VOL. 54 NO. 11, NOV. 2001 THE JOURNAL OF ANTIBIOTICS pp. 904 — 910

CJ-15,183, a New Inhibitor of Squalene Synthase Produced by a Fungus, *Aspergillus aculeatus*

SHUZO WATANABE*, HIDEO HIRAI, MASARU ISHIGURO, TAKAHITO KAMBARA, YASUHIRO KOJIMA, TOMOYUKI MATSUNAGA, HIROYUKI NISHIDA, YUMIKO SUZUKI, AYUMU SUGIURA, H. JAMES HARWOOD, Jr.[†], LIANG HSIUNG HUANG[†] and NAKAO KOJIMA[††]

Exploratory Medicinal Sciences, PGRD, Nagoya Laboratories, Pfizer Pharmaceuticals Inc., 5-Gochi 2, Taketoyo-cho, Chita-gun, Aichi 470-2393, Japan
[†] PGRD, Groton Laboratories, Pfizer Inc., Eastern Point Road, Groton, CT 06340, USA
[††] Faculty of Pharmacy, Meijo University, 150 Yagotoyama, Tempaku-ku, Nagoya 468-8503, Japan

(Received for publication June 29, 2001)

A new squalene synthase (SSase) inhibitor, CJ-15,183 (**I**) was isolated from the fermentation broth of a fungus, *Aspergillus aculeatus* CL38916. The compound potently inhibited rat liver and *Candida albicans* microsomal SSases and also inhibited the human enzyme. It also showed antifungal activities against filamentous fungi and a yeast. The structure was determined to be an aliphatic tetracarboxylic acid compound consisting of an alkyl γ-lactone, malic acid and isocitric acid moieties by spectroscopic studies.

Sterol biosynthesis has been shown to be an attractive target for not only cholesterol lowering agents but also antifungal agents, and a number of inhibitors of sterol biosynthesis have been discovered. Pravastatin[1], a 3-hydroxy-3-methylglutaryl coenzyme A reductase (EC 1.1.1.34) inhibitor, is one of a group of very active antihypercholesterolemic agents. Terbinafine[2], a squalene epoxidase (EC 1.14.99.7) inhibitor, and zaragozic acids (squalestatins)[3,4], SSase (EC 2.5.1.21) inhibitors, are a new group of potent antifungal agents. In the course of our screening program for discovery of sterol biosynthesis inhibitors, a fungus, *Aspergillus aculeatus* CL38916 was found to produce a new SSase inhibitor, CJ-15,183 (**I**). In this paper we report the taxonomy of the producing organism, and the fermentation, isolation, structure elucidation and biological activities of **I**.

Results

Taxonomy

Cultural characteristics were as follows: Colonies on Czapek sucrose agar, attaining 7.5 cm diam. in 11 days, dark vinaceous drab, dark purple-drab, dusky brown to blackish brown (XLV); deeply velutinous, smooth, with concentric zones of heavy sporulation, no exudate; reverse colorless; no soluble pigment. Colonies on malt extract agar, attaining 7.0 cm diam. in 11 days, dark purple-drab, deep brownish drab, to dusky drab (XLV); velutinous, smooth, moderate sporulation, without exudate; reverse colorless to pale cream; no soluble pigment. Colonies on cornmeal agar, attaining 7.0 cm diam. in 11 days, deep brownish drab to dusky drab (XLV); velutinous, smooth, with concentric zones of heavy sporulation; reverse colorless; no soluble pigment. Morphological properties were observed after 11 days of incubation on Czapek sucrose agar. Conidial heads were globose at first, then splitting into 3 to 8 defined columns which measured 15~40×5~25 μm, with small heads measuring 10~40 μm diam. and with conidial columns easily fallen off. Conidiophores were hyaline, but pale brown to brown below the vesicles, smooth, with a thick wall, measuring 0.9~2.3(~3.2) mm×10~17 μm. Vesicles were often elliptical when young, but globose or nearly so when

* Corresponding author: shuzo.watanabe@japan.pfizer.com

matured, brown, and measured 40~80 μm diam. They were fertile over the entire surface. Phialides were in a single series, and closely packed, measuring 7~13×3~7 μm. Conidia were elliptical to globose, 4~5×3~4 μm or 3~4.2 μm diam., conspicuously echinulate, and dark brown to purplish black in mass.

Under the alternate period of 12 hours light and 12 hours darkness, the strain CL38916 is characterized by the radiate conidial heads, the long conidiophores, the globose vesicles, the compact phialides arranged in a single series, the echinulate globose to elliptical spores, and the purplish black spores in mass. The growth was good from 20 to 37°C but was none at 45 and 50°C. Under complete darkness, the spores in mass were brown-grayish black rather than purplish black. The conidiophores were generally shorter. The vesicles and the phialides were smaller. In darkness hyaline exudates were produced on cornmeal agar, potato dextrose agar, yeast extract-soluble starch agar, and phytone yeast extract agar; whereas they were absent under the alternate light and darkness. In darkness ochraceous buff or white sclerotia were produced on yeast extract-soluble starch agar and phytone yeast extract agar, respectively, but they were absent when incubated under the alternate light and darkness. Except for the production of sclerotia the strain CL38916 fits into the description of *Aspergillus aculeatus*, as defined by RAPER and FENNELL[5]. Thus, the strain CL38916 is identified as a new strain of *Aspergillus aculeatus* Iizuka.

Isolation

The detection of **I** was monitored by HPLC using an ODS column as described in the experimental section. The fermentation broth (20 liters) was filtered with the aid of cerite, and then applied to a Diaion HP-20 column (500 ml, Mitsubishi Chemical Industries Ltd.). After washing the column with 50% aqueous acetone (2 liters), **I** was eluted with acetone (2 liters). The eluate was concentrated to dryness to yield an oily residue. The residue was dissolved in MeCN (10 ml) and subjected to preparative HPLC on an ODS column (YMC-Pack ODS AM-343, 20×250 mm, YMC Co., Ltd.) with MeOH-0.1% H_3PO_4 in H_2O (75:25) at a flow rate of 10 ml/minute. The active eluate was applied to a Sephadex LH-20 column (500 ml, Amersham Pharmacia Biotech) with MeOH, and the fractions containing **I** were applied to preparative HPLC on the same ODS column again with MeOH-0.1% TFA in H_2O (80:20) at a flow rate of 10 ml/minute. The eluted peak was collected and concentrated to yield 32.2 mg of **I** as colorless oil.

Physico-chemical Properties

The physico-chemical properties of **I** are summarized in Table 1. The molecular formula of **I** was determined to be $C_{28}H_{38}O_{13}$ by negative ion HRFAB-MS. The UV spectrum showed no characteristic UV absorption above 210 nm. The IR spectra exhibited the presence of a hydroxyl (3450 cm^{-1}) and a carbonyl (1740 cm^{-1}) group.

Structure Elucidation

The ^{13}C NMR spectrum of **I** showed total 28 carbons which were classified to one methyl, ten methylenes, two methines, two oxymethines, six sp^2 methines, one oxyquarternary carbon and six carbonyl carbons, respectively by DEPT experiments. The NMR spectral data of **I** are summarized in Table 2.

Table 1. Physico-chemical properties of CJ-15,183 (**I**).

Appearance	Colorless oil
$[\alpha]_D$ (26°C)	23.3° (*c* 0.06, MeOH)
Molecular formula	$C_{28}H_{38}O_{13}$
Molecular weight	582
HRFAB-MS (*m/z*)	
Found:	581.2237 $(M-H)^-$
Calcd.:	581.2234 (for $C_{28}H_{37}O_{13}$)
UV λ_{max} (MeOH)	End absorption
IR ν_{max} (KBr) cm^{-1}	3450, 2920, 1740, 1400, 1255, 1180
Solubility	
Soluble:	MeOH, MeCN, Acetone, DMSO
Insoluble:	H_2O

Table 2. ^{1}H and ^{13}C NMR data for CJ-15,183 (**I**).

Position	^{13}C Chemical shift δ (multiplicity)	^{1}H Chemical shift δ (multiplicity, J = Hz)
1	175.5 (s)	
2	41.9 (t)	3.15 (1H, d, 16.5), 3.56 (1H, d, 16.5)
3	76.5 (s)	
4	47.7 (d)	3.08 (1H, dd, 5.9, 9.5)
5	31.9 (t)	2.06 (1H, m), 2.40 (1H, m)
6	81.3 (d)	4.98 (1H, m)
7	129.8 (d)	5.55 (1H, dd, 7.0, 15.0)
8	135.6 (d)	5.80 (1H, dd, 6.6, 15.0)
9	33.1 (t)	2.14 (2H, m)
10	27.6 (t)	2.17 (2H, m)
11	129.8 (d)	5.55 (1H, m)
12	129.7 (d)	5.35 (1H, m)
13	26.5 (t)	2.77 (2H, dd, 5.1, 7.7)
14	128.8 (d)	5.32 (1H, m)
15	131.1 (d)	5.35 (1H, m)
16	28.1 (t)	2.05 (2H, m)
17	30.4 (t)	1.34 (2H, m)
18	32.9 (t)	1.34 (2H, m)
19	23.6 (t)	1.34 (2H, m)
20	14.4 (q)	0.90 (3H, t, 6.9)
21	170.7 (s)	
22	177.6 (s)	
23	171.1 (s)	
24	73.2 (d)	5.42 (1H, d, 3.7)
25	44.0 (d)	3.50 (1H, ddd, 3.7, 5.1, 9.2)
26	32.6 (t)	2.54 (1H, dd, 5.1, 17.2), 2.75 (1H, dd, 9.2, 17.2)
27	175.0 (s)	
28	173.4 (s)	

Chemical shifts are shown with reference to CD_3OD as 3.30 ppm for ^{1}H NMR and as 49.8 ppm for ^{13}C NMR.

From the analysis of ^{1}H-^{1}H DQF-COSY spectral data, four partial structures drawn with bold lines in Fig. 1, were confirmed. The connections between these partial structures, and six carbonyl carbons, one methylene (C-2) and one oxyquarternary carbon (C-3) were examined by HMBC and selective INEPT experiments.

As shown in Fig. 1, the unsaturated alkyl chain (C-7~20) was determined by correlations from H-16 (δ 2.05) and H-20 (δ 0.90) to C-18 (δ 32.9), and from H-9 (δ 2.14) to C-10 (δ 27.6) and C-11 (δ 129.8) in the HMBC experiments. Correlations from H-4 (δ 3.08), H-5 (δ 2.06 and 2.40) and H-6 (δ 4.98) to C-22 (δ 177.6) indicated the presence of a γ-lactone moiety. The malic acid moiety (C-1~C-3 and C-21) was determined by correlations from H-2 (δ 3.15) to C-1 (δ 175.5), C-3 (δ 76.5) and C-21 (δ 170.7). The connection between the γ-lactone and malic acid moieties (linkage between C-3 and C-4) was determined by correlations from H-4 to C-3 and C-21 in the selective INEPT experiments. Correlations from H-26 (δ 2.54 and 2.75) to C-27 (δ 175.0) and C-28 (δ 173.4), from H-25 (δ 3.50) to C-23 (δ 171.1), C-27 and C-28, and from H-24 (δ 5.42) to C-23 and C-28 supported the presence of an isocitric acid moiety (C-23~C-28). The connection between the isocitric acid and malic acid moieties (linkage between C-21 and C-24) was confirmed by correlations from H-24 to C-21.

The geometry of double bond at C-7 was assigned as *E* configuration because of the coupling constant ($J_{7,8}$=15.0 Hz). In addition, the geometries of double bonds at C-11 and C-14 were deduced to be both *Z* configuration on the basis of the chemical shift value of the allyic methylene at C-13 (δ 26.5)[6]. The results described above and the molecular formula for **I** ($C_{28}H_{38}O_{13}$) show five additional hydrogens, and therefore suggest the presence of one

Fig. 1. Summary of ^{1}H-^{1}H DQF-COSY, HMBC and selective INEPT experiments of CJ-15,183 (**I**).

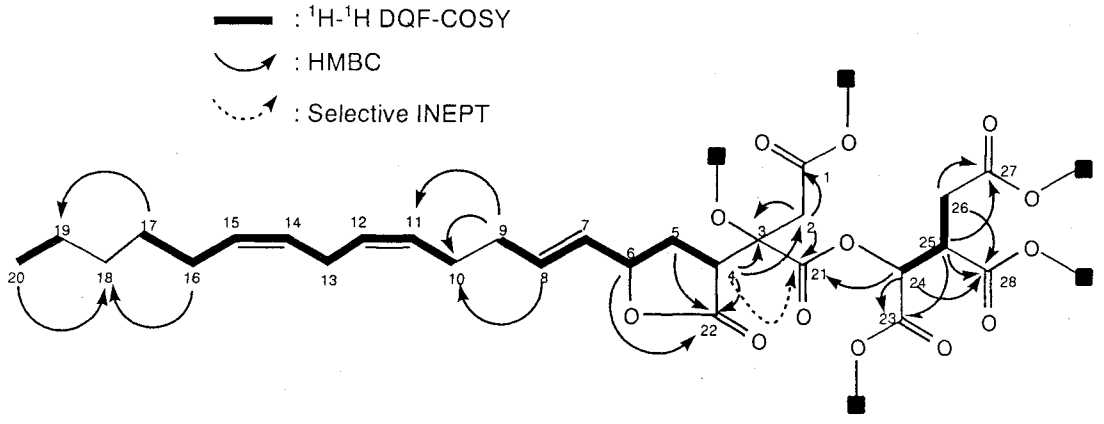

Fig. 2. Structures of CJ-15,183 (**I**) and its related compounds.

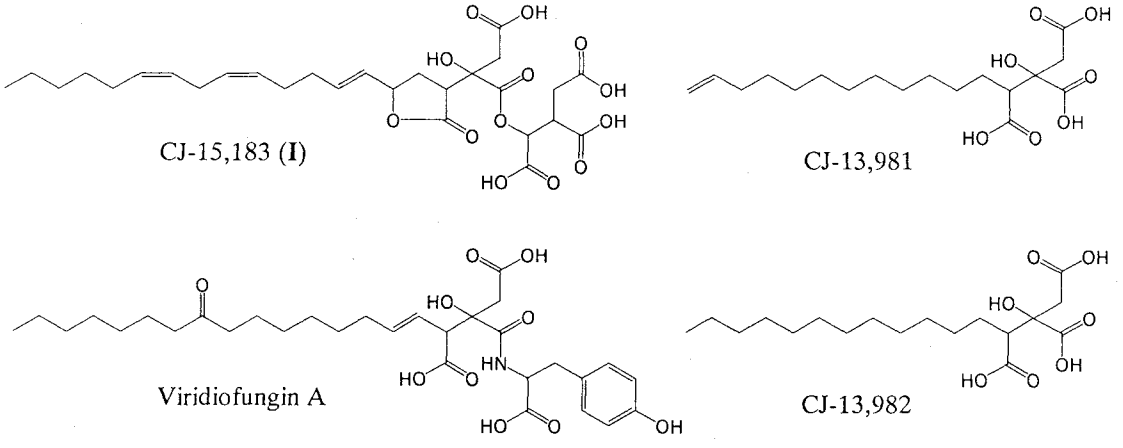

Table 3. SSase inhibitory activities of CJ-15,183 (**I**) and its-related compounds.

	SSase inhibition (IC_{50}, μM)		
	Rat liver	Human liver	*Candida albicans*
CJ-15,183 (**I**)	5.2	98	8.6
CJ-13,981	4.2	2.8	25
CJ-13,982	2.2	1.1	5.6

hydroxyl group and four carboxylic acid residues in the molecule. Thus, the structure of **I** was determined as shown in Fig. 2.

Biological Activities

As shown in Table 3, **I** inhibited rat liver, human liver and *Candida albicans* microsomal SSases at the IC_{50}s of

Table 4. Antifungal activities of CJ-15,183 (**I**).

Tested strain	Diameter of inhibition zone[1] (mm)			
	CJ-15,183 (**I**)	ZA[2]	FLZ[3]	AMB[4]
Aspergillus flavus ATCC 26938	10	37	nz[5]	12
Aspergillus fumigatus ATCC 24681	22	43	nz	nz
Aspergillus niger ATCC 16404	nz	nz	nz	15
Fusarium oxysporum ATCC 48112	12	12	nz	10
Byssochlamys fulva ATCC 28799	21	10	nz	24
Candida albicans ATCC 58716	9	14	34	23
Candida parapsilosis ATCC 22019	9	22	34	14
Candida tropicalls ATCC 18213	11	9	24	11
Cryptococcus albidus ATCC 32420	9	19	31	14
Saccharomyces cerevisiae ATCC 12341	21	33	9	20

1) Paper disc (8 mm) with 50 μg of sample
2) Zaragozic acid A
3) Fluconazole
4) Amphotericin B
5) No zone

5.2, 98 and 8.6 μM, respectively.

Compound **I** showed antifungal activities against *Aspergillus fumigatus* ATCC 24681, *Byssochlamys fulva* ATCC 28799 and *Saccharomyces cerevisiae* ATCC 12341 (Table 4).

Discussion

The new SSase inhibitor, CJ-15,183 (**I**), structurally belongs to the type of alkylcitric acid compounds such as CJ-13,981[7], CJ-13,982[7] and viridiofungin A[8] (Fig. 2). The biosynthetic pathway of these compounds is considered to arise by condensation of the carbonyl group of oxaloacetate with the α-methylene group of a fatty acid[9,10], suggesting that **I** also arises by the same biosynthetic pathway with the formation of γ-lactone ring and the addition of isocitrate (24-OH) to oxaloacetate (C-21).

Compound **I** inhibited rat liver microsomal SSase with an IC_{50} of 5.2 μM, but showed 20 times less activity against human enzyme, whereas CJ-13,981 and CJ-13,982 exhibited same potency to each SSase. It is assumed that the difference of affinity to each SSase or stability of **I** in human SSase assay might cause the low potency to human SSase of **I**.

Viridiofungin A inhibited rat and *Candida albicans* microsomal SSases with IC_{50}s of 15 and 11.8 μM, respectively[11]. Additionally it showed a broad antifungal spectrum against filamentous fungi and a yeast[12]. These results lead to the conclusion that the alkylcitric acid compounds have same potency to rat and *Candida albicans* microsomal SSases, and show broad antifungal spectrum against filamentous fungi and a yeast, but show less activity compared to the zaragozic acids.

Experimental

General

Spectral and physico-chemical data were obtained on the following instruments: UV, JASCO Ubest-30; IR, Shimadzu IR-470; optical rotations, JASCO DIP-370 with a 10 cm cell; NMR, JEOL JNM-GX270 updated with an LSI-11/73 host computer, TH-5 tunable probe and version 1.6 software; and LRFAB- and HRFAB-MS, JEOL MS-700 with a mastation data processing system. All NMR spectra were measured in CD_3OD unless otherwise indicated and peak positions are expressed in parts per million (ppm) based on the internal standard of the MeOH peak at 3.30 ppm for ^1H NMR and 49.8 ppm for ^{13}C NMR. All FAB-MS spectra were measured using glycerol-matrix.

Producing Microorganism

The producing organism, strain CL38916 was isolated

from a soil sample collected in Kira-cho, Hazu-gun, Aichi Prefecture, Japan. The culture was three-spot inoculated from a spore suspension of 0.2% agar onto plates of identification media, and the plates were incubated at 25°C for up to two weeks under compete darkness or cycles of alternate 12-hour lights and 12-hour darkness. The results were read at 11 days for cultural characteristics and 14 days for temperature studies. The colors were determined by comparisons with color chips from RIDGWAY[13]. Identification media used are Czapek-sucrose agar[5], cornmeal agar[14], malt extract agar[5], phytone yeast extract agar (BBL), potato dextrose agar (peeled potato 100 g, dextrose 10 g, agar 20 g, tap water 1 liter), yeast extract-soluble starch agar[15]. Malt extract agar was used for temperature studies.

Fermentation

Aspergillus aculeatus CL38916 was maintained on potato dextrose agar slant (Difco). A vegetative cell suspension from the slant was used to inoculate a 500-ml Erlenmeyer flask containing 100 ml of a seed medium (potato dextrose broth 2.4%, yeast extract 0.5% and agar 0.1%). The flask was shaken at 26°C for 4 days on a rotary shaker (7-cm throw at 250 rpm). Five ml aliquot was inoculated into a 500-ml Erlenmeyer flask containing 150 ml of the seed medium and shaken at 26°C for 3 days. The second seed culture was inoculated into a 6-liter jar fermentor containing 3 liters of a production medium (sucrose 2%, potato starch 10%, casamino acid 1%, KH_2PO_4 0.5% and $MgSO_4 \cdot 7H_2O$ 0.03%). The fermentation was carried out at 26°C for 14 days at an aeration rate of 3 liters/minute and an agitation rate of 1,750 rpm.

HPLC Analysis

HPLC analysis was performed on a Hewlett Packard HP1090 system. Samples were subjected to an ODS column (YMC-pack ODS AM-312, 6.0×150 mm, YMC Co., Ltd.) maintained at 42°C and eluted with MeOH-0.1% TFA in H_2O (80 : 20) at a flow rate of 0.8 ml/minute. Compound **I** was monitored by absorbance at 210 nm. Under these conditions, **I** was eluted at the retention time of 5.8 minutes.

Preparation of Rat Hepatic Microsomes

Livers freshly obtained from Wistar rats (Charles River) were rinsed in ice-cold PBS and briefly homogenized in buffer A (50 mM MOPS-NaOH; pH 7.4, 10 mM $MgCl_2$, 1 mM EDTA, 10 mM dithiothreitol) containing the protease inhibitors (500 μM PMSF, 10 μM aprotinin, 10 μM leupeptin, 10 μM chymostatin). The homogenate was centrifuged at 3,000×g for 20 minutes at 4°C. The supernatant was recentrifuged at 20,000×g for 30 minutes at 4°C. The supernatant was again recentrifuged at 100,000×g for 60 minutes at 4°C. After centrifugation, the supernatant was removed and the pellet was suspended in buffer A. This microsomal preparation had a protein concentration of about 13.2 mg/ml. The microsomal suspensions were stored at −70°C. Under these conditions, SSase activity was stable for at least several months.

SSase Activity

Assays were performed on 96-well microtiter plates in a total volume of 50 μl containing 50 mM MOPS-NaOH (pH 7.4), 10 mM KF, 10 mM $MgCl_2$, 2 mM CHAPS, 10 mM dithiothreitol, 0.5 mM NADPH, 50 mM ascorbate, 20 units/ml ascorbate oxidase, 2 mM glucose-6-phosphate, 20 units/ml glucose-6-phosphate dehydrogenase, rat hepatic microsomes and 6 μM [1-^{3}H] FPP. After incubation at room temperature for 30 minutes, the reaction was terminated by the addition of 20 μl of 1-propanol containing unlabeled 1% squalene. Thirty microliters of reaction mixture was applied onto a polyester-backed silica gel TLC sheet (Sigma; 10 cm×10 cm) which was developed with hexane-EtOAc (7 : 3). After drying, radioactivity in zone containing the squalene (Rf=0.8) was scraped and measured by a scintillation counter[16,17].

Antifungal Activity

Antifungal assay was tested using paper discs (i.d. 8 mm, ADVANTEC). *Aspergillus*, *Fusarium*, *Byssochlamys*, *Candida* and *Saccharomyces* species were grown on potato dextrose agar medium (Difco) and *Cryptococcus albidus* ATCC 32420 was grown on sabouraud agar medium (Nissui). Antimicrobial activity was observed after 24-hour incubation at 27°C for yeasts and after 48-hour incubation for fungi.

References

1) TSUJITA, Y.; M. KURODA, Y. SHIMADA, K. TANZAWA, M. ARAI, I. KANEKO, M. TANAKA, H. MASUDA, C. TARUMI, Y. WATANABE & S. FUJII: CS-514, a competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase: tissue-selective inhibition of sterol synthesis and hypolipidemic effect on various animal species. Biochem. Biophys. Acta 877: 50~60, 1986
2) PETRANYI, G.; N. S. RYDER & A. STUETZ: Allylamine derivatives: new class of synthetic antifungal agents inhibiting fungal squalene epoxidase. Science 224: 1239~1241, 1984
3) BAXTER, A.; B. J. FITZGERALD, J. L. HUTSON, A. D. MCCARTHY, J. M. MOTTERAM, B. C. ROSS, M. SAPRA, M. A. SNOWDEN, N. S. WATSON, R. J. WILLIAMS & C.

WRIGHT: Squalestatin 1, a potent inhibitor of squalene synthase, which lowers serum cholesterol *in vivo*. J. Biol. Chem. 267: 11705~11708, 1992

4) BERGSTROM, J. D.; M. M. KURTZ, D. J. REW, A. M. AMEND, J. D. KARKAS, R. G. BOSTEDOR, V. S. BANSAL, C. DUFRESNE, F. L. VANMIDDLESWORTH, O. D. HENSENS, J. M. LIESCH, D. L. ZINK, K. E. WILSON, J. ONISHI, J. A. MILLIGAN, G. BILLS, L. KAPLAN, M. N. OMSTEAD, R. G. JENKINS, L. HUANG, M. S. MEINZ, L. QUINN, R. W. BURG, Y. L. KONG, S. MOCHALES, M. MOJENA, I. MARTIN, F. PELAEZ, M. T. DIEZ & A. W. ALBERTS: Zaragozic acids: a family of fungal metabolites that are picomolar competitive inhibitors of squalene synthase. Proc. Natl. Acad. Sci. USA 90: 80~84, 1993
5) RAPER, K. B. & D. I. FENNELL: The genus *Aspergillus*, Baltimore. The Williams and Wilkins: 686, 1965
6) WENKERT, E.; B. L. BUCKWALTER, I. R. BURFITT, M. J. GASIC, H. E. GOTTLIEB, E. W. HAGAMAN, F. M. SCHELL & P. M. WOVKULICH: Carbon-13 nuclear magnetic resonance spectroscopy of naturally occurring substances. Topics in Carbon-13 NMR Sectroscopy 2: 81~121, 1975
7) WATANABE, S.; H. HIRAI, N. YOSHIKAWA, A. SUGIURA, T. KAMBARA, H. NISHIDA, Y. YAMAUCHI, Y. KOJIMA, H. J. HARWOOD, Jr., L. H. HUANG & N. KOJIMA: CJ-13,981 and CJ-13,982, new squalene synthase inhibitors. J. Antibiotics, in preparation
8) HARRIS, G. H.; E. T. T. JONES, M. S. MEINZ, M. N. OMSTEAD, G. L. HELMS, G. F. BILLS, D. ZINK & K. E. WILSON: Isolation and structure elucidation of viridiofungins A, B and C. Tetrahedron Lett. 34: 5235~5238, 1993
9) TANABE, M.; T. HAMASAKI, Y. SUZUKI & F. L. JONSON: Biosynthetic studies with carbon-13. Fourier transform nuclear magnetic resonance spectra of the metabolite avenaciolide. J. C. S. Chem. Comm. 212~213, 1973
10) MCCORKINDALE, N. J.; W. P. BLACKSTOCK, G. A. JOHNSTON, T. P. ROY & J. A. TROKE: Biosynthesis of canadensolide, ethisolide and related metabolites. 11th IUPAC Int. Symp. Chem. Nat. Prod. 1: 151~154, 1978
11) HARRIS, G. H.: Biological active compounds isolated from aerobic fermentation of *Trichoderma viride*. WO9418157-A1, Jan. 25, 1994
12) ONISHI, J. C.; J. A. MILLIGAN, A. BASILIO, J. BERGSTROM, J. CUROTTO, L. HUANG, M. MEINZ, M. NALLIN-OMSTEAD, F. PELAEZ, D. REW, M. SALVATORE, J. THOMPSON, F. VICENTE & M. B. KURTZ: Antimicrobial activity of viridiofungins. J. Antibiotics 50: 334~337, 1997
13) RIDGWAY, R.: Color standards and color nomenclature. Publ. by the author, Washington, D.C., p. 43, 1912
14) CARMICHAEL, J. W.: *Geotrichum candidum*. Mycologia 49: 820~830, 1957
15) EMERSON, R.: Mycological organization. Mycologia 50: 589~621, 1958
16) HARWOOD, Jr., H. J.: Measurement of enzymatic activity in 96-well format using TopCount microplate scintillation counting technology. Anal. Biochem. 226: 268~278, 1995
17) PETRAS, S. F.; S. LINDSEY & H. J. HARWOOD, Jr.: HMG-CoA reductase regulation: use of structurally diverse first half-reaction squalene synthetase inhibitors to characterize the site of mevalonate-derived nonsterol regulator production in cultured IM-9 cells. J. Lipid Res. 40: 24~38, 1999