g, 0.002 95 mol) in phosphorus oxychloride (10 mL) was stirred and heated to the reflux temperature of $POCl_3$ for 30 min. At this time TLC indicated that the desired reaction was complete. The reaction solution was poured into ice water and ether, and the pH of the aqueous phase was adjusted to ~8 by the addition of solid potassium carbonate. The ether layer was separated, and the aqueous phase was extracted 3 more times with ether. The ether extracts were pooled, washed with water, dried over sodium sulfate, filtered, and concentrated under reduced pressure. The residual oil was chromatographed over two Merck size B Lobar silica gel columns, using 20% ethyl acetate–hexane followed at fraction 113 by 40% ethyl acetate–hexane to elute the column. Fractions of 25 mL were collected, and the products eluted in the following order of increasing polarity: isomer **22** (0.295 g, 0.000 93 mol, 31%) was obtained in fractions 31–43; isomer **23** (0.271 g, 0.000 86 mol, 29%) was obtained in fractions 47–56; isomer **24** (0.017 g, 0.000 054 mol, 2%) was obtained in fractions 78–87; and isomer **25** (0.114 g, 0.000 36 mol, 12%) was obtained in fractions 145–154. After pooling the appropriate fractions and removing solvent, all the products crystallized. The individual isomers were recrystallized and analyzed as follows.

Recrystallization of fractions 31–43 from acetone–hexane gave 0.272 g of **22** as colorless crystals: mp 112–112.5 °C; IR (Nujol) 1734, 1723, 1563, 1439, 1320, 1304, 1288, 1214, 1194, 1145, 1140, 1097, 1025, 836, 812, 767, 746 cm^{-1}, ^1H NMR ($CDCl_3$) δ 8.36 (d, 1 H, J = 2.5 Hz, pyridine C_2 proton), 7.18–7.65 (m, 6 H, aromatic protons), 4.45 (q, 2 H, J = 7 Hz, OCH_2CH_3), 4.07 (s, 2 H, Py–CH_2–Ar), 1.42 (t, 3 H, J = 7 Hz, OCH_2CH_3); mass spectrum, 315.0649 (100%) (calcd for $C_{17}H_{14}{}^{35}ClNO_3$: 315.0662), m/e 270 (28), 242 (30), 214 (21). Anal. ($C_{17}H_{14}ClNO_3$) C, H, N.

Recrystallization of fractions 47–56 from hexane gave 0.243 g of **23** as colorless crystals: mp 82–83 °C; IR (Nujol) 1727, 1568, 1564, 1409, 1322, 1311, 1298, 1214, 1200, 1193, 1146, 1098, 1063, 1020, 843, 811, 786, 767 cm^{-1}; ^1H NMR δ 8.32 (d of d, 1 H, J_{H_5} = 5 Hz, J_{H_4} = 2 Hz, pyridine C_6 proton), 7.10–7.68 (m, 6 H, aromatic protons), 4.43 (q, 2 H, J = 7 Hz, OCH_2CH_3), 4.17 (s, 2 H, Py–CH_2–Ar), 1.40 (t, 3 H, J = 7 Hz, OCH_2CH_3); mass spectrum, 315.0658 (100%) (calcd for $C_{17}H_{14}{}^{35}ClNO_3$: 315.0662), m/e 270 (30), 242 (13), 214 (14). Anal. C, H, N.

The crystalline fractions 79–87 of **24** were used for analysis without recrystallization: mp 90–91 °C; ^1H NMR ($CDCl_3$) δ 8.48 (m, 2 H, pyridine C_2 and C_6 protons), 7.23–7.73 (m, 5 H, aromatic protons), 4.48 (q, 2 H, J = 7 Hz, OCH_2CH_3), 4.09 (s, 2 H, Py–CH_2–Ar), 1.43 (t, 3 H, J = 7 Hz, OCH_2CH_3); mass spectrum, 315.0661 (100%) (calcd for $C_{17}H_{14}{}^{35}ClNO_3$: 315.0662), m/e 271 (37), 242 (13), 214 (25).

Recrystallization of fractions 145–154 from hexane gave 0.098 g of **25** as colorless crystals: mp 103–104 °C; IR (Nujol) 1714, 1578, 1556, 1436, 1408, 1347, 1318, 1302, 1267, 1221, 1199, 1134, 1125, 1084, 1012, 943, 882, 833, 825, 820, 804, 782, 762, 741, 695 cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.52 (s, 1 H, pyridine C_2 proton), 8.47 (d, 1 H, J = 5.5 Hz, pyridine C_6 proton), 7.23–7.73 (m, 5 H, aromatic protons), 4.48 (q, 2 H, J = 7 Hz, OCH_2CH_3), 4.09 (s, 2 H, Py–CH_2–Ar), 1.43 (t, 3 H, J = 5 Hz, OCH_2CH_3); mass spectrum, 315.0655 (100%) (calcd for $C_{17}H_{14}{}^{35}ClNO_3$: 315.0662), m/e 270 (25), 242 (15), 214 (22). Anal. C, H, N.

5-(3-Pyridinylcarbonyl)-2-benzofurancarboxylic Acid (29). A mixture of potassium superoxide (0.20 g, 0.002 82 mol) in dimethylsulfoxide (10 mL) containing dicyclohexyl-18-crown-6 (0.10 g) was stirred for 30 min at room temperature. The ester **8b** (0.140 g, 0.000 50 mol) was added directly to the mixture and stirring was continued. The reaction was followed by TLC (1:1 acetone–hexane or 75% ethyl acetate–hexane) until no starting material remained in the reaction mixture. After 4 h the reaction was poured into water (75 mL), and the pH of the resulting solution was adjusted to 4–5 by addition of 1 N aqueous hydrochloric acid. A white precipitate gradually began to form. The solution was cooled on ice, and after several hours the solid was collected by filtration. The dry solid weighed 0.049 g (0.000 183 mol, 36%) and did not melt below 300 °C. The solid could be crystallized from either acetic acid–water or methanol. A sample dissolved in hot glacial acetic acid and crystallized by the addition of water was submitted for analysis: IR (Nujol) 1731 (br), 1655, 1613, 1598, 1584, 1572, 1321, 1254, 1180, 1143, 1125, 1116, 1097, 1053, 754 cm^{-1}; mass spectrum, m/e 267 (74%), 222 (31), 189 (100), 151 (25). Anal. C, H, N.

Peptidyl Carbamates Incorporating Amino Acid Isosteres as Novel Elastase Inhibitors

George A. Digenis,* Bushra J. Agha, Kiyoshi Tsuji,† Masayuki Kato,† and Masaki Shinogi‡

College of Pharmacy, University of Kentucky, Lexington, Kentucky 40536-0053. *Received August 12, 1985*

The design and synthesis of 13 novel peptidyl carbamates are described. When tested for inhibitory activity toward porcine pancreatic elastase, trypsin, and chymotrypsin, six compounds were found to specifically inhibit elastase without affecting the other two serine proteases. All the active inhibitors had an amino acid isostere at the P_1 position. Kinetic studies indicated that the inhibition was competitive with K_i values ranging from 4.23×10^{-5} to 2.4×10^{-6} M. The degree of inhibition was found to be dependent on the specificity of the peptide chain for the extended subsites on the enzyme as well as on the nature of P_1'. Preliminary work on one inhibitor indicates that the inhibition is reversible and proceeds via the rapid formation of a strong enzyme–inhibitor complex, followed by slow acylation of the serine residue on the active site of the enzyme. Peptidyl carbamates represent a novel class of elastase inhibitors.

Proteinases from polymorphonuclear leukocytes and macrophages, especially elastases (human leukocyte (HL) elastase and cathepsin G), appear to be responsible for the chronic tissue destruction associated with inflammation, arthritis, and emphysema.[1-4] During infection or inflammation, the normal lung is protected from proteolytic digestion by the protease inhibitor, α_1-antitrypsin. This protective mechanism appears to be nonoperative in individuals with an α_1-antitrypsin deficiency due to genetic or other causes. Synthetic elastase inhibitors capable of replacing α_1-antitrypsin would therefore be expected to be potentially useful in the treatment of pulmonary emphysema and related diseases.[5]

†Present address: Fujisawa Pharmaceutical Co., Ltd., 1-6, 2-Chome, Kashima, Yodogawa-ku, Osaka 532, Japan.

‡Present address: Kobe Women's College of Pharmacy, 4-19-1, Motoyamakita-Machi, Higashinadada-KU, Kobe Shi 658, Japan.

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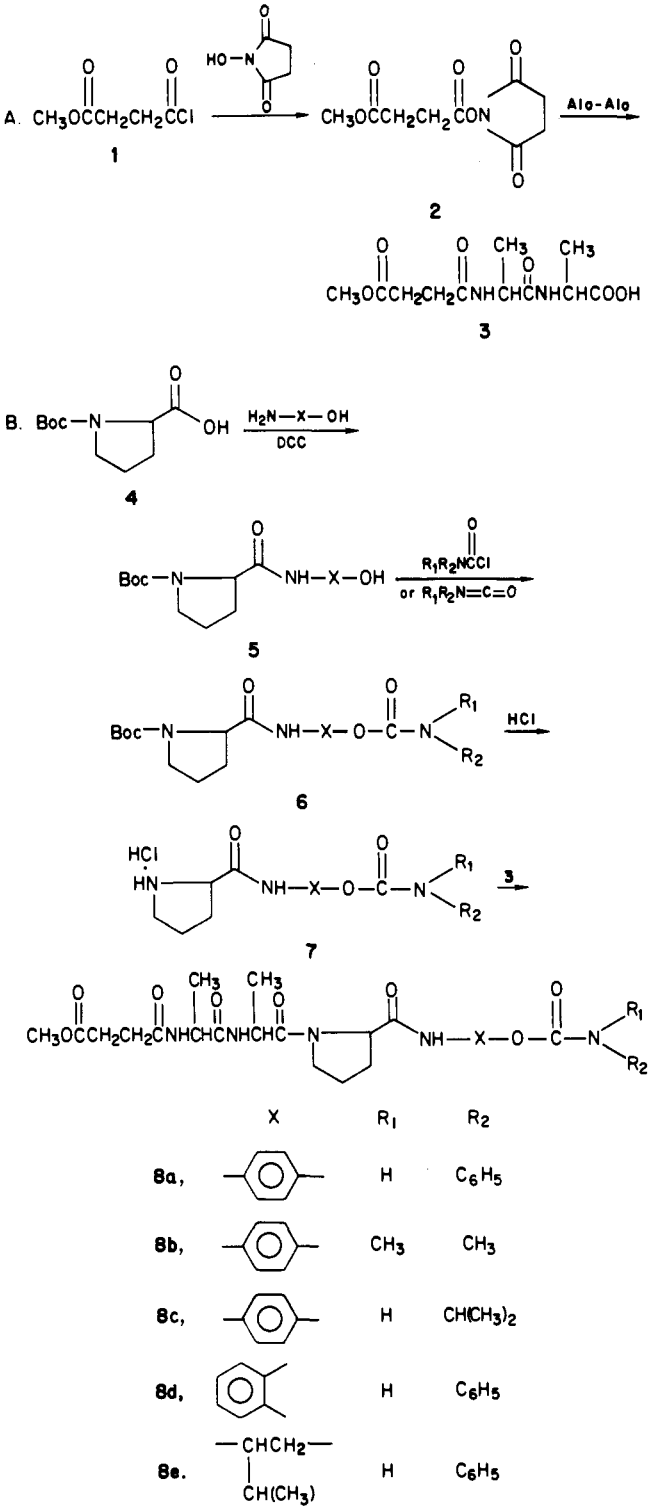
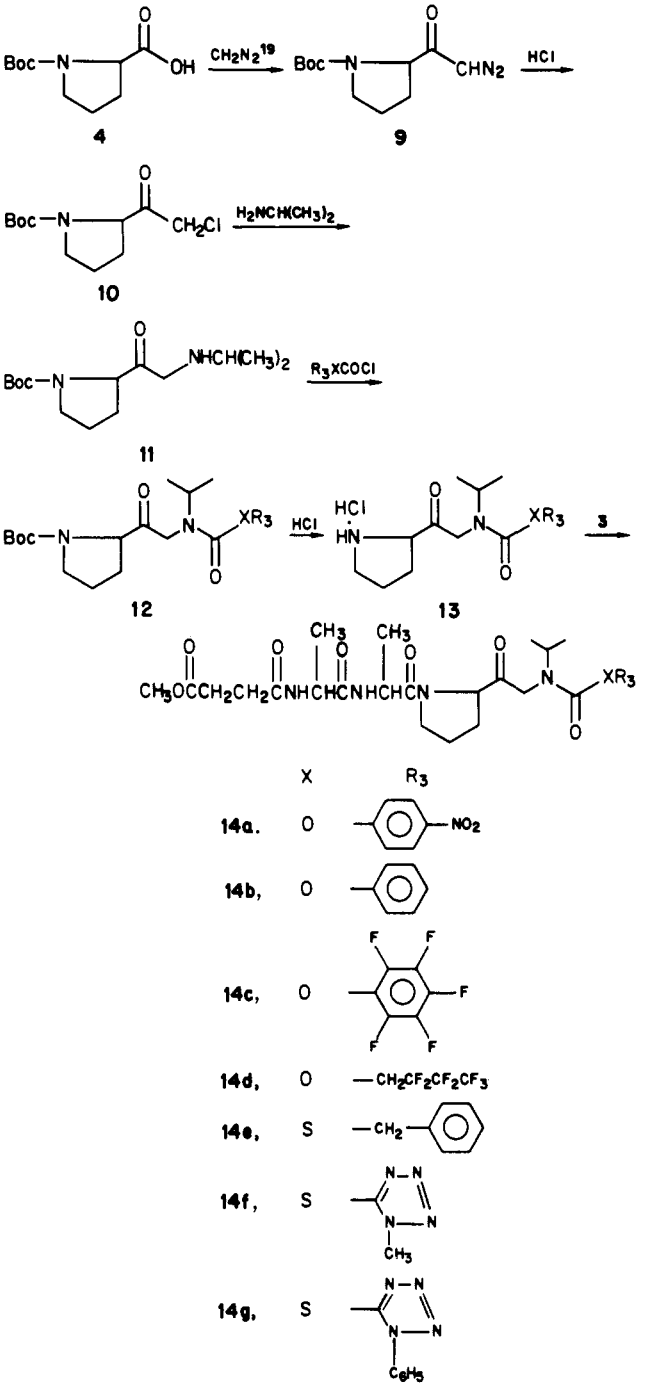
Scheme I



Several types of elastase inhibitors have been reported in the literature. These include, among others, peptide chloromethyl ketones,[6,7] azapeptides,[8,9] imidazole-*N*-carboxamides,[10] sulfonyl fluorides,[11,12] *p*-nitrophenyl *N*-alkylcarbamates,[13] and heterocyclic acylating agents.[14,15] Although some peptide chloromethyl ketones[6,7] have been shown[16] to be effective in preventing elastase-induced emphysema in animal models, there is considerable question whether such reactive reagents could be effective

Scheme II



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in treating emphysema in humans. This is not surprising since the alkylating moieties in these inhibitors might render them toxic when used on a continuous basis.[12] To be suitable for human use, an enzyme inhibitor has to show a high degree of selectivity and must have minimal toxic side effects. As a result, many drugs are molecules that reversibly bind to specific enzymes or receptor sites; examples are the carbamate esters physostigmine and neostigmine, which have been clinically used as inhibitors of acetylcholine esterases.[17] In this paper we illustrate the use of the carbamate functionality (RXCONR'R'') in the design of active site directed inhibitors of elastase with potential application in humans. This class of compounds will provide the opportunity to (a) incorporate chemical moieties (peptide portion) that would optimize the affinity of the inhibitor toward the enzyme and (b) transfer an acylating moiety (CONR'R'') to the active site of the enzyme. The nature of the latter could be varied to optimize the duration of enzymatic inactivation.

Results

Chemistry. The peptidyl carbamates (RXCONR'R'') investigated in this study are of two types: (a) those that have the tripeptide on the alcohol or thiol part (RX) of the molecule and (b) those where the peptide portion comprises the amine part (NR'R'') of the carbamate ester. Schemes I and II depict the synthetic routes followed in the preparation of these compounds.

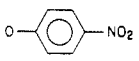
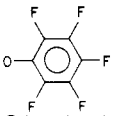
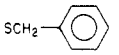
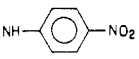
In both classes of compounds the MeO-Suc-Ala-Ala (3) portion of the molecule was obtained by reacting Ala-Ala with methyl succinic acid *N*-hydroxysuccinimide ester (2) (Scheme I). Subsequent coupling of 3 with either carbamate esters 7 (Scheme I) or 13 (Scheme II), using the mixed anhydride method,[18] afforded the desired carbamates **8a–e** and **14a–g**, respectively.

In the synthesis of 7, Boc-L-proline (4) was reacted with the appropriate amino alcohol utilizing *N,N'*-cyclohexylcarbodiimide (DCC) to activate the carboxylic group of proline. Carbamate esters 6 were formed by treating the resulting alcohol 5 with the required isocyanate or carbamyl chloride. Deprotection in acidic media yielded the intermediate carbamates 7.

The carbamate intermediates 13 were synthesized from Boc-L-proline (4) by the sequence of reactions shown in Scheme II. Reaction with diazomethane by the method of Penke et al.[19] gave the diazomethyl ketone **9**, which upon treatment with HCl yielded the α-chloromethyl ketone **10**. This was then reacted with isopropylamine in the presence of NaI to give the amine 11, which was subsequently converted to a carbamate or thiocarbamate ester by the reaction with the appropriate chloroformate. Deprotection under acidic conditions afforded the esters 13.

Enzymatic Studies. The inhibitory activity of the above peptidyl carbamates was tested toward porcine pancreatic (PP) elastase, and kinetic studies were carried out to determine the mode and potency (K_i values) of the inhibition. Enzymatic activity was assayed with the use of the synthetic substrate Boc-Ala-ONp[20] and spectrophotometrical monitoring of the rate of production of *p*-nitrophenolate anion at 398 nm. Compounds **8a–e** did not inhibit PP elastase under the experimental conditions used, whereas carbamate esters **14a–d**, **14f**, and **14g** (Table I) exhibited good inhibitory activity toward the enzyme as indicated by their K_i values (82.1–2.4 μM). These inhibitors were found to be specific for PP elastase as demonstrated by their lack of activity toward the serine-dependent proteases bovine pancreatic trypsin, chymotrypsin, and acetylcholine esterase.

Table I. Inhibition of PP Elastase by Peptidyl Carbamates of the General Formula MeO-Suc-Ala-Ala-$\overline{NCH_2CH_2CH_2CH}$COCH$_2$N(*i*-Pr)COP$_1$': Effect of Variations in P$_1$'

compd	P$_1$'	K_i, μM
14a		42.5
14b		31.5
14c		35.0
14d	$OCH_2CF_2CF_2CF_3$	82.1
14e		NI[a]
14f		19.5
14g		2.4
14h		NI

[a] NI: no inhibition at [I]/[E] = 260×.

With the use of five different inhibitor concentrations (19.3–2.4 μM) and three substrate concentrations (160–700 μM), initial velocities were recorded and used to plot Lineweaver–Burk[21] and Dixon[22] curves. The mode of inhibition, as determined by these plots, appears to be *uncompetitive*.

In another experiment, PP elastase was preincubated with the inhibitor, and aliquots of the incubate were taken at different time intervals and assayed for remaining enzyme activity. Thus, it was found that after 1-min preincubation of the enzyme (3 mg/150 mL of buffer) with **14a** or **14f** (3.8 μM), 89% and 93% of the enzymatic activity was lost, respectively. The residual activity of PP elastase was determined utilizing the synthetic substrate Boc-Ala-ONp.

The reversibility of inhibition of PP elastase by **14a** was examined by conducting dialysis experiments: the enzyme was incubated with a 100-fold excess of inhibitor for 2 h, and the reaction mixture was dialyzed. After removal of excess inhibitor (24 h), 95% inhibition was still observed; dialysis for additional 74 h resulted in full restoration (98%) of enzymatic activity. A control experiment showed that the activity of the native enzyme was not affected under the dialysis conditions described above.

Discussion

The present paper describes a novel class of elastase inhibitors that are peptidyl carbamates of the general formula RXCONR'R''. This class of compounds was designed so that the peptide portion of the inhibitor would provide specificity for the enzyme, whereas the carbamate functionality would act as the acylating moiety. The

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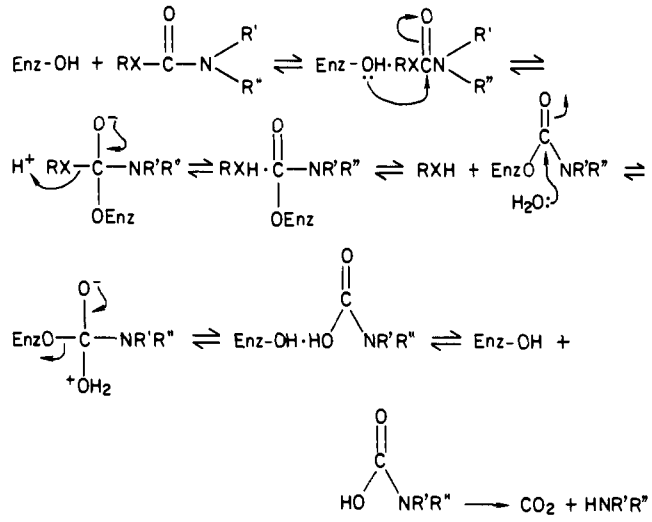
Scheme III[a]



[a] X = S, O.

peptide portion could be placed on either the alcohol (RX) or the amine (NR′R″) part of the molecule.

The proposed mechanism for the inhibition of PP elastase by peptidyl carbamates is depicted in Scheme III. The inactivation involves the carbamoylation of a serine residue at the active site of the enzyme via a tetrahedral intermediate. The carbamyl enzyme thus formed is a carbamate ester that is analogous to the acyl enzyme formed when a serine protease reacts with a normal peptide substrate. However, in contrast to a normal acyl enzyme (a carboxylic acid ester), the carbamyl enzyme is much more stable toward deacylation due to the resonance effect of the nitrogen adjacent to the carbonyl, which makes the latter less susceptible toward nucleophilic attack. Hence, the enzyme would be inhibited for a certain period of time, and slow deacylation would lead to the recovery of enzymatic activity.

As shown from the above mechanism, two factors are to be considered in the design of peptidyl carbamates as elastase inhibitors: (a) specificity of the inhibitor for the enzyme and (b) reactivity of the carbamate moiety of the inhibitor. The first factor would lead to an optimum "fit" and thus the formation of a strong Michaelis–Menton-type complex between the enzyme and the inhibitor, whereas the second factor would lead to a faster acylation of the serine residue on the active site of the enzyme. The latter was found to be highly dependent on the nature and nucleofugicity of the leaving group RX.

Throughout this report the terminology of Schechter and Berger[23] will be used where ...S_2-S_1-S_1'-S_2'... refer to subsites on both sides of the catalytic site of the enzyme, and the notation P on the inhibitor (or substrate) denotes amino acid residues that bind to these enzyme subsites such that P_1-P_1' represents the bond that is cleaved.

The design of the peptidyl carbamates reported here was based on substrate specificity studies with HL elastase,[7,18] which showed that substrates with the sequence MeO-Suc-Ala-Ala-Pro-Val (representing P_6-P_5-P_4-P_3-P_2-P_1) are highly reactive with the enzyme. The same sequence, although not ideal for PP elastase, was found to react with it effectively. In order to maintain specificity, the above sequence of P_6-P_5-P_4-P_3-P_2 was kept unchanged whereas P_1 and P_1' were varied and chosen in such a manner as to form a carbamate ester.

Compounds **8a–e** (Scheme I) are peptidyl carbamates where the peptide resides on the alcohol part of the molecule. Therefore, P_1 was chosen to be an amino alcohol. Reports by Powers et al. have shown, using molecular models, that the phenyl ring in anthranilic acid derivatives[24] and arenesulfonyl fluorides[11,12] fits the S_1 subsite of PP elastase. Furthermore, it has been shown in our laboratories[25] that carbamate esters of aromatic alcohols are hydrolyzed easier by PP elastase than those derived from aliphatic alcohols. Thus, the choice of an aromatic amino alcohol at P_1 was expected to fulfill the specificity as well as the reactivity requirements. The P_1' moiety (NR′R″) in these compounds was varied to include primary aliphatic and aromatic amines as well as secondary aliphatic amines. However, when carbamate esters **8a–d** were tested for inhibitory activity toward PP elastase, the enzymatic hydrolysis of the synthetic substrate Boc-Ala-ONp was unaffected. This lack of inhibitory activity may be due to the following two factors. Firstly, small molecule substrates such as anthranilic acid derivatives may interact at the S_1 subsite in a favorable conformation, due to the uncomplicated nature of their structures, i.e., lack of steric complexity in the molecule. However, in the case of peptidyl carbamates **8a–d**, the specificity of the extended peptide chain (P_6-P_5-P_4-P_3-P_2), for the corresponding subsites, dictates a certain conformation of the aromatic ring (P_1) that may make its "fit" for the S_1 subsite unfavorable due to steric considerations. Secondly, the distance between the carbonyl groups of P_2 and P_1 (site of proposed attack by the serine reside on the enzyme) seems to be of crucial importance. This is not surprising since in the natural substrate (or inhibitor) of elastase the P_1 residue is always an amino acid. With use of molecular models, this distance was found to be 3.0–3.7 Å as compared to 6.7 Å for the peptidyl carbamates **8a–d**. Therefore, it appears that an active peptide-type inhibitor of elastase should have an amino acid or an isostere of an amino acid as the P_1 residue.

Consequently, compound **14a** (Table I) was prepared such that the peptide resided on the amine part (NR′R″) of the carbamate functionality, and P_1 was an isostere of valine, thus satisfying the specificity requirement in the design. In order to satisfy the reactivity factor, the good leaving group *p*-nitrophenol was chosen as P_1'. Indeed, incubation of PP elastase with excess of **14a** for 1 min at 25 °C and pH 6.5 led to 89% loss of enzymatic activity. In order to study the effect of structural variations in P_1' on the inhibitory activity of this class of compounds toward elastase, carbamates **14b–g** (Table I) were prepared. The P_6-P_1 sequence in these compounds was kept constant, as in **14a**, and P_1' was varied to include aromatic alcohols (**14a–c**) and aliphatic alcohols and thiols (**14d–e**), as well as aromatic heterocycles (**14f–g**). When these carbamates were tested for inhibitory activity toward elastase, results showed that compounds where RX (P_1') was a good leaving group, such as an aromatic alcohol or thiol, exhibited the highest potency. Thiobenzyl carbamate **14e** was found to be inactive, wherease **14d**, where RX is a polyfluorinated alcohol, was active. The fact that **14i**, the urea analogue of **14a**, did not inhibit the enzyme tends to underline the importance of the reactivity factor in the choice of the P_1' moiety.

The specificity of peptidyl carbamate inhibitors for PP elastase was demonstrated by their lack of activity toward trypsin and chymotrypsin. These enzymes were chosen

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because X-ray crystallographic studies of the active-site regions of these enzymes and PP elastase have revealed a remarkable degree of homology.[26] In fact, it has been shown[27] that the catalytic sites in these enzymes are essentially identical, all containing a charge relay system composed of a histidine, an aspartic acid, and two serine residues.

Kinetic studies were carried out to determine the mode of inhibition and the relative activity of these inhibitors. Dixon,[22] Cornish-Bowden,[28] and Lineweaver–Burk[21] plots showed, using linear regression, that the inhibition of PP elastase by peptidyl carbamates is of the mixed type or uncompetitive (both V_{max} and K_m were affected).

Incubation of PP elastase with excess concentrations of inhibitors **14a** and **14f** led to the respective loss of 89% and 93% of enzymatic activity in 1 min. However, when equivalent amounts of inhibitor and enzyme were preincubated, only 80% and 31% of enzymatic activity were lost in 30 min with **14a** and **14f**, respectively. The nonstoichiometric nature of inhibition indicates that the mechanism of inhibition may be more complex than that depicted in Scheme III. Therefore, preliminary studies were carried out, with use of **14a** as a model compound, in an attempt to understand the inhibition mechanism.

Thus, PP elastase was incubated with a 100-fold excess of inhibitor (**14a**) and the following factors were tested: (a) reversibility of inhibition, (b) stability of enzyme–inhibitor complex, and (c) involvement of covalent bonding between enzyme and inhibitor.

The reversibility of inhibition was tested by conducting dialysis experiments. The obtained results indicate that inhibition of PP elastase by peptidyl carbamates is a slowly reversible process. Such reversibility is desirable for pharmacological purposes, as suggested by Abeles,[29] since it permits more flexibility in controlling the time period during which the activity of the target enzyme is repressed.

The stability of the inhibited enzyme species was tested by subjecting the inhibition mixture to gel filtration with subsequent isolation of the inhibitor–enzyme complex. Incubation of the isolated complex in distilled water (pH 6.4) for 72 h at 25 °C led to 25% recovery of enzymatic activity, thus indicating that the inhibitor binds strongly to the enzyme. If the mechanism in Scheme III is operative and there is acylation of the enzyme, the release of *p*-nitrophenol from the enzyme–inhibitor incubation mixture can be followed to monitor the rate of acylation. Indeed, when such an experiment was conducted, it was found that after 2-h incubation only 80% of the expected *p*-nitrophenol was released. No nonenzymatic hydrolysis of the inhibitor (to give *p*-nitrophenol) was observed after 2-h incubation in phosphate buffer (pH 6.5). Comparison of the data shows that enzymatic inactivation by **14a** is much faster than enzymatic hydrolysis of the same compound. This discrepancy in percent loss of activity as compared to the percent release of *p*-nitrophenol might be due to an initial rapid formation of strong Michaelis–Menton-type enzyme–inhibitor complex (leading to enzyme inhibition) that slowly acylates the enzyme (leading to *p*-nitrophenol release). More work is underway to elucidate the proposed mechanism and to unequivocally prove that a carbamoylated enzyme is formed.

In conclusion, a new class of specific elastase inhibitors are reported. Although, at present, the mechanism of inactivation is not clear, there is an indication that a stable acyl enzyme is formed that slowly recovers activity. A similar mechanism is believed to be operative in the inhibition of serine proteases by their natural protease inhibitors.[30] In both cases, the acylation of the enzyme results in the transfer of a peptide chain to the enzyme. The similarities in the mechanism of action of peptidyl carbamate inhibitors and natural protease inhibitors suggest that such synthetic inhibitors have a potential to be used in vivo. Moreover, preliminary data from our laboratories show that the active peptidyl carbamate inhibitors listed here exhibit high inhibitory activity toward HL elastase.[31] Thus, for example, compound **14f** (Table I) exhibited a K_i value of 4×10^{-6} M against HL elastase, the enzyme believed to be responsible for major tissue destruction associated with several disease states.

Experimental Section

Melting points were determined on a Thomas-Hoover Uni-Melt apparatus and are uncorrected. Microanalyses were performed by Atlantic Microlab, Inc., Atlanta, GA, or by Microanalysis, Inc., Wilmington, DE. Analytical results obtained for all compounds were within ±0.4% of the theoretical values unless otherwise stated. ^{1}H NMR spectra were recorded on a Varian EM-360 (60 MHz) or EM-390 (90 MHz) spectrometer; tetramethylsilane was used as an internal standard. IR spectra were determined as KBr disks, Nujol mulls, or liquid films on a Perkin-Elmer 567 spectrophotometer. Reactions were routinely followed by thin-layer chromatography (TLC) with Whatman MK6F silica gel plates; spots were detected by UV irradiation at 254 nm, iodine or HBr–ninhydrin spraying. Column chromatography was carried out with silica gel 60 from E. Merck, Darmstadt, Germany.

The following abbreviations have been used in the text: MeO-Suc = methoxysuccinyl; Ala = L-alanine; Pro = L-proline; Val = L-valine; Boc-Ala-ONp = (*tert*-butyloxycarbonyl)alanine *p*-nitrophenyl ester; Bz-Arg-OEt = benzoylarginine ethyl ester; Bz-Tyr-OEt = benzoyltyrosine ethyl ester.

Methyl Succinimido Succinate (2). To an ice-cooled solution of *N*-hydroxysuccinimide (1.15 g, 10 mmol) and methyl succinyl chloride (1.23 mL, 10 mmol) in ethyl acetate (50 mL) was added, dropwise, triethylamine (1.4 mL, 10 mmol). The mixture was stirred at 4 °C for 10 min and at room temperature for 5 min. Filtration of insoluble materials, followed by evaporation of the filtrate, yielded 2.1 g of pale yellow crystals of **2** (92%); mp 86–88 °C.

(Methoxysuccinyl)-L-alanyl-L-alanine (3). Without further purification, compound **2** (1.48 g, 6.46 mmol) was slowly added to a stirred solution of L-alanyl-L-alanine (862 mg, 5.38 mmol) and sodium bicarbonate (452 mg, 5.38 mmol) in water (5 mL) and acetone (5 mL). The mixture was stirred at room temperature for 4 h, and the acetone was evaporated. The aqueous solution was washed with EtOAc (8 mL), acidified with citric acid (1.3 g), saturated with NaCl, and extracted with EtOAc (4 × 30 mL). The organic layers were washed with NaCl solution (3 mL), dried ($MgSO_4$), and concentrated. The residue was washed with EtOAc to give colorless crystals of **3** (920 mg, 62%); mp 134–135 °C; NMR (Me_2SO-d_6) δ 1.16 (d, 3 H, J = 7 Hz), 1.24 (d, 3 H, J = 7 Hz), 2.40 (t, 4 H, J = 7 Hz), 3.54 (s, 3 H), 4.16 (m, 1 H), 4.29 (m, 1 H), 8.01 (d, 2 H, J = 7 Hz); IR (Nujol) 3280, 1735, 1690, 1630, 1540 cm^{-1}. Anal. ($C_{11}H_{18}N_2O_6$) C, H, N.

4-(*N*-Boc-L-prolinamido)phenol (5a). A mixture of *N*-Boc-L-proline (4) (2.5 g, 11.6 mmol) and *N*,*N*′-dicyclohexylcarbodiimide (DCC) (2.63 g, 12.8 mmol) in THF (30 mL) was stirred at room temperature for 30 min; *p*-aminophenol (1.27 g, 11.6 mmol) was added, and the resulting mixture was stirred overnight. The solvent was evaporated, and the residue obtained was dissolved in DMF (30 mL) and filtered. The filtrate was

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poured into water (300 mL), and the precipitate was filtered, washed with THF, and dried in vacuo to give colorless crystals of **5a** (1.4 g, 40%); mp 235–236 °C dec; NMR (Me_2SO-d_6) δ 1.25 (s, 9 H), 1.60–2.30 (m, 4 H), 3.13–3.53 (m, 2 H), 4.16 (m, 1 H), 6.64 (d, 2 H, J = 9 Hz), 7.34 (d, 2 H, J = 9 Hz), 9.10 (s, 1 H), 9.62 (s, 1 H); IR (Nujol) 3330, 1670, 1630, 1575 cm^{-1}. Anal. ($C_{16}H_{22}N_2O_4$) C, H, N.

2-(*N*-Boc-L-prolinamido)phenol (5b). This compound was prepared in the same manner as **5a**. Yield from *N*-Boc-L-proline, 39%; mp 136–138 °C; NMR ($CDCl_3$) δ 1.47 (s, 9 H), 1.76–2.46 (m, 4 H), 3.44 (t, 2 H, J = 6 Hz), 4.50 (m, 1 H), 6.66–7.40 (m, 4 H), 8.20–9.53 (br s, 2 H); IR (Nujol) 3280, 1680, 1665, 1590, 1540 cm^{-1}. Anal. ($C_{16}H_{22}N_2O_4$) C, H, N.

DL-2-(*N*-Boc-L-prolinamido)-3-methyl-1-butanol (5c). A mixture of *N*-Boc-L-proline (1.0 g, 4.65 mmol) and dicyclohexylcarbodiimide (1.05 g, 5.11 mmol) in THF (10 mL) was stirred at room temperature for 40 min. DL-2-Amino-3-methyl-1-butanol (0.479 g, 4.65 mmol) was added, and the resulting mixture was stirred at room temperature overnight, cooled in a refrigerator, and filtered. The filtrate obtained was evaporated and the residue purified by column chromatography ($CHCl_3$/EtOAc, 5:1). Fractions that contained two spots attributed to two diastereomers of **5c** were combined and evaporated to give colorless crystals of **5c** (0.60 g, 43%); mp 140–150 °C; NMR ($CDCl_3$) δ 0.97 (d, 3 H, J = 7 Hz), 1.00 (d, 3 H, J = 7 Hz), 1.50 (s, 9 H), 1.70–2.40 (m, 5 H), 3.00 (br s, 1 H), 3.33–3.85 (m, 5 H), 4.30 (m, 1 H), 6.66 (br s, 1 H); IR (film) 3380, 3320, 1690, 1660, 1545 cm^{-1}. Anal. ($C_{15}H_{28}N_2O_4$) C, H, N.

4-(*N*-Boc-L-prolinamido)phenyl *N*-Phenylcarbamate (6a). A solution of **5a** (900 mg, 2.94 mmol), phenyl isocyanate (0.35 mL, 3.23 mmol), and pyridine (0.2 mL) in DMF (9 mL) was stirred at 55 °C for 4 h. The reaction mixture was poured into ice–water (100 mL), and the precipitate was filtered, washed with water, and dried in vacuo. The crude compound thus obtained was purified by column chromatography ($CHCl_3$/EtOAc, 30:1) to give colorless crystals of **6a** (930 mg, 74%); mp 207–209 °C dec; NMR (Me_2SO-d_6) δ 1.30 (br s, 9 H), 1.66–2.36 (m, 4 H), 3.10–3.60 (m, 2 H), 4.23 (m, 1 H), 6.90–7.80 (m, 9 H), 10.00 (s, 1 H), 10.13 (s, 1 H); IR (Nujol) 3350, 3230, 1730, 1690, 1660, 1605 cm^{-1}. Anal. ($C_{23}H_{27}N_3O_5 \cdot {}^1/_2H_2O$) C, H, N.

4-(*N*-Boc-prolinamido)phenyl *N*,*N*-Dimethylcarbamate (6b). A mixture of **5a** (500 mg, 1.63 mmol) and dimethylcarbamoyl chloride (0.67 mL, 7.31 mmol) in pyridine (5 mL) was sealed and stirred at 90 °C for 4 h. The mixture was poured into ice water (80 mL) and extracted twice with dichloromethane (50 mL). The extracts were combined, successively washed with 10% citric acid and 4% $NaHCO_3$ solutions, dried ($MgSO_4$), and concentrated. The oil obtained was purified by column chromatography ($CHCl_3$–EtOAc, 30:1) and washed with petroleum ether (40–60 °C) to give colorless crystals of **6b** (440 mg, 72%); mp 140–142 °C; NMR ($CDCl_3$) δ 1.42 (s, 9 H), 1.66–2.40 (m, 4 H), 3.02 (s, 6 H), 3.43 (m, 2 H), 4.53 (m, 1 H), 7.09 (d, 2 H, J = 9 Hz), 7.58 (d, 2 H, J = 9 Hz), 8.66–9.80 (br s, 1 H); IR (Nujol) 3320, 1740, 1720, 1700, 1680, 1540 cm^{-1}. Anal. ($C_{19}H_{27}N_3O_5$) C, H, N.

4-(*N*-Boc-prolinamido)phenyl *N*-Isopropylcarbamate (6c). A mixture of **5a** (500 mg, 1.63 mmol), isopropyl isocyanate (0.8 mL, 8.14 mmol), and pyridine (6 drops) in DMF (5 mL) was sealed and stirred at 70 °C for 8 h. The mixture was poured into a mixture of water (50 mL) and EtOAc (30 mL). The precipitate obtained was filtered and washed with cold EtOAc to give 0.15 g of colorless crystals of **6c**. The organic layer from the filtrate was separated, dried ($MgSO_4$), filtered, and evaporated to give an oil. Trituration with ether yielded another 0.22 g of **6c** (60%); mp 214–215 °C; NMR (Me_2SO-d_6) δ 1.15 (d, 6 H, J = 6 Hz), 1.32 (br s, 9 H), 1.70–2.33 (m, 4 H), 3.20–3.90 (m, 3 H), 4.23 (m, 1 H), 7.03 (d, 2 H, J = 9 Hz), 7.40–7.80 (m, 3 H), 9.96 (s, 1 H); IR (Nujol) 3350, 3290, 1740, 1670, 1530 cm^{-1}. Anal. ($C_{20}H_{29}N_3O_5$) C, H, N.

2-(*N*-Boc-prolinamido)phenyl *N*-Phenylcarbamate (6d). This compound was prepared following the procedure used for **6a**. Yield from **5b**, 65%; mp 127–129 °C; NMR ($CDCl_3$) δ 1.40 (s, 9 H), 1.80–2.50 (m, 4 H), 3.50 (m, 2 H), 4.73 (m, 1 H), 6.90–7.73 (m, 9 H), 8.26–8.60 (m, 2 H); IR (Nujol) 3300, 3260, 1750, 1685, 1665, 1610, 1540 cm^{-1}. Anal. ($C_{23}H_{27}N_3O_5$) C, H, N.

DL-2-(*N*-Boc-L-prolinamido)-3-methyl-1-butyl *N*-Phenylcarbamate (6e). A solution of **5c** (0.52 g, 1.73 mmol), phenyl isocyanate (0.21 mL, 1.91 mmol), and pyridine (a few drops) in THF (7.5 mL) was stirred at 55 °C overnight. The residue obtained by evaporation was purified by column chromatography ($CHCl_3$) to give **6e** as a white powder (0.59 g, 81%); mp 60 °C; NMR ($CDCl_3$) δ 0.86–1.13 (m, 6 H), 1.53 (s, 9 H), 1.70–2.30 (m, 5 H), 3.30–3.66 (m, 2 H), 4.00–4.50 (m, 4 H), 6.93–7.60 (m, 7 H); IR (film) 3300, 1710, 1685, 1650, 1600, 1535 cm^{-1}. Anal. ($C_{22}H_{33}N_3O_5$) C, H, N.

4-(L-Prolinamido)phenyl *N*-Phenylcarbamate Hydrochloride (7a). A mixture of **6a** (700 mg, 1.65 mmol) in formic acid (5 mL) and 1 N solution of hydrogen chloride in THF (2 mL) was stirred at room temperature for 1 h. The mixture was evaporated and the oil obtained was triturated with ether to give pale yellow crystals of **7a** (520 mg, 87%); mp 159–163 °C dec; NMR (Me_2SO-d_6) δ 1.70–2.20 (m, 4 H), 3.10–3.66 (m, 2 H), 4.50 (m, 1 H), 6.90–7.85 (m, 9 H), 9.40 (br s, 1 H), 10.25 (br s, 1 H); IR (Nujol) 3190, 3130, 1720, 1690, 1600, 1540, 1500 cm^{-1}. Anal. ($C_{18}H_{20}ClN_3O_3 \cdot {}^1/_2H_2O$) C, H, N.

4-(L-Prolinamido)phenyl *N*,*N*-Dimethylcarbamate Hydrochloride (7b). This compound was prepared in a similar manner to **7a**; yield from **6b**, 96%; mp 265–267 °C dec; NMR (Me_2SO-d_6) δ 1.80–2.30 (m, 4 H), 3.04 (s, 3 H), 3.12 (s, 3 H), 3.23–3.60 (m, 2 H), 4.63 (t, 1 H, J = 7 Hz), 7.18 (d, 2 H, J = 9 Hz), 7.85 (d, 2 H, J = 9 Hz), 8.90–10.15 (br s, 2 H); IR (Nujol) 3300, 3260, 3130, 1740, 1700, 1625, 1570, 1520 cm^{-1}. Anal. ($C_{14}H_{20}ClN_3O_3$) C, H, N.

4-(L-Prolinamido)phenyl *N*-Isopropylcarbamate Hydrochloride (7c). This compound was prepared following the same procedure as for **7a**; yield from **6c**, 94%; mp 230–232 °C; NMR (Me_2SO-d_6) δ 1.15 (d, 6 H, J = 6 Hz), 1.80–2.20 (m, 4 H), 3.10–3.90 (m, 3 H), 4.47 (t, 1 H, J = 7 Hz), 7.08 (d, 2 H, J = 9 Hz), 7.50–7.80 (m, 1 H), 7.66 (d, 2 H, J = 9 Hz), 9.50 (br s, 1 H); IR (Nujol) 3400, 3300, 2790, 1730, 1700, 1620, 1555, 1520 cm^{-1}. Anal. ($C_{15}H_{22}ClN_3O_3 \cdot {}^1/_4H_2O$) C, H, N, Cl.

2-(L-Prolinamido)phenyl *N*-Phenylcarbamate Hydrochloride (7d). This compound was prepared in the same manner as **7a**. Yield from **6d**, 100%; mp 145–155 °C; NMR ($CDCl_3$) δ 1.60–2.20 (m, 4 H), 3.00–3.50 (m, 2 H), 5.10 (m, 1 H), 6.80–8.10 (m, 9 H), 9.60 (s, 1 H), 9.90 (s, 1 H); IR (Nujol) 3180, 3120, 1750, 1690, 1600, 1540 cm^{-1}. Anal. ($C_{18}H_{20}ClN_3O_3$) C, H, N, Cl.

DL-2-(L-Prolinamido)-3-methyl-1-butyl *N*-Phenylcarbamate Hydrochloride (7e). This compound was prepared by the same procedure used for the synthesis of **7a**. Yield from **6e**, 86%; mp 128–130 °C; NMR ($CDCl_3$) δ 0.90–1.20 (m, 6 H), 1.66–2.50 (m, 5 H), 3.20–3.70 (m, 2 H), 3.80–4.73 (m, 4 H), 6.70–7.70 (m, 5 H), 7.80–9.00 (m, 2 H); IR (film) 3230, 1725, 2680, 1600, 1540 cm^{-1}. Anal. ($C_{17}H_{26}ClN_3O_3 \cdot {}^1/_2H_2O$) C, H, N, Cl.

4-[(Methoxysuccinyl)-L-alanyl-L-alanyl-L-prolinamido]phenyl *N*-phenylcarbamate (8a). A solution of isobutyl chloroformate (63 μL, 0.486 mmol) in THF (1 mL) was added dropwise to a stirred mixture of **3** (133 mg, 0.486 mmol) and *N*-methylmorpholine (53 μL, 0.486 mmol) in THF (2 mL) at −15 to −20 °C, and the resulting reaction mixture was stirred for 10 min at the same temperature. A solution of **7a** (160 mg, 0.442 mmol) and *N*-methylmorpholine (49 μL, 0.442 mmol) in THF (3 mL) was added dropwise to the above mixture at −15 to −30 °C and then warmed gradually and stirred at room temperature overnight. The reaction mixture was concentrated in vacuo, purified by column chromatography ($CHCl_3$–CH_3OH, 20:1), and washed with ether to give colorless crystals of **8a** (185 mg, 72%); mp 160–163 °C; NMR ($CDCl_3$) δ 0.80–1.50 (m, 6 H), 1.60–2.30 (m, 4 H), 2.58 (br s, 4 H), 3.30–3.80 (m, 2 H), 3.57 (s, 3 H), 4.30–4.90 (m, 3 H), 6.50–7.70 (m, 11 H), 8.10 (m, 1 H), 9.33 (s, 1 H); IR (Nujol) 3300, 1750, 1735, 1650, 1630, 1600, 1530 cm^{-1}. Anal. ($C_{29}H_{35}N_5O_8$) C, H, N.

4-[(Methyloxysuccinyl)-L-alanyl-L-alanyl-L-prolinamido]phenyl *N*,*N*-Dimethylcarbamate (8b). To a stirred mixture of **3** (213 mg, 0.778 mmol) and *N*-methylmorpholine (86 μL, 0.778 mmol) in THF (2.5 mL) at −15 to −30 °C was added, dropwise, a solution of isobutyl chloroformate (101 μL, 0.778 mmol) in THF (1 mL) and the mixture was stirred for 30 min at the same temperature. A solution of **7b** (244 mg, 0.779 mmol), *N*-methylmorpholine (86 μL, 0.778 mmol), and bis(trimethylsilyl)acetamide (1 mL) in THF (3 mL) was then added dropwise to the above mixture at −50 °C. The reaction mixture obtained was warmed gradually, stirred at room temperature overnight, diluted with $CHCl_3$ (10 mL), successively washed with 10% citric

acid and 4% $NaHCO_3$ solution, and evaporated. The resulting oil was purified by column chromatography ($CHCl_3$–MeOH, 50:1) to give colorless crystals of **8b** (234 mg, 56%), mp 100–102 °C; NMR ($CDCl_3$) δ 1.10–1.40 (m, 6 H), 1.90–2.30 (m, 4 H), 2.61 (m, 4 H), 3.02 (s, 6 H), 3.50–3.90 (m, 2 H), 3.65 (s, 3 H), 4.50–5.10 (m, 3 H), 6.7–7.20 (m, 3 H), 7.53 (d, 2 H, J = 8 Hz), 8.23 (d, 1 H, J = 8 Hz), 9.38 (br s, 1 H); IR (Nujol) 3330, 1735, 1640, 1550, 1510 cm^{-1}. Anal. ($C_{25}H_{35}N_5O_8$) C, H, N.

4-[(Methoxysuccinyl)-L-alanyl-L-alanyl-L-prolinamido]-phenyl *N*-Isopropylcarbamate (8c). This compound was prepared by the same procedure used for the synthesis of **8b**; yield from **7c**, 49%; mp 108–110 °C; NMR ($CDCl_3$) δ 1.00–1.66 (m, 12 H), 1.80–2.30 (m, 4 H), 2.60 (m, 4 H), 3.40–4.00 (m, 6 H), 4.40–5.30 (m, 3 H), 6.70–7.70 (m, 5 H), 8.20 (m, 2 H), 9.35 (br s, 1 H); IR (Nujol) 3290, 1730, 1635, 1540, 1500 cm^{-1}. Anal. ($C_{26}H_{37}N_5O_8 \cdot H_2O$) C, H, N.

2-[(Methoxysuccinyl)-L-alanyl-L-alanyl-L-prolinamido]-phenyl *N*-Phenylcarbamate (8d). This compound was prepared in a similar manner to that for **8a**. Yield from **7d**, 69%; mp 160–163 °C; NMR ($CDCl_3$) δ 1.24 (d, 3 H, J = 7 Hz), 1.36 (d, 3 H, J = 7 Hz), 1.60–2.00 (m, 4 H), 2.58 (br s, 4 H), 3.30–3.70 (m, 2 H), 3.57 (s, 3 H), 4.20–4.90 (m, 3 H), 6.70–7.76 (m, 10 H), 8.33 (m, 1 H), 8.83 (s, 1 H), 9.13 (s, 1 H); IR (Nujol) 3300, 1750, 1735, 1650, 1630, 1600, 1530 cm^{-1}. Anal. ($C_{29}H_{35}N_5O_8$) C, H, N.

DL-2-[(Methoxysuccinyl)-L-alanyl-L-alanyl-L-prolinamido]-3-methyl-1-butyl *N*-Phenylcarbamate (8e). This compound was prepared following the same procedure used for **8a**. Yield from **7e**, 29%; mp 125–130 °C; NMR ($CDCl_3$) δ 0.76–1.50 (m, 12 H), 1.70–2.30 (m, 5 H), 2.60 (br s, 4 H), 3.30–4.30 (m, 5 H), 3.60 (s, 3 H), 4.40–5.10 (m, 3 H), 6.60 (d, 1 H, 8 Hz), 6.80–7.60 (m, 7 H), 8.33 (d, 1 H, J = 8 Hz); IR (Nujol) 3300, 1730, 1630, 1530 cm^{-1}. Anal. ($C_{28}H_{41}N_5O_8$) C, H, N.

***N*-Boc-L-prolyl Chloromethyl Ketone (10).** Into an ice-cooled solution of **9** (11.0 g) in ether (240 mL) was introduced HCl gas until the yellow solution turned colorless. The mixture was evaporated and purified by column chromatography (hexane–$CHCl_3$, 1:1) to give colorless crystals of **10** (9.8 g, 51.7% from Boc-L-proline); mp 47–49 °C; NMR ($CDCl_3$) δ 1.47 (s, 9 H), 1.80–2.20 (m, 4 H), 3.56 (m, 2 H), 4.36 (s, 2 H), 4.40–4.80 (m, 1 H); IR (film) 1735, 1690 cm^{-1}. Anal. ($C_{11}H_{18}ClNO_3$) C, H, N.

***N*-[(*N*-Boc-L-propyl)methyl]isopropylamine (11).** To an ice-cooled solution of **10** (3.0 g, 12.1 mmol) in 95% EtOH (30 mL) was added sodium iodide (1.95 g, 12.8 mmol) and isopropylamine (10.2 mL, 60 mmol). The mixture was shaken at 65 °C for 15 h, and saturated $NaHCO_3$ solution was added. The mixture was extracted into ether, the solvent evaporated, and the residue purified by column chromatography ($CHCl_3$–CH_3OH, 50:1) to give **11** as a brown oil (1.84 g, 56%); NMR ($CDCl_3$) δ 1.12 (d, 6 H, J = 6 Hz), 1.50 (s, 9 H), 1.80–2.30 (m, 4 H), 2.83 (m, 1 H), 3.40–3.80 (m, 5 H), 4.30 (m, 1 H); IR (film) 3330 (br), 1695 (br), 1695 (br) cm^{-1}.

***p*-Nitrophenyl *N*-[(*N*-Boc-L-prolyl)methyl]-*N*-isopropylcarbamate (12a).** A solution of *p*-nitrophenyl chloroformate (0.88 g, 4.34 mmol) in dry THF (10 mL) was added dropwise to a solution of **11** (10.98 g, 3.63 mmol) and triethylamine (0.604 mL, 4.34 mmol) in THF at 5 °C. The mixture was stirred at 5 °C for 2 h and filtered. The filtrate was evaporated and purified by column chromatography ($CHCl_3$–EtOAc, 100:1) to give a yellow oil of **12a** (1.28 g, 81%); NMR ($CDCl_3$) δ 1.06–1.30 (m, 6 H), 1.46 (m, 9 H), 1.70–2.33 (m, 4 H), 3.33–3.70 (m, 2 H), 4.10–4.80 (m, 4 H), 7.33 (dd, 2 H, J = 4, 9 Hz), 8.33 (d, 2 H, J = 9 Hz); IR (film) 1740–1690, 1590, 1520 cm^{-1}.

Phenyl *N*-[(*N*-Boc-L-prolyl)methyl]-*N*-isopropylcarbamate (12b). This compound was prepared in a similar manner to that for **12a**. Yield from **11** 95%; NMR ($CDCl_3$) δ 1.10–1.47 (m, 6 H), 1.47 (s, 9 H), 1.70–2.10 (m, 4 H), 3.40–3.70 (m, 2 H), 3.80–4.70 (m, 4 H), 7.00–7.60 (m, 5 H); IR (film) 1750–1680, 1595 cm^{-1}.

Pentafluorophenyl *N*-[(*N*-Boc-L-prolyl)methyl]-*N*-isopropylcarbamate (12c). This compound was prepared in a similar manner to **12a**. Yield from **11** 80%; NMR ($CDCl_3$) δ 1.10–1.40 (m, 6 H), 1.48 (s, 9 H), 1.76–2.30 (m, 4 H), 3.53 (m, 2 H), 4.15–4.80 (m, 4 H); IR (film) 1750, 1690, 1515 cm^{-1}.

2,2,3,3,4,4,4-Heptafluorobutyl *N*-[(*N*-Boc-L-prolyl)-methyl]-*N*-isopropylcarbamate (12d). This compound was prepared in a similar manner to that for **11a**. Yield from **11** 57%; NMR ($CDCl_3$) δ 1.13 (d, 6 H, J = 7 Hz), 1.49 (s, 9 H), 1.80–2.33 (m, 4 H), 3.53 (m, 2 H), 4.00–5.90 (m, 6 H); IR (film) 1740–1690 cm^{-1}.

1-Methyl-5-tetrazolyl Chlorothioformate. A solution of triethylamine (0.65 mL, 4.64 mmol) in THF (2 mL) was added dropwise to a mixture of 1-methyl-5-mercaptotetrazole (450 mg, 3.87 mmol) in THF (5 mL) and 12.5% phosgene in benzene (4.7 mL, 6 mmol) at 0–5 °C. The mixture was stirred at 0.5 °C for 30 min and at room temperature for 2 h, followed by filtration of the mixture. The filtrate obtained was evaporated to give the product (0.62 g, 90%); NMR ($CDCl_3$) δ 4.23 (s, 3 H); IR (film) 1770 cm^{-1}.

***S*-Benzyl *N*-[(*N*-Boc-L-prolyl)methyl]-*N*-isopropylthiocarbamate (12e).** This compound was prepared according to the procedure described for **12a** with use of *S*-benzyl chlorothioformate as the starting material. Yield from **11** 75%; NMR ($CDCl_3$) δ 1.18 (d, 6 H, J = 7 Hz), 1.50 (s, 9 H), 1.80–2.40 (m, 4 H), 3.60 (m, 2 H), 4.26 (s, 2 H), 4.20–4.60 (m, 4 H), 7.45 (s, 5 H); IR (film) 1740, 1690, 1640 cm^{-1}.

***S*-1-Methyl-5-tetrazolyl *N*-[(*N*-Boc-L-prolyl)methyl]-*N*-isopropylthiocarbamate (12f).** To a solution of **11** (1.95 g, 7.2 mmol) and triethylamine (9.3 mL, 1.36 mmol) in THF (15 mL) at 5 °C was added a suspension of 1-methyl-5-tetrazoyl chlorothioformate (1.67 g, 9.36 mmol) in THF (10 mL) over 10 min, and the mixture was stirred for 1 h at 5 °C. The reaction mixture was diluted with cold water and extracted with $CHCl_3$. The organic layer was dried over anhydrous $MgSO_4$ and evaporated in vacuo to give an oil. The oil was purified by column chromatography (silica gel, 40 g) with $CHCl_3$ as an eluent to give 1.86 g of the product as an oil (74%). The oil was crystallized from ethyl acetate–hexane, mp 145–150 °C; NMR ($CDCl_3$) δ 1.30 (d, 6 H, J = 6 Hz), 1.46 (s, 9 H), 1.80–2.30 (m, 4 H), 3.53 (m, 2 H), 4.10 (s, 3 H), 4.00–4.60 (m, 4 H); IR (film) 1740, 1690, 1670 cm^{-1}. Anal. ($C_{17}H_{28}N_6O_4S$) C, H, N.

***S*-1-Phenyl-5-tetrazolyl *N*-[(*N*-Boc-L-prolyl)methyl]-*N*-isopropylthiocarbamate (12g).** The title compound was prepared following the procedure used for **12f** and was obtained as an oil. Yield, 74%; NMR ($CDCl_3$) δ 1.17 (d, 6 H, J = 7 Hz), 1.47 (s, 9 H), 1.95 (m, 4 H), 3.46 (m, 2 H), 3.90–4.60 (m, 4 H), 7.70 (s, 5 H); IR (film) 1735, 1690, 1590, 1495, 1400 cm^{-1}.

***N*-[(*N*-Boc-L-prolyl)methyl]-*N*-isopropyl-*N'*-(*p*-nitrophenyl)urea (12h).** A mixture of **11** (0.26 g, 0.96 mmol) and *p*-nitrophenyl isocyanate (0.16 g, 0.06 mmol) in THF (3 mL) was stirred overnight at room temperature. The reaction mixture was concentrated in vacuo and purified by silica gel column chromatography ($CHCl_3$) to give 350 mg of **12h** (84%); NMR ($CDCl_3$) δ 1.30 (d, 6 H, J = 7 Hz), 1.45 (s, 9 H), 1.70–2.20 (m, 4 H), 3.20–3.70 (m, 4 H), 4.10–4.70 (m, 2 H), 8.10–8.70 (m, 4 H), 8.99 (s, 1 H); IR (film) 3200, 1710, 1650, 1600 cm^{-1}.

***p*-Nitrophenyl *N*-(L-prolylmethyl)-*N*-isopropylcarbamate Hydrochloride (13a).** This compound was prepared in a similar manner to that for **7a**, yield from **12a** 68%; mp 190–193 °C dec; NMR (Me_2SO-d_6) δ 1.14 (d, 6 H, J = 6 Hz), 1.60–2.33 (m, 4 H), 3.10–4.80 (m, 6 H), 7.52 (d, 2 H, J = 9 Hz), 8.48 (d, 2 H, J = 9 Hz); IR (Nujol) 1740, 1725, 1615, 1595, 1520 cm^{-1}. Anal. ($C_{16}H_{22}ClN_3O_5$) C, H, N.

Phenyl *N*-(L-prolylmethyl)-*N*-isopropylcarbamate Hydrochloride (13b). This compound was prepared in a similar manner to that for **7a**, yield from **12b** 58%; mp 207–208 °C dec; NMR (Me_2SO-d_6) δ 1.00–1.43 (m, 6 H), 1.70–2.20 (m, 4 H), 3.00–3.70 (m, 2 H), 4.10–5.00 (m, 4 H), 7.10–7.70 (m, 5 H); IR (Nujol) 1750, 1725, 1600, 1550 cm^{-1}. Anal. ($C_{16}H_{23}ClN_2O_3$) C, H, N.

Pentafluorophenyl *N*-(L-Prolylmethyl)-*N*-isopropylcarbamate Hydrochloride (13c). This compound was prepared in a similar manner to that for **7a**, yield from **12c** 80%; mp 189–190 °C; NMR (Me_2SO-d_6) δ 1.05–1.40 (m, 6 H), 1.70–2.30 (m, 4 H), 3.10–3.50 (m, 2 H), 4.00–4.90 (m, 6 H); IR (Nujol) 1750, 1515 cm^{-1}. Anal. ($C_{16}H_{18}ClF_5N_2O_3$) C, H, N, Cl.

2,2,3,3,4,4,4-Heptafluorobutyl *N*-(L-prolylmethyl)-*N*-isopropylcarbamate Hydrochloride (13d). This compound was prepared in a similar manner to that for **7a**. Yield from **12d** 80%; mp 149–150 °C; NMR (Me_2SO-d_6) δ 1.11 (d, 6 H, J = 7 Hz), 1.70–2.30 (m, 4 H), 3.00–3.50 (m, 2 H), 3.90–5.20 (m, 4 H), 4.40 (s, 2 H), 9.70 (br s, 2 H); IR (Nujol) 3410, 1745, 1710 cm^{-1}. Anal. ($C_{14}H_{20}ClF_7N_2O_3$) C, H, N, Cl.

S-Benzyl N-(L-Prolylmethyl)-N-isopropylthiocarbamate Hydrochloride (13e). This compound was prepared according to the procedure described for **7a**. Yield from **13f** 72%; mp 193–194 °C; NMR (Me_2SO-d_6) δ 1.10 (d, 6 H, J = 7 Hz), 1.60–2.30 (m, 4 H), 3.00–3.50 (m, 2 H), 4.16 (s, 2 H), 4.46 (s, 2 H), 4.20–4.80 (m, 2 H), 7.41 (s, 5 H), 9.20–10.30 (br s, 2 H); IR (Nujol) 1745, 1635, 1545 cm^{-1}. Anal. ($C_{17}H_{25}ClN_2O_2S$) C, H, N, Cl, S.

S-1-Methyl-5-tetrazolyl N-(L-prolylmethyl)-N-isopropylthiocarbamate Hydrochloride (13f). The title compound was prepared by the same method as that for **7a**. Yield from **12g** 52%, mp 160–162 °C dec; NMR (Me_2SO-d_6) δ 1.26 (d, 6 H, J = 7 Hz), 1.70–2.33 (m, 4 H), 3.30 (m, 2 H), 4.07 (s, 3 H), 3.90–4.80 (m, 4 H); IR (Nujol) 1745, 1665 cm^{-1}.

S-1-Phenyl-5-tetrazolyl N-(L-Prolylmethyl)-N-isopropylthiocarbamate Hydrochloride (13g). The title compound was prepared in a similar manner to that for **13f** and was obtained as an amorphous powder. Yield 46%; NMR (Me_2SO-d_6) δ 1.13 (m, 6 H), 1.70–2.30 (m, 4 H), 3.30 (m, 2 H), 3.70–4.80 (m, 4 H), 7.50–8.10 (m, 5 H); IR (Nujol) 3400, 1760, 1730, 1670, 1590 cm^{-1}.

N-(L-Prolylmethyl)-N-isopropyl-N′-(p-nitrophenyl)urea Hydrochloride (13h). This compound was prepared in a similar manner to that for **7a**. Yield from **12h** 80%; mp 221–223 °C; NMR (Me_2SO-d_6) δ 1.38 (d, 6 H, J = 6 Hz), 1.80–2.40 (m, 4 H), 2.90–3.70 (m, 4 H), 4.30–4.70 (m, 2 H), 7.89 (d, 2 H, J = 9 Hz), 8.57 (d, 2 H, J = 9 Hz), 9.00–10.00 (br s, 2 H); IR (Nujol) 3130, 2660, 1690, 1680, 1630, 1615, 1600, 1530 cm^{-1}.

p-Nitrophenyl N-[(Methoxysuccinyl)-L-alanyl-L-alanyl-L-prolylmethyl]-N-isopropylcarbamate (14a). This compound was prepared in a similar manner to that for **8b**. Yield from **13a** 82%; mp 173–175 °C; NMR ($CDCl_3$) δ 1.10–1.50 (m, 12 H), 1.80–2.20 (m, 4 H), 2.63 (m, 4 H), 3.50–4.00 (m, 2 H), 3.73 (s, 3 H), 4.10–5.00 (m, 6 H), 6.33 (d, 1 H, J = 8 Hz), 7.00 (d, 1 H, J = 8 Hz), 7.33 (dd, 2 H, J = 4, 9 Hz), 8.33 (d, 2 H, J = 9 Hz); IR (Nujol) 3350, 1737, 1720, 1688, 1655, 1645, 1605, 1585, 1525 cm^{-1}. Anal. ($C_{27}H_{37}N_5O_{10}$) C, H, N.

Phenyl N-[(Methoxysuccinyl)-L-alanyl-L-alanyl-L-prolylmethyl]-N-isopropylcarbamate (14b). This compound was prepared in a similar manner to that for **8b**. Yield from **13b** 67%; mp 60–65 °C; NMR ($CDCl_3$) δ 1.00–1.50 (m, 12 H), 1.80–2.20 (m, 4 H), 2.63 (m, 4 H), 3.77 (s, 3 H), 3.40–3.80 (m, 2 H), 4.10–4.90 (m, 6 H), 6.33 (d, 1 H, J = 8 Hz), 6.80–7.55 (m, 6 H); IR (Nujol 3350, 1740, 1690, 1655, 1590, 1530 cm^{-1}. Anal. ($C_{27}H_{38}N_4O_8$) C, H, N.

Pentafluorophenyl N-[(Methoxysuccinyl)-L-alanyl-L-alanyl-L-prolylmethyl]-N-isopropylcarbamate (14c). This compound was prepared according to the procedure described for compound **8a**. Yield from **13c** 44%; mp 152–154 °C; NMR ($CDCl_3$) δ 1.00–1.50 (m, 12 H), 1.80–2.33 (m, 4 H), 2.56 (m, 4 H), 3.40–3.90 (m, 2 H), 3.75 (s, 3 H), 4.10–5.00 (m, 6 H), 6.50 (m, 1 H), 7.10 (m, 1 H); IR (Nujol) 3350, 1765, 1740, 1690, 1660, 1640, 1520 cm^{-1}. Anal. ($C_{27}H_{33}F_5N_4O_8$) C, H, N.

2,2,3,3,4,4,4-Heptafluorobutyl N-[(Methoxysuccinyl)-L-alanyl-L-alanyl-L-prolylmethyl]-N-isopropylcarbamate (14d). This compound was prepared in a similar manner to that for **8a**. Yield from **13d** 43%; mp 165–166 °C; NMR ($CDCl_3$) δ 1.16 (d, 6 H, J = 7 Hz), 1.38 (d, 6 H, J = 7 Hz), 1.80–2.30 (m, 4 H), 2.60 (m, 4 H), 3.75 (s, 3 H), 3.60–4.00 (m, 2 H), 4.21 (s, 2 H), 4.21 (s, 2 H), 4.00–5.00 (m, 6 H), 6.46 (d, 1 H, J = 7 Hz), 7.08 (d, 1 H, J = 7 Hz); IR (Nujol) 3350, 1735, 1690, 1655, 1640, 1525 cm^{-1}. Anal. ($C_{25}H_{35}F_7N_4O_8$) C, H, N.

S-Benzyl N-[(Methoxysuccinyl)-L-alanyl-L-alanyl-L-prolylmethyl]-N-isopropylthiocarbamate (14e). This compound was prepared according to the procedure described for **8a**. Yield from **13e** 41%; mp 125–127 °C; NMR ($CDCl_3$) δ 1.00–1.50 (m, 12 H), 1.70–2.30 (m, 4 H), 2.66 (m, 4 H), 3.40–3.90 (m, 2 H), 3.80 (s, 3 H), 4.0–4.9 (m, 9 H), 7.50 (s, 5 H); IR (Nujol) 3360, 1735, 1680, 1655, 1640, 1530 cm^{-1}. Anal. ($C_{28}H_{40}N_4O_7S$) C, H, N, S.

S-1-methyl-5-tetrazolyl N-[(Methoxysuccinyl)-L-alanyl-L-alanyl-L-prolylmethyl]-N-isopropylthiocarbamate (14f). This compound was prepared by the same method as for **8a**. Yield from **13f** 21%; mp 110–113 °C; NMR ($CDCl_3$) δ 1.00–1.50 (m, 12 H), 1.80–2.20 (m, 4 H), 2.60 (m, 4 H), 3.50–3.80 (m, 2 H), 3.76 (s, 3 H), 4.10 (s, 3 H), 4.00–4.90 (m, 6 H), 6.56 (d, 1 H, J = 8 Hz), 7.20 (d, 1 H, J = 8 Hz); IR (film) 3300, 1735, 1680–1640, 1520 cm^{-1}. Anal. ($C_{23}H_{36}N_8O_7S$) C, H, N.

S-1-Phenyl-5-tetrazolyl N-[(Methoxysuccinyl)-L-alanyl-L-alanyl-L-prolylmethyl]-N-isopropylthiocarbamate (14g). This compound was prepared in a similar manner to that for **14f**. Yield 44%; mp 103–108 °C; NMR ($CDCl_3$) δ 0.90–1.60 (m, 12 H), 2.03 (m, 4 H), 2.60 (m, 4 H), 3.70 (s, 3 H), 3.50–3.90 (m, 2 H), 4.00–5.00 (m, 6 H), 6.46 (d, 1 H, J = 8 Hz), 7.07 (d, 1 H, J = 8 Hz), 7.63 (s, 5 H); IR (Nujol) 3340, 1735, 1680, 1655, 1530 cm^{-1}. Anal. ($C_{28}H_{38}N_8O_7S$) C, H, N.

N-[(Methoxysuccinyl)-L-alanyl-L-alanyl-L-prolylmethyl]-N-isopropyl-N′-(p-nitrophenyl)urea (14h). This compound was prepared in a similar manner to that for **8a**. Yield from **13h** 39%; mp 60–70 °C; NMR ($CDCl_3$) δ 1.10–1.70 (m, 12 H), 1.80–2.30 (m, 4 H), 2.66 (m, 4 H), 3.50–4.00 (m, 4 H), 3.77 (s, 3 H), 4.20–4.90 (m, 4 H), 6.66 (m, 1 H), 7.33 (m, 1 H), 7.90 (m, 2 H), 8.50 (m, 2 H); IR (film) 3300 (br), 1735, 1640–1690, 1600 cm^{-1}. Anal. ($C_{27}H_{38}N_6O_9$) C, H, N.

Enzymatic Studies. Materials. PP elastase, bovine trypsin, chymotrypsin, and their respective substrates were purchased from Sigma Chemical Co., St. Louis, MO. Dialysis cellulose tubing with molecular weight cut-off of 12 000 was also obtained from Sigma. Sephadex-G25 was purchased from Pharmacia Fine Chemicals, Inc.

Enzyme Assays. All enzyme assays were performed spectrophotometrically at 25 °C on a Cary 219 Varian spectrophotometer. The activities of PP elastase, trypsin, and chymotrypsin were assayed with use of Boc-Ala-ONp, Bz-Arg-OEt, and Bz-Tyr-OEt as the substrates and monitoring of the absorbance at 398, 253, and 256 nm, respectively.

Screening for Inhibitory Activity. General Procedure. In a typical experiment, the inhibitor (0.1 mL, 1 mM in Me_2SO) and the substrate (0.1 mL, 10 mM) were added to 2.7 mL of buffer in a quartz cuvette and thermally equilibrated in the spectrophotometer for 2 min, and the absorbance was balanced at the desired wavelength. The enzyme (0.1 mL, 3.85 μM) was added to the sample cuvette, the mixture shaken for 20 s, and the increase in absorbance monitored for 30 min.

PP Elastase. The substrate, Boc-Ala-ONp, was dissolved in CH_3OH and the enzyme in 0.05 M phosphate buffer (pH 6.5), which is also used as the buffer medium. The absorbance was monitored at 398 nm and 25 °C.

Trypsin. The substrate, Bz-Arg-OEt, was dissolved in 0.01 M Tris·HCl buffer (pH 8.0), which is also used as the buffer medium. The enzyme was dissolved in 2 M HCl and the absorbance monitored at 253 nm and 25 °C.

Chymotrypsin. The substrate, Bz-Tyr-OEt, was dissolved in 50% aqueous MeOH and the enzyme in 0.001 M HCl, and the buffer medium used was 0.1 M Tris·HCl containing 0.1 M $CaCl_2$ (pH 7.8). Absorbance was monitored at 256 nm and 30 °C.

Kinetic Studies. In a typical experiment, 0.1 mL of the inhibitor (19.3–2.4 μM in Me_2SO) and 0.1 mL of Boc-Ala-ONp (700–160 μM in CH_3OH) were added to 2.7 mL of 0.05 M phosphate buffer (pH 6.5) in a quartz cuvette and thermally equilibrated in the spectrophotometer for 2 min, and the absorbance was balanced at 398 nm. PP elastase (0.1 mL of a 0.3 mg/mL in buffer) was added to the sample cuvette, the mixture shaken for 20 s, and the increase in absorbance monitored for 2 min. K_i values were determined by plotting the slopes from Lineweaver–Burk curves vs. inhibitor concentration.

Dialysis Experiments. Dialysis bags were washed in buffer before use. Dialysis was carried out in stirred 0.05 M sodium phosphate buffer containing 8.3% Me_2SO (250 mL, pH 6.5) at 1–5 °C. The dialysate was changed three times during the first 24 h and twice in the following 42 h.

PP elastase (6.25×10^{-6} M) was incubated with or without (control) inhibitor **14a** (6.26×10^{-4} M) in phosphate buffer (pH 6.5) at 25 °C for 2 h. The enzymatic activity was assayed as usual with Boc-Ala-ONp as the substrate: 98% of the activity was lost. Three milliliters of each incubation solution was diluted to 5 mL with buffer and dialyzed for 24 h to get rid of any excess inhibitor. At specified time intervals the activity of the enzyme inside the dialysis bag was assayed: 98% enzymatic activity was recovered in 74 h.

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Registry No. 1, 1490-25-1; 2, 52787-46-9; 3, 102284-27-5; 4, 15761-39-4; 5a, 102284-28-6; 5b, 102284-29-7; 5c (diastereomer 1), 102284-30-0; 5c (diastereomer 2), 102284-64-0; 6a, 102284-31-1; 6b, 102284-32-2; 6c, 102284-33-3; 6d, 102284-34-4; 6e, 102284-35-5; 7a, 102284-36-6; 7b, 102284-37-7; 7c, 102284-38-8; 7d, 102284-39-9; 7e, 102284-40-2; 8a, 92279-27-1; 8b, 92279-28-2; 8c, 92279-29-3; 8d, 92279-30-6; 8e, 92279-31-7; 9, 101130-03-4; 10, 102284-41-3; 11, 102284-42-4; 12a, 102284-43-5; 12b, 102284-44-6; 12c, 102284-45-7; 12d, 102284-46-8; 12e, 102284-47-9; 12f, 102284-48-0; 12g, 102284-49-1; 12h, 102284-50-4; 13a, 102284-51-5; 13b, 102284-52-6; 13c, 102284-53-7; 13d, 102306-03-6; 13e, 102284-54-8; 13f, 102284-55-9; 13g, 102284-56-0; 13h, 102284-57-1; 14a, 92279-32-8; 14b, 92279-33-9; 14c, 102284-58-2; 14d, 102306-04-7; 14e, 102284-59-3; 14f, 102284-60-6; 14g, 102284-61-7; 14h, 102284-62-8; Ala-Ala, 1948-31-8; *p*-aminophenol, 123-30-8; DL-2-amino-3-methyl-1-butanol, 16369-05-4; phenyl isocyanate, 103-71-9; dimethylcarbamoyl chloride, 79-44-7; isopropyl isocyanate, 1795-48-8; isopropylamine, 75-31-0; *p*-nitrophenyl chloroformate, 7693-46-1; *S*-benzyl chlorothioformate, 37734-45-5; 1-methyl-5-tetrazolyl chlorothioformate, 102284-63-9; 1-methyl-5-mercaptotetrazole, 13183-79-4; phosgene, 75-44-5; *o*-aminophenol, 95-55-6; *p*-nitrophenyl isocyanate, 100-28-7; elastase, 9004-06-2.

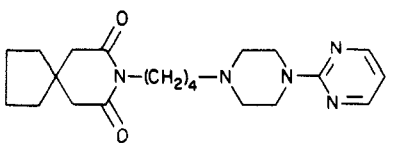
Buspirone Analogues. 2. Structure–Activity Relationships of Aromatic Imide Derivatives

James S. New,* Joseph P. Yevich, Michael S. Eison, Duncan P. Taylor, Arlene S. Eison, Leslie A. Riblet, Cam P. VanderMaelen, and Davis L. Temple, Jr.

Preclinical CNS Research, Pharmaceutical Research and Development Division, Bristol-Myers Research Center, Wallingford, Connecticut 06492-7660. *Received September 20, 1985*

Several analogues of the novel anxiolytic buspirone were synthesized and evaluated in vivo for tranquilizing activity and their ability to reverse neuroleptic-induced catalepsy. The in vitro binding affinities of these compounds were also examined for both the α_1 and dopamine D_2 receptor systems. The general structure–activity relationships of this series highlight compounds 17, 21, and 32 as having anticonflict activity. Each of these structures contains the 1-(2-pyrimidinyl)piperazine moiety linked by a tetramethylene chain to a variable cyclic imide moiety. Compound 32 (4,4-dimethyl-1-[4-[4-(2-pyrimidinyl)-1-piperazinyl]butyl]-2,6-piperidinedione) was found to be equipotent with buspirone in its anxiolytic activity and was therefore selected for extensive preclinical characterization. The pharmacology of buspirone and 32 is contrasted, and the potent serotonin agonist properties of 32 are discussed with reference to its potential contribution to the anxioselective mechanism of this compound.

Anxiolytic drugs that express their antianxiety activity without the anticonvulsant, sedative, or muscle relaxant effects normally associated with the use of the benzodiazepines are termed anxioselective. Buspirone (1) is unique in this class since it does not share a mechanism of anxiolysis mediated through the benzodiazepine–GABA–chloride ionophore receptor complex utilized by other anxioselective drugs. The diverse neuropharmacologic effects of buspirone, which include subtle involvement with the dopamine, GABA, serotonin, and adrenergic neurotransmitter systems, may collectively facilitate its mechanism of anxiolysis. Elucidation of this mechanism has been the subject of numerous pharmacologic investigations which have evolved a midbrain modulator role to define the anxiolytic specificity of this novel drug.[1,2] This working hypothesis postulates the anxioselective vector of buspirone alleviates anxiety through the summation of its various polysynaptic actions, which modify the diffuse assemblage of neurochemical inputs involved in the etiology of this disease.



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The anxiolytic effects of buspirone are known to be dependent on two distinct substructural pharmacophores within the molecular framework: the azaspirodecanedione imide and the arylpiperazine moiety found in its pyrimidinylpiperazine fragment. Previous structure–activity studies have focused on carbocyclic imides as potential surrogates for the azaspirodecanedione imide present in 1.[3-5] This resulted in adaptation of a generalized topology for a potential receptor site that can accommodate the critical molecular features of this structural class. This current report extends the structure–activity relationships (SAR) of buspirone and explores replacement of both imide and pyrimidinylpiperazine fragments in this drug with new aromatic imide moieties and arylpiperazine groups.

Chemistry. The synthesis of target structures 10–24, which incorporate a pyrrolidinedione ring, is accomplished with standard published procedures. Briefly, condensation of commercially available aldehydes or ketones (2) with ethyl cyanoacetate under either Cope or Knoevenagel reaction conditions yields a variety of α,β-unsaturated cyanoacetates, 3 (Scheme I). The reaction of 3 (R^2 = H, phenyl, or $(CH_2)_n$-Ar) with potassium cyanide in aqueous ethanol yields the dicyano intermediates 4. Either acid or base hydrolysis of 4 generates the succinic acid derivatives 7, which on conversion to their respective anhydrides react with ammonia and are cyclized to the imides represented by 8.

An alternative synthesis of 8 involves the Emmons–Horner condensation of triethyl phosphonoacetate with the appropriate ketone forming the α,β-unsaturated ethyl ester 5. Reaction of 5 with potassium cyanide in DMF (60 °C) yields 6 whose hydrolysis also affords 7. The subsequent elaboration of either the anhydrides or intermediary

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