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F-12509A, a New Sphingosine Kinase Inhibitor, Produced by a Discomycete

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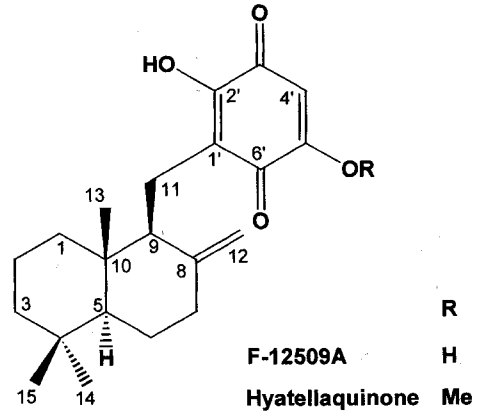
In the course of our screening for inhibitors of sphingosine kinase, we found an active compound from a culture broth of a discomycete, *Trichopezizella barbata* SANK 25395. The structure of the compound, named F-12509A, was elucidated by a combination of spectroscopic analyses, to be a new sesquiterpene quinone consisting of a drimane moiety and a dihydroxybenzoquinone. Enzyme kinetic analyses showed that F-12509A inhibits sphingosine kinase activity in a competitive manner with respect to sphingosine, with a K_i value of 18 μM.

Sphingosine-1-phosphate (SPP) was initially described as an intermediate in the metabolic pathway of long-chain sphigoid bases[1]. However, it is now widely accepted to be a unique bioactive lipid messenger[2,3]. SPP has been shown to be involved in a variety of cellular functions, including mediation of FcεRI receptor signaling in mast cells[4], nerve growth factor-mediated neuronal survival and differentiation[5], regulation of cell motility[6,7], platelet activation[8], activation of muscarinic K^+ currents[9], neurite retraction[10] and cell proliferation, especially in response to the stimulus of platelet-derived growth factor[11,12].

Sphingosine kinase, which catalyzes phosphorylation of sphingosine (SPH) on its primary hydroxyl group, is a key enzyme that regulates cellular SPP levels. Intervention of SPH kinase activity with specific inhibitors may give us some insight on the roles of SPP and SPH kinase in cellular physiology. Moreover, these inhibitors would have clinical value since SPP generation might be implicated in pathogenic states such as arteriosclerosis[6,11], thrombosis[8,13], and inflammation[4]. SPH analogs such as D,L-*threo*-dihydroSPH and *N*,*N*-dimethylSPH (DMS) were reported to be potent SPH kinase inhibitors and have been used as biochemical tools[4,11,13]. Application of these reagents, however, should be made with great care since they were also reported to have several other pharmacological activities[14~16] on account of their structural similarity to SPH. Thus, there is still a need for specific inhibitors of SPH kinase.

Based on these arguments, we screened for inhibitors of SPH kinase, and isolated a novel SPH kinase inhibitor, named F-12509A (Fig. 1), from a culture broth of a discomycete, *Trichopezizella barbata* SANK 25395. Here we report on the taxonomy of the producing organism, fermentation, isolation, physico-chemical properties, structure determination and enzyme inhibitory activities of the new compound.

Fig. 1. Structure of F-12509A.



Materials and Methods

Materials

[3-^{3}H]D-*erythro*-SPH (20 Ci/mmol) was purchased from Du Pont-New England Nuclear. SPH and SPP were from Matreya. DMS was from Calbiochem.

General Experimental Procedures

Spectral and physico-chemical data were obtained by the following instruments: UV, Shimadzu UV-265FW; IR, JASCO FT/IR-8300; NMR, Bruker AMX 360; HREI-MS, JASCO VMS-HX110; Specific rotations, JASCO DIP-370 with a 10 cm cell.

Preparation of an Enzyme Source

As a source of SPH kinase, we used rat liver cytosol. The cytosol prepared from livers of male rats (Wistar-Imamichi, 8~10 weeks old; Japan SLC Co.) was fractionated by ammonium sulfate precipitation. The 25~55% saturated fraction was dissolved in buffer A (100 mM potassium phosphate (pH 7.4), 20% glycerol, 1 mM mercaptoethanol, 1 mM EDTA, 1 mM sodium orthovanadate, 15 mM NaF, 0.5 mM 4-deoxypyridoxine, 1 mM phenylmethylsulfonyl fluoride and 1 μg/ml leupeptin) and dialyzed against the same buffer.

Assay of SPH Kinase Activity in a Cell-Free System

For the assay of SPH kinase activity in a cell-free system, we used the method of LOUIE *et al.*[17] with some modifications outlined as follows: reactions were carried out in polypropylene U-bottom microtubes (Greiner). Test samples and enzyme were mixed with buffer B (100 mM potassium phosphate (pH 7.4), 10 mM ATP, 10 mM $MgCl_2$, 1 mM mercaptoethanol, 1 mM EDTA, 1 mM sodium orthovanadate, 15 mM NaF, and 0.5 mM 4-deoxypyridoxine) to a total volume of 190 μl. The reactions were started by the addition of 10 μl of [3-^{3}H]D-*erythro*-SPH (50 nCi, 0.28 μM in propyleneglycol) and then the mixtures were incubated for 20 minutes at 37°C. The reactions were terminated by the addition of 50 μl of concentrated ammonium hydroxide followed by 750 μl of chloroform/methanol (2 : 1). After repeated pipetting, the phases were separated by centrifugation. Finally, appropriate portions of the upper aqueous phases containing the enzymatically released SPP were transferred onto Luma-plates (Packard). After drying the plates, radioactivities were determined with a TopCount scintillation counter (Packard). The labeled product in the upper aqueous phase was confirmed to be SPP, using TLCs developed with the following solvent systems, 1-butanol/water/acetic acid (3 : 1 : 1, Rf=0.47), chloroform/methanol/water (60 : 35 : 8, Rf=0.23) and chloroform/methanol/ammonium hydroxide (13 : 7 : 1, Rf=0.00)[12].

Metabolism of [^{3}H]SPH in Platelets

Metabolism of [^{3}H]SPH in washed human platelets was determined by the method of YATOMI *et al.*[13] Briefly, washed human platelets were suspended (3×10^8/ml) in 0.44 ml of buffer C (20 mM HEPES (pH 7.4), 138 mM NaCl, 3.3 mM NaH_2PO_4, 2.9 mM KCl, 1.0 mM $MgCl_2$ and 1 mg/ml glucose). They were pre-incubated with indicated concentrations of inhibitors for 5 minutes at 37°C, and were incubated with 1 μM [^{3}H]SPH (0.2 μCi) for 5 minutes more at 37°C. The reactions were terminated by the addition of 1.8 ml of ice-cold chloroform/methanol/concentrated HCl (100 : 200 : 1). Then the lipids were extracted by the method of BLIGH and DYER[18]. Portions of lipids obtained from the lower organic phases were resolved by TLC on Silica Gel HPTLC plates (Merck) with 1-butanol/water/acetic acid (3 : 1 : 1). The plates were visualized with an Imaging analyzer (BAS2000; Fuji Film) and radioactivities corresponding to authentic SPP and SPH were quantified with BAS2000.

Other Enzyme Assays

Other enzyme assays were carried out as follows: neutral sphingomyelinase (SMase) activity was evaluated by the method of NARA *et al.*[19] using rat brain microsomes as the enzyme source. Phosphatidylinositol (PI) 3-kinase activity was evaluated by the method of GOLD *et al.*[20] using Jurkat cell lysates as the enzyme source. Protein kinase C (PKC) activity was evaluated by a commercial PKC assay kit (Amersham Pharmacia Biotech) using partially purified rat brain PKC preparation (enriched in α, β and γ isoforms; Upstate Biotechnology).

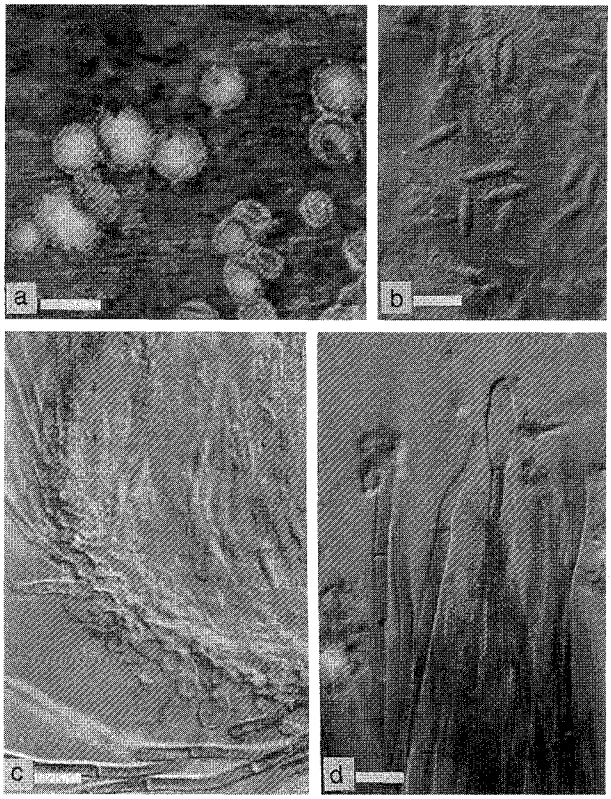
Results

Taxonomy of the Producing Organism

The F-12509A producing organism, SANK 25395, was derived from a single ascospore discharged from a fresh apothecium occurring on an unidentified piece of wood collected in May, 1994 in Aomori Pref., Japan using a single-ascospore isolation technique. As this discomycete could not be identified by its properties in culture, the dried apothecia were examined for identification. The mycological properties were as follows (Fig. 2).

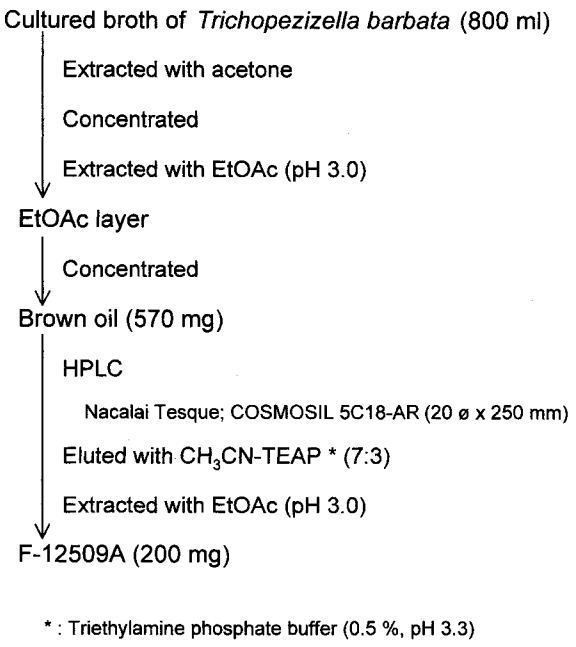
Apothecia were sessile to stipitate, globose or patelliform with incurving margin, grayish orange disc with brown

Fig. 2. Morphological characteristics of *Trichopezizella barbata* SANK 25395.



a. Fresh apothecia, b. Ascospores, c. Vertical section showing ectal excipulum, d. Hairs with thin-walled glandular apices. Scales: a. 1 mm, b~d. 10 μm.

Fig. 3. Isolation procedure of F-12509A.



hairs. Ectal excipulum was light to pale brown and two-layered; the outer layer was "textura angularis" to "textura globulosa", composed of cells of 5~8 μm across, whereas the inner layer was composed of closely interwoven, intricate hyphae of 2~3 μm wide. Hairs were mostly straight, but often flexuous, undulate or strongly curved, multi-septate, up to 250 μm long, with walls 3~5 μm thick. The apices of the hairs were blunt, occasionally capped with resinous matter, rarely showing glandular expansion and up to 5 μm wide. Asci were 60~70.5×4.5~5 μm, cylindrical clavate, arising from croziers. Ascospores were 10~13×2~3 μm, elliptic to fusiform and mostly single septate. Paraphyses were narrowly lanceolate, 3~3.5 μm thick at the widest point and exceeding the asci for 10~15 μm. These mycological properties showed good agreement with the descriptions given for *Trichopezizella barbata* (Kunze: Fr.) Raitv.[21] Hence, the strain was identified as *Trichopezizella barbata*. We have focused our attention on the discomycetes as unexplored screening resources. We have confirmed their potential by discovering some interesting compounds[22].

Fermentation

A mature slant culture of *Trichopezizella barbata* SANK 25395 was inoculated into a 500 ml-Erlenmeyer flask containing 80 ml of the medium composed of glycerol 3.0%, glucose 3.0%, soybean meal 1.0%, yeast extract 0.25%, starch 2.0% and 0.02% of the anti-foaming agent, CB-442. The flask was shaken under aerobic condition for 7 days at 20°C, 200 r.p.m. One ml of each seed culture was transferred into 500-ml Erlenmeyer flasks containing 80 ml of the same medium and shaken for 10 days at 20°C, 200 r.p.m.

Isolation

The isolation procedure of F-12509A is summarized in Fig. 3. The cultured broth (800 ml) of *Trichopezizella barbata* SANK 25395 was extracted with an equivalent volume of acetone. The extract was concentrated *in vacuo* to remove the acetone, and the resulting aqueous solution was further extracted with ethyl acetate at pH 3.0. The ethyl acetate extract was washed with brine, dried over Na_2SO_4

Table 1. Physico-chemical properties of F-12509A.

Appearance	pale orange powder
Molecular formula	$C_{21}H_{28}O_4$
HREI-MS *m/z*	
Found:	344.1985 (M + H)$^+$
Calcd.:	344.1987
UV λ_{max} nm (ε)	
MeOH	290 (14700), 477 (500)
MeOH-0.01N NaOH	235 sh (9700), 327 (22400), 530 (200)
IR ν_{max} (KBr) cm^{-1}	3310, 2965, 1645, 1620, 1360, 1320, 1190
$[\alpha]_D^{25}$	-96° (*c* 0.25, MeOH)

and concentrated *in vacuo* to give 570-mg of brown oil. Further purification was accomplished by HPLC with an ODS column (Nacalai Tesque, COSMOSIL 5C18-AR, 20i.d.×250 mm, flow rate: 9.9 ml/minute, mobile phase: acetonitrile/triethylamine phosphate buffer pH 3.3 (7 : 3), detection: 210 nm). The eluent containing F-12509A (retention time: 26 minutes) was concentrated to remove the organic solvent and the resulting aqueous solution was extracted twice with ethyl acetate at pH 3.0. After concentration and drying, 200 mg of F-12509A was obtained in pure form.

Physico-chemical Properties

The physico-chemical properties of F-12509A are summarized in Table 1. F-12509A was obtained as a pale orange powder and was soluble in most organic solvents including methanol, acetone, chloroform and ethyl acetate but insoluble in water. The molecular formula of F-12509A was determined to be $C_{21}H_{28}O_4$ on the basis of high resolution FABMS spectral analyses. F-12509A was optically active and exhibited UV absorption at 290 and 477 nm that shifted in alkaline solution to 327 and 530 nm. IR absorption bands at 3310, 1645 and 1620 cm^{-1} revealed the presence of a hydroxyl group and two conjugated carbonyl groups.

Structure Determination

^{1}H- and ^{13}C-NMR spectral data are summarized in Table 2. In the ^{13}C-NMR spectrum of F-12509A, four carbon signals were not observed presumably because of extensive line broadening due to exchange between tautomers. Similar phenomenon has been reported on stealthin A[23]. Therefore, F-12509A was treated with diazomethane and the ^{13}C-NMR spectrum of the resulting dimethyl derivative showed all of the carbon signals as expected (Table 2). Analyses of the DQF-COSY and HMBC spectra of the dimethyl derivative suggested the existence of the 8(12)-drimene skeleton and NOESY data confirmed the relative configuration as shown in Fig. 4. A literature search based on the partial structure hit two compounds, zonarol[24] and hyatellaquinone[25]. The physico-chemical properties and spectral data of the dimethyl derivative and hyatellaquinone were very close except for the presence of a methoxy group at 2′ position in the former. Therefore the structure of the dimethyl derivative was decided to be 2′-*O*-methyl hyatellaquinone, and thus that of the original active compound, F-12509A, was proved as shown in Fig. 1.

Enzyme Inhibitory Activities

To characterize the biological activities of F-12509A, we first examined the effect of F-12509A on SPH kinase activity in the cell-free system. In the subsequent experiments, we used DMS as a positive control, as it is a known competitive inhibitor of SPH kinase[13]. As shown in Fig. 5, F-12509A inhibited rat liver SPH kinase in a dose-dependent manner with an IC_{50} value of 18 μM and it inhibited the enzyme completely at 50 μM. On the other hand, the dimethyl derivative of F-12509A showed only 30% inhibition even at 250 μM. Kinetic analyses of F-12509A were performed using the rat liver cytosol fraction as the SPH kinase source. The increasing K_m values but no effect on the V_{max} value in the presence of increasing concentrations of F-12509A, shown in the Lineweaver-Burke plot (Fig. 6A), indicate that the inhibition was competitive with respect to SPH. Moreover, the Dixon plot also indicates that F-12509A inhibited SPH kinase in a competitive manner with respect to SPH, with a K_i value of 18 μM (Fig. 6B).

Next, we examined the effect of F-12509A on the metabolism of [^{3}H]SPH in human platelets to verify whether it would permeate the plasma membrane and inhibit SPH kinase in intact cells. As reported previously by YATOMI *et al.*[8], platelets are good tools for analyzing

Table 2. ^{13}C- and ^{1}H- NMR spectral data of F-12509A and the dimethyl derivative.

	F-12509A		Dimethyl derivative	
No.	δC	δH	δC	δH
1	38.8(t)	1.79(br dt 12.5, 3Hz), 1.35(m)	38.7(t)	1.78(dt 12, 3.5 Hz), 1.36(m)
2	19.5(t)	1.68-1.50(m)	19.5(t)	1.63-1.46(m)
3	42.1(t)	1.41(ddd 13, 3.5, 1.5Hz), 1.25(m)	42.2(t)	1.39(m), 1.21(m)
4	33.6(s)		33.6(s)	
5	55.6(d)	1.17(dd 12, 2.5Hz)	55.6(d)	1.14(dd 13, 3Hz)
6	24.5(t)	1.72(m), 1.32(m)	24.5(t)	1.70(m), 1.29(m)
7	38.3(t)	2.32(ddd 13, 4, 2.5Hz), 1.94(dt 13, 5Hz)	38.4(t)	2.29(m), 1.90(dt 13, 5Hz)
8	148.6(s)		148.9(s)	
9	54.1(d)	2.40(br d 11Hz)	55.0(d)	2.29(m)
10	40.1(s)		40.3(s)	
11	18.8(t)	2.66(dd 14, 11Hz), 2.54(dd 14, 3Hz)	19.2(t)	2.68(dd 13.5, 10.5Hz), 2.47(dd 13.5, 3Hz)
12	106.6(t)	4.68(d 1.5Hz), 4.66(d, 1.5Hz)	106.7(t)	4.68(br d 1.5Hz), 4.66(br d, 1.5Hz)
13	14.1(q)	0.77(s)	14.1(q)	0.75(s)
14	33.6(q)	0.88(s)	33.6(q)	0.86(s)
15	21.8(q)	0.82(s)	21.7(q)	0.80(s)
1'	117.0(s)		131.0(s)	
2'	ND		156.1(s)	
3'	ND		183.4(s)	
4'	102.1(d)	5.97(s)	105.3(d)	5.69(s)
5'	ND		158.9(s)	
6'	ND		182.9(s)	
2'-OMe			61.3(q)	4.06(s)
5'-OMe			56.4(q)	3.78(s)
OH		7.78(br s)		

ND : not detected

δ= ppm in $CDCl_3$

SPH kinase activity in intact cells because they lack SPP degradation pathway. Therefore, [^{3}H]SPH added extracellularly is rapidly converted to [^{3}H]SPP by SPH kinase and remains unchanged as [^{3}H]SPP in the platelets. As shown in Fig. 7, F-12509A inhibited the SPP formation in a dose-dependent manner suggesting that F-12509A permeate the plasma membrane and inhibit SPH kinase in intact cells. In the control study, DMS inhibited the SPP formation.

Specificity

In order to assess the specificity of F-12509A, we evaluated the effects of the compound on other kinases (PI 3-kinase and PKC) and mammalian neutral SMase, a key enzyme of sphingolipid metabolism. F-12509A showed no inhibitory activity towards these enzymes at 100 μM, which indicates that F-12509A is a specific inhibitor for SPH kinase.

Fig. 4. Structural determination of the dimethyl derivative.

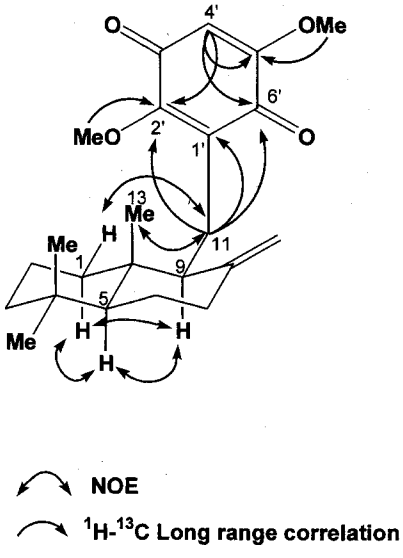


Fig. 5. Inhibitory effect of F-12509A on rat liver SPH kinase.

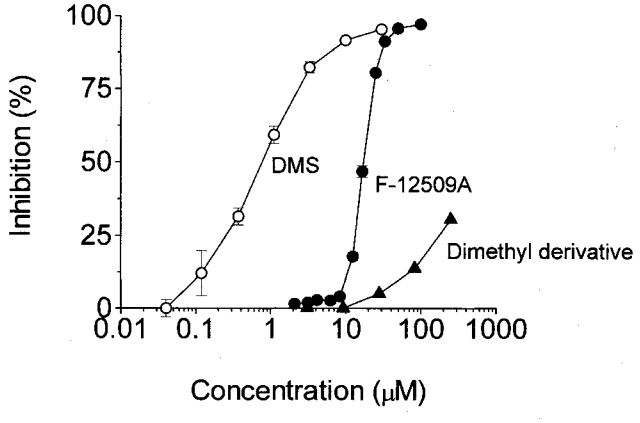
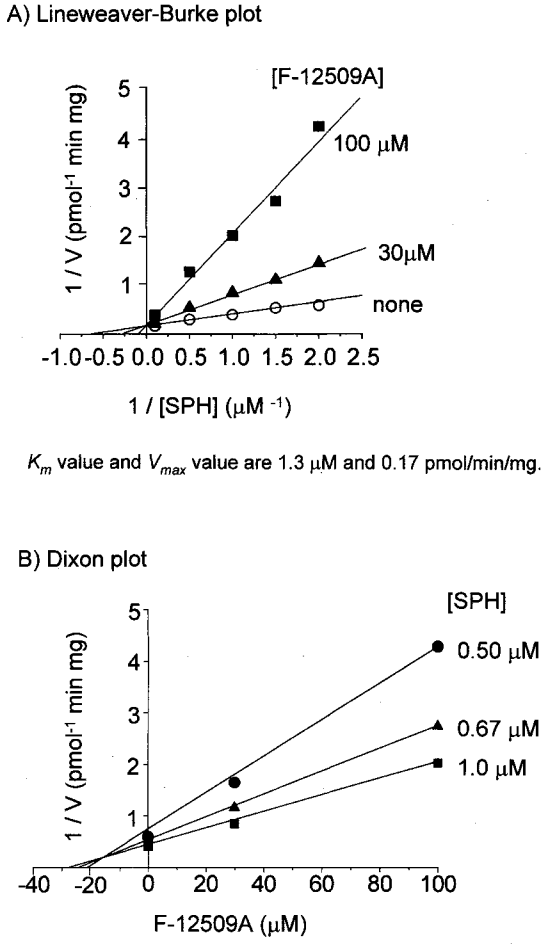


Fig. 6. Kinetic analyses of the SPH kinase inhibition.



Discussion

A new compound, F-12509A is the first SPH kinase inhibitor isolated from natural sources, and not an analog of SPH. F-12509A inhibits rat liver SPH kinase in a dose-dependent manner with the IC_{50} value of 18 μM and the inhibition is complete at 50 μM (Fig. 5). It also inhibits endogenous SPH kinase activity in platelets even when added extracellularly (Fig. 7). This fact shows that F-12509A permeates the plasma membrane and inhibits SPH kinase even in intact cells. Moreover, F-12509A is a specific inhibitor for SPH kinase, since it shows no inhibitory activity towards other enzymes (*i.e.* neutral SMase, PI 3-kinase and PKC) even at 100 μM. Therefore, F-12509A may fit our requirement for the inhibition of SPH kinase to study the biological functions of SPP.

Kinetic analyses of F-12509A show that the inhibition is competitive with respect to SPH (Fig. 6). The results suggest that the sesquiterpene moiety of F-12509A may mimic the conformation of SPH in the binding to the active site of SPH kinase. Though we do not have much evidences on the inhibitory mechanism of this compound, hydroxyl groups are necessary to inhibit SPH kinase since the inhibitory effect of the dimethyl derivative of F-12509A is significantly lower than that of F-12509A (Fig. 5).

DMS, an analog of SPH, has been used as a potent inhibitor of SPH kinase. In practice, DMS had higher inhibitory activity than F-12509A as shown in Fig. 5 and

Fig. 7. Inhibitory effect of F-12509A on SPP formation in human platelets.

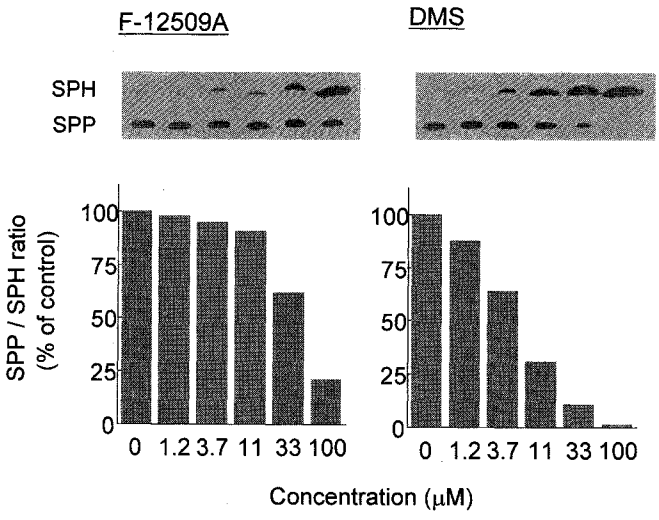


Fig. 7. However, DMS has been reported to have several pharmacological activities other than the inhibition of SPH kinase on account of its structural similarity to SPH. For example, DMS acts as a potent inhibitor of protein kinase C[14,15] and it induces apoptosis in a variety of cell lines[16]. Furthermore, it is reported that DMS can be phosphorylated in activated platelets[26]. Thus, application of this reagent should be made with great care. Accordingly, we still needed a new inhibitor of SPH kinase and screened for inhibitors of SPH kinase. Hence we isolated the novel and specific SPH kinase inhibitor, F-12509A.

In summary, a new compound, F-12509A is the first SPH kinase inhibitor from natural sources that is not an analog of SPH. Therefore, it is hoped that F-12509A will be an effective tool to understand the role of SPP in the cellular physiological functions.

Acknowledgment

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