VOL. 56 NO. 7, JULY 2003 THE JOURNAL OF ANTIBIOTICS pp. 603 — 609

EI-1941-1 and -2, Novel Interleukin-1β Converting Enzyme Inhibitors Produced by *Farrowia* sp. E-1941

II. Taxonomy of Producing Strain, Fermentation, Isolation, Physico-chemical Properties, and Biological Properties

FUMITO KOIZUMI, HIROKI ISHIGURO, KATSUHIKO ANDO, HIDEMASA KONDO[a], MAYUMI YOSHIDA[b], YUZURU MATSUDA[c] and SATOSHI NAKANISHI[d]

·Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., Ltd.,
3-6-6 Asahimachi, Machida-shi, Tokyo 194-8533, Japan

(Received for publication December 24, 2002)

EI-1941-1 and -2, novel interleukin-1β converting enzyme (ICE) inhibitors, were isolated from the culture broths of *Farrowia* sp. E-1941. EI-1941-1 and -2 selectively inhibited the human recombinant ICE activity with IC_{50} values of 0.086 and 0.006 μM, respectively. Taxonomy and fermentation of the producing strain and isolation, physico-chemical properties, structure elucidation, and biological properties of EI-1941-1 and -2 are described.

Interleukin-1β (IL-1β) is an important mediator of pathogenesis of rheumatoid arthritis, septic shock, inflammation, and other physiological situations[1,2]. IL-1β converting enzyme (ICE) is a cysteine-containing protease which cleaves a biologically-inactive precursor to biologically-active IL-1β[3,4]. IL-1β is released from macrophage-like cells in an inflammatory situation, and is the major form of IL-1 in diseases. ICE inhibitors have been shown to prevent inflammation in several acute models[5,6], suggesting that ICE inhibitors would be useful as anti-inflammatory drugs.

To discover novel anti-inflammatory drugs, we initiated a screening program to obtain ICE inhibitors from microbial sources[7~10]. In the course of this screening, we found the metabolites of *Farrowia* sp. E-1941 inhibited ICE. In this article, we describe the taxonomy and fermentation of the producing strain and isolation, physico-chemical properties, and biological properties of two novel compounds, EI-1941-1 and -2.

Materials and Methods

Material

Human recombinant ICE was prepared by heterologous expression in *Escherichia coli* and *in vitro* refolding as described[11]. The enzyme was purified by ion exchange chromatography[11]. Acetyl-Tyr-Val-Ala-Asp-amino-4-methylcoumarin (Ac-Tyr-Val-Ala-Asp-AMC)[3], a fluorescent substrate was purchased from Peptide Institute, Inc., Osaka, Japan. All other chemicals were of analytical grade.

[a] Present address: Pharmaceutical Research laboratories, Kyowa Hakko Kogyo Co., Ltd., 1188 Shimotogari, Nagaizumi-cho, Sunto-Gun, Shizuoka 411-8731, Japan

[b] Present address: Genomic Sciences Center, RIKEN,1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama-shi, Kanagawa, 230-0045, Japan

[c] Present address: Corporate Plannning Department, Kyowa Hakko Kogyo Co., Ltd.,1-6-1 Ohtemachi, Chiyoda-ku, Tokyo 100-8185, Japan

[d] Present address: Strategic Planning Department, Pharmaceuticals Company, Kyowa Hakko Kogyo Co., Ltd.,1-6-1 Ohtemachi, Chiyoda-ku, Tokyo 100-8185, Japan

* Corresponding author: fumito.koizumi@kyowa.co.jp

Taxonomy

The producing strain E-1941 was isolated from soil collected in Aichi prefecture, Japan. E-1941 was cultured on malt extract agar (malt extract 2%, glucose 2%, peptone 0.1% and agar 2%) and potato-dextrose agar (Difco 0549) at 25°C. Culture characteristics were determined after cultivation for 7 and 14 days. Morphological observations of strain E-1941 grown on potato-dextrose agar for 7 days at 25°C were ascertained by light microscopy. The temperature and pH range of growth was determined after cultivation with malt extract broth (malt extract 2%, glucose 2% and peptone 0.1%) for 7 days.

Culture and Medium Conditions

A loopful of spores of E-1941, grown on an agar slant, was inoculated into each of five 50-ml test tubes containing 10 ml of a seed medium composed of dried mashed potatoes 3%, glucose 10% and yeast extract 0.5% in deionized water (pH 7.2 before sterilization). The inoculated test tubes were incubated on a reciprocating shaker for 3 days at 25°C. Five ml of the seed culture were added to a 300-ml Erlenmeyer flask containing 45 ml of the same medium. Eight inoculated flasks were incubated for 2 days on a rotary shaker (200 rpm) at 25°C. Fifty ml of the seed culture were added to a 2-liter Erlenmeyer flask containing 500 ml of the same medium. Eight inoculated flasks were incubated for 2 days on a rotary shaker (200 rpm) at 25°C. The 1.8 liters of the seed culture was transferred into a 30-liter fermenter containing 18 liters of a fermentation medium composed of glucose 2%, dried mashed potatoes (Snow Brand Milk Products) 2%, peptone (Kyokuto) 0.5%, dry yeast (Asahi Brewery) 0.5%, KH_2PO_4 0.5% and $Mg_3(PO_4)_2 \cdot 8H_20$ 0.05% in deionized water (pH 6.0 before sterilization). The fermentation was carried out for 5 days at 25°C with agitation of 250 rpm and aeration of 18 liters per minute. The growth of microorganism was monitored during fermentation by the measurement of packed cell volume (PCV). Production of EI-1941-1 and -2 was determined by HPLC. For this measurement, 1 ml of the culture broth was extracted with 1 ml of methyl ethyl ketone, the extracts evaporated and dissolved in 200 μl of MeOH. The concentrated extract (10 μl) was provided for HPLC analysis.

Determination of EI-1941-1 and -2 by HPLC

HPLC analysis was performed on an ODS-AQ-312 (6 mm i.d.×150 mm, YMC Co., Ltd.). The column was eluted with 25% aqueous acetonitrile, at a flow rate of 1.0 ml per minute. The effluent was monitored at a wavelength of 220 nm. The retention times of EI-1941-1 and EI-1941-2 were 11.6 minutes and 18.6 minutes, respectively.

Assay of ICE Activity

ICE activities were measured as described by THORNBERRY *et al.*[3] with a slight modification. An assay mixture (sodium phosphate buffer: pH 6.8 10 mM, bovine serum albumin 0.2%, substrate Ac-Tyr-Val-Ala-Asp-AMC 10 μM, refolded ICE 1×10^{-12} I.U., test sample solution 1%; final volume 100 μl) was incubated for 2 hours at room temperature. One I.U. was defined as the amount of enzymatic activity which produced 1 μmol AMC per minute. Then, the change in fluorescent intensity of liberated AMC (excitation-wave length: 370 nm, emission-wave length: 440 nm) was measured.

Antimicrobial Activity

The *in vitro* antimicrobial activities of EI-1941 and -2 were determined on nutrient agar by 2-fold serial dilution methods. The lowest concentration that inhibited growth of a bacterial strain after 18 hours incubation at 37°C was recorded as the MIC.

Results

Characterization of the Producing Strain E-1941

When the strain was cultured at 25°C on malt extract agar media, the diameter of a colony reached 43~46 mm on the seventh day of culturing. On the fourteenth day, the color of the colony was mustard brown and was beige at the periphery; and the color of the reverse side was oatmeal and was slate tan at the center. When the strain was cultured at 25°C on potato-dextrose agar media, the diameter of a colony reached 50~56 mm on the seventh day of culturing. On the fourteenth day, the color of the colony was covert brown and covert tan at the periphery; and the color of the reverse side was light mustard tan and was deep brown at the center. The growth temperature range for this strain was 12~34.5°C and optimum growth temperature was about 26°C. The pH range which allows its growth was 2~11 and optimum growth pH was around 8.

Hyphae were septate and branched well. Many perithecia were formed on the surface of agar media. The perithecium was small lageniform and its color was brown to dark brown. It was 126~274 μm long and 53~116 μm wide and had a short conical ostiole at the tip. Setae were slightly formed on the perithecium as terminal hair and lateral hair, which were difficult to distinguish from each other. The seta was light brown to brown, straight without branching,

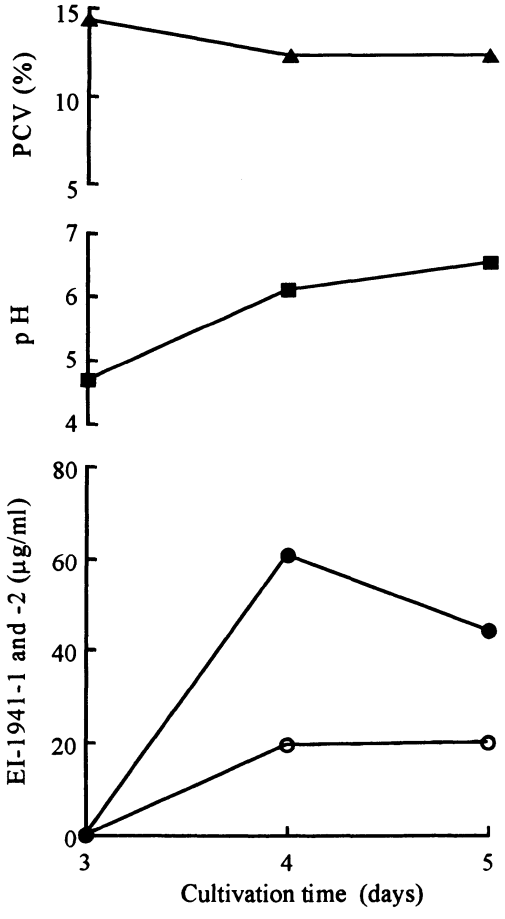
and septate. It was 85~205 μm long and 4~5.5 μm wide at the base and tapers. Asci were formed within the perithecium and were colorless, smooth and clavate. The ascus were unitunicate, rapidly deliquescing and eight-spored. The ascospore was olive brown, smooth and unicellular, and had an unclear germ pore. The shape of ascospore was wide ellipsoidal or limoniform with both ends being slightly pointed. The ascospore was 8~10 μm long and 6~9.5 μm wide and was released from the ostiole at the tip of perithecium.

On the basis of the above mycological properties, the strain was classified in the genus *Farrowia*[12]. The strain was distinguished from *Chaetomium* sp. since lateral and terminal hairs of *Chaetomium* sp. were variously branched or contorted. The strain has been deposited at the National Institute of Bioscience and Human-Technology, Agency of industrial Science and Technology, Tsukubashi, Ibaraki, Japan, as *Farrowia* sp. E-1941 with the accession number FERM BP-5258.

Production of EI-1941-1 and -2 by Fermentation

The production of EI-1941-1 and -2 in the culture broth initiated on day 4, and the amount of both EI-1941 compounds reached maximum on day 4 (Fig. 1). The EI-1941 compounds were produced in both culture supernatant and mycelia, but accumulated mainly in culture supernatant.

Fig. 1. Time course of EI-1941-1 and -2 production in a 30-liter fermenter.

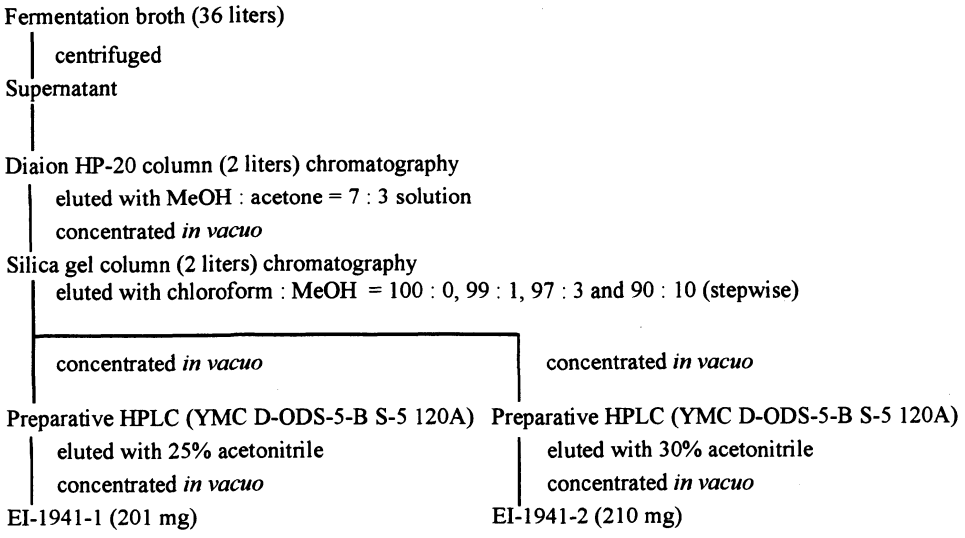


Production of EI-1941-1 (●) and EI-1941-2 (○), pH of the culture broth (■) and packed cell volume (▲) were indicated.

Isolation and Purification

The isolation procedure for the EI-1941-1, -2, and -3 is shown schematically in Fig. 2. The producing strain E-1941 was cultured according to the method described in the Materials and Methods. Culture broth (36 liters) was centrifuged to obtain culture supernatant, and this supernatant was applied on a Diaion HP-20 column (2 liters). After washing the column with 20% aqueous methanol (8 liters) and 40% aqueous methanol (8 liters) sequentially, the active principles were eluted from the column with methanol - acetone (7 : 3) solution (8 liters). Active fractions were concentrated *in vacuo* to give a crude material. This material was dissolved with chloroform. 100 g of Radiolite #600 (Showa Kagaku Kogyo Co., Ltd.) was added to this solution, and the solution was concentrated *in vacuo*. This residual material adsorbed on Radiolite was applied onto a silica gel column (2 liters) equilibrated with chloroform, and eluted with chloroform and chloroform - methanol (99 : 1, 97 : 3 and 90 : 10 (8 liters each)), sequentially. EI-1941-2 was eluted with chloroform and chloroform - methanol (99 : 1), and EI-1941-1 was eluted with chloroform - methanol (97 : 3). Active fractions containing EI-1941-2 were combined and concentrated *in vacuo* to give a partially purified material (1.7 g). This material was dissolved in methanol and purified by preparative HPLC using a column (D-ODS-5-B S-5 120A, 30 i.d.×250 mm) with 30% aqueous acetonitrile as elution solvent. Active fractions containing EI-1941-1 were concentrated *in vacuo* to give a partially purified material (2.1 g). This material was dissolved in methanol and purified by preparative HPLC using the same column with 25% acetonitrile as elution solvent. Active fractions were evaporated, and EI-1941-1 (201 mg) and EI-1941-2 (210 mg) were obtained as brown oil and red oil, respectively. The decomposed product of EI-1941-2 (EI-1941-3) was isolated from EI-1941-2 stored for 8 days at

Fig. 2. Purification procedure for EI-1941-1 and -2.



room temperature in the dry state. EI-1941-2 (80 mg) which had been stored was dissolved in methanol, and this solution was purified by preparative HPLC using the same column with 30% acetonirile as elution solvent to separate EI-1941-2-containing fractions and EI-1941-3-containing fractions. EI-1941-3-containing fractions were evaporated, and EI-1941-3 (13 mg) was obtained as red oil.

Physico-chemical Properties

Physico-chemical properties of EI-1941-1, -2, and -3 are summarized in Table 1. The structures of EI-1941-1, -2, and -3 were determined (Fig. 3) from their physico-chemical properties and spectral data. These compounds were found to be novel compounds. Details of structural elucidation studies will be reported elsewhere.

Biological Properties

EI-1941-1 and -2 inhibited human recombinant ICE activities with IC_{50} values of 0.086 and 0.006 μM, respectivly. On the other hand, EI-1941-3, which is the decomposed compound of EI-1941-2, was inactive against human recombinant ICE at concentrations up to 10 μM (Table 2). EI-1941-2 had weak antimicrobial activities against *Enterococcus hirae*, *Staphylococcus aureus* subsp. *aureus*, *Bacillus subtilis* and *Proteus vulgaris*. MIC values were 88, 88, 42 and 21 μM, respectively. On the other hand, EI-1941-1 had no antimicrobial activities at the concentrations we tested (Table 3).

Discussion

We have isolated novel compounds, EI-1941-1 and -2, from the culture broths of *Farrowia* sp. E-1941. EI-1941-1 and -2 are new compounds, the structure of which are close to that of cycloepoxydon[13,14] produced by the deuteromycete strain *45-93*. It has been reported that cycloepoxydon inhibited the activation of NF-κB by preventing the phosphorylation of the IκBα, a key mediator of NF-κB activation[15]. However, it remains unknown whether or not this compound inhibits ICE activities.

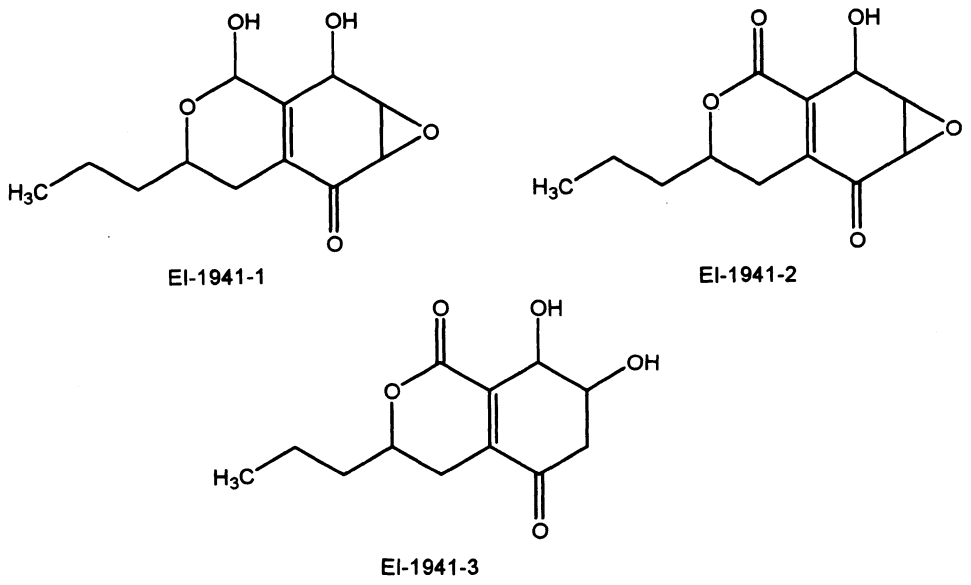
Several peptide-related compounds have been reported as ICE inhibitors, and the role of ICE in the pathology of inflammation has been clarified with these inhibitors[3,5,6]. L-741,498[16] and pentenocins A and B[17] have been also reported as ICE inhibitors from microbial sources. In addition, we also have previously demonstrated that benz[α]anthracene-related EI-1501s and manumycin-related EI-1511s and EI-1625-2 from microbial sources inhibited ICE activities[7~10]. Although EI-1941-1 and -2 had different chemical skeletons from these other ICE inhibitors from microbial sources, pentenocins A, EI-1501s, EI-1511s, EI-1625-2 and EI-1941s have the epoxy

Table 1. Physico-chemical properties of EI-1941-1, -2, and -3.

	EI-1941-1	EI-1941-2	EI-1941-3
Appearance	Brown oil	Red Oil	Red Oil
Optical rotation	$[\alpha]_D^{27}$ -193.7° c 0.314, MeOH	$[\alpha]_D^{23}$ -307.5° c 0.568, MeOH	$[\alpha]_D^{23}$ -87.5° c 0.314, MeOH
Molecular formula	$C_{12}H_{16}O_5$	$C_{12}H_{14}O_5$	$C_{12}H_{16}O_5$
FAB-MS *m/z*	241 $(M+H)^+$	239 $(M+H)^+$	241 $(M+H)^+$
HR FAB-MS Found.	241.1058 $(M+H)^+$	239.0928 $(M+H)^+$	241.1061 $(M+H)^+$
Calcd.	241.1076	239.0919	241.1076
UV λ_{max} (MeOH) (ε)	209.0 (6800), 240.5 (5500)	225.0 (8340)	232.0 (6800)
IR ν_{max} (KBr)	3419, 2960, 2933, 2873 1682, 1456, 1281, 1026 874, 725	3367, 2959, 2873,1717, 1696, 1409, 1240,1040,	3419, 2960, 2933, 2873, 1716, 1693, 1410, 1230, 1024
TLC (Rf)			
$CHCl_3$-MeOH (9 :1)[a]	0.43	0.68	0.41
Color reaction			
Positive	I_2, H_2SO_4	I_2, H_2SO_4	I_2, H_2SO_4
Solubility			
Soluble	MeOH, acetonitrile	MeOH, acetonitrile	MeOH, acetonitrile

[a] Silica gel $60F_{254}$ plate (Merck)

Fig. 3. Structures of EI-1941-1, -2, and -3.



structure in their molecule. This mutual structure might be important for the inhibition of ICE activity. In addition, decomposed compound of EI-1941-2 (EI-1941-3), which has no epoxy structure, was inactive against human recombinant ICE at concentrations up to 10 μM. These results indicated that epoxy structure of EI-1941-1 and -2 might play important role to inhibit ICE activity.

EI-1941-2 had weak antimicrobial activities against

Table 2. ICE inhibitory activities of EI-1941-1, -2, and -3.

Enzyme	IC_{50} value (μM)		
	EI-1941-1	EI-1941-2	EI-1941-3
ICE	0.086	0.006	>10

Table 3. The antibiotic activities of EI-1941-1 and -2.

Strains	MIC (μM)	
	EI-1941-1	EI-1941-2
Candida albicans ATCC10231	>346	>349
Enterococcus hirae ATCC10541	>346	88
Pseudomonas aeruginosa BHM No.1	>346	>349
Staphylococcus aureus subsp. *aureus* ATCC6538P	>346	88
Escherichia coli ATCC26	>346	>349
Bacillus subtilis No. 10707	>346	42
Proteus vulgaris ATCC6897	>346	21
Shigella sonnei ATCC9290	>346	>349
Klebsiella pneumoniae subsp. *choleraesuis* ATCC9992	>346	>349

Gram-positive bacteria and moderate activity against *Proteus vulgaris*, whereas EI-1941-1 had no antimicrobial activities against all bacteria and yeast we tested. Among ICE inhibitors from microbial sources, EI-1507-2, EI-1511s and EI-1625-2 also had weak antimicrobial activities against Gram-positive bacteria. As observed for EI-1941-2, EI-1507-1 had weak antimicrobial activities against Gram-positive bacteria and *Proteus vulgaris*.

Acknowledgment

We would like to express thanks to Ms. TOSHIKO NAKANO and KIYOMI YOSHIKAWA for their expert assistance.

References

1) DINARELLO, C. A.: Interleukin-1 and interleukin-1 antagonism. Blood 77: 1627~1652, 1991
2) DINARELLO, C. A. & S. M. WOLFF: The role of interleukin-1 in disease. N. Engl. J. Med. 328: 106~113, 1993
3) THORNBERRY, N. A.; H. G. BULL, J. R. CALAYCAY, K. T. CHAPMAN, A. D. HOWARD, M. J. KOSTURA, D. K. MILLER, S. M. MOLINEAUX, J. R. WEIDNER, J. AUNINS, K. O. ELLISTON, J. M. AYALA, F. J. CASANO, J. CHIN, G. J.-F. DING, L. A. EGGER, E. P. GAFFNEY, G. LIMJUCO, O. C. PALYHA, S. M. RAJU, A. M. ROLANDO, J. P. SALLEY, T.-T. YAMIN, T. D. LEE, J. E. SHIVELY, M. MACCROSS, R. A. MUMFORD, J. A. SCHMIDT & M. J. TOCCI: A novel heterodimeric cysteine protease is required for interleukin-1β processing in monocytes. Nature 356: 768~774, 1992
4) GERRETTI, D. P.; C. J. KOZLOSKY, B. MOSLEY, N. NELSON, K. V. NESS, T. A. GREENSTREET, C. J. MARCH, S. R. KRONHEIM, T. DRUCK, L. A. CANNIZZARO, K. HUEBNER & R. A. BLACK: Molecular cloning of the interleukin-1β converting enzyme. Science 256: 97~100, 1992
5) ELFORD, P. R.; R. HENG, L. RÉVÉSZ & A. R. MACKENZIE: Reduction of inflammation and pyrexia in the rat by oral administration of SDZ 224-015, an inhibitor of the interleukin-1β converting enzyme. British J. Pharmacol. 115: 601~606, 1995
6) KU, G.; T. FAUST, L. L. LAUFFER, D. J. LIVINGSTON & M. W. HARDING: Interleukin-1β converting enzyme inhibition blocks progression of type II collagen-induced arthritis in mice. Cytokine 8: 377~386, 1996
7) TSUKUDA, E.; T. TANAKA, K. OCHIAI, H. KONDO, M. YOSHIDA, T. AGATSUMA, Y. SAITOH, S. TESHIBA & Y. MATSUDA: EI-1507-1 and -2, novel interleukin-1β converting enzyme inhibitors produced by *Streptomyces* sp. E-1507. J. Antibiotics 49: 333~339, 1996

8) TANAKA, T.; E. TSUKUDA, K. OCHIAI, H. KONDO, S. TESHIBA & Y. MATSUDA: EI-1511-3, -5 and EI-1625-2, novel interleukin-1β converting enzyme inhibitors produced by *Streptomyces* sp. E-1511 and E-1625. I. Taxonomy of producing strain, fermentation and isolation. J. Antibiotics 49: 1073~1078, 1996

9) UOSAKI, Y.; T. AGATSUMA, T. TANAKA & Y. SAITOH: EI-1511-3, -5 and EI-1625-2, novel interleukin-1β converting enzyme inhibitors produced by *Streptomyces* sp. E-1511 and E-1625. II. Structure determination. J. Antibiotics 49: 1079~1084, 1996

10) TANAKA, T.; E. TSUKUDA, Y. UOSAKI & Y. MATSUDA: EI-1511-3, -5 and EI-1625-2, novel interleukin-1β converting enzyme inhibitors produced by *Streptomyces* sp. E-1511 and E-1625. III. Biochemical properties of EI-1511-3, -5 and EI-1625-2. J. Antibiotics 49: 1085~1090, 1996

11) WALKER, N. P. C.; R. V. TALANIAN, K. D. BRADY, L. C. DANG, N. J. BUMP, C. R. FERENZ, S. FRANKLIN, T. GHAYUR, M. C. HACKETT, L. D. HAMMILL, L. HERZOG, M. HUGUNIN, W. HOUY, J. A. MANKOVICH, L. MCGUINESS, E. ORLEWICZ, M. PASKIND, C. A. PRATT, P. REIS, A. SUMMANI, M. TERRANOVA, J. P. WELCH, L. XIONG, A. MÖLLER, D. E. TRACEY, R. KAMEN & W. W. WONG: Crystal structure of the cysteine protease interleukin-1β-converting enzyme: A $(p20/p10)_2$ homodimer. Cell 78: 343~352, 1994

12) HAWKSWORTH, D. L.: *Farrowia*, a new genus in the Chaetomiaceae. Persoonia 8: 167~185, 1975

13) GEHRT, A.; G. ERKEL, H. ANKE, T. ANKE & O. STERNER: New hexaketide inhibitors of eukaryotic signal transduction. Natl. Prod. Lett. 9: 259~264, 1997

14) GEHRT, A.; G. ERKEL, T. ANKE & O. STERNER: Cycloepoxydon, 1-hydroxy-2-hydroxymethyl-3-pent-1-enylbenzene and 1-hydroxy-2-hydroxymethyl-3-pent-1,3-dienylbenzene, new inhibitors of eukaryotic signal transduction. J. Antibiotics 51: 455~463, 1998

15) BAEUERLE, P. A. & T. HENKEL: Function and activation of NF-κB in the immune system. Annu. Rev. Immunol. 12, 141~179, 1994

16) SALVATORE, M. J.; O. D. HENSENS, D. L. ZINK, J. LIESCH, C. DUFRESNE, J. G. ONDEYKA, T. M. JÜRGENS, R. P. BORRIS, S. RAGHOOBAR, E. MCCAULEY, L. KONG, S. E. GARTNER, G. E. KOCH, F. PELAEZ, M. T. DIEZ, C. CASCALES, I. MARTIN, J. D. POLISHOOK, M. J. BALICK, H. T. BECK, S. R. KING, A. HSU & R. B. LINGHAM: L-741,494, a fungal metabolite that is an inhibitor of interleukin-1β converting enzyme. J. Natur. Prod. 57: 755~760, 1994

17) MATSUMOTO, T.; A. ISHIYAMA, Y. YAMAGUCHI, R. MASUMA, H. UI, K. SHIOMI, H. YAMADA & S. OMURA: Novel cyclopentanone derivatives pentenocins A and B, with interleukin-1β converting enzyme inhibitory activity, produced by *Trichoderma hamatum* FO-6903. J. Antibiotics 52: 754~757, 1999