Amidino-Containing Schiff Base Copper(II) and Iron(III) Chelates as a Thrombin Inhibitor

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Four series of Schiff base copper(II) and iron(III) chelates were synthesized from 4-formyl-3-hydroxybenzamidine or 3-formyl-4-hydroxybenzamidine and various L- or D-amino acids. Their inhibitory activities for bovine α-thrombin (abbreviated as thrombin) were determined. The most potent thrombin inhibitor in this series is copper(II) chelate (1g′) derived from 4-formyl-3-hydroxybenzamidine and D-Trp. Its K_i value, 2.7×10^{-8} M, is comparable to that of Argatroban (MD-805), which is a clinically used compound. The iron(III) chelates derived from 4-formyl-3-hydroxybenzamidine and hydrophobic L-amino acids (Val, Ile, Leu, Phe, Trp, Met) also exhibited higher inhibitory potency. It appears that coordination geometry composed of metal ion, amidino group, amino acid side chain is well accommodated to the thrombin active site. From the K_i values of Schiff base metal chelates for thrombin, the structure–activity relationships between the chelates and active site of thrombin were discussed.

Key words   thrombin inhibitor; Schiff base metal chelate; benzamidine derivative; inhibitory potency; structure–activity relationship

Thrombin is a trypsin-like serine proteinase that plays a critical role in the blood coagulation cascade. Thrombin converts fibrinogen into fibrin which forms part of the blood clot,[1,2] and activates other blood coagulation factors such as V, VIII, XIII, and protein C.[3] Moreover, thrombin also activates blood platelets,[4,5] and acts as a mitogen for various cell types.[6—8] Thus, thrombin has become a special target in the design and development of antithrombotic agents.

Various naturally occurring thrombin inhibitors have been found: hirudin (from the leech *Hirudo medicinalis*),[9,10] cyclotheonamide A, B, and nazumamide A (from the marine sponge *Theonella* sp.),[11—14] aeruginosin 298-A, aeruginosins 98-A and 98-B (from the blue-green alga *Microcystis aeruginosa*).[15—17] On the other hand, many compounds having benzamidine or arginine moiety as a partial structure have been synthesized and their inhibitory effects on thrombin examined.[18—23] Argatroban (MD-805) proposed by Okamoto *et al.*[18] is one of most potent synthetic thrombin inhibitors ($K_i = 1.9 \times 10^{-8}$ M) and is clinically used. Complexes of human or bovine α-thrombin with synthetic arginyl peptides have been analyzed by X-ray diffraction studies.[24—30] The X-ray data suggested that the thrombin active site consists of the specificity (S1) pocket, the hydrophobic proximal pocket (P-pocket), and the hydrophobic distal pocket (D-pocket). The guanidino nitrogen atoms of inhibitors form a salt bridge with Asp189 (numbering of the thrombin amino acid residues is based on the chymotrypsinogen nomenclature introduced by Bode *et al.*)[24,28] in S1 pocket, and additional favorable interactions between the inhibitor and D-pocket or P-pocket enhance the inhibitory potency.

Cationic organic molecules such as benzamidine and phenylguanidine derivatives are potent inhibitors of trypsin and trypsin-like enzymes,[31—33] owing to the electrostatic interaction between the cationic group of inhibitor and an anionic residue at S1 site in the trypsin active site. It is known that salicylaldehyde spontaneously forms a very stable Schiff base metal chelate with an α-amino acid and metal ion. Therefore, we designed and synthesized amidino- or guanidino-containing Schiff base metal chelates (Chart 1) as trypsin-like enzyme inhibitors.[34—36] The manifold compounds in the series of **A** and **B** can be prepared by changing the combination of salicylaldehyde derivatives, various α-amino acids, and metal ions, respectively.

Recently we proposed a new binding mode between trypsin active site and amidino-containing Schiff base metal chelate by X-ray diffraction study.[37] The cationic amidino group of chelates was shown to form a salt bridge with the carboxylate of Asp189 in the S1 pocket of trypsin as expected. In addition, it was evidenced that the metal ion and the phenolic oxygen of the Schiff base contribute additional interactions with His57 and Ser195 of trypsin.

In this report, the inhibitory effect of amidino-containing Schiff base copper(II) and iron(III) chelates for thrombin was analyzed and the structure–activity relationship between thrombin and inhibitor was also discussed based on the K_i values.

Results and Discussion

Syntheses and Characterization of Schiff Base Metal Chelates   Syntheses of the amidino-containing Schiff base copper(II) chelates (Chart 2; series **1**) were achieved by mixing equimolar amounts of 4-formyl-3-hydroxybenzamidine hydrochloride, D- or L-amino acids, and copper(II) acetate,
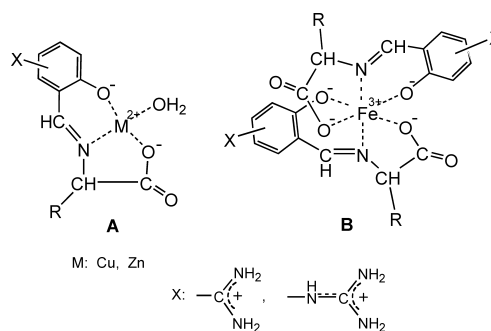

M: Cu, Zn

Chart 1

respectively. Syntheses of the copper(II) chelates (Chart 2; series **2**) were also carried out in a similar manner using 3-formyl-4-hydroxybenzamidine hydrochloride. The corresponding iron(III) chelates (Chart 2; series **3** and **4**) were prepared from 4-formyl-3-hydroxy-benzamidine hydrochloride or 3-formyl-4-hydroxybenzamidine hydrochloride, D- or L-amino acids, and iron(II) acetate, respectively. The formation of the Schiff base metal chelates was ascertained by the appearance of a marked absorption band at 360—380 nm for copper(II) chelates and at 430—480 nm for iron(III)
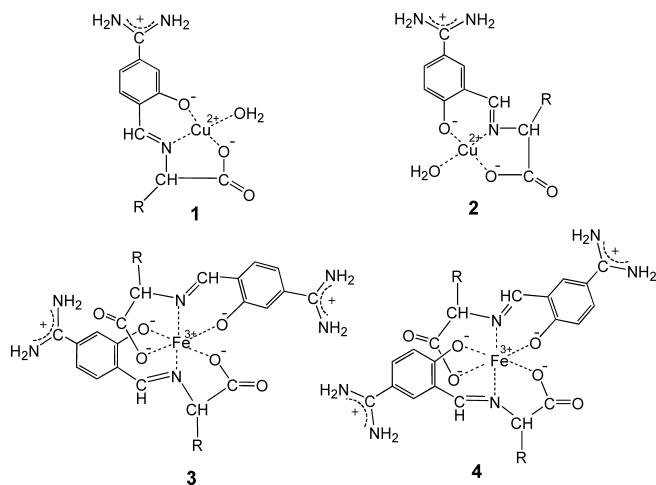

Chart 2

chelates,[34—36)] which can be attributed to a $\pi \rightarrow \pi^*$ transition originating mainly from the azomethine chromophore.

Inhibition Constant (K_i) of Schiff Base Metal Chelates for Thrombin The inhibition constants (dissociation constants) of the Schiff base metal chelates with the thrombin were determined from a rate assay employing benzoyl-L-arginine p-nitroanilide (BAPA) as the substrate, and the K_i values are listed in Table 1. All the chelates behaved as competitive inhibitors for thrombin with K_i values in the range of 10^{-4}—10^{-8} M.

Effect of Metal Ions From the results of our previous studies,[34—36)] the coordination geometry was shown to be an octahedral configuration for iron(III) chelates and a square planar configuration for copper(II) chelates, as is shown in Chart 2. The most striking difference between the iron(III) chelates and the copper(II) chelates is the presence of a second Schiff base ligand in iron(III) chelates. The iron(III) chelates (**3e**—**i**) derived from 4-formyl-3-hydroxybenzamidine hydrochloride and L-amino acid have higher inhibitory potency ($K_i = 10^{-7}$ M) than corresponding copper(II) chelates (**1e**—**i**, 10^{-5}—10^{-6} M), indicating that there is an additional favorable interaction between the thrombin active site and the second Schiff base ligand of iron(III) chelate. The chelates derived from 3-formyl-4-hydroxybenzamidine, however, behaved differently. The inhibitory potency ($K_i = 10^{-4}$—10^{-5} M) of the iron(III) chelates with L-amino acid (**4c**—**i**) and D-amino acid (**4c′**—**i′**) are lower than that ($K_i = 10^{-5}$—10^{-6} M) of corresponding copper(II) chelates **2c**—**i** and **2c′**—**i′**, re-

Table 1. Inhibition Constants of Copper(II) and Iron(III) Chelates for Bovine α-Thrombin-Catalyzed Hydrolysis of Benzoyl-L-arginine p-Nitroanilide at pH 8.2

Amino acid	Copper(II) chelates				Iron(III) chelates			
	Comp. No.	K_i (M)	Comp. No.	K_i (M)	Comp. No.	K_i (M)	Comp. No.	K_i (M)
Gly	**1a**	4.9×10^{-5}	**2a**	8.1×10^{-5}	**3a**	4.7×10^{-5}	**4a**	5.6×10^{-5}
L-Ala	**1b**	2.3×10^{-5}	**2b**	8.5×10^{-5}	**3b**	7.5×10^{-6}	**4b**	2.4×10^{-5}
L-Val	**1c**	1.1×10^{-6}	**2c**	4.2×10^{-5}	**3c**	2.1×10^{-6}	**4c**	2.3×10^{-4}
L-Leu	**1d**	3.1×10^{-6}	**2d**	5.5×10^{-5}	**3d**	8.9×10^{-6}	**4d**	1.9×10^{-4}
L-Ile	**1e**	4.4×10^{-6}	**2e**	8.7×10^{-5}	**3e**	4.6×10^{-7}	**4e**	1.4×10^{-4}
L-Phe	**1f**	1.1×10^{-6}	**2f**	1.8×10^{-5}	**3f**	1.8×10^{-7}	**4f**	2.1×10^{-4}
L-Trp	**1g**	1.0×10^{-6}	**2g**	1.8×10^{-5}	**3g**	7.9×10^{-7}	**4g**	6.8×10^{-4}
L-Met	**1h**	1.5×10^{-6}	**2h**	4.1×10^{-5}	**3h**	4.3×10^{-7}	**4h**	6.1×10^{-4}
L-Ser	**1i**	2.3×10^{-5}	**2i**	5.6×10^{-5}	**3i**	4.1×10^{-7}	**4i**	6.0×10^{-4}
L-Thr	**1j**	1.2×10^{-5}	**2j**	4.3×10^{-5}	**3j**	1.5×10^{-6}	**4j**	7.5×10^{-5}
L-Asn	**1k**	$a)$	**2k**	$a)$	**3k**	3.8×10^{-5}	**4k**	4.5×10^{-5}
L-Gln	**1l**	1.4×10^{-5}	**2l**	3.2×10^{-5}	**3l**	5.9×10^{-6}	**4l**	1.5×10^{-5}
L-Lys	**1m**	2.3×10^{-5}	**2m**	1.2×10^{-5}	**3m**	1.9×10^{-5}	**4m**	1.6×10^{-5}
L-Arg	**1n**	2.3×10^{-5}	**2n**	4.7×10^{-6}	**3n**	4.0×10^{-5}	**4n**	5.1×10^{-5}
L-His	**1o**	1.3×10^{-6}	**2o**	1.1×10^{-5}	**3o**	1.9×10^{-5}	**4o**	4.5×10^{-5}
D-Ala	**1b′**	1.4×10^{-5}	**2b′**	1.2×10^{-5}	**3b′**	9.5×10^{-5}	**4b′**	8.9×10^{-5}
D-Val	**1c′**	3.2×10^{-5}	**2c′**	7.6×10^{-5}	**3c′**	1.4×10^{-5}	**4c′**	1.5×10^{-4}
D-Leu	**1d′**	1.0×10^{-5}	**2d′**	9.6×10^{-6}	**3d′**	2.5×10^{-5}	**4d′**	1.3×10^{-4}
D-Ile	**1e′**	1.4×10^{-5}	**2e′**	5.0×10^{-6}	**3e′**	6.4×10^{-5}	**4e′**	2.8×10^{-4}
D-Phe	**1f′**	4.0×10^{-5}	**2f′**	6.1×10^{-6}	**3f′**	3.4×10^{-5}	**4f′**	1.4×10^{-4}
D-Trp	**1g′**	2.7×10^{-8}	**2g′**	4.0×10^{-6}	**3g′**	8.8×10^{-6}	**4g′**	1.8×10^{-4}
D-Met	**1h′**	1.3×10^{-5}	**2h′**	1.1×10^{-5}	**3h′**	3.4×10^{-5}	**4h′**	6.0×10^{-5}
D-Ser	**1i′**	2.7×10^{-5}	**2i′**	1.5×10^{-5}	**3i′**	2.8×10^{-5}	**4i′**	7.7×10^{-5}
D-Thr	**1j′**	1.1×10^{-5}	**2j′**	1.4×10^{-5}	**3j′**	2.4×10^{-5}	**4j′**	7.1×10^{-5}
D-Asn	**1k′**	$a)$	**2k′**	1.4×10^{-5}	**3k′**	3.2×10^{-5}	**4k′**	1.2×10^{-5}
D-Gln	**1l′**	3.0×10^{-5}	**2l′**	$a)$	**3l′**	$a)$	**4l′**	1.6×10^{-5}
D-Lys	**1m′**	1.7×10^{-5}	**2m′**	3.3×10^{-5}	**3m′**	7.6×10^{-5}	**4m′**	6.8×10^{-5}
D-Arg	**1n′**	3.3×10^{-5}	**2n′**	3.1×10^{-6}	**3n′**	5.7×10^{-5}	**4n′**	8.0×10^{-5}
D-His	**1o′**	7.8×10^{-6}	**2o′**	4.4×10^{-5}	**3o′**	1.5×10^{-5}	**4o′**	5.9×10^{-5}

$a)$ Not measured, since the solubility of the chelate was low.

spectively, as shown in Table 1. This result indicates that the second Schiff base ligand in the iron(III) chelates may have unfavorable interaction with the thrombin active site for the compounds of series **4**.

Effect of the Position of the Amidino Group In this study, we used two amidinosalicylaldehyde isomers, 4-formyl-3-hydroxybenzamidine and 3-formyl-4-hydroxybenzamidine. Difference of the position of the amidino group in the salicylaldehyde caused a dramatic change in the metal chelating structure, as shown in Chart 2. The amino acid side chain (R group) of series **1** is situated to the left of copper(II) ion, as shown in Chart 2. In contrast, for the compounds of series **2**, the side chain (R group) of amino acid moiety is to the right of copper(II) ion. When the amidino group of chelate forms a salt bridge with the carboxylate of Asp189 at the thrombin active site, the localization of the R group for the two thrombin–chelate complexes was different. Therefore, the effect of position of the R group on inhibitory potency was apparent. This effect did, in fact, appear in the K_i values of copper(II) and iron(III) chelates. The copper(II) and iron(III) chelates (series **1** and **3**) derived from 4-formyl-3-hydroxybenzamidine and L-amino acids exhibit higher inhibitory potency compared to the chelates (series **2** and **4**) derived from 3-formyl-4-hydroxybenzamidine and L-amino acids, as shown in Table 1. Especially, the iron(III) chelates **3c**—**i** have 100—1000 times higher inhibitory potency than chelates **4c**—**i**. Similarly the iron(III) chelates (**3c′**—**i′**) derived from 4-formyl-3-hydroxybenzamidine and D-amino acids exhibit higher inhibitory potency than the chelates (**4c′**—**i′**) derived from 3-formyl-4-hydroxybenzamidine and D-amino acids. In the copper(II) chelates with D-amino acid, however, this tendency was not appreciable. The result suggests that the conformation of the thrombin active site is complementary for the chelates derived from 4-formyl-3-hydroxybenzamidine to a greater extent than those derived from 3-formyl-4-hydroxybenzamidine.

Effect of Amino Acid Inhibitory effect of the side chains and absolute configuration of the α-carbon was examined using both L- and D-enantiomers for 20 different α-amino acids. The Asp, Glu, Cys, Tyr, and Pro, however, did not form the Schiff base metal chelate. A control experiment was carried out with chelates with Gly (**1a**—**4a**), and their K_i values were found to be less than 10^{-5} M. Both copper(II) and iron(III) chelates (**1c**—**h**, **3c**—**h**) derived from 4-formyl-3-hydroxybenzamidine and hydrophobic L-amino acids (Val, Leu, Ile, Phe, Trp, Met) exhibited a strong inhibitory effect on thrombin activity ($K_i = 10^{-6}$—10^{-7} M). In contrast, for the copper(II) and iron(III) chelates (**1c′**—**h′**, **3c′**—**h′**) derived from D-amino acid, no pronounced inhibitory potency was seen except for chelate **1g′** from D-Trp, indicating that L-configuration has better interaction with the thrombin active site than D-configuration for compounds of series **1** and **3**. No such phenomenon was observed, however, in the copper(II) and iron(III) chelates (series **2** and **4**).

The low inhibitory potency ($K_i = 10^{-4}$ M) of the iron(III) chelates (**4c**—**i**, **4c′**—**i′**) for thrombin suggests that octahedrally coordinated iron(III) chelates derived from 3-formyl-4-hydroxybenzamidine and amino acid with bulky side chain could not be accommodated in the thrombin active site, *i.e.*, unfavorable interactions with the active site residues occured.[36]

Structure–Activity Relationship The copper(II) chelate **1g′** derived from 4-formyl-3-hydroxybenzamidine and D-Trp is the most potent inhibitor tested in this study. Its K_i value is comparable to that (1.9×10^{-8} M) of Argatroban (MD-805) which is a clinically used compound.

Various X-ray diffraction studies[24—30] of thrombin-inhibitor clarified that the thrombin active site is mainly defined by the specificity S1 pocket, the hydrophobic P-pocket, and the hydrophobic D-pocket, as is shown in Figs. 1 and 2. The S1 pocket consists of the carboxylate of Asp189 and backbone carbonyl in the bottom of the pocket. The P-pocket consisted of the Tyr60A and Trp60D[38] side chains, the imidazole ring of His57 of the catalytic triad, and the isobutyl group of Leu99, while the D-pocket consisted of the indole ring of Trp215 side chain, Ile174 side chain, and Glu97A-Leu99 side chains.

Nilsson *et al.* recently reported the X-ray crystal structure of thrombin-inhibitor complex, which contains benzamidine moiety.[30] Based on this structure, the schematic model for the most potent compound, **1g′**, binding to the thrombin active site is shown in Fig. 1A. The strongly basic amidino
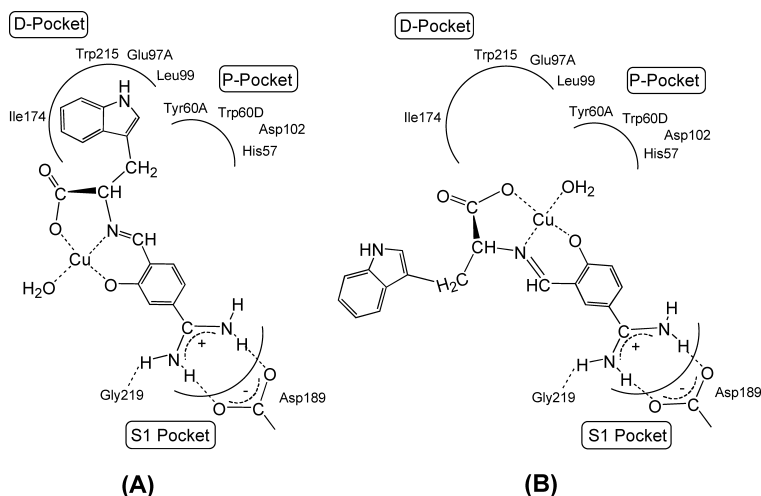

Fig. 1. Binding Modes of Thrombin-**1g′** (A) and Thrombin-**2g′** (B)

This model was described based on the thrombin-inhibitor binding model in ref. 30.
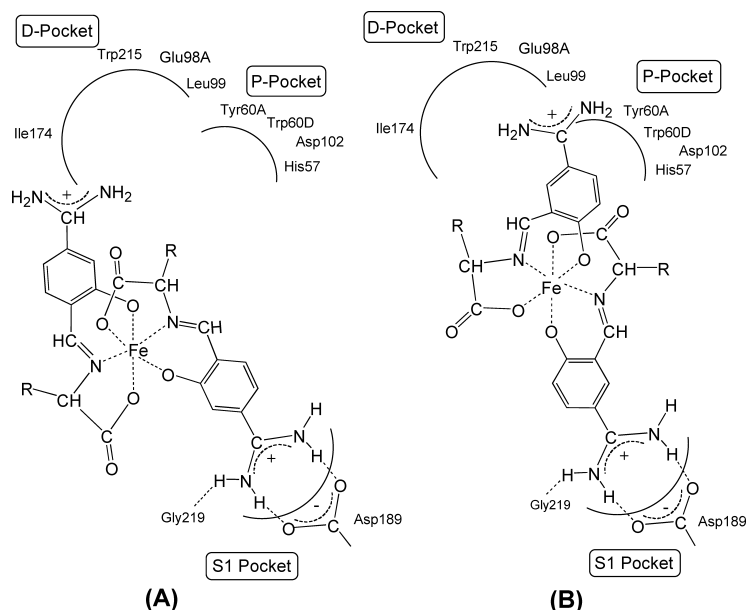
Fig. 2.   Binding Modes of Thrombin-**3** (A) and Thrombin-**4** (B)
This model was described based on the thrombin-inhibitor binding model in ref. 30.

group in **1g′** forms a salt bridge with carboxylate of Asp189, and forms a hydrogen bond with carbonyl oxygen of Gly219 in the S1 pocket. Then the indole ring of **1g′** results in close contact with the D-pocket. The location of the indole ring of **1g′** must allow better fits into the D-pocket than that of **1g**. On the other hand, the amino acid side chain of **2g′** oriented away from the D-pocket, as shown in Fig. 1B. Therefore, no interaction could occur between this side chain and the D-pocket. This can explain why amino acid side chains have no remarkable effect on K_i value in series **2** chelates.

In the iron(III) chelates, chelates **3c—i** exhibited strong inhibitory potency, whereas those of **4c—i** were decreased. The amino acid side chains in chelates **3c—i** probably cause favorable interaction with the P-pocket, whereas that in chelates **4c—i** cause unfavorable interactions with the P-pocket and D-pocket in the thrombin active site, as shown in Fig. 2.

In this study we found a potent thrombin inhibitor **1g′**. Further details of structural requirements for the thrombin-**1g′** complex using the X-ray diffraction method will be described in a future paper.

Experimental

Materials and Instruments   All chemicals for synthetic work were purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.), Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan), and Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Amino acids were purchased from Peptide Institute Inc. (Osaka). Bovine α-thrombin (EC 3.4.21.5) was purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). Absorption spectra were recorded on a U-2000 spectrophotometer (Hitachi).

Synthesis of Inhibitors   *p*-Amidinosalicylidene-L-amino acidato)(aqua)copper(II) (**1b—o**), *p*-amidinosalicylidene-D-amino acidato)(aqua)copper(II) (**1b′—o′**), *m*-amidinosalicylidene-L-amino acidato(aqua)copper(II) (**2b—o**), and *m*-amidinosalicylidene-D-amino acidato)(aqua)copper(II) (**2b′—o′**) were synthesized according to the procedure reported for *p*- or *m*-amidinosalicylidene-L-alaninato(aqua)copper(II) (**1b, 2b**).[34] Bis(*p*-amidinosalicylidene-L-amino acidato)iron(III) (**3b—o**), bis(*p*-amidinosalicylidene-D-amino acidato)iron(III) (**3b′—o′**), bis(*m*-amidinosalicylidene-L-amino acidato)iron(III) (**4b—o**), and bis(*m*-amidinosalicylidene-D-amino acidato)iron(III) (**4b′—o′**) were synthesized according to the procedure

reported for bis(*p*-amidinosalicyliden-L-alaninato)iron(III) or bis(*m*-amidinosalicylidene-L-alaninato)iron(III) (**3b, 4b**).[35]

Determination of Inhibitory Constant (K_i) of Inhibitors   Enzyme activity was determined in 50 mM Tris–HCl buffer (pH 8.2) using BAPA as a substrate. Determination of K_i values was carried out as follows: Concentrations of the inhibitor and thrombin used in the kinetic analysis were 10^{-4}—10^{-7} M and 10^{-6} M, respectively. Hydrolytic rates in the presence of inhibitor were determined and the K_i values were calculated according to the method of Dixon[39] using a linear regression program.

References and Notes
1)   Blombäck B., Blombäck M., Hessel B., Iwanaga S., *Nature* (London), **215**, 1445—1448 (1967).
2)   Blombäck B., Hessel B., Hogg D., Therkildsen L., *Nature* (London), **275**, 501—505 (1978).
3)   Fenton J. W. II, *Ann. N.Y. Acad. Sci.*, **370**, 468—495 (1981).
4)   Davey M. G., Lüsher E. F., *Nature* (London), **216**, 857—858 (1967).
5)   Shuman M. A., Levine S. P., *J. Clin. Invest.*, **61**, 1102—1106 (1978).
6)   Gurwitz D., Cunningham D. D., *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 3440—3444 (1988).
7)   Vu T.-K. H., Hung D. T., Wheaton V. I., Coughlin S. R., *Cell*, **64**, 1057—1068 (1991).
8)   McNamara C. A., Sarembock I. J., Gimple L. W., Fenton J. W. II, Coughlin S. R., Owens G. K., *J. Clin. Invest.*, **91**, 94—98 (1993).
9)   Markwardt F., *Methods Enzymol.*, **19**, 924—932 (1970).
10)   Rydel T. J., Tulinsky A., Bode W., Huber R., *J. Mol. Biol.*, **221**, 583—601 (1991).
11)   Fusetani N., Matsunaga S., Matsumoto H., Takebayashi Y., *J. Am. Chem. Soc.*, **112**, 7053—7054 (1990).
12)   Maryanoff B. E., Qiu X., Padmanabhan K. P., Tulinsky A., Almond H. R., Jr., Andrade-Gordon P., Greco M. N., Kauffman J. A., Nicolaous K. C., Liu A., Brungs P. H., Fusetani N., *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 8048—8052 (1993).
13)   Fusetani N., Nakao Y., Matsunaga S., *Tetrahedron Lett.*, **32**, 7073—7074 (1991).
14)   Hayashi K., Hamada Y., Shioiri T., *Tetrahedron Lett.*, **33**, 5075—5076 (1992).
15)   Murakami M., Okita Y., Matsuda H., Okino T., Yamaguchi K., *Tetrahedron Lett.*, **35**, 3129—3132 (1994).
16)   Murakami M., Ishida K., Okino T., Okita Y., Matsuda H., Yamaguchi K., *Tetrahedron Lett.*, **36**, 2785—2788 (1995).
17)   Rios Steiner J. L., Murakami M., Tulinsky A., *J. Am. Chem. Soc.*, **120**,

597—598 (1998).

18) Okamoto S., Hijikata A., Kikumoto R., Tonomura S., Hara H., Ninomiya K., Maruyama A., Sugano M., Tamao Y., *Biochem. Biophys. Res. Commun.*, **101**, 440—446 (1981).

19) Stürzebecher J., Prasa D., Hauptmann J., Vieweg. H., Wikström P., *J. Med. Chem.*, **40**, 3091—3099 (1997).

20) Wagner J., Kallen J., Ehrhardt C., Evenou J.-P., Wagner D., *J. Med. Chem.*, **41**, 3664—3674 (1998).

21) Steinmetzer T., Renatus M., Künzel S., Eichinger A., Bode W., Wikström P., Hauptmann J., Stürzebecher J., *Eur. J. Biochem.*, **265**, 598—605 (1999).

22) Hauel N. H., Nar H., Priepke H., Ries Uwe., Stassen J.-M., Wienen W., *J. Med. Chem.*, **45**, 1757—1766 (2002).

23) Lee K., Park C. W., Jung W.-H., Park H. D., Lee S. H., Chung K. H., Park S. K., Kwon O. H., Kang M., Park D.-H., Lee S. K., Kim E. E., Yoon S. K., Kim A., *J. Med Chem.*, **46**, 3612—3622 (2003).

24) Bode W., Mayr I., Baumann U., Huber R., Stone S. R., Hofsteenge J., *EMBO J.*, **8**, 3467—3475 (1989).

25) Bode W., Turk D., Stürzebecher J., *Eur. J. Biochem.*, **193**, 175—182 (1990).

26) Banner D. W., Hadvary P., *J. Biol. Chem.*, **266**, 20085—20093 (1991).

27) Brandstetter H., Turk D., Hoeffken H. W., Grosse D., Stürzebecher J., Martin P. D., Edwards B. F. P., Bode W., *J. Mol. Biol.*, **226**, 1085—1099 (1992).

28) Bode W., Turk D., Karshikov A., *Protein Science*, **1**, 426—471 (1992).

29) Tabernero L., Chang C. Y., Ohringer S. L., Lau W. F., Iwanowicz E. J., Han W.-C., Wang T. C., Seiler S. M., Roberts D. G. M., Sack J. S., *J. Mol. Biol.*, **246**, 14—20 (1995).

30) Nilsson J. W., Kvarnström I., Musil D., Nilsson I., Samulesson B., *J. Med. Chem.*, **46**, 3985—4001 (2003).

31) Mares-Guia M., Shaw E., *J. Biol. Chem.*, **240**, 1579—1585 (1965).

32) Mares-Guia M., Shaw E., Cohen W., *J. Biol. Chem.*, **242**, 5777—5781 (1967).

33) Mares-Guia M., Shaw E., *J. Biol. Chem.*, **242**, 5782—5788 (1967).

34) Toyota E., Chinen C., Sekizaki H., Itoh K., Tanizawa K., *Chem. Pharm. Bull.*, **44**, 1104—1106 (1996).

35) Toyota E., Miyazaki H., Itoh K., Sekizaki H., Tanizawa K., *Chem. Pharm. Bull.*, **47**, 116—119 (1999).

36) Toyota E., Sekizaki H., Itoh K., Tanizawa K., *Chem. Pharm. Bull.*, **51**, 625—629 (2003).

37) Toyota E., Ng K. K. S., Sekizaki H., Itoh K., Tanizawa K., James M. N. G., *J. Mol. Biol.*, **305**, 471—479 (2001).

38) Nine overlapping amino acid residues are numbered as the same 60th residue based on chymotrypsinogen number. Tyr60A and Trp60D represent tyrosine and tryptophan at the 1st and 4th sequential position of the overlapping nona-peptide, respectively.

39) Dixon M., *Biochem. J.*, **55**, 170—171 (1953).