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# S-15183a and b, New Sphingosine Kinase Inhibitors, Produced by a Fungus

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In the course of our screening for inhibitors of sphingosine kinase, we found two active compounds in a culture broth of a fungus, *Zopfiella inermis* SANK 15183. The structures of the compounds, named S-15183a and b, were elucidated by a combination of spectroscopic analyses to be new azaphilone-type metabolites. S-15183a and b inhibited sphingosine kinase from rat liver with $IC_{50}$ values of 2.5 and 1.6 $\mu$M, respectively. S-15183a also inhibited endogenous SPH kinase activity in intact platelets.

Sphingosine-1-phosphate (SPP) was initially described as an intermediate in the metabolic pathway of long-chain sphingoid bases[1]. However, it is now widely accepted to be a unique bioactive lipid messenger. It acts as a second messenger to regulate proliferation and survival intracellulary, and as a ligand for G protein-coupled receptors of the Edg-1 subfamily extracellularly[2,3]. SPP is involved in a variety of cellular functions, including vascular maturation[4], angiogenesis[5], TNF-$\alpha$ signalling[6], mediation of Fc$\varepsilon$RI signaling in mast cells[7], nerve growth factor-mediated neuronal survival and differentiation[8], regulation of cell motility[9,10], platelet activation[11], activation of muscarinic $K^+$ currents[12], neurite retraction[13] and cell proliferation, especially in signal transduction pathways of platelet-derived growth factor (PDGF)[14,15].

Sphingosine kinase, which catalyzes phosphorylation of sphingosine (SPH) on its primary hydroxyl group, is a key enzyme that regulates the cellular SPP level[16,17]. Intervention of SPH kinase activity with specific inhibitors may give us insight into the regulation mechanism of SPH kinase and the roles of SPP. Moreover, these inhibitors may have clinical value, since SPP generation has been implicated in pathogenic states such as angiogenesis[4,5] arteriosclerosis[9,14], thrombosis[11,18], and inflammation[6,7].

SPH analogs, such as D,L-*threo*-dihydroSPH and *N,N*-dimethylSPH (DMS), have been used as SPH kinase inhibitors[7,14,18]. However, due to structural similarity to SPH, they are also reported to have several other physiological functions[19~21] which may not be responsible for SPH kinase inhibition. Thus, specific inhibitors for SPH kinase are desired.

We therefore screened for inhibitors of SPH kinase, and have previously isolated novel SPH kinase inhibitors, named F-12509A[22] and B-5354s[23,24]. Continuing efforts to screen new SPH kinase inhibitors led to the discovery of new compounds, named S-15183a and b (Fig. 4), in a culture broth of a fungus, SANK 15183. Herein, we report on the taxonomy of the producing organism as well as the fermentation, isolation, physico-chemical properties, structure determination and enzyme inhibitory activities of the new compounds.

## Materials and Methods

### Materials

Unless otherwise specified, all reagents were obtained from Sigma. [3-$^3$H]D-*Erythro*-SPH (20 Ci/mmol) was

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purchased from DuPont-New England Nuclear. SPH and SPP were from Matreya. DMS was from Calbiochem.

### General Experimental Procedures

Spectral and physico-chemical data were obtained using the following instruments: UV, Shimadzu UV-265FW; IR, JASCO FT/IR-8300; NMR, Bruker AMX 360 and HRFAB-MS, JASCO VMS-HX110.

### SPH Kinase Assay in a Cell-free System

As the source of SPH kinase, we used rat liver cytosol as previously described[22]. For the assay of SPH kinase activity in a cell-free system, we used the method of LOUIE *et al.*[25] with some modifications as previously described[22].

### [$^3$H]SPP Formation from [$^3$H]SPH in Platelets

[$^3$H]SPP formation from [$^3$H]SPH in intact human platelets was determined by the method of YATOMI *et al.*[18]

### Other Enzyme Assays

Other enzyme assays were carried out as follows: Neutral sphingomyelinase (SMase) activity was evaluated by the method of NARA *et al.*[26] using rat brain microsomes as the enzyme source. Phosphatidylinositol (PI) 3-kinase activity was evaluated by the method of GOLD *et al.*[27] using Jurkat cell lysates as the enzyme source. Protein kinase C (PKC) activity was evaluated by a commercial PKC assay kit (Amersham Pharmacia Biothech) using partially purified rat brain PKC preparation (enriched in $\alpha$, $\beta$ and $\gamma$ isoforms; Upstate Biotechnology).

### S-15183a

Yellow oil. Molecular formula: $C_{25}H_{36}O_5$ (HR-FABMS $(M+H)^+$, *m/z* 417.2652, $\Delta$ +1.1 mmu). UV spectrum: $\lambda_{max}$ nm in MeOH ($\varepsilon$) 220 (17100), 320 (22500), 455 (800). IR spectrum: $\nu_{max}$ cm$^{-1}$ (liquid film) 2960, 2930, 2860, 1740, 1720, 1670, 1650. Specific rotation: $[\alpha]_D^{25}$ −220° (*c* 0.2 in MeOH). $^1$H NMR (360 MHz, $CDCl_3$): $\delta$ 7.88 (1H, d, *J*=1.1 Hz), 6.09 (1H, s), 5.52 (1H, d, *J*=1.1 Hz), 2.48~2.38 (4H, m), 1.69~1.38 (4H, m), 1.52 (3H, s), 1.43~1.23 (16H, m), 0.96~0.84 (6H, m). $^{13}$C NMR (90 MHz, $CDCl_3$): $\delta$ 193.5 (s), 192.9 (s), 173.1 (s), 162.4 (s), 154.0 (d), 142.7 (s), 115.2 (s), 108.6 (d), 106.8 (d), 84.1 (s), 33.2 (t), 33.1 (t), 31.6* (t), 28.9* (t), 26.5 (t), 24.6 (t), 22.6* (t), 22.2 (q), 14.1* (q). (* signals were overlapped)

### S-15183b

Yellow oil. Molecular formula: $C_{27}H_{40}O_5$ (HR-FABMS $(M+H)^+$, *m/z* 445.2959, $\Delta$+0.5 mmu). UV spectrum: $\lambda_{max}$ nm in MeOH ($\varepsilon$) 220 (16900), 320 (21600), 455 (1200). IR spectrum: $\nu_{max}$ cm$^{-1}$ (liquid film) 2960, 2930, 2860, 1740, 1720, 1670, 1650. Specific rotation: $[\alpha]_D^{25}$ −227° (*c* 0.3 in MeOH). $^1$H NMR (360 MHz, $CDCl_3$): $\delta$ 7.87 (1H, d, *J*=1.1 Hz), 6.08 (1H, s), 5.51 (1H, d, *J*=1.1 Hz), 2.48~2.38 (4H, m), 1.74~1.55 (4H, m), 1.52 (3H, s), 1.43~1.23 (20H, m), 0.95~0.83 (6H, m). $^{13}$C NMR (90 MHz, $CDCl_3$): $\delta$ 193.5 (s), 192.9 (s), 173.1 (s), 162.4 (s), 154.0 (d), 142.7 (s), 115.2 (s), 108.6 (d), 106.8 (d), 84.1 (s), 33.3 (t), 33.1 (t), 31.9 (t), 31.6 (t), 29.4 (t), 29.3 (t), 29.0* (t), 28.9* (t), 26.6 (t), 24.7 (t), 22.7 (t), 22.6 (t), 22.2 (q), 14.1 (q), 14.05 (q). (* signals were overlapped)

### Hydrogenation of S-15183a

A solution of S-15183a (10 mg) in 5 ml of MeOH was subjected to hydrogenation over 10% palladium carbon (5 mg) for 3 hours under atmospheric pressure at room temperature. The catalyst was filtered off and the solvent was evaporated. The residue was purified by preparative TLC (Hexane - EtoAc 4 : 1, Rf: 0.55) to give 4 mg (60%) of the isochroman derivative. Isochroman derivative: White powder. Molecular formula: $C_{17}H_{26}O_3$ (FABMS: $(M+H)^+$, *m/z* 279). $^1$H NMR (360 MHz, $CDCl_3$): $\delta$ 6.17 (1H, s), 4.90 (1H, d, *J*=15.8 Hz), 4.61 (1H, d, *J*=15.8 Hz), 3.55 (1H, m), 2.58 (2H, m), 2.10 (3H, s), 1.70~1.18 (12H, m), 0.95 (3H, t, *J*=6.5 Hz). $^{13}$C NMR (90 MHz, $CDCl_3$): $\delta$ 152.6 (s), 150.3 (s), 132.8 (s), 114.4 (s), 107.3 (d), 107.0 (s), 74.5 (d), 64.5 (t), 35.9 (t), 33.9 (t), 31.8 (t), 29.7 (t), 29.3 (t), 25.5 (t), 22.7 (t), 14.1 (q), 7.6 (q).

## Results and Discussion

### Taxonomy

The fungal strain SANK 15183 grew rapidly on modified Weitzman and Silva-Hutner's medium (oat-meal 1.0%, $MgSO_4 \cdot 7H_2O$ 0.1%, $KH_2PO_4$ 0.1%, $NaNO_3$ 0.1% and agar 2.0%) to form grayish orange colonies attaining a diameter of 80 mm in 3 weeks at 23°C. The colony consisted of thin basal felt with floccose hyphae, grayish orange, developing cleistothecia on the felt and more abundantly submerging colony areas. Cleistothecia were 125~270 $\mu$m in diameter, globose, non-ostiolate and gray to griseous. Peridium was composed of pale-colored and pseudoparenchymatous cells. Asci were 8-spored, clavate, 60~105×19~50 $\mu$m, broadly rounded above, thin-walled, evanescent and without a distinct structure in apex. Ascospores were at first aseptate, hyaline and clavate to elongate. Mature ascospores were two-celled and totally 31~38 $\mu$m. Upper cells were fusiform-ellipsoidal, 26~31×19~22 $\mu$m, dark brown, with truncate base and an apical germ pore. Lower cells were

conical, 5~10×4~8 $\mu$m, hyaline to pale brown and thin-walled. Anamorphs were not observed in this strain. Based on these characteristics, SANK 15183 was identified as *Zopfiella inermis* (Cailleux) Malloch & Cain[28]. This strain has been deposited in the National Institute of Bioscience and Technology, Japan as *Zopfiella inermis* SANK 15183 with the accession number FERM BP-5737.

## Fermentation

A mature slant culture of SANK 15183 was inoculated into 500 ml-Erlenmeyer flasks 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 8 days at 23°C, 200 r.p.m..

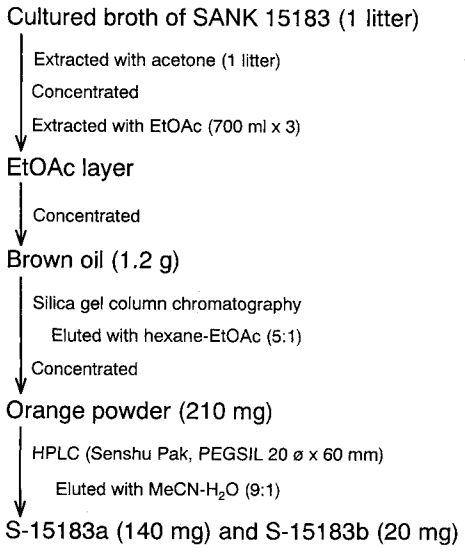
## Isolation

The isolation procedure for S-15183a and b is summarized in Fig. 1. The cultured broth (1 liter) of SANK 15183 was extracted with equivalent volume of acetone. After centrifugation, the extract was concentrated *in vacuo* to remove the acetone, and the residue was extracted three times with 700 ml of ethyl acetate at pH 3.0. The ethyl acetate extract was washed with brine, dried over $Na_2SO_4$ and concentrated *in vacuo* to give a 1.2 g of brown oil. The oil was applied onto a silica gel column (100 ml) equilibrated with hexane - ethyl acetate (5 : 1). The column was eluted with the same solvent, and the fractions showing SPH kinase inhibitory activity were combined. After concentration, 210 mg of orange powder was obtained. Further purification was accomplished by HPLC (column: Senshu Pak, PEGASIL 20 i.d.×60 mm, flow rate: 6 ml/minute, mobile phase: 90% aqueous acetonitrile, detection: UV absorption at 210 nm) to give S-15183a (140 mg) and S-15183b (20 mg). The retention times of these congeners were 8 minutes and 13 minutes, respectively.

## Physico-chemical Properties

The physico-chemical properties of S-15183a and b are summarized in Table 1. They were obtained as yellow oils, which have characteristic UV absorption bands at 220, 320 and 455 nm in methanol. The IR spectra suggested the existence of an ester (1720 $cm^{-1}$) and an $\alpha,\beta,\gamma,\delta$-conjugated carbonyl (1670 $cm^{-1}$) groups. The molecular formulae of the compounds were determined mainly by high resolution FAB-MS analyses. $^1H$ and $^{13}C$ NMR spectral data of the S-15183s are summarized in the experimental section.

Fig. 1. Isolation procedure for S-15183a and b.




## Structure Determination

The physico-chemical data suggested that S-15183a and b are closely related to each other, and structural studies were first carried out on the major component S-15183a. In the $^1H$ NMR of S-15183a, resonances arising from three olefinic protons ($\delta_H$ 7.88, 6.09 and 5.52) and a singlet methyl group ($\delta_H$ 1.52) were observed. The $^{13}C$ NMR and DEPT spectra showed the presence of a linear saturated fatty acid ester and two carbonyl carbons ($\delta_C$ 193.5, 192.9). A literature search revealed that these data were similar to those of a known azaphilone, daldinin A[29]. The presence of the chromophore was supported by the UV and IR spectral data (*vide supra*). In consideration of the molecular formula, $C_{27}H_{36}O_5$, the structure of S-15183a was proposed as shown in Fig. 2, while m and n values remained to be solved due to the signal overlap in the aliphatic region of $^1H$ NMR.

Azaphilones are known to give isochroman derivatives by catalytic hydrogenation[30]. As expected, S-15183a was converted to an isochroman-type compound by hydrogenation over 10% palladium carbon under atmospheric pressure at room temperature. The product was purified by preparative TLC and the hydrogenation product structure is shown in Fig. 3. Taking into account the molecular formula $C_{27}H_{36}O_5$, only the gross structure of

Table 1. Physico-chemical properties of S-15183a and b.

| | S-15183a | S-15183b |
|---|---|---|
| Appearance | Yellow oil | Yellow oil |
| Molecular formula | $C_{25}H_{36}O_5$ | $C_{27}H_{40}O_5$ |
| HRFAB-MS (*m/z*) Found | 417.2652 $(M+H)^+$ | 445.2959 $(M+H)^+$ |
| Calcd. | 417.2641 | 445.2954 |
| UV $\lambda_{max}$ nm ($\varepsilon$) | 220 (17100), 320 (22500), | 220 (16900), 320 (21600), |
| in MeOH | 455 (800) | 455 (1200) |
| IR $\nu_{max}$ $cm^{-1}$ (liquid film) | 2960, 2930, 2860, 1740, | 2960, 2930, 2860, 1740, |
| | 1720, 1670, 1650 | 1720, 1670, 1650 |
| $[\alpha]_D^{25}$ (MeOH) | -220° (*c* 0.2) | -227° (*c* 0.3) |

Fig. 2. Possible structure of S-15183a and b.

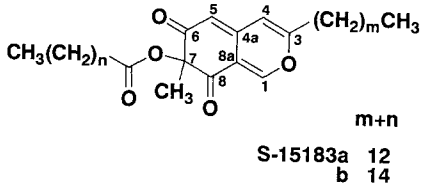
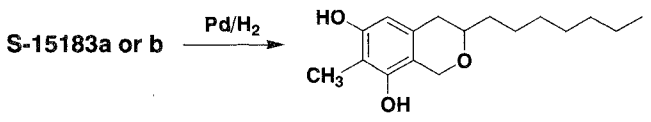
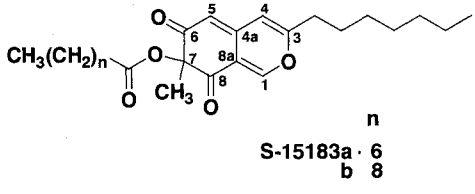



Fig. 3. Catalytic hydrogenation of S-15183a and b.




S-15183a could be proposed at this stage leaving the side chain m and n values yet to be determined due to overlapping signals in the $^1H$ NMR. Since S-15183b differs from S-15183a only in an increase of $2\times CH_2$ units, the final structures could not be confirmed by NMR alone. The chain lengths were established for both S-15183a and b by reduction of each compound to the isochroman. In each case the same hydrogenated compound was produced possessing the same C-3 side chain. Therefore if the C-3 side chain contains seven carbones in S-15183a, this leaves C-7 side chain as an octanoate and in S-15183b the side chain is therefore a decanoate, to account for the difference in molecular weight. The structure of the isochroman derivative was determined as shown in Fig. 3, therefore, the structures of S-15183a and b were determined as shown in Fig. 4. Fragment ions (*m/z* 291 and 290) assigned to the azaphilone skeleton with no acyl group were detected in FAB-MS spectra of both compounds. These data also confirmed the structures.

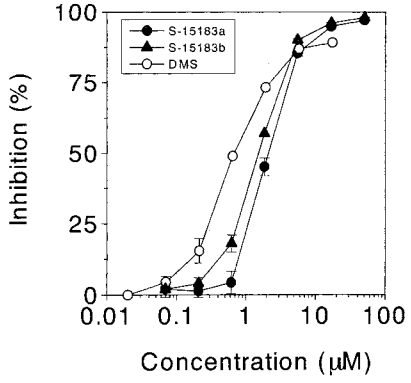
Fig. 4. Structures of S-15183a and b.




## SPH Kinase Inhibitory Activity in a Cell-Free System

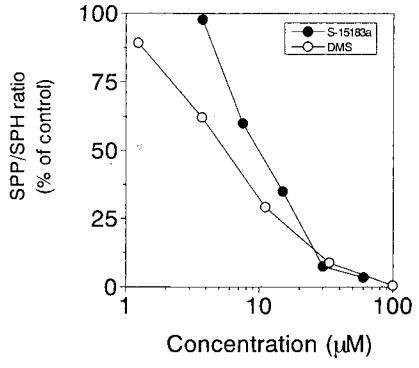
We evaluated effects of the S-15183s on rat liver SPH kinase activity in a cell-free system. As shown in Fig. 5, S-15183a and b inhibited the SPH kinase in a dose-dependent manner, with $IC_{50}$ values of 2.5 and 1.6 $\mu$M, respectively. As a positive control, DMS[18] inhibited the SPH kinase with an $IC_{50}$ value of 1.0 $\mu$M in the assay system.

Fig. 5. Effects of S-15183a and b on the rat liver SPH kinase activity.




SPH kinase activity from rat liver cytosol was measured with varying concentrations of inhibitors in a cell-free assay system as described in "Materials and Methods". Data are means±S.E. (n=3).

Fig. 6. Inhibitory effect of S-15183a on SPP formation in intact human platelets.




## SPH Kinase Inhibition in Intact Human Platelets

In order to evaluate inhibitory activity in intact cells, we examined the effect of S-15183a on [$^3$H]SPP formation from [$^3$H]SPH in human platelets. As reported previously by YATOMI *et al.*[18], platelets are good tools for analyzing SPH kinase activity in intact cells because they lack the SPP degradation pathway. Therefore, the radioactivity added as [$^3$H]SPH is rapidly converted to [$^3$H]SPP by SPH kinase and remains unchanged as [$^3$H]SPP in the platelets. As shown in Fig. 6, S-15183a inhibited the SPP formation in a dose-dependent manner even when added extracellularly. The result suggests that S-15183a permeates the plasma membrane and inhibits SPH kinase in intact cells. In a control study, DMS also inhibited SPP formation.

## Specificity

In order to assess the specificity, we evaluated the effects of S-15183a on other sphingolipid metabolic enzyme, neutral SMase, and on other kinases, PI 3-kinase and PKC. S-15183a showed virtually no inhibitory activity towards any of the enzymes even at 100 $\mu$M. These results indicate that S-15183a is a specific inhibitor of SPH kinase.

In summary, S-15183a, a new azaphilone, is a potent and specific inhibitor of mammalian SPH kinase. S-15183a may be an effective tool in understanding the role of SPP in the cellular physiological functions.

## Acknowledgment

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