Relationship between Structure and Papain Inhibitory Activity of Epoxysuccinyl Amino Acid Derivatives*

Masaharu TAMAI, Takashi ADACHI, Kiyoshi OGUMA, Shigeo MORIMOTO,
Kazunori HANADA, Sadafumi OHMURA and Masahiro OHZEKI

Research Laboratories of Taisho Pharmaceutical Co., Ltd.,
1-403, Yoshinocho, Omiya, Saitama 330, Japan

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A number of amino acid derivatives of DL-*trans*-epoxysuccinic acid, with a general formula of $R_1O-ES-AA-OR_2$ (ES, DL-*trans*-epoxysuccinyl group; AA, amino acid residue) were newly synthesized and used for the study of structure-activity relationships of papain inhibition. Branched-alkyl amino acids, such as Leu, Ile and Val, as AA and hydrogen or an alkyl group substituted with a phenyl or cycloalkyl group as R_1 were desirable for activity, respectively. However, R_2 or the optical activities of ES and AA not so much influenced on the activity.

Research on the physiological function of mammalian thiolproteases has been promoted recently.^{1~8)} Potent inhibitors having high specificity may be used in therapy of diseases caused by abnormal elevation of proteolytic activities and may serve as a powerful tool for biological analysis to make the physiological roles of these proteases clear.

In the previous papers, the biochemical properties and the structure of E-64, a specific thiolprotease inhibitor obtained from a mold, were clarified.^{4,5)} As described before, thiolproteases such as papain,⁴⁾ calcium activated neutral protease⁶⁾ and cathepsin B⁷⁾ were inhibited strongly but other thiol enzymes and proteases were not affected by E-64. It consists of one mol each of L-leucine, agmatine and (2*S*,3*S*)-*trans*-epoxysuccinic acid, as shown in Fig. 1. It was clarified that *trans*-epoxysuccinic acid was essential for the activity and

its di- and monoesters also inhibited thiolproteases.⁸⁾

FIG. 1. Structure of E-64.

In the present paper, the preparation of various kinds of amino acid derivatives of DL-*trans*-epoxysuccinic acid and the structure-activity relationship are described. Moreover, the diastereoisomers of some derivatives were resolved by silica gel column chromatography and the effects of the L(2*S*,3*S*)- and D(2*R*,3*R*)-*trans*-epoxysuccinic acid moieties of the derivatives against papain were examined.

Figure 2 shows the synthetic scheme of the amino acid derivatives of DL-*trans*-epoxysuccinic acid. The monoesters of this acid were prepared by the same method described previously.⁸⁾ They were coupled to various amino acid esters in the usual manner used for peptide synthesis to give compound I ($R_1O-ES-AA-OR_2$).

The comparison of the kinds of amino acids for the inhibitory activity against papain is summarized in Table I. The group of amino acids having an alkyl side-chain such as Leu, Ile and Val was the most effective, followed

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Abbreviations: ES, $\begin{array}{c} \text{H} \\ | \\ \text{C} \\ / \quad \backslash \\ \text{O} \quad \text{C} \\ | \quad | \\ \text{H} \quad \text{CO-} \end{array}$; AA,

amino acid residue. Symbols for amino acids and protecting groups are according to the IUPAC-IUB Commission [J. Biol. Chem., 241, 2491 (1966)].

FIG. 2. Synthetic Scheme of the *trans*-Epoxysuccinyl Amino Acid Derivatives.

TABLE I. PAPAIN INHIBITORY ACTIVITIES OF VARIOUS KINDS OF AMINO ACID DERIVATIVES

EtO-ES-AA-OR ₂			
AA	R ₂ =Bz	R ₂ =Me	R ₂ =Et
Gly	142.90		
L-Ala		79.71	
L-Val	9.95		
L-Leu	9.93	3.95	1.28
D-Leu	9.74		
L-Ile	4.30		
L-Ser			84.18
L-Thr	25.44		
L-Pro	138.00		
L-Phe	24.66		9.33
L-Trp			19.10
L-Tyr	24.70		
L-Met		30.00	
L-Glu(OBz)	29.40		
L-Asp(OBz)	65.41		
L-Orn(Z)	47.25		
L-Lys(Z)	76.21		8.86
L-Arg(NO ₂)		50.51	
L-His		45.18	
ID ₅₀ (nmol)			

TABLE II. PAPAIN INHIBITORY ACTIVITIES OF ALKYL AMINO ACID DERIVATIVES

EtO-ES-AA-OBz	
AA	ID ₅₀ (nmol)
-NH-CH ₂ -CO-	142.90
-NH-CH-CH ₂ -CH ₃ -CO-	29.60
-NH-CH-CH(CH ₃) ₂ -CO-	9.95
-NH-CH-(CH ₂) ₂ -CH ₃ -CO-	17.09
-NH-CH-CH ₂ -CH(CH ₃) ₂ -CO-	9.93
-NH-CH-(CH ₂) ₃ -CH ₃ -CO-	14.03
-NH-(CH ₂) ₂ -CO-	146.10
-NH-(CH ₂) ₃ -CO-	310.10
-NH-(CH ₂) ₅ -CO-	116.90

TABLE III. EFFECT OF R₁ ON PAPAIN INHIBITORY ACTIVITY

R ₁ O-ES-L-Lys(Z)-OBz			
R ₁	ID ₅₀ (n mol)	R ₁	ID ₅₀ (n mol)
-K	1.88	-C ₆ H ₅	5.44
-CH ₃	89.55	-CH ₂ -C ₆ H ₅	5.06
-CH ₂ CH ₃	76.21	-CH ₂ -C ₆ H ₄ -F	5.62
-CH ₂ CH=CH ₂	39.10	-CH ₂ -C ₆ H ₄ -CH ₃	5.65
-CH ₂ C≡CH	19.94	-(CH ₂) ₂ -C ₆ H ₅	4.25
-CH(CH ₃) ₂	18.56	-(CH ₂) ₃ -C ₆ H ₅	10.92
-(CH ₂) ₂ CH ₃	13.18	-C ₆ H ₁₁	1.32
-CH(CH ₃)CH ₂ CH ₃	7.45	-CH ₂ -C ₆ H ₁₁	1.57
-(CH ₂) ₃ CH ₃	5.98	-CH ₂ -C ₆ H ₁₃	1.80
-CH ₂ C(CH ₃) ₃	3.57	-(CH ₂) ₂ -C ₆ H ₁₃	1.06
-CH ₂ -C(CH ₃) ₂ -C(CH ₃) ₂ -CH ₃	7.08	-(CH ₂) ₃ -C ₆ H ₁₃	2.56
		-(CH ₂) ₄ -C ₆ H ₁₃	1.62

by the group of aromatic amino acids such as Phe and Tyr. The effect of the alkyl side-chain group is shown in Table II. Lengthening of the alkyl chain had more effect and a branched-alkyl group was more favorable than a straight-alkyl group. Table II also shows that the best location of the amino group is the α -position. From these results, it was found that an α -amino acid having a branched-

alkyl side-chain showed potent activity.

Table III shows the effect of R₁ in compound I on the inhibitory activity. When R₁ and ES formed esters, the activity was highly dependent on the kind of R₁. The lower alkyl esters of ES were considerably less potent as inhibitors of papain, but lengthening of the alkyl-chain, especially the branched-alkyl group, resulted in elevation of their activities. The phenyl-

alkyl and cycloalkyl esters of ES showed higher activities than the simple alkyl esters did, but the additional substituent group on the phenyl had no influence on the activity. Among them, the cyclohexyl-alkyl esters were more favorable for strong activity. From the above results, it seems likely that the spread and the steric factor of R_1 are more important than the length. This is similar to the results of the structure-activity relationship study of esters in the previous paper. On the other hand, when R_1 and ES formed the carboxylate ion, they exhibited potent activity, the intensity of which was identical to or more potent than cyclohexyl-alkyl esters.

The effect of R_2 in compound I on the inhibitory activity is shown in Table IV. Among these esters the ethyl group was most effective, although their influence was far less than that of R_1 . In addition, when R_1 was the carboxylate ion, R_2 was favorable if not the free form.

Table V shows the effects of the blocking groups (Z and NO_2) of the basic amino acid derivatives on the activity. Generally, removal of the blocking groups resulted in a decrease in some activity. However, when R_1 and R_2 were the free forms (OH or OK) together, the activity was elevated by deblocking of the basic group. It seems likely that the neutralization of R_2 with the formed basic group produces the effect shown in Table IV, that is, protection of the carboxyl group of the amino acid elevates the activity when R_1 is the carboxylate ion.

The stereoisomers of EtO-Leu-OBz were resolved by silica gel column chromatography. The configuration of ES was determined from the specific rotation and PMR spectrum (chemical shifts of two methin protons on an oxirane ring) and shown in Table VI. The (2*S*,3*S*)-ES-L-Leu derivative showed stronger activity than the (2*R*,3*R*)-ES-L-Leu derivative, but in the D-Leu series the (2*R*,3*R*)-ES derivative was superior to that of (2*S*,3*S*)-ES one. Among the four isomers, the (2*S*,3*S*)-ES-L-Leu derivative which had the same configuration as natural E-64 was the best. But the differences among them were rather small.

TABLE IV. EFFECT OF R_2 ON PAPAIN INHIBITORY ACTIVITY

EtO-ES-L-Leu-OR ₂		KO-ES-L-Leu-OR ₂	
R ₂	ID ₅₀ (nmol)	R ₂	ID ₅₀ (nmol)
Me	3.95	K	0.63
Et	1.28	Et	0.37
<i>n</i> -Pro	2.03	Bz	0.51
<i>n</i> -Bu	2.62	KO-ES-L-Phe-OR ₂	
<i>t</i> -Bu	1.62		
Bz	9.93	K	4.96
NH ₂	6.94	Et	0.52

TABLE V. EFFECTS OF THE BASIC GROUPS OF L-ARGININE AND L-ORNITHINE DERIVATIVES ON PAPAIN INHIBITORY ACTIVITY

R ₁ O-ES-L-Arg(X)-OR ₂				R ₁ O-ES-L-Orn(X)-OR ₂			
R ₁	R ₂	X	ID ₅₀ (nmol)	R ₁	R ₂	X	ID ₅₀ (nmol)
K	K	NO ₂	152.7	H	Et	Z	0.47
H	H	—	76.2	H	Et	—	9.92
K	Me	NO ₂	0.41	Me	Me	Z	45.74
K	Me	—	2.91	Me	Me	—	95.87

TABLE VI. PAPAIN INHIBITORY ACTIVITIES OF FOUR STEREOISOMERS OF *trans*-EPOXYSUCCINYL-LEUCINE DERIVATIVES
EtO-ES-Leu-OBz

Configuration of ES	Configuration of Leu	Chemical Shifts of 2- and 3-Methine Protons (PMR)	$[\alpha]_D^{25}$ ($c=1$, EtOH)	ID ₅₀ (nmol)
2 <i>R</i> , 3 <i>R</i>	L(<i>S</i>)	3.44, 3.64	-83.6	14.34
2 <i>S</i> , 3 <i>S</i>	L(<i>S</i>)	3.40, 3.64	+31.5	7.82
2 <i>R</i> , 3 <i>R</i>	D(<i>R</i>)	3.40, 3.64	-31.9	9.05
2 <i>S</i> , 3 <i>S</i>	D(<i>R</i>)	3.44, 3.64	+86.1	10.98

TABLE VII. RELATIVE ACTIVITIES TO E-64 OF POTENT EPOXYSUCCINYL AMINO ACID DERIVATIVES

Compounds	ID ₅₀ (nmol)	Relative activity to E-64(%)
KO-ES-L-Leu-OEt	0.37	59
KO-ES-L-Leu-OBz	0.51	43
BzO-ES-L-Leu-OEt	0.40	55
KO-ES-L-Ile-OBz	0.48	46
KO-ES-L-Phe-OEt	0.52	42
KO-ES-L-Orn(Z)-OEt	0.47	48
KO-ES-L-Arg(NO ₂)-OMe	0.41	54
E-64	0.22	100

Table VII shows the relative activities to

FIG. 3. Similarity of the Inhibitors to the Specific Substrate of Papain.

E-64 of the derivatives having high potency in this investigation. Judging from the above results, it was necessary for exhibition of strong activity that AA was a branched-alkyl amino acid such as Leu, Ile or Val, and R_1 was the free form or an alkyl group substituted with a cyclic compound such as a phenyl or cyclo-alkyl group, and furthermore, R_2 was an ethyl group.

We described previously that E-64 inhibited papain irreversibly⁴⁾ and the *trans*-epoxysuccinic acid moiety was essential for the activity.⁸⁾ The results obtained in the present investigation suggest that the structure around ES also contribute to the inhibitory activity. Therefore, it is necessary to take into account the competitive effects of the inhibitor as an analogue of the peptide substrate. Figure 3 shows schematically the similarity of the inhibitors to the specific substrates of papain. Berger *et al.*⁹⁾ reported that the Phe residue showed strong affinity for the subsite S_2 and a peptide containing Phe as the second residue from the C-terminal was a strong competitive inhibitor of papain. It seems reasonable that the effect of a benzyl or cyclohexyl group corresponds to that of the Phe residue.

There are many reports about the affinity for subsite S_1 or S_2 , but little about that for S_1' . From the results shown in Table I and Fig. 3, it can be considered that an amino acid residue, AA, corresponds to position P_1' , that is, the branched-alkyl amino acids have strong affinity for subsite S_1' .

The preponderance of the carboxylate form, however, can not be accounted for reasonably enough by the above interpretation. We can

speculate that the carboxylate of ES participates in the dissociation of the ion pair formed between Cys²⁵ and His^{159, 10, 11)} thus potentiating the subsequent reaction of the active thiol group. We feel that this problem requires further investigation.

We have obtained some other derivatives available for further discussion on the structure-activity relationship. The details will be reported in a subsequent paper.

EXPERIMENTAL

Assay method of papain inhibitory activity. Inhibitory activity against papain was determined by the method described previously.⁴⁾

General analyses. PMR and MS spectra were recorded on a Hitachi-Perkin-Elmer R-20 spectrometer and a Shimadzu LKB-9000 mass spectrometer, respectively. Optical rotation was measured with a Japan Spectroscopic DIP-4 automatic polarimeter.

*Acid chlorides.** To a stirred solution of the potassium salt of DL-*trans*-epoxysuccinic acid monoester (10 mmol) and triethylamine (11 ml) in ethylether (10 ml), was added dropwise oxalylchloride (11 mmol) in ethylether (10 ml) under ice cooling, and stirring continued at room temperature for 3 hr. After removing a deposited white precipitate, the solvent was evaporated to give the oily acid chloride.

Coupling procedure-1. To a stirred solution of an amino acid ester (10 mmol, acidic group of Asp and Glu, and basic group of Orn, Lys and Arg were blocked) and triethylamine (11 ml) in ethylether (60 ml), was added dropwise acid chloride (10 mmol) in ethylether (12 ml) at 0°C, and stirring continued at room temperature for 3 hr. And then the reaction mixture was poured into ice-water and extracted with ethylacetate. The organic layer was washed with 2N HCl, sat. NaHCO₃ solution and water successively and dried over anhyd. MgSO₄. After evaporation of the solvent, the residue was purified by silica gel column chromatography to give compound I.

Coupling procedure-2. To a stirred solution of amino acid ester *p*-toluensulfonate (or amino acid ester hydrochloride, 10 mmol), DL-epoxysuccinic acid monoester* (10 mmol), *N*-methylmorpholine (10 mmol) and 1-hydroxysuccinimide (or 1-hydroxybenzotriazol, 12 mmol) in dimethylformamide (or tetrahydrofuran, 40 ml) was added dropwise dicyclohexylcarbodiimide (or 1-ethyl-(3-diethylaminopropyl)carbodiimide, 11-

* See Fig. 2.

mmol) in dimethylformamide (10 ml), at $-5 \sim -10^{\circ}\text{C}$, and stirring continued at room temperature for 2 hr. The reaction mixture was allowed to stand overnight at room temperature. The post-treatment of this reaction mixture was similar to that of coupling procedure-1.

Ester-exchange reaction. To a solution of diester* (1 mmol) in various alcohols (30 ml) was added two drops of conc. H_2SO_4 under ice cooling, and the mixture was stirred at room temperature for a few hr. The reaction mixture was poured into water and extracted with ethylacetate. The organic layer was washed with sat. NaHCO_3 solution and water, and then dried over anhyd. MgSO_4 . After evaporation of the solvent, the residue was purified by silica gel column chromatography to give another diester.

Hydrolysis procedure-1. To a solution of diester* (1 mmol) in ethanol (5 mmol) was added dropwise alcoholic KOH (1 mmol) under ice cooling to give the monopotassium salt* as a crystal (or a precipitate). In this reaction, the ester of the amino acid residue was changed into the ethyl ester. When water-acetone was used as a solvent, no ester-exchange reaction occurred.

Hydrolysis procedure-2. To a solution of diester* (1.7 mmol) in ethanol (3 ml) and water (1 ml) was added dropwise KOH (5.1 mmol) in water (3 ml) under ice cooling, and the mixture was stirred at room temperature for 2 hr. Additional ethanol gave the dipotassium salt* as a crystal.

Catalytic reduction procedure. A solution of blocked amino acid derivatives (1 mmol) with benzyloxy-carbonyl, a nitro or benzyl ester group in methanol

(16 ml), acetic acid (4 ml) and water (2 ml) was stirred vigorously under a hydrogen atmosphere in the presence of 5% Pd-C or Pd-black (0.15 g) at room temperature for 3~5 hr. After removal of the catalyst by filtration, the filtrate was evaporated. The aqueous solution of the residue was washed with ethylacetate and evaporated to give the corresponding deblocked compound.

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* See Fig. 2.