Application of Spontaneous Schiff Base Copper Chelates Formation Process to the Design of a Trypsin Inhibitor

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Salicylaldehyde derivatives carrying an amidinium group react spontaneously with α-amino acids in copper-containing aqueous media to afford very stable Schiff base copper chelates. A variety of Schiff base chelates were prepared from various α-amino acids. Each α-amino acid provides enantiomeric isomers due to the asymmetric α-carbon, except for glycine. Inhibitory activity of these amidinium chelates toward trypsin was generally very strong. Thus these compounds represent a novel series of potent trypsin inhibitors. The structure–activity relationship of the inhibitors is discussed based on their inhibition constants, though the variations are not large.

Key words Schiff base copper chelate; trypsin; synthetic inhibitor; benzamidine derivative

Studies of the mechanisms of enzyme catalysis have mostly been carried out using proteolytic enzymes. Trypsin is the enzyme studied most intensively because of its distinct substrate specificity. Analysis of the interaction between the enzyme active site and synthetic small molecular competitive inhibitors or substrates has afforded useful information on the characteristics of the enzymatic process. Cationic organic molecules such as amidine and guanidine derivatives are potent inhibitors of trypsin, owing to electrostatic interaction between the cationic group of inhibitor and an anionic residue at the enzyme binding site. Hydrophobic interaction between the hydrocarbon moiety of an inhibitor and the binding cavity of enzyme also significantly affects the binding affinity.

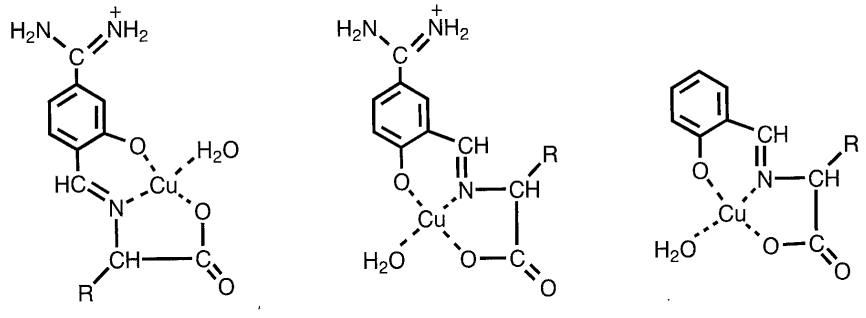
Studies on trypsin-specific compounds can aid the design of clinically useful compounds, since a variety of physiologically important enzymes such as plasmin, thrombin and urokinase have trypsin-like specificity. In this regard we have found a new series of compounds which were termed as "inverse substrates" for trypsin.[1]

We were also interested in the well-known formation of very stable chelates from α-amino acids, salicylaldehyde and copper ion. The process was applied for immobilization of protein on an insoluble support, affording a new method for protein immobilization.[2,3] The method is based on the fact that Schiff bases composed of an α-amino acid and salicylaldehyde are not stable in aqueous media, but dissociate into the original components, while both components are associated with each other *via* a highly stable copper(II) chelate, of which the dissociation constant is as small as 10^{-15} M, when copper(II) is present.[4] The spontaneously formed immobilized protein could be released from the matrix by the addition of ethylenediaminetetraacetate (EDTA). This reversible process has also been applied to the resolution of racemic α-amino acid mixtures.[5]

In the present paper, an application of the spontaneous chelate formation process to the preparation of a variety of trypsin inhibitors is described. Schiff base copper chelates carrying an amidino group (**1**, **2**) have been prepared and the structure–inhibitory activity relationship of these derivatives has been studied. The inhibitory activity of Schiff base copper chelate lacking an amidine group (**3**) has also been examined.

Synthesis of the amidine-containing chelate (**1**) was carried out by mixing equimolar amounts of 4-formyl-3-hydroxybenzamidine hydrochloride, an α-amino acid and copper acetate as follows: Copper acetate hydrate (100 mg, 0.5 mmol) was added to a solution of α-amino acid (0.5 mmol) and 4-formyl-3-hydroxybenzamidine hydrochloride (100 mg, 0.5 mmol) in 5 ml of water. The reaction mixture was stirred at 50—60 °C for several hours and kept at room temperature overnight. The resulting greenish crystalline powder was collected and recrystallized from water. Chelates prepared as above were characterized by infrared and ultraviolet(visible) absorption spectroscopy and also by elemental analysis. Synthesis of the chelate (**2**) was carried out in a similar manner using 3-formyl-4-hydroxybenzamidine hydrochloride.

The absorption spectrum of **1** (prepared from L-



1 2 3

Chart 1

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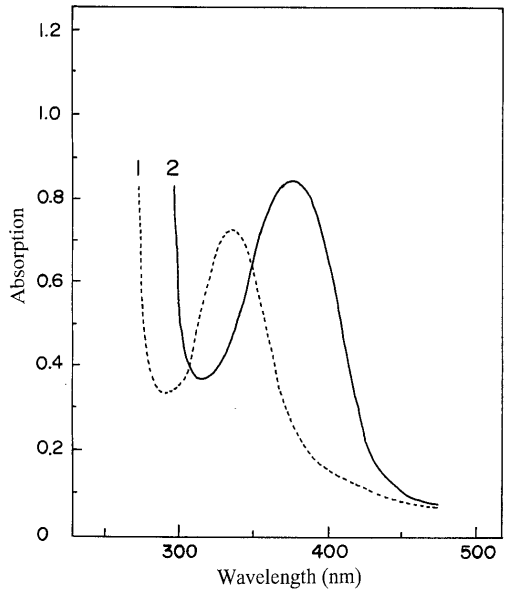

Fig. 1. Absorption Spectra of 4-Formyl-3-hydroxybenzamidine Hydrochloride and Schiff Base Copper Chelate in Water

1, 4-Formyl-3-hydroxybenzamidine hydrochloride (0.2 mM); 2, 4-formyl-3-hydroxybenzamidine hydrochloride (0.2 mM), L-Phe (0.2 mM) and copper(II) ion (0.2 mM).

Table 1. Inhibition Constants of a Series of Chelates Derived from Amidinosalicylaldehyde for Trypsin-Catalyzed Hydrolysis of Benzoyl-L-arginine *p*-Nitroanilide at pH 8.0

1		**2**	
Inhibitor R=	K_i (M)	Inhibitor R=	K_i (M)
L-Ala	4.2×10^{-6}	L-Ala	2.8×10^{-6}
L-Val	4.5×10^{-6}	L-Val	3.2×10^{-6}
L-Leu	7.4×10^{-6}	L-Leu	2.8×10^{-6}
L-Phe	4.6×10^{-6}	L-Phe	1.4×10^{-6}
L-Met	3.7×10^{-6}	L-Met	1.1×10^{-6}
L-Glu	3.2×10^{-5}	L-Glu	3.5×10^{-6}
L-Ser	1.1×10^{-5}	L-Ser	2.4×10^{-6}
D-Ala	1.5×10^{-5}	D-Ala	2.8×10^{-6}
D-Val	2.0×10^{-5}	D-Val	3.1×10^{-6}
D-Leu	1.0×10^{-5}	D-Leu	2.0×10^{-6}
D-Phe	1.5×10^{-5}	D-Phe	3.5×10^{-6}
D-Met	2.7×10^{-5}	D-Met	3.7×10^{-6}
D-Ser	1.0×10^{-5}	D-Ser	2.4×10^{-6}
D-Glu	3.0×10^{-5}	D-Glu	5.7×10^{-6}
Gly	8.2×10^{-6}	Gly	3.2×10^{-6}
4-Formyl-3-hydroxy benzamidine	2.8×10^{-5}	3-Formyl-4-hydroxy benzamidine	1.2×10^{-5}

phenylalanine; $R=CH_2C_6H_5$) is shown, together with that of 4-formyl-3-hydroxybenzamidine, in Fig. 1. In our previous study of the chelate derived from salicylaldehyde, formation of the Schiff base copper chelate was ascertained by the appearance of a marked absorption at 360—380 nm. The result in Fig. 1 is consistent with that observation.[6]

Inhibitory activity of amidine-containing chelates toward bovine trypsin was determined according to the reported procedure.[7] The enzyme activity was determined at pH 8.0 using benzoyl-L-arginine *p*-nitroanilide as the substrate. Determination of K_i values was carried out following the method of Dixon.[8] Concentrations of the inhibitors used in the kinetic analysis were in the range of 10^{-5}—10^{-6} M, corresponding to their K_i values. A control experiment was carried out with *N*-salicylideneglycinato(aquo)copper(II) (**3**) lacking an amidinium group. The tryptic activity was entirely unaffected by **3** in this concentration range. At a higher concentration of **3**, competitive inhibition with a K_i value of 2.6×10^{-3} M was found. This value is reasonable for an aromatic compound which may interact hydrophobically with the enzyme active site.[9] Table 1 lists the K_i values for 30 chelates. All the chelates behaved as potent competitive inhibitors. They are all stronger inhibitors than the parent salicylaldehydes. For the trypsin-catalyzed reaction, K_i values in the range of 10^{-6} M indicate exceptionally strong inhibition. The strongest low molecular-weight competitive inhibitor for trypsin so far reported is *p*-aminobenzamidine, with a K_i value of 8.25×10^{-6} M.[10] About two-thirds of the compounds reported in this work were more potent inhibitors than *p*-aminobenzamidine, and the chelate derived from L-Met and 3-formyl-4-hydroxybenzamidine hydrochloride (K_i: 1.1×10^{-6} M) is the most potent inhibitor presently known.

No pronounced difference in the inhibitory activity in relation to the α-substituent was seen. L-Amino acids afforded strong inhibitors with either 3-formyl or 4-formyl derivatives (chelates from 3-formyl-4-hydroxybenzamidine tended to be more potent). Chelates with D-amino acids, however, were considerably different, *i.e.*, chelates in series **1** are relatively less potent. Their K_i values are only slightly better than that of 4-formyl-3-hydroxybenzamidine itself, *i.e.*, in the range of 10^{-5} M.

The amidinium group is the conjugate acid of a strong base. The reported pK_a value for benzamidine hydrochloride is 11.3.[11] Therefore in the medium used for K_i determination (pH 8.0) compounds of series **1** and **2** exist exclusively as protonated forms. It is clear that the electronic character of the amidinium group is similar within **1** and **2** since the Hammett substituent constants for hydroxy and formyl groups are small.[12] However, the isomers differ in the geometrical alignment of the asymmetric carbon atom relative to the amidinophenyl portion. The difference in the positioning of the asymmetric α-carbon atoms between **1** and **2** is 2.2—2.5 Å when their amidinophenyl parts are superimposed. It is assumed that the molecular structure of **2** is preferable for the formation of a tight enzyme-inhibitor complex and that the environment of the enzyme in the region where the asymmetric carbon atom of **1** interacts is critical for the binding affinity, because the K_i values for the compounds of series **1** are variable.

Analysis of the enzyme-inhibitor interaction was carried out by using a trypsin model constructed based on the atom co-ordinate data.[13,14] In the model-fitting, the amidine nitrogen of **1** or **2** was oriented to the carbonyl carbon of the Asp-189 residue at the bottom of the enzyme binding cavity, and the distance between two atoms was kept at 2.9 Å.[14] In addition to this primary electrostatic interaction, hydrophobic and hydrophilic interactions contribute to the binding affinity. Molecular model analysis of the enzyme-inhibitor complex revealed that the

orientation of the hydrocarbon moiety (including the chelate portion) of the inhibitor was not necessarily restricted. The cavity could be sufficiently large to accomodate the inhibitor easily and steric repulsion from the cavity surface is not appreciable. It can be assumed, however, that the inhibitors **1** and **2** exist in a conformation that affords favorable interaction between their hydrocarbon moiety and functional groups in the enzyme binding cavity. Considering the rigid fused ring structure, this could be attained by rotation of the carbon–carbon bond between the amidine group and the benzene ring. The stronger affinity of **1** and **2** than *p*-aminobenzamidine can be explained in terms of the additional, favorable contribution resulting from the hydrocarbon moiety containing the chelate portion and hetero atoms.

Observation of the stronger binding of **1** than **2** may suggest the involvement of a common structure intrinsic to series **1** or **2**. In this respect, it would be interesting to know why an enantiomeric difference of inhibitory activity was seen for series **1**, but not for series **2**. At present, we have no information on the dynamic structure of the inhibitor at the binding cavity. From the analysis of the static structure of the inhibitor and trypsin model, it was concluded that the asymmetric carbon atoms of **1** and **2** differently approach the region composed of amino acid residues His-57, Gln-192, Ser-214, Ser-195, and Gly-216. It seems that the α-carbon atom of **1** cannot come close to these residues, whereas that of **2** can come close to one of these amino acid residues. Further investigation is needed using a variety of compounds.

Experimental

Materials All chemicals for synthetic work were obtained from Wako Pure Chemicals and Aldrich Chemical Co.

Instruments Proton nuclear magnetic resonance (^{1}H-NMR) spectra were obtained on JNM-EX 400 and JNM FX-100 spectrometers (JEOL). Infrared (IR) spectra were recorded on a 270-30 spectrometer (Hitachi) and an IRA-1 spectrometer (JASCO).

Synthesis of 4-Formyl-3-hydroxybenzamidine Hydrochloride 4-Formyl-3-hydroxybenzonitrile (8.68 g, 59.0 mmol) was dissolved in dry ethanol. The solution was saturated with dry hydrogen chloride and stirred at room temperature for 21 h. Removal of the solvent afforded crude ethyl 4-formyl-3-hydroxybenzimidate. The residue was dried in a vacuum desiccator without purification. ^{1}H-NMR (DMSO-d_6) δ: 1.47 (3H,t), 4.6 (2H, q), 7.68 (2H, m), 7.82 (1H, d), 10.37 (1H, s), 11.46 (1H, br s).

4-Formyl-3-hydroxybenzimidate was dissolved in dry ethanol. The solution was saturated with dry ammonia and stirred at room temperature for 24 h. After removal of solvent, the residue was dissolved in water and treated with hydrochloric acid. The water was evaporated and the resulting crystals were collected. Recrystallization from water gave a yellow crystalline powder in 65% yield; mp 190—192 °C. ^{1}H-NMR (DMSO-d_6) δ: 7.29 (1H, d), 7.49 (1H, s), 7.80 (1H, d), 9.49 (4H, d), 10.83 (1H, s), 11.56 (1H, s): IR (Nujol): 1650, 1680, 2960—3240 cm^{-1}. *Anal.* Calcd for $C_8H_9ClN_2O_2$: C, 47.86; H, 4.52; Cl, 17.67; N, 13.96. Found: C, 47.66; H, 4.67; Cl, 17.45; N, 13.77.

Synthesis of 3-Formyl-4-hydroxybenzamidine Hydrochloride Synthesis was carried out in the same manner as above, using 3-formyl-4-hydroxybenzonitrile. Recrystallization from water gave a colorless crystalline powder in 42% yield, mp 213—214 °C (dec.). ^{1}H-NMR (DMSO-d_6) δ: 7.30 (1H, d), 7.98 (1H, dd), 8.17 (1H, d), 9.20 (4H, br d), 10.35 (1H, s), 12.04 (1H, s). IR (Nujol): 1610, 1670, 3000—3360 cm^{-1}. *Anal.* Calcd for $C_8H_9ClN_2O_2$: C, 47.86; H, 4.52; Cl, 17.67; N, 13.96. Found: C, 47.88; H, 4.46; Cl, 17.69; N, 13.89.

Synthesis of *p*-Amidinosalicylidene-L-alaninato(aquo)copper(II) Hemihydrate (1) Preparation of the chelate was carried out following the procedure reported for *N*-salicylideneglycinato(aquo)copper(II).[15] A solution of 0.045 g (0.5 mmol) of L-alanine in 5 ml of water was prepared, and 0.1 g (0.5 mmol) of 4-formyl-3-hydroxybenzamidine hydrochloride was added. The mixture was stirred and heated at about 60 °C. Upon the addition of 0.1 g (0.5 mmol) of copper(II) acetate, the solution became dark green. It was stirred and heated for several hours, and then filtered. Recrystallization of the product from water gave a dark green powder in 34% yield; mp 212—215 °C (dec.). IR (KBr): 1640 cm^{-1}. UV $\lambda_{max}^{H_2O}$ nm (ε): 663 (310), 374 (5610). *Anal.* Calcd for $C_{11}H_{15}ClCuN_3O_{4.5}$: C, 36.67; H, 4.12; N, 11.67. Found: C, 36.50; H, 3.67; N, 11.56.

Synthesis of *m*-Amidinosalicylidene-L-alaninato(aquo)copper(II) (2) Synthesis was carried out in the same manner as above. Yield 57%, mp 230 °C. IR (KBr): 1640 cm^{-1}. UV $\lambda_{max}^{H_2O}$ nm (ε): 672 (310), 347 (5270).

Determination of Inhibitory Activity of 1 and 2 Inhibition constants of **1**, **2**, and **3** toward bovine trypsin were determined according to the reported procedure.[7] The enzyme activity was determined at pH 8.0 in 0.05 M Tris buffer containing 0.02 M $CaCl_2$ using benzoyl-L-arginine *p*-nitroanilide as the substrate. The hydrolytic rates in the presence of the chelate were determined and the reciprocals of the rates were plotted as a function of the chelate concentration following the method of Dixon.[8]

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