Structure-Based Design of a General Class of Mechanism-Based Inhibitors of the Serine Proteinases Employing a Novel Amino Acid-Derived Heterocyclic Scaffold†

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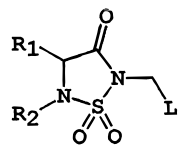
ABSTRACT: We describe in this paper the structure-based design of a general class of heterocyclic mechanism-based inhibitors of the serine proteinases that embody in their structure a novel peptidomimetic scaffold (1,2,5-thiadiazolidin-3-one 1,1-dioxide). Sulfone derivatives of this class (**I**) were found to be time-dependent, potent, and highly efficient irreversible inhibitors of human leukocyte elastase, cathepsin G, and proteinase 3. The partition ratios for a select number of inhibitors were found to range between 0 and 1. We furthermore demonstrate that these inhibitors exhibit remarkable enzyme selectivity that is dictated by the nature of the P_1 residue and is consistent with the known substrate specificity reported for these enzymes. Thus, inhibitors with small hydrophobic side chains were found to be effective inhibitors of elastase, those with aromatic side chains of cathepsin G, and those with a basic side chain of bovine trypsin. Taken together, the findings cited herein reveal the emergence of a *general* class of stable mechanism-based inhibitors of the serine proteinases which can be readily synthesized using amino acid precursors. Biochemical and high-field NMR studies show that the interaction of this class of inhibitors with a serine proteinase results in the formation of a stable acyl complex(es) and the release of benzenesulfinate, formaldehyde, and a low molecular weight heterocycle. The data are consistent with initial formation of a Michaelis−Menten complex, acylation of Ser^{195}, and tandem loss of the leaving group. The initial HLE−inhibitor complex reacts with water generating formaldehyde and a stable HLE−inhibitor complex. Whether the initial HLE−inhibitor complex also reacts with His^{57} to form a third complex is not known at this point. The desirable salient parameters associated with this class of inhibitors, including the expeditious generation of structurally diverse libraries of inhibitors based on **I**, suggest that this class of mechanism-based inhibitors is of general applicability and can be used in the development of inhibitors of human and viral serine proteinases of clinical relevance.

An array of inflammatory diseases are associated with a massive influx of neutrophils, the release of chemokines, the production of reactive oxygen species, and a compromised proteinase/antiproteinase screen (Weiss, 1989; Harada et al., 1994; Yoshimura et al., 1994; Piccioni et al., 1992). The inflammatory process also involves the extracellular release of the lysosomal serine endopeptidases elastase (HLE), cathepsin G (Cat G), and proteinase 3 (PR 3). Poor control of the activity of these enzymes due to depressed levels of their endogenous protein inhibitors (α-1-proteinase inhibitor, α-1-antichymotrypsin, secretory leukoproteinase inhibitor, elafin) is believed to result in the destruction of the major components of connective tissue, including elastin (Barrett, 1994; Janusz & Doherty, 1991; Birrer, 1993).

The aberrant activity of these enzymes in disease states has provided the impetus behind efforts related to the design of agents capable of modulating the activity of these enzymes and redressing the proteinase/antiproteinase imbalance. Thus, there has been an intense and ongoing interest in the design of inhibitors, including mechanism-based inhibitors, of these and related serine endopeptidases (Edwards & Bernstein, 1994). Examples of inactivators of this type include haloenol and ynenol lactones (Katzenellenbogen et al., 1992; Copp et al., 1987), substituted isocoumarins (Hernandez et al., 1992), 3-alkyl-*N*-hydroxysuccinimide derivatives (Groutas et al., 1989, 1991, 1993a), substituted dihydrouracils (Groutas et al., 1994a), *β*-lactams (Underwood et al., 1995), and saccharin derivatives (Hlasta et al., 1995; Groutas et al., 1993b, 1996a). Herein we wish to describe the biochemical rationale underlying the design of a novel and general class of mechanism-based inhibitors of the serine proteinases (structure **I** (Groutas et al., 1994b), as well as the results of



structure I

pertinent biochemical and mechanistic studies related to the inhibition of HLE, Cat G, and PR 3 by sulfone derivatives of 1,2,5-thiadiazolidin-3-one 1,1-dioxide (**I**). We furthermore demonstrate that the heterocyclic ring in **I** is a highly effective peptidomimetic scaffold suitable for appending peptidyl and nonpeptidyl recognition elements that allow the optimization of binding interactions with both the S_n and

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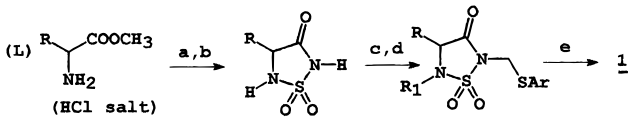
Scheme 1: Synthesis of **I**[a]



[a] Reagents: a, NH_2SO_2Cl/TEA; b, NaH/THF; c, $ArSCH_2Cl$/TEA; d, NaH/R_1X/THF; e, *m*-chloroperbenzoic acid/CH_2Cl_2.

Table 1: Inhibitory Activity of Compounds **1**−**13** against Human Leukocyte Elastase, Cathepsin G, and Proteinase 3

			k_{inact}/K_I (M^{-1} s^{-1})		
compound[a]	R_1	R_2	HLE	PR 3	Cat G
1	DL-ethyl	methyl	190[b]	200[b]	NA[c]
2	DL-ethyl	benzyl	810[b]	80[b]	NA
3	*n*-propyl	methyl	780	1830	NA
4	*n*-propyl	benzyl	7620	4960	130
5	isopropyl	methyl	NA	NA	NA
6	isopropyl	benzyl	NA	NA	NA
7	isobutyl	methyl	9490	2250	NA
8	isobutyl	benzyl	95190	5200	110
9	*n*-butyl	methyl	1080	NA	NA
10	*n*-butyl	benzyl	8060	NA	610
11[d]	benzyl	methyl	NA	NA	120
12[d]	benzyl	benzyl	NA	NA	11210
13[e]	$(CH_2)_4NH_2$	methyl	NA	NA	NA

[a] All compounds are derived from the corresponding L-amino acid esters unless indicated otherwise, and L = SO_2Ph. [b] $k_{obs}/[I]$ (M^{-1} s^{-1}) was determined by the incubation method. [c] NA: no activity. [d] Alternate substrate inhibitor of α-chymotrypsin. [e] Potent inhibitor of bovine trypsin (k_{inact}/K_I = 16 820 M^{-1} s^{-1}).

S_n' subsites (Schecter & Berger, 1967) and yields efficient and highly specific inhibitors of serine proteinases.

MATERIALS AND METHODS

Materials. MeOSuc-AAPV-pNA,[1] Suc-AAPF-pNA, BOC-L-Ala *p*-nitrophenyl ester, *N*-acetyl-L-tyrosine methyl ester, and N^{α}-benzoyl-L-arginine *p*-nitroanilide were purchased from Sigma Chemical Co., St. Louis, MO. Aldrich 230−400 mesh silica gel was used for flash chromatography. Human leukocyte elastase was purchased from Elastin Products Co., Owensville, MO. Human leukocyte cathepsin G and proteinase 3 were purchased from Athens Research and Technology Co., Athens, GA. Substrate and inhibitor stock solutions were prepared in DMSO. Chymotrypsin and trypsin were purchased from Sigma Chemical Co., St. Louis, MO. Active site titration of chymotrypsin (Schonbaum et al., 1961) yielded 80−90% active sites.

The infrared and NMR spectra of the synthesized compounds were recorded on a Perkin-Elmer 1330 infrared spectrophotometer and a Varian XL-300 NMR spectrometer, respectively. The enzyme−inhibitor NMR studies were performed using a 500 MHz Varian Unity Plus NMR spectrometer. A Hewlett-Packard diode array UV/vis spectrophotometer was used in the enzyme assays and inhibition studies.

Synthesis. Scheme 1 was used in the synthesis of inhibitors **1**−**12**. These are listed in Table 1. A representative synthesis (compound **7** in Table 1) is described below.

[1] Abbreviations: MeOSuc-AAPV-pNA, methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide; DMSO, dimethyl sulfoxide; MPCBA, *m*-chloroperbenzoic acid; TEA, triethylamine; DBU, 1,8-diazabicyclo-[5.4.0]undec-7-ene; NCS, *N*-chlorosuccinimide.

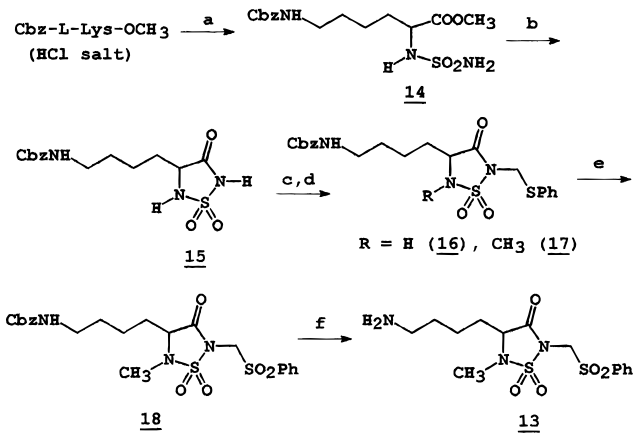
(S)-Methyl 2-Sulfamido-4-methylpentanoate. To a mixture of L-Leu-OCH_3 hydrochloride (7.23 g; 0.04 mol) and sulfamoyl chloride (4.62 g; 0.04 mol) in 50 mL of methylene chloride was added dropwise a solution of triethylamine (8.1 g; 0.08 mol) in 30 mL of methylene chloride at 0 °C. After the mixture was stirred at room temperature for 2 h, the triethylamine hydrochloride salt was filtered off, and the filtrate was washed with water, 5% HCl, and brine and dried over anhydrous sodium sulfate. Removal of the solvent yielded 6.4 g (71% yield) of an oily product. ^{1}H NMR ($CDCl_3$): δ 0.99 (dd, 6H), 1.57 (t, 2H), 1.85 (m, 1H), 3.78 (s, 3H), 4.10 (m, 1H), 5.12 (s, 2H), 5.73 (d, 1H). ^{13}C NMR ($CDCl_3$): δ 21.41, 22.70, 24.44, 41.53, 52.65, 54.89, 174.55. $[\alpha]_D$ −26.9 (*c* 1, CH_2Cl_2) (Dewynter et al., 1996).

(S)-4-Isobutyl-1,2,5-thiadiazolidin-3-one 1,1-Dioxide. A solution of (*S*)-methyl 2-sulfamido-4-methylpentanoate (6.4 g; 0.285 mol) in 60 mL of dry THF was treated portionwise with 60% sodium hydride (1.5 g; 0.037 mol) at 0 °C. The reaction mixture was stirred at room temperature overnight. The solvent was removed *in vacuo*, and the residue was dissolved in 30 mL of cold water. The pH was adjusted to 6−7 with concentrated HCl and extracted with ethyl acetate (30 mL). The aqueous layer was separated and acidified with concentrated HCl to pH 1. The product was extracted with ethyl acetate (3 × 30 mL), and the combined organic extracts were dried over anhydrous sodium sulfate and evaporated. The crude product (4.2 g; 77% yield) was recrystallized from methanol, mp 196−197 °C. ^{1}H NMR (DMSO-d_6): δ 0.96 (dd, 6H), 1.72 (m, 2H), 1.84 (m, 1H), 6.95 (s, 1H),7.36 (s, 1H). ^{13}C NMR ($CDCl_3$): δ 21.04, 22.83, 24.60, 39.87, 59.71, 172.48 (Dewynter et al., 1996).

(S)-4-Isobutyl-2-[(phenylthio)methyl]-1,2,5-thiadiazolidin-3-one 1,1-Dioxide. A solution of (*S*)-4-isobutyl-1,2,5-thiadiazolidin-3-one 1,1-dioxide (1.92 g; 10 mmol), chloromethyl phenyl sulfide (1.80 g; 12 mmol), and triethylamine (1.01 g; 10 mmol) in 15 mL of dry acetonitrile was refluxed for 20 h. The solvent was removed *in vacuo*, and the residue was taken up in 30 mL of ethyl acetate, washed with water, 5% aqueous HCl (10 mL), and 5% aqueous sodium bicarbonate, and dried over anhydrous sodium sulfate. Evaporation of the solvent left a crude product which was purified by flash chromatography, using a hexane/methylene chloride gradient, mp 48−49 °C (1.64 g; 52% yield). ^{1}H NMR ($CDCl_3$): δ 0.94 (dd, 6H), 1.60 (m, 1H), 1.75 (m, 2H), 4.08 (m, 1H), 4.94 (d, 2H), 5.05 (d, 1H), 7.33 (m, 3H), 7.56 (m, 2H). ^{13}C NMR ($CDCl_3$): δ 21.17, 22.73, 25.00, 39.88,45.64, 59.15, 128.44, 129.14, 132.33, 132.89, 168.63. Anal. Calcd for $C_{13}H_{18}N_2O_3S_2$: C, 49.66; H, 5.77; N, 8.91. Found: C, 49.61; H, 5.81; N, 8.87.

(S)-4-Isobutyl-5-methyl-2-[(phenylthio)methyl]-1,2,5-thiadiazolidin-3-one 1,1-Dioxide. Methyl iodide (3.92 g; 27.6 mmol) was added to a solution of (*S*)-4-isobutyl-2-[(phenylthio)methyl]-1,2,5-thiadiazolidin-3-one 1,1-dioxide (1.74 g; 5.52 mmol) in dry acetonitrile (100 mL). The solution was cooled in an ice bath, and 60% NaH (2.21 g; 5.52 mmol) was added in small portions over a period of 5 min. The reaction mixture was stirred at room temperature overnight, and the solvent and excess methyl iodide were removed using a rotary evaporator. The residue was dissolved in methylene chloride (25 mL) and washed with water (10 mL). The organic layer was separated and dried over anhydrous sodium sulfate. Removal of the solvent left a crude product which was purified by flash chromatography (hexane/methylene

Scheme 2: Synthesis of Compound **13**[a]



[a] Reagents: a, NH_2SO_2Cl/TEA/CH_2Cl_2 (51%); b, NaH/THF, then HCl (88%); c, $PhSCH_2Cl$/TEA/CH_3CN (55%); d, NaH, then CH_3I/CH_3CN (52%); e, *m*-chloroperbenzoic acid (100%); f, $(CH_3)_3SiI$/CH_3CN (90%).

chloride), yielding an oily product (1.41 g; 78% yield). ^{1}H NMR ($CDCl_3$): δ 0.92 (dd, 6H), 1.60−1.88 (m, 3H), 2.84 (s, 3H), 3.72 (t, 1H), 4.97 (s, 2H), 7.30−7.60 (m, 5H). ^{13}C NMR ($CDCl_3$): δ 22.37, 22.48, 24.58, 33.36, 38.53, 45.38, 65.18, 128.44, 129.07, 132.32, 133.14, 166.34. Anal. Calcd for $C_{14}H_{20}N_2S_2O_3$: C, 51.19; H, 6.14; N, 8.53. Found: C, 51.34; H, 6.40; N, 8.44.

*(S)-4-Isobutyl-5-methyl-2-[(phenylsulfonyl)methyl]-1,2,5-thiadiazolidine 1,1-Dioxide (***7***).* A solution of (*S*)-4-isobutyl-5-methyl-2-[(phenylthio)methyl]-1,2,5-thiadiazolidin-3-one 1,1-dioxide (0.3 g; 0.74 mmol) and *m*-chloroperbenzoic acid (0.58 g; 1.85 mmol) in 10 mL of methylene chloride was stirred at room temperature overnight. The mixture was diluted with 10 mL of methylene chloride, washed with 5% sodium bicarbonate (10 mL) and water (10 mL), and dried over anhydrous sodium sulfate. Removal of the solvent left a crude product, which was purified by flash chromatography (hexane/methylene chloride) to yield 0.31 g (99% yield) of pure product, mp 133−134 °C. ^{1}H NMR ($CDCl_3$): δ 0.85 (dd, 6H), 1.55−1.80 (m, 3H), 2.81 (s, 3H), 4.75 (t, 1H), 4.82 (s, 2H), 7.50−7.68 (m, 3H), 7.93 (d, 2H). ^{13}C NMR ($CDCl_3$): δ 22.20, 22.43, 24.65, 34.41, 38.64, 60.08, 65.62, 129.20, 129.26, 134.67, 136.88, 166.57. Anal. Calcd for $C_{14}H_{20}N_2O_5S_2$: C, 46.65; H, 5.59; N, 7.77. Found: C, 46.49; H, 5.77; N, 7.58. The synthesis of compound **13** was accomplished using Scheme 2.

*(S)-Methyl 6-[(benzyloxycarbonyl)amino]-2-sulfamidohexanoate (***14***).* N^{ϵ}-Cbz-L-lysine methyl ester hydrochloride (25.00 g; 75.6 mmol) was added to a stirred solution of sulfamoyl chloride, prepared by adding dropwise 95% formic acid (3.14 g; 68.3 mmol) to chlorosulfonyl isocyanate (11.77 g; 83 mmol) in methylene chloride (Kristinsson et al., 1994), at 0 °C under nitrogen. A solution of triethylamine (15.27 g; 151.2 mmol) in 40 mL of methylene chloride was then added dropwise, and the resulting mixture was stirred at room temperature for 1 h. The solvent was removed *in vacuo*, and the residue was taken up in ethyl acetate (100 mL). The organic layer was then washed with 5% HCl (50 mL), 5% $NaHCO_3$ (50 mL), and brine (50 mL) and dried over anhydrous sodium sulfate. Evaporation of the solvent left a crude product which was purified by flash chromatography (hexane/methylene chloride) to give 14.5 g (51% yield) of pure product, mp 90−91 °C. ^{1}H NMR ($CDCl_3$): δ 1.38−1.58 (m, 4H), 1.69 (m, 1H), 3.18 (q, 2H), 3.75 (s, 3H), 4.05 (m, 1H), 4.98 (br t, 1H), 5.04 (br s, 2H), 5.09 (s, 2H), 5.62 (d, 1H), 7.34 (m, 5H). ^{13}C NMR ($CDCl_3$): δ 22.02, 29.10, 31.85, 40.36, 52.75, 56.02, 66.70, 128.07, 128.49, 136.52, 156.64, 173.52.

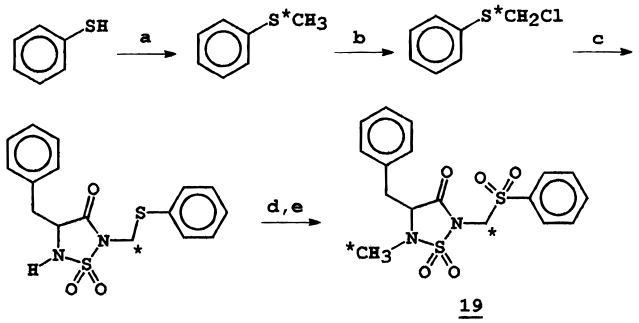
*(S)-4-[4-[(Benzyloxycarbonyl)amino]butyl]-1,2,5-thiadiazolidin-3-one 1,1-Dioxide (***15***).* A solution of compound **14** (12.35 g; 33.1 mmol) in 50 mL of dry THF kept at 0 °C was treated portionwise with 60% sodium hydride (1.7 g; 1.3 equiv) with stirring. The solution was stirred at room temperature overnight, and the solvent was removed on the rotary evaporator. The residue was dissolved in 40 mL of cold water and the solution extracted with ethyl acetate (40 mL). The aqueous layer was cooled in ice and carefully acidified with concentrated HCl to pH <2. The aqueous solution was extracted with ethyl acetate (2 × 40 mL), and the combined organic extracts were dried over anhydrous sodium sulfate. Evaporation of the solvent left a pure product (10.0 g; 88% yield). ^{1}H NMR (DMSO-d_6): δ 1.40 (m, 4H), 1.60 (m, 1H), 1.75 (m, 1H), 2.99 (m, 2H), 4.14 (m, 1H), 5.01 (s, 2H), 6.55 (br s, 1H), 7.22 (br t, 1H), 8.21 (br s, 1H). ^{13}C NMR(DMSO-d_6): δ 22.32, 28.79, 30.64, 59.70, 61.07, 65.09, 127.66, 128.29, 137.24, 156.05, 171.76.

*(S)-4-[4-[(Benzyloxycarbonyl)amino]butyl]-2-[(phenylthio)methyl]-1,2,5-thiadiazolidin-3-one 1,1-Dioxide (***16***).* Chloromethyl phenyl sulfide (7.0 g; 44.1 mmol) was added to a solution of compound **15** (10.0 g; 29.3 mmol) and triethylamine (3.0 g; 29.7 mmol) in dry acetonitrile (40 mL), and the solution was refluxed overnight. The solvent was removed *in vacuo*, and the residue was taken up in methylene chloride (100 mL). The organic layer was washed with 5% HCl (50 mL), saturated $NaHCO_3$ (50 mL), and brine (50 mL) and dried over anhydrous sodium sulfate. The solvent was removed and the crude product purified by flash chromatography (hexane/methylene chloride), yielding 7.52 g (55% yield) of pure product, mp 87−89 °C. ^{1}H NMR ($CDCl_3$) (recorded at 55 °C due to its low solubility): δ 1.37 (m, 2H), 1.46 (m, 2H), 1.73 (m, 1H), 1.84 (m, 1H), 3.12 (q, 2H), 4.03 (br m, 1H), 4.93 (q, 2H), 5.10 (br s, 2H), 6.46 (br s, 1H), 7.32 (m, 8H), 7.55 (m, 2H). ^{13}C NMR ($CDCl_3$): δ 21.51, 29.09, 29.91, 39.84, 45.31, 60.21, 66.86, 127.88, 128.06, 128.22, 128.43, 129.00, 132.42, 132.74, 136.26, 157.04, 168.11.

*(S)-4-[4-[(Benzyloxycarbonyl)amino]butyl]-2-[(phenylthio)methyl]-5-methyl-1,2,5-thiadiazolidin-3-one 1,1-Dioxide (***17***).* A solution of compound **16** (7.4 g; 16 mmol) in dry acetonitrile (30 mL) was cooled to 0 °C and then treated with methyl iodide (4.5 g; 32 mmol) and 60% sodium hydride (0.65 g; 16 mmol). The resulting mixture was stirred at room temperature for 2 h. The solvent was removed, and the residue was taken up in ethyl acetate (100 mL). The organic layer was washed with water (50 mL) and brine (50 mL) and dried over anhydrous sodium sulfate. Flash chromatography using hexane/methylene chloride gave a pure oily product (4.0 g; 52% yield). ^{1}H NMR ($CDCl_3$): δ 1.5 (m, 4H), 1.77 (m, 1H), 1.90 (m, 1H), 2.80 (s, 3H), 3.16 (m, 2H), 3.70 (t, 1H), 4.71 (br s, 1H), 4.95 (s, 2H), 5.09 (s, 2H), 7.30 (m, 8H), 7.63 (m, 2H). ^{13}C NMR ($CDCl_3$): δ 21.18, 28.68, 29.56, 31.95, 40.65, 45.44, 66.04, 66.71, 128.03, 128.07, 128.42, 128.51, 129.12, 133.07, 136.80, 156.43, 165.45.

(S)-4-[4-[(Benzyloxycarbonyl)amino]butyl]-2-[(phenylsulfonyl)methyl]-5-methyl-1,2,5-thiadiazolidin-3-one 1,1-Di-

Scheme 3: Synthesis of Compound **19**[a]



[a] Reagents: a, DBU/*CH_3I/benzene; b, NCS/benzene; c, (*S*)-4-benzyl-1,2,5-thiadiazolidin-3-one 1,1-dioxide/TEA/acetonitrile; d, NaH/*CH_3I; e, MCPBA/CH_2Cl_2.

*oxide (***18***).* *m*-Chloroperbenzoic acid (70%) (4.8 g; 19.5 mmol) was added to a solution of sulfide **17** (3.7 g; 7.7 mmol) in 20 mL of methylene chloride at 0 °C. The mixture was stirred at room temperature overnight. Methylene chloride (80 mL) was added, and the mixture was washed with saturated $NaHCO_3$ (30 mL), 5% Na_2SO_3 (30 mL), and brine (30 mL). The organic layer was dried over anhydrous sodium sulfate and the solvent removed *in vacuo*, leaving a pure product (3.87 g; 100% yield), mp 123−125 °C. ^{1}H NMR ($CDCl_3$): δ 1.25−1.60 (m, 4H), 1.72−1.98 (m, 2H), 2.83 (s, 3H), 3.20 (m, 2H), 3.81 (t, 1H), 4.85 (br m, 1H), 4.87 (s, 2H), 5.08 (s, 2H), 7.34 (s, 5H), 7.57 (t, 2H), 7.69 (t, 1H), 7.97 (d, 2H). ^{13}C NMR ($CDCl_3$): δ 21.02, 28.40, 29.34, 32.39, 40.34, 59.92, 66.18, 66.59, 127.99, 128.06, 128.47, 129.17, 129.27, 134.69, 136.59, 136.97, 156.43, 165.57.

*(S)-4-(4-Aminobutyl)-5-methyl-2-(phenylsulfonyl)-1,2,5-thiadiazolidin-3-one 1,1-Dioxide (***13***).* Sulfone **18** (1.0 g; 2 mmol) in methylene chloride (6 mL) was treated with iodotrimethylsilane (0.6 g; 3 mmol) with stirring. After 10 min, methanol (2 mL) was added, at which point a precipitate formed. Stirring was continued for an additional 10 min. The solvent was removed *in vacuo*, leaving a solid residue which was then washed with methylene chloride/ethyl ether (1:1). The solid was dissolved in dry methanol (5 mL), followed by the addition of charcoal and sodium thiosulfate (0.5 g). The mixture was stirred for 5 min and then passed through a Celite pad. The filtrate was evaporated off, leaving a white crystalline solid (0.7 g; 95% yield), mp 180 °C dec. ^{1}H NMR (DMSO-d_6): δ 1.18 (m, 1H), 1.36 (m, 1H), 1.52 (m, 2H), 1.76 (m, 2H), 2.75 (br m, 2H), 2.82 (s, 3H), 4.31 (t, 1H), 5.23 (d, 2H), 7.63 (br s, 2H), 7.68 (m, 2H), 7.80 (m, 1H), 7.91 (m, 2H). ^{13}C NMR (DMSO-d_6): δ 20.54, 26.46, 27.96, 32.19, 38.52, 59.47, 65.23, 128.63, 129.30, 134.62, 137.03, 165.86.

Synthesis of ^{13}C-Labeled Inhibitor ***19***

Compound **19** was synthesized according to Scheme 3.

Methyl-^{13}C Phenyl Sulfide (Ono et al., 1980). A solution of thiophenol (3.1 g; 2.8 mmol) in benzene (80 mL) was treated with DBU (4.26 g; 28 mmol). The reaction mixture was cooled in an ice bath, and $^{13}CH_3I$ (4.0 g; 28 mmol) was added. The reaction mixture was stirred for 2 h at room temperature and then washed with 5% aqueous HCl (30 mL), saturated sodium bicarbonate (25 mL), and brine (35 mL). The solution was dried over anhydrous sodium sulfate, filtered, and evaporated *in vacuo*, yielding 3.0 g (86% yield) of an oily product. ^{13}C NMR ($CDCl_3$): δ 15.90 (*), 125.04, 126.72, 128.82.

Chloromethyl-^{13}C Phenyl Sulfide (Tuleen & Stephens, 1969). Methyl-^{13}*C* phenyl sulfide (3.0 g; 24 mmol) in 40 mL of benzene was treated with *N*-chlorosuccinimide (3.53 g; 26.4 mmol), and the solution was stirred at room temperature for 20 h. The precipitate was filtered off, and the filtrate was washed with 10% aqueous sodium bicarbonate and saturated sodium chloride and dried over anhydrous magnesium sulfate. The solvent was filtered off and the solvent removed *in vacuo*, leaving 3.8 g (100%) of an oil. ^{13}C NMR ($CDCl_3$): δ 50.99 (*), 127.94, 129.20, 130.85, 133.30.

(S)-4-Benzyl-2-[(phenylthio)methyl-^{13}C]-1,2,5-thiadiazolidin-3-one 1,1-Dioxide. A solution of (*S*)-4-benzyl-1,2,5-thiadiazolidin-3-one 1,1-dioxide (2.57 g; 11.3 mmol), chloromethyl-^{13}*C* phenyl sulfide (1.80 g; 11.3 mmol), and triethylamine (1.14 g; 11.3 mmol) in 15 mL of dry acetonitrile was refluxed for 10 h. The solvent was removed *in vacuo*, and the residue was taken up in methylene chloride (40 mL), washed with 5% HCl, saturated sodium bicarbonate, and brine, and dried over anhydrous sodium sulfate. Removal of the solvent *in vacuo* left a crude product which was purified by flash chromatography (hexane/CH_2Cl_2), yielding 2.3 g (58% yield) of pure product. ^{1}H NMR ($CDCl_3$): δ 3.12 (ddd, 2H), 4.28 (m, 1H), 4.67 (dd, 1H), 5.18 (d, 1H), 7.14−7.55 (m, 10H). ^{13}C NMR: δ 36.29, 45.80 (*), 61.08, 127.87, 128.47, 129.13, 129.18, 129.33, 132.97, 134.20, 167.26.

(S)-4-Benzyl-2-[(phenylthio)methyl-^{13}C]-5-methyl-^{13}C-1,2,5-thiadiazolidin-3-one 1,1-Dioxide. A solution of (*S*)-4-benzyl-2-[(phenylthio)methyl-^{13}C]-1,2,5-thiadiazolidin-3-one 1,1-dioxide (1.0 g; 2.87 mmol) in 9 mL of dry acetonitrile was treated with 60% sodium hydride (0.115 g) at 0 °C. After the solution was stirred for 5 min, $^{13}CH_3I$ (1.0 g; 7 mmol) was added, and the reaction mixture was stirred for 4 h at room temperature. The solvent was removed *in vacuo*, and the residue was taken up in methylene chloride. The organic layer was washed with water (25 mL) and dried over anhydrous sodium sulfate. Removal of the solvent left a crude product which was purified by flash chromatography (0.90 g; 97% yield), mp 69−70 °C. ^{1}H NMR ($CDCl_3$): δ 2.37 and 2.84 (s, 3H), 3.01 (dd, 1H), 3.19 (dd, 1H), 3.92 (m, 1H), 4.64 (q, 1H), 5.16 (q, 1H), 7.17−7.54 (m, 10H). ^{13}C NMR ($CDCl_3$): δ 33.51 (*), 36.63, 45.53 (*), 67.75, 127.36, 128.42, 128.66, 129.03, 129.40, 132.24, 133.06, 135.02, 164.95.

*(S)-4-Benzyl-2-[(phenylsulfonyl)methyl-^{13}C]-5-methyl-^{13}C-1,2,5-thiadiazolidin-3-one 1,1-Dioxide (***20***).* (*S*)-4-Benzyl-2-[(phenylthio)methyl-^{13}*C*]-5-methyl-^{13}*C*-1,2,5-thiadiazolidin-3-one 1,1-dioxide (0.82 g; 2.27 mmol) in 10 mL of dry methylene chloride was treated with 65% *m*-chloroperbenzoic acid (1.5 g; 2.5 equiv) and stirred for 15 h at room temperature. Methylene chloride (30 mL) was added, and the solution was washed with saturated $NaHCO_3$ containing 10% sodium sulfite. Evaporation of the solvent *in vacuo* left a crude product, which was purified by flash chromatography (hexane/methylene chloride), yielding 0.82 g (92% yield) of pure product, mp 137−138 °C. ^{1}H NMR ($CDCl_3$): δ 2.41 and 2.89 (s, 3H), 3.03 (dd, 1H), 3.20 (dd, 1H), 4.01 (m, 1H), 4.57 (d, 1H), 5.09 (d, 1H), 7.18−7.38 (m, 5H), 7.52−7.73 (m, 3H), 7.92 (m, 2H). ^{13}C NMR ($CDCl_3$): δ 34.39 (*), 36.64, 60.11 (*), 68.26, 127.58, 128.83, 129.27, 129.45, 134.71, 165.25.

Biochemical Studies

Enzyme Assays and Inhibition Studies: Incubation Method. (A) Human Leukocyte Elastase (HLE). HLE was assayed by mixing 10 μL of a 20.9 μM enzyme solution in 0.05 M sodium acetate buffer, pH 5.5, 10 μL of dimethyl sulfoxide, and 980 μL of 0.1 M HEPES buffer, pH 7.2, in a thermostated test tube. A 100 μL aliquot was transferred to a thermostated cuvette containing 880 μL of HEPES buffer and 20 μL of a 4.25 mM solution of MeOSuc-Ala-Ala-Pro-Val-*p*-NA, and the change in absorbance was monitored at 410 nm for 1 min. In a typical inhibition run, 10 μL of inhibitor (104 μM) in dimethyl sulfoxide was mixed with 10 μL of a 20.9 μM enzyme solution and 980 μL of 0.1 M HEPES buffer, pH 7.25, and placed in a constant temperature bath. Aliquots (100 μL) were withdrawn at different time intervals and transferred to a cuvette containing 20 μL of MeOSuc-Ala-Ala-Pro-Val-*p*-NA (4.25 mM) and 880 μL of HEPES buffer. The absorbance was monitored at 410 nm for 1 min. The pseudo-first-order rate constants were obtained from plots of $\ln(v_t/v_o)$ vs time and are the average of two or three determinations. The data were analyzed as described in the Data Analysis section.

(B) Human Leukocyte Cathepsin G (Cat G). Cat G was assayed by mixing 20 μL of a 63.5 μM enzyme solution in 0.05 M sodium acetate buffer, pH 5.5, 10 μL of dimethyl sulfoxide, and 970 μL of 0.1 M HEPES buffer, pH 7.5, and placed in a thermostated test tube. A 100 μL aliquot was transferred to a thermostated cuvette containing 880 μL of HEPES buffer and 20 μL of a 42.5 mM solution of Suc-Ala-Ala-Pro-Phe-*p*-NA, and the change in absorbance was monitored at 410 nm for 1 min. In a typical inhibition run, 10 μL of a 0.634 mM solution of the inhibitor in dimethyl sulfoxide was mixed with 20 μL of a 63.5 μM enzyme solution and 970 μL of 0.1 M HEPES buffer, pH 7.5, and placed in a constant temperature bath. Aliquots (100 μL) were withdrawn at different time intervals and transferred to a cuvette containing 20 μL of a 42.5 mM substrate solution and 880 μL of HEPES buffer. The absorbance was monitored at 410 nm for 1 min.

(C) Human Leukocyte Proteinase 3 (PR 3). PR 3 was assayed by mixing 10 μL of a 37.3 μM enzyme solution in 0.1 M phosphate buffer, pH 6.5, 10 μL of dimethyl sulfoxide, and 980 μL of 0.1 M phosphate buffer, pH 6.5, in a thermostated test tube. A 100 μL aliquot was transferred to a thermostated cuvette containing 875 μL of 0.1 M phosphate buffer and 25 μL of 20 mM Boc-L-Ala *p*-nitrophenyl ester in acetonitrile. The change in absorbance was monitored at 348 nm for 1 min (Kao et al., 1988). In a representative inhibition run, 10 μL of a 0.75 mM solution of the inhibitor in dimethyl sulfoxide was mixed with 10 μL of a 37.3 μM enzyme solution and 980 μL of 0.1 M phosphate buffer, pH 6.5, and placed in a constant temperature bath. Aliquots (100 μL) were withdrawn at different time intervals and transferred to a cuvette containing 25 μL of 20 mM BOC-L-Ala *p*-nitrophenyl ester in acetonitrile and 875 μL of phosphate buffer. The absorbance was monitored at 348 nm for 1 min.

Progress Curve Method. The rates of inactivation of HLE by compounds **3**−**13** were too fast to measure by ordinary sampling techniques, and consequently, the progress curve method was used (Morrison & Walsh, 1988). Thus, 10 μL of a 2.0 μM HLE solution were added to a solution containing 10 μL of inhibitor (10.0 μM solution in dimethyl sulfoxide), 26 μL of substrate (MeOSuc-Ala-Ala-Pro-Val-*p*-NA, 38.6 mM), and 954 μL of 0.1 M HEPES buffer, pH 7.25, and the absorbance was monitored at 410 nm for 1000 s. Likewise, 100 μL of Cat G (680 nM) was added to a solution containing inhibitor (10 μL of a 68 μM solution in DMSO), Suc-Ala-Ala-Pro-Phe-*p*-NA (20 μL of a 42.5 mM solution), and 870 μL of 0.1 M HEPES buffer, pH 7.5. The absorbance was monitored at 410 nm for 1000 s. Finally, in a separate experiment, 50 μL of PR 3 (373 nM) was added to a solution containing 20 μL of inhibitor (18.6 μM in DMSO), 50 μL of MeOSuc-Ala-Ala-Pro-Val-*p*-NA (60 mM), and 880 μL 0.1 M Tris buffer, pH 7.5. The absorbance was monitored at 410 nm for 10 000 s.

Data Analysis. The apparent second-order rate constants k_{inact}/K_I were determined by the progress curve method.

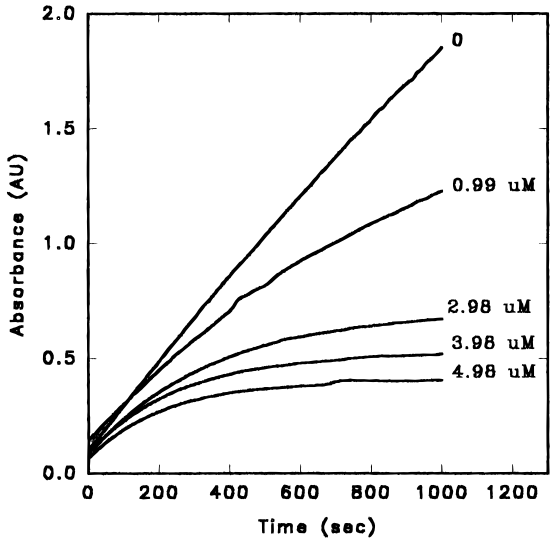


FIGURE 1: Progress curves for the inhibition of human leukocyte elastase by compound **7**. Absorbance was recorded at 410 nm for reaction solutions containing 20.0 nM HLE, 1 mM MeOSuc-AAPV-pNA, and the indicated concentrations of inhibitor in 0.1 M HEPES buffer, pH 7.25, and 3.6% DMSO. The temperature was maintained at 25 °C, and the reactions were initiated by the addition of enzyme.

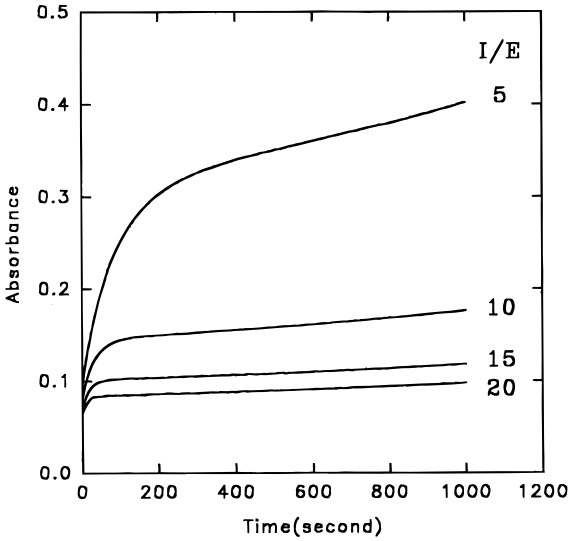


FIGURE 2: Progress curves for the inhibition of bovine trypsin by inhibitor **13**. Absorbance was monitored at 410 nm for reaction solutions containing 500.0 nM bovine trypsin, 0.6 mM N^{α}-benzoyl-L-Arg *p*-nitroanilide, and the indicated concentrations of inhibitor in 0.025 M phosphate buffer, pH 7.51, and 2% DMSO. The temperature was maintained at 25 °C, and the reactions were initiated by the addition of enzyme.

Typical progress curves for the hydrolysis of MeOSucc-AAPV-*p*-NA (1 mM) by HLE (20.0 nM) in the presence of inhibitor **7** and for the hydrolysis of N^{α}-benzoyl-L-Arg-*p*-NA (0.6 mM) by trypsin (500 nM) are shown in Figures 1 and 2. The release of *p*-nitroaniline was continuously monitored at 410 nm. Control curves in the absence of inhibitor were linear. The pseudo-first-order rate constants, k_{obs}, for the inhibition of HLE, Cat G, PR 3, and trypsin as a function of time by inhibitor (**I**) were determined according to eq 1, where A is the absorbance at 410 nm, v_0 is the reaction velocity at $t = 0$, v_s is the final steady-state velocity, k_{obs} is the observed first-order rate constant, and A_0 is the absorbance at $t = 0$ (Morrison & Walsh, 1988). This involved fitting by nonlinear regression analysis the $A \sim t$ data into eq 1 (SigmaPlot, Jander Scientific) to determine k_{obs}. The second-order rate constants (k_{inact}/K_I) were determined in duplicate or triplicate by calculating k_{obs}/[I] and then correcting for the substrate concentration and Michaelis constant using eq 2. The ratio k_{inact}/K_I is an index of inhibitory potency. The values of these ratios for inhibitors **3**−**13** are listed in Table 1.

$$A = v_s t + \{(v_0 - v_s)(1 - e^{-k_{obs}t})/k_{obs}\} + A_0 \quad (1)$$

$$k_{obs}/[I] = k_{inact}/K_I\{(1 + [S]/K_m)\} \quad (2)$$

The kinetics data obtained by using the incubation method (compounds **1** and **2**) were analyzed by determining the slopes of the semilogarithmic plots of enzymatic activity remaining vs time using eq 3, where $[E]_t/[E]_0$ is the amount of active enzyme remaining at time t (Kitz & Wilson, 1962).

$$\ln([E_t]/[E]_0) = k_{obs}t \quad (3)$$

Efficiency of Inactivation. HLE (212 nM) was incubated at 25 °C with various concentrations of inhibitor **7** in 0.1 M HEPES buffer, pH 7.25, containing 1% DMSO in a final volume of 500 μL. After a 30 min incubation, a 100 μL aliquot of the enzyme solution was removed and assayed for catalytic activity using MeOSuc-AAPV-*p*-NA (77.2 mM) in 0.1 M HEPES buffer, pH 7.25. The partition ratio was calculated as described by Knight and Waley (1985) by plotting the fraction of remaining enzyme activity ($[E]_f/[E]_0$) versus the initial ratio of inhibitor to enzyme ($[I]/[E]_0$).

Hydroxylamine Reactivation of Inactivated HLE. HLE (10 μL, 15.6 μM) was incubated with a 5-fold excess of inhibitor **7** (10 μL of a 78 μM solution in dimethyl sulfoxide) and 980 μL of 0.1 M HEPES buffer, pH 7.25, for 10 min. The enzyme was totally inactivated (as shown by withdrawing an aliquot and assaying for remaining enzyme activity). Excess hydroxylamine hydrochloride (100 μL of a 0.5 M solution) was then added to the fully inactivated enzyme. Aliquots (100 μL) were removed at various time intervals (from 1 min to 24 h) and assayed for enzyme activity by mixing with MeOSuc-Ala-Ala-Pro-Val-*p*-NA (20 μL, 4.25 mM) and 0.1 M HEPES buffer (880 μL), pH 7.25, and monitoring the absorbance at 410 nm. Enzyme activity was determined by comparing the activity of an enzyme solution containing no inhibitor (control) with the activity of an enzyme solution containing inhibitor at the same time point.

Reactivation of the HLE−Inhibitor **7** *Complex.* In a typical experiment, 21.9 μM HLE (100 μL) was incubated with excess inhibitor **7** (10 μL of a 4.38 mM solution in DMSO) and 890 μL of 0.1 M HEPES buffer, pH 7.25 at 25 °C. After 10 min, a 100 μL aliquot was removed and assayed for enzymatic activity (the enzyme was completely inhibited). Excess inhibitor was removed via Centricon-10 filtration by centrifuging at 6000*g* for 1 h at 5 °C. Buffer (750 μL) was added to the HLE−inhibitor complex and the centrifugation was repeated under the same conditions. The HLE−inhibitor complex was dissolved in 500 μL of 0.1 M HEPES buffer, pH 7.25, and 10 μL aliquots were withdrawn at different time intervals and added to a cuvette containing 20 μL of 4.25 mM MeOSuc-Ala-Ala-Pro-Val-*p*-NA and 970 μL of 0.1 M HEPES buffer, pH 7.25. The amount of active enzyme was determined by monitoring the release of *p*-nitroaniline at 410 nm. A control containing HLE (100 μL, 21.9 μM), buffer (890 μL), and DMSO (10 μL) was run under the same conditions.

NMR Spectroscopy of the Chymotrypsin−Inhibitor Complex. A solution containing 200 μL of 4-benzyl-2-[(phenylsulfonyl)methyl-^{13}C]-5-methyl-1,2,5-thiadiazolidin-3-one 1,1-dioxide (**19**) (20 mM), dimethyl sulfoxide (100 μL), and deuterium oxide (200 μL) was mixed with 100 μL of chymotrypsin (2 mM) in deuterium oxide and 600 μL of phosphate buffer, pH 7.5, and the resulting solution was incubated at 25 °C for 8 h. The 125.70 MHz ^{13}C DEPT-45 NMR spectra were recorded on a 500 MHz Varian UNITY Plus NMR spectrometer at different time intervals using the conditions listed in the caption of Figure 7.

Turnover of Compound **20** *by Chymotrypsin.* Compound **20** (**I**, R = R_1 = benzyl, L = $PhSO_2$) (45 mg) in 0.5 mL of acetonitrile was added to 10 mL 0.025 M phosphate buffer, pH 7.25, containing chymotrypsin (25 mg). Acetonitrile (1 mL) and deuterated dimethyl sulfoxide (1 mL) were added to keep the inhibitor in solution, and the mixture was stirred at room temperature for 3 days. The solution was extracted with ethyl acetate (3 × 10 mL) dried over anhydrous sodium sulfate, and the solvent was removed on the rotary evaporator. The residue was dissolved in 0.5 mL of DMSO-d_6, and the ^{1}H NMR spectrum was recorded.

The aqueous solution was concentrated *in vacuo* to about 1 mL, acidified with 5% HCl, and extracted with ethyl acetate (3 × 10 mL). The combined ethyl acetate extracts were dried over anhydrous sodium sulfate and evaporated *in vacuo*. The residue was dissolved in DMSO-d_6, and the ^{1}H and ^{13}C NMR spectra were recorded. ^{1}H NMR: δ 2.99 (d, 2H), 4.30 (d, 2H), 4.45 (t, 1H), 7.12−7.36 (m, 5H), 7.47−7.80 (m, 5H), 9.00 (br s, 1H). ^{13}C NMR: δ 35.46, 49.73, 66.58, 124.47, 126.62, 127.71, 127.98, 128.25, 128.78, 128.97, 129.67, 131.36, 135.30, 135.54, 148.75.

Assay and Inhibition of Bovine Trypsin. Bovine trypsin was assayed spectrophotometrically using N^{α}-benzoyl-L-Arg *p*-nitroanilide. Enzyme inhibition was carried out using the progress curve method. Thus, 10 μL of a 6×10^{-2} M substrate solution in DMSO was mixed with 10 μL of inhibitor **13** in DMSO (inhibitor stock concentrations ranged between 2.5×10^{-4} and 1.0×10^{-3} M) and 970 μL of 0.025 M phosphate buffer, pH 7.51, containing 0.1 M sodium chloride. Bovine trypsin (10 μL of a 5×10^{-5} M solution in 1 mM HCl) was added, and the change in absorbance was monitored at 410 nm as a function of time. The final concentration of DMSO was 2%. The data were analyzed using eqs 1 and 2 above and a substrate K_m of 0.86 mM.

Molecular Modeling. Enzyme−inhibitor modeling studies were performed using the Tripos force field of SYBYL,

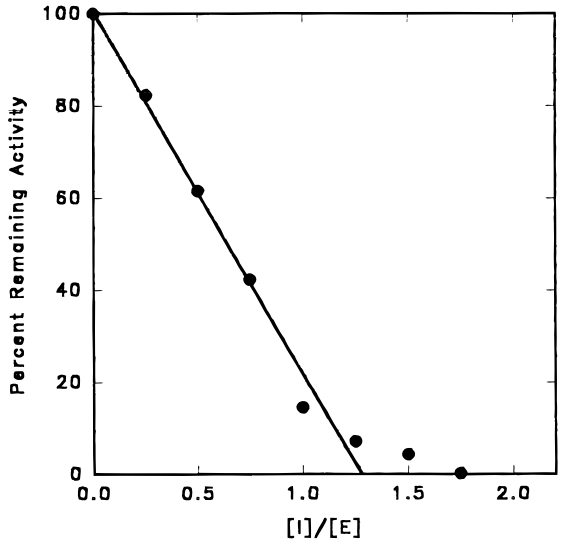


FIGURE 3: Inactivation of human leukocyte elastase as a function of the molar ratio of inhibitor **7** to enzyme. HLE (20 nM) and various amounts of inhibitor **7** (1.00−4.97 μM) in 0.1 M HEPES buffer, pH 7.25, were incubated at 25 °C for 30 min, and aliquots were withdrawn for assay. The fractional activity remaining is proportional to the molar ratio of inhibitor to enzyme.

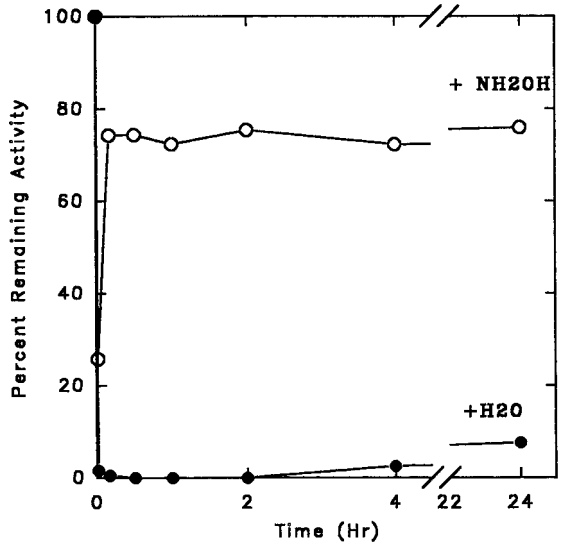


FIGURE 4: Effect of hydroxylamine on enzyme reactivation. Human leukocyte elastase (156 nM) was totally inactivated by incubating with a 5-fold excess of inhibitor **7** (0.78 μM) for 10 min in 0.1 M HEPES buffer, pH 7.25. Excess hydroxylamine (0.045 M) was then added, and aliquots were removed at different time intervals and assayed for enzyme activity using MeOSuc-Ala-Ala-Pro-Val-*p*-NA. Enzyme activity was determined by comparison with a control at the same time point.

version 6.1a (Tripos Associates, St. Louis, MO), and a Silicon Graphics INDY workstation.

Stability. The stability of a representative member of this class of compounds (compound **7**) in 0.1 M HEPES buffer, pH 7.25, was investigated using HPLC. Inhibitor **7** in acetonitrile was mixed with buffer (the final concentration of the inhibitor was 10 mM), and aliquots (15 μL) were removed at different time intervals and injected into a C-18 column (15 cm × 4.6 mm). The final concentration of the inhibitor was 10 mM. The mobile phase used consisted of 60% acetonitrile and 40% water and a flow rate of 1.5 mL/min. The decrease in **7** was monitored at a wavelength of 254 nm over a 24 h period.

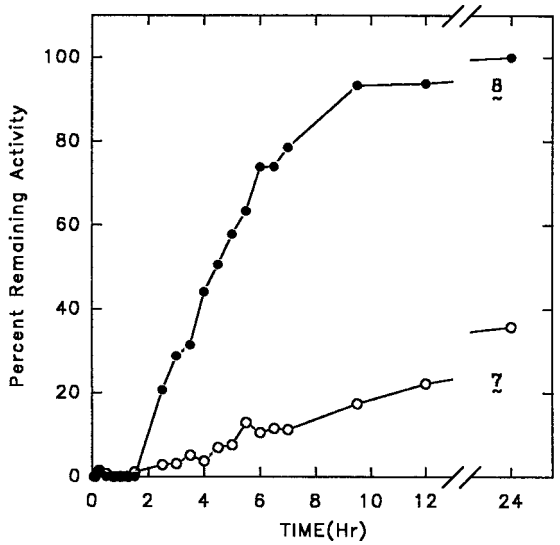


FIGURE 5: Reactivation of the HLE−inhibitor **7** complex. (a) Human leukocyte elastase was incubated with excess inhibitor **7** in 0.1 M HEPES buffer, pH 7.25. After 10 min, excess inhibitor was removed by Centricon-10 filtration. Buffer was added to the HLE−inhibitor complex, and the Centricon-10 filtration was repeated. The HLE−inhibitor complex was then dissolved in buffer, and aliquots were withdrawn at different time intervals and assayed for enzyme activity using MeOSuc-Ala-Ala-Pro-Val-*p*-NA (see Materials and Methods for details). (b) The experiment was repeated using inhibitor **8**. A control was run under the same conditions in both experiments.

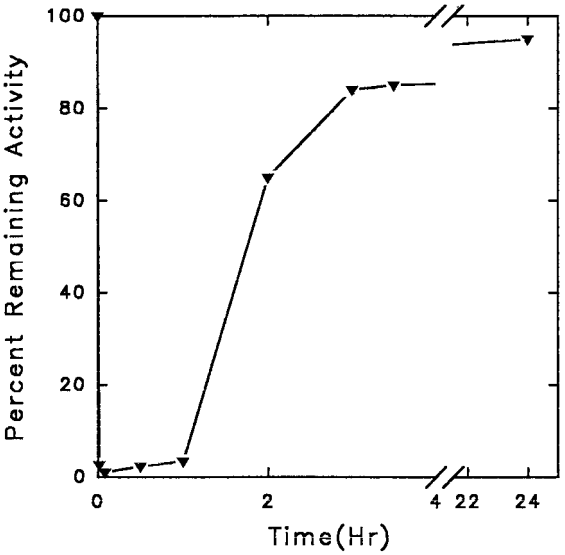


FIGURE 6: Inhibition of chymotrypsin by compound **20**. A 20-fold excess of inhibitor **20** (1.16 μM) was incubated with chymotrypsin (57.8 nM) in 0.1 M phosphate buffer, pH 7.5. Aliquots were withdrawn at different time intervals, and enzyme activity was assayed using *N*-benzoyl-L-tyrosine ethyl ester.

RESULTS

Synthesis. Inhibitors **1**−**12** were synthesized using Scheme 1 starting with the readily available L-amino acid esters. Specificity for a target serine proteinase can be tailored through the use of an appropriate starting amino acid ester, the selection of which is based on the known substrate specificity of the enzyme under investigation. Since the preferred primary specificity residues (P_1) for HLE, PR 3, and Cat G are Val (or Leu), Abu (or Norval), and Phe, respectively (Bode et al., 1989; Brubaker et al., 1992; Rao et al., 1991; Kam et al., 1992), an R_1 group corresponding

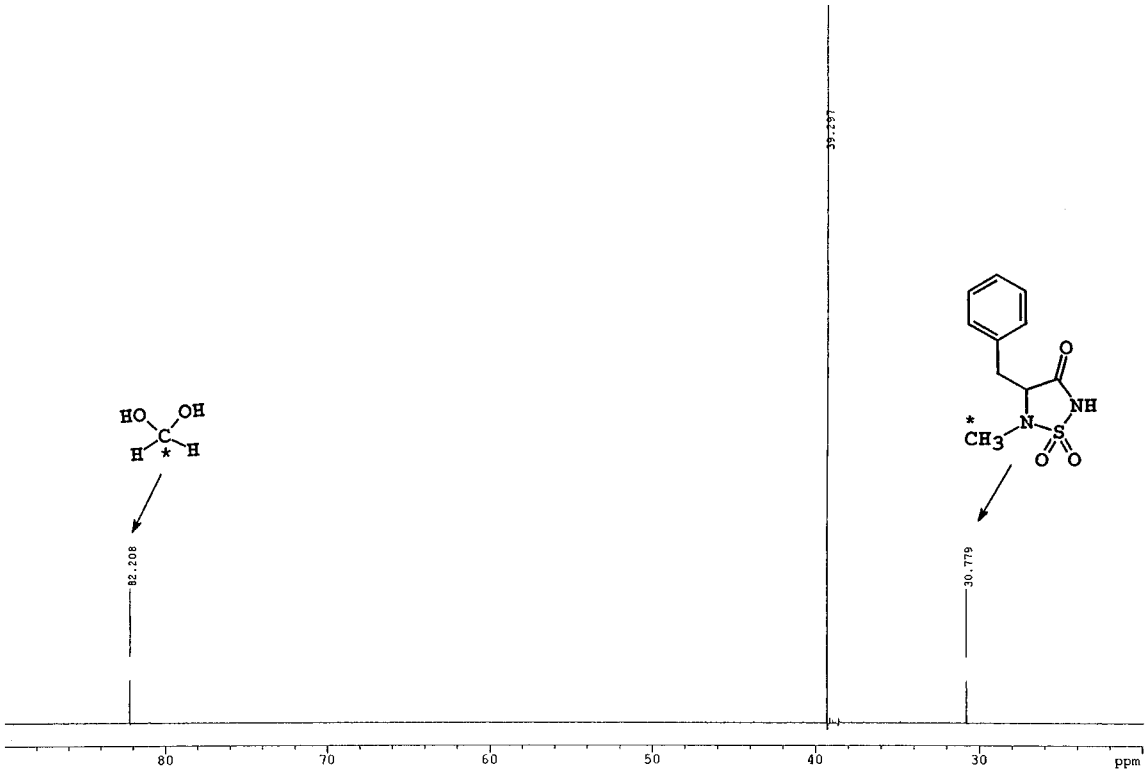


FIGURE 7: A 20-fold excess of inhibitor **19** (20 mM) was incubated with chymotrypsin (2 mM) in D_2O, 0.1 M phosphate buffer, pH 7.5, and deuterated dimethyl sulfoxide for 8 h. The 125.70 MHz DEPT-45 NMR spectra (Doddrell et al., 1982) were recorded on a 500 MHz UNITY Plus NMR spectrometer using the following conditions: 1.0 s repetition time, 1000 scans, 27K sweep width, 32K data points, 45° variable pulse (^{1}H) to excite all protonated carbons, and broad-band ^{1}H decoupling. The signal at 39.5 ppm is due to dimethyl sulfoxide.

to the side chain of the appropriate P_1 residue was selected in order to achieve high potency and specificity for a target proteinase. Thus, compounds **1**−**4** were directed at PR 3, **5**−**10** for HLE, **11** and **12** for Cat G, and compound **13** for trypsin (Table 1). Compound **13** was synthesized according to Scheme 2 and the ^{13}C-labeled inhibitor **19** according to Scheme 3.

Inactivation Kinetics. Compounds **1**−**13** were synthesized and evaluated for their inhibitory activity toward human leukocyte elastase, cathepsin G, and proteinase 3. Several derivatives of **I** were found to function as time-dependent inhibitors of the three target enzymes. The apparent second-order rate constants k_{inact}/K_I were determined in duplicate or triplicate by the progress curve method (Figures 1 and 2) and are listed in Table 1. Figures 1 and 2 also clearly demonstrate that the inactivation process involves the active site. The preincubation method under pseudo-first-order conditions was used in the evaluation of inhibitors **1** and **2**, yielding the corresponding $k_{obs}/[I]$ values for these inhibitors.

Partition Ratio Determination. The partition ratio, a parameter that corresponds to the number of molecules of inhibitor necessary to inactivate a single molecule of enzyme, and thus describes how efficiently a mechanism-based inhibitor inactivates an enzyme (Silverman, 1995), was determined by plotting the fraction of remaining enzyme activity ($[E]_t/[E]_0$) after a 30-min incubation period versus the initial ratio of inhibitor to enzyme($[I]/[E]_0$). The extent of inactivation was found to be linearly dependent on the inhibitor to enzyme molar ratio (Figure 3). Extrapolation of the linear part of the curve to the line of complete inactivation yielded a partition ratio for inhibitor **7** that was close to zero, attesting to the high inhibitory prowess of this class of inhibitors.

Hydroxylamine Reactivation. HLE was fully inactivated using a 5-fold excess of inhibitor **7**. The effect of adding excess hydroxylamine on the HLE−inhibitor complex(es) was examined by removing aliquots at different time intervals and monitoring the regain in enzymatic activity (Figure 4). The enzyme regained rapidly about 80% of its activity and then leveled off.

Reactivation Kinetics. The reactivation of the complex(es) formed between HLE and inhibitor **7** was examined in order to ascertain the extent of reactivation. Thus, HLE was totally inactivated via treatment with excess inhibitor **7**. The excess inhibitor was then removed by Centricon-10 filtration, and the regain in enzymatic activity was monitored (Figure 5). The experiment was repeated using inhibitor **8**. Biphasic reactivation kinetics were observed with both inhibitors, suggesting the presence of two different complexes (Green et al., 1995). Furthermore, the complex(es) formed with inhibitor **7** exhibited much higher stability and led to partial regain (37%) of enzymatic activity after 24 h. In contrast, the complex(es) derived from inhibitor **8** had lower stability which ultimately led to full regain of enzymatic activity. The deacylation constants (k_{deacyl}) for the HLE−inhibitor complexes derived from **7** and **8**, which correspond to the rapid recovery phase, were determined to be 5.44×10^{-5} and 8.43×10^{-4} s^{-1}, respectively. A control consisting of an identical solution of enzyme without inhibitor was subjected to the same process.

Products Formed When Inhibitor **20** *Is Incubated with Chymotrypsin.* The interaction of chymotrypsin with inhibitor **20** is transient, leading to full regain of enzymatic activity (Figure 6). The identity of the products generated when inhibitor **20** is turned over by the enzyme was determined. In addition to the formation of formaldehyde hydrate (vide

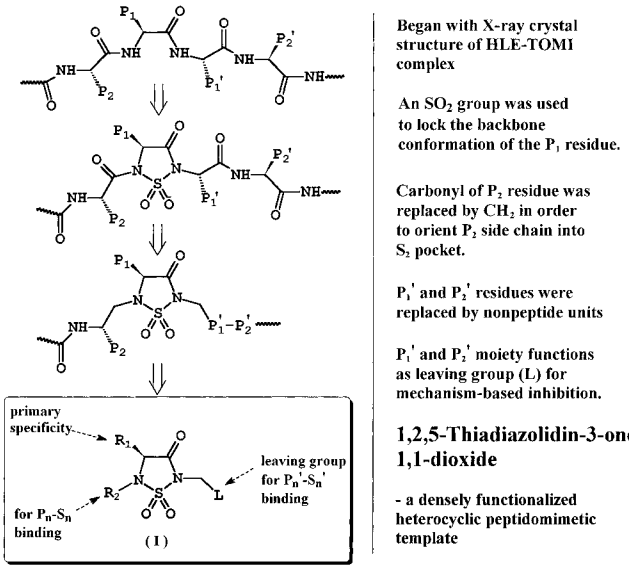


FIGURE 8: Design rationale and comparison of a natural peptide with the 1,2,5-thiadiazolidin-3-one 1,1-dioxide peptidomimetic scaffold of **I**.

infra), two other major products were formed in approximately 1:1 ratio. These were identified as phenylsulfinic acid and 4,5-dibenzyl-1,2,5-thiadiazolidin-3-one 1,1-dioxide using authentic samples.

NMR Studies Using ^{13}C-Labeled Inhibitor **19** *and Chymotrypsin.* The mechanism of action of **I** was explored further using inhibitor **19** and chymotrypsin. Thus, incubation of **19** with chymotrypsin resulted in the formation of formaldehyde hydrate and 4-benzyl-5-methyl-^{13}C-1,2,5-thiadiazolidin-3-one 1,1-dioxide (Figure 7). The formation of benzenesulfinate was also demonstrated using reverse-phase HPLC and an authentic sample.

Specificity. The primary determinant of specificity for a particular serine proteinase resides with the nature of the residue (P_1) which interacts with the S_1 pocket. Consequently, it was hypothesized that inhibitors derived from amino acids with small hydrophobic side chains would inhibit HLE and proteinase 3, those derived from aromatic amino acids would inhibit cathepsin G and chymotrypsin, and those derived from basic amino acids would inhibit trypsin. The results obtained with compounds **1**−**13** (Table 1) validate the proposed hypothesis and suggest that compound **I** constitutes a *general* class of mechanism-based inhibitors of the serine proteinases.

Stability. The stability of compound **7** in 0.1 M HEPES buffer, pH 7.25, was investigated using HPLC. After 24 h, there was no change in the structural integrity of inhibitor **7**, attesting to the high stability of compound **I**.

DISCUSSION

Design Rationale. On the basis of what is currently known about the mechanism of action and substrate specificity of the target proteinases (Stein et al., 1987; Bode et al., 1989) and the chemistry of 1,2,5-thiadiazolidin-3-one 1,1-dioxides, we reasoned that the heterocyclic ring system in **I** might function as a highly effective scaffold for appending peptidyl and nonpeptidyl recognition elements, making possible the exploitation of favorable binding interactions with the S_n *and* S_n' subsites of the enzyme. The design process utilized the available X-ray crystal structures of the HLE−turkey ovomucoid inhibitor complex (HLE−TOMI) (Bode et al., 1986) and that of the complex of HLE with methoxysuccinyl-Ala-Ala-Pro-Val chloromethyl ketone (Navia et al., 1989) in constructing the template to gain insight into the binding of **I** to the active site of HLE (Figure 8). The modeling studies suggested that the binding of **I** to the active site of HLE might be similar to that of a peptidyl substrate, with R_1 being accommodated at the primary specificity site (S_1) of the target enzyme, with R_2 and L extending into the S_2−S_n and S_n' subsites, respectively (Figure 8). Indeed, modeling studies that docked energy-minimized inhibitor **8** into the active site of HLE showed the benzyl group of the inhibitor nestled into the hydrophobic S_2 pocket and the benzenesulfonyl ring extending into the hydrophobic S_n' pocket (Figure 9). Of special note is the hydrogen bond between Ser^{195} and one of the oxygen atoms of the ring SO_2 (vide infra). Finally, mechanistic considerations suggested that **I** might function as a suicide inhibitor of serine proteinases via a cascade of steps involving nucleophilic ring opening by a target proteinase, followed by tandem loss of the leaving group (L) to form a highly reactive electrophilic species (an *N*-sulfonylimine). Subsequent reaction with an active site nucleo-

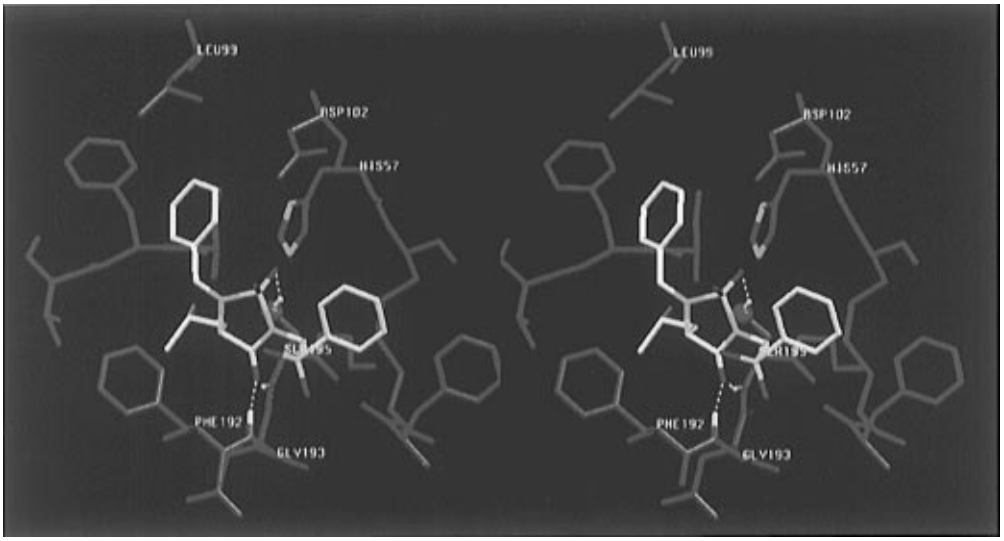


FIGURE 9: Energy-minimized structure of inhibitor **7** with human leukocyte elastase detailing the binding and catalytic sites. Hydrogen bonds are indicated by dotted lines.

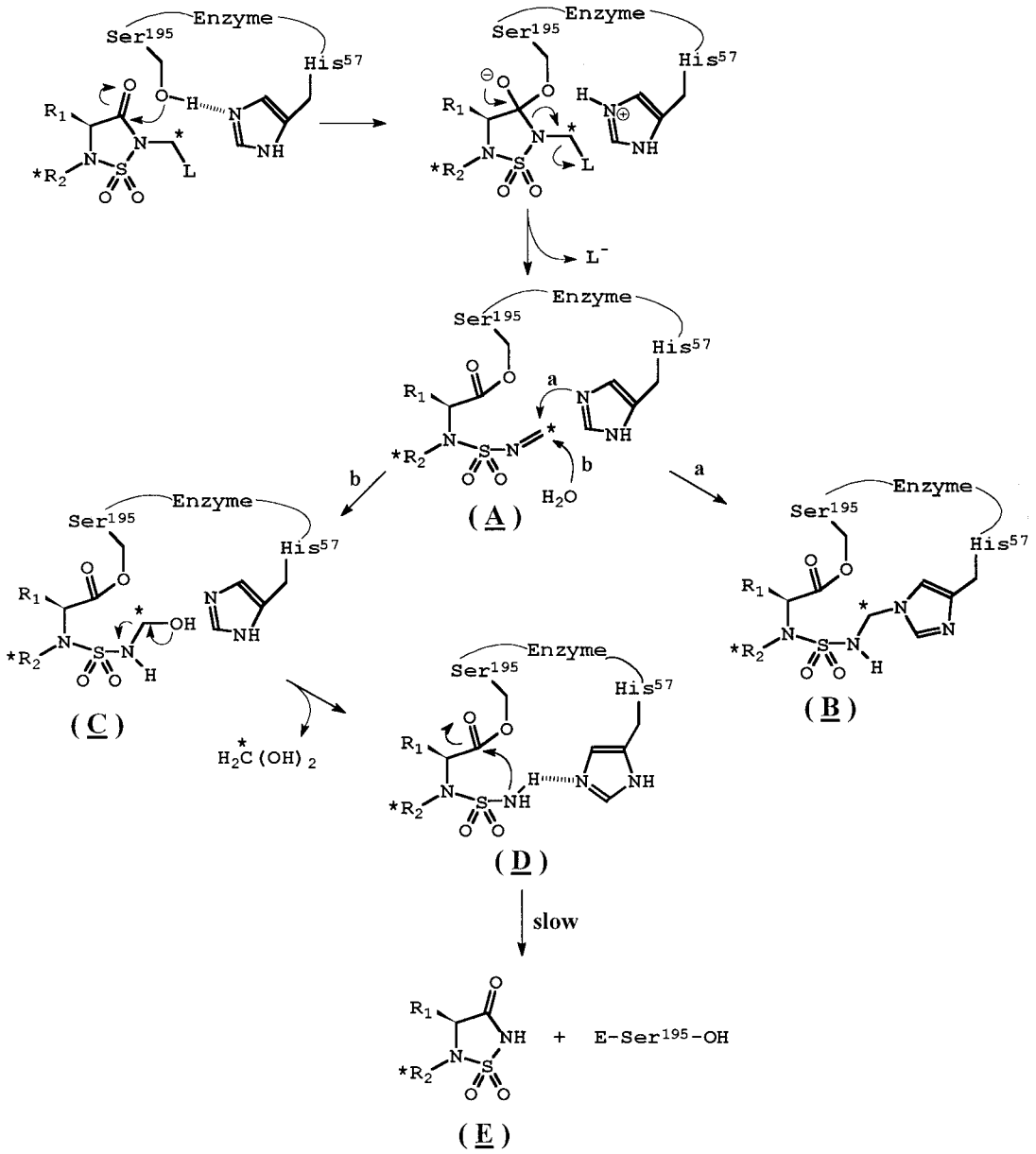


FIGURE 10: Postulated mechanism of action of **I**.

philic residue was anticipated to lead to irreversibly inactivated enzyme (Figure 10).

Inactivation of HLE, Cat G, and PR 3 by Derivatives of **I***: (A) Relationship of Structure to Inhibitory Potency and Specificity.* Several derivatives of **I** were found to be highly efficient, time-dependent inhibitors of HLE, Cat G, and PR 3 (Table 1). The interaction of the inhibitors with these enzymes involves the active site. Furthermore, the densely functionalized heterocyclic scaffold in **I** was found to provide considerable flexibility in terms of probing the effect of structure on inhibitory activity and specificity. Thus, differential inhibition among the three closely related proteinases could be achieved by varying the nature of R_1, believed to be accommodated at the primary specificity site (S_1). The observed relative inhibitory potency of the inhibitors was found to be in agreement with the known substrate specificity of the three enzymes, suggesting that the interaction of **I** with a target proteinase is similar to that of a peptidyl substrate. Remarkably, near absolute specificity for a proteinase could be achieved by using appropriate derivatives of **I** (compounds **9**, **12**, and **13**). The observation that the presence of a basic side chain leads to a potent inhibitor of bovine trypsin (k_{inact}/K_I 16 820 M^{-1} s^{-1}), but not HLE, Cat G, or PR 3, is strongly supportive of the notion that **I** binds to the active site with the R_1 nestled into the S_1 subsite and suggests that **I** constitutes a *general class* of mechanism-based inhibitors of serine proteinases, augmenting considerably the scope and importance of this class of inhibitors.

The R_2 group in **I** is accommodated at the S_2 subsite. In the case of HLE, inhibitory potency increased by an order ofmagnitude in going from R_2 = methyl to R_2 = benzyl (Table 1, compounds **1** and **2**, **3** and **4**, **7** and **8**). The beneficial effect of the benzyl group was even more pronounced in the case of Cat G (Table 1, **11** and **12**). The benzenesulfonyl group in **I** extends into the S_n' subsites; consequently, binding interactions involving these subsites can be exploited either by introducing structural variations in the sulfone leaving group or by utilizing different leaving groups. Indeed, the uniqueness of the heterocyclic template in **I** is further evidenced by (a) the unparalleled degree of flexibility afforded by the use of an array of leaving groups, including L = halogen, SOR, OOCR, OOCCHRNHCbz,

BOC, etc. (unpublished observations), suggesting that the inherent structure of L is important for enzyme affinity, (b) the observation that inhibitory potency is related to the pK_a of L, and (c) the finding that the corresponding hydantoin sulfones were inactive (unpublished observations).

In general, compounds that inhibited HLE also inhibited PR 3, attesting to the close similarity of the active sites of the two enzymes. It is likely that highly specific inhibitors of PR 3 derived from **I** can be realized by exploiting some of the subtle differences in the active sites of PR 3 and HLE (Fujinaga et al., 1996). In this regard, the structural diversification afforded by the heterocyclic template in **I** via the construction of biased combinatorial libraries (Thomson et al., 1996; Gallop et al., 1994) is anticipated to yield highly specific inhibitors of PR 3.

The surprising lack of inhibitory activity of compounds **5** and **6** toward HLE may be due to the restricted conformational mobility of the isopropyl group, as opposed to the isobutyl group, which prevents proper juxtaposition of **I** with the catalytic residues. Lastly, in addition to their high potency and specificity, these inhibitors were found to be highly stable in buffer solution.

(B) Mechanism of Inactivation. The postulated mechanism of action of **I** (Figure 10) was explored using biochemical studies and ^{13}C NMR. Thus, the incubation of inhibitor **19** with chymotrypsin led to the formation of formaldehyde hydrate and 4-benzyl-5-methyl-^{13}C-1,2,5-thiadiazolidin-3-one 1,1-dioxide (Figure 7). The formation of a third product, benzenesulfinate was also demonstrated. It was not possible to establish the formation of an enzyme−inhibitor complex(es). The low aqueous solubility of **19** did not allow the attainment of the millimolar concentrations needed for the detection of any enzyme−inhibitor complex(es).

The low molecular weight turnover products formed from the interaction of chymotrypsin with **20** were found to be formaldehyde hydrate, benzenesulfinate, and 4,5-dibenzyl-3-oxo-1,2,5-thiadiazolidine 1,1-dioxide (the last two in a 1:1 ratio). These results suggest that enzymatic processing of **19** (or **20**) by chymotrypsin results in acylation of the active site serine and the formation of a highly reactive *N*-sulfonylimine A and benzenesulfinate. Furthermore, the formation of formaldehyde hydrate indicates that one of the pathways followed by species A involves reaction with water to form C, which is rapidly transformed to acyl enzyme D, formaldehyde hydrate, and 4-benzyl-5-methyl-^{13}C-1,2,5-thiadiazolidin-3-one 1,1-dioxide E.

In addition to the NMR data, the reactivation experiments provided valuable insights regarding the mechanism of action of **I**. Thus, when excess hydroxylamine was added to HLE that was fully inactivated by inhibitor **7**, the enzyme regained about 80% of its activity after 24 h, suggesting the presence of *two* inactive forms of the enzyme, one having a labile acyl linkage that leads to active enzyme upon treatment with hydroxylamine and a second form that is unaffected by hydroxylamine. The reactivation of the HLE−inhibitor **7** complex(es) was also investigated following the removal of excess inhibitor. Biphasic reactivation kinetics were observed, suggesting the presence of two different complexes (Green et al., 1995). Similar observations were made when inhibitor **8** was used. Taken together, the NMR and biochemical data suggest that the inhibition of HLE by **I** leads to the formation of two HLE−inhibitor complexes that differ in stability. The two complexes might be B and D, the former arising from a "double hit" mechanism. Alternatively, the two complexes might be two different forms of D, by analogy with the reported mechanism of action of β-lactam inhibitors of HLE (Chabin et al., 1993; Underwood et al., 1995). Interestingly, the mechanistic inferences cited herein parallel closely those recently reported for saccharin derivatives (Groutas et al., 1996a).

In summary, the findings cited herein (a) describe the discovery of a general class of heterocyclic mechanism-based inhibitors of serine proteinases, (b) demonstrate that the incorporation of a nonpeptidyl scaffold into the structure of this class of inhibitors provides an effective means for appending recognition elements in a well-defined spatial arrangement and is well suited to using multiple binding sites to effect inhibitor potency and enzyme selectivity, and (c) suggest that a balance between such salient parameters as inhibitor reactivity, enzyme selectivity, hydrolytic instability, and stability of the enzyme−inhibitor complex can be achieved. Further studies aimed at exploiting the full potential of the heterocyclic scaffold in **I** are currently in progress.

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