34±9
	1	8±2
	0.1	6±2
	0.01	12±7
	0.001	82±44
SPN-1-P[c]	5	41±10
	1	10±1
	0.1	12±1
	0.01	17±2
	0.001	122±39
SPN	5	12±3
	1	78±10
	0.1	90±22

[a] mean±S.D. (n= 3 or 4). Control value (defined as 100%) represented $2.2x10^4$ migrated cells. See footnote 9 for experimental details.
[b] Enzymatically synthesized.
[c] Chemically synthesized.

synthetic product gave the correct molecular ion, and its fragmentation pattern is identical to that of enzymatically-derived material. The ^{1}H-NMR (500 MHz) spectrum (Figure 2) showed only one set of resonances in H-1 and H-3, indicating that there was no racemization.

Synthetic SPN-1-P was tested *in vitro* for inhibition of chemotactic motility of mouse melanoma B16/F1 cells, using a Matri-gel-coated transwell assay system[9]. The results (summarized in Table I) show that both chemically- and enzymatically-prepared SPN-1-P have a similar dose-dependent inhibitory effect on cell motility. SPN-1-P at a concentration of 10 nM blocked penetration of F1 cells through the Matri-gel-coated filter, while a much higher concentration (5 μM) was required for SPN.

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7 All intermediates were purified by silica-gel chromatography. Their structures were supported (unless otherwise indicated) by ^{1}H-NMR and low resolution FAB-MS.

8 In each system, SPN-1-P was positive with orcinol, ninhydrin, and phosphate sprays.

9 Chemical motility of F1 cells in a Matri-gel-coated transwell chamber was assayed as follows. The upper chamber filter was precoated with 1 μg Matri-gel. F1 cells suspended in DMEM with 2% fetal calf serum ($5x10^4$ cells/ml) were placed on the filter and incubated for 30 min in a CO_2 incubator. The upper chamber with attached cells was fitted onto the lower chamber containing 0.6 ml of splenic stromal cell-derived conditioned medium as a chemoattractant, and incubated for 20 h to allow the cells to penetrate the filter. After incubation, cells remaining in the upper chamber were wiped off with a cotton swab, and cells migrating to the lower side of the filter were fixed in methanol and stained with 0.05% toluidine blue. The stain was solubilized in 10% acetic acid, and OD_{630} was measured with an ELISA reader. A linear relationship was observed between number of cells migrating to the lower chamber and OD_{630}.