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SYMMETRICAL ANHYDRIDE-TYPE SERINE PROTEASE INHIBITORS: STRUCTURE-ACTIVITY RELATIONSHIP STUDIES OF HUMAN CHYMASE INHIBITORS

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Abstract: We prepared a potent and relatively selective human chymase inhibitor **9** (-), based on the study of SAR of a symmetrical anhydride-type serine protease inhibitor **1**. Kinetic studies suggested that **9** (-) reacts with the Ser residue at the active site of the enzyme, forming a stable acyl enzyme complex. We also showed the importance of the tri-substituted β-amino acid structure for the potent anti-enzymatic activity.

Chymase is a chymotrypsin-like serine protease found in mast cells.[1] Although several studies have suggested the possible involvement of chymase in inflammatory diseases such as arthritis, skin disorders or allergy, the physiological and pathological roles of chymase have not yet been elucidated.[2] Recently, chymase of several species, including humans, dogs, monkeys and hamsters, has been shown to generate angiotensin II from its inactive precursor angiotensin I.[3,4] Specific inhibitors of chymase are probably suitable tools for investigation of the physiological functions of chymase and are potentially useful therapeutically. Several anhydride inactivators of serine proteases such as enol lactones,[5] isatoic anhydride,[6] and isocoumarines,[7] have been reported. These inhibitors are involved in acyl transfer to the active site Ser residue in the enzymes as a substrate, which generates a stable acyl-enzyme. We have recently described the synthesis of *N*-modified tri-substituted β-amino acid anhydride **1** [2,2-dimethyl-3-(*N*-4-cyanobenzoyl)amino-5-phenylpentanoic anhydride] and examined its inhibitory activities against several serine proteases.[8]

As a basis for the development of a potent and selective human chymase (h-chymase) inhibitor, we investigated in the present study the structure activity relationship (SAR) of **1** in order to elucidate the structural factors responsible for the selective inhibition of h-chymase. We also compared the inhibitory effects of the new derivatives toward other serine proteases, such as bovine pancreatic α-chymotrypsin (α-CT), porcine pancreatic elastase (PPE), human leukocyte cathepsin G (h-CG), and porcine pancreatic trypsin (TRP), with those toward h-chymase. We also investigated the effect of a tri-substituted β-amino acid structure as a potent anti-enzymatic activity.

Chemistry

Preparation of compound **1** derivatives is shown in Scheme 1. The α,α-dimethyl-β–monosubstituted β-amino acids **2**,[9] which were synthesized according to the ester-imine condensation[10] and subsequent acid hydrolysis, were coupled with benzoyl-, 4-cyanobenzoyl- or butanoly-chloride to obtain the corresponding *N*-acylated tri-substituted β-amino acids **3**. PyBroP reagent was used for the synthesis of the targeted symmetrical anhydride from **3**.[11] The final anhydrides were then purified by column chromatography on silica.

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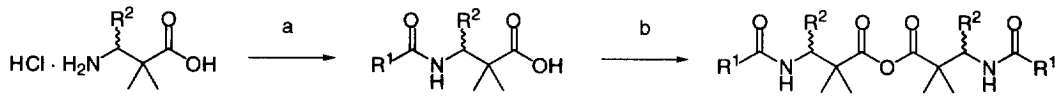
Scheme 1



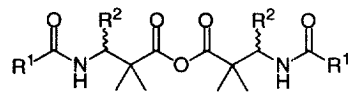
Reagents and conditions : a) benzoyl-, 4-cyanobenzoyl- or butanoly-chloride, pyridine, 0°C, overnight; b) PyBroP reagent, Et_3N, CH_2Cl_2, 0°C, 3 h.

Results and Discussion

Structure-activity relationship studies

Although **1** showed a markedly potent inhibitory activity against α-CT and h-CG, with an IC_{50} value of 2.1 and 1.4 nM, respectively, its inhibitory effect on recombinant human chymase was less potent (IC_{50} = 270 nM, Table 1). To increase the inhibitory activity and selectivity toward h-chymase, we synthesized a series of derivatives of **1** by focusing on two positions on its structure. These were a 4-cyanophenyl group and a β-substituent (phenylethyl) at the tri-substituted β-amino acid residue in **1**. The IC_{50} values[12] of the synthesized derivatives against h-chymase, α-CT, h-CG, PPE and TRP are summarized in Table 1.

Table 1. Inhibition of serine proteases by derivatives of **1**



			IC_{50} (nM)[a]			
compd	R^1	R^2	h-chymase	α-CT	h-CG	PPE	TRP
1	4-CN-Ph[b]	2-phenylethyl	270 ±43[c]	2.1± 0.6	1.4 ± 0.2	36 ± 5.3	300 ±39
5	4-CN-Ph	Ph	13000	15 ± 3.5	15 ± 2.0	210 ±10	1800
6	4-CN-Ph	2-Me-Ph	8700	190 ±66	190 ±40	2100	18000
7	4-CN-Ph	2-Cl-Ph	47000	250 ±51	140 ±25	1100	8200
8	4-CN-Ph	3-cyclohexenyl	130 ±18	220 ±80	69 ± 7.3	180 ±36	1600
9	Ph	2-phenylethyl	20 ± 4.5	18 ± 2.8	64 ± 5.7	64 ± 8.9	2700
10	Ph	ethyl	37 ± 5.1	72 ± 4.9	160 ±36	210 ±18	2200
11	Ph	*n*-propyl	58 ± 8.6	49 ±18	75 ±11	58 ± 7.5	8800
12	Ph	*n*-butyl	5.0 ± 0.3	5.3± 0.6	6.4 ± 0.5	34 ± 5.7	2900
13	Ph	*n*-pentyl[d]	13 ± 1.1	31 ± 3.5	48 ± 2.5	61 ± 3.6	3900
14	Ph	*n*-hexyl	32 ± 2.9	44 ± 3.9	80 ± 4.4	180 ±32	3300
15	*n*-propyl	2-phenylethyl	2600	530 ±71	520 ±53	65 ± 6.6	1800
16	*n*-propyl	*n*-pentyl	29000	810 ±110	1600	55 ± 3.9	18000

[a] IC_{50} values of chymostatin for chymase, α-chymotrypsin and cathepsin G, elastatinal for elastase and leupeptin for trypsin are 0.44, 0.02, 0.025, 0.22 and 0.44 μM, respectively. [b] 4-CN-Ph : 4-cyanophenyl. [c] Values are means ± SEM of three experiments. When the mean value was higher than 1 μM, SEM value was omitted. [d] *n*-pentyl : *normal*-pentyl.

In the first SAR study, substitution of the 2-phenylethyl group (R^2 site) of **1** with the phenyl group (**5**) resulted in a decrease in the inhibitory activity (about 5-50 fold) against all tested enzymes, especially against h-chymase, and **5** was about 50 fold less active than **1**. These results suggest that the pocket for the R^2 site may be

less tolerable with regard to the position of the aromatic ring. Compounds **6** and **7**, which have more balky 2-methyl- and 2-chlorophenyl groups at the R^2 site, also showed reduced inhibitory activities against all proteases. However, when the 3-cyclohexenyl group was introduced at this site (compound **8**), anti-chymase activity was slightly (2-fold) increased compared with that of **1** with a large reduction in the inhibitory activity (5-100 fold) against other tested proteases, suggesting that the aliphatic structure is preferred to aromatic ring at this site for selective activity to chymase.

In the second SAR study, we examined the effects of substituting the 4-cyanophenyl group (R^1 site). Compound **9**, in which the 4-cyano group of **1** was eliminated, exhibited inhibitory activity that was 10 times more potent than **1** against h-chymase and a marked loss of activity against other enzymes, suggesting that modification of the R^1 site affects chymase selectivity on enzyme inhibition. Replacement of this cyano group by -Cl, $-NO_2$ or $-OCH_3$, however, resulted in a decrease in the inhibitory activity, particularly against h-chymase (data not shown), and compounds in which the 4-cyanophenyl group was replaced with alkyl groups such as the *n*-propyl group (**15** and **16**) also showed a marked decrease in anti-chymase activity. These results suggest that, at the R^1 site, h-chymase prefers an aromatic structure without any substituted groups that affect the distribution of π electrons on the ring.

The results of these SAR studies indicated that the phenyl group at the R^1 site and aliphatic group at the R^2 site are preferable for increasing the inhibitory activity and selectivity toward h-chymase. To prepare a more preferable structure for R^2, we modified the R^2 site of compound **9** with several alkyl groups. The inhibitory activity for h-chymase increased following the introduction of a *normal*-pentyl group (**13**) although the selectivity was the same as that of **9**. Compound **12**, with an *n*-butyl group, exhibited the most potent inhibitory activity against h-chymase (IC_{50} = 5.0 nM) in this series. However, the inhibitory activity of this compound against α-CT and h-CG also increased, indicating reduced selectivity.

Since the 2-phenylethyl group at the R^2 site of compound **1** corresponds to the benzyl group of phenylalanine residue (P1 site) in peptide substrates, it is probable that this group may be recognized by the S1 site of the enzymes. The present results suggest that the S1 site of h-chymase may prefer not only the phenylalkyl structure but also the alkyl group as a counterpart at this position in these symmetrical anhydrides, whereas phenylalkyl structure may be preferable for other serine proteases.

Table 2. Inhibition of serine proteases by enantiomers of compound **9**

	IC_{50} (nM)[a]				
compd	h-chymase	α-CT	h-CG	PPE	TRP
9	20 ± 4.5[b]	18 ± 2.8	64 ± 5.7	64 ± 8.9	2700
9 (+)	91 ± 4.6	87 ± 6.4	250 ±59	15 ± 1.3	490 ±42
9 (-)	5.6 ± 0.5	12 ± 1.3	21 ± 9.0	3300	1200

[a] see Table 1. [b] Values are means ± SEM of three experiments.

Since these symmetrical anhydrides contain a pair of asymmetric carbons, there are three isomers (a couple of enantiomers and a meso form compound). In the next step, we synthesized two enantiomers [(+) and (-) forms] of **9** from each optically active β-amino acid derivative,[13] and evaluated their inhibitory activity against proteases. As shown in Table 2, only **9** (-)[14] exhibited a potent and relatively selective inhibitory activity against h-chymase and a weak activity against PPE, indicating that the anti-PEE activity of **9** is mainly attributed to its (+)

or the meso form. Although **9** (-) was still not a fully selective inhibitor for h-chymase, this compound showed an inhibitory activity (nanomolar level) that was 50 fold more potent than that of our starting compound **1**. Further modification of **9**, including a change from an anhydride structure to other system such as diketone structure with a new S' site recognition, should yield more practical inhibitors for the investigation of the physiological functions of chymase and for therapeutic uses.

Kinetic studies of compound 9

In order to investigate the chymase inhibitory activity of **9**, we performed a series of kinetic studies using hamster chymase (ham-chymase), which has a substrate selectivity similar to h-chymase.[15] The IC_{50} values of **9** against ham-chymase was 6.5 nM, which was almost the same as that against h-chymase (5.6 nM). In competitive binding assays using various concentrations of the chromogenic substrate, *N*-succinyl-Ala-Ala-Pro-Phe-pNA, and analysis using the Lineweaver-Burk method shown in Figure 1, **9** behaved as a competitive inhibitor of chymase. These results suggested that **9** interacts with the active site of chymase and forms a stable acyl-enzyme.

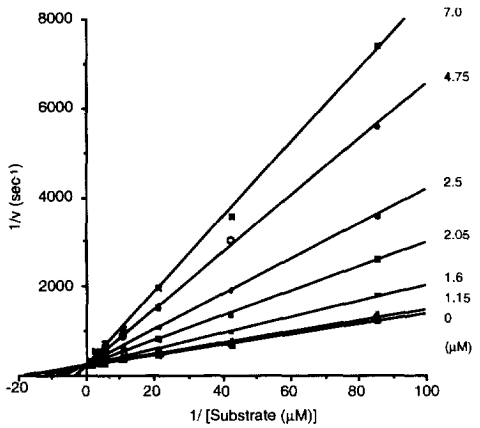


Figure 1.
Competitive inhibition of ham-chymase by **9**

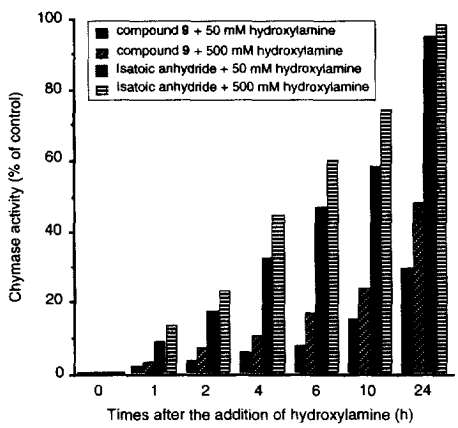


Figure 2.
Recovery of chymase activity by hydroxylamine

To examine such possibility, we compared the recovery of chymase activity from **9**- or isatoic anhydride[6]-inactivated chymase. Ham-chymase, fully inactivated by treatment with **9** or isatoic anhydride, was dialyzed overnight at 4°C against Tris buffer (pH 8.0) and the activity of the recovered chymase was measured by the addition of the chromogenic substrate in the presence of hydroxylamine. As shown in Figure 2, the acyl-chymase formed with **9** was stable against overnight dialysis and a slow deacylation was induced by treatment with hydroxylamine, suggesting that **9** inhibits chymase by forming an irreversible acyl-enzyme, probably by reacting with the hydroxyl group of the active site Ser residue.

As shown in Figure 3A, inhibition of ham-chymase by the (-)-form of **9** was time-dependent and the hydrolysis rate of the substrate did not reach a steady state by the end of the observation period (300 sec), suggesting that **9** (-) is a slow binding inhibitor of chymase. Therefore, Ki and k_{inact} values were calculated by the progress curve method described by Hart and O'Brien.[16] As shown in Figure 3B, the relationship between the reciprocal of [**9** (-)] and $1/\pi$ was linear. In this plot, the x- and y-intercepts represent -1/Ki and $1/k_{inact}$,

respectively. The estimated Ki and k_{inact} values of **9** (-) were 1.45×10^{-7} M and 0.031 s^{-1}, respectively, with a k_{inact}/ Ki value of 220000 $M^{-1}s^{-1}$.

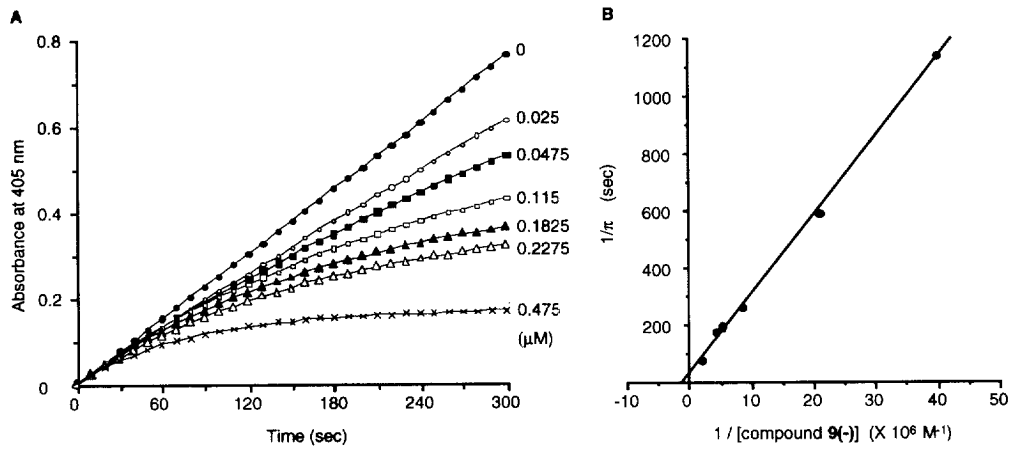
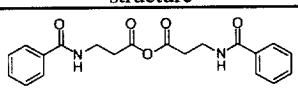
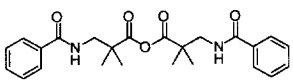
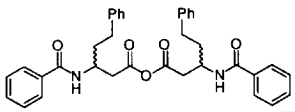


Figure 3. Time-dependent inhibition of ham-chymase by compound **9** (-). A; Progress curves of the substrate hydrolysis. B; 1/ [I] vs.1/ π plot.

Importance of tri-substituted β-amino acid structure

To understand the importance of α,α-dimethyl-β-monosubstituted β-amino acid structure, we synthesized compounds **17-19**, which were devoid of part of the tri-substituted β-amino acid structure of **9**, using almost the same method described in Scheme 1, and examined their anti-enzymatic activities. As shown in Table 3, these compounds showed very weak inhibitory activity, compared to **9**, against h-chymase, α-CT, h-CG and PPE. These results indicate that both the α,α-dimethyl group and β-monosubstitution in tri-substituted β-amino acids are important for the potent inhibitory activity.

Table 3. Inhibition of serine proteases by *N*-benzoyl-β-amino acid anhydrides

		IC_{50} (nM)[a]				
compd	structure	h-chymase	α-CT	h-CG	PPE	TRP
17		40000	4600	3100	1900	1500
18		3000	140 ± 23	170 ± 28	2600	990 ± 75
19		$>10^6$	10000	21000	$>10^6$	$>10^6$

[a] see Table 1. Values are means ± SEM of three experiments.

We have recently [9] shown that in peptidomimetics with a tri-substituted β-amino acid structure, the free rotation of its backbone structure is prevented by the steric repulsion caused by three side chains of the tri-

substituted β-amino acid residue, which is sandwiched between two amide bonds. Therefore, it is probable that, in the anhydrides described here, the tri-substituted β-amino acid structure also plays a role in restricting the conformational flexibility of anhydrides and fixation of the spatial position of the side chain at the β-position that may interact with S1 site of the enzyme.

In conclusion, we described here the synthesis of a series of new anhydride-type inhibitors against chymotrypsin-like serine proteases. The results of SAR showed that **9** (-) was a very potent and relatively selective inhibitor of human and hamster chymase, and the kinetics studies suggested that this compound reacts with the active site Ser residue of the enzyme, forming a stable acyl enzyme. The tri-substituted β-amino acid structure in these anhydride-type compounds was important for the potent inhibitory effect of these compounds against chymotrypsin-like serine proteases. The structural factors responsible for the selective inhibition of chymase that were identified in the present study will be utilized to develop more selective inhibitors necessary for elucidating the physiological roles of chymase.

References and Notes

1. Lagunoff, D.; Benditt, E. P. *Ann. NY Acad. Sci.*, **1963**, *103*, 185.
2. Schechter, N. M.; Fräki, J.E.; Geesin, J. C.; Lazarus, G.S. *J. Biol. Chem.* **1983**, *258*, 2973.
3. Okunishi, H.; Miyazaki, M.; Okamura, T.; Toda, N. *Biochem. Biophys. Res. Commun.* **1987**, *149*,1186.
4. Urata, H.; Kinoshita, A.; Misono, K. S.; Bumpus, F. M.; Husain, A. *J. Biol. Chem.* **1990**, *265*, 22348.
5. Baek, D.-J.; Reed, P. E.; Daniels, S. B.; Katzenellenbogen, J. A. *Biochemistry* **1990**, *29*, 4305.
6. Moorman, A. R.; Abeles, R. H. *J. Am. Chem. Soc.* **1982**, *104*, 6785.
7. Hernandez, M. A.; Powers, J. C.; Glinski, J.; Oleksyszyn, J.; Vijayalakshmi, J.; Meyer, Jr. E. F. *J. Med. Chem.* **1992**, *35*, 1121.
8. Ito, K.; Igarashi, K.; Muramatsu, M.; Harada, T.; Hayashi, Y.; Katada, J.; Uno, I. *Biochem. Biophys. Res. Commun.* **1997**, *240*, 850.
9. Hayashi, Y.; Katada, J.; Harada, T.; Tachiki, A.; Iijima, K.; Takiguchi, Y.; Muramatsu, M.; Miyazaki, H.; Asari, T.; Okazaki, T.; Sato, Y.; Yasuda, E.: Yano, M.; Uno, I.; Ojima, I. *J. Med. Chem.* **1998**, *41*, 2345.
10. Ha, D.; Hart, D. J.; Yang, T. *J. Am. Chem. Soc.* **1984**, *106*, 4819.
11. PyBroP reagent; Bromo-tris-pyrrolidino-phosphonium hexafluorophosphate. Coste, J.; Dufour, M.-N.; Pantaloni, A.; Castro, B. *Tetrahedron Lett.* **1990**, *31*, 669.
12. Enzyme assay; The inhibitory effects of each compound on the enzymatic activities of five serine proteases were evaluated using the purified enzymes and chromogenic substrates. The enzymes and those substrates used here were as follow, *N*-succinyl-Ala-Ala-Pro-Phe-pNA (1.5 mM) for human recombinant chymase and bovine pancreatic α-chymotrypsin (Sigma Type I-S; 47 ng/mL); *N*-succinyl-Ala-Ala-Pro-Phe-pNA (3 mM) for human leukocyte cathepsin G (Elastin Products Co., Inc.; 2.5 U/mL); *N*-succinyl-Ala-Ala-Ala-pNA (1 mM) for porcine pancreatic elastase (Sigma Type III; 0.8 mg/mL); Bz-L-Arg-pNA (1 mM) for porcine pancreatic trypsin (Wako Pure Chemicals; 1 U/mL). All experiments were carried out using 50 mM HEPES buffer (pH 7.4) containing 0.1 mM NaCl, except for the buffer used for chymase, which contained 50 mM Tris-HCl (pH 8.0) and 1 M KCl.
13. Each enantiomer of 2,2-dimethyl-3-(*N*-benzoyl)amino-5-phenylpentanoic acid was isolated by HPLC using chiral column (CHIRALCEL OD, 10 × 250 mm) eluted with a mixture of hexane and 2-propanol (3:1) and was used for the anhydride formation according to the procedure shown in Scheme 1.
14. **9** (-) [(-)-2,2-dimethyl-3-(*N*-benzoyl)amino-5-phenylpentanoic anhydride] : white solid; mp 60-62 °C; ^{1}H NMR (270 MHz, $CDCl_3$) δ 1.18 (s, 6H), 1.31 (s, 6H), 1.68-1.83 (m, 2H),1.89-2.01 (m, 2H), 2.74-2.85 (m, 2H), 3.03-3.13 (m, 2H), 3.51 (dd, *J* = 3.4, 11.2 Hz, 2H), 7.17-7.34 (m, 10H), 7.42-7.56 (m, 6H), 8.06-8.11 (m, 4H); $[\alpha]^{25}_D$ = -52.0° (c 0.25, MeOH); Anal. ($C_{40}H_{44}N_2O_5$·0.5hexane) C, H, N.
9 (+) : $[\alpha]^{25}_D$ = +51.0° (c 0.25, MeOH).
15. Takai, S.; Shiota, N.; Yamamoto, D.; Okunushi, H.; Miyazaki, M. *Life Sci.* **1996**, *58*, 591.
16. Hart, G. J.; O'Brien, R. D. *Biochemistry* **1973**, *12*, 2940.