Synthesis, Structure−Activity Relationships, and Pharmacokinetic Profiles of Nonpeptidic Difluoromethylene Ketones as Novel Inhibitors of Human Chymase

Fumihiko Akahoshi,*,† Atsuyuki Ashimori,† Hiroshi Sakashita,† Takuya Yoshimura,† Masahiro Eda,† Teruaki Imada,† Masahide Nakajima,† Naoko Mitsutomi,† Shigeki Kuwahara,† Tatsuyuki Ohtsuka,† Chikara Fukaya,‡ Mizuo Miyazaki,§ and Norifumi Nakamura#

Drug Discovery Laboratories, Welfide Corporation, 2-25-1, Shodai-Ohtani, Hirakata, Osaka 573-1153, Japan, and Department of Pharmacology, Osaka Medical College, 2-7, Daigaku-cho, Takatsuki, Osaka 569-8686, Japan

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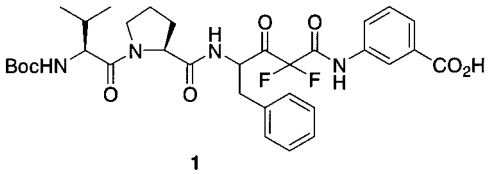
Potent human chymase inhibitors with high enzymatic selectivity and satisfactory metabolic stability were obtained by replacing the Val-Pro (P_3-P_2) dipeptide portion of the previously described inhibitor **1** with a nonpeptidic pyrimidinone skeleton. The potency of the novel compounds was further enhanced by the introduction of carbamoyl-substituted difluoromethylene ketone moieties. The most potent chymase inhibitor of the newly created series was **2u** (Y-40018), which had a K_i of 2.62 nM. Compound **2u** possessed high selectivity for human chymase since it lacked significant activity toward other representative human proteolytic enzymes. Moreover its strict specificity for human chymase suggested that **2u** strongly inhibited human and canine chymases but not rat and mouse ones. Pharmacokinetic studies in rats and dogs indicated that **2u** was absorbed rapidly after oral administration and had satisfactory bioavailability in these experimental animal species (rat, 17%; dog, 32%). In conclusion, **2u** is a novel, potent, and orally active chymase inhibitor which would prove very useful in revealing the precise roles of the latter in various pathophysiological processes.

Introduction

Chymase (EC 3.4.21.39) is a chymotrypsin-like serine protease localized mainly in mast cell secretory granules.[1−3] Recent reports suggest that this enzyme might intervene in numerous important physiological processes, such as stimulation of submucosal gland secretion,[4] hormone and cytokine processing,[5,6] growth factor release,[7] parasite expulsion,[8] and tissue remodeling.[9,10] However, its precise physiological functions as well as its intrinsic substrates are still unclear. Although several chymase inhibitors have been reported,[11] one which is nonpeptidic, specific, and metabolically stable remains to be found. This is one of the reasons why the pathophysiological roles of chymase have not yet been clearly elucidated.

Previously we described a peptidic human chymase inhibitor incorporating a difluoromethylene ketone (DFMK) moiety which, by interacting with the S′ subsite of the enzyme, increases affinity and selectivity for human chymase compared to other chymotrypsin-like serine proteases.[12,13] In particular compound **1**, bearing a carboxyphenylamide group at the N-terminus, showed potent chymase-inhibitory activity (K_i = 5.6 nM) and high human chymase selectivity and was 10-fold more potent than the corresponding trifluoromethyl ketone (TFMK) derivative.

The construction of a three-dimensional model of human chymase utilizing the coordinate data of rat mast cell protease-II[14] allowed analysis of its docking behavior with compound **1** and revealed three favorable interactions of the carboxyphenylamide group with the S′ subsite. These were (1) a hydrogen bond of the anilide nitrogen atom with the Phe-41 carbonyl oxygen atom, (2) an aromatic−aromatic interaction with the Phe-41 side chain, and (3) an electrostatic interaction of the terminal carboxylic acid moiety with the side chain of Lys-40.



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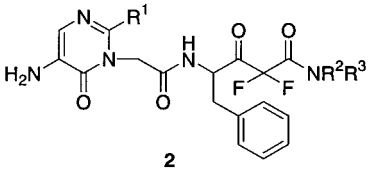

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In the search for human chymase inhibitors characterized by greater potency, higher enzymatic selectivity, and metabolic stability, we designed nonpeptidic compounds based on the DFMK (DIMK) derivative **1**. Although it was possible to modify substantially the peptidic portion (P_3-P_1) of **1**, the P_1 phenylalanine residue had to be conserved because it functions as a critical recognition element for chymase activity. X-ray crystallographic analysis of the irreversible inhibitor succinyl-Ala-Ala-Pro-Phe-CH_2Cl complexed with human chymase indicated that the P_3-P_1 residue forms an anti-

* To whom correspondence should be addressed. Tel: +81-72-856-9283. Fax: +81-72-868-9597. E-mail: akahoshi@welfide.co.jp.
† Welfide Corp.
§ Osaka Medical College.
‡ Present address: Production Division, Welfide Corp., 2-6-9, Hiranomachi, Chuo-ku, Osaka 541-0046, Japan.
# Present address: Welfide International Corp., 2410 Lillyvale Ave., Los Angeles, CA 90032.

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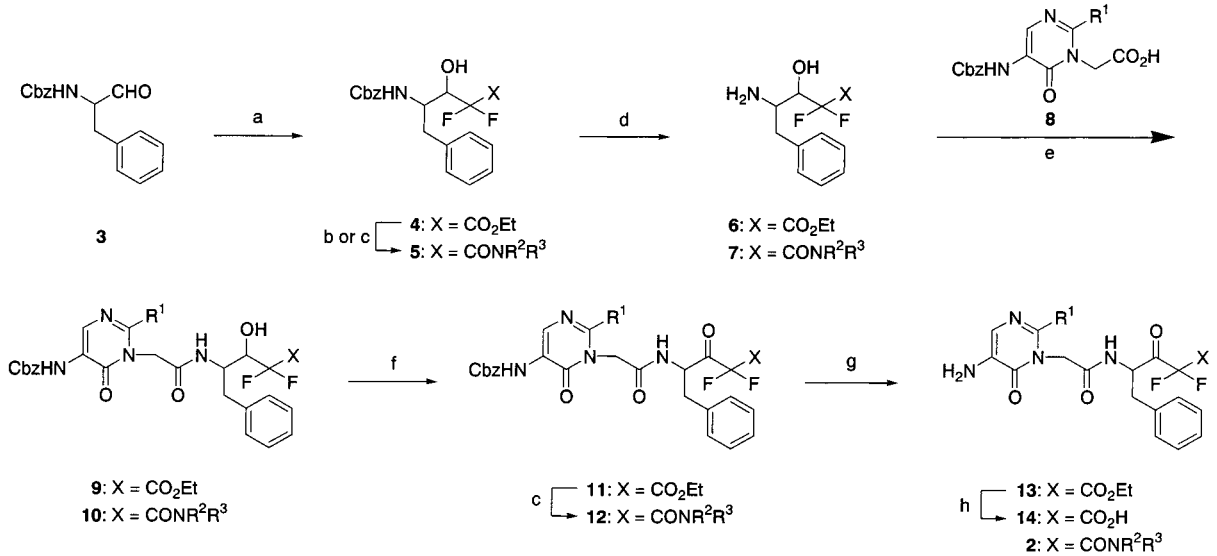
Scheme 1[a]



[a] Generic groups X and R^1 are defined in Tables 1 and 2. Reagents: (a) $BrCF_2CO_2Et$, Zn, THF; (b) (i) 0.1 N NaOH, THF, (ii) R^2R^3NH, EDC, HOBT, DMF; (c) R^2R^3NH, THF; (d) H_2, Pd/C, CH_3OH; (e) EDC, HOBT, *N*-ethylmorpholine, DMF; (f) Dess−Martin periodinate, DMF or EDC, Cl_2CHCO_2H, DMSO, toluene; (g) H_2, Pd/C, CH_3OH or CF_3SO_3H, anisole, CH_2Cl_2; (h) 0.1 N NaOH, THF.

parallel β-sheet binding arrangement with Ser-214 to Gly-216.[15] This suggested that the corresponding portion of **1** may bind to chymase in a similar manner. Meanwhile, the X-ray crystal structure of a complex of porcine pancreatic elastase with the peptidic inhibitor acetyl-Ala-Pro-Val-CF_3 indicated that this binding is characterized by an analogous antiparallel β-sheet hydrogen-bonding pattern.[16] On the basis of these findings then, we hypothesized that pyrimidinone structures, which have been successfully used in the design of elastase inhibitors,[17,18] would be effective P_3-P_2 mimetics in our chymase inhibitors, and likewise, the same results allowed us to predict that the combination of nonpeptidic pyrimidinone skeletons with DFMK moieties capable of interacting with the S′ subsite (general structure **2**) would yield orally active chymase inhibitors.

The present report describes the synthesis and structure−activity relationships (SARs) of a series of 5-amino-6-oxo-1,6-dihydropyrimidine-containing DFMK moieties bearing a variety of substituents at the position corresponding to the P′ site. We also describe the pharmacokinetic profile and the inhibitory profile against several representative proteases of the *N*-benzylcarbamoyl-substituted DFMK **2u** (Y-40018), the most potent compound of the series.

Chemistry

The general method of synthesis for DFMK-substituted 5-aminopyrimidin-6-one derivatives is summarized in Scheme 1. Introduction of a difluoromethylene moiety to the phenylalaninal **3**[19] was performed by Reformatsky reaction, which usually requires some type of activation, e.g. reflux,[20,21] ultrasonic irradiation,[22,23] electrolysis,[24,25] or addition of various metals.[26−28] Although yields were good, except for with reflux, this method was found to be unsuitable and impractical for large-scale reaction. We therefore developed a modified Reformatsky reaction which produces good yields of difluoromethylene compounds with high reproducibility. Reformatsky reaction was promoted by initial activation of zinc with a small amount of ethyl bromodifluoroacetate and subsequent addition of a solution of residuary acetate and the aldehyde **3** in THF at room temperature, followed by stirring for a few hours. The desired compound **4** was produced at 82% yield (cf. Experimental Section).

The ethyl ester **4** was hydrolyzed and the resulting carboxylic acid condensed with several amines to give the amide **5**. Alternatively, direct reaction of the ethyl ester **4** with amines also gave the desired amide **5**. The amines **6** and **7**, prepared by hydrogenolysis of **4** and **5**, were condensed with the oxopyrimidinylacetic acids **8**[18] followed by Dess−Martin or modified Pfitzner−Moffatt oxidation to furnish compounds **11** and **12**. Treatment of **11** with various amines also afforded compound **12**. Removal of the carbonylbenzyloxy group of **11** and **12** by hydrogenolysis or trifluoromethanesulfonic acid in the presence of anisole afforded the target compounds **13** and **2**, respectively. Basic hydrolysis of the ethyl ester **13** gave the carboxylic acid **14**.

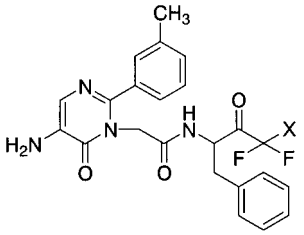
Biological Assays

All compounds were evaluated for in vitro inhibitory activity using purified chymase from human heart and bovine pancreas α-chymotrypsin purchased from Sigma Chemical Co. The assay was based on a published method.[12] Hydrolysis of the substrate succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide was monitored spectrophotometrically by measuring the release of *p*-nitroaniline at 405-nm absorbance.

Results and Discussion

First of all, using a [5-amino-6-oxo-2-(*m*-tolyl)-1,6-dihydro-1-pyrimidinyl]acetyl moiety as the basic framework, we investigated the effect of substitution at the C-terminus, which is thought to interact with the S′ subsite of the enzyme (Table 1). The nonpeptidic TFMK compound **15** had K_i values of 50.6 and 455 nM toward human chymase and bovine pancreatic α-chymotrypsin, respectively. Introduction of ethoxycarbonyl and car-

Table 1. Enzyme Inhibitory Activities of DFMK Analogues



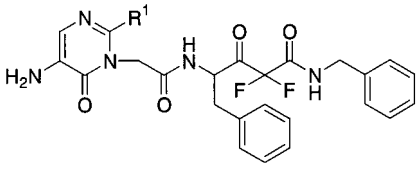
compd	X	inhibitory activity: K_i (nM)[a] chymase	inhibitory activity: K_i (nM)[a] chymotrypsin	selectivity: chymotrypsin/chymase
15	F	50.6 ± 14.2	455 ± 117	8.99
13	CO_2Et	>10000	27800 ± 5000	
14	CO_2H	>10000	17900 ± 8800	
2a	$CONH_2$	13.3 ± 1.3	12.6 ± 0.3	0.947
2b	$CONH-CH_3$	90.7 ± 15.4	348 ± 82	3.84
2c	$CON(CH_3)_2$	489 ± 113	13700 ± 2300	28.0
2d	$CONH-C_6H_4$-*m*-(CO_2H)	9.7 ± 0.3	780 ± 62	80.4
2e	$CONH-C_6H_4$-*p*-(CO_2H)	16.6 ± 1.6	282 ± 21	17.0
2f	$CONH-CH_2-CO_2CH_3$	10.2 ± 1.1	147 ± 13	14.4
2g	$CONH-CH_2-CO_2H$	16.9 ± 1.6	1830 ± 320	108
2h	$CONH-CH_2-CONH_2$	12.0 ± 0.0	1440 ± 40	120
2i	$CONH-CH_2-CONHEt$	7.71 ± 1.57	1400 ± 330	182
2j	$CONH-CH_2-CONEt_2$	9.32 ± 0.84	1960 ± 120	210
2k	$CONH-CH_2-CO(^cNC_5H_{10})$	10.2 ± 2.2	3570 ± 480	350
2l	$CONH-CH_2-CO(^cNC_6H_{12})$	3.56 ± 0.89	3220 ± 170	904
2m	$CONH-(CH_2)_2-CO_2H$	23.0 ± 2.6	1430 ± 112	62.2
2n	$CONH-(CH_2)_2-CONEt_2$	33.7 ± 2.6	295 ± 107	8.75
2o	$CONH-^nC_5H_{11}$	18.5 ± 1.8	451 ± 162	24.4
2p	$CONH-CH_2-C_6H_5$	16.9 ± 4.6	1920 ± 100	114
2q	$CONH-CH_2-C_5H_4N$	28.9 ± 4.1	1090 ± 50	37.7
1		5.6 ± 1.9	364 ± 27	65
chymostatin		13.1 ± 1.4	9.36 ± 2.19	0.715

[a] Values are means ± SEM of three independent experiments.

boxyl groups to the DFMK moiety completely canceled chymase-inhibitory activity (**13**, **14**). Since an electron-withdrawing property in the functional group adjacent to the P_1 carbonyl group would normally increase reactivity toward the Ser-195 of the enzyme, and since the ethoxycarbonyldifluoromethyl group has a similar electron-withdrawing ability to the trifluoromethyl group, the reason for the complete loss of activity is unclear. The carbamoyl analogue **2a** showed strong activity, with a K_i of ca. 13 nM toward both chymase and chymotrypsin, and is superior in potency to the TFMK compound **15**. We propose that this carbamoyl group interacts with the carbonyl of the Phe-41 of chymase as observed in the corresponding peptidic inhibitor.[13] To improve enzymatic selectivity (selectivity against chymotrypsin), we next explored further substitution on this carbamoyl nitrogen atom. Introduction of a methyl group resulted in a 7-fold decrease in chymase-inhibitory activity but a 4-fold increase in selectivity against chymotrypsin (**2b**). Introduction of a dimethyl group induced a further decrease in inhibitory activity (**2c**). These results suggest that at least one hydrogen atom on this carbamoyl nitrogen is essential to maintain potent chymase-inhibitory activity. Next, we carried out substitutions on this carbamoyl nitrogen atom with carboxyphenyl and carboxymethyl moieties, which in our previous study had provided potent peptidic chymase inhibitors with high selectivity against chymotrypsin.[12] The *m*- and *p*-carboxylphenyl analogues **2d**,**e** did not greatly affect chymase-inhibitory activity. However, as in the corresponding series of peptidic analogues,[12] they improved selectivity; in particular the *meta*-substituted analogue **2d** showed a comparable chymotrypsin/chymase selectivity ratio to the peptidic compound **1**. Meanwhile, the glycine methyl ester, glycine, and glycine amide derivatives **2f**−**l** retained potent chymase-inhibitory activity, with the *N*-homopiperidinylglycine analogue **2l** being the most potent (K_i = 3.56 nM). On the other hand, these glycyl derivatives had considerably decreased chymotrypsin-inhibitory activity compared with **2a**, with a chymase/chymotrypsin selectivity ratio of almost 1000 in compound **2l**. These results suggest that chymase has some space where a large hydrophobic group can comfortably settle but that chymotrypsin does not. Incorporation of an additional methylene in the glycine moiety resulted in a decrease in activity (**2m**,**n**). Substitution with a benzyl group resulted in unchanged chymase-inhibitory activity but significant loss of chymotrypsin-inhibitory activity (**2p**, chymotrypsin/chymase selectivity ratio 114). Replacement of the benzyl group with a pyridylmethyl group (**2q**) had little effect on either potency. It is noteworthy that chymotrypsin/chymase selectivity can be enhanced by increasing the interaction between the chymase S′ subsite and either a polar carboxylic acid or a nonpolar hydrophobic moiety. Due to its high intrinsic stability in plasma, the benzyl amide analogue **2p** was selected for further SAR studies.

Next, we focused on study of SAR at the 2-position of the pyrimidinone ring of the selected *N*-benzylcarbamoyldifluoromethyl derivative **2p**, which had both high chymase-inhibitory activity and high selectivity against chymotrypsin. The results are summarized in Table 2. The nonsubstituted phenyl analogue **2r** was 2-fold less

Table 2. Enzyme Inhibitory Activities of 2-Substituted Pyrimidinone Analogues



compd	R^1	inhibitory activity: K_i (nM)[a] chymase	inhibitory activity: K_i (nM)[a] chymotrypsin	selectivity: chymotrypsin/chymase
2p	3-$(CH_3)C_6H_4$	16.9 ± 4.6	1920 ± 100	114
2r	C_6H_5	32.5 ± 3.4	177 ± 10	5.45
2s	3-FC_6H_4	15.7 ± 1.4	306 ± 20	19.5
2t	4-FC_6H_4	440 ± 36	3280 ± 346	7.45
2u	3-ClC_6H_4	2.62 ± 0.39	458 ± 19	175
2v	3-$(CH_3O)C_6H_4$	13.8 ± 1.7	109 ± 21	7.90
2w	3-pyridyl	74.3 ± 0.9	171 ± 32	2.30
2x	4-pyridyl	84.7 ± 3.7	240 ± 46	2.83

[a] Values are means ± SEM of three independent experiments.

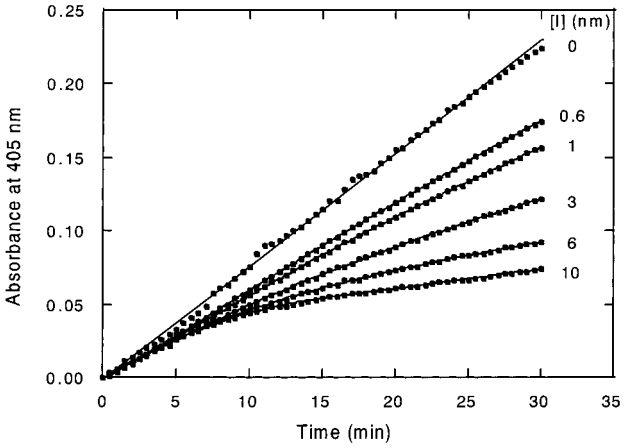


Figure 1. Progress curve of succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide hydrolysis by human chymase in the presence of various concentrations of **2u**. Measurement was done in 20 mM Tris-HCl (pH 7.5) containing 0.1 mM aprotinin and 2 M KCl in the presence of 2.5 mM substrate. The temperature was maintained at 37 °C, and reactions were initiated by the addition of enzyme. Values are shown corrected for background absorbance. Numbers on the right correspond to the molar concentration of **2u**.

potent than **2p**. Introduction of a fluoro atom at the 4-position of the phenyl ring resulted in a significant decrease in chymase-inhibitory activity (**2t**), whereas introduction at the 3-position gave unchanged activity (**2s**). The 3-chlorophenyl analogue **2u** showed potent chymase-inhibitory activity (K_i = 2.62 nM) and a high chymotrypsin/chymase selectivity ratio of 175. The 3-methoxy analogue **2v** did not significantly improve inhibitory activity, and the pyridyl analogues **2w**,**x** were inferior to **2r**. Clearly, introduction of substituents at the 3-position of the phenyl ring enhances selectivity for chymase as observed in heterocyclic inhibitors.[29] The results suggest that this series of inhibitors utilizes S_2–P_2 interaction to discriminate between these enzymes.

To further investigate chymase-inhibitory activity, we performed kinetic studies on compound **2u** and obtained a progress curve for the hydrolysis of succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide by human chymase[29] as shown in Figure 1. A biphasic curve was observed at various concentrations of **2u** with a first phase (pre-steady-state)

Table 3. Enzyme Selectivity Data for Compound **2u**

enzyme	K_i (nM)[a]
human heart chymase	2.62 ± 0.39
canine skin chymase	28.0 ± 1.6
rat peritoneal chymase	4240 ± 560
mouse peritoneal chymase	1260 ± 300
bovine pancreatic chymotrypsin	458 ± 19
human cathepsin G	1390 ± 230
human leukocyte elastase	NI (10 μM)[b]
human plasma thrombin	NI (10 μM)[b]
human angiotensin-converting enzyme	NI (10 μM)[b]

[a] Values are means ± SEM of three independent experiments. [b] NI = no inhibition at highest concentration (in parentheses) tested.

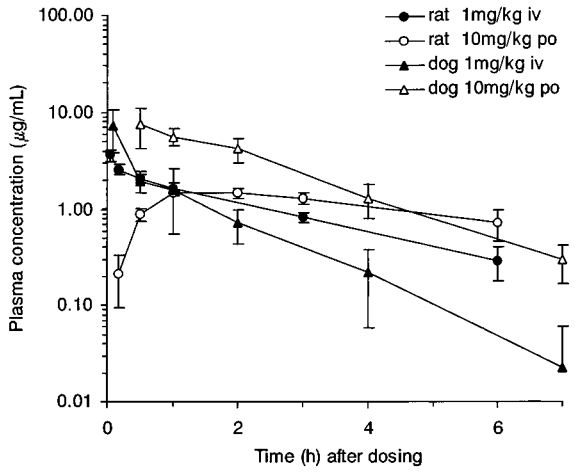


Figure 2. Plasma concentration profile of **2u** in rats and dogs.

due to a time-dependent inactivation of chymase and ending in a steady-state phase. This phenomenon is typical of slow-binding kinetics[30] and suggests that **2u** binds to the predicted site of chymase in a manner similar to that of peptidic compounds.[15] The inhibitory constant K_i, the association rate constant k_{on}, and the dissociation constant k_{off} were calculated from the progress curve as 1.53 nM, 8.80×10^7 M^{-1} s^{-1}, and 0.050 s^{-1}, respectively. This K_i value differed little from one determined from the conventional 'stopped' method (incubation of enzyme with inhibitor and substrate for a given time followed by discontinuance of the reaction[12] as used in Tables 1 and 2).

Compound **2u**, the most potent inhibitor of chymase, was measured for its inhibitory activity toward nonhuman origin (canine, rat, and mouse) chymases and other representative human proteolytic enzymes (Table 3). Only chymotrypsin-type serine proteases were inhibited by **2u**, which showed 10-fold weaker activity toward canine chymase and drastically reduced potency toward rat and mouse chymases and human cathepsin G, thus demonstrating very high selectivity for human chymase.

Finally, we examined plasma levels of **2u** after intravenous (iv, 1 mg/kg) and oral administration (po, 10 mg/kg) to rats and dogs. Changes in plasma concentration are shown in Figure 2 and pharmacokinetic parameters summarized in Tables 4 and 5. This compound was absorbed rapidly after oral administration and had moderate plasma clearance. Although the pharmacokinetic half-life was relatively short and plasma levels were below the quantification limit at 24 h after dosing, the high values for maximum plasma concentration and oral bioavailability (BA) allow investigation of

Table 4. Pharmacokinetic Parameters after Intravenous Administration of **2u** to Rats and Dogs at 1 mg/kg

species	n^a	$AUC_{iv}{}^b$ (μg·h/mL)	$t_{1/2}{}^c$ (h)	CL^d (mL/h/kg)	$V_{d(ss)}{}^e$ (mL/kg)
rat	3	7.33 ± 0.61	2.1 ± 0.7	137 ± 11	369 ± 94
dog	3	5.72 ± 2.17	1.4 ± 0.5	195 ± 82	257 ± 114

[a] n = number of animals. [b] AUC_{iv} = integrated area under plasma concentration vs time curve from time 0 to time infinity. [c] $t_{1/2}$ = half-life. [d] CL = plasma clearance. [e] $V_{d(ss)}$ = steady-state volume of distribution.

Table 5. Pharmacokinetic Parameters after Oral Administration of **2u** to Rats and Dogs at 10 mg/kg

species	n^a	$C_{max}{}^b$ (μg/mL)	$T_{max}{}^c$ (h)	$AUC_{po}{}^d$ (μg·h/mL)	BA^e (%)
rat	3	1.52 ± 0.09	1.3 ± 0.6	12.1 ± 4.9	17 ± 7
dog	3	7.32 ± 2.20	0.7 ± 0.3	18.5 ± 4.8	32 ± 8

[a] n = number of animals. [b] C_{max} = maximum plasma concentration taken directly from measured values. [c] T_{max} = time to reach C_{max}. [d] AUC_{po} = integrated area under plasma concentration vs time curve from time 0 to time infinity. [e] BA = oral bioavailability: $(AUC_{po} \times dose_{iv})/(AUC_{iv} \times dose_{po}) \times 100$.

the function of chymase in vivo ($t_{1/2}$ = 1.4 ± 0.5 h, C_{max} = 7.32 ± 2.20 μg/mL, BA = 32 ± 8%).

We generated the nonpeptidic chymase inhibitor **2u**, which showed potent inhibitory activity, high selectivity for chymase, and a satisfactory pharmacokinetic profile in rats and dogs. This orally active, specific chymase inhibitor will help further our understanding of the roles of chymase in numerous pathophysiological processes.

Conclusion

Starting from the peptidic DFMK chymase inhibitor **1** reported previously, we synthesized a series of the nonpeptidic compounds **2** and evaluated their human chymase-inhibitory activity. We discovered that carbamoyl-substituted DFMK derivatives brought about enhanced chymase-inhibitory activity, while introduction of hydrophobic groups at the N-terminus led to increased selectivity for chymase over chymotrypsin. The compound **2u** (Y-40018), in particular, had a K_i of 2.62 nM toward human chymase, a K_i of 28.0 nM toward canine chymase, and high selectivity against chymase of other species and other representative human proteolytic enzymes. Pharmacokinetic studies in rat and dog indicated that **2u** was absorbed rapidly following oral administration and had satisfactory bioavailability. Given its favorable biological profile, this compound was selected for further evaluation to determine its therapeutic potential against chymase-induced diseases.

Experimental Section

Chemistry. Melting points were determined with a Yanaco melting point apparatus and are uncorrected. ^{1}H NMR spectra were measured on either a Bruker DPX-300 or AMX-500 instrument with tetramethylsilane as the internal standard; chemical shifts are reported in parts per million (ppm, δ units). Splitting patterns are designated as s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; or br, broad. Mass spectra (MS) were recorded on a Hitachi M-2000 or PE Sciex API-165 instrument operating in the secondary ionization mass spectrometry (SIMS) and electrospray ionization (ESI) modes, respectively. Elemental analyses for carbon, hydrogen, and nitrogen were conducted with a Yanaco MT-6 and are within ±0.4% of theory for formulas given. Chromatography refers to flash chromatography conducted on Kieselgel 60 230−400 mesh (E. Merck, Darmstadt) using the indicated solvents. All chemicals and solvents were reagent grade unless otherwise specified. Reactions were run under a nitrogen atmosphere at ambient temperature unless otherwise noted. The following abbreviations are used: THF, tetrahydrofuran; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; EDC, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride; HOBT, 3-hydroxybenztriazole hydrate.

Ethyl 4-Benzyl-4-benzyloxycarbonylamino-2,2-difluoro-3-hydroxybutylate (4). To a suspension activated zinc (1.51 g, 23.1 mmol) in THF (2.5 mL) was added dropwise ethyl bromodifluoroacetate (0.50 mL, 3.9 mmol). After the reaction was started, a solution of residuary ethyl bromodifluoroacetate (2.50 mL, 19.5 mmol) and phenylalaninal (**3**) (2.18 g, 7.70 mmol) in THF (12 mL) added dropwise for 2 min. After stirring at room temperature for 30 min, the reaction mixture was cooled by ice−water and treated with saturated NH_4Cl (10 mL). The mixture was poured into saturated NH_4Cl (40 mL), and then extracted with ethyl acetate. The extract was washed with brine and dried ($MgSO_4$). The solvent was removed and the residue purified by silica gel column chromatography (67:33 hexane−ethyl acetate) to give **4** (2.57 g, 82% yield) as a white solid: ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.50−6.93 (m, 11H, ArH, NH), 6.39 (d, J = 7.7 Hz, 0.4H, OH), 6.29 (d, J = 7.5 Hz, 0.6H, OH), 5.02−4.77 (m, 2H, CH_2−O), 4.28−4.15 (m, 2H, CH_2−O), 4.10−3.80 (m, 2H, N−CH−CH−O), 3.07 (dd, J = 13.8, 2.6 Hz, 0.4H, CH*H*-Ph), 2.90−2.70 (m, 1.2H, CH_2−Ph × 0.6), 2.62 (m, 0.4H, CH*H*-Ph), 1.30−1.14 (m, 3H, CH_3).

4-Benzyl-4-benzyloxycarbonylamino-2,2-difluoro-3-hydroxy-*N*-methylbutanamide (5b). To a solution of **4** (10.2 g, 25.0 mmol) in THF (100 mL) and methanol (150 mL) was added a solution of K_2CO_3 (13.8 g, 100 mmol) in water (100 mL). The resulting mixture was stirred at room temperature for overnight, at which time organic solvents were evaporated. The residual solution was poured into water (100 mL) and washed with diethyl ether. The aqueous layer was acidified with concentrated HCl to pH 3 and extracted with ethyl acetate. The extract was washed with brine, dried ($MgSO_4$) and concentrated. The residue was crystallized from ethyl acetate−hexane to give 4-benzyl-4-benzyloxycarbonylamino-2,2-difluoro-3-hydroxybutanoic acid (8.21 g, 86% yield) as a pale yellow solid. To a solution of this intermediate (733 mg, 1.93 mmol), methylamine hydrochloride (141 mg, 2.09 mmol) and *N*-ethylmorpholine (0.27 mL, 2.1 mmol) in DMF (7 mL) were added HOBT (600 mg, 3.92 mmol) and EDC (419 g, 2.19 mmol). After stirring at room temperature for 18 h, the reaction mixture was poured into water (30 mL), and then extracted with ethyl acetate. The extract was washed successively with 10% citric acid, saturated $NaHCO_3$ and brine, and dried ($MgSO_4$). The solvent was removed and the residue purified by silica gel column chromatography (ethyl acetate) to give **5b** (294 mg, 39% yield) as a white solid: ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.55 (m, 1H, NH), 7.40−7.06 (m, 10H, ArH), 6.13 (d, J = 7.1 Hz, 0.3H, OH), 6.05 (d, J = 7.2 Hz, 0.7H, OH), 5.02−4.75 (m, 2H, CH_2−O), 4.12−3.80 (m, 2H, N−CH−CH−O), 3.07−2.55 (m, 2H, CH_2−Ph), 2.63 (s, 3H, CH_3−N).

4-Amino-4-benzyl-2,2-difluoro-3-hydroxy-*N*-methylbutanamide Hydrochloride (7b). To a solution of **5b** (290 mg, 0.739 mmol) in methanol (16 mL) were added 1 N HCl (0.77 mL) followed by 10% palladium−carbon (100 mg) under a nitrogen atmosphere. The resulting mixture was stirred at room temperature for 15 h under a hydrogen atmosphere. Palladium−carbon was removed by filtration and washed with methanol. The filtrate was concentrated to give **7b** (217 mg, quant.) as a white solid: ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.95 (m, 0.7H, NH), 8.89 (m, 0.3H, NH), 8.11 (br s, 3H NH_3), 7.40−7.26 (m, 5H, ArH), 7.20 (m, 0.7H, OH), 6.76 (m, 0.3H, OH), 4.45 (m, 0.3H, CH−N), 3.94 (m, 0.7H, CH−N), 3.60 (m, 0.7H, CH−O), 3.54 (m, 0.3H, CH−O), 3.08 (dd, J = 13.8, 4.0 Hz, 1H, CH*H*-Ph), 2.92−2.84 (m, 1H, CH*H*-Ph), 2.71 (d, J = 4.5 Hz, 0.9H, CH_3−N × 0.3), 2.66 (d, J = 4.5 Hz, 2.1H, CH_3−N × 0.7).

2-[5-Benzyloxycarbonylamino-6-oxo-2-(*m*-tolyl)-1,6-dihydro-1-pyrimidinyl]-*N*-[1-benzyl-3,3-difluoro-2-hydroxy-3-(*N*-methylcarbamoyl)propyl]acetamide (10b). To a so-

lution of [5-benzyloxycarbonylamino-6-oxo-2-(*m*-tolyl)-1,6-dihydro-1-pyrimidinyl]acetic acid (**8a**) (290 mg, 0.737 mmol), **7b** (217 mg, 0.736 mmol) and *N*-ethylmorpholine (0.10 mL, 0.79 mmol) in DMF (2.5 mL) were added HOBT (227 mg, 1.48 mmol) and EDC (159 mg, 0.828 mmol). After stirring at room temperature for 16 h, the reaction mixture was poured into water (10 mL), and then extracted with ethyl acetate. The extract was washed successively with 10% citric acid, saturated $NaHCO_3$ and brine, and dried ($MgSO_4$). The solvent was removed and the residue purified by recrystallization (2:5 ethyl acetate−hexane) to give **10b** (383 mg, 82% yield) as a white solid: ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.95 (m, 0.7H, NH), 8.86 (m, 0.3H, NH), 8.62 (m, 0.3H, NH), 8.45 (s, 0.7H, CH═N), 8.41 (m, 0.3H, CH═N), 8.33 (m, 0.7H, NH), 8.23 (d, J = 8.9 Hz, 0.3H, NH), 8.15 (d, J = 9.0 Hz, 0.7H, NH), 7.49−7.03 (m, 14H, ArH), 6.33 (d, J = 7.1 Hz, 0.7H, OH), 6.20 (d, J = 7.1 Hz, 0.3H, OH), 5.19 (s, 1H, CH_2−O), 4.55−4.35 (m, 2H, CH_2−N), 4.29−3.86 (m, 2H, N−CH−CH−O), 2.94−2.60 (m, 2H, CH_2−Ph), 2.57 (s, 0.9H, CH_3−N × 0.3), 2.56 (s, 2.1H, CH_3−N × 0.7), 2.31 (s, 2.1H, CH_3−Ar × 0.7), 2.29 (s, 0.9H, CH_3−Ar × 0.3).

2-[5-Benzyloxycarbonylamino-6-oxo-2-(*m*-tolyl)-1,6-dihydro-1-pyrimidinyl]-*N*-[1-benzyl-3,3-difluoro-3-(*N*-methylcarbamoyl)-2-oxopropyl]acetamide (12b). To a solution of **10b** (360 mg, 0.568 mmol) in DMF (2.5 mL) was added Dess−Martin periodinate (370 mg, 0.872 mmol). The reaction mixture was stirred at room temperature for 14 h, after which saturated $NaHCO_3$ (10 mL) containing sodium thiosulfate (2.5 g) and ethyl acetate were added. After stirring for 2 h, the mixture was poured into water (10 mL) and extracted with ethyl acetate. The extract was washed with saturated $NaHCO_3$ and brine, dried ($MgSO_4$) and concentrated. The residue was purified by silica gel column chromatography (60:40 dichloromethane−ethyl acetate) and recrystallization (1:5 ethyl acetate−hexane) to give **12b** (248 mg, 69% yield) as a white solid: mp 157−162 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.07 (q, J = 4.4 Hz, 1H, NH), 8.94 (s, 1H, NH), 8.84 (d, J = 7.4 Hz, 1H, NH), 8.43 (s, 1H, CH═N), 7.49−7.08 (m, 14H, ArH), 5.18 (s, 1H, CH_2−O), 4.96 (m, 1H, CH−N), 4.41 (m, 2H, CH_2−N), 3.15 (dd, J = 14.4, 3.9 Hz, 1H, CH*H*-Ph), 2.79−2.60 (m, 4H, CH_3−N, CH*H*-Ph), 2.31 (s, 3H, CH_3−Ar).

2-[5-Amino-6-oxo-2-(*m*-tolyl)-1,6-dihydro-1-pyrimidinyl]-*N*-[1-benzyl-3,3-difluoro-3-(*N*-methylcarbamoyl)-2-oxopropyl]acetamide (2b). To a solution of **12b** (209 mg, 0.331 mmol) in methanol (15 mL) was added 10% palladium−carbon (62 mg) under a nitrogen atmosphere. The resulting mixture was stirred at room temperature for 16 h under a hydrogen atmosphere. Palladium−carbon was removed by filtration and washed with methanol. The filtrate was concentrated and the residue purified by recrystallization (1:2 ethyl acetate−hexane) to give **2b** (139 mg, 84% yield) as white crystals: mp 199−201 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.07 (m, 1H, NH), 8.78 (d, J = 7.3 Hz, 1H, NH), 7.38−7.05 (m, 10H, ArH, CH═N), 5.15 (br s, 1H, NH_2), 4.95 (m, 1H, CH−N), 4.38 (m, 2H, CH_2−N), 3.15 (dd, J = 14.3, 4.0 Hz, 1H, CH*H*-Ph), 2.79−2.61 (m, 4H, CH_3−N, CH*H*-Ph), 2.29 (s, 3H, CH_3−Ar); MS (SIMS) m/z 498 (MH^+). Anal. ($C_{25}H_{25}F_2N_5O_4$·0.7H_2O) C, H, N.

2-[5-Benzyloxycarbonylamino-6-oxo-2-(*m*-tolyl)-1,6-dihydro-1-pyrimidinyl]-*N*-[1-benzyl-3-[*N*-(3-carboxyphenyl)carbamoyl]-3,3-difluoro-2-oxopropyl]acetamide (12d). To a solution of 2-[5-benzyloxycarbonylamino-6-oxo-2-(*m*-tolyl)-1,6-dihydro-1-pyrimidinyl]-*N*-[1-benzyl-3-[*N*-(3-*tert*-butyloxycarbonylphenyl)carbamoyl]-3,3-difluoro-2-oxopropyl]acetamide (352 mg, 0.443 mmol) in dichloromethane (7 mL) was added trifluoroacetic acid (3.4 mL). After stirring at room temperature for 5.5 h, the reaction mixture was concentrated. The residue was dissolved in ethyl acetate, washed with water and brine, and concentrated. The residue was purified by silica gel column chromatography (70:30 ethyl acetate−methanol) and crystallization (diethyl ether−hexane) to give **12d** (282 mg, 86% yield) as white crystals: mp 157−165 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.99 (br s, 1H, NH), 8.94 (d, J = 6.6 Hz, 1H, NH), 8.93 (s, 1H, NH), 8.43 (s, 1H, CH═N), 8.24 (s, 1H, ArH), 7.78−7.65 (m, 2H, ArH), 7.47−7.10 (m, 15H, ArH), 5.18 (s, 2H, CH_2−O), 5.05 (m, 1H, CH−N), 4.43 (s, 2H, CH_2−N), 3.19 (dd, J = 14.0, 3.6 Hz, 1H, CH*H*-Ph), 2.78 (dd, J = 14.0, 10.1 Hz, 1H, CH*H*-Ph), 2.29 (s, 3H, CH_3−Ar); MS (SIMS) m/z 738 (MH^+).

2-[5-Amino-6-oxo-2-(*m*-tolyl)-1,6-dihydro-1-pyrimidinyl]-*N*-[1-benzyl-3-[*N*-(3-carboxyphenyl)carbamoyl]-3,3-difluoro-2-oxopropyl]acetamide (2d). Compound **12d** was deprotected using method similar to that described for **2b** to give **2d** as pale yellow crystals: mp >177 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.01 (br s, 1H, NH), 8.88 (d, J = 7.2 Hz, 1H, NH), 8.26 (s, 1H, ArH), 7.75 (d, J = 7.7 Hz, 1H, ArH), 7.72 (d, J = 8.7 Hz, 1H, ArH), 7.38 (t, J = 7.9 Hz, 1H, ArH), 7.32−7.06 (m, 10H, ArH, CH═N), 5.15 (s, 2H, NH_2), 5.05 (m, 1H, CH−N), 4.40 (s, 2H, CH_2−N), 3.19 (m, 1H, CH*H*-Ph), 2.79 (dd, J = 14.2, 9.5 Hz, 1H, CH*H*-Ph), 2.28 (s, 3H, CH_3−Ar); MS (SIMS) m/z 604 (MH^+). Anal. ($C_{31}H_{27}F_2N_5O_6$·0.8$CHCl_3$·1.0H_2O) C, H, N.

Other analogues **2** were prepared using the same procedures as described above.

2-[5-Benzyloxycarbonylamino-6-oxo-2-(*m*-tolyl)-1,6-dihydro-1-pyrimidinyl]-*N*-[1-benzyl-3-(ethoxycarbonyl)-3,3-difluoro-2-hydroxypropyl]acetamide (9). Compound **8a** (3.52 g, 8.95 mmol) and ethyl 4-benzyl-4-benzyloxycarbonylamino-2,2-difluoro-3-hydroxybutylate hydrochloride (**6**) (2.59 g, 8.36 mmol) were coupled using a method to that described for **10b** to give **9** (4.28 g, 73% yield) as a pale brown solid: ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.86 (s, 1H, NH), 8.43 (s, 1H, CH═N), 8.16 (d, J = 8.9 Hz, 1H, NH), 7.44 (d, J = 7.1 Hz, 2H, ArH), 7.39 (t, J = 7.1 Hz, 2H, ArH), 7.36−7.18 (m, 10H, ArH), 6.50 (d, J = 7.0 Hz, 1H, OH), 5.19 (s, 2H, CH_2−O), 4.55 (d, J = 16.5 Hz, 1H, CH*H*-N), 4.40 (d, J = 16.5 Hz, 1H, CH*H*-N), 4.23 (m, 1H, CH−O), 4.18 (q, J = 7.0 Hz, 2H, CH_2−O), 3.87 (m, 1H, CH−N), 2.78 (dd, J = 13.4, 8.6 Hz, 1H, CH*H*-Ph), 2.63 (dd, J = 13.4, 6.2 Hz, 1H, CH*H*-Ph), 2.31 (s, 3H, CH_3−Ar), 1.17 (t, J = 7.0 Hz, 3H, CH_3).

2-[5-Benzyloxycarbonylamino-6-oxo-2-(*m*-tolyl)-1,6-dihydro-1-pyrimidinyl]-*N*-[1-benzyl-3-(ethoxycarbonyl)-3,3-difluoro-2-oxopropyl]acetamide (11). To a solution of **9** (4.04 g, 5.72 mmol) in DMSO (30 mL) and toluene (30 mL) were added EDC (5.48 g, 28.6 mmol) and then dichloroacetic acid (0.90 mL, 11 mmol) with ice−water cooling. After stirring at 0 °C for 3 h, the reaction mixture was poured into 1 N HCl (120 mL), and then extracted with ethyl acetate. The extract was washed with saturated $NaHCO_3$ and brine, and dried ($MgSO_4$). The solvent removed and the residue purified by silica gel column chromatography (80:20 dichloromethane−ethyl acetate) to give pale yellow crystals. Recrystallization from 1:1 ethyl acetate−hexane afforded **11** (1.97 g, 53% yield) as white crystals: mp 186−188 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.95 (d, J = 6.8 Hz, 1H, NH), 8.88 (s, 1H, NH), 8.42 (s, 1H, CH═N), 7.44 (d, J = 7.1 Hz, 2H, ArH), 7.39 (t, J = 7.1 Hz, 2H, ArH), 7.36−7.17 (m, 10H, ArH), 5.19 (s, 2H, CH_2−O), 4.86 (m, 1H, CH−N), 4.48 (d, J = 16.6 Hz, 1H, CH*H*-N), 4.41 (d, J = 16.6 Hz, 1H, CH*H*-N), 4.25 (q, J = 7.1 Hz, 2H, CH_2−O), 3.09 (dd, J = 14.2, 5.0 Hz, 1H, CH*H*-Ph), 2.79 (dd, J = 14.2, 9.0 Hz, 1H, CH*H*-Ph), 2.32 (s, 3H, CH_3−Ar), 1.16 (t, J = 7.1 Hz, 3H, CH_3).

2-[5-Amino-6-oxo-2-(*m*-tolyl)-1,6-dihydro-1-pyrimidinyl]-*N*-[1-benzyl-3-(ethoxycarbonyl)-3,3-difluoro-2-oxopropyl]acetamide (13). Compound **11** was deprotected using method similar to that described for **2b** to give **13** (682 mg, 92% yield) as white crystals: mp 146−147 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.89 (d, J = 6.8 Hz, 1H, NH), 7.31−7.09 (m, 10H, ArH, CH═N), 5.12 (s, 2H, NH_2), 4.85 (m, 1H, CH−N), 4.44 (d, J = 16.5 Hz, 1H, CH*H*-N), 4.36 (d, J = 16.5 Hz, 1H, CH*H*-N), 4.25 (q, J = 7.1 Hz, 2H, CH_2−O), 3.09 (dd, J = 14.1, 5.0 Hz, 1H, CH*H*-Ph), 2.81 (dd, J = 14.1, 9.0 Hz, 1H, CH*H*-Ph), 2.30 (s, 3H, CH_3−Ar), 1.18 (t, J = 7.1 Hz, 3H, CH_3); MS (SIMS) m/z 513 (MH^+). Anal. ($C_{26}H_{26}F_2N_4O_5$·0.5H_2O) C, H, N.

2-[5-Amino-6-oxo-2-(*m*-tolyl)-1,6-dihydro-1-pyrimidinyl]-*N*-[1-benzyl-3-carboxy-3,3-difluoro-2-oxopropyl]acetamide (14). To a solution of **13** (90.2 mg, 0.176 mmol) in THF (2.5 mL) was added 0.1 N NaOH (2.5 mL). The resulting

mixture was stirred at room temperature for 1.5 h, at which time THF was evaporated and 0.1 N HCl (5 mL) and ethyl acetate (10 mL) were added. After the reaction mixture was stirred at room temperature for 0.5 h, the precipitated solid was collected by filtration, washed with water (20 mL) and dried in vacuo to give **14** (71 mg, 83% yield) as white crystals: mp 118−121 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.79 (d, J = 7.3 Hz, 1H, NH), 7.31−7.10 (m, 10H, ArH, CH=N), 4.94 (m, 1H, CH−N), 4.42 (d, J = 16.5 Hz, 1H, CH*H*-N), 4.37 (d, J = 16.5 Hz, 1H, CH*H*-N), 3.13 (dd, J = 14.2, 4.1 Hz, 1H, CH*H*-Ph), 2.75 (dd, J = 14.2, 9.3 Hz, 1H, CH*H*-Ph), 2.30 (s, 3H, CH_3−Ar); MS (SIMS, negative) m/z 483 ($M-H^+$). Anal. ($C_{24}H_{22}F_2N_4O_5$·2.0H_2O) C, H, N.

Protease Inhibition Assay. Human heart chymase was purified according to the method of Urata et al.[31] Bovine pancreas α-chymotrypsin and human cathepsin G were purchased from Sigma Chemical Co., St. Louis, MO, and Athens Research and Technologies Inc., respectively. Human thrombin, elastase and angiotensin-converting enzyme (ACE) were from Welfide Corp., Osaka, Japan, Elastins Products Co., and Nippon Zoki Pharmaceutical Co., Osaka, Japan, respectively.

Inhibitory activities of compounds for chymase, chymotrypsin, and cathepsin G were measured by the methods described previously using a synthetic substrate, succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Sigma Chemical Co.). In the case of thrombin and elastase, the methods used were previously described method[32] involving the synthetic substrates S-2238 (Chromogenix AB, Mölndal, Sweden) and CH_3O-Suc-Ala-Ala-Pro-Val-*p*-nitroaniline (Sigma Chemical Co.), respectively. Enzymatic activity of ACE was measured by the methods described,[33] and calculation of K_i's was performed by the same method.[12]

Pharmacokinetic Measurements. Male Sprague−Dawley rats (weight 204−222 g) and male beagle dogs (weight 11.4−12.5 kg) were used for pharmacokinetic studies. The test compound **2u** was administered at a dose of 1 mg/kg intravenously (via bolus injection into the tail vein of rats and into the cutaneous vein of dogs) or 10 mg/kg orally (via gavage to the stomach). For intravenous administration the test compound was formulated as a solution (0.4 mg/mL) in DMSO−PEG-400−1 M HCl−saline (1:8:10:81, v/v). For oral administration in rats and dogs the test compound was formulated as a suspension (10 mg/mL) in 0.5% hydroxypropylmethyl cellulose and a solution (2.5 mg/mL) in DMSO−PEG-400−1 M HCl−saline (2:38:10:50, v/v), respectively. Rats and dogs were fasted for 18 h before dosing and 4 h after dosing. Blood samples were collected from the jugular vein of rats and cutaneous vein of dogs into a heparinized syringe at several time points up to 24 h after dosing. Plasma samples were separated by centrifugation and kept frozen (−20 °C) until analysis.

Plasma concentration of the test compound was determined by the internal standard method. Calibration standards were prepared in plasma by adding known amounts of the test compound to control plasma. Plasma samples (100 μL) were mixed with 0.5 M H_3PO_4 solution (0.4 mL) and internal standard solution (10 μg/mL **2p**; 10 μL). The samples were passed through Bond Elut C2 (100 mg, Varian) columns that had been conditioned with methanol (1 mL) and 0.5 M H_3PO_4 (1 mL). The columns were washed with 2 mL of water and finally eluted with 1 mL of water−acetonitrile (20:80, v/v). The eluate was concentrated to dryness under nitrogen steam. The residue was reconstituted in 100 μL of 10 mM ammonium acetate (pH 6.8)−acetonitrile (50:50, v/v) and 50 μL was injected onto an LC−MS/MS system. Chromatographic separation was performed using a Symmetry Shield RP_8 (100 mm × 2.1 mm i.d., 3.5 μm, Waters) column at a flow rate of 0.25 mL/min. The mobile phase consisted of 10 mM ammonium acetate (pH 6.8) (A) and acetonitrile (B). The following gradient system was used: 10% B to 90% B linearly over the first 10 min. Protonated analyte ions were generated using electron spray ionization. For quantitation, ion signal was recorded by selective reaction monitoring. Pharmacokinetic parameters were determined by noncompartmental methods.

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Supporting Information Available: Experimental data and elemental analyses. This material is available free of charge via the Internet at http://pubs.acs.org.

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