J. Enzyme Inhibition, 1996, Vol. 11, pp. 39–49
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A NOVEL SYNTHETIC INHIBITOR OF ENDOPEPTIDASE-24.15

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(Received 26 June 1995; in final form 22 February 1996)

A novel synthetic inhibitor of endopeptidase-24.15 (EP-24.15, EC 3.4.24.15), *N*-[(2*R*,4*R*)-2-(2-hydroxyphenyl)-3-(3-mercaptopropionyl)-4-thiazolidinecarbonyl]-L-phenylalanine (SA898) is described. This compound inhibited rat EP-24.15 competitively with an IC_{50} of 23 nM and K_i of 9.1 nM. These values were, respectively, 9.6 times and 6.3 times smaller than those for *N*-(1-carboxy-3-phenylpropyl)-alanyl-alanyl-phenylalanyl-*p*-aminobenzoate (cFP-AAF-*p*AB), which was one of the most potent inhibitors thus far reported. The inhibitory effect of SA898 on other endopeptidases, angiotensin converting enzyme (ACE, EC.3.4.15.1) and endopeptidase-24.11 (EP-24.11, EC.3.4.24.11) was also studied. SA898 inhibited ACE significantly, but the potency was about 20-fold lower than that for EP-24.15 in terms of the K_i value. The inhibitory effect of SA898 on EP-24.11 was almost negligible (K_i = 28 μM). In addition, the inhibitory activities of several SA898-related compounds were examined. Based on these data, the structure-activity relationships for EP-24.15 inhibitors are discussed.

Keywords: Endopeptidase-24.15, SA898, metalloendopeptidase, inhibition

INTRODUCTION

Many physiologically important peptides such as hormones and cytokines exist in the human body. The synthesis and degradation of these peptides is regulated by various proteolytic enzymes. For instance, membrane-bound metalloendopeptidase-24.11 (EP-24.11, EC.3.4.24.11) hydrolyzes and inactivates enkephalins[1] and a kind of cystein-proteinase has been defined as a key enzyme in the production of IL-1β from its precursor.[2] One of such endopeptidases, i.e. endopeptidase-3.4.24.15 (EP-24.15, EC.3.4.24.15), was initially discovered as a soluble metalloendopeptidase in rats.[3] This enzyme is found widely distributed in the body and exists

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Abbreviations: IL-1β, interleukin-1β; LHRH, luteinizing hormone-releasing hormone; *p*AB, *p*-aminobenzoic acid; Glt, glutaryl; 4MN, 4-methoxy-2-naphthylamine; Hip, hippuric acid; Bz, α-*N*-benzoyl.

with a relatively high content in the brain and the testis.[4] In *in vitro* experiments, this enzyme has been shown to cleave various bioactive peptides, such as LHRH,[5] neurotensin,[3,5] bradykinin[3,5] and substance P.[3] Furthermore, this soluble peptidase was reported to hydrolyze enkephalin precursors and convert them into active, enkephalin, *in vitro*.[5] Thus, EP-24.15 is possibly involved in the regulatory systems of activation and inactivation of these bioactive peptides. This soluble peptidase may also have a role in regulating the actions of other peptides of the peripheral organs and to clarify further the function of this enzyme *in vivo*, a specific inhibitor would be a useful tool. Substrate-related *N*-(1-carboxy-3-phenylpropyl) peptide derivatives have already been reported as EP-24.15 inhibitors[6] and one of the derivatives, *N*-(1-carboxy-3-phenylpropyl)-alanyl-alanyl-phenylalanyl-*p*-aminobenzoate (cFP-AAF-*p*AB), has been used to examine the function of EP-24.15 *in vivo*. In our laboratories, we have synthesized many sulfhydryl moiety-containing compounds. Since EP-24.15 contains a zinc atom in its active center, we assumed that the sulfhydryl moieties of these compounds could chelate to zinc and show enzyme inhibitory activities. Consequently we have examined the inhibitory activities of our sulfhydryl compounds on EP-24.15 and found one compound, *N*-[(2*R*,4*R*)-2-(2-hydroxyphenyl)-3-(3-mercaptopropionyl)-4-thiazolidinecarbonyl]-L-phenylalanine (SA898) (Figure 1), to have a more potent inhibitory effect than cFP-AAF-*p*AB. In this paper, we describe the characterization of this new inhibitor and discuss the structure-activity relationships using the inhibitory potency data of SA898 and its derivatives.

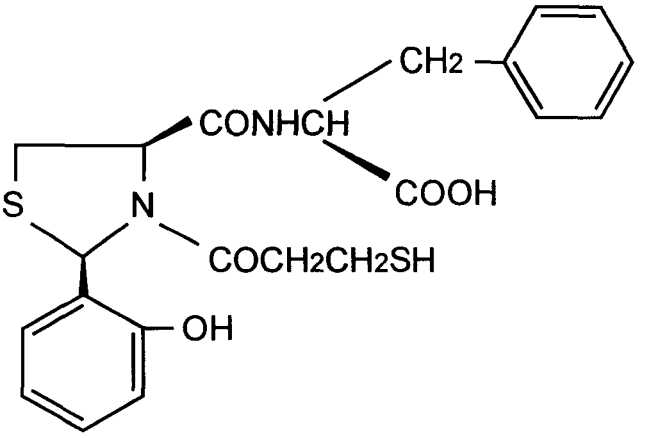


FIGURE 1 Chemical structure of SA898.

MATERIALS AND METHODS

Materials

Angiotensin converting enzyme (ACE, EC.3.4.15.1, rabbit lung), *p*-aminobenzoic acid, hippuric acid, Glt-Ala-Ala-Phe-4MN and Fast Garnet GBC were obtained from Sigma Chemical Co. (St. Louis, MO. U.S.A). Aminopeptidase M (EC.3.4.11.2) was purchased from Boehringer/Mannheim Inc. (Tokyo, Japan). Phosphoramidon and Hip-His-Leu · H_2O were from Peptide Institute Inc. (Osaka, Japan). DEAE-cellulose (DE-52) and BW-300 were from Whatman Inc. (Tokyo, Japan) and Fuji Silysia Chemical Ltd. (Aichi, Japan), respectively. A Nucleosil $7C_{18}$ column was obtained from Chemco Scientitic Co. Ltd. (Osaka, Japan).

Preparation of Enzymes

EP-24.15 was purified from rat whole brain according to the method of Orlowski *et al.*[3] Whole brains (about 40 g) were collected from male Wistar rats weighing about 300 g and immediately homogenized in 4 volumes of 0.01 M Tris-HCl buffer containing 0.32 M sucrose and 0.5 mM 2-mercaptoethanol (pH 7.6). The homogenate was centrifuged at 30,000 ×g for 120 min. The supernatant was applied to a DEAE-cellulose column (DE-52; 55 ml) equilibrated with 180 ml of the same buffer without sucrose. Proteins were eluted with 200 ml of a linear gradient of 0–0.3 M NaCl in 0.01 M Tris-HCl containing 0.5 mM 2-mercaptoethanol (pH 7.6) at a flow rate of 1.2 ml/min. Active fractions were collected and concentrated to about 5 ml by ultrafiltration using an Amicon PM-10 filter. EP-24.11 was prepared from rat kidneys as described by Malfroy and Schwartz.[7] All procedures for enzyme preparation were carried out at 4°C.

Synthesis of Substrate and Inhibitors

Bz-Gly-Ala-Ala-Phe-*p*AB and cFP-AAF-*p*AB were synthesized as described by Orlowski *et al.*,[3,6] respectively. SA898, *N*-[(2*R*,4*R*)-2-(2-hydroxyphenyl)-3-(3-mercaptopropionyl)-4-thiazolidinecarbonyl]-L-phenylalanine, was synthesized as follows.

Step 1: N-[(2R,4R)-3-[3-(benzoylthio)propionyl]-2-(2-hydroxyphenyl)-4-thiazolidinecarbonyl]-L-phenylalanine. (2*R*, 4*R*)-3-[3-(benzoylthio)propionyl]-2-(2-hydroxyphenyl)-4-thiazolidinecarboxylic acid[8] (8.00 g, 16.1 mmol) was dissolved in anhydrous tetrahydrofuran (100 ml) with *N*-methylmorpholine (1.77 ml, 16.1 mmol). Isobutyl chloroformate (2.07 ml, 16.1 mmol) was then added dropwise

to the solution with stirring at −18°C. After 5 min a solution of L-phenylalanine (5.32 g, 32.2 mmol) and triethylamine (4.49 ml, 32.2 mmol) in water (100 ml) was added and the reaction mixture was stirred for 1 h at room temperature. After the addition of water (200 ml), the solution was acidified with 2 M hydrochloric acid (30 ml) and extracted with ethyl acetate (extra pure reagent; 200 ml × 2). The organic layer was washed with 50 ml of saturated sodium chloride solution, dried over $MgSO_4$, and concentrated *in vacuo*. The residue was chromatographed on silica gel (BW-300; 300 g) using benzene-ethyl acetate (4:1) to give 8.25 g (yield 90.7%) of the title compound.

Step 2: N-[(2R,4R)-2-(2-hydroxyphenyl)-3-(3-mercaptopropionyl)-4-thiazolidinecarbonyl]-L-phenylalanine (SA898). Aqueous ammonia (28% w/v, 60 ml) was added to *N*-[(2*R*,4*R*)-3-[3-(benzoylthio)propionyl]-2-(2-hydroxyphenyl)-4-thiazolidinecarbonyl]-L-phenylalanine (7.62 g, 13.5 mmol) in methanol (60 ml), and the mixture was stirred for 2 h at room temperature. Excess ammonia and methanol were removed *in vacuo* and the by-product, benzamine, was extracted with ethyl acetate (100 ml × 2). The aqueous layer was acidified with 6 M hydrochloric acid (20 ml) and the separated oil was extracted with ethyl acetate (150 ml × 2). The extract was washed with saturated sodium chloride solution (50 ml × 2), dried over $MgSO_4$, and concentrated *in vacuo*. The residue was chromatographed on silica gel (BW-300; 85 g) using benzene-ethyl acetate (3:2) to give 4.82 g (yield 77.5%) of the title compound.

Enzyme Assay

EP-24.15 activity was determined according to the procedure described by Orlowski *et al.*[3] using Bz-Gly-Ala-Ala-Phe-*p*AB as substrate. The standard reaction mixture (0.2 ml) contained 0.5 mM substrate, enzyme preparation (10 μg of protein), 5 μM phosphoramidon and 0.2 M Tris-HCl (pH 7.0) with or without EP-24.15 inhibitor unless otherwise stated. Phosphoramidon was included in the reaction mixture to inhibit EP-24.11 activity which was a possible contaminant in the EP-24.15 preparation.[5] It was confirmed that phosphoramidon did not affect the activity of EP-24.15. The reaction was started by addition of the enzyme preparation and the mixture was incubated at 37°C for 15 min and then placed in a boiling water bath for 2 min to terminate the enzyme reaction. After cooling to room temperature, the reaction mixture was incubated at 37°C for 120 min with 20 μg of Aminopeptidase M (50 μl). The reaction was then terminated by the addition of 100 μl of 25% trichloroacetic acid. *p*AB released from the substrate was determined using the modified diazotization procedure.[9] *p*AB formation in the presence of an excess amount (10 μM) of cFP-AAF-*p*AB was used as a blank and

was subtracted from the values of the test samples. The *p*AB formation in samples without EP-24.15 inhibitor was evaluated as 100% enzyme activity.

ACE activity was determined using Hip-His-Leu · H_2O as substrate according to Horiuchi's method.[10] The reaction mixture containing 25 μl of enzyme preparation (2.5 mU), 0.6 mM of substrate, 300 mM NaCl and 100 mM phosphate buffer (pH 8.3) was incubated in a total volume of 125 μl at 37°C for 10 min. The reaction was stopped by the addition of 375 μl of 3% HPO_3 and the mixture was then centrifuged at 750 ×g for 5 min. The supernatant (20 μl) was analyzed with a Nucleosil $7C_{18}$ column (4.6 × 250 mm) at 40°C using methanol/10 mM KH_2PO_4 (1/1) (pH 3.0) as the mobile phase (flow rate 0.7 ml/min).

EP-24.11 activity was determined using Glt-Ala-Ala-Phe-4MN as substrate according to Orlowski's method.[11] The reaction mixture (0.25 ml) contained the substrate (0.25~0.5 mM), an excess amount of aminopeptidase M (10 μg) and Tris-HCl (0.5 M; pH 7.6). The reaction was started by addition of 50 μl of the enzyme preparation. After 10 min incubation at 37°C, the reaction was terminated by addition of a solution containing 4-chloromercuribenzoic acid and Fast Garnet GBC according to Barrett's procedure.[12] The released 4 MN, which was diazotized, was determined by measuring the absorption at 520 nm using a spectrophotometer.

RESULTS

Inhibition of EP-24.15 by SA898 and cFP-AAF-*p*AB

SA898 (*N*-[(2*R*,4*R*)-2-(2-hydroxyphenyl)-3-(3-mercaptopropionyl)-4-thiazolidine-carbonyl]-L-phenylalanine), the chemical structure of which is shown in Figure 1, was synthesized in our laboratory and its inhibitory effect on rat brain EP-24.15 was examined. The substrate for EP-24.15 assay used in these experiments was a synthetic peptide, Bz-Gly-Ala-Ala-Phe-*p*AB. Under the assay condition described in Materials and Methods, the V_{max} was 0.5 nmol · min^{-1}· mg $protein^{-1}$ and the K_m value of this substrate was 0.3 mM. Figure 2 shows the inhibitory curve of EP-24.15 by SA898 along with the data of the known inhibitor, cFP-AAF-*p*AB. SA898 inhibited EP-24.15 in a concentration-dependent manner similar to cFP-AAF-*p*AB. The IC_{50} values of SA898 and cFP-AAF-*p*AB on EP-24.15 calculated from these curves were 23 nM and 220 nM, respectively. The Lineweaver-Burk plot analysis indicated that the inhibition by SA898 was in a substrate-competitive manner (Figure 3). Figure 4 shows the Dixon plot of SA898 on EP-24.15 activity. The K_i value calculated from this plot was 9 nM. The K_i value of cFP-AAF-*p*AB calculated from the same assay was 57 nM. The inhibitory potency of SA898 for EP-24.15 was stronger by 9.6-fold in terms of IC_{50} and 6.3-fold in terms of K_i than that of cFP-AAF-*p*AB.

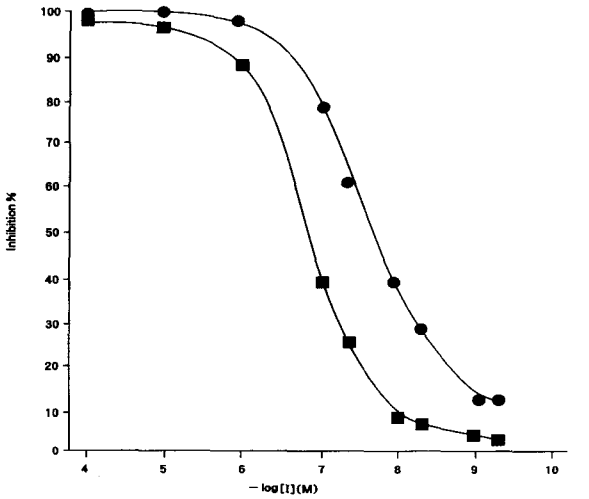


FIGURE 2 Inhibition curve of EP-24.15 by SA898 and cFP-AAF-*p*AB. The enzyme assay was performed with 0.5 mM substrate and either SA898 (●) or cFP-AAF-*p*AB (■) with other conditions as described under Materials and Methods section. Each point indicates the mean of duplicate measurements in a single experiment.

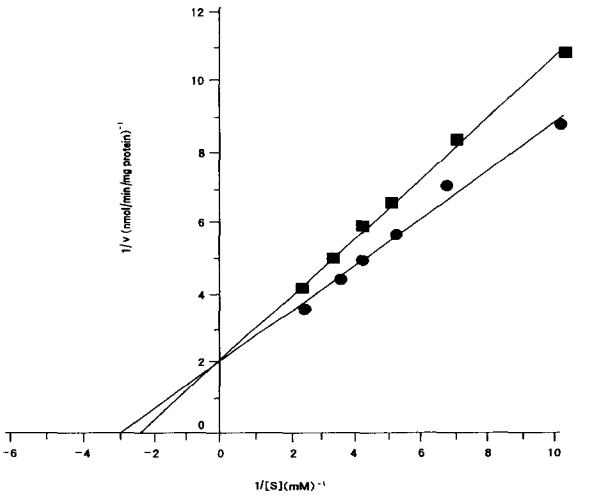


FIGURE 3 Lineweaver-Burk plot of EP-24.15 inhibition by SA898. The inhibitory assay in this plot was performed with 0.1 ~ 0.4 mM substrate in the absence (●) and presence (■) of 5 nM SA898. Each point indicates the mean of duplicate measurements in a single experiment. Reaction velocity (v) is expressed as A_{555} nm per min.

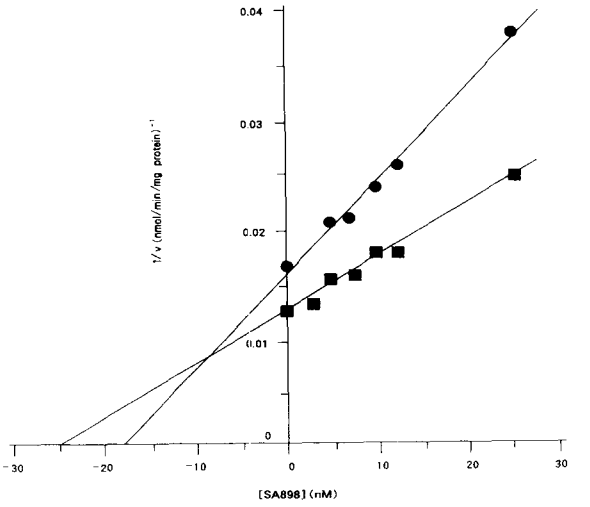


FIGURE 4 Dixon plot of EP-24.15 inhibition by SA898. The inhibitory assay in this plot was performed with 0.4 mM (•) and 0.6 mM (■) substrate. Each point indicates the mean of duplicate measurements. The K_i value obtained in this plot was 9 nM.

The effects of SA898 on other metalloendopeptidases, ACE and EP-24.11, were also examined. Because these peptidases also have a zinc atom in their active sites, SA898 having a sulfhydryl moiety which could chelate to zinc was expected to have some inhibitory effects on these enzymes. Table I shows the inhibitory effects of SA898 and cFP-AAF-*p*AB on these two enzymes. The inhibitory potency of SA898 for rabbit ACE was stronger by about 20-fold in terms of IC_{50} and 7.6-fold in terms of K_i than that of cFP-AAF-*p*AB. The K_i value of SA898 for ACE was about 20-fold lower than that for EP-24.15. In addition, the inhibitory potency of SA898 for rat EP-24.11 was less than that for EP-24.15. The K_i of SA898 for EP-24.11 was calculated as 10^{-5} molar from the Dixon plots.

Inhibitory Activities of SA898-related Compounds

In order to determine the structure-activity relationships, we studied the inhibitory actions of some SA898 derivatives. Table II shows the inhibitory effects of SA898-related compounds on EP-24.15, ACE and EP-24.11. When the phenylalanine moiety of SA898 was deleted (compound **1**), the inhibitory effects on EP-24.15 decreased dramatically. However, this compound showed a very strong inhibitory action on ACE. In the case of compounds lacking phenylalanine moiety at the

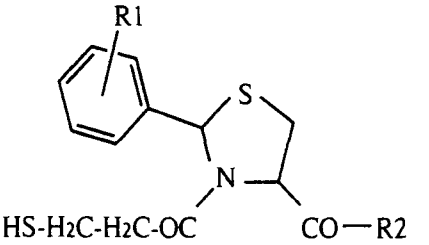
TABLE I Inhibition of metalloendopeptidases by SA898 and cFP-AAF-*p*AB

	IC_{50} (μM)[a]			K_i (μM)[b]		
	EP-24.15	*ACE*	*EP-24.11*	*EP-24.15*	*ACE*	*EP-24.11*
SA898	0.023	0.17	28	0.0091 ± 0.0007	0.17 ± 0.05	18 ± 2
cPF-AAF-*p*AB	0.22	3.7	500	0.057 ± 0.004	1.3 ± 0.3	–

[a] IC_{50} values were obtained from inhibition curves of percentage inhibition *vs* log concentration.

[b] K_i values were obtained from Dixon plots at two substrate concentrations. Data for the K_i values are expressed as the mean value ± S.E. of three to six determinations.

TABLE II Inhibitory effects of SA898-derivatives on EP-24.15, ACE and EP-24.11 activity



			IC_{50} (μM)		
Compound	*R1*	*R2*	*EP-24.15*	*ACE*	*EP-24.11*
1	*o*-OH	OH	360	0.019	>100
2	*p*-OCH_3	OH	32	0.12	>100
3	*p*-CH_3	OH	12	0.17	42
4	*p*-CH_3	Phe	0.7	1.6	13
5	*o*-OH	Ser	0.37	7.7	63
6	*o*-OH	Phe-NH_2	0.52	0.47	49
SA898	*o*-OH	Phe	0.023	0.17	28

R2 position as in compound **1**, the replacement of an *o*-OH moiety in the phenyl ring of compound **1** with *p*-OCH_3 (compound **2**) or *p*-CH_3 (compound **3**) resulted in an order increase in inhibitory action on EP-24.15. When R1 was *p*-CH_3, the phenylalanine at R2 (compound **4**) increased the activity about 20-fold.

The replacement of phenylalanine of SA898 with serine (compound **5**) reduced the inhibitory effect of SA898 on EP-24.15 more than 10-fold. This replacement resulted in a slight reduction of the inhibitory action on EP-24.11. Furthermore when

the free COOH in the phenylalanine moiety was esterified with NH_2 (compound **6**), the inhibitory effect on EP-24.15 was also reduced more than 20-fold.

Regarding the inhibition of ACE, these seven compounds showed relatively high activities. The most potent, compound **1**, showed almost the same activity as captopril, which is a representative ACE inhibitor. The IC_{50} value of captopril in our assay system was about 5 nM. It is interesting that compound **1** is the least potent for EP-24.15 inhibition and the most potent for ACE inhibition. It would seem that an amino acid moiety at the R2 position was not necessary for ACE inhibitory activity. With regard to the R1 position, *o*-OH seemed better than *p*-OCH_3 or *p*-CH_3 for ACE inhibition.

These seven compounds showed low inhibitory actions on EP-24.11. Among them, compound **4** was the most potent EP-24.11 inhibitor with an IC_{50} value of 13 μM. This value seems too small to exert an inhibitory action on EP-24.11 *in vivo* because the reported IC_{50} value of the representative EP-24.11 inhibitor, thiorphan, is 80 nM.[13]

DISCUSSION

Although more than 10 years have passed since endopeptidase EP-24.15 was first reported in 1983, there have been only a few studies about its inhibition. Actually, only *N*-(1-carboxy-3-phenylpropyl) peptide derivatives have been reported thus far as inhibitors of EP-24.15.[6] We report here a novel synthetic inhibitor of EP-24.15. The inhibitory effect of this compound, SA898, was more potent than that of cFP-AAF-*p*AB. The Lineweaver-Burk plot and Dixon plot analyses indicated that EP-24.15 inhibition by SA898 was substrate competitive. Considering that both SA898 and cFP-AAF-*p*AB are substrate-competitive, we can assume that these two inhibitors interact with EP-24.15 in a similar manner, although the structures of these inhibitors are different.

Because EP-24.15 is a zinc-containing endopeptidase, it can be reasonably postulated that the sulfhydryl moiety of SA898 chelates to the zinc atom in EP-24.15. In addition, SA898 has a L-phenylalanine at the carboxyl position of the thiazolidine carboxylic acid, without which its inhibitory effect is reduced by more than 10,000-fold. In the case of the substrate-related inhibitor cFP-AAF-*p*AB, the phenylalanine moiety was reported to be important in the inhibitory action by interacting with one of the enzyme subsites at its active site.[6] Because SA898 showed substrate-competitive inhibition on EP-24.15, the phenylalanine moiety is thought to interact with the substrate-recognition site of the enzyme.

However, replacement of phenylalanine by serine caused a slight decrease in the inhibitory activity. Serine has a structure in which the phenyl group of phenylalanine

is replaced with hydroxide. In terms of hydrophobicity, phenylalanine and serine are very different so perhaps the size of the amino acid residue at R2 position of SA898 is important and the hydrophobicity at this site not influential. Furthermore, esterification of COOH in phenylalanine with NH_2 reduced the activity about 20-fold. This reduction was almost the same as the replacement of phenylalanine with serine. The presence of a free carboxylic acid in the amino acid residue at R2 seemed to be as much important as the size of the amino acid residue. These findings suggest that EP-24.15 recognizes a relatively wide variety of amino acids. Actually, the recognition site of endogenous substrates for EP-24.15 have not been narrowly specified and this enzyme has been reported to hydrolyze several kinds of peptide bonds in *in vitro*.[3–5] For example, Orlowski *et al.*[3] showed that EP-24.15 cleaved the bonds of Phe-Ser of bradykinin, Arg-Arg of neurotensin, His-Trp and Tyr-Gly of luliberin and Pro-Gln, Phe-Phe and Phe-Gly of substance P as well as several other peptide bonds in synthetic peptides.[3–5] Peptide sequences recognized by EP-24.15 are not strictly limited and this enzyme seems to hydrolyze a relatively broad spectrum of peptides.

cFP-AAF-*p*AB has three aromatic residues in its structure and all of these aromatic residues are thought to fit the hydrophobicity-recognized pockets of the active center of EP-24.15.[6] Although SA898 has only two benzene rings in its structure, this compound showed a more potent inhibitory action than cFP-AAF-*p*AB. Therefore, at least one of the three pockets does not seem to be important in substrate recognition.

In this study, the inhibitory actions of SA898 on ACE and EP-24.11 activities was also investigated. We found a weak but significant inhibitory activity of SA898 against ACE and a very weak activity against EP-24.11. The K_i value of SA898 for ACE was approximately 10-fold larger than that for EP-24.15. These results suggest that the inhibitory specificity of SA898 on these metalloendopeptidases is similar to that of cFP-AAF-*p*AB.

The actions of SA898-related compounds on ACE and EP-24.11 inhibition were also studied. The inhibitory activities of these compounds on EP-24.11 were much less than those on EP-24.15. However, some compounds showed relatively strong inhibitory activities on ACE. It is clear from the data of Table 2 that EP-24.15 and ACE inhibition could not be correlated. Therefore, the structure of the active center of EP-24.15 seems to be different from that of ACE. However, the molecular size of the compounds tested as enzyme inhibitors in our study was not very different. It may be possible to design a compound which has dual inhibitory actions on both EP-24.15 and ACE.

In the present study, we describe a novel potent inhibitor of EP-24.15. The physiological function of this enzyme has not been well-characterized, but it has been reported that EP-24.15 is a key enzyme in the degradation of LHRH in *in vivo*.[14]

Therefore, an inhibitor of this enzyme like SA898 may be useful for the treatment of disorders involving EP-24.15.

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