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

I. Biochemical Characterization of EI-1941-1 and -2

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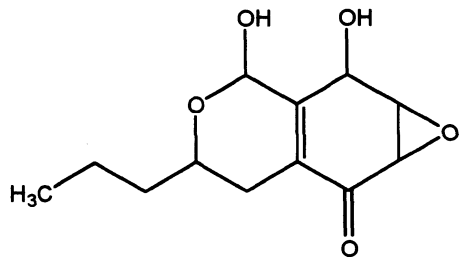
EI-1941-1 and -2 isolated from the culture broths of *Farrowia* sp. selectively inhibited the human recombinant ICE activity with IC_{50} values of 0.086 and 0.006 μM, respectively, without inhibiting elastase and cathepsin B. EI-1941-1 and -2 also inhibited mature interleukin-1β secretion from THP-1 cells induced by LPS with IC_{50} values of 5.0 and 10.3 μM, respectively. Biochemical characterizations of EI-1941-1 and -2 are described in this article.

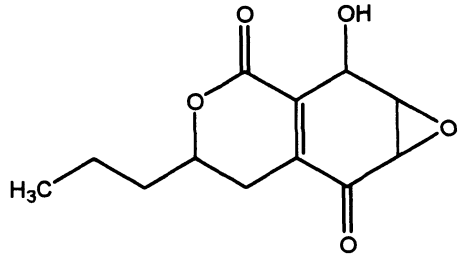
The IL-1β converting enzyme (ICE) is a cysteine protease which cleaves biologically-inactive 31 kDa precursor to biologically-active IL-1β[1,2], a key mediator of inflammation[3,4]. Thus, ICE inhibitors would be useful as anti-inflammatory agents.

To discover novel anti-inflammatory drugs, we initiated a screening program to obtain ICE inhibitors from microbial sources[5~8]. We isolated novel ICE inhibitory compounds, EI-1941-1 and -2, from culture broths of *Farrowia* sp. strain E-1941 (Fig. 1). In this article, we describe the biochemical characterization of EI-1941-1 and -2. The taxonomy and fermentation of producing strain, the isolation, structure elucidation and biological properties will be described in a separate paper[9].

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



EI-1941-1



EI-1941-2

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Material and Methods

Material

Human recombinant ICE was prepared by heterologous expression in *Escherichia coli* and *in vitro* refolding as described[10]. The enzyme was purified by ion exchange chromatography[10]. Acetyl-Tyr-Val-Ala-Asp-amino-4-methylcoumarin (Ac-Tyr-Val-Ala-Asp-AMC)[1], a fluorescent substrate was purchased from Peptide Institute, Inc., Osaka, Japan. All other chemicals were of analytical grade.

Determination of Stability of EI-1941s by HPLC

HPLC analysis was performed on a ODS-AQ-312 (6 mm i.d.×150 mm, YMC Co., Ltd.). The column was eluted with 25% aqueous acetonitrile (EI-1941-1) or 30% aqueous acetonitrile (EI-1941-2), at a flow rate of 1 ml per minute. The effluent was monitored at a wavelength of 220 nm.

Assay of ICE Activity

ICE activities were measured as described by THORNBERRY *et al.*[1] 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.

Assay of Cathepsin B and Elastase Activities

The enzymatic activities of cathepsin B and elastase were assayed according to the method of BARRETT & KIRSCHKE[11] and that of MUMFORD *et al.*[12], respectively.

Measurement of Interleukin-1β Secretion

THP-1 cells were suspended in RPMI1640 medium (Nissui Pharmaceutical Co., Ltd.) supplemented with 10% fetal bovine serum, and were distributed into 24-well plates as inocula of 1×10^5 cells/well. The cells were differentiated with phorbol-12-myristate-13-acetate (PMA: 30 nM) for 72 hours. After the plates were rinsed with serum-free RPMI1640 medium to remove unadherent cells, adherent cells were stimulated with lipopolysaccharide (LPS: 25 μg/ml) for 4 hours in the presence of various concentrations of EI-1941-1 or -2. The culture media were harvested, and mature IL-1β was measured by an ELISA method using IL-1β assay kit (Amersham). RPMI1640 medium without cysteine was prepared using select amine kit (Gibco).

Detection of Cell Survival

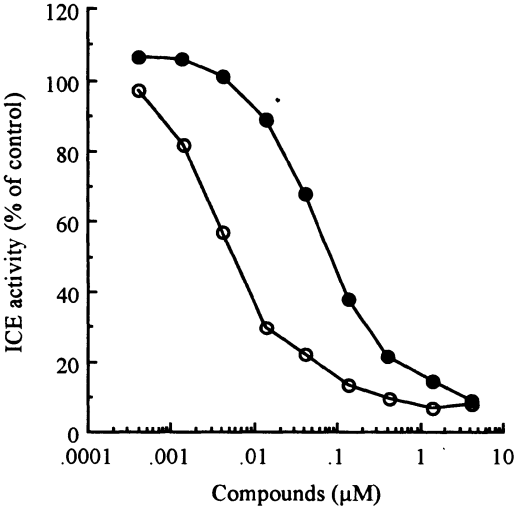
The cytotoxicities of EI-1941-1 and -2 against THP-1 cells were examined by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) method[13]. MTT solution (10 μl/well; final concentration 1 mg/ml) was added to the cells at the time of EI-1941's application. The culture medium was removed after 4 hours incubation, and dimethyl sulfoxide (50 μl/well) was added to dissolve formazan. The absorbance of formazan in DMSO solution was measured at 560 nm.

Results

Inhibition of ICE

EI-1941-1 and -2 inhibited the enzymatic activities of human recombinant ICE in a dose-dependent manner (Fig. 2); IC_{50} values were calculated to be 0.086 and 0.006 μM, respectively. The enzyme specificity of EI-1941-1 and -2 was examined by testing for inhibition of cathepsin B (another thiol-containing protease) and elastase. EI-1941-1 and -2 were inactive against these two enzymes at

Fig. 2. Inhibition of ICE by EI-1941-1 and -2.



The assay mixture was incubated for 2 hours at room temperature after which the fluorescent intensity (excitation-wave length: 370 nm, emission-wave length: 440 nm) of the assay mixtures was measured. Symbols indicate EI-1941-1 (●) and EI-1941-2 (○).

Table 1. Effect of EI-1941-1 and -2 on various enzyme activities.

Enzyme	IC_{50} value (μM)	
	EI-1941-1	EI-1941-2
ICE	0.086	0.006
Elastase	>40	>40
Cathepsin B	>40	>40

concentrations up to 40 μM. These data showed the selective inhibitory activities of EI-1941-1 and -2 against ICE and are summarized in Table 1.

Effect of DTT on ICE Inhibitory Activities of EI-1941-1 and -2

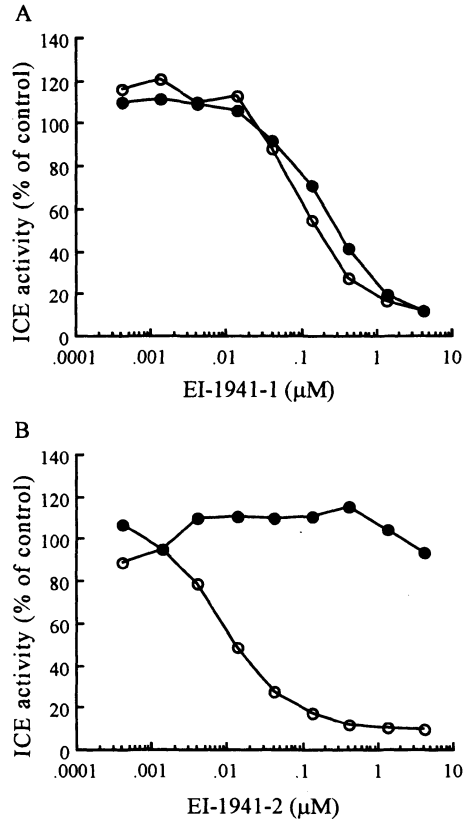
EI-1941-2 inhibited the ICE activity in the absence of DTT as described above. The addition of 1 mM DTT to the reaction mixture completely reduced the ICE inhibitory activity of EI-1941-2. On the other hand, the ICE inhibitory activity of EI-1941-1 was not affected by the presence of 1 mM DTT (Fig. 3).

Stability of EI-1941-1 and -2 in DTT, Cysteine, and Glutathione

Since the ICE inhibitory activity of EI-1941-2 was decreased in the presence of DTT, the stability of these compounds in the presence or absence of DTT was examined by detecting the amount of EI-1941-1 and -2 by HPLC analysis (Fig. 4A). EI-1941-1 and -2 were incubated in a sodium phosphate buffer, pH 6.8 containing various concentrations of DTT for 2 hours at room temperature. The amount of EI-1941-2 decreased by 33% in the presence of 0.01 mM DTT, and completely disappeared in the presence of 0.1 and 1 mM DTT. In contrast, the amount of EI-1941-1 did not decrease in the presence of 0.1 mM DTT, but did in the presence of 1 mM DTT.

Next, we examined the stability of these compounds in the presence of 0.4 mM cysteine or 0.3 mM glutathione which are included in RPMI1640 medium (Fig. 4B). The amount of EI-1941-1 and -2 decreased in phosphate-buffered saline containing 0.4 mM cysteine but neither of the compounds were affected when incubated in phosphate-buffered saline containing 0.3 mM glutathione.

Fig. 3. Effect of DTT on ICE inhibitory activity of EI-1941-1 and -2.



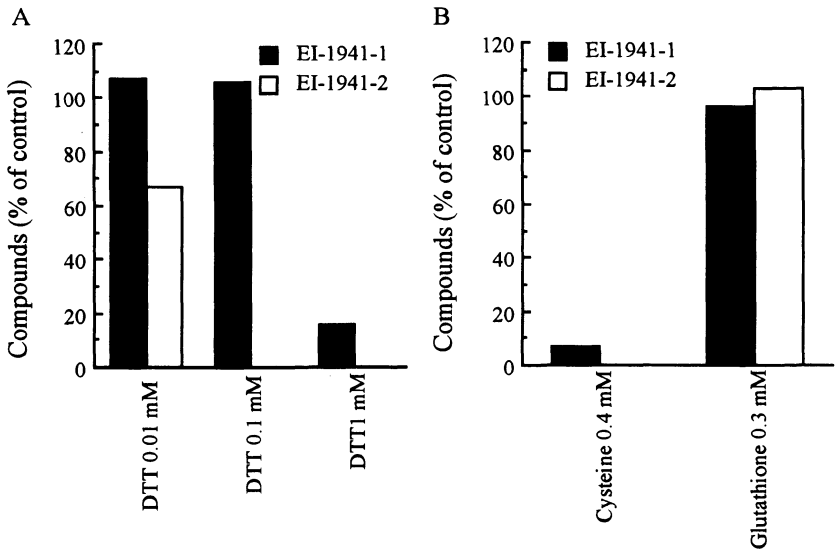
The assay mixture was incubated for 2 hours at room temperature in the presence of 1 mM DTT and various concentration of either EI-1941-1 (A) or -2 (B). Fluorescent intensity of the assay mixtures was measured. Symbols indicate control (○) and 1 mM DTT (●).

Since the amount of EI-1941-1 and -2 was decreased by cysteine, we examined the stability of these compounds in the cysteine-free and cysteine-containing RPMI1640 media. The amount of both EI-1941-1 and -2 decreased in RPMI1640 medium containing cysteine. On the other hand, neither of the compounds was decreased in cysteine-free medium (Fig. 5).

Biological Properties

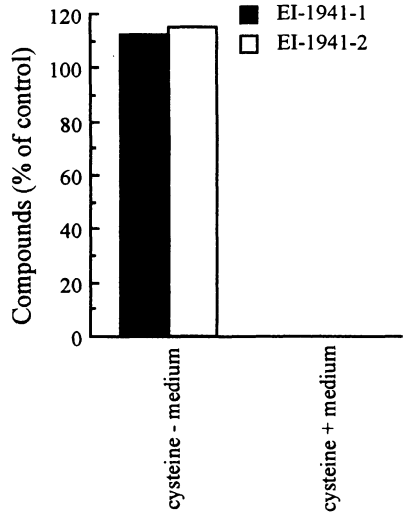
In order to determine whether EI-1941-1 and -2 were efficacious in intact cells, we investigated the effects of EI-1941-1 and -2 on extracellular release of IL-1β from THP-1

Fig. 4. Stability of EI-1941-1 and -2 in the presence of thiol compounds.



10 μg/ml of EI-1941-1 and -2 were incubated for 2 hours at room temperature in a sodium phosphate buffer, pH 6.8 containing various concentration of DTT (A). 10 μg/ml of EI-1941-1 and -2 were incubated for 2 hours at room temperature in phosphate-bufferd saline containing 0.4 mM cysteine and 0.3 mM glutathione (B). EI-1941-1 and -2 were analyzed by HPLC, and the concentration of the compounds were determined from their peak area.

Fig. 5. Stability of EI-1941-1 and -2 in the cysteine-free or cysteine-containing RPMI1640 medium.

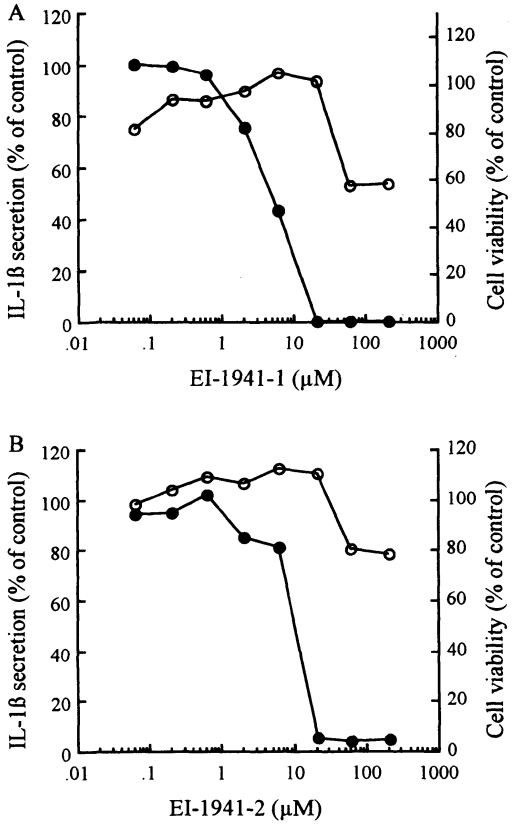


10 μg/ml of EI-1941-1 and -2 were incubated for 2 hours at room temperature in RPMI1640 medium in the presence (cysteine+medium) or absence (cysteine−medium) of cysteine. EI-1941-1 and -2 were determined by HPLC analysis.

cells. EI-1941-1 and -2 inhibited IL-1β secretion in a dose-dependent manner (Fig. 6); IC_{50} values of EI-1941-1 and -2 were calculated to be 5.0 and 10.3 μM, respectively. On the other hand, these inhibitors did not affect the cell survival at 20 μM and lower concentrations (as shown in Fig. 6). However, these inhibitors slightly affected the cell survival at 60 and 200 μM. EI-1941-1 and -2 inhibited IL-1β secretion from THP-1 cells induced by LPS in the concentration ranges where cell viability was not affected.

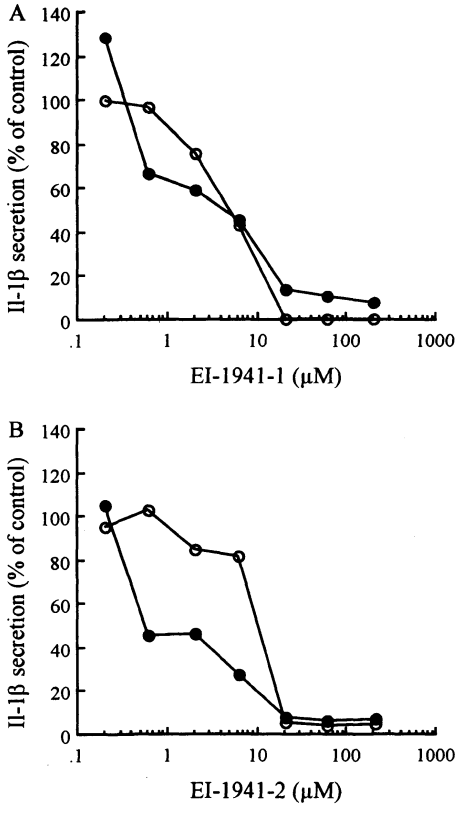
It was speculated that since the amount of EI-1941-1 and -2 decreased in the presence of cysteine, these inhibitors did not inhibit the IL-1β secretion in the concentration range where they inhibited recombinant ICE. In order to obtain evidence for this speculation, we examined whether EI-1941-1 and -2 inhibited IL-1β secretion from THP-1 cells induced by LPS under the conditions where cysteine was removed from the culture medium. Removal of cysteine from the culture medium shifted inhibition curves of EI-1941-2 to the left, but did not shift that of EI-1941-1 (Fig. 7). However, the concentrations needed to inhibit IL-1β secretion from THP-1 cells were around 10 times higher than that needed to inhibit the isolated enzyme in a cell free system, even in the absence of cysteine.

Fig. 6. Effect of EI-1941-1 and -2 on IL-1β secretion from LPS-stimulated THP-1 cells and on cell viabilities of THP-1 cells.



Symbols indicate IL-1β secretion (●) and percentage of viable cells (○).

Fig. 7. Effect of EI-1941-1 and -2 on IL-1β secretion from LPS-stimulated THP-1 cells in the cysteine-free or cysteine-containing RPMI1640 medium.



Symbols indicate IL-1β secretion from THP-1 cells in the absence of cysteine (●) and in the presence of cysteine (○).

Discussion

EI-1941-1 and -2 inhibited human recombinant ICE in a dose-dependent fashion. The ICE inhibitory activities of these compounds were potent and selective in cell free systems as shown in Table 1.

We previously reported that manumycin-related compounds containing epoxy structure in their molecules failed to inhibit ICE in the presence of DTT[8]. Although both EI-1941-1 and -2 have an epoxy structure in their molecules, EI-1941-1 inhibited ICE activity in the presence of DTT, while EI-1941-2 did not. To clarify this difference, we examined the stability of these compounds in a DTT containing buffer. EI-1941-2 disappeared completely after 2-hour incubation in the presence of 0.1 mM DTT at room temperature, whereas EI-1941-1 did not decrease under the same conditions. The DTT concentration needed to decrease EI-1941-1 was higher than that needed to decrease EI-1941-2, indicating that EI-1941-1 is more resistant to DTT than EI-1941-2. This may explain that EI-1941-1 inhibited ICE in the presence of DTT, and that EI-1941-2 did not. Although even 1 mM DTT did not affect the inhibition of ICE by EI-1941-1, the amount of EI-1941-1 decreased by 84% in the presence of such a high concentration of DTT. This may be due to the difference in assay mixtures of the stability test and the activity test. However, we could not exclude the possibility that degradation product of EI-1941-1 inhibits ICE activity.

The inhibition of IL-1β secretion by EI-1941-1 and -2 (IC_{50} values of 5.0 and 10.3 μM, respectively) was not due to toxic effects of the compounds as shown in Fig. 6. EI-1941-1 inhibited IL-1β secretion from THP-1 cells in the

absence of cysteine at almost the same concentration range it inhibited in the presence of cysteine. On the other hand, removal of cysteine from the culture medium shifted the inhibition curve of EI-1941-2 to the left. These results indicate that EI-1941-2 is labile to cysteine even in the cell system, whereas EI-1941-1 is not. The difference of lability of these compounds to SH-containing reagents in the cell system corresponds with that in the cell free system. These results suggest that their inhibition of IL-1β secretion in the THP-1 cells is due to ICE inhibition. However, the concentration ranges of these compounds needed to inhibit IL-1β secretion in the absence of cysteine were higher than that needed to inhibit human recombinant ICE in a cell-free system. One possibility of the less effectiveness in the cell system is that the inhibitors do not completely penetrate the plasma membrane of THP-1 cells to reach intracellular ICE. Another possibility is that the compounds reacted with and are inactivated by the thiol-containing amino acid residues of proteins in THP-1 cells. However, the latter possibility may not be the case in EI-1941-1, since EI-1941-1 is resistant to the reaction with thiol reagents, such as DTT and cysteine. Alternatively, it is possible that EI-1941-1 and -2 may inhibit the activation of NF-κB, since cycloepoxydon[14,15], which structure was close to that of EI-1941-1 and -2, inhibited the activation of NF-κB, a key transcription factor regulating the expression of inflammatory cytokines such as IL-1β and TNFα[16].

In this paper, we described that EI-1941-1 and -2 inhibited human recombinant ICE together with IL-1β secretion from THP-1 cells induced by LPS. EI-1941-1 and -2 would be another sets of useful tools to elucidate the physiological and pathological roles of the ICE and could be lead compounds to synthesize selective ICE inhibitors.

Acknowledgment

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References

1) 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
2) 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
3) DINARELLO, C. A.: Interleukin-1 and interleukin-1 antagonism. Blood 77: 1627~1652, 1991
4) DINARELLO, C. A. & S. M. WOLFF: The role of interleukin-1 in disease. N. Engl. J. Med. 328: 106~113, 1993
5) 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
6) 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
7) 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
8) 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
9) KOIZUMI, F.; H. ISHIGURO, K. ANDO, H. KONDO, M. YOSHIDA, Y. MATSUDA & S. NAKANISHI: J. Antibiotics, in preparation
10) 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
11) BARRETT, A. J. & H. KIRSCHKE: Cathepsin B, cathepsin H and cathepsin L. *In* Methods in Enzymol. Vol. 80 Proteolytic enzymes. Part C. *Ed.*, L. LORAND, pp. 535~561, Academic Press, New York, 1981
12) MUMFORD, R. A.; A. W. STRAUSS, J. C. POWERS, P. A. PIERZCHALA, N. NISHINO & M. MIMMERMAN: A zinc methalloendopeptidase associated with dog pancreatic membranes. J. Biol. Chem. 255: 2227~2230, 1980
13) MOSMANN, T.: Colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J. Immnol. Methods 65: 55~63, 1983
14) GEHRT, A.; G. ERKEL, H. ANKE, T. ANKE & O. STERNER: New hexaketide inhibitors of eukaryotic signal transduction. Natl. Prod. Lett. 9: 259~264, 1997
15) 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
16) BAEUERLE, P. A. & T. HENKEL: Function and activation of NF-κB in the immune system. Annu. Rev. Immunol. 12: 141~179, 1994