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EI-2128-1, a Novel Interleukin-1β Converting Enzyme Inhibitor Produced by *Penicillium* sp. E-2128

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EI-2128-1, a novel interleukin-1β converting enzyme (ICE) inhibitor, was isolated from the culture broths of *Penicillium* sp. E-2128. EI-2128-1 selectively inhibited human recombinant ICE activity with IC_{50} value of 0.59 μM, without inhibiting elastase and cathepsin B. EI-2128-1 also inhibited mature interleukin-1β secretion from THP-1 cells induced by LPS with IC_{50} value of 0.28 μM.

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[5~9]. We isolated novel ICE inhibitory compound, EI-2128-1, from culture broths of *Penicillium* sp. strain E-2128. In this article, we describe the taxonomy and fermentation of the producing strain and isolation, structure elucidation, and biological properties of EI-2128-1.

Material and Methods

Material

Human recombinant ICE was prepared by heterologous expression of p10 and p20 subunits 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.

Taxonomy

The producing strain E-2128 was isolated from a soil sample. E-2128 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 14 days. Morphological observations of strain E-2128 grown on potato-dextrose agar for 7 days at 25°C were ascertained by light microscopy. The temperature 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-2128, grown on an agar slant, was inoculated into each of three 50-ml test tubes containing 10 ml of a seed medium composed of dried mashed potatoes 3%, glucose 10% and yeast extract 0.5%

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in deionized water (pH 7.2 before sterilization). The inoculated test tubes were incubated on a reciprocating shaker for 4 days at 25°C. Five ml of the seed culture was added to a 300-ml Erlenmeyer flask containing 50 ml of the same medium. Four inoculated flasks were incubated for 2 days on a rotary shaker (200 rpm) at 25°C. Five ml of the seed culture was transferred into a 300-ml Erlenmeyer flask containing 50 ml of the fermentation medium composed of glucose 1%, soluble starch 1%, corn starch 1%, corn steep liquor 0.5%, Pharmamedia (IWAKI) 0.5%, dried yeast 0.5%, and calcium carbonate 0.2% in deionized water (pH 6.0 before sterilization). The fermentation was carried out for 6 days at 25°C with agitation of 200 rpm.

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. The change in fluorescent intensity of liberating 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 and 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-2128-1. The culture media were harvested, and mature IL-1β was measured by an ELISA method using IL-1β assay kit (Amersham).

Detection of Cell Survival

The cytotoxicity of EI-2128-1 against THP-1 cells was examined by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) method[13]. THP-1 cells were suspended in RPMI1640 medium (Nissui Pharmaceutical Co., Ltd.) supplemented with 10% fetal bovine serum, and were distributed into 96-well plates as inocula of 4×10^4 cells/well. The cells were differentiated with phorbol-12-myristate-13-acetate (PMA: 30 nM) for 72 hours. After cells were stimulated with LPS (25 μg/ml) for 3 hours in the presence of various concentrations of EI-2128-1, MTT solution (10 μl/well; final concentration 1 mg/ml) was added to the cells and cells were incubated for 1 hour. The culture medium was removed, and dimethyl sulfoxide (50 μl/well) was added to dissolve formazan. The absorbance of formazan in DMSO solution was measured at 560 nm.

Results

Characterization of the Producing Strain E-2128

Colonies on malt extract agar are about 60 mm in diameter after culturing for 14 days at 25°C. The color of the colony is dark greenish blue with tint of gray and is white to pale blue at the periphery. The color of the reverse of the colony is pale yellowish white and is cream at the periphery and the center. Colonies on potato - dextrose agar are about 70 to 75 mm in diameter after culturing for 14 days at 25°C. The color of the colony is grayish olive, and the color of the reverse of the colony is dark reddish brown. The growth temperature range for this strain is 6~38.5°C and the optimum growth temperature is about 22°C.

Hyphae are septate, smooth, and colorless to pale yellowish brown, and branch well. Conidiophores are mononematous, smooth, and colorless to pale yellowish brown; they are 1.8~3.5 μm wide and sometimes as long as 160 μm. Penicilli are irregular in shape and size, but many of them are biverticillate-asymmetrica. At the tip of the penicillus, three to four metulae are formed. The metula is cuneiform or rectangular and is 6.5~13.5 μm long and 1.5~3.5 μm wide. Three to six phialides are formed at the tip of the metula. The phialide is lecythiform or lageniform and is 5.5~9.5 μm long and 2~3 μm wide at the widest part and 1.2 μm wide at the tip. The mode of ontogeny of conidia is enteroblastic, and the conidia are formed from the tip of the phialide in catenation. Phialoconidium is single cell type, smooth, globose or subglobose, and 2.5~4.3 μm in diameter; it is colorless to light brown but appears olive brown in a mass. In this strain, the foregoing anamorph alone is observed and no teleomorph is observed.

On the basis of the above mycological properties, the strain was classified as *Penicillium* sp. The strain has been

deposited at the National Institute of Bioscience and Human-Technology, Agency of industrial Science and Technology, Tsukubashi, Ibaraki, Japan, as *Penicillium* sp. E-2128 with the accession number FERM BP-5488.

Production of EI-2128-1 by Fermentation

E-2128 was cultured in 300-ml Erlenmeyer flasks containing the fermentation medium described in Materials and Methods. The production of active materials in the culture broth initiated on day 3, and the amount of active materials reached maximum on day 6. Active materials were produced mainly in mycelia.

Isolation and Purification

The isolation procedure for the EI-2128-1 is shown schematically in Fig. 1. The producing strain E-2128 was cultured according to the method described in the Materials and Methods. EI-2128-1 was purified from mycelia obtained by filtration of fermentation broth. The mycelia cake was extracted with 2 liters of methanol. The extract was diluted with 8 liters of deionized water and applied on a Diaion HP-20 column (400 ml). After washing the column with 50% aqueous methanol (1.6 liters), the active principles were eluted from the column with methanol (1.6 liters). Active fractions containing EI-2128-1 were combined and diluted with an equal amount of deionized water. This aqueous solution was applied onto an ODS gel column (ODS-AQ-S50, 353 ml) equilibrated with 50% methanol, and eluted with 75% methanol. Active fractions containing EI-2128-1 were pooled and concentrated *in vacuo* to yield crude EI-2128-1 (193 mg). This crude EI-2128-1 was dissolved in 19 ml of methanol and purified by preparative HPLC using a column (D-ODS-5-B S-5 120A, YMC) with 80% methanol as elution solvent. Active fractions containing EI-2128-1 were evaporated, and EI-2128-1 (107.6 mg) was obtained as white powder.

Fig. 1. Purification procedure for EI-2128-1.

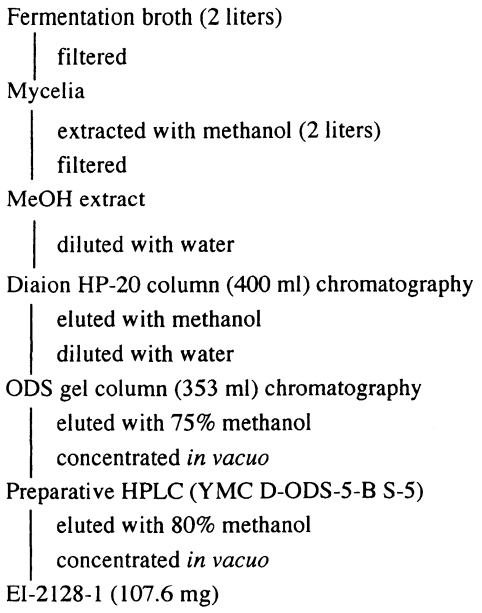


Table 1. Physico-chemical properties of EI-2128-1.

Appearance	White powder
Optical rotation	$[\alpha]_D^{27}$ +13.9° c 0.17, $CHCl_3$
Molecular formula	$C_{23}H_{35}NO_6$
HR FAB-MS Found.	422.2538 $(M+H)^+$
Calcd.	422.2542
UV λ_{max} (MeOH) (ε)	220 (sh)
IR ν_{max} (KBr)	3390, 1668, 1635, 1539, 984, 930
TLC (Rf)	
$CHCl_3$-MeOH (85 :15)[a]	0.71
Color reaction	
Positive	I_2, H_2SO_4
Solubility	
Soluble	Methanol, acetonitrile, and chloroform

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

Table 2. NMR spectral data for EI-2128-1.

No.	δC[a] (ppm)	δH[b] (ppm, multi-, *J* in Hz)
2	96.6	5.64, d, *J*=4.6
2-OH		4.75, m
3	52.0	4.75, m
3-NH		6.09, d, *J*=8.1
4	36.0	2.05, dd, *J*=10.5, 13.1
		2.63, dd, *J*=8.3, 13.1
5	79.1	
6	64.3	3.69, dd, *J*=3.7, 3.9
7	55.9	3.42, dd, *J*=2.7, 3.9
8	198.3	
9	55.6	3.46, dd, *J*=2.7, 3.9
10	62.9	3.57, dd, *J*=3.7, 3.9
1'	166.2	
2'	121.1	5.77, d, *J*=15.4
3'	151.9	6.74, dd, *J*=8.3, 15.4
4'	34.2	2.40, m
4'-CH_3	20.5	1.03, d, *J*=6.8
5'	43.9	1.12, m
		1.37, m
6'	30.4	1.39, m
6'-CH_3	19.5	0.84, d, *J*=6.6
7'	37.4	1.08, m
		1.25, m
8'	26.8	1.25, m
9'	29.7	1.25, m
10'	31.9	1.25, m
11'	22.7	1.25, m
12'	14.1	0.88, t, *J*=6.8

[a] ^{13}C NMR spectra were recorded at 100 MHz in $CDCl_3$.
[b] ^{1}H NMR spectra were recorded at 400 MHz in $CDCl_3$.

Physico-chemical Properties and Structure Elucidation

Physico-chemical properties of EI-2128-1 are summarized in Table 1. EI-2128-1 was readily soluble in methanol, acetonitrile, and chloroform. The molecular formula of EI-2128-1 was determined by the high resolution FAB-MS to be $C_{23}H_{35}NO_6$. The ^{13}C NMR spectrum (Table 2) showed 23 carbon signals which were assigned to seven methines, seven methylenes, three methyls, one quaternary (δ 43.9), one acetal (δ 96.6), two olefinic (δ 121.1, δ 151.9), and two carbonyl (δ 166.2, δ 198.3) carbons. ^{1}H-^{1}H COSY and HMBC data revealed the structure of the acyl side chain as shown in Figure 2. HMBC spectrum exhibited long-range correlation from NH (δ 6.09) to C1′ (δ 166.2), indicating the presence of an amide bond in the side chain. ^{1}H-^{1}H COSY and HMBC data showed connectivity from C2 to C4 and HMBC spectrum exhibited long-range correlation from H-2 hemiacetal proton (δ 5.64) to C5 (δ 79.1) and from H-4 methylene proton (δ 2.05, δ 2.63) to C5. These results indicated the presence of a 5-membered lactol ring system. ^{1}H-^{1}H COSY and HMBC data showed the connectivity of the acyl side chain to the lactol ring (C3-N-C1′-C2′) which was also supported by the NOESY data correlation between 3-NH and H-4. The remaining unaccounted ^{13}C signals were one carbonyl and four oxymethine carbons. Four oxymethine proton signals, $\delta_{H\text{-}6}$ 3.69, $\delta_{H\text{-}7}$ 3.42, $\delta_{H\text{-}10}$ 3.57 and $\delta_{H\text{-}9}$ 3.46, were coupled with the remaining two oxymethine proton ($J_{H6\text{-}H7}$=3.9 Hz, $J_{H6\text{-}H10}$=3.7 Hz, $J_{H7\text{-}H9}$= 2.7 Hz, and $J_{H9\text{-}H10}$=3.9 Hz). These long range coupling (H-7 and H-9, H-6 and H-10) and ^{13}C chemical shift of C6 (δ 64.3), C7 (δ 55.9), C9 (δ 55.6), and C10 (δ 62.9) indicated these carbons to be attached to oxygen atom forming two epoxide groups. Detailed analysis of COSY and HMBC data revealed the presence of cyclohexanone bisepoxide spiro lactol system as shown in Fig. 2. ^{1}H and ^{13}C NMR spectrum of EI-2128-1 were similar to those reported for the known fungal metabolite, aranorosin[14~17]. Upon comparison of these data (chemical shifts of ^{1}H and ^{13}C and ^{1}H-^{1}H coupling patterns) with those of aranorosin, cyclohexanone bisepoxide spiro lactol system, amide group, and alkyl moiety (–CH(CH_3)–CH_2–CH_2–CH_2–CH_2–CH_2–CH_3) were found as common partial structure. However, significant difference between EI-2128-1 and aranorosin were observed at H-4′ (EI-2128-1: 2.40 ppm; aranorosin: not observed) and H-5′ (EI-2128-1: 1.12 and 1.37 ppm; aranorosin: 5.67 ppm), indicating EI-2128-1 should be the corresponding 4′-5′-dihydro derivative of aranorosin.

The relative stereochemistry of the spiro ring system was established by the NOESY experiment. Cross peaks were observed between H-6 and H-7, H-9 and H-10, H-6 and H_b-4, H_b-4 and N-H, H-10 and H_a-4, H-10 and H-3, H-2 and H-3, H-2 and H_a-4, and H_a-4 and H-3, respectively. These observations indicated that H-2, H-3, and H_a-4 are on the same face, and the two epoxide groups and lactol ring oxygen were on the same orientation. The relative stereochemistry of the spiro ring system of EI-2128-1 were same as aranorosin. The total synthesis of aranorosin[17] revealed the absolute as well as the relative configuration of the natural product, including C6′ configuration. Stereochemical studies of C4′ and C5′ configuration of EI-2128-1 are now in progress.

Fig. 2. Summary of COSY, HMBC, and NOESY data for EI-2128-1.

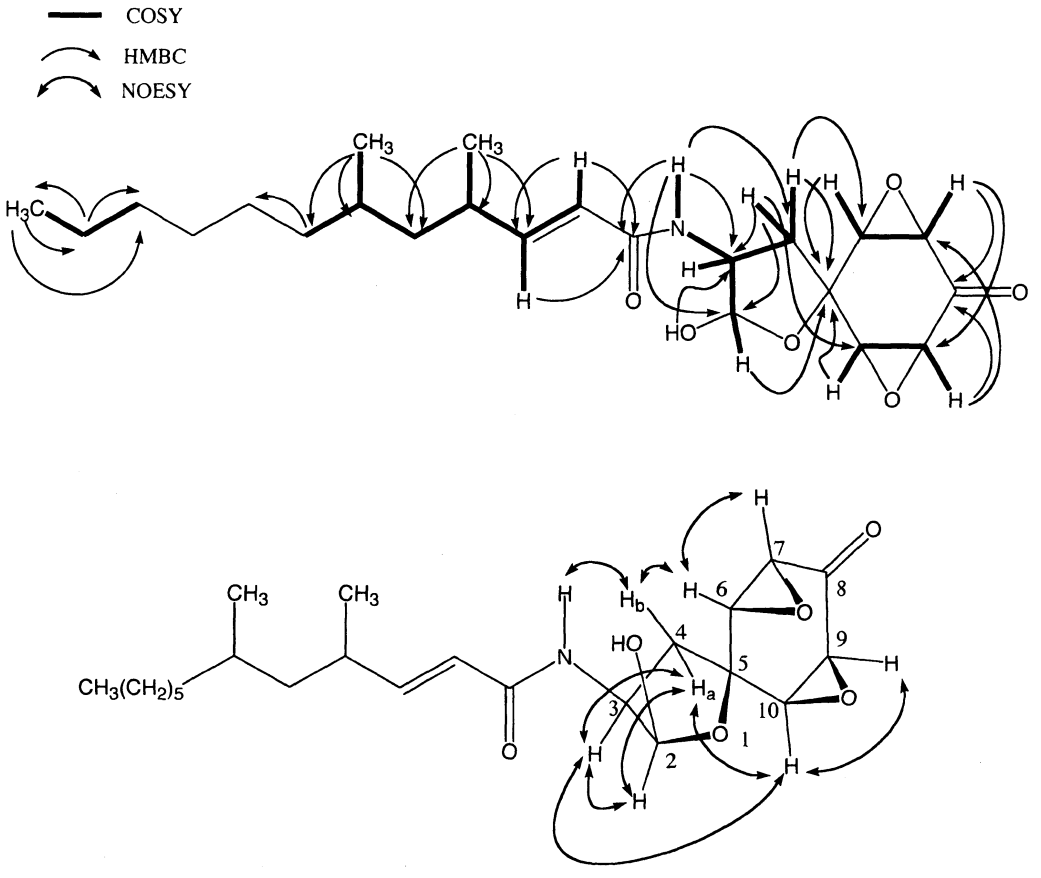
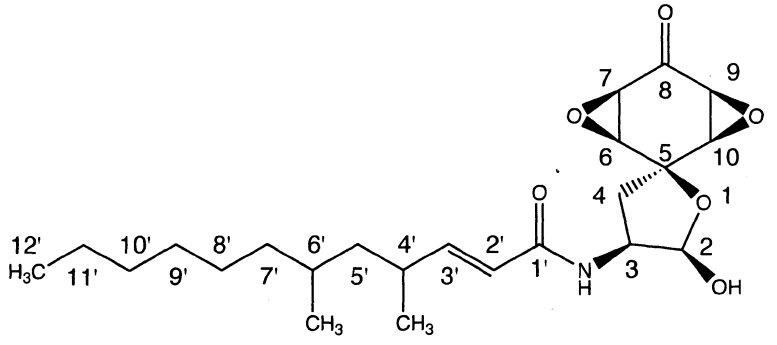


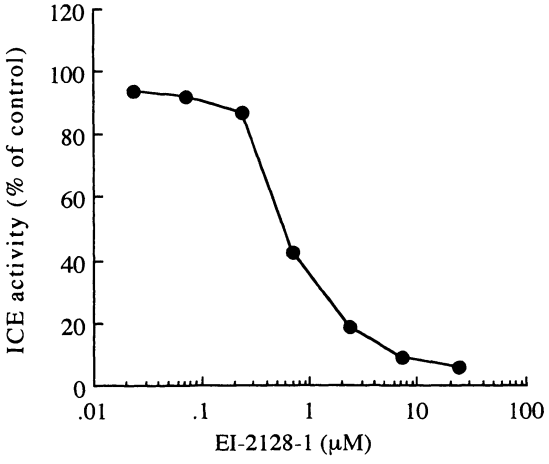
Fig. 3. Structure of EI-2128-1.



Inhibition of ICE

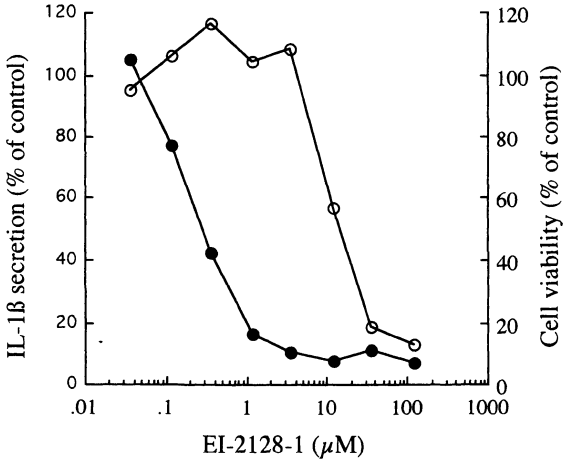
EI-2128-1 inhibited the enzymatic activities of human recombinant ICE in a concentration-dependent manner; IC_{50} value was calculated to be 0.59 μM (Fig. 4). The enzyme specificity of EI-2128-1 was examined by testing for inhibition of cathepsin B (another cysteine protease) and elastase. EI-2128-1 was inactive against these two enzymes at concentrations up to 24 μM. These data showed the selective inhibitory activities of EI-2128-1 against ICE and are summarized in Table 3.

Fig. 4. Inhibition of ICE by EI-2128-1.



The assay mixture was incubated for 2 hours at room temperature. Then the fluorescent intensity (excitation-wave length: 370 nm, emission-wave length: 440 nm) of the assay mixtures was measured. Symbols indicate the ICE inhibition %.

Fig. 5. Effect of EI-2128-1 on IL-1β secretion from LPS-stimulated THP-1 cells and on cell viabilities of THP-1 cells.



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

Table 3. Effect of EI-2128-1 on various enzyme activities.

Enzyme	IC_{50} value (μM)
ICE	0.59
Elastase	>24
Cathepsin B	>24

Biological Properties

We determined ICE inhibitory activity of EI-2128-1 in intact cells by testing its effect on the extracellular release of IL-1β from THP-1 cells. EI-2128-1 inhibited IL-1β secretion in a concentration-dependent manner (Fig. 5); IC_{50} value of EI-2128-1 was calculated to be 0.28 μM. This inhibitor affect the cell survival with IC_{50} value of 14.5 μM (as shown in Fig. 5). However, cell viability at a concentration of 3.6 μM, at which EI-2128-1 completely inhibited IL-1β secretion from THP-1 cells were more than 90%, showing that EI-2128-1 inhibited IL-1β secretion from THP-1 cells induced by LPS in the concentration ranges where cell viability was not affected. These data indicated that EI-2128-1 inhibited mature IL-1β secretion from THP-1 cells without cell toxicity at concentrations lower than 12 μM.

EI-2128-1 had strong antimicrobial activities against *Enterococcus hirae*, *Staphylococcus aureus* subsp. *aureus*, and *Bacillus subtilis*. MIC values were 0.78 μM, for both strains (Table 4). EI-2128-1 had weak antimicrobial activities against *Candida albicans*, *Proteus vulgaris*, and *Klebsiella pneumoniae* subsp. *pneumoniae*. MIC values were 100, 100, and 50 μM, respectively (Table 4).

Discussion

We have isolated a novel cyclohexanone bisepoxide-related compound, termed EI-2128-1, from the culture broths of *Penicillium* strain E-2128, and demonstrated that this compound is an ICE inhibitor. EI-2128-1 is a new compound, the structure of which is close to that of aranorosin. It has been reported that aranorosin showed both antibacterial and antifungal activities[14]. However, the ICE inhibitory acitivities of aranorosin have not yet been reported. EI-2128-1 has a cyclohexanone bisepoxide structure in its molecule. Several natural compounds from microbial sources have been reported as ICE inhibitors[5~9,18]. Among them, EI-1507s, EI-1511s, EI-1941s and pentenocin A have epoxy structures[5,6,9,18]. Thus,

Table 4. The antibiotic activities of EI-2128-1.

	Strains	MIC (μM)
Gram-positive bacteria	*Bacillus subtilis* No. 10707	0.78
	Staphylococcus aureus subsp. *aureus* ATCC6538P	0.78
	Enterococcus hirae ATCC10541	0.78
Gram-negative bacteria	*Escherichia coli* ATCC26	>100
	Klebsiella pneumoniae subsp. *pneumonie* ATCC10031	50
	Pseudomonas aeruginosa BHM No.1	>100
	Proteus vulgaris ATCC6897	100
	Shigella sonnei ATCC9290	>100
Yeast	*Candida albicans* ATCC10231	100

the epoxy structure of EI-2128-1 might play an important role to inhibit ICE activity.

We have demonstrated that EI-2128-1 inhibited the enzymatic activity of ICE, without inhibiting cathepsine B and elastase, showing ICE selective inhibition. We demonstrated also that EI-2128-1 inhibited IL-1β secretion from LPS-induced THP-1 cells. This inhibition was not due to toxic effects of EI-2128-1 as shown in Fig. 5. These results suggest that inhibition of IL-1β secretion from THP-1 cells could be due to ICE inhibition. EI-2128-1 had strong antimicrobial activities against *Enterococcus hirae*, *Staphylococcus aureus* subsp. *aureus*, and *Bacillus subtilis*. Aranorosin also had strong antimicrobical activities against *Staphylococcus aureus* and *Bacillus subtilis*. Among ICE inhibitors from microbial sources, EI-1507-2, EI-1511s and EI-1625-2 also had weak antimicrobial activities against Gram-positive bacteria.

In this paper, we described that EI-2128-1 inhibited human recombinant ICE together with IL-1β secretion from THP-1 cells induced by LPS. EI-2128-1 would be a useful tool to understand the physiological and pathophysiological roles of ICE.

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