New Orally Active Serine Protease Inhibitors

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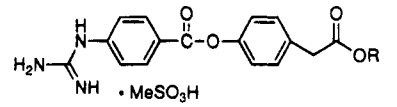
Orally active synthetic inhibitors of serine proteases have attracted a great deal of attention because of their important role in a number of biological processes. Among the serine proteases, those with trypsin-like specificity are most frequently involved in physiological regulation. Trypsin is one of the digestive enzymes and serves to activate its proenzyme as well as the other zymogens in pancreatic tissue. Inhibitors of trypsin could potentially be used for the treatment of pancreatitis, reflux esophagitis after gastrectomy, or hyperproteolytic conditions.[1]

A number of benzamidine,[2] naphthoamidine,[3] arginine,[4] and benzguanidine[5] derivatives have been reported as potent trypsin inhibitors, and several acyl derivatives of 4-hydroxybenzamidine[6] have been reported as "inverse substrates" having an amidine function in their leaving group. However, there have been very few examples of orally active antitrypsin drugs mainly because of their metabolic instability and very poor oral absorption. The guanidinobenzoate protease inhibitor **1a** [(*N,N*-dimethylcarbamoyl)methyl *p*-[(*p*-guanidinobenzoyl)oxy]phenylacetate methanesulfonate, FOY-305)[7] is one notable exception (Chart 1).

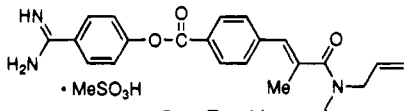
Results and Discussion. In this paper, we report the synthesis and evaluation of oral activity of a new series of benzamidine derivatives, **2a** and **3a**. These inhibitors selectively inactivate trypsin and plasma kallikrein (Table 1).

The preparation of **2a** and **3a** is illustrated in Scheme 1. Synthesis was started with reaction of telephthaladehydic acid esters **4** and **5** with (diethylphosphono)-acetates to produce **6** and **7** after acid-mediated deprotection. Acylation of the corresponding esters of *N*-allylglycine with the acid chlorides derived from **6** and **7** resulted in amides **8** and **9**, respectively, in good yields. Deprotection of the benzyl ester of **8** was successfully carried out under acidic conditions to produce the carboxylic acid **10**, which was esterified with *p*-amidinophenol hydrochloride using DCC to provide one of the target compounds as a hydrochloride salt **3b**. Hydrolysis of the methyl ester of **9** afforded carboxylic acid **11**, which was converted to **13** under the same conditions as described above for **3b**. Deprotection of the *tert*-butyl ester of **13** provided **2b**. Both compounds (**2b** and **3b**) were converted to the corresponding methanesulfonates **2a** and **3a**,[8] respectively.

Chart 1



1a : R = CH_2CONMe_2 (FOY-305)
1b : R = H (FOY-251)



2a : R = H
3a : R = Et

The compounds prepared are listed in Table 1. Benzamidine derivatives **2a** and **3a** were evaluated for their ability to inhibit hydrolysis of each chromogenic substrate by various kinds of serine proteases. These inhibitors (**2a** and **3a**) were found to possess analogous inhibitory spectra. As shown in Table 1, these compounds showed especially good selectivity for trypsin and plasma kallikrein over thrombin, pancreas kallikrein, and plasmin but did not exhibit any inhibitory activity against chymotrypsin, cathepsin G, S. elastase, or P. elastase at 100 μM.

Evaluation of their oral activity in rats was carried out based on the plasma antitrypsin activity which inhibits hydrolysis of a chromogenic substrate, Boc-Phe-Ser-Arg-AMC (AMC = 7-amino-4-methylcoumarin), by trypsin (Figure 1 and 2). Both of the inhibitors (**2a** and **3a**) exhibited good oral activity and long duration of action in rats. Interestingly, only **2a** was detected in rat plasma after oral administration of **3a**.[9] Potency of the plasma antitrypsin activity, which corresponds to

Table 1. *In Vitro* Inhibition[a] of Proteolytic Enzymes by Benzamidine Derivatives **2a** and **3a** and Guanidinobenzoate **1a,b**[b]

	IC_{50} (μM)			
enzyme	**1a**	**1b**	**2a**	**3a**
trypsin	0.0506 ± 0.00152	0.055 ± 0.0031	0.00413 ± 0.000224	0.0112 ± 0.00068
thrombin	31.6 ± 1.62	107.5 ± 1.71	35 ± 3.6	3.66 ± 0.118
pancreatic kallikrein	34.1 ± 3.06	>100	>100	15.7 ± 0.69
plasma kallikrein	1.48 ± 0.110	2.32 ± 0.061	0.0965 ± 0.00185	0.19 ± 0.065
plasmin	2.62 ± 0.190	12.5 ± 1.44	1.83 ± 0.072	0.873 ± 0.0155
chymotrypsin	>100	>100	>100	>100
cathepsin G	>100	>100	>100	>100
sputum elastase	>100	>100	>100	>100
pancreatic elastase	>100	>100	>100	>100

[a] Concentration required to inhibit enzymatic cleavage of the chromogenic substrates by 50%. The following compounds were used as the synthetic substrates of each protease: Boc-Phe-Ser-Arg-AMC for trypsin; H-D-Phe-Pip-Arg-pNA for thrombin; H-D-Val-Leu-Arg-pNA for pancreatic kallikrein; H-D-Pro-Phe-Arg-PNA for plasma kallikrein; H-D-Val-Leu-Lys-pNA for plasmin; Suc-Ala-Ala-Pro-Phe-pNA for chymotrypsin; MeO-Suc-Ala-Ala-Pro-Met-pNA for cathepsin G; MeO-Suc-Ala-Ala-Pro-Val-pNA for sputum elastase; Suc-Ala-Ala-Ala-AMC for pancreatic elastase (AMC = 7-amino-4-methylcoumarin, pNA = *p*-nitroaniline). The assay was carried out according to ref 13. All IC_{50} values were expressed as means ± SEM (n = 4). [b] All compounds discussed here were characterized by ^1H and ^{13}C NMR and IR mass spectrometry, elemental analysis, or high-resolution mass spectrometry.

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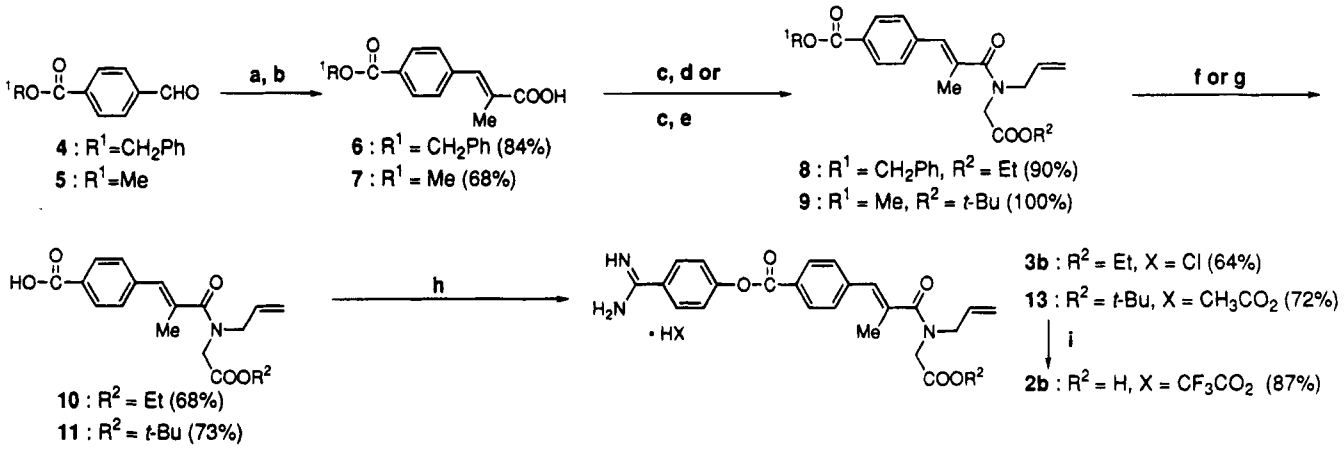
Scheme 1[a]



[a] Reagent: (a) *t*-BuOCOCH$_2$P=O(EtO)$_2$, NaH, THF, 25 °C, 0.5 h; (b) CF_3COOH, anisole, 25 °C, 2 h; (c) $SOCl_2$, reflux, 1 h; (d) *N*-allylglycine ethyl ester, py, CH_2Cl_2, 5–25 °C, 1 h; (e) *N*-allylglycine *tert*-butyl ester, py, CH_2Cl_2, 5–25 °C, 1 h; (f) $MeSO_3H$, anisole, 25 °C, 2 h; (g) NaOH, 1,4-dioxane, 25 °C, 3 h; (h) DCC, 4-amidinophenol hydrochloride, py, 25 °C, 15 h; (i) CF_3COOH, 1.5 h.

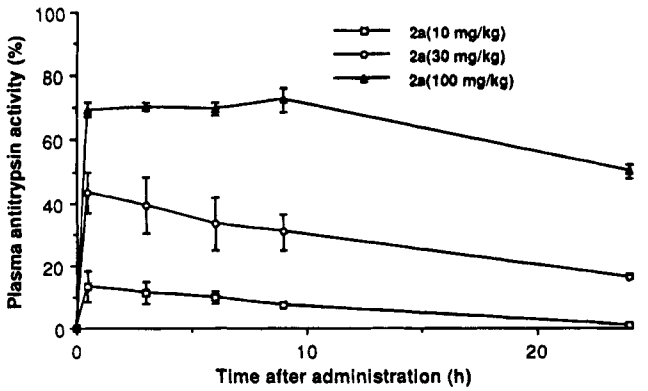


Figure 1. Plasma antitrypsin activity in rats after oral administration of **2a** to rats ($n = 3$). After oral administration of **2a** to fasted rats, blood was taken from the jugular vein at regular intervals. To the plasma (0.1 mL) was added bovine pancreatic trypsin (2 μM/mL, 0.1 mL) followed by the substrate Boc-Phe-Ser-Arg-AMC (0.1 mM, 0.8 mL). The reaction mixture was incubated at 37 °C for 15 min and the reaction quenched by addition of 30% acetic acid (1 mL) to obtain samples for analysis. Fluorescence of the released 7-amino-4-methylcoumarin (AMC) in the samples was measured (excitation, 380 nm; emission, 460 nm) using a HITACHI 850 fluorescence spectrometer. Plasma antitrypsin activity (%) was calculated according to the following equation:

$$\text{inhibition (\%)} = \frac{\text{Pre (F)} - \text{Test (F)}}{\text{Pre (F)}} \times 100$$

where Pre (F) is the fluorescence of the control group (no drug administration) and Test (F) is the fluorescence of the tested group.

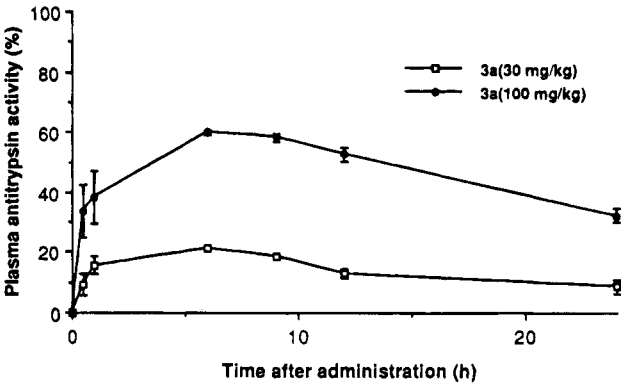


Figure 2. Plasma antitrypsin activity in rats ($n = 3$) after oral administration of **3a**. Plasma antitrypsin activity of **3a** was evaluated as described in the legend of Figure 1.

the plasma concentration of the absorbed inhibitor, was found to be dose-dependent in po studies using rats (Figure 1 and 2).

Oral activities of **2a** and **3a** exhibited clear time differences to reach maximum plasma antitrypsin activity after administration (Figure 1 and 2). Oral administration of **2a** gave the maximum inhibitory activity from 30 min to 9 h (Figure 1), while administration of **3a** showed the maximum activity after 6 h (Figure 2). To obtain more detailed information to explain the difference between the two absorption profiles in rats, plasma concentrations of these inhibitors were measured after their oral administration in beagle dogs (Figure 3).

Compound **2a** exhibited good oral absorption and good AUC in beagle dogs (Figure 3), whereas the corresponding ethyl ester **3a** showed very poor absorption.[10] The maximum plasma concentration of (**3a** + **2a**)[10] after 15 min was less than 1 μg/mL.[11] The detailed *in vitro* examination of their metabolic pathway disclosed a remarkable difference in the metabolic profile of **3a** between rats and dogs. Although rat small intestine homogenate hydrolyzed **3a** to **2a**, that from dogs did not. This crucial difference in the metabolism of these compounds in the small intestine was a key finding in solving the above discrepancy.[12]

The long time lag of **3a** in rats for oral absorption, the *in vitro* enzymatic conversion of **3a** to **2a** in rat small intestine, the inability of dog small intestine to convert **3a** to **2a**, and the very poor absorption of **3a** in dogs suggested to us that the actual orally active compound was **2a**, that **3a** was absorbed orally after its enzymatic conversion to **2a** in rats, and that dogs could not absorb **3a** because of their lack of the enzyme required to convert **3a** to **2a** in their small intestine (Table 2). The time lag for oral absorption of **3a** in rats is presumed to be necessary for the enzymatic conversion of **3a** to **2a**. We also presumed that the longer duration of action of **2a** in rats was largely dependent upon its greater metabolic stability in rat plasma compared to that in dog plasma (Table 2a vs 2b). Another large difference was observed in the metabolism of **3a** in both rat and dog plasma. Rat plasma specifically converted **3a** to **2a** within 1 min. Trace amounts of absorbed **3a** could have been detected as **2a** after oral absorption in rats. Of course, the possibility of the immediate conversion of

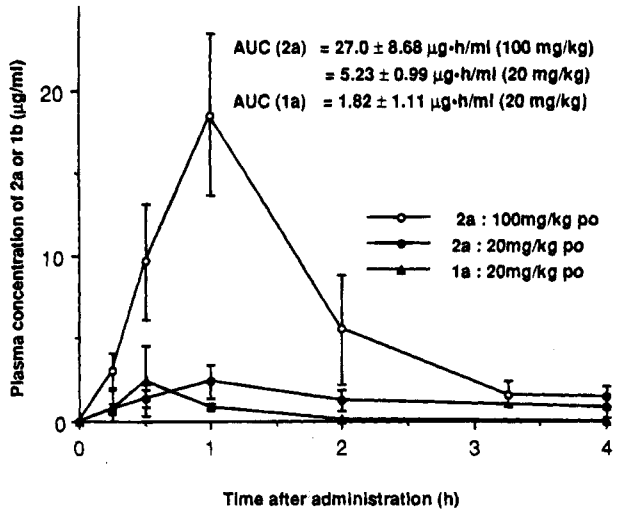


Figure 3. Plasma concentrations of **1a** and **2a** after administration (20 or 100 mg/kg, respectively) to beagle dogs. Values are shown as means ±SD (n = 3). Plasma concentration of **1b** and **2a** in fasted beagle dogs was determined by high-performance liquid chromatography (HPLC) using n-propyl or *iso*-amyl 4-hydroxybenzoate as an internal standard (IS), after plasma (0.2 mL) had been treated with acetonitrile containing 1% methanesulfonic acid (3 mL) at 5 °C and centrifuged for 10 min at 3000 rpm at 5 °C to give protein-free samples. HPLC analyses were carried out using a Uvidec 100III (JASCO) apparatus equipped with a Capcell pak C18 column at 275 nm. A solution consisting of MeOH:H_2O:acetic acid: 5% sodium n-dodesyl sulfate in H_2O–MeOH (1:1):5% sodium n-heptyl sulfate in H_2O–MeOH (1:1) (v/v, 250:170:1:3:8 for **1b**, 330:210:1:3:8 for **2a**) was used as the mobile phase at a flow rate of 1.2 mL/min. The area under the plasma concentration curves (AUC) was calculated by the trapezoidal method.

Table 2. Metabolism[a] of **2a** and **3a** in Rat and Dog Tissue Homogenates

	small intestine homogenate		plasma		liver homogenate	
compd	$t_{1/2}$[b]	products[c]	$t_{1/2}$[b]	products[c]	$t_{1/2}$[b]	products[c]
			(a) Rat			
2a	>60	AP[d] + C[e]	>60	**2a**	>60	**2a**
3a	11	**2a** + AP[d] + **10**	<1	**2a**	<1	**2a**
			(b) Dog			
2a	>60	**2a**	15	AP[d] + C[e]	>60	**2a**
3a	>60	**3a**	25	AP[d] + **10**	<1	**2a**

[a] Enzymatic hydrolysis rates of **2a** and **3a** were evaluated as their half-lives on HPLC analyses described in the legend of Figure 3. **2a** and **3a** in MeOH (1 mg/mL, 40 μL) were treated with tissue samples (2 mL) at 37 °C for 120 min. The reaction mixture sampled at regular intervals was added to acetonitrile containing 1% of methanesulfonic acid (3 mL) at 5 °C and centrifuged at 10 000 rpm for 1 min. [b] $t_{1/2}$ = half-life (min). [c] Structures of all the metabolites were confirmed based on their retention time on HPLC using the synthesized authentic compounds. [d] 4-Amidinophenol. [e] C = *N*-allyl-*N*-(4-carboxy-α-methylcinnamoyl)glycine.

3a to **2a** in rat plasma after absorption cannot be excluded at this stage. However, the presumed similar extent of mucous membrane permeability of rats and dogs would support our estimation of the different absorption profiles of **3a** between rats and dogs.

Dog plasma specifically cleaved the phenol ester moieties of **3a** ($t_{1/2}$ = 25 min) and **2a** ($t_{1/2}$ = 15 min) to produce the inactive products, whereas **2a** in the rat plasma was much more stable. This result was thought to be related to the shorter duration of action of **2a** in dogs. Liver homogenates of both species specifically converted to **3a** to **2a**. Compound **2a** showed good stability ($t_{1/2}$ > 60 min) in both rat and dog liver homogenates.

In conclusion, we have discovered new orally active synthetic inhibitors of trypsin-like serine proteases and disclosed their metabolic profile in rats and dogs. One of these compounds, **2a**, was orally active in both of species tested, while **3a** was presumed to be orally active after its enzymatic conversion to **2a** prior to absorption in the rat small intestine. As a result, **3a** could be predicted to show good oral absorption also in the other species such as pigs and monkeys possessing the enzyme which converts **3a** to **2a** in their intestine. These compounds are expected to be more effective than FOY-305 (**1**) for the treatment of inflammatory diseases such as pancreatitis and reflux esophagitis after gastrectomy mainly because of their longer duration of action. Further biological and pharmacological evaluation of **2a** and **3a** will be the subject of future reports. Further details of structural requirements for their oral absorption will be described in a future paper.

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(8) The methanesulfonates were confirmed by their elemental analyses.

(9) HPLC analysis of the blood plasma was carried out at regular intervals (2 h, 4 h, 6 h).

(10) Two active compounds, **3a** and **2a**, which were presumed to be produced by the metabolism of **3a** with dog liver, were not detected besides the inactive metabolites amidinophenol and **10**.

(11) The limit of detection in HPLC analysis was 50 ng/mL.

(12) Both of the compounds **2a** and **3a** were considered to be stable to metabolism in the stomach. More than 95% of the parent compounds were detected unaffected after treatment with gastric juice or gastric homogenate for 30 min.

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