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B-5354a, b and c, New Sphingosine Kinase Inhibitors, Produced by a Marine Bacterium; Taxonomy, Fermentation, Isolation, Physico-chemical Properties and Structure Determination

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In the course of our screening for inhibitors of sphingosine kinase, we found a series of active compounds in a culture broth of a novel marine bacterium, SANK 71896. The structures of the compounds, named B-5354a, b and c, were elucidated by a combination of spectroscopic analyses to be new esters of 4-amino-3-hydroxybenzoic acid with long-chain unsaturated alcohols. B-5354a, b and c inhibit sphingosine kinase activity with IC_{50} values of 21, 58 and 38 μM, respectively.

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 signal transduction pathways of platelet-derived growth factor (PDGF)[11,12].

Sphingosine kinase, which catalyzes phosphorylation of sphingosine (SPH) on its primary hydroxyl group, is a key enzyme that regulates the cellular SPP level[13,14]. Intervention of SPH kinase activity with specific inhibitors may give us insight on the regulation mechanism of SPH kinase and the roles of SPP. Moreover, these inhibitors may have clinical value, since SPP generation might be implicated in pathogenic states such as arteriosclerosis[6,11], thrombosis[8,15], and inflammation[4].

SPH analogs, such as D,L-*threo*-dihydroSPH and *N,N*-dimethylSPH (DMS), have been used as SPH kinase inhibitors[4,11,15]. However, probably on account of their structural similarity to SPH, they are also reported to have several physiological functions[16~18] which may not be responsible for SPH kinase inhibition. Thus, specific inhibitors for SPH kinase are desired.

Under these circumstances, we have screened for inhibitors of SPH kinase, and have previously isolated a novel SPH kinase inhibitor, named F-12509A, from a culture broth of a discomycete, *Trichopezizella barbata* SANK 25395[19]. Continuing efforts to screen new SPH kinase inhibitors led a discovery of new compounds, named B-5354a, b and c, in a culture broth of a novel marine bacterium, SANK 71896. Here we report on the taxonomy of the producing organism as well as the fermentation, isolation, physico-chemical properties and structure determination of the new compounds.

Results and Discussion

Taxonomy

The producing strain, SANK 71896 is a kind of Gram-negative bacteria isolated from seawater collected in 1996 at Ibaraki Prefecture, Japan. The strain could be called a marine bacterium since it absolutely required seawater for its growth. The bootstrapped 16S rDNA sequence-based neighbor-joining phylogeny of SANK 71896 suggested that

the strain is a new species in the genus Ruegeria. The details of taxonomic studies will be reported in elsewhere[20].

Fermentation

A mature slant culture of SANK 71896 was inoculated into 500 ml-Erlenmyer flasks containing 80 ml of the medium composed of glucose 1.0%, Bactopeptone 1.0%, yeast extract 0.1%, $NaNO_3$ 0.1%, $(NH_4)_2SO_4$ 0.1%, Mg_2SO_4 0.1%, $FeCl_2$ 2×10^{-7}%, $MnSO_4$ 2×10^{-7}%, 2×10^{-5}% of anti-foaming agent, CB-442 and artificial sea water (Jamarin S: Jamarin Labs.). The flasks were shaken under aerobic conditions for 3 days at 23°C, 200 r.p.m.

Isolation

The isolation procedure for B-5354a, b and c is summarized in Fig. 1. The cultured broth (2 liters) of SANK 71896 was extracted with equivalent volume of acetone. After centrifugation, the extract was concentrated *in vacuo* to remove the acetone, and the resulting aqueous solution was extracted twice with 2 liters 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 415 mg of yellow oil. The yellow oil was applied onto a silica gel column equilibrated with hexane - ethyl acetate (4 : 1). The column was eluted with the same solvent, and the fractions showing SPH kinase inhibitory activity were combined. Further purification was accomplished by HPLC (column: Senshu Pak, PEGASIL 20 i.d.×60 mm, flow rate: 6 ml/minute, mobile phase: 80% aqueous acetonitrile, detection: UV absorption at 210 nm) to give B-5354a (12.2 mg), B-5354b (5.8 mg) and B-5354c (320 mg). The retention times of these congeners were 10 minutes, 13 minutes and 15 minutes, respectively.

Physico-chemical Properties

The physico-chemical properties of the B-5354c are summarized in Table 1. The B-5354s are colorless powders, which have characteristic UV absorption bands at 203, 229, 281 and 309 nm. The compounds were positive in the ninhydrin spot tests in which unusual blue-green colors appeared. The IR spectra showed the absorption band at 1680 cm^{-1}, which could be assigned to arylic ester carbonyl group. The molecular formulae of the B-5354s were determined mainly by high resolution FAB-MS analyses. ^1H and ^{13}C NMR spectral data of B-5354s are summarized in the experimental section.

Structure Determination

The physico-chemical data suggested that each of the B-5354s are closely related compounds and the structural studies were first carried out on B-5354c, the major component of the fermentation broth by interpreting the

Fig. 1. Isolation procedure for the B-5354s.

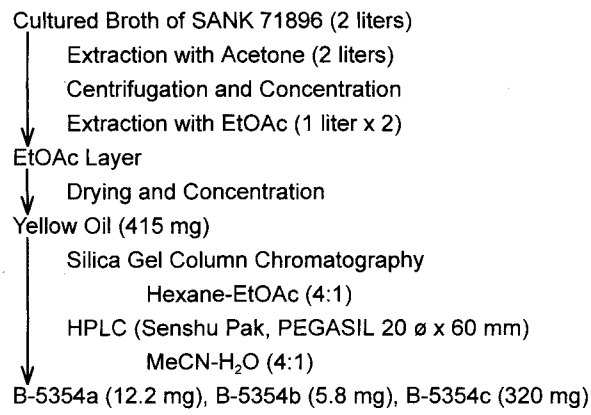


Table 1. Physico-chemical properties of B-5354c.

	B-5354c
Appearance	White powder
Molecular formula	$C_{21}H_{33}NO_3$
HRFAB-MS (*m/z*) Found	347.2436 ($M^{+\cdot}$)
Calcd.	347.2460
UV λ_{max} nm (ε) in MeOH	203 (16300), 229 (9700), 281 (8500), 309 (11800)
IR ν_{max} cm^{-1} (KBr)	3400, 3350, 2920, 2850, 1680, 1615, 1450, 1310, 1290, 1230, 1120, 765

NMR spectra taken in DMSO-d_6. In ^{1}H NMR, the resonance arising from three aromatic protons were observed, and analysis of spin-spin couplings of these signals suggested the existence of a 1,2,4-trisubstituted benzene ring. The substituents were deduced to be an amino group, a carbonyl group and a hydroxyl group. The substituting pattern of the three functional groups were determined mainly based on the HMBC analysis of B-5354c *N*-acetate obtained by the treatment with acetic anhydride in pyridine. As shown in Fig. 2, long-range correlations of the amide proton (9.33 ppm) with one of the aromatic methine carbons (120.4 ppm) and with two aromatic quaternary carbons (131.3, 146.6 ppm) were observed. While long-range correlations of two aromatic protons (7.38, 7.46 ppm) with the ester carbonyl carbon (165.4 ppm) were observed. Therefore, the substituting pattern on the benzene ring of B-5354c was determined as shown in Fig. 4.

The molecular formula and DEPT spectrum indicated that the remaining portion of the compound was an aliphatic chain consisting of a methyl group, two olefinic methine groups, ten methylene groups and an oxymethylene group. Therefore, B-5354c was deduced to be an ester of 4-amino-3-hydroxybenzoic acid with a long chain alcohol. Because of the signal overlapping, the position and geometry of the double bond could not be determined by interpreting the NMR spectra. Thus, we made use of the charge-remote fragmentation[21,22] in the mass spectral analysis of B-5354c. As shown in Fig. 3, a series of ions (*m/z* 330, 316, 302, 288, 275, 274, 220, 206) were observed in the negative ion collisionally activated dissociation spectrum, and the existence of a 7-tetradecen-1-oxyl group was proved.

The geometry of the double bond could be determined by the comparison of the spectral data with that of the synthetically obtained *Z* isomer. Dehydration of 4-amino-3-hydroxybenzoic acid with *cis*-7-tetradecen-1-ol in the presence of DCC/DMAP gave the *Z* isomer. The spectral data of the natural compound and the synthetic product were identical, and thus, the structure of B-5354c was determined as shown in Fig. 4.

^{1}H and ^{13}C NMR spectral data of B-5354a and b (see the experimental section) are similar to those of B-5354c, showing that each of them contains the same chromophore. In the case of B-5354a, the molecular formula and DEPT spectrum indicated the presence of a dodecen-1-oxyl group. The location of the double bond was readily determined to be at C-5 of the alkoxyl group by interpretation of DQF-

Fig. 2. Partial structure of B-5354c-*N*-acetate.

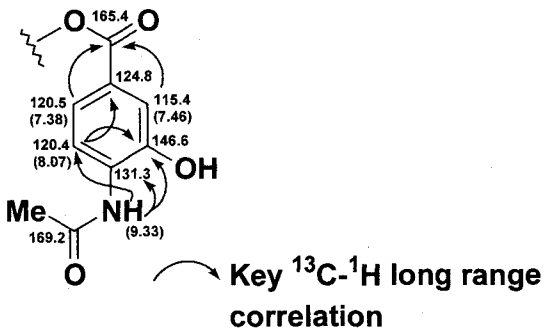


Fig. 3. Charge-remote fragmentation of B-5354c.

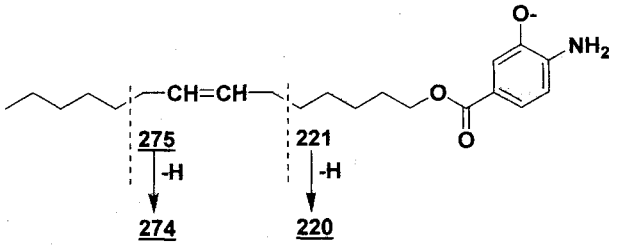
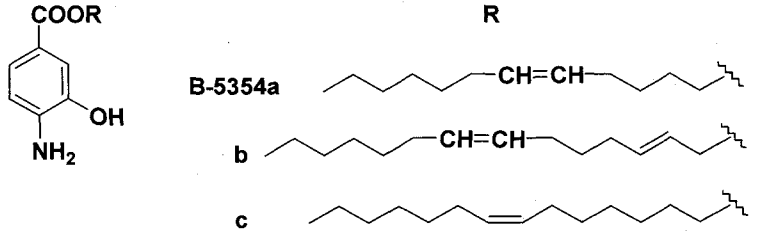


Fig. 4. Structures of the B-5354s.



COSY spectrum while the geometry was remained to be solved. In the case of B-5354b, the molecular formula and DEPT spectrum indicated the presence of a tetradecadien-1-oxyl group. DQF-COSY spectrum showed that one of the double bonds was located at C-2 of the alkoxyl group, whose geometry was determined to be *E* based on the large coupling constant (15 Hz) between the olefinic proton signals. The location of the remaining double bond was at C-7 based on the analysis of the charge-remote fragmentation in the mass spectrum.

Inhibitory Potency of the B-5354s on SPH Kinase

We evaluated effects of the B-5354s on rat liver SPH kinase activity in a cell-free system. B-5354a, b and c inhibited the SPH kinase in a dose-dependent manner, with IC_{50} values of 21, 58 and 38 μM, respectively. The synthetically obtained B-5354c showed the same IC_{50} value with the natural compound. On the other hand, B-5354c *N*-acetate did not inhibit the SPH kinase even at 500 μM. The kinetic studies and preliminary structure-activity relationships of these compounds are described in a subsequent paper[23].

Experimental

Materials

Unless otherwise specified, all reagents were obtained from Sigma. [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. The 4-amino-3-hydroxybenzoic acid and di-*tert*-butyl dicarbonate were from Tokyo Chemical Industry.

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 HREI-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[19]. For the assay of SPH kinase activity in a cell-free system, we used the method of LOUIE *et al.*[24] with some modifications as previously described[19].

B-5354a

White powder. Molecular formula: $C_{19}H_{29}NO_3$ (HR-EIMS ($M^{+\cdot}$), *m/z* 319.2122, Δ−2.5 mmu). UV spectrum: λ_{max} nm in MeOH (ε) 205 (16100), 229 (10200), 281 (9200), 309 (12700). ^{1}H NMR (360 MHz, CD_3OD): δ 7.36 (1H, dd, *J*=8, 2 Hz), 7.33 (1H, d, *J*=2 Hz), 6.67 (1H, d, *J*=8 Hz), 5.43~5.33 (2H, m), 4.22 (2H, t, *J*=6.5 Hz), 2.12 (2H, m), 2.04 (2H, m), 1.75 (2H, m), 1.51 (2H, m), 1.38~1.21 (8H, m), 0.88 (3H, t, *J*=6.7 Hz). ^{13}C NMR (90 MHz, CD_3OD): δ 166.7 (s), 142.6 (s), 141.0 (s), 129.1 (d), 128.0 (d), 121.8 (d), 117.4 (s), 113.7 (d), 112.3 (d), 63.0 (t), 30.6 (t), 28.5 (t), 27.7 (t), 27.2 (t), 25.9 (t), 25.4 (t), 25.0 (t), 21.4 (t), 12.1 (q).

B-5354b

White powder. Molecular formula: $C_{21}H_{31}NO_3$ (HR-EIMS ($M^{+\cdot}$), *m/z* 345.2284, Δ−2.0 mmu). UV spectrum: λ_{max} nm in MeOH (ε) 203 (16600), 229 (10000), 283 (8800), 309 (12200). ^{1}H NMR (360 MHz, CD_3OD): δ 7.37 (1H, dd, *J*=8, 2 Hz), 7.33 (1H, d, *J*=2 Hz), 6.67 (1H, d, *J*=8 Hz), 5.84 (1H, m), 5.67 (1H, m), 5.40~5.30 (2H, m), 4.66 (2H, dd, *J*=6, 0.9 Hz), 2.14~1.97 (6H, m), 1.47 (2H, m), 1.36~1.21 (8H, m), 0.88 (3H, t, *J*=7.2 Hz). ^{13}C NMR (90 MHz, CD_3OD): δ 166.4 (s), 142.6 (s), 141.0 (s), 134.3 (d), 129.0 (d), 128.0 (d), 123.7 (d), 121.9 (d), 117.3 (s), 113.8 (d), 112.2 (d), 63.7 (t), 30.6 (t), 30.4 (t), 28.5 (t), 27.8 (t), 27.7 (t), 25.9 (t), 25.2 (t), 21.4 (t), 12.1 (q).

B-5354c

^{1}H NMR (360 MHz, DMSO-d_6): δ 9.37 (1H, s), 7.26 (1H, d, *J*=1.5 Hz), 7.25 (1H, dd, *J*=8, 1.5 Hz), 6.59 (1H, d, *J*=8 Hz), 5.40~5.30 (2H, m), 4.14 (2H, t, *J*=6.5 Hz), 2.0 (4H, m), 1.65 (2H, m), 1.44~1.21 (14H, m), 0.86 (3H, t, *J*=7 Hz). ^{13}C NMR (90 MHz, DMSO-d_6): δ 166.0 (s), 142.8 (s), 142.3 (s), 129.7 (d), 129.6 (d), 122.4 (d), 116.6 (s), 114.5 (d), 112.5 (d), 63.5 (t), 31.1 (t), 29.1 (t), 29.0 (t), 28.4 (t), 28.3 (t), 28.3 (t), 26.6 (t), 26.5 (t), 25.4 (t), 22.1 (t), 13.9 (q)

Synthesis of B-5354c

To a solution of 4-amino-3-hydroxybenzoic acid (1 g) in 50 ml of CH_2Cl_2 was added 3.7 ml of di-*tert*-butyl dicarbonate and 5 ml of pyridine. The reaction mixture was stirred for 1 hour at room temperature. After aqueous workup and extraction, 1.5 g of *N*-Boc-4-amino-3-hydroxybenzoic acid was obtained. Hydrolysis of *cis*-7-tetradecen-1-yl acetate (1 g) under basic conditions (1 N NaOH in MeOH) gave *cis*-7-tetradecen-1-ol. To a solution of *N*-Boc-4-amino-3-hydroxybenzoic acid (25 mg), *cis*-7-tetradecen-1-ol (40 mg) and DMAP (8 mg) in 1 ml of CH_2Cl_2, 20 mg of DCC dissolved in 1 ml of CH_2Cl_2 was added. The reaction mixture was stirred for 2 hours at room temperature, then 2 ml of H_2O was added. After removing

CH_2Cl_2 *in vacuo*, the resulting aqueous solution was extracted with ethyl acetate and washed with brine followed by drying over Na_2SO_4. The condensation product was purified on a preparative TLC plate eluted with *n*-hexane-EtOAc 4:1. The major product (Rf 0.6) was re-extracted and deprotected by TFA at 0°C. After purification by HPLC as the natural compound was done, then 7 mg of the synthetic Z isomer of B-5354c was obtained.

N-Acetylation of B-5354c

To a solution of B-5354c (10 mg) in 1.5 ml of pyridine was added 1.2 equivalent of acetic anhydride. The mixture was stirred at room temperature for 1 hour. After concentration *in vacuo* and purification by HPLC (column: Senshu Pak, PEGASIL 20 i.d.×60 mm, flow rate: 6 ml/minute, mobile phase: 80% aqueous acetonitrile, detection: UV absorption at 210 nm, retention time: 18 minutes), 8 mg of B-5354c-*N*-acetate was obtained. B-5354c-*N*-acetate: white powder. Molecular formula: $C_{23}H_{35}NO_4$ (HR-EIMS (M^+), *m/z* 389.2557, Δ −0.9 mmu). UV spectrum: λ_{max} nm in MeOH (ε) 203 (16700), 219 (12800), 268 (11300), 305 (7800). IR spectrum: ν_{max} cm^{-1} 3410, 3300, 3110, 2925, 2855, 1710, 1680, 1670, 1600, 1520, 1425, 1335, 1230, 770. ^1H NMR (360 MHz, DMSO-d_6): δ 10.28 (1H, br s), 9.33 (1H, s), 8.07 (1H, d, J=8 Hz), 7.46 (1H, d, J=1.3 Hz), 7.38 (1H, dd, J=8, 1.3 Hz), 5.32 (2H, m), 4.20 (2H, t, J=6.5 Hz), 2.13 (3H, s) 2.02~1.93 (4H, m), 1.67 (2H, m), 1.43~1.16 (14H, m), 0.83 (3H, t, J=7 Hz). ^{13}C NMR (90 MHz, DMSO-d_6): δ 169.2 (s), 165.4 (s), 146.6 (s), 131.3 (s), 129.7 (d), 129.5 (d), 124.8 (s), 120.5 (d), 120.4 (d), 115.4 (d), 64.3 (t), 31.1 (t), 29.1 (t), 29.0 (t), 28.3 (t), 28.3 (t), 28.2 (t), 26.6 (t), 26.5 (t), 25.3 (t), 24.0 (q), 22.1 (t), 13.9 (q).

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References

1) STOFFEL, W. & G. ASSMANN: Metabolism of sphingosine bases. XV. Enzymatic degradation of 4t-sphingenine 1-phosphate (sphingosine 1-phosphate) to 2t-hexadecen-1-al and ethanolamine phosphate. Hoppe Seyler's Z. Physiol. Chem. 51: 1041~1049, 1970
2) SPIEGEL, S. & A. H. MERRILL, Jr.: Sphingolipid metabolism and cell growth regulation. FASEB J. 10: 1388~1397, 1996
3) SPIEGEL, S. & S. MILSTIEN: Sphingolipid metabolites: members of a new class of lipid second messengers. J. Membr. Biol. 146: 225~237,1995
4) CHOI, O. H.; J. H. KIM & J. P. KINET: Calcium mobilization *via* sphingosine kinase in signalling by the FcεRI antigen receptor. Nature 380: 634~636, 1996
5) EDSALL, L. C.; G. G. PIRIANOV & S. SPIEGEL: Involvement of sphingosine 1-phosphate in nerve growth factor-mediated neuronal survival and differentiation. J. Neurosci. 17: 6952~6960, 1997
6) BORNFELDT, K. E.; L. M. GRAVES, E. W. RAINES, Y. IGARASHI, G. WAYMAN, S. YAMAMURA, Y. YATOMI, J. S. SIDHU, E. G. KREBS, S. HAKOMORI & R. ROSS: Sphingosine-1-phosphate inhibits PDGF-induced chemotaxis of human arterial smooth muscle cells: spatial and temporal modulation of PDGF chemotactic signal transduction. J. Cell Biol. 130: 193~206, 1995
7) YAMAMURA, S.; Y. YATOMI, F. RUAN, E. A. SWEENEY, S. HAKOMORI & Y. IGARASHI: Sphingosine 1-phosphate regulates melanoma cell motility through a receptor-coupled extracellular action and in a pertussis toxin-insensitive manner. Biochemistry 36: 10751~10759, 1997
8) YATOMI, Y.; F. RUAN, S. HAKOMORI & Y. IGARASHI: Sphingosine-1-phosphate: a platelet-activating sphingolipid released from agonist-stimulated human platelets. Blood 86: 193~202, 1995
9) BUNEMANN, M.; B. BRANDTS, D. M. HERINGDORF, C. J. VAN KOPPEN, K. H. JAKOBS & L. POTT: Activation of muscarinic K^+ current in guinea-pig atrial myocytes by sphingosine-1-phosphate. J. Physiol. 189: 701~707, 1995
10) POSTMA, F. R.; K. JALINK, T. HENGEVELD & W. H. MOOLENAAR: Sphingosine-1-phosphate rapidly induces Rho-dependent neurite retraction: action through a specific cell surface receptor. EMBO J. 15: 2388~2392, 1996
11) OLIVERA, A. & S. SPIEGEL: Sphingosine-1-phosphate as second messenger in cell proliferation induced by PDGF and FCS mitogens. Nature 365: 557~560, 1993
12) ZHANG, H.; N. N. DESAI, A. OLIVERA, T. SEKI, G. BROOKER & S. SPIEGEL: Sphingosine-1-phosphate, a novel lipid, involved in cellular proliferation. J. Cell Biol. 114: 155~167, 1991
13) OLIVERA, A.; T. KOHAMA, Z. ZU, S. MILSTIEN & S. SPIEGEL: Purification and characterization of rat kidney sphingosine kinase. J. Biol. Chem. 273: 12576~12583, 1998
14) KOHAMA, T.; A. OLIVERA, L. EDSALL, M. M. NAGIEC, R. DICKSON & S. SPIEGEL: Molecular cloning and functional characterization of murine sphingosine kinase. J. Biol. Chem. 273: 23722~23728, 1998
15) YATOMI, Y.; F. RUAN, T. MEGIDISH, T. TOYOKUNI, S. HAKOMORI & Y. IGARASHI: *N,N*-dimethylsphingosine inhibition of sphingosine kinase and sphingosine 1-phosphate activity in human platelets. Biochemistry 35: 626~633, 1996
16) IGARASHI, Y.; S. HAKOMORI, T. TOYOKUNI, B. DEAN, S. FUJITA, M. SUGIMOTO, T. OGAWA, K. E. GHENDY & E. RACKER: Effect of chemically well-defined sphingosine and its *N*-methyl derivatives on protein kinase C and src kinase activities. Biochemistry 28: 6796~6800, 1989
17) KHAN, W. A.; R. DOBROWSKY, S. TOUNY & Y. A. HANNUN: Protein kinase C and platelet inhibition by D-*erythro*-sphingosine: comparison with *N,N*-dimethyl-sphingosine and commercial preparation. Biochem.

Biophys. Res. Commun. 172: 683~691, 1990

18) SWEENEY, E. A.; C. SAKAKURA, T. SHIRAHAMA, A. MASAMUNE, H. OHTA, S. HAKOMORI & Y. IGARASHI: Sphingosine and its methylated derivative *N,N*-dimethylsphingosine (DMS) induce apoptosis in a variety of human cancer cell lines. Int. J. Cancer 66: 358~366, 1996

19) KONO, K.; M. TANAKA, T. OGITA, T. HOSOYA & T. KOHAMA: F-12509A, a new sphingosine kinase inhibitor, produced by a discomycete. J. Antibiotics 53: 459~466, 2000

20) MIZUNO, T.; H. HARADA & K. KODAMA, "In preparation"

21) TOMER, K. B.; F. W. CROW & M. L. GROSS: Location of double bond position in unsaturated fatty acids by negative ion MS/MS. J. Am. Chem. Soc. 105: 5487~5488, 1983

22) JENSEN, N. J.; K. B. TOMER & M. L. GROSS: Collisional activation decomposition mass spectra for locating double bonds in polyunsaturated fatty acids. Anal. Chem. 57: 2018~2021, 1985

23) KONO, K.; M. TANAKA, T. OGITA & T. KOHAMA: Characterization of B-5354c, a new sphingosine kinase inhibitor, produced by a marine bacterium. J. Antibiotics 53: 759~764, 2000

24) LOUIE, D. D.; A. KISIC & G. J. SCHROEPFER, Jr.: Sphingolipid base metabolism. J. Biol. Chem. 251: 4557~4564, 1976