EI-1507-1 and -2, Novel Interleukin-1β Converting Enzyme Inhibitors Produced by *Streptomyces* sp. E-1507

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EI-1507-1 and -2, novel interleukin-1β converting enzyme (ICE) inhibitors, were isolated from the culture broths of *Streptomyces* sp. E-1507. EI-1507-1 and EI-1507-2 selectively inhibited the recombinant human ICE activity with IC_{50} values of 0.23 and 0.42 μM, respectively. EI-1507-1 and EI-1507-2 also inhibited mature interleukin-1β secretion from THP-1 cells with IC_{50} values of 1.1 and 1.4 μM, respectively.

The cytokine interleukin-1 (IL-1) has been implicated in rheumatoid arthritis, septic shock, inflammation and other physiological situations[1,2]. Two different IL-1 gene sequences (IL-1α and IL-1β) have been described[3,4]. Interleukin-1β converting enzyme (ICE) is a unique cysteine-containing heterodimeric protease which cleaves the inactive precursor of IL-1β into biologically active IL-1β[5,6]. IL-1β is released from macrophage-like cells following inflammatory stimulation, and is the major form of IL-1 in diseases. ICE inhibitors therefore might be useful as anti-inflammatory agents[7].

During the course of our screening to obtain ICE inhibitors from microorganisms, we found metabolites of *Streptomyces* sp. E-1507 which inhibited ICE. The compounds, EI-1507-1 and -2, were isolated from culture broths. In this article, we describe the taxonomy and fermentation of the producing strain, as well as the isolation, chemical structures and some biological properties of EI-1507-1 and -2.

Materials and Methods

Materials

Recombinant human ICE was prepared by heterologous expression in *Escherichia coli* and *in vitro* refolding as described[8]. The enzyme was purified by ion exchange chromatography[8]. Acetyl-Tyr-Val-Ala-Asp-amino-4-methylcoumarin (Ac-Tyr-Val-Ala-Asp-AMC)[5], fluorescent substrate, was purchased from Peptide Institute, Inc., Osaka, Japan. All other chemicals were of analytical grade.

Microorganism

The producing strain E-1507 was isolated from soil collected in Yamagata prefecture, Japan. The strain has been deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Tsukubashi, Ibaraki, Japan, as *Streptomyces* sp. E-1507 with the accession number FERM BP-4623.

Taxonomical Characterization

Cultural and physiological characteristics of strain E-1507 were determined by the methods of the International *Streptomyces* Project[9] (ISP). Color codes were assigned to the substrate and aerial mycelial pigments according to the Color Harmony Manual[10]. Morphology of the strain was ascertained by light and scanning electron microscopy (HITACHI S-570). The temperature ranges for growth of the strain were determined after submerged cultivation using ISP No. 5 medium for 7 days. Analysis of diaminopimelic acid was performed on the hydrolysate of aerial mycelia grown on solid ISP No. 4 medium as described previously[11].

Culture and Media Conditions

A loopful of cells from a mature slant culture was inoculated into each of five 50-ml test tubes containing 10 ml of seed medium composed of glucose 1%, soluble starch 1%, yeast extract (Nihon-Seiyaku) 0.5%, Bacto-Tryptone (Difco) 0.5%, beef extract (Kyokuto) 0.3%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05% and $Mg_3(PO4)_2 \cdot 8H_2O$ 0.05% in deionized water (pH adjusted to 7.0 with NaOH before sterilization). The inoculated tubes were incubated on a reciprocating shaker at 28°C for 3 days. Five ml of the seed culture were added to a 300-ml

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Erlenmeyer flask containing 45 ml of the same medium. Eight inoculated flasks were incubated for 2 days on a rotary shaker (200 rpm) at 28°C. Fifty ml of the seed culture were added to a 2-liter Erlenmeyer flask containing 350 ml of the same medium. Six inoculated flasks were incubated for 2 days on a rotary shaker (200 rpm) at 28°C. The 1.8 liter seed culture was transferred into a 30-liter fermentor containing 17 liters of a fermentation medium composed of Diaion HP-20 10% (v/v), soluble starch 4%, soybean meal 1%, cornsteep liquor 0.5%, dry yeast (Asahi brewery) 0.5%, KH_2PO_4 0.05%, $ZnSO_4 \cdot 7H_2O$ 10 μg/ml, $CoCl_2 \cdot 6H_2O$ 1 μg/ml, $NiSO_4$ 1 μg/ml and $Mg_3(PO_4)_2 \cdot 8H_2O$ 0.05% in deionized water (pH adjusted to 7.0 with NaOH before sterilization). The fermentation was carried out for 4 days at 28°C with agitation of 250 rpm and aeration of 18 liters per minute.

Assay of ICE Activity

ICE activities were measured essentially as described by THORNBERRY *et al.*[5]. Assay mixtures (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} IU, test sample solution 1%; final volume 100 μl) were incubated for 2 hours at room temperature. 1 IU was defined as the amount of enzymic activity which produced 1 μmol AMC per minute. Then the change in fluorescent intensity due to liberated AMC (excitation-wave length: 370 nm, emission-wave length: 440 nm) was measured.

Assay of Cathepsin B and Elastase Activities

The enzymic activities of cathepsin B and elastase were assayed according to the methods of BARRETT and KIRSCHKE[12] and MUMFORD *et al.*[13], respectively.

Measurement of Interleukin-1β Secretion

THP-1 (ATCC TIB 202) 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 in a humidified atmosphere of 5% CO_2 in air at 37°C. After the cells were rinsed with serum-free RPMI1640 medium to remove unadherent cells, adherent cells were stimulated for 4 hours with lipopolysaccharide (LPS: 25 μg/ml) containing various concentrations of EI-1507-1 or -2. The culture media were harvested and mature IL-1β was measured by an ELISA method using an IL-1β assay kit (Amersham).

Detection of Cell Survival

The cytotoxicities of EI-1507-1 and -2 against THP-1 cells were examined by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) method[14]. MTT solution (10 μl/well; final concentration 1 mg/ml) was added at the time of EI-1507's application. The culture medium was removed and dimethyl sulfoxide (50 μl/well) was added to dissolve formazan. The absorbance of soluble formazan was then measured.

Analytical Conditions

Melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. Optical rotations were measured on a Jasco DIP-370 digital polarimeter. Low resolution FAB-MS and HRFAB-MS spectra were measured on a JEOL HX110A mass spectrometer using *m*-nitrobenzyl alcohol as a matrix. UV spectra were recorded on a Shimadzu UV-2200 spectrophotometer and IR spectra on a JEOL JIR-RFX3001 FT-IR spectrophotometer. ^1H and ^{13}C NMR spectra were obtained on a Bruker AM500 spectrometer in $CDCl_3$ solution. Chemical shifts are given in ppm using TMS as an internal standard and coupling constants are in Hz.

Results

Characterization of the Producing Strain E-1507

Strain E-1507 grew well or moderately on seven agar media, but not on nutrient agar as shown in Table 1. The color of the aerial mycelia was white to putty on the agar media. Brown or light brown soluble pigment was observed on ISP No. 2, No. 4, No. 5, No. 7 and glucose-asparagine agar media after 14 days (Table 1).

Table 1. Cultural characteristics of strain E-1507.

Medium	Amount of growth	Color of: Aerial mycelium	Substrate mycelium	Soluble pigment
Yeast extract-malt extract agar (ISP No.2)	abundant	White	Yellowmaple	brown
Oatmeal agar (ISP No.3)	abundant	White	Mustard	none
Inorganic salts-starch agar (ISP No.4)	abundant	Putty	Lt Mustard Tan	light brown
Glycerol-asparagine agar (ISP No.5)	abundant	White	Mustard	brown
Tyrosine agar (ISP No.7)	abundant	White	Clove Brown	brown gold
Sucrose-nitrate agar	abundant	Putty	Lt Maize	none
Glucose-asparagine agar	moderate	Putty	Lt Mustard Tan	light brown
Nutrient agar	poor	none	Lt Olive Gray	none

The aerial mycelia were moderately short with simple branches and formed spiral spore chains of 10 or more. The spores were ellipsoidal, smooth and 0.7 to 0.9 μm by 1.0 to 1.2 μm. No fragmentation of substrate mycelia was observed, and sclerotia, sporangia, or flagellated spores were not formed in cultures. The physiological characteristics of strain E-1507 are shown in Table 2. Analysis of cell hydrolysates of the strain revealed that the cell walls contained LL-diaminopimelic acid. These taxonomic observations indicated that strain E-1507 belongs to the genus *Streptomyces*.

Table 2. Physiological properties of strain E-1507.

Characteristics	E-1507
Temperature range for growth	8 ~ 40 °C
Optimum temperature range	25~30 °C
Liquefaction of gelatin	negative
Hydrolysis of starch	positive
Coagulation of milk	negative
Peptonization of milk	positive
Formation of melanin	positive
Utilization of	
D-Glucose	positive
L-Arabinose	positive
D-Xylose	positive
D-Fructose	positive
L-Rhamnose	positive
Sucrose	positive
Raffinose	negative
D-Mannitol	positive
Inositol	positive

Fig. 1. Purification procedure for EI-1507-1 and -2.

Fermentation broth (17liters)
| filtered
Filtrate
| iso-PrOH (10 %) added
Diaion HP-20 column (3 liters) chromatography
| eluted with MeOH : acetone = 7 : 3 solution
| extracted with ethyl acetate
Ethyl acetate extract
| concd *in vacuo*
Silica gel column (500 ml) chromatography
| eluted with chloroform : MeOH =
| 99:1, 93:7, 90:10 and 100% MeOH (stepwise)
| concd *in vacuo*
YMC ODS A60-230/70 column (2.5 liters) chromatography
| eluted with 50% aqueous MeOH
| diluted with water
Preparative HPLC (YMC ODS AQ 120-S50)
| eluted with 50% aqueous MeOH
| diluted with water
Diaion HP-20 column (50 ml) chromatography
| eluted with ethyl acetate
| triturated in hexane
EI-1507-1 (320 mg), EI-1507-2 (340 mg)

Isolation and Purification

The isolation procedure for the EI-1507s is shown schematically in Fig. 1. The producing strain E-1507 was cultured according to the method described. Culture broth (17 liters) was filtered with the aid of diatomaceous earth. Isopropanol was added to the filtrate to a final concentration of 10%, and the filtrate was adsorbed on a Diaion HP-20 column (3 liters). After washing with 30% aqueous methanol (12 liters), the active principles were eluted with methanol-acetone 7:3 solution (10 liters). The active fractions were combined and extracted with ethyl acetate (5 liters). The ethyl acetate layer was concentrated *in vacuo* to give crude material. This material was dissolved in 30 ml of chloroform. The solution was adsorbed onto a silica gel column (50 mm i.d.; 500 ml) equilibrated with chloroform. The column was eluted stepwise with chloroform-methanol 99:1, 93:7, 90:10 and 100% methanol solution (2 liters each), sequentially. The active fraction was concentrated *in vacuo* to give partially purified material. This material was dissolved in 50% aqueous methanol and applied to an octadecylated silica gel (ODS) column (ODS A60-230/70, 80 mm i.d.; 2.5 liters). The column was eluted with 50% aqueous methanol (flow rate; 40 ml/minute). The active eluates were further purified by preparative HPLC using a column (AQ 120-S50, 20 i.d. × 500 mm) with 50% aqueous methanol elution (flow rate; 10 ml/minute), and the active eluates were readsorbed on an HP-20 column (25 mm i.d.; 50 ml) equilibrated 10% aqueous methanol solution. After the column was washed with water, the active compounds were eluted with ethyl acetate and then triturated in hexane. EI-1507-1 (320 mg) and EI-1507-2 (340 mg) were obtained as gray-yellow powders.

Physico-chemical Properties and Structure Elucidation

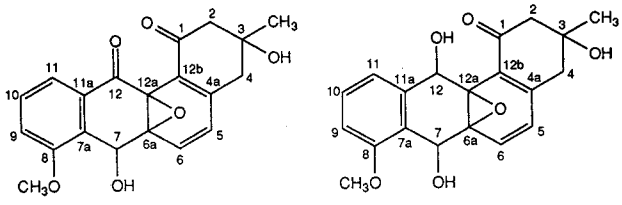
Physico-chemical properties of EI-1507-1 (**1**) and -2 (**2**) are summarized in Table 3. The structures of **1** and **2** were determined as shown in Fig. 2 and found to be novel compounds.

The molecular formula of **1** was determined by high resolution FAB-MS to be $C_{20}H_{18}O_6$. The ^{13}C NMR spectrum (Table 4) showed twenty carbon signals appropriate for the molecular formula, indicating the presence of two carbonyl carbons and ten olefinic and/or aromatic carbons. The structure elucidation of **1** was based principally on 2D NMR analyses. Analysis of

Table 3. Physico-chemical properties of **1** and **2**.

		1	2
Appearance		Pale brown powder	Pale brown powder
Melting point		177~178°C	192~194°C
Optical rotation		$[\alpha]_D^{27}$ +464° (*c* 0.136, MeOH)	$[\alpha]_D^{28}$ +135° (*c* 0.158, MeOH)
Molecular formula		$C_{20}H_{18}O_6$	$C_{20}H_{20}O_6$
FAB-MS	*m/z*	355 $(M+H)^+$	357 $(M+H)^+$
HR FAB-MS	Found.	355.1198 $(M+H)^+$	339.1231 $(M-H_2O+H)^+$
	Calcd.	355.1181	339.1232
Anal	Found.	C 67.37, H 5.20 %	C 67.04, H 5.75 %
	Calcd.	C 67.78, H 5.13 % (for $C_{20}H_{18}O_6$)	C 67.40, H 5.67 % (for $C_{20}H_{20}O_6$)
UV	λ_{max} (MeOH) (ε)	215 (24,700), 256 (8,200), 300 (7,400) nm	220 (15,100), 283 (9,600), 300 (7,500) nm
IR	ν_{max} (KBr)	3421, 1685, 1672, 1300, 1265, 1026, 752 cm^{-1}	3357, 1670, 1592, 1473, 1315, 1261, 752 cm^{-1}

Fig. 2. Structures of EI-1507-1 (**1**) and EI-1507-2 (**2**).



EI-1507-1 (**1**) EI-1507-2 (**2**)

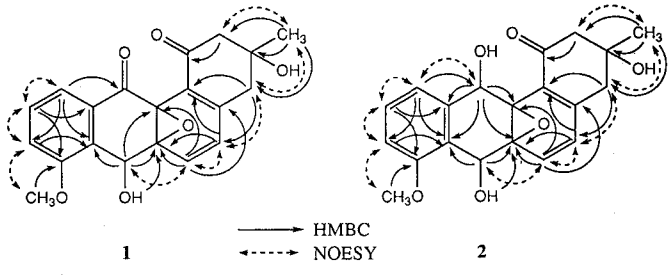
Table 4. ^{13}C and ^1H NMR data for **1** and **2**.

	1		2	
No.	δC[a]	δH[b]	δC[a]	δH[b]
1	192.8		200.5	
2	51.8	2.60 (1H, d, 16.3)	53.4	2.78 (2H, m)
		3.00 (1H, d, 16.3)		
3	70.7		72.0	
3-CH_3	28.5	1.38 (3H, s)	29.4	1.42 (3H, s)
3-OH		2.11 (1H, br s)		1.77 (1H, br s)
4	44.4	2.73 (1H, d, 17.8)	45.8	2.80 (1H, d, 18.0)
		2.80 (1H, d, 17.8)		2.96 (1H, d, 18.0)
4a	146.6		150.7	
5	132.2	6.48 (1H, d, 9.6)	131.5	6.51 (1H, d, 9.5)
6	132.8	6.99 (1H, d, 9.6)	138.8	7.19 (1H, d, 9.5)
6a	65.7		63.1	
7	63.6	5.91 (1H, d, 5.1)	64.0	5.74 (1H, d, 9.4)
7-OH		2.24 (1H, d, 5.1)		2.46 (1H, d, 9.4)
7a	127.2		122.8	
8	156.7		157.3	
8-OCH_3	56.0	3.94 (3H, s)	55.8	3.90 (3H, s)
9	114.9	7.12 (1H, dd, 1.2, 7.8)	111.2	6.93 (1H, dd, 0.8, 8.1)
10	130.6	7.46 (1H, t, 7.8)	130.4	7.36 (1H, dd, 7.7, 8.1)
11	119.4	7.41 (1H, dd, 1.2, 7.8)	122.9	7.02 (1H, dd, 0.8, 7.7)
11a	133.2		136.0	
12	193.1		69.9	6.14 (1H, br s)
12-OH				3.96 (1H, br s)
12a	61.3		64.3	
12b	127.7		128.9	

[a] ^{13}C NMR spectra were recorded at 125 MHz in $CDCl_3$.
[b] ^1H NMR spectra were recorded at 500 MHz in $CDCl_3$.
Coupling constants in Hz are given in parentheses.

COSY data readily showed the existence of a three proton spin system (9-H to 11-H) and a two proton spin system (5-H and 6-H). One bond proton-carbon connectivities were determined by a heteronuclear single quantum coherence (HSQC) experiment, which was then followed by a heteronuclear multiple bond correlation (HMBC) experiment to assign the long range proton-carbon couplings. The long range couplings observed in the HMBC spectrum revealed that the methoxyl group (3.94 ppm) was attached to the aromatic carbon at 156.7 ppm (C-8) and the methyl group (1.38 ppm) to the oxygen-bearing quarternary carbon at 70.7 ppm (C-3). A NOESY experiment further supported these partial structures by showing cross peaks between the following pairs of protons: methoxyl at C-8 to 9-H (7.12 ppm), methyl at C-3 to 2-H_2 (2.60, 3.00 ppm) and 4-H_2 (2.73, 2.80 ppm). Detailed analyses of HSQC, HMBC and NOESY data summarized in Fig. 3 revealed the presence of the benz[a]anthracene skeleton for **1**. ^{13}C chemical shifts of C-6a (65.7 ppm) and C-12a (61.3 ppm) indicated these quarternary carbons to be attached to oxygen forming the epoxide. Several benz[*a*]anthracene antibiotics bearing oxygen at C-6a[15,16] and/or C-12a[17,18] have been reported; SF2315B[19,20] possesses a similar 6a,12a-epoxide functional group.

Fig. 3. Summary of HMBC and NOESY data for **1** and **2**.



1 **2**

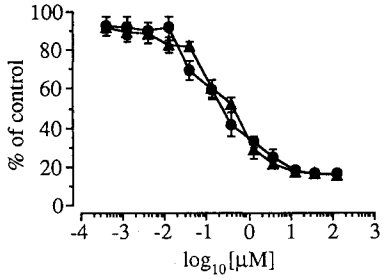
^1H and ^{13}C NMR spectra of **2** were almost similar to **1**, but significant differences between them were found at C-12 (**1**: 193.1 ppm; **2**: 69.9 ppm) and 12-H (**2**: 6.14 ppm) indicating the carbonyl carbon of **1** was replaced by the hydroxymethine carbon in **2**. This finding was confirmed by FAB-MS data of **2** (*m/z* 357 $(M+H)^+$) appropriate for the dihydro derivative of **1**. This structure was supported by the HMBC and NOESY data summarized in Fig. 3.

Inhibition of ICE

EI-1507-1 and -2 inhibited the enzymic activity of human ICE in a dose-dependent manner (Fig. 4); IC_{50}

Fig. 4. Inhibition of ICE by the EI-1507s.

Symbols indicate EI-1507-1 (●) and EI-1507-2 (▲).



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. All experiments were performed in triplicate.

Table 5. Effects of EI-1507-1 and -2 on various enzyme activities.

Enzyme	IC_{50} value (μM)	
	EI-1507-1	EI-1507-2
ICE	0.23	0.42
Elastase	>28	>28
Cathepsin B	>28	>28

values were calculated to be 0.23 and 0.42 μM, respectively. The specificity of EI-1507-1 and -2 was examined by testing them against cathepsin B (another thiol-containing protease) and elastase. EI-1507-1 and -2 were inactive against these two enzymes at concentrations up to 28 μM. These data affirm the specificity of EI-1507-1 and -2 against ICE and are summarized in Table 5.

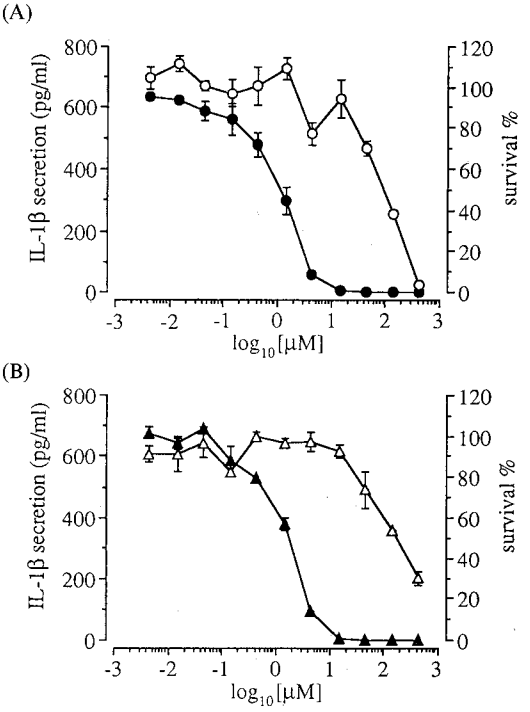
Biological Properties

In order to determine whether EI-1507-1 and -2 were efficacious even in intact cells, we investigated the effects of EI-1507-1 and -2 on the extracellular release of IL-1β from THP-1 cells. EI-1507-1 and -2 inhibited IL-1β secretion in dose-dependent manners (Fig. 5); IC_{50} values were calculated to be 1.1 and 1.4 μM, respectively. On the other hand, 50% cell survival doses of EI-1507-1 and -2 were calculated to be 90 and 163 μM, respectively (as shown in Fig. 5). Cell viabilities at a concentration of 14 μM, at which EI-1507-1 and -2 completely inhibited IL-1β secretion from THP-1 cells were more than 90%. These data indicate that EI-1507-1 and -2 inhibited mature IL-1β secretion from THP-1 cells without showing cell toxicity at concentrations lower than 14 μM.

EI-1507-1 had little antimicrobial activity against

Fig. 5. Effects of the EI-1507s on IL-1β secretion from LPS-stimulated THP-1 cells and on cell viabilities of THP-1 cells.

EI-1507-1 (A) and EI-1507-2 (B) were applied to the LPS-stimulated THP-1 cells. IL-1β secretion (●, ▲) and percentage of cell survival (○, △) were measured.



Determinations of IL-1β secretion were performed in duplicate and cell survival in triplicate.

Enterococcus faecium, *Bacillus subtilis* and *Proteus vulgaris*; MIC value were 120, 60 and 60 μM, respectively. EI-1507-2 also had little antimicrobial activity against *B. subtilis* with an MIC of 60 μM.

Discussion

We isolated two novel benz[*a*]anthracene-related compounds, termed EI-1507-1 and -2, from the culture broths of *Streptomyces* strain E-1507, and we have demonstrated that EI-1507-1 and -2 selectively inhibited the enzymic activity of ICE. These compounds are unique among the benz[*a*]anthracene-related compounds, as they have an epoxy structure in their molecules. Some members of the benz[*a*]anthraquinone family have been reported to have antimicrobial activities[21,22], but these compounds have no epoxy structures and no inhibitory activities against ICE. Several peptide-related compounds have been reported as ICE inhibitors[5,23]. L-741,498 is the only ICE inhibitor isolated previously from a microbial source[24], but its activity is less than those of EI-1507-1 and -2.

In this paper, we showed also that EI-1507-1 and -2 inhibited IL-1β secretion from LPS-stimulated THP-1

cells. This inhibition was not due to toxic effects of the EI-1507s as shown in Fig. 4. These results suggest that EI-1507s would be effective even *in vivo*.

IL-1β, generated by ICE, is implicated in the pathophysiology of various diseases[2], but details of this implication are still unclear. Recently, it was reported that ICE was homologous to the *Caenorhabditis elegans* cell death gene and responsible for apoptosis[25,26]. EI-1507s would be useful for clarifying the true pathophysiological and physiological roles of ICE.

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