Journal of Enzyme Inhibition and Medicinal Chemistry, June 2008; 23(3): 352–356

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Novel peptide-based pepsin inhibitors containing an epoxide group

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(*Received 22 February 2007; accepted 10 July 2007*)

Abstract

1,2-Epoxy-3-(*p*-nitrophenoxy)propane (EPNP) is known to inhibit pepsin A and other aspartic proteinases by reacting with the active site aspartic acid residue(s). However, the reaction is considerably slow in general, and therefore, it is desirable to develop similar reagents that are capable of inhibiting these enzymes more rapidly. In the present study, we synthesized a series of novel inhibitors which have a reactive epoxide group linked with peptide by a hydrazide bond, with a general structure: Iva-L-Val-L-Val-(L-AA)$_n$-N_2H_2-ES-OEt (n = 0 ~ 2) (Iva, isovaleryl; AA, bulky hydrophobic or aromatic amino acid residue; ES, epoxysuccinyl). These inhibitors were shown to inhibit porcine pepsin A remarkably faster than EPNP.

Keywords: *EPNP, epoxide, peptide-based inhibitor, pepsin A, pepstatin A*

Introduction

Pepsin is the major proteinase involved in protein digestion in the stomach under acidic conditions, and belongs to the aspartic proteinase family, possessing two catalytic aspartic acid residues at the active site [1]. There are two major types of pepsin, i.e. pepsin A [2] and pepsin C (or gastricsin) [3], in the adult stomach, and the former is the major pepsin in human and many other animals. So far, there are three kinds of potent low-molecular-weight pepsin inhibitors described in literature; diazoacetyl-DL-norleucine methyl ester (DAN) (and related diazo reagents) [4–6] and 1,2-epoxy-3-(*p*-nitrophenoxy)propane (EPNP) [7] are irreversible pepsin inhibitors, and pepstatin A (isovaleryl-L-valyl-L-valyl-statyl-L-alanyl-statine) is a reversible pepsin inhibitor obtained from Actinomysates [8] (Figure 1). Porcine pepsin A was shown to be inhibited by specific esterification of the carboxyl group of the active site Asp215 with DAN or related diazo reagents in the presence of cupric ions [4–6] and by that of the active site Asp32 or Asp215 with EPNP [9]. On the other hand, pepstatin A is known to bind non-covalently to the active site of porcine pepsin A, as a transition-state analog inhibitor [10], the K_i value being estimated to be around 10^{-10} M [11]. Human pepsin A was also shown to be strongly inhibited by these inhibitors like porcine pepsin A [7,12]. Pepsin C is also inhibited by DAN and EPNP like pepsin A, although it is less sensitive to pepstatin A. Thus, these inhibitors have been widely used as specific inhibitors of pepsin and other aspartic proteinases. So far DAN- and EPNP-related inhibitors have not been used pharmacologically, but many attempts have been made to make pharmacological use of pepstatin A-related (statin-containing) inhibitors as specific inhibitors toward related aspartic proteinases such as human renin [13] and human immunodefficiency virus proteinase [14].

DAN reacts with pepsin and other aspartic proteinases very rapidly; porcine pepsin A is inactivated completely within 10 min in the presence

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ISSN 1475-6366 print/ISSN 1475-6374 online
DOI: 10.1080/14756360701611910

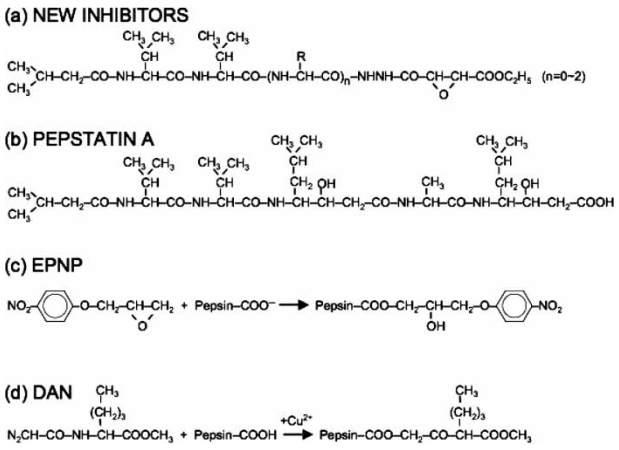

Figure 1. The structures of pepsin inhibitors. (a) New inhibitors: Isovaleryl-L-Val-L-Val-(L-AA)$_n$-N_2H_2-ES-OEt (n = 0 ~ 2), (b) Pepstatin A: Isovaleryl-L-Val-L-Val-Sta-L-Ala-Sta. Sta, statine (4-amino-3-hydroxy-6-methylheptanoic acid). (c) EPNP. (d) DAN. For EPNP and DAN, the reaction schemes with an active site carboxyl group of pepsin are also shown.

of about 40- and 33-fold molar excess of DAN and cupric ions, respectively, at pH 5.0 and 14°C [4]. This rapid reaction is thought to be partly due to the formation from DAN of a Cu^{2+}-complexed reactive carbene intermediate [15]. On the other hand, EPNP reacts with them very slowly; it took over 70 h for complete inactivation of human pepsin with an excess EPNP at pH 4.6 and 25°C [7]. Therefore it is desirable to develop similar inhibitors which are capable of inhibiting these enzymes much more rapidly.

In the present study, we synthesized a series of novel inhibitors carrying a reactive epoxide group. These inhibitors have a general structure: Iva-L-Val-L-Val-(L-AA)$_n$-NHNH-ES-OEt (n = 0 ~ 2) (Iva, isovaleryl; AA, bulky hydrophobic or aromatic amino acid residue; ES, epoxysuccinyl), in which the epoxide group is linked with the peptide moiety by a hydrazide bond. They were prepared by reacting Iva-Val-Val-(AA)$_n$-$NHNH_2$ with ethyl L-trans-epoxysuccinate. All these compounds thus prepared were shown to inhibit porcine pepsin A at much faster rates than EPNP and

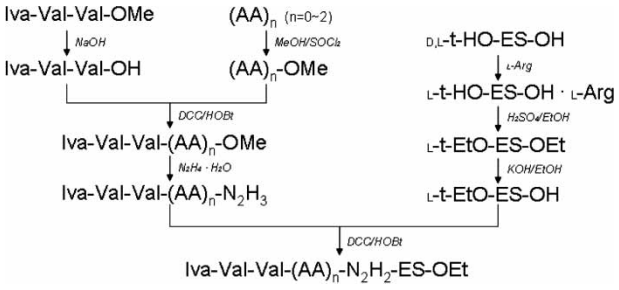

Figure 2. Outline of the procedures for the synthesis of the inhibitors. DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; Iva, isovaleryl; $N_2H_4{\cdot}H_2O$, hydrazine hydrate; $SOCl_2$, thionylchloride; ES, epoxysuccinyl; t, trans.

suggested to be useful as a novel series of active-site-directed irreversible inhibitors for pepsin and other aspartic proteinases.

Materials and methods

Materials

Porcine pepsin A, bovine hemoglobin and EPNP were obtained from Sigma, and pepstatin A from Peptide Instiute, Osaka. Porcine pepsin A was used in the present study instead of human pepsin A since the former is the only commercially available pepsin and is thought to be very similar to human pepsin A in various characteristics. Amino acids were obtained from Nippon Rika Co., Tokyo and the other reagents used were mostly from Wako Pure Chem. Ind. Tokyo except for hydrazine hydrate and dicyclohexylcarbodiimide (Nacalai Tesque Co., Kyoto) and 1-hydroxybenzotriazole monohydrate (Kokusan Chemical Co., Toyo). Other reagents used were of analytical grade.

Synthesis of inhibitors

The compounds Iva-Val-Val-(AA)$_n$-N_2H_2-ES-OEt (n = 0 ~ 2) were synthesized from Iva-Val-Val-(AA)$_n$-N_2H_3 (n = 0 ~ 2) and ethyl L-trans-epoxysuccinate by the following general procedures (see Figure 2).

Iva-Val-Val-OH was conventionally prepared and reacted with H-(AA)$_n$-OMe (n = 0 ~ 2) to yield Iva-L-Val-L-Val-(AA)$_n$-OMe. Iva-Val-Val-(AA)$_n$-OMe (5 mmol) was dissolved in 200 ml of methanol (or dimethylformamide) and to this was added 4.8 ml (100 mmol) of 100% hydrazine hydrate. The mixture was stirred for 4 days at room temperature, then concentrated under reduced pressure. To the residue was added 300 ml of deionized water and the solution was chilled with ice. The resulting precipitates were collected by filtration and dried in a vacuum desiccator over silica gel. The peptide hydrazides Iva-Val-Val-(AA)$_n$-N_2H_3 (n = 0 ~ 2) thus prepared were purified by preparative HPLC on a Shiseido Capcell Pak C18S column (20 × 250 mm) essentially as described below. The overall yields of the purified peptide hydrazides from Iva-Val-Val-(AA)$_n$-OMe were 19 to 42%.

Diethyl L-trans-epoxysuccinate was prepared from trans-D,L-epoxysuccinic acid via trans-L-epoxysuccinic acid/L-Arg salt as described [16], and then converted to ethyl L-trans-epoxysuccinate as described [17]. Ethyl L-trans-epoxysuccinate (0.16 g, 1.0 mmol) dissolved in 5 ml of tetrahydrofuran was ice-cooled and mixed with Iva-Val-Val-(AA)$_n$-N_2H_3 (0.5 mmol), 0.076 g (0.5 mmol) of 1-hydroxybenzotriazole monohydrate and 0.11 g (0.5 mmol) of dicyclohexylcarbodiimide, and the mixture was stirred for 1 h in an ice bath, then overnight at room

Table I. Analytical data of the inhibitors.

Inhibitors						
Name[a]	Structure	Yield[b] (%)	MW	$[M + H]^+$	Amino acid composition (mol/mol)[c]	Retention time (min)[d]
VV	Iva-Val-Val-N_2H_2-ES-OEt	40	456.7	457	Val nd	20.7
VVF	Iva-Val-Val-Phe-N_2H_2-ES-OEt	29	603.7	604	Val 1.95, Phe 1.05	26.8
VVW	Iva-Val-Val-Trp-N_2H_2-ES-OEt	36	642.7	643	Val 1.88, Trp nd	26.6
VVI	Iva-Val-Val-Ile-N_2H_2-ES-OEt	(35)	569.7	570	Val 2.16, Ile 0.83	(17.2)
VVL	Iva-Val-Val-Leu-N_2H_2-ES-OEt	16	569.7	570	Val 1.99, Leu 1.13	26.4
VVM	Iva-Val-Val-Met-N_2H_2-ES-OEt	14	587.7	588	Val 1.98, Met 0.72	24.0
VVLW	Iva-Val-Val-Leu-Trp-N_2H_2-ES-OEt	(62)	755.9	756	Val 1.87, Leu 0.97, Trp nd	(23.1)
VVLM	Iva-Val-Val-Leu-Met-N_2H_2-ES-OEt	(71)	700.9	701	Val 2.05, Leu 1.21, Met 0.91	(21.4)

[a] The inhibitors are named based on the amino acid sequences of the peptide portions, using the one-letter codes for amino acid residues. [b] Overall yield after HPLC purification from the corresponding peptide hydrazide except for the values in parenthesis which were determined before HPLC. [c] Each inhibitor was hydrolyzed in 6N HCl at 110°C for 24 h for the analysis. nd, not determined. [d] Retention time was obtained with a Shimadzu LC-10A HPLC system using a JEOL Crest Pak C18S column (4.6 × 150 mm) with a 0-80% actonitrile gradient except for the values in parenthesis which were determined using a Shiseido Capcell Pak C18 column (4.6 × 150 mm) with a 30–90% acetonitrile gradient as described in the text.

temperature. To this was added a few drops of glacial acetic acid and the mixture was stirred for 10 min, and dried under reduced pressure, then in a vacuum desiccator over silica gel. The residue (0.55–0.69 g) was dissolved in dimethylsulfoxide to 20 mg/mL, and 1.0 mL of this solution was submitted to HPLC. Preparative HPLC was performed in a Shimadzu LC-6A HPLC system using a Shiseido Capcell Pak C18S column (20 × 250 mm) eluted with a linear gradient of acetonitrile (from 20 to 80% in 2 h) in 0.1% trifluoroacetic acid at a flow rate of 3.2 mL per min and monitored at 210 nm. The major peak fraction was pooled and lyophilized.

Purity check

The molecular weight determination was performed by mass spectrometry using a JEOL (JMS-SX102/JMA-DA6000) mass spectrometer. HPLC was carried out with a Shimadzu LC-10A HPLC system using a JASCO Crest Pak C18S column (4.6 × 150 mm) eluted with a linear gradient of acetonitrile (0–80% in 40 min) in 0.1% trifluoroacetic acid at a flow rate of 1.0 mL/min or using a Shiseido Capcell Pak C18 column (4.6 × 150 mm) eluted with a linear gradient (30–90% in 30 min) in 0.1% trifluoroacetic acid at a flow rate of 1.0 mL/min. Amino acid analysis was performed with a Waters AccQTag amino acid analysis system after acid hydrolysis in 6N HCl at 110°C for 24 h.

Pepsin assay

The pepsin activity was determined with bovine hemoglobin as a substrate as described [18] when the inhibition of pepsin A by the epoxide inhibitors was investigated. On the other hand, the inhibition of pepsin A by Iva-Val-Val-OH and Iva-Val-Val-N_2H_3 was examined using the chromogenic peptide substrate Lys-Pro-Ile-Glu-Phe⋆Phe(NO_2)-Arg-Leu [19].

Measurement of pepsin inhibition with the synthetic inhibitors

The inhibition of pepsin A by each inhibitor was examined as follows. A mixture of 400 μL of 2.9 μM (0.1 mg/ml) solution of porcine pepsin A dissolved in 0.01M sodium acetate buffer, pH 5.0, 400 μL of 0.1 M sodium acetate buffer, pH 3.5, and 400 μL of deionized water was preincubated at 37°C for 3 min. To this was added 400 μL of 1.75 mM inhibitor solution in dimethylsulfoxide. The reaction mixture (pepsin: Inhibitor = 1: 600, mol/mol; final pepsin concentration, approximately 0.73 μM) was kept at 37°C and at appropriate intervals an aliquot of 80 μL was pipetted for assay. The assay was performed with bovine hemoglobin as a substrate. Pepsin A was also treated with EPNP (Sigma) under the same conditions for comparison.

In addition, the reversible inhibition of the enzyme by Iva-Val-Val-OH and Iva-Val-Val-N_2H_3 was also measured. A mixture of 29 nM porcine pepsin A and each peptide at various concentrations was preincubated at 37°C for 10 min in 0.1M sodium formate buffer, pH 3.1, and then the activity was determined with the chromogenic peptide substrate.

Results and discussion

The general structure of the inhibitors is illustrated in Figure 1a and the structures of pepstatin A, EPNP and DAN are also shown for comparison (Figures 1b–d). Figure 2 shows schematically the outline of the synthetic procedures of the inhibitors. The yields, molecular weights, amino acid compositions and

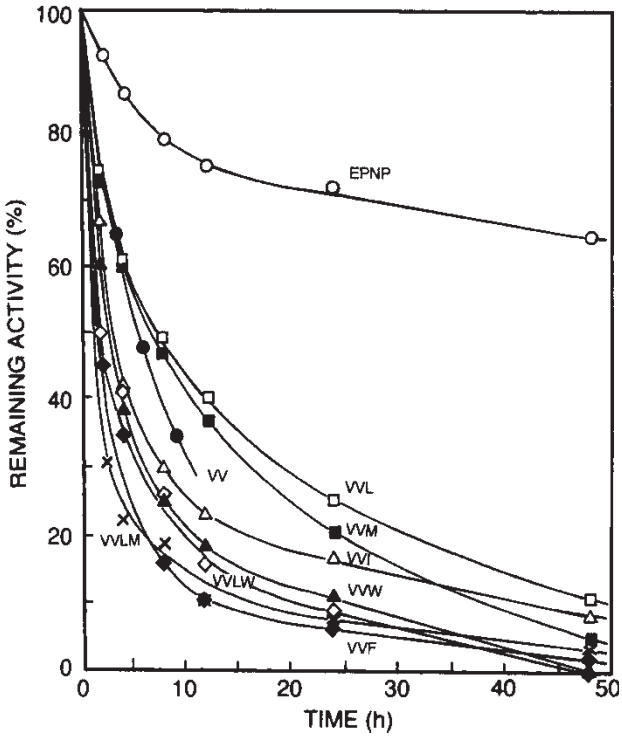

Figure 3. Time courses of inhibition of porcine pepsin A by the new inhibitors and EPNP. ○, EPNP; ●, Inhibitor VV; □, Inhibitor VVL; ■, Inhibitor VVM; △, Inhibitor VVI; ▲, Inhibitor VVW; ◆, Inhibitor VVF; ◇, Inhibitor VVLW; and ×, Inhibitor VVLM.

HPLC retention times of the inhibitors are summarized in Table I. Each inhibitor gave a molecular weight essentially identical with the theoretical one and an expected amino acid composition, and also gave a single peak on HPLC.

The peptide Iva-Val-Val constitutes part of the pepstatin A molecule and was shown to bind to the S3-S1 subsites of pepsin A when pepstatin forms a tight complex with the enzyme [20]. This peptide portion was therefore thought to bind strongly to pepsin. However, Iva-Val-Val-OH and Iva-Val-Val-N_2H_3 showed rather weak reversible inhibition of pepsin A; about 1 mM concentration of each peptide was necessary to obtain over 50% inhibition (data not shown). This result indicated that the binding to the active site of these peptides alone was not so strong and specific as expected. Moreover, the subsites of pepsin A outside S1 and S1' are known to be in general quite non-specific, and to be able to accommodate different types of residues [2]. This suggested the possibility that the inhibitor might bind to the active site cleft more strongly when the peptide portion was C-terminally extended with a bulky hydrophobic or aromatic residue or residues, such as Phe and Leu, which are known to be more preferred than Val as the P1 residue [21,22]. Therefore, such inhibitors were also synthesized.

The time courses of inhibition of pepsin A by these epoxide inhibitors are shown in Figure 3 together with that of EPNP. All the inhibitors prepared in this study were shown to inhibit pepsin A considerably faster than EPNP. Approximate times required for 50% inhibition for the individual inhibitors were as follows: VVLM, 1.3 h; VVF, 1.6 h; VVLW, 2.0 h; VVW, 2.6 h; VVI, 3.0 h; VV, 5.5 h; VVM, 6.8 h; VVL, 7.5 h; and EPNP, >50 h. Inhibitor VV has the structure most similar to part (Iva-Val-Val-Sta, $K_i = \sim 10^{-6}$ M [23]) of pepstatin, in which statin is replaced with N_2H_2-ES-OEt. Therefore, when the Iva-Val-Val portion of Inhibitor VV is bound to the enzyme like that of pepstatin, this N_2H_2-ES-OEt group might come close to the S1-S1' subsite of the enzyme, to be favorable for reaction with the catalytic residue(s). In the case of Inhibitor VV, about 65% of the original activity was lost in 9 h of reaction while the inhibition was about 20% with EPNP (Figure 3).

Many of the other inhibitors tended to show somewhat stronger inhibition than Inhibitor VV as expected. Thus, under the conditions used, over 70% inhibition occurred in 24 h with each inhibitor, whereas it was less than 30% in 24 h with EPNP (Figure 3). Against expectation, Inhibitors VVL and VVM were somewhat less inhibitory than Inhibitor VV. Anyway, it is evident that the peptide moiety of each inhibitor helps to increase the reactivity of the epoxide group more effectively than the *p*-nitrophenoxy moiety of EPNP, although there are some variations in the inhibitory activity among the inhibitors. The differences in the inhibitory activity among the inhibitors may be partly due to some differences in the affinity and mode of binding of the inhibitors to the active site.

Meanwhile, we synthesized some additional inhibitors related with those described above and investigated their inhibitory effects on porcine pepsin A (Ito H, Hirono T, Takahashi K. unpublished). Benzyloxycarbonyl-L-Leu-N_2H_2-ES-OEt was almost as inhibitory as EPNP, indicating the importance of the extended peptide portion in the epoxide inhibitors for stronger inhibition. On the other hand, the compound L-trans-EtO-ES-L-Val-L-Val-L-Leu, which has the epoxide moiety at the N-terminus of the peptide, hardly inhibited pepsin. Thus it seems important to have the epoxide moiety at the C-terminus; the peptide portion of the above compound might not be able to effectively bind to the S1'-S3' subsite of pepsin. Furthermore, we synthesized DAN-analogs, diazoacetyl-L-Leu-L-Val-L-Val-O-benzyl (or methyl) ester and diazoacetyl-L-Val-L-Val-L-Leu-O-benzyl (or methyl) ester, which however inhibited pepsin rather weakly. Under the conditions where DAN inhibited pepsin A almost completely, these compounds inhibited the enzyme to the extent of only 10-30%.

The sites of reaction of EPNP have been elucidated so far for porcine pepsin [9], penicillopepsin [24,25], bovine chymosin [26], and rhizopuspepsin [27], and in most cases EPNP was shown to react fairly

specifically with the catalytic Asp residues corresponding to Asp32 and Asp215 in porcine pepsin A. The reaction involves specific esterification of the β-carboxyl group of Asp by the epoxy group of EPNP as shown in Figure 1c, resulting in irreversible inhibition of the enzyme. In the present study, the site(s) of reaction of the inhibitors in the pepsin molecule has not been determined; however it should be reasonable to assume that they could most probably esterify the active site Asp32 or Asp215.

Taken together, the novel series of inhibitors synthesized in the present study and similar epoxide inhibitors are thought to be useful as active-site-directed irreversible inhibitors not only of pepsin A but also of aspartic proteinases in general. The site(s) of reaction of each inhibitor in pepsin A and other aspartic proteinases remains to be elucidated in future studies.

Acknowledgements

This study was supported in part by grants-in-aid for Scientific Research from the Japan Society for the Promotion of Science.

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