Mechanism-Based Isocoumarin Inhibitors for Human Leukocyte Elastase. Effect of the 7-Amino Substituent and 3-Alkoxy Group in 3-Alkoxy-7-amino-4-chloroisocoumarins on Inhibitory Potency†

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A series of 3-alkoxy-7-amino-4-chloroisocoumarins with various 3-alkoxy substituents have been prepared and evaluated as inhibitors of human leukocyte elastase (HLE). In addition, a new series of acyl, urea, and carbamate derivatives of 7-amino-4-chloro-3-methoxyisocoumarin (**1**), 7-amino-4-chloro-3-propoxyisocoumarin (**3**), and 7-amino-4-chloro-3-(2-bromoethoxy)isocoumarin (**6**) have been synthesized. Most of the synthesized compounds are very potent inhibitors of HLE with k_{obs}/[I] values between 10^4 and 10^6 M^{-1} s^{-1}. Hydrophobic substituents on the 7-amino position of the isocoumarin ring afford the best selectivity and inhibitory potency for HLE. In the 2-bromoethoxy series, compound **24** with a PhNHCONH 7-substituent had a k_{obs}/[I] value of 1.2×10^6 M^{-1} s^{-1}, was very selective for HLE, and was the most potent inhibitor of HLE tested. Of the extended chain L-phenylalanyl derivatives, the Bz-L-Phe compound **66** with a k_{obs}/[I] value of 1.8×10^5 M^{-1} s^{-1} was the most potent inhibitor of HLE in the 3-methoxyisocoumarin series and was also very selective for HLE. Our results indicate that a high degree of selectivity, along with potency, can be introduced into mechanism-based isocoumarin inhibitors.

Introduction

Human leukocyte elastase (HLE) is an important serine protease that is responsible for phagocytosis and defense against invading microorganisms.[1] HLE cleaves elastin and other connective tissue proteins during phagocytosis. Normally, the destructive properties of HLE are kept in balance by α-1-protease inhibitor (α1-PI), a natural inhibitor present in the blood plasma. Deficiency of this natural inhibitor (α1-PI) has been associated with the lung tissue breakdown observed in the disease emphysema.[2,3] The absence of human secretory leukocyte protease inhibitor, another specific natural HLE inhibitor, has also been linked to bronchial secretory cell metaplasia.[4,5] Cigarette smoke oxidatively inactivates α1-PI *in vitro*,[6] and it is therefore believed that cigarette smoking leads to a deficiency of α1-PI in the lungs. This is the primary cause of emphysema in cigarette smokers.[6] Normal levels of α1-PI are restored upon cessation of cigarette smoking. Other disease states in which HLE is involved include rheumatoid arthritis, adult respiratory distress syndrome, infantile respiratory distress syndrome, glomerulonephritis, arteriosclerosis, and psoriasis. The destruction of lung tissue commonly found in the genetic disease cystic fibrosis (CF) has also been linked to HLE.[7] The development of effective specific elastase inhibitors is an active area of research due to their considerable potential for use in therapy.

Many synthetic inhibitors of serine proteases are known[8] including heterocyclic structures such as β-lactams,[9,10] *N*-substituted saccharins,[11] haloenol lactones,[12] 6-chloropyrones,[13] isatoic anhydrides,[14] oxazine-2,6-diones,[15] benzopyran-1,4-diones,[16] benzoxazin-4-ones,[17] 3,4-dichloroisocoumarin,[18] and 3-alkoxy-7-amino-4-chloroisocoumarins.[19] Some of these inhibitors, after initial acylation of the active-site serine (Ser-195), can "unmask" a reactive functional group (e.g., halo ketone in the case of haloenol lactones, an acyl chloride in the case of chloropyrones, and 3,4-dichloroisocoumarin or quinone imine methide in the case of 3-alkoxy-7-amino-4-chloroisocoumarins). The unmasked functional group can then react with other active-site residues such as His-57. These inhibitors are commonly referred to as mechanism-based or suicide inactivators.

Isocoumarins as a structure class are very effective mechanism-based inhibitors of serine proteases.[19,20] Changes in both the 3-alkoxy group and the substituent at the 7-position can profoundly influence the reactivity and binding mode of isocoumarin inhibitors. The binding mode of isocoumarin inhibitors to porcine pancreatic elastase (PPE) has been extensively investigated due to the relative ease of crystalizing PPE–inhibitor complexes in comparison with HLE–inhibitor complexes.[20–22] The crystal structures of individual isocoumarin inhibitors of PPE show dramatic differences in contrast to peptide derivatives, which have similar binding modes to PPE and HLE since both enzymes have similar primary substrate specificities.[23] However, the S′ subsites have enhanced hydrophobicity as a result of differences in the primary sequence between HLE and PPE,[23] and the increased reactivity of certain hydrophobic isocoumarins for HLE is probably a reflection on HLE's increased active-site hydrophobicity.[20] The crystal structure of 4-chloro-7-guanidino-3-methoxyisocoumarin complexed to PPE[21] clearly shows the 3-alkoxy group interacting with the S_1 pocket of the enzyme.[19] However, the crystal structure of 7-amino-3-(2-bromoethoxy)-4-chloroisocoumarin complexed with PPE indicates that the 3-bromoethoxy side chain is directed toward the S_3 site with the bromine atom in hydrogen bond distance to the Nε2 of Gln-192.[22] The bromoethoxy

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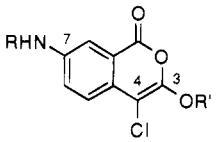
Table 1. Physical Properties of 7-(Substituted-amino)-4-chloro-3-alkoxyisocoumarins

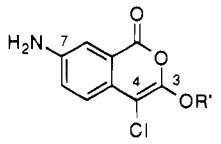


compd	R	R′	yield (%)	mp (°C)	method[a]	formula[b]
10	CH_3CO	$CH_2CH_2CH_3$	68	158–160	B	$C_{14}H_{14}NO_4Cl$[c]
11	$CH_3CH_2CH_2CO$	$CH_2CH_2CH_3$	68	183–184	B	$C_{16}H_{18}NO_4Cl$[d]
12	$(CH_3)_2CHCH_2CO$	$CH_2CH_2CH_3$	70	173–174	B	$C_{17}H_{20}NO_4Cl$[c]
13	$PhCH_2CH_2CO$	$CH_2CH_2CH_3$	81	182–184	A	$C_{21}H_{20}NO_4Cl \cdot {}^1/_2H_2O$[d]
14	CH_3SCH_2CO	$CH_2CH_2CH_3$	54	175–177	B	$C_{15}H_{16}NO_4Cl \cdot {}^1/_2THF$
15	Boc-L-Val	$CH_2CH_2CH_3$	48	171–173	B	$C_{22}H_{29}N_2O_6Cl$[c]
16	$3\text{-}O_2N\text{-}C_6H_4CO$	$CH_2CH_2CH_3$	65	222–224	A	$C_{19}H_{15}N_2O_6Cl \cdot {}^1/_4H_2O$[d]
17	CH_3CH_2OCO	$CH_2CH_2CH_3$	55	172–174	A	$C_{15}H_{16}NO_5Cl$[d]
18	CH_3CH_2HNCO	$CH_2CH_2CH_3$	45	189–191	C	$C_{15}H_{17}N_2O_4Cl$
19	PhHNCO	$CH_2CH_2CH_3$	56	235–236	C	$C_{19}H_{17}N_2O_4Cl$[c]
20	$PhCH_2HNCS$	$CH_2CH_2CH_3$	46	171–172	A	$C_{20}H_{19}N_2O_3ClS$[c]
21	Tos-L-Phe	$CH_2CH_2CH_3$	48	220–222	A	$C_{28}H_{27}N_2O_6ClS$[c]
22	PhHNCS	$CH_2CH_2CH_3$	52	167–168	C	$C_{19}H_{17}N_2O_3ClS$[c]
24	PhHNCO	CH_2CH_2Br	41	215–217	C	$C_{18}H_{14}N_2O_4BrCl$[c]
25	$PhCH_2HNCO$	CH_2CH_2Br	44	200–203	C	$C_{19}H_{16}N_2O_4BrCl$[d]
26	$PhCH_2CO$	CH_2CH_2Br	72	165–169	A	$C_{19}H_{15}NO_4BrCl$[d]
27	Boc-D-Phe	CH_2CH_2Br	81	180–182	B	$C_{25}H_{26}N_2O_6BrCl$[c]
28	Boc-L-Phe	CH_2CH_2Br	81	184–185	B	$C_{25}H_{26}N_2O_6BrCl$[d]
29	Boc-Ala-Ala	CH_2CH_2Br	65	147–151	B	$C_{22}H_{27}N_3O_7BrCl$[d]
30	(Np)HNCO	CH_2CH_2Br	75	234–235	C	$C_{22}H_{16}N_2O_4BrCl$[d]
31	$(CH_3)_3CNHCO$	CH_2CH_2Br	45	165–166	C	$C_{16}H_{18}N_2O_4BrCl$[d]
32	$(CH_3)_2CHNHCO$	CH_2CH_2Br	47	205–206	C	$C_{15}H_{16}N_2O_4BrCl$[d]
33	$(CH_3)_3CCH_2CO$	CH_2CH_2Br	49	150–152	A	$C_{17}H_{19}NO_4BrCl$[d]
34	$(R)\text{-}Ph(CH_3)CHNHCO$	CH_2CH_2Br	66	183–185	C	$C_{20}H_{18}N_2O_4BrCl$[d]
35	$(S)\text{-}Ph(CH_3)CHNHCO$	CH_2CH_2Br	59	186–188	C	$C_{20}H_{18}N_2O_4BrCl$[d]
36	PhNHCS	CH_2CH_2Br	53	157–158	C	$C_{18}H_{14}N_2O_3BrClS \cdot {}^1/_4THF$[d]
37	$m\text{-}(HOOC)PhNHCS$	CH_2CH_2Br	64	157–158	C	$C_{19}H_{14}N_2O_5BrClS$[d]
38	$p\text{-}(HOOC)PhNHCS$	CH_2CH_2Br	51	190–192	C	$C_{19}H_{14}N_2O_5BrClS$[d]
39	$Ph(CH_2)_2CO$	CH_2CH_2Br	36	195	A	$C_{20}H_{17}NO_4BrCl$
40	$PhCH_2OCO(CH_2)_2CO$	CH_2CH_2Br	30	216	B	$C_{22}H_{19}NO_6BrCl$
41	$CH_3OCO(CH_2)_3CO$	CH_2CH_2Br	24	174	B	$C_{17}H_{17}NO_6BrCl \cdot {}^1/_4THF$
42	$CH_3OCO(CH_2)_2CO$	CH_2CH_2Br	53	218	A	$C_{16}H_{15}NO_6BrCl$
43	Indo	CH_2CH_2Br	41	201	F	$C_{25}H_{24}N_2O_6BrCl$[e]
44	$Ph_2CHNHCO$	CH_2CH_2Br	48	183	D	$C_{25}H_{20}N_2O_4BrCl \cdot {}^3/_4H_2O$
45	succinyl-L-Phe	CH_2CH_2Br	77	191	H	$C_{24}H_{22}N_2O_7BrCl$
46	$\text{-}(OCCH_2CHPhCH_2CO)\text{-}$	CH_2CH_2Br	33	208	E	$C_{22}H_{17}NO_5BrCl$
47	Boc-L-Trp	CH_2CH_2Br	52	139	F	$C_{27}H_{27}N_3O_6BrCl \cdot {}^1/_2H_2O$
48	$(R)\text{-}(Np)(CH_3)CHNHCO$	CH_2CH_2Br	48	182	I	$C_{24}H_{20}N_2O_4BrCl \cdot {}^1/_2H_2O$
49	$(S)\text{-}(Np)(CH_3)CHNHCO$	CH_2CH_2Br	58	183	I	$C_{24}H_{20}N_2O_4BrCl$
50	$(S)\text{-}(PhNHCOO)(CH_3)CHCO$	CH_2CH_2Br	36	171	F	$C_{21}H_{18}N_2O_6BrCl$
51	$C_6H_5CO\text{-}L\text{-}Phe$	CH_2CH_2Br	86	213	G	$C_{27}H_{22}N_2O_5BrCl \cdot {}^1/_2H_2O$
52	$m\text{-}(CH_3)C_6H_4CO\text{-}L\text{-}Phe$	CH_2CH_2Br	77	196	G	$C_{28}H_{24}N_2O_5BrCl \cdot H_2O$
53	$p\text{-}(CH_3)C_6H_4CO\text{-}L\text{-}Phe$	CH_2CH_2Br	86	198	G	$C_{28}H_{24}N_2O_5BrCl \cdot H_2O$
54	Nic-L-Phe	CH_2CH_2Br	99	195	G	$C_{26}H_{21}N_3O_5BrCl \cdot {}^9/_{10}H_2O$
55	$m\text{-}(PhHNCONH)C_6H_4CO\text{-}Phe$	CH_2CH_2Br	15	159	G	$C_{34}H_{28}N_4O_6BrCl \cdot {}^1/_2H_2O$
56	$p\text{-}(PhHNCONH)C_6H_4CO\text{-}Phe$	CH_2CH_2Br	30	188	G	$C_{34}H_{28}N_4O_6BrCl$
57	PhNHCO	$CH_2(CH_2)_2Br$	38	192–193	C	$C_{19}H_{16}N_2O_4BrCl$[d]
58	$PhCH_2NHCO$	$CH_2(CH_2)_2Br$	38	188–189	C	$C_{20}H_{18}N_2O_4BrCl$[d]
59	CH_3CO	$CH_2(CH_2)_2Br$	90	170–172	A	$C_{14}H_{13}NO_4BrCl$[d]
60	$PhCH_2CO$	$CH_2(CH_2)_2Br$	74	151–153	A	$C_{20}H_{17}NO_4BrCl$[d]
61	$Ph(CH_2)_2CO$	$CH_2(CH_2)_2Br$	85	184–185	A	$C_{21}H_{19}NO_4BrCl$[d]
62	Boc-D-Phe	$CH_2(CH_2)_2Br$	74	162–164	B	$C_{26}H_{28}N_2O_6BrCl \cdot {}^1/_2THF$[d]
63	Boc-L-Phe	$CH_2(CH_2)_2Br$	68	162–163	B	$C_{26}H_{28}N_2O_6BrCl \cdot {}^1/_4THF$[d]
65	H	$CH_2CHBrCH_3$	22	87–88	J	$C_{12}H_{11}NO_3BrCl$[c]
66	$C_6H_5CO\text{-}L\text{-}Phe$	CH_3	37	203–206	G	$C_{26}H_{21}N_2O_5Cl$
67	$m\text{-}(CH_3)C_6H_4CO\text{-}L\text{-}Phe$	CH_3	55	202	G	$C_{27}H_{23}N_2O_5Cl$
68	$p\text{-}(CH_3)C_6H_4CO\text{-}L\text{-}Phe$	CH_3	35	186–188	G	$C_{27}H_{23}N_2O_5Cl$
69	Nic-L-Phe	CH_3	41	142–144	G	$C_{25}H_{20}N_3O_5Cl \cdot H_2O$
70	$m\text{-}(PhHNCONH)C_6H_4CO\text{-}Phe$	CH_3	23	188	G	$C_{33}H_{27}N_4O_6Cl \cdot {}^1/_2H_2O$
71	$p\text{-}(PhHNCONH)C_6H_4CO\text{-}Phe$	CH_3	29	210	G	$C_{33}H_{27}N_4O_6Cl \cdot {}^1/_2H_2O$
72	$p\text{-}(O_2N)C_6H_4CO\text{-}L\text{-}Phe$	CH_3	74	197	G	$C_{26}H_{20}N_3O_7Cl \cdot {}^1/_2H_2O$
73	Boc-L-Trp	CH_3	23	116	F	$C_{26}H_{26}N_3O_6Cl \cdot {}^3/_{10}H_2O$
74	$BrCH_2CO$	CH_3	31	204	A	$C_{12}H_9NO_4BrCl \cdot {}^3/_4H_2O$

[a] Synthetic method as described in the Experimental Section. [b] Except where indicated, all compounds were analyzed for C, H, and N and their results agreed to ±0.4% of the theoretical values. [c] Analyzed for C, H, N, and Cl. [d] Analyzed for C and H only. [e] HRMS: calcd for $C_{25}H_{24}N_2O_6BrCl$, 562.0506; found, 562.0092.

group is too big to fit into the S_1 site of the elastases. Hernandez and Powers have reported an X-ray crystal structure of the complex between PPE and 7-(Tos-L-Phe-amino)-4-chloro-3-methoxyisocoumarin, which clearly shows the 7-amino Tos-L-Phe group resting in the S′ site of PPE.[20]

Table 2. Inhibition of Human Leukocyte Elastase by 3-Alkoxy-7-amino-4-chloroisocoumarins[a]



compd	inactivator R′ (3-alkoxy group)	[I] (μM)	k_{obs}/[I][b] (M^{-1} s^{-1})
1	CH_3	6	10 000
2	CH_2CH_3	4	9400
3	$CH_2CH_2CH_3$	2.6	54 000
4	$CH(CH_3)_2$	26	2000
5	$CH_2CH(CH_3)_2$	50	2700
6	CH_2CH_2Br	0.07	200 000[c]
7	$CH_2CH_2CH_2Br$	3.4	4000
8	$CH_2CH_2OCH_3$	7.8	390
9	$CH_2CH_2OCH_2CH_2OCH_3$	710	33

[a] Conditions were as follows: 0.1 M HEPES, 0.5 M NaCl, 2.5% Me_2SO, pH 7.5, at 25 °C. Rate constants were obtained as described in Materials and Methods. [b] The first-order rate (k_{obs}) plots were linear for at least three to four half-lives. [c] Apparent rate constants were obtained by the progress curves method at substrate concentration [S] = 193 μM.

In this paper we report the design and synthesis of new HLE inactivators based on the modification of the 7-amino substituent and 3-alkoxy group of 3-alkoxy-7-amino-4-chloroisocoumarins. We sought to increase the potency and selectivity of isocoumarin elastase inhibitors by optimizing interactions between the 3-alkoxy group and the 7-substituent with the enzyme. A specific and potent inhibitor of HLE may be useful in the treatment of emphysema.[24]

Results and Discussion

Chemistry. In general, the appropriate 3-alkoxy-7-amino-4-chloroisocoumarins were prepared by previously reported methods.[19,21] Acylation, carbamylation, and thiocarbamylation of these isocoumarins produced the series of inhibitors **10**–**74**. Their structures and characterizations are listed in Table 1. The synthesis of compounds **1**-**9** has been previously reported.[21]

Inactivation of HLE by 3-Alkoxy-7-amino-4-chloroisocoumarins. We tested compounds **1**–**9**, which contain different 3-alkoxy groups, as inhibitors of HLE. The second-order inactivation rate constants, k_{obs}/[I], for isocoumarins **1**–**9** are reported in Table 2. The best inactivator in Table 1 is the 3-(2-bromoethoxy)-isocoumarin **6**, with k_{obs}/[I] = 200 000 M^{-1} s^{-1}. Compound **6** is 50-fold more potent than the corresponding 3-bromopropoxy isocoumarin **7**.

For the other 3-alkoxy derivatives, the inhibitory potency toward HLE is clearly related to the size of the 3-alkoxy substituent. The 3-propoxyisocoumarin **3** had a k_{obs}/[I] value of 54 000 M^{-1} s^{-1}, compared to 10 000 M^{-1} s^{-1} for the 3-methoxy derivative **1** and 9400 M^{-1} s^{-1} for the 3-ethoxy derivative **2**. Isocoumarins with longer 3-alkoxy substituents such as **7**–**9** had lower inactivation rate constants (e.g., compound **9** had a k_{obs}/[I] of only 33 M^{-1} s^{-1}). Isocoumarins **4** and **5** with branched 3-alkoxy substituents similar to the valine side chain, which is preferred in the S_1 pocket of HLE,[23] are not good inhibitors of HLE. The more potent inhibitors of HLE, **1**, **3**, and **6**, from Table 2 were selected for further investigation involving chemical modification at the 7-amino substituent.

Table 3. Inhibition of Human Leukocyte Elastase by 7-Substituted 7-Amino-4-chloro-3-propoxyisocoumarins[a]

compd	k_{obs}/[I] (M^{-1} s^{-1}) HNE[b]	PPE[c]	chymotrypsin[d]
10	>190 000		
11	>120 000		
12	>220 000		
13	>250 000		
14	>150 000		
15	64 000		
16	>210 000		
17	>180 000		
18	>280 000	220	12 000
19	140 000	1600	5200
20	>130 000	79	120
21	33 000	520	6100
22	>170 000	59	240

[a] Conditions were as follows: 0.1 M HEPES, 0.5 M NaCl, 2.5% Me_2SO, pH 7.5, at 25 °C. Rate constants were obtained as described in Materials and Methods. [b] Inhibitor concentrations were 0.7–1.7 μM. [c] Inhibitor concentrations were 34–56 μM. [d] Inhibitor concentrations were 3.4–70 μM.

Inactivation of HLE by Derivatives of 7-Substituted 7-Amino-4-chloro-3-propoxyisocoumarin. Several new acyl and ureido derivatives of **3** (structures in Table 1; R = acyl or ureido, R′ = propyl) were next synthesized and evaluated as HLE inactivators. The second-order inactivation rate constants, k_{obs}/[I], are reported in Table 3. In general, most of the compounds in Table 3 are potent inhibitors of human leukocyte elastase with inactivation rate constants (k_{obs}/[I]) >120 000 M^{-1} s^{-1}. The inhibition rates with most of the isocoumarin inactivators were so fast that they could not be measured accurately under the pseudo-first-order reaction condition of [I] ≫ [E]. The inactivation of HLE by **10**–**14**, **16**–**18**, **20**, and **22** was very rapid with less than 20% of the residual enzymatic activity being observed at the first time points (0.15–0.25 min). Therefore, the calculated inactivation rate constants should be considered to be lower limits. Alternate kinetics methods like the progress curve method could not be applied due to the instability of the acyl–enzyme complex.

Human leukocyte elastase has previously been shown to be effectively inhibited by derivatives of **1** (3-methoxy),[19] the best inhibitor being the derivative with a Tos-L-Phe substituent on the 7-amino group giving a k_{obs}/[I] of 200 000 M^{-1} s^{-1}.[19,20] However, the corresponding 3-propoxyisocoumarin derivatives, 7-Boc-Val-amido **15** and 7-Tos-Phe-amido **21**, are the slowest inhibitors in Table 3. Clearly the combination of the Tos-Phe substituent at the 7-amino position and the propyloxy group in the 3-position is not an optimum choice for interaction with the enzyme. A bulky hydrophobic group at the 7-position, responsible for hydrophobic interactions with the enzyme's S′ sites, may prevent the strong interaction of the propyloxy substituent with the S_1 pocket of elastase.

Compounds in the 3-propyloxy series with short aliphatic substituents at the 7-position such as **10**–**12**, **14**, **17**, and **18** are the best inhibitors of HLE. However, it seems that phenyl groups at the 7-position in compounds **13**, **16**, **20**, and **21** could be tolerated by the enzyme, and these derivatives are as active as those with short aliphatic chains in the 7-position. It is likely that the phenyl ring in the inhibitors is interacting with the enzyme through hydrophobic contacts. However, it is not clear what kind of interaction is responsible for

Table 4. Inhibition Rates of Serine Proteases by 7-Substituted-4-chloro-3-(2-bromoethoxy)isocoumarins[a]

compd	7-substituent	k_{obs}/[I] (M^{-1} s^{-1})			
		PPE[b]	HLE[c]	chymotrypsin[d]	cathepsin G[e]
6	NH_2	1000	200 000[f]	1160	410
23	NO_2	6330	65 600	98 000[g]	710
24	PhNHCONH	36	1 200 000[g]	12	NI[h]
25	$PhCH_2NHCONH$	3010	480 000[g]	890	23%[i]
26	$PhCH_2CONH$	4950	480 000[g]	82 000[g]	70
27	Boc-D-Phe-NH	30	160 000[g]	150	19%[i]
28	Boc-L-Phe-NH	50	146 000[g]	400	19%[i]
29	Boc-Ala-Ala-NH	1670	230 000[g]	2750[j] 810[j]	46
30	naphthyl-NHCONH	76	390 000[g]	80	22%[i]
31	*t*-BuNHCONH	6600		320	56
32	isopropyl-NHCONH	4470	646 000[g]	1340[j] 380[j]	77
33	$(CH_3)_3CCH_2CONH$	3650		1070	240
34	(*R*)-$(C_6H_5)(CH_3)CHNHCONH$	9900	>440 000[g]	180[j] 90[j]	77
35	(*S*)-$(C_6H_5)(CH_3)CHNHCONH$	2660	>570 000[g]	440	21%[j]
36	PhNHCSNH	1250	>480 000[g]	39 000[g]	200
37	*m*-COOH-PhNHCSNH		>240 000[g]	1960	320
38	*p*-COOH-PhNHCSNH		>390 000[g]	1720	450
39	$Ph(CH_2)_2CONH$	11		11	
40	$PhCH_2OCO(CH_2)_2CONH$	NI	3210	NI	
41	$CH_3OCO(CH_2)_3CONH$	2730	>170 000	1950	
42	$CH_3OCO(CH_2)_2CONH$	1500		4100	
43	Indo-NH	4		17	
44	$Ph_2CHNHCONH$	6	4410	24	
45	succinyl-L-Phe-NH	1900	40 000	690	
46	$-(OCCH_2CHPhCH_2CO)-N$	75	85 000	270	
47	Boc-L-Trp-NH	12	170 000	35	
48	(*R*)-$(C_{10}H_7)(CH_3)CHNHCONH$	40	170 000[k]	56	
49	(*S*)-$(C_{10}H_7)(CH_3)CHNHCONH$	20	310 000[k]	58	
50	(*S*)-$(PhNHCOO)(CH_3)CHCONH$	13	140 000[k]	69	
51	C_6H_5CO-L-Phe-NH	11	30 000	9	
52	*m*-$(CH_3)C_6H_4CO$-L-Phe-NH	3500[f]	31 000	2400	
53	*p*-$(CH_3)C_6H_4CO$-L-Phe-NH	32	49 000	16 000[f]	
54	Nic-L-Phe-NH	2700	36 000	36	
55	*m*-$(PhHNCONH)C_6H_4CO$-Phe-NH	300	21 000	610	
56	*p*-$(PhHNCONH)C_6H_4CO$-Phe-NH	130	6100	130	

[a] Inhibition rates were measured in 0.1 M HEPES, 0.5 M NaCl, pH 7.5, buffer and 8–9% Me_2SO at 25 °C. [b] Inhibitor concentrations were 0.04–2.0 mM. [c] Inhibitor concentrations were 0.07–710 μM. [d] Inhibitor concentrations were 1.7–58 μM. [e] Inhibitor concentrations were 35–710 μM. [f] The progress curve method was used according to ref 33. [g] Second-order rate constant was obtained using equimolar concentrations of inhibitor and enzyme. [h] NI = no inhibition. [i] Percentage of inhibition was obtained after 5 min of incubation of inhibitor with enzyme. [j] Biphasic plot was obtained, and two inhibition rates were observed. [k] Data were obtained from ref 29.

the very high inactivation rate constants observed in the case of those compounds with short aliphatic substituents at the 7-position.

The crystal structures of two enzyme–inhibitor complexes, trypsin inhibited by 7-guanidino-4-chloro-3-methoxyisocoumarin[25] and porcine pancreatic elastase with this same inhibitor,[21] clearly show that both the 7-amino substituent and the 3-alkoxy substituent can interact with the S_1 pocket. Thus two different modes of binding are possible. In the case of elastase, the 3-alkoxy group is usually directed into or toward the S_1 binding site. This binding mode is also supported by the crystal structure of HLE inhibited by a benzoxazinone,[26] where the methyl group attached to the phenyl ring is found in the S_1 pocket of the enzyme. However, it is possible that the derivatives of 7-amino-4-chloro-3-propoxyisocoumarin which have short aliphatic substituents at the 7-position could interact with the S_1 pocket of the enzyme either through the 3-propyloxy substituent or through the small 7-acylamino substituent. This second binding mode is clearly not likely when the 7-position has substitutents larger than short alkyl groups.

Inactivation of HLE by Derivatives of 7-Substituted 7-Amino-3-(bromoalkoxy)-4-chloroisocoumarins. Acyl and ureido derivatives of **6** and **7** (structures in Table 1) were also synthesized and evaluated as inhibitors of HLE, PPE, chymotrypsin (ChT), and cathepsin G. The inactivation rate constants, k_{obs}/[I], are shown in Tables 4 and 5. All of the derivatives of **6** were very potent inhibitors of HLE with k_{obs}/[I] values in the 10^4–10^6 M^{-1} s^{-1} range. These compounds are more potent inhibitors of HLE than derivatives of **7** by 1–2 orders of magnitude. These results clearly indicate that the the 3-bromoethoxy chain in derivatives of **6** makes a better fit into the primary specificity pocket of HLE than the 3-bromopropoxy chain in derivatives of **7**. Compound **65** with a branched 3-bromoisopropoxy chain is a better inhibitor of HLE than compound **7**.

Within the 3-(2-bromoethoxy) derivatives, HLE prefers aromatic hydrophobic chains in the 7-position (e.g., **24–26**, **30**, and **34–36**). However, it will also tolerate alkyl chains since compound **32** with an isopropylurea 7-substituent and compound **41** with a methylglutaryl 7-substituent are both very good inhibitors of HLE.

Among the aromatic substituents which contain chirality, HLE prefers the *S* stereocenter as is indicated by the comparison of compound **48** with **49**. Compound **49** with the *S* chirality is almost 2-fold better than compound **48** with the *R* chirality.

Inactivation of HLE by 7-Substituted-4-chloro-3-methoxyisocoumarins. Several new acyl and ureido derivatives of 1 (structures in Table 2) were synthesized and evaluated as inhibitors of HLE, PPE, and

Table 5. Inhibition Rates of Serine Proteases by 7-Substituted-4-chloro-3-(3-bromopropoxy)isocoumarins[a]

compd	7-substituent	R′ (3-alkoxy)	k_{obs}/[I] (M^{-1} s^{-1}) PPE[b]	HLE[c]	ChT[d]	Cat G[e]
7	NH_2	$CH_2(CH_2)_2Br$	10	4000	790	210
57	PhNHCONH	$CH_2(CH_2)_2Br$	4	13 750[f] 2890[f]	180	17%[g]
58	$PhCH_2NHCONH$	$CH_2(CH_2)_2Br$	13	15 650	440	21%[g]
59	CH_3CONH	$CH_2(CH_2)_2Br$	24	24 400	3980	170
60	$PhCH_2CONH$	$CH_2(CH_2)_2Br$	28	32 350	140 000[h]	28%[g]
61	$PhCH_2CH_2CONH$	$CH_2(CH_2)_2Br$		35 650[f] 9870[f]	600	NI[i]
62	Boc-D-Phe-NH	$CH_2(CH_2)_2Br$		1480	70	NI[i]
63	Boc-L-Phe-NH	$CH_2(CH_2)_2Br$		1320	490	NI[i]
64	NO_2	$CH_2CHBrCH_3$	1060		200 000[h]	1660
65	NH_2	$CH_2CHBrCH_3$	62	24 000	320	150

[a] Inhibition rates were measured in 0.1 M HEPES, 0.5 M NaCl, pH 7.5, buffer and 8–9% Me_2SO at 25 °C. [b] Inhibitor concentrations were 0.04–2.0 mM. [c] Inhibitor concentrations were 0.07–710 μM. [d] Inhibitor concentrations were 1.7–58 μM. [e] Inhibitor concentrations were 35–710 μM. [f] Biphasic plot was obtained, and two inhibition rates were observed. [g] Percentage of inhibition was obtained after 5 min of incubation of inhibitor with enzyme. [h] Second-order rate constant was obtained using equimolar concentrations of inhibitor and enzyme. [i] NI = no inhibition.

Table 6. Inhibition Rates of Serine Proteases by 7-Substituted-4-chloro-3-methoxyisocoumarins[a]

compd	7-substituent	k_{obs}/[I] (M^{-1} s^{-1}) PPE[b]	HLE[c,d]	chymotrypsin[e]
66	C_6H_5CO-L-Phe-NH	530	180 000	55
67	m-$(CH_3)C_6H_4CO$-L-Phe-NH	1600	130 000	64
68	p-$(CH_3)C_6H_4CO$-L-Phe-NH	83	170 000	130
69	Nic-L-Phe-NH	5600	62 000	49
70	m-$(PhHNCONH)C_6H_4CO$-Phe-NH	54	28 000	110
71	p-$(PhHNCONH)C_6H_4CO$-Phe-NH	68	15 000	10
72	p-$(O_2N)C_6H_4CO$-L-Phe-NH	64	130 000	30
73	Boc-L-Trp-NH	5800[d]	79 000	33
74	$BrCH_2CONH$	3500	39 000	350

[a] Inhibition rates were measured in 0.1 M HEPES, 0.5 M NaCl, pH 7.5, buffer and 8–9% Me_2SO at 25 °C. [b] Inhibitor concentrations were 2–600 μM. [c] Inhibitor concentrations were 0.4–4 μM. [d] Second-order rate constant(s). [e] Inhibitor concentrations were 360–570 μM.

ChT (see Table 6). In the 3-methoxy series, the Bz-L-Phe-NH derivative **66** was the best of the nine new inhibitors. It is almost as good as the previously reported tosyl-L-phenylalanyl derivative.[19,20] The presence of a *m*-toluoyl group (**67**) gave good inhibition of HLE; however, the *p*-toluoyl compound **68** was slightly better. As with the bromoethoxy series, the *m*-*N*-(phenylureido)-*N*-benzoyl compound **70** is a 2-fold better inhibitor of HLE than is the *p*-*N*-(phenylureido)-*N*-benzoyl compound **71**.

Curiously, all of the bromoethoxy derivatives with extended L-Phe 7-substituents (**51**–**56**) listed in Table 4 were poorer inhibitors of HLE than their methoxy counterparts (**66**–**71**). The longer 3-(2-bromoethoxy) group may cause crowding of the extended 7-substituent into the S_n' subsites. This would make binding of the inhibitor to the enzyme more difficult.

Inhibition of PPE, Chymotrypsin, and Cathepsin G. Several derivatives of **6** such as **25**, **26**, and **31**–**34** are good inhibitors of PPE; however, compounds **24**, **27**, **28**, and **30** and derivatives of **7** are poor PPE inhibitors. The X-ray structure of compound **6** complexed to PPE showed that the bromoethoxy chain is directed toward the S_3 site of the enzyme and that the bromine atom is in hydrogen bond distance to Nε of Gln-192.[22] Therefore, the low inactivation rates of derivatives of **7** indicate that the 3-bromopropoxy chain is most likely too long to achieve a proper fit in the active site of PPE.

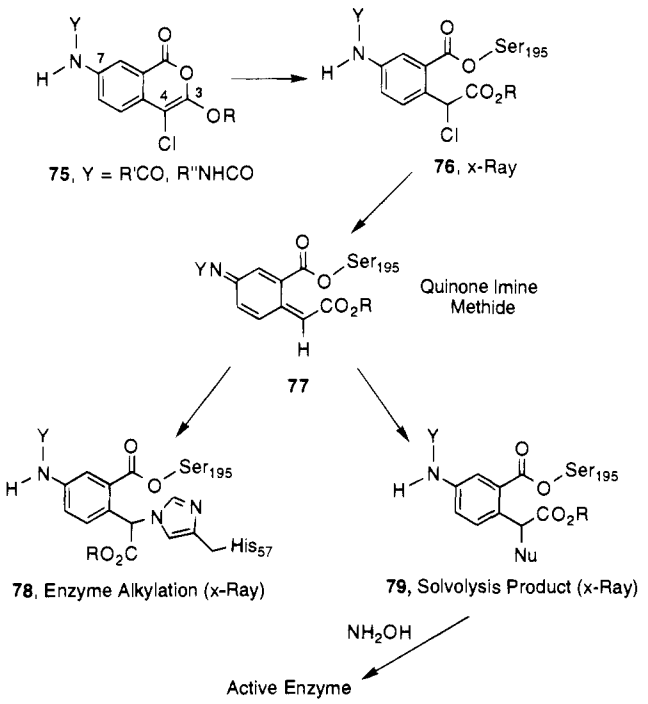
Compounds **6** and **7** and most of their derivatives inhibited chymotrypsin moderately; however, compounds **26** and **60** with a 7-phenylacetamino substituent inhibited chymotrypsin with k_{obs}/[I] values of 82 000 and 140 000 M^{-1} s^{-1}, respectively. This may suggest that there is a strong hydrophobic interaction of the 7-amino substituent of compounds **26** and **60** with the S_1 site of chymotrypsin. The *m*-toluoyl-L-Phe compound **52** is a good inhibitor of chymotrypsin, and the *p*-toluoyl-L-Phe compound **53** is an excellent inhibitor of ChT. All of the compounds in Tables 4 and 5 are poor to moderate inhibitors of cathepsin G.

Specificity of the Inhibitors for HLE. Six compounds, **24**, **30**, and **47**–**50**, are very specific for HLE. They are at least 10 000 times better inhibitors of HLE than of PPE, chymotrypsin, and cathepsin G. Another example is compound **19**, which has a k_{obs}/[I] for HLE of 143 000 M^{-1} s^{-1} and a k_{obs}/[I] of only 79 and 120 M^{-1} s^{-1} for PPE and chymotrypsin, respectively. Therefore, **19** is a better inhibitor of HLE as compared to PPE by 1800-fold. Compound **19** is almost 1200-fold better for HLE than for chymotrypsin. Clearly, a very high specificity is observed for these compounds.

The 7-[(substituted-L-phenylalanyl)amino]-3-(bromoethoxy)-4-chloroisocoumarins (**51**–**56**) inactivate HLE better than they do PPE (Table 4). This was also observed with the tosyl amino acid derivatives described by Hernandez and Powers.[20] With the exception of compounds **52**–**54**, the new isocoumarins are very selective for HLE. The *p*-toluoyl compound **53** is the best HLE inhibitor of this series.

In the methoxy series, the nicotinoyl-L-Phe compound **69** (Table 6) gave good inhibition of both HLE and PPE and was not very selective. After examining the X-ray structure of 4-chloro-3-methoxy-7-[(tosyl-L-phenylalanyl)amino]isocoumarin complexed to PPE,[20] we hypothesized that the pyridine nitrogen on the nicotinoyl ring of compound **69** may be in proximity to hydrogen bond with the hydroxyl hydrogen of the Tyr-35 of PPE in the

Scheme 1



S_3' pocket. The added hydrogen bond along with hydrophobic contacts may explain the observed enhanced inhibition of PPE by compound **69**. Compound **73** with a Boc-L-tryptophanyl 7-substituent (see Table 6) also gave good inhibition of PPE probably due to hydrophobic contacts combined with a hydrogen bond to the indole NH of the tryptophan chain.

Inhibition Mechanism. The mechanism of inactivation of elastase by 7-(substituted-amino)-4-chloro-3-alkoxyisocoumarins **75** is shown in Scheme 1.[19] The acyl enzyme intermediate **76** is formed by attack of the Ser-195 hydroxyl on the ring carbonyl group of the isocoumarin. The latent quinone imine methide functional group (**77**) is unmasked with elimination of HCl from the acyl–enzyme. The quinone imine methide can react either with a solvent nucleophile to give the stable acyl–enzyme **79** or with an enzyme nucleophile (His-57) to give the alkylated enzyme **78**. The acyl–enzymes **76** and **79** and the alkylated acyl–enzyme **78** have been detected in X-ray crystal structures.[21,22,27] Partial reactivation of the enzyme has been observed by addition of hydroxylamine. Hydroxylamine reacts with any one of the acyl–enzyme forms (**79** or **76**) to give the active enzyme. However, hydroxylamine cannot reactivate the alkylated enzyme **78**.

Deacylation Kinetics. The deacylation of the HLE– and PPE–inhibitor complexes was determined for the most potent and specific compounds from Tables 2–6. The half-lives for deacylation of the enzyme after removal of excess of inhibitor by centrifugation using a centricon-10 microconcentrator are summarized in Table 7. The finding that in most cases less than about 5% of the specific activity was regained during the centrifugation period is consistent with the formation of a covalent acyl–enzyme adduct. Further standing of the acyl–enzyme adduct at 25 °C in buffer resulted in slow deacylation of enzyme in some cases.

With the exception of **12** and **15**, all of the inhibitors tested with PPE gave stable complexes. For example, compound **18** slowly recovered 30% enzyme activity after a 24 h incubation period and did not show further reactivation. Compounds **49** and **50** appear to form very stable acyl–enzymes with PPE which required long incubation times for reactivation with hydroxylamine.

However, the complexes of the 3-methoxyisocoumarins (**66**, **68**, and **72**) with HLE were not nearly as stable. We were unable to test the stability of the 1:1 complexes of the 3-(2-bromoethoxy) compounds with HLE due to difficulties in isolating these complexes due to their rapid deacylation. The succinyl-L-Phe compound **45** complexed to HLE turned out to be the most stable to hydroxylamine reactivation and had a half-life of 1 h.

The experiments provide support for the formation of a quinone imine methide which can alkylate His-57 in the case of the parent compound **6**. The new compounds have the amine of the isocoumarin acylated which effectively ties up the lone pair of nitrogen into an amide bond. As a result, these acylated compounds may be very slow "enzyme alkylators".

A modified version of the hydroxylamine experiment was performed to see if enzyme alkylation occurs with several of the new inhibitors upon longer incubation (Table 8). Knowing that the 1:1 PPE–inhibitor complexes of compounds **66**, **68**, and **72** are very stable to buffer hydrolysis (Table 7), we decided to preincubate the complexes for 20 h at room temperature in buffer followed by addition of excess hydroxylamine. Only 6–31% of enzyme activity was restored after 20 h which indicates formation of the alkylated enzyme **78**. No additional enzyme activity was regained upon further incubation with hydroxylamine for 1–17 h. In contrast, addition of hydroxylamine restored 91% of enzyme activity if the inhibitor was not preincubated with enzyme. Thus, with compound **66**, at least 69% of the PPE–inhibitor complex isolated consists of alkylated enzyme after a 20 h preincubation period.

Stability of Inhibitors in Buffer. The half-life for the hydrolysis of various 7-amino-4-chloro-3-alkoxyisocoumarins in HEPES buffer is shown in Table 9. The 3-methoxy compounds (**66–68**) all exhibit excellent stability in HEPES buffer; they are far more stable than the parent compound 7-amino-4-chloro-3-methoxyisocoumarin (**1**). The stabilities of the 3-bromoethoxy derivatives in HEPES buffer were more variable. Compounds **46** and **49** are very stable in HEPES buffer, while compounds **45** and **50** are unstable. It is not altogether obvious as to why there is a such difference in stability between compounds **49** and **50**. In the case of the succinyl-L-Phe compound **45**, the carboxylate may be attacking the isocoumarin ring carbonyl leading to its own decomposition at pH 7.5.

Conclusion

The introduction of different substituents at the 3-position of 3-alkoxy-7-amino-4-chloroisocoumarins had a significant effect on the inhibitory potency of isocoumarin inactivators of HLE. The three best compounds in this series, 7-amino-4-chloro-3-methoxyisocoumarin (**1**) ($k_{obs}/[I]$ = 10 000 M^{-1} s^{-1}), 7-amino-4-chloro-3-propoxyisocoumarin (**3**) ($k_{obs}/[I]$ = 54 000 M^{-1} s^{-1}), and 7-amino-4-chloro-3-(2-bromoethoxy)isocoumarin (**6**) ($k_{obs}/[I]$ = 200 000 M^{-1} s^{-1}), were further modified on the 7-amino group, and a new series of acyl, urea, and carbonate derivatives of **1**, **3**, and **6** were synthesized and evaluated as inhibitors of HLE. Most of these compounds are very potent irreversible inhibitors of

Table 7. Rates of Deacylation and Hydroxylamine Reactivation of 7-Substituted-4-chloro-3-propoxyisocoumarins, 7-Substituted-4-chloro-3-(2-bromoethoxy)isocoumarins, and 7-Substituted-4-chloro-3-methoxyisocoumarins

			deacylation[a] $t_{1/2}$ (h)		NH_2OH reactivation[b] $t_{1/2}$ (h)	
compd	R	R′ (3-alkoxy)	PPE	HLE	PPE	HLE
12	$(CH_3)_2CHCH_2CO$	$CH_2CH_2CH_3$	16			
15	Boc-L-Val	$CH_2CH_2CH_3$	17			
18	CH_3CH_2HNCO	$CH_2CH_2CH_3$	>48[c]			
19	PhNHCO	$CH_2CH_2CH_3$	>48			
22	PhHNCS	$CH_2CH_2CH_3$	>48			
45	succinyl-L-Phe	CH_2CH_2Br	>48		0.2	1
46	$-(OCCH_2CHPhCH_2CO)-$	CH_2CH_2Br			1.3	seconds
49	(*S*)-(Np)$(CH_3)CHNHCO$	CH_2CH_2Br	>48		20	0.8
66	C_6H_5CO-L-Phe	CH_3	>48	3	0.8	0.2
68	*p*-$(CH_3)C_6H_4CO$-L-Phe	CH_3	>48	4	2.7	1
72	*p*-$(O_2N)C_6H_4CO$-L-Phe	CH_3	>48	4	0.8	0.5

[a] Conditions: [I]/[E], 1:1, 0.1 M HEPES, 0.5 M NaCl, pH 7.5, buffer and 8% DMSO at 25 °C. [b] Conditions: 0.5 M NH_2OH, 0.1 M HEPES, 0.5 M NaCl, pH 7.5, buffer at 25 °C. [c] 30% of reactivation after 24 h, and no further progress was observed.

Table 8. PPE Preincubation Study of 7-[(Substituted-L-phenylalanyl)amino]-4-chloro-3-methoxyisocoumarins[a]

	percent activity regained	
compd	non-preincubated PPE/[I][b]	preincubated PPE/[I][c]
66, C_6H_5CO	91	31
68, *p*-$(CH_3)C_6H_4CO$	91	14
72, *p*-$(O_2N)C_6H_4CO$	91	6

[a] Conditions: 0.5 M NH_2OH, 0.1 M HEPES, 0.5 M NaCl, pH 7.5, buffer at 25 °C. [b] Activity regained after 17 h of incubation with hydroxylamine. The hydroxylamine was added immediately after inhibition of the enzyme, [I]/PPE = 200:1. [c] Preincubated 20 h after inhibition using a 1:1 [I]/PPE ratio before addition of hydroxylamine. The percent activity regained remained constant after 1 h through 17 h.

Table 9. Half-Life for Hydrolysis of 3-Alkoxy-7-(substituted-amino)-4-chloroisocoumarins[a]

compd	7-substituent	R′ (3-alkoxy)	$t_{1/2}$ (h)
1	H	CH_3	3.3
2	H	CH_2CH_3	3.6
3	H	$CH_2CH_2CH_3$	4.6
6	H	CH_2CH_2Br	2.4
7	H	$CH_2(CH_2)_2Br$	3.2
45	succinyl-L-Phe-NH	CH_2CH_2Br	2.5
46	$-(OCCH_2CHPhCH_2CO)-N$	CH_2CH_2Br	>48
47	Boc-L-Trp-NH	CH_2CH_2Br	26
49	(*S*)-$(C_{10}H_7)(CH_3)CHNHCONH$	CH_2CH_2Br	>48
50	(*S*)-$PhNHCOOCH(CH_3)CONH$	CH_2CH_2Br	6.4
66	C_6H_5CO-L-Phe-NH	CH_3	>48
67	*m*-$(CH_3)C_6H_4CO$-L-Phe	CH_3	>48
68	*p*-$(CH_3)C_6H_4CO$-L-Phe	CH_3	>48

[a] Conditions: 0.1 M HEPES, 0.5 M NaCl, pH 7.5, buffer with 10% DMSO at 25 °C.

HLE with second-order rate constants between 10^4 and 10^6 M^{-1} s^{-1}. This demonstrates that elaboration on the 7-substituent combined with the better fitting 3-alkoxy substituent in compound **6** can lead to highly potent and selective inhibitors of HLE. However, extended and rigid side chains on the 7-substituent in the case of the 3-(2-bromoethoxy) compounds lead to reduced inhibitor potency and selectivity. Additional inhibition kinetic studies with chymotrypsin, PPE, and cathepsin G show that some of the derivatives of **1**, **3**, and **6** are very specific HLE inhibitors. Thus, a high degree of HLE potency and selectivity can be introduced into isocoumarin inhibitors.

Experimental Section

Synthesis. Reactions that required anhydrous conditions were performed in flame or oven-dried glassware which was cooled under argon. Solvents which were distilled from CaH_2 before use include benzene, methylene chloride (CH_2Cl_2), and triethylamine (Et_3N). Tetrahydrofuran (THF) was distilled from sodium benzophenone ketyl. Reaction mixtures were concentrated by rotary evaporation with a Büchi Rotavapor-R at water aspirator pressure followed by evacuation of the flask at 2.5 mmHg for 30 min. Thin layer chromatography (TLC) was performed on glass plates coated with 254 nm fluorescent indicator with a layer thickness of 250 μm. Melting points were determined on a Thomas-Hoover apparatus using open capillary tubes and are uncorrected.

Proton nuclear magnetic resonance (^1H NMR) spectra were done on a Varian Gemini 300 FT-NMR spectrometer (300 MHz). Mass spectra (MS) and FABMS were performed on a VG analytical 70-SE mass spectrometer. Microanalyses were performed by Atlantic Microlab, Inc., Norcross, GA.

Compounds **1**–**9** were synthesized using known methods.[21] Compounds **10**–**74** were synthesized via methods A–J below, except where noted.

Acylation of 3-Alkoxy-7-amino-4-chloroisocoumarins (Method A). An equimolar mixture of 7-amino-4-chloro-3-methoxyisocoumarin (**1**), 7-amino-4-chloro-3-propoxyisocoumarin (**3**), or 7-amino-4-chloro-3-(2-bromoethoxy)isocoumarin (**6**), the appropriate acyl chloride, and triethylamine in dry THF or CH_2Cl_2 was stirred overnight at room temperature. The solution was washed with water and 4% $NaHCO_3$, dried ($MgSO_4$), and concentrated. The yellow solid obtained was crystallized from THF–pentane.

Acylation of 3-Alkoxy-7-amino-4-chloroisocoumarins (Method B). An equimolar amount of **3** or **6** and the corresponding anhydride in THF was stirred overnight at room temperature. The workup as described above gave a yellow solid which was triturated from THF–pentane.

Carbamylation and Thiocarbamylation of 3-Alkoxy-7-amino-4-chloroisocoumarins (Method C). An equimolar amount of **3** or **6** and the corresponding isocyanate or isothiocyanate in a small amount of dry THF was allowed to stir for 2 days at room temperature. During this time, the product slowly precipitated out of solution. After filtration, the compounds were triturated from THF–pentane.

3-(2-Bromoethoxy)-4-chloro-7-[[*N*-(diphenylmethyl)carbamoyl]amino]isocoumarin (44) (Method D). To a THF solution (3 mL) of 7-amino-3-(2-bromoethoxy)-4-chloroisocoumarin (200 mg, 0.63 mmol) at room temperature was added a THF solution (3 mL) of triphosgene (124 mg, 0.46 mmol). The mixture was stirred for 2 h followed by evaporation of solvent using a KOH trap. The yellow solid obtained was taken up in THF (5 mL) followed by addition of aminodiphenylmethane (0.24 mL, 1.4 mmol). The mixture was stirred at room temperature overnight. The suspension obtained was diluted in $CHCl_3$ (50 mL), washed with 1 N HCl (20 mL) and H_2O (2 × 20 mL), dried ($MgSO_4$), and concentrated. The yellow solid was triturated from THF with hexane to give the urea as a yellow powder, mp 183 °C dec (159 mg, 48%): FABMS ($M^+ + H$) 527. Anal. ($C_{25}H_{20}N_2O_4BrCl \cdot {}^3/_4H_2O$) C, H, N.

3-(2-Bromoethoxy)-4-chloro-7-(4-phenyl-2,6-diketopiperidinyl)isocoumarin (46) (Method E). To thionyl chloride (2.5 mL, 28.9 mmol) neat was added 3-phenylglutaric acid

(500 mg, 2.4 mmol). The mixture was refluxed for 1 h followed by concentration *in vacuo* to give a dark colored solid. The solid was diluted with THF (4.0 mL) followed by addition of **6** (191 mg, 0.6 mmol) at room temperature. The mixture was stirred at room temperature for 5 h. A solid precipitated out upon addition of water to the reaction mixture. The crude solid obtained was triturated from acetone/H_2O to give the product as a yellow powder, mp 208 °C dec (100 mg, 33%): FABMS ($M^+ + 1$) *m/e* 490. Anal. ($C_{22}H_{17}NO_5BrCl$) C, H, N.

Acylation of 3-Alkoxy-7-amino-4-chloroisocoumarins via the Mixed-Anhydride Method[28] (Method F). To a THF solution (5 mL) of the appropriate organic acid (0.9 mmol) at −20 °C was added *N*-methylmorpholine (0.1 mL, 0.9 mmol) and isobutyl chloroformate (0.1 mL, 0.9 mmol). The mixture was stirred for 10 min followed by addition of **1** or **6** (0.9 mmol) at −20 °C as a THF solution (5 mL). The mixture was warmed to room temperature over a period of 15 min. The mixture was diluted in EtOAc (100 mL), washed with 1 M HCL (2 × 33 mL), 5% $NaHCO_3$ (33 mL), and saturated NaCl (33 mL), dried ($MgSO_4$), and concentrated. The crude solid obtained was recrystallized from MeOH/H_2O.

General Procedure for Synthesis of Extended Chain Derivatives of 3-Alkoxy-4-chloro-7-(L-phenylalanylamino)isocoumarin Hydrochloride (Method G). The appropriate acid chloride was added to a CH_2Cl_2 solution of 4-chloro-3-methoxy-7-(L-phenylalanylamino)isocoumarin hydrochloride or 3-(2-bromoethoxy)-4-chloro-7-(L-phenylalanylamino)isocoumarin hydrochloride and triethylamine (TEA) at 0 °C. The mixtures were warmed to room temperature and stirred for 1 h. The products were purified by trituration from MeOH with H_2O, except where noted.

3-(2-Bromoethoxy)-4-chloro-7-[(succinyl-L-phenylalanyl)amino]isocoumarin (45) (Method H). To a pyridine solution (4.0 mL) of 7-(L-phenylalanylamino)-3-(2-bromoethoxy)-4-chloroisocoumarin hydrochloride (100 mg, 0.2 mmol) at room temperature was added succinic anhydride (80 mg, 0.8 mmol). The mixture was stirred at room temperature for 2 h. Trituration with acetone/H_2O/MeOH (2:2:1) gave the desired product as a colorless powder, mp 191 °C (87 mg, 77%): FABMS ($M^+ + 1$) *m/e* 565. Anal. ($C_{24}H_{22}N_2O_7BrCl$) C, H, N.

3-(2-Bromoethoxy)-4-chloro-7-[(*R*)-[[1-(1-naphthyl)ethyl]carbamoyl]amino]isocoumarin[29] (48) (Method I). To a THF solution (5 mL) of **6** (200 mg, 0.6 mmol) at room temperature was added (*R*)-(−)-1-(1-naphthyl)ethyl isocyanate. The mixture was stirred at room temperature for 2 h. The mixture was concentrated *in vacuo* to give a crude yellow solid. The solid was washed with benzene and ethyl ether to give the pure urea, mp 182 °C dec (154 mg, 48%): FABMS ($M^+ + 1$) *m/e* 515. Anal. ($C_{24}H_{20}N_2O_4BrCl \cdot {}^1/_2H_2O$) C, H, N.

3-(2-Bromopropoxy)-4-chloro-7-nitroisocoumarin (64). This compound was prepared from 1-methyl-2-(bromoethyl)-2-carboxyphenylacetate, previously reported by Harper et al.[19] The product was obtained by cyclization of 1-methyl-2-(bromoethyl)-2-carboxyphenylacetate with PCl_5, mp 103−105 °C (47): MS *m/e* 362 ($M^+ + 1$).

7-Amino-3-(2-bromopropoxy)-4-chloroisocoumarin (65) (Method J). This compound was prepared from **64** via hydrogenolysis and purified by column chromatography (CH_2Cl_2), mp 87−88 °C (22%): MS *m/e* 332 ($M^+ + 1$). Anal. ($C_{12}H_{11}NO_3ClBr$) C, H, N, Cl.

Materials and Methods. HLE was a generous gift from Dr. James Travis and his research group at the University of Georgia. Chymotrypsin and PPE were purchased from Sigma Chemical Co., St. Louis, MO. HEPES was purchased from Research Organics, Inc., Cleveland, OH.

Enzyme Inhibition Kinetics. The method of Kitz and Wilson was used for most of the enzyme inhibition assays.[30] The enzyme was preincubated with inhibitor by adding an aliquot (50 μL) of the inhibitor in DMSO to the enzyme (50 μL of a 4.0 or 20 μM solution) in HEPES (0.5 mL) buffer. The inhibition rates were measured spectrophotometrically on a Beckman 35 spectrophotometer by removing aliquots (50 μL) of the enzyme−inhibitor solution with time and diluting into the substrate solution (50 μL of substrate into a cuvette with 2 mL of HEPES buffer). The chymotrypsin− and PPE−enzyme solutions were prepared in 1 mM HCl at a concentration of 20 μM. The HLE−enzyme solution was prepared in 1 M NaCl, 0.4 M NaOAc, pH 5.5, buffer at a concentration of 4 μM.

The inhibition rates were monitored by following the increase in absorbance at 410 nm of the *p*-nitroaniline group (ϵ_{410} = 8800 M^{-1} cm^{-1}) released from the substrate upon hydrolysis catalyzed by the enzyme.[31] The buffer solution used for these experiments was 0.1 M HEPES, 0.5 M NaCl, pH 7.5. Inhibitor concentrations are listed in the appropriate tables. The peptide-4-nitroanilide (peptide-*p*NA) substrates used for the assays were chymotrypsin and cathepsin G, Suc-Val-Pro-Phe-*p*NA (0.4−0.5 mM); PPE, Suc-Ala-Ala-Ala-*p*NA (0.4−0.5 mM); and HLE, MeO-Suc-Ala-Ala-Pro-Val-*p*NA (0.2−0.5 mM).[32] First-order inactivation constants (k_{obs}/[I]) were obtained from plots of $\ln(v_t/v_o)$ vs time. All inhibition rates are the average of two determinations. In general the standard errors for the inhibition rates are ≤15%, and if rate differences were >15%, additional rate determinations were performed. In cases where the inhibition rates were too fast to measure using pseudo-first-order conditions ([I] ≫ [E]), second-order constants were measured at equal concentrations of inhibitor and enzyme. With very fast inhibitors, we were only able to put lower limits on the rates. Second-order inactivation plots were linear for 1.9−2.7 half-lives, while the first-order inactivation plots were linear for 3.5−5.0 half-lives. For the HLE data, all correlation factors ranged from 0.93 to 1.00.

Hydrolysis Rates of Inhibitors in HEPES Buffer. An aliquot (20 μL of a 2.9−5.0 mM solution in DMSO) of the inhibitor solution was added to a mixture of HEPES buffer (2 mL) and DMSO (200 μL) such that the final concentration of DMSO was 10% v/v. The spontaneous hydrolysis rates were monitored by following the decrease in absorbance at 385 nm of the isocoumarin ring (ϵ_{385} = 3300 M^{-1} cm^{-1}) due to ring opening. First-order hydrolysis rates were obtained from plots of $\ln[(A_t - A_f)/(A_o - A_f)]$ vs time, where A_t and A_f are absorbance after time *t* and final, respectively. The kinetic constants were converted to half-lives. The hydrolysis rates are based on two determinations, and the standard errors are ±10% or less.

Deacylation Kinetics. To HEPES buffer (1 mL) in a centricon-10 tube was added the enzyme (100 μL of a 4.0−20.0 μM solution) followed by addition of the inhibitor (100 μL) in DMSO. The enzyme−inhibitor solutions were assayed spectrophotometrically for complete loss of enzyme activity. The solutions were diluted to 2 mL with HEPES buffer. The excess inhibitor was removed by centrifugation with the centricon-10 microconcentrators two to three times at 0 °C for 1 h. Enzymatic activity of the solution upon standing at 25 °C was assayed as described previously. The first-order deacylation rate (k_{deacyl}) was obtained from plots of $\ln(v_o - v_t)$ vs time, where v_o is the enzymatic rate under the same conditions without inhibitor present. The hydrolysis rates are based on two determinations, and the standard errors are ±15% or less.

Determination of Inactivation Rates in the Presence of Substrate. Progress Curve Method. In some cases, k_{obs}/[I] values were determinated in the presence of substrate as described by Tian and Tsou.[33] For example, inactivation of human leukocyte elastase (0.02 μM) by **6** (0.07 μM) in the presence of 0.193 mM MeO-Suc-Ala-Ala-Pro-Val-*p*NA was measured by addition of a 0.01 mL aliquot of the enzyme solution to a mixture of substrate and inhibitor containing 5% Me_2SO. The increase in absorbance was monitored (410 nm) with time until no further release of *p*-nitroaniline was observed. The k_{obs}/[I] values were calculated from plots of $\log([P] - [P]_t)$ vs time, where [P] and $[P]_t$ are the concentrations of *p*-nitroaniline after total inactivation and at time *t*, respectively. The reaction rates are based on two determinations, and the standard errors are ±10% or less.

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(1) Abbreviations: α1-PI, α-1-protease inhibitor; Boc, *tert*-butyloxycarbonyl; Bz, benzoyl; Cat G, cathepsin G; ChT, chymotrypsin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; HLE, human leukocyte elastase; Indo, (*S*)-*N*-(isobutoxycarbonyl)-indolinyl; Nic, nicotinoyl; Np, 1-naphthyl; *p*NA, *p*-nitroanilide; PPE, porcine pancreatic elastase; Tos, tosyl.

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