Mechanism-Based Isocoumarin Inhibitors for Blood Coagulation Serine Proteases. Effect of the 7-Substituent in 7-Amino-4-chloro-3-(isothioureidoalkoxy)isocoumarins on Inhibitory and Anticoagulant Potency

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A series of 7-amino-4-chloro-3-(3-isothioureidopropoxy)isocoumarin (NH_2-CiTPrOIC) derivatives with various substituents at the 7- and 3-positions have been synthesized as inhibitors of several blood coagulation enzymes. Isocoumarins substituted with basic groups such as guanidino or isothioureidoalkoxy groups were previously shown to be potent irreversible inhibitors of blood coagulation enzymes [Kam et al. *Biochemistry* **1988**, *27*, 2547–2557]. Substituted isocoumarins with an isothioureidoethoxy group at the 3-position and a large hydrophobic group at the 7-position are better inhibitors for thrombin, factor VIIa, factor Xa, factor XIa, factor XIIa, and factor IXa than NH_2-CiTPrOIC (**4**). PhNHCONH-CiTEtOIC (**14**), (*S*)-Ph(CH_3)CHNHCONH-CiTEtOIC (**25**), and (*R*)-Ph(CH_3)CHNHCONH-CiTEtOIC (**26**) inhibit thrombin quite potently and have k_{obs}/[I] values of (1–4) × 10^4 M^{-1} s^{-1}. Modeled structures of several isocoumarins noncovalently complexed with human α-thrombin suggest that H-bonding between the 7-substituent and the Lys-60F NH_3^+ relates to the inhibitory potency. Thrombin inhibited by **14**, **25**, or **26** is quite stable, and only 4–16% of enzymatic activity is regained after incubation for 20 days in 0.1 M Hepes, pH 7.5 buffer. However, 100, 67, and 65% of enzyme activity, respectively, is regained with the addition of 0.38 M hydroxylamine. With normal citrated pig or human plasma, these isocoumarin derivatives prolong the prothrombin time ca. 1.3–3.1-fold and also prolong the activated partial thromboplastin time more than 3–7-fold at 32 μM. Thus, these compounds are effective anticoagulants *in vitro* and may be useful *in vivo*.

Introduction

Blood coagulation is involved in thrombotic disorders such as vascular clotting and myocardial infarction and thrombin acts as a trigger in thrombus formation. Current treatment of thrombosis has concentrated on platelet suppression, anticoagulant therapy, and fibrinolytic therapy. Considerable recent effort has been made in the development of new thrombin inhibitors,[1] which include the arginine derivative (2*R*,4*R*)-MQPA,[2,3] the benzamidine derivative NAPAP,[4] the peptide aldehyde D-Phe-Pro-Arg-H,[5] the peptide boronic acid D-Phe-Pro-boroArg-OH,[6] the peptide trifluoromethyl ketone D-Phe-Pro-Arg-CF_3,[7] the peptide α-keto ester D-Phe-Pro-Lys-COOMe,[8] the peptide chloromethyl ketone D-Phe-Pro-Arg-CH_2Cl,[9] peptide phosphonates,[10] several active ester acylating agents which contain guanidine or amidine functional groups,[11] and mechanism-based inhibitors.[12–17] A few inhibitors have also been reported for other coagulation enzymes. Several benzamidine derivatives such as DABE, TAPAM, and BABCH are potent inhibitors of factor Xa.[18] Peptide chloromethyl ketones such as Dns-Glu-Gly-Arg-CH_2Cl and Ile-Glu-Gly-Arg-CH_2Cl inhibit factor Xa and factor IXa,[19,20] and Ile-Glu-Gly-Arg-CH_2Cl also inhibits factor VIIa.[21] Factor IXa and factor Xa are also inhibited by *p*-amidinophenyl esters.[11]

Mechanism-based inhibitors, also called suicide inhibitors, contain a masked functional group which is unmasked upon reaction with an active-site residue of the target enzyme. Only a few mechanism-based inhibitors have been reported for trypsin-like enzymes. Thrombin and several trypsin-like enzymes are inhibited by 7-(aminomethyl)-1-benzylisatoic anhydride,[12] 3,4-dihydro-3-benzyl-6-(chloromethyl)coumarin,[13] and guanidinophenyl-substituted enol lactones.[14] DCI (**1**), a general serine protease inhibitor, also inhibits thrombin, but the inhibition rate is very slow.[15] Mechanism-based isocoumarin inhibitors substituted with basic groups such as aminoalkoxy, guanidino, and isothioureidoalkoxy groups have been reported to be potent inhibitors of several blood coagulation enzymes.[16,17] Among these compounds, 4-Cl-3-EtO-7-GuaIC (**2**) and NH_2-CiTPrOIC (**4**) showed anticoagulant activity *in vitro*,[16,17] but only NH_2-CiTPrOIC was effective in an *in vivo* rabbit coagulation model.[22] Here we report the syntheses of a series of new compounds (Figure 1) with various substituents in the 7- and 3-position of the isocoumarin ring that inhibit several coagulation enzymes. The more potent inhibitors were modeled into the active site of α-thrombin and were also tested as anticoagulant agents *in vitro*.

Results

Inhibition of Coagulation Enzymes by Isocoumarins. Inhibition rates of several coagulation enzymes including thrombin, factor Xa, factor XIa, factor XIIa, factor VIIa and factor IXa by substituted isocoumarins are shown in Table 1. Inhibition rates of four coagulation enzymes by compounds 1–4 have been reported previously[16,17] and are included here for comparison. Compound 4 was modified by placing a hydrophobic substituent at

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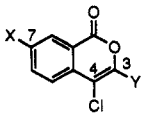
Table 1. Inhibition Rates of Coagulation Enzymes by 7-Substituted 4-Chloro-3-(isothioureidoalkoxy)isocoumarins[a]

	$k_{obs}/[I]$ (M^{-1} s^{-1})						
compound	bovine thrombin[b]	human thrombin[c]	human factor Xa[d]	human factor XIa[e]	human factor XIIa[f]	human factor VIIa[g]	porcine factor IXa[h]
1, DCI	25[i]	10[i]			64[i]	31	10
2, 4-Cl-3-EtO-7-GuaIC	55000[j]		11000[j]	60000[j]	22000[j]	2200	2850
			7-Substituted CiTPrOIC				
3, 7-H	1400[j]			47000[j]	27000[j]	450	90
4, 7-NH_2	630[j]	760	60[j]	22000[j]	6200[j]	430	80
5, 7-$PhCH_2NHCONH$	420	700					
6, 7-PhNHCONH	970	1840	50		7720	720	110
7, 7-CH_3CONH	420	310					
8, 7-$PhCH_2CH_2CONH$	820	630					
9, 7-$PhCH_2CONH$	600	610	610	610	610	610	610
10, Boc-L-Phe-NH	330	520					
11, L-Phe-NH	400	470					
12, Boc-D-Phe-NH	190	230					
13, D-Phe-NH	180	220					
			7-Substituted CiTEtOIc				
14, 7-PhNHCONH	25000	22400	4740	104000	50000	3140	1790
15, 7-$PhCH_2NHCONH$	16800	11680	2340	105000	45000	1810	2320
16, 7-$PhCH_2CONH$	15800	6730	3630		59000	1100	2620
17, 7-Boc-D-Phe-NH	1040	1090					
18, 7-D-Phe-NH	4240	3070	3070		82000		
19, Boc-L-Phe-NH	1090	1140	1620				
20, L-Phe-NH	1280	1340	3770		107000	4010	1590
21, Boc-Ala-Ala-NH	1530	970					
22, Ala-Ala-NH	1070	880	1490				
23, $(CH_3)_2CHNHCONH$	4100	5000					
24, 1-naphthyl-NHCONH	17500	5800					
25, (*S*)-$Ph(CH_3)CHNHCONH$	41300	21000				1830	3980
26, (*R*)-$Ph(CH_3)CHNHCONH$	29500	12000					
27, $(Ph)_2CHNHCONH$		7050					
28, $CH_3COCH_2CH_2CONH$		1770					
29, $PhCH_2CH_2CONH$		2150					

[a] Inhibition rates were measured in 0.1 M Hepes, 0.01 M $CaCl_2$, pH 7.5 buffer, 8% Me_2SO, and at 25 °C. [b] Inhibitor concentrations were 1.2–54 μM. [c] Inhibitor concentrations were 1.2–54 μM. [d] Inhibitor concentrations were 3.6–44 μM. [e] Inhibitor concentrations were 0.7–0.8 μM. [f] Inhibitor concentrations were 3.6–4.9 μM. [g] Inhibitor concentrations were 7.2–44 μM. [h] Inhibitor concentrations were 8.3–82 μM. [i] Data were obtained from ref 15. [j] Data were obtained from ref 16.



1 X = H, Y=Cl
2 X = $NHC(=NH_2^+)NH_2$, Y = OEt
Y = $O(CH_2)_3SC(=NH_2^+)NH_2$
3 X = H
4 X = NH_2
5 X = $PhCH_2NHCONH$
6 X = PhNHCONH
7 X = CH_3CONH
8 X = $PhCH_2CH_2CONH$
9 X = $PhCH_2CONH$
10 X = Boc-L-Phe-NH
11 X = L-Phe-NH
12 X = Boc-D-Phe-NH
13 X = D-Phe-NH

Y = $O(CH_2)_2SC(=NH_2^+)NH_2$
14 X = PhNHCONH
15 X = $PhCH_2NHCONH$
16 X = $PhCH_2CONH$
17 X = Boc-D-Phe-NH
18 X = D-Phe-NH
19 X = Boc-L-Phe-NH
20 X = L-Phe-NH
21 X = Boc-Ala-Ala-NH
22 X = Ala-Ala-NH
23 X = $(CH_3)_2CHNHCONH$
24 X = 1-Naphthyl-NHCONH
25 X = S-$Ph(CH_3)CHNHCONH$
26 X = R-$Ph(CH_3)CHNHCONH$
27 X = $(Ph)_2CHNHCONH$
28 X = $CH_3COCH_2CH_2CONH$
29 X = $PhCH_2CH_2CONH$

Figure 1. Structures of substituted isocoumarins.

the 7-position and a shorter isothioureidoethoxy group at the 3-position. The resulting compounds (**14–29**) are more potent inhibitors toward thrombin than **4**. Compounds **14** and **25** are the best thrombin inhibitors with $k_{obs}/[I]$ values of (2.1–4.1) × 10^4 M^{-1} s^{-1} and they inhibit thrombin more potently than **4** by 28–65-fold. PhNHCONH-CiTPrOIC (**6**) inhibits both human and bovine thrombin better than the parent compound, NH_2-CiTPrOIC (**4**), by ca. 2-fold. PhNHCONH-CiTEtOIC (**14**) with a shorter 3-substituent inhibits thrombin, factor Xa, factor XIIa, factor VIIa, and factor IXa more potently than does PhNHCONH-CiTPrOIC (**6**). Several isocoumarins with 3-isothioureidoethoxy substituents (**14–16**, **18–20**, **22**) inhibit factor Xa moderately with $k_{obs}/[I]$ values of (1.5–4.7) × 10^3 M^{-1} s^{-1}, and the best inhibitor is PhNHCONH-CiTEtOIC (**14**). Only two compounds, PhNHCONH-CiTEtOIC (**14**) and $PhCH_2NHCONH$-CiTEtOIC (**15**), were tested with factor XIa, and they inhibit this enzyme quite potently with $k_{obs}/[I]$ values of 10^5 M^{-1} s^{-1}. The best inhibitor of factor XIIa and factor VIIa is L-Phe-NH-CiTEtOIC (**20**), and the best factor IXa inhibitor is (*S*)-$Ph(CH_3)CHNHCONH$-CiTEtOIC (**25**) among the tested isocoumarins.

Hydrolysis of Isocumarins in Buffer. The half-lives for the hydrolysis of substituted isocoumarins were measured in pH 7.5 buffer (Table 2). Among the series, only PhNHCONH-CiTPrOIC (**6**), $PhCH_2NHCONH$-CiTPrOIC (**5**), PhNHCONH-CiTEtOIC (**14**), and $(Ph)_2$-CHNHCONH-CiTEtOIC (**27**) are more stable than the parent compound NH_2CiTPrOIC (**4**). The isocoumarins L-Phe-NH-CiTEtOIC (**20**) and D-Phe-NH-CiTEtOIC (**18**) are the least stable compounds with half-lives of 21 and 18 min, respectively. Generally, the isocoumarins with the shorter 3-isothioureidoethoxy group are less stable than those with a 3-isothioureidopropoxy substituent.

Stability of Inhibited Thrombin. The stabilities of thrombin inhibited by PhNHCONH-CiTEtOIC (**14**), (*S*)-$Ph(CH_3)CHNHCONH$-CiTEtOIC (**25**), and (*R*)-$Ph(CH_3)$-CHNHCONH-CiTEtOIC (**26**) were monitored in a 0.1 M Hepes, 0.01 M $CaCl_2$, pH 7.5 buffer, and at 25 °C by measuring the residual enzymatic activities. Thrombin

Table 2. Hydrolysis Rates of 7-Substituted 4-Chloro-3-(isothioureidoalkoxy)isocoumarins[a]

compd		$t_{1/2}$ (min)
4	NH_2-CiTPrOIC	90
5	$PhCH_2NHCONH$-CiTPrOIC	148
6	PhNHCONH-CiTPrOIC	148
7	CH_3CONH-CiTPrOIC	68
8	$PhCH_2CH_2CONH$-CiTPrOIC	66
9	$PhCH_2CONH$-CiTPrOIC	61
10	Boc-L-Phe-NH-CiTPrOIC	55
11	L-Phe-NH-CiTPrOIC	46
12	Boc-D-Phe-NH-CiTPrOIC	51
13	D-Phe-NH-CiTPrOIC	42
14	PhNHCONH-CiTEtOIC	108
17	Boc-D-Phe-NH-CiTEtOIC	39
18	D-Phe-NH-CiTEtOIC	18
19	Boc-L-Phe-NH-CiTEtOIC	42
20	L-Phe-NH-CiTEtOIC	21
21	Boc-Ala-Ala-NH-CiTEtOIC	40
25	(S)-$Ph(CH_3)CHNHCONH$-CiTEtOIC	56
26	(R)-$Ph(CH_3)CHNHCONH$-CiTEtOIC	62
27	$(Ph)_2CHNHCONH$-CiTEtOIC	168
28	$CH_3COCH_2CH_2CONH$-CiTEtOIC	28
29	$PhCH_2CH_2CONH$-CiTEtOIC	39

[a] Half-lives for the hydrolysis reactions were measured in 0.1 M Hepes, 0.01 M $CaCl_2$, pH 7.5 buffer, 8% Me_2SO, and at 25 °C.

inhibited by compounds **14**, **25**, or **26** regains less than 3% of the original activity after 5 days and regains 4, 16, and 10% of activity, respectively, after 20 days. The reactivation of human thrombin inhibited by PhNHCONH-CiTEtOIC (**14**), $PhCH_2CONH$-CiTEtOIC (**16**), (S)-$Ph(CH_3)CHNHCONH$-CiTEtOIC (**25**), and (R)-$Ph(CH_3)CHNHCONH$-CiTEtOIC (**26**) was also measured in Hepes buffer after the addition of 0.38 M NH_2OH. Thrombin inhibited by **14** or **16** regains full activity after 1 day; however, thrombin inhibited by **25** or **26** only regains 67 and 65% of the enzymatic activity, respectively.

Effect of Factor VIIIa and Phospholipid Vesicles on the Inhibition of Factor IXa by Three Inhibitors. The possible effect of porcine factor VIIIa and phospholipid vesicles on the inhibition of porcine factor IXa by three inhibitors (4-Cl-3-EtO-7-GuaIC, **2**, NH_2-CiTPrOIC, **4**, and Dns-Glu-Gly-Arg-CH_2Cl) was studied, and the results indicate that factor VIIIa and phospholipid have no effect on the inhibition. One of the three inhibitors, Dns-Glu-Gly-Arg-CH_2Cl, was reported to inhibit factor IXa with a $k_{obs}/[I]$ value of 280 M^{-1} s^{-1}.[19] We have found that Dns-Glu-Gly-Arg-CH_2Cl inhibits porcine factor IXa and human factor VIIa with $k_{obs}/[I]$ values of 180 and 20 M^{-1} s^{-1}, respectively. The other two inhibitors **2** and **4** also inhibit factor IXa with $k_{obs}/[I]$ values of 2900 and 80 M^{-1} s^{-1}, respectively (see Table 1).

Anticoagulant Activity of Isocoumarins. The anticoagulant activity of the most potent inhibitors of thrombin in the series (**14**, **15**, **25**, **26**) and three other isocoumarins (**27**, **28**, **29**) were tested in pig or human plasma, and the data are shown in Table 3. All seven compounds prolong the APTT by more than 3–7-fold and six of them prolong the PT by 1.3–3.1-fold.

Table 3. Effect of 7-Substituted 4-Chloro-3-(2-isothioureidoethoxy)isocoumarins on PT and APTT[a]

compd		[I] (μM)	PT (s)	APTT (s)
	control	0	18.6[b]	17.7[b]
		0		29.0[c]
14	PhNHCONH-CiTEtOIC	16	28.5[b]	>120[b]
		32	58.3[b]	
		9.4		47.7[c]
		31		87.3[c]
15	$PhCH_2NHCONH$-CiTEtOIC	32	31.1[b]	>120[b]
		9.4		60.1[c]
		31		115.4[c]
25	(S)-$Ph(CH_3)CHNHCONH$-CiTEtOIC	32	30.0[b]	>120[b]
		9.4		57.6[c]
		31		111.2[c]
26	(R)-$Ph(CH_3)CHNHCONH$-CiTEtOIC[b]	32	26.2	>120
	control[d]	0	18.3	35.2
27	$(Ph)_2CHNHCONH$-CiTEtOIC[d]	31	18.4	121
28	$CH_3COCH_2CH_2CONH$-CiTEtOIC[d]	31	42.5	>140
29	$PhCH_2CH_2CONH$-CiTEtOIC	9.4		52.4[c]
		31		76.2[c]
		31	24.4[d]	140[d]

[a] Average of two or three trials. [b] Coagulant assays were performed with pig plasma, Dade thromboplastin (PT), and Ortho-activated thrombofax (APTT). [c] APTT assays were performed with Dade human plasma Ci-Trol and Dade Actin FS reagent. [d] Coagulant assays were performed with Dade human plasma Ci-Trol, Dade thromboplastin (PT), and Ortho thrombosil 1 (APTT).

Discussion

Inhibition Studies. Substituted isocoumarins with basic groups such as guanidino and isothioureidoalkoxy groups are potent inhibitors of several coagulation enzymes.[16,17] NH_2-CiTPrOIC inhibits various coagulation enzymes with $k_{obs}/[I]$ values of 60–22 000 M^{-1} s^{-1} (Table 1). This compound is reasonably stable in plasma with a half-life of 165 min and is an effective anticoagulant *in vitro* and *in vivo*.[16,17,22] We have modified the structure of NH_2-CiTPrOIC by the addition of various substituents at the 3- and 7-positions in order to increase the inhibitory potency and selectivity of these isocoumarins toward various coagulation enzymes.

Isocoumarins with a large hydrophobic group at the 7-position and a shorter isothioureidoethoxy group at the 3-position are better inhibitors for coagulation enzymes than the parent compound NH_2-CiTPrOIC (**4**). For example, compounds **14** and **25** are more potent thrombin inhibitors than the various 3-(isothioureidopropoxy)-isocoumarins (**4**–**13**). Compound **14** with a (phenylcarbamoyl)amino group at the 7-position inhibits factor Xa more potently than other derivatives with the same 3-substituent. Compound **20** with a L-Phe-NH group in the 7-position is the best inhibitor in the series against factor XIIa and factor VIIa. The *S*-isomer **25** inhibits factor IXa most potently. Factor VIIa and factor IXa are known to hydrolyze synthetic substrates more slowly than other coagulation enzymes.[17,23] Thus, it is not surprising that these isocoumarins inhibit factor VIIa and factor IXa more slowly than thrombin, factor Xa, factor XIa, and factor XIIa with the exception of **20**. However, these isocoumarins still inhibit factor VIIa and factor IXa more potently than Dns-Glu-Gly-Arg-CH_2Cl. Some of the isocoumarins reported here are probably the most potent synthetic inhibitors for the individual coagulation enzyme (e.g., **20** for factor VIIa).

Effect of Factor VIIIa and Phospholipid Vesicles on the Inhibition of Factor IXa. Several of the serine proteases in blood coagulation require the presence of accessary components for the efficient catalysis of their natural substrates. For example, the rate of activation of factor X by factor IXa is increased in the presence of factor VIIIa, Ca^{2+}, and an anionic phospholipid surface (intrinsic factor X activation complex). The catalytic advantage resulting from the formation of this complex is manifested by a 3000-fold decrease in the K_M and a 200 000-fold increase in the k_{cat}.[24] The K_M effect is predominantly achieved by the phospholipid interaction while the k_{cat} effect is contributed by factor VIIIa.[24] The mechanism by which factor VIIIa produces this increase in k_{cat} is not

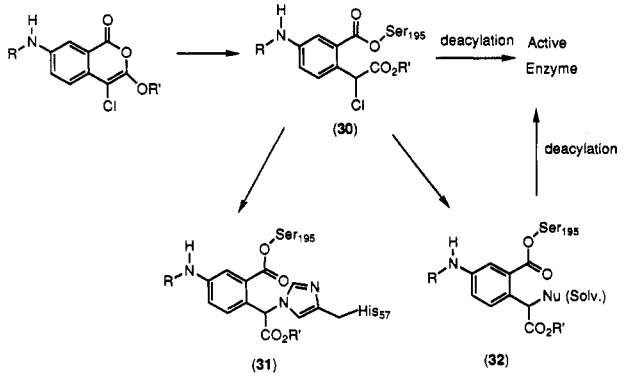


Figure 2. Inhibition mechanism of serine proteases by substituted isocoumarins.

known. Fluorescence studies performed with the analogous prothrombinase complex indicate that the binding of cofactor to the enzyme may alter the active site of the enzyme.[25] If factor VIIIa functions primarily by altering the nature of the active site and making factor IXa more reactive toward its native substrate, then one might expect factor IXa to show increased reactivity toward a variety of substrates and inhibitors upon complex formation. However, we show here that the formation of the intrinsic factor X activating complex does not increase the reactivity of factor IXa toward three active site directed inhibitors. These findings are in agreement with a study in the prothrombinase complex in which the reactivity of the enzyme factor Xa was not enchanced toward a tetrapeptide substrate by prothrombinase complex formation.[26]

Inhibition Mechanism. The inhibition mechanism of serine proteases by substituted isocoumarins has been described previously (Figure 2).[16,27] Isocoumarins react with the active site Ser-195 initially to form an acyl enzyme derivative (**30**), which can deacylate to regenerate the active enzyme. Alternately, the acyl enzyme (**30**) could eliminate chlorine to form a quinone imine methide intermediate (not shown) which can then react with a nearby enzyme nucleophile such as His-57 to give an alkylated acyl enzyme (**31**) or with a solvent molecule to give an acyl enzyme derivative (**32**). The acyl enzyme derivatives (**30, 32**) can be reactivated by NH_2OH, while the alkylated acyl enzyme derivative (**31**) is stable toward reactivation. Several acyl enzyme derivatives and the alkylated acyl enzyme derivatives have been observed in X-ray structures of complexes of isocoumarins with trypsin or PPE.[28-30]

The reactivation of thrombin inhibited by **14**, **16**, **25**, and **26** in the presence of NH_2OH indicates that a stable acyl enzyme derivative is formed upon inhibition of thrombin by **14** and **16** since full enzyme activity can be regenerated. Both the acyl enzyme and the alkylated acyl enzyme derivatives are formed upon inhibition of thrombin by **25** and **26** since 65–67% of activity was regained in the presence of NH_2OH.

Molecular Modeling. The actual binding modes of these inhibitors to enzymes are not known since X-ray structures of NH_2-CiTPrOIC derivatives with trypsin or thrombin have not been obtained. However, these complexes have sufficient stability for X-ray studies since thrombin inhibited by compounds **14**, **25**, or **26** regained very little enzyme activity after 20 days of incubation in pH 7.5 buffer. We are hopeful that crystal structures with either thrombin or trypsin will be available in the future.

The X-ray structures of complexes of bovine trypsin and PPE with another isocoumarin, 4-Cl-3-EtO-7-GuaIC (**2**), have been determined.[28,29] In the trypsin–isocoumarin crystal structure, both the acyl enzyme and the alkylated acyl enzyme derivatives are observed in the E–I complex. The guanidino group goes into the S_1 pocket[31] and interacts with Asp-189. In the case of the complex of PPE with compound **2**, the alkoxy group of the inhibitor interacts with the S_1 pocket while the 7-guanidino group forms hydrogen bonds with Thr-41 in the S′ binding sites of the enzyme. We speculate that the positive charged isothioureidoalkoxy group of PhNHCONH-CiTEtOIC will probably fit into the S_1 pocket of thrombin and interact with Asp-189, and the hydrophobic group at the 7-position will go into the S′ subsites of the enzyme.

Table 4. Steric Energies for Modeled Productive Structures of Human α-Thrombin Complexed with Isocoumarins[a]

	isocoumarins	energy (kcal/mol)
25	(*S*)-Ph(CH_3)CHNHCONH-CiTEtOIC	−1490
		−1560
26	(*R*)-Ph(CH_3)CHNHCONH-CiTEtOIC	−1660
		−1500
15	$PhCH_2NHCONH$-CiTEtOIC	−1520
		−1630
		−1660
27	$(Ph)_2CHNHCONH$-CiTEtOIC	−1600
		−1640
16	$PhCH_2CONH$-CiTEtOIC	−1590
		−1590
		−1610
		−1500

[a] Productive structures are those with an erngy ≤ −1490 kcal/mol and $d < 2.85$ Å where d refers to the distance between Ser-195 O_γ and the isocoumarin benzoyl ester carbonyl carbon. Isocoumarins are listed in decreasing order of potency (see Table 1).

In order to gain additional information on the binding modes of the new isocoumarins, we have modeled five of the most potent isocoumarins into the active site of thrombin. Table 4 lists all of the low-energy structures which we discovered where the isocoumarin carbonyl group is within reaction distance of Ser-195. With one exception, at least one of the NH_2 hydrogens of the isothioureido moiety in each structure is within 2.4 Å of one of the Asp-189 carboxylate oxygens in thrombin's S_1 pocket. The lowest complex energies (−1660 kcal/mol) are observed for the orientation where both nitrogens of the isothioureido substituent are respectively equidistant from O_{δ_1} and O_{δ_2} of the Asp-189 carboxylate. The highest complex energies (−1560 to −1490 kcal/mol) are observed where only one NH_2 is hydrogen bonded (2.4 Å) to a single carboxylate oxygen. For all structures listed in Table 4, the benzoyl ester carbonyl oxygen of the isocoumarin is directed into the oxyanion hole (i.e., the oxygen is within 2.3 Å of either the Ser-195 or the Gly-193 >NH hydrogen or both). At least one of the phenyl hydrogens of the isocoumarin's 7-substituent, with the exception of some complex structures of compound **15**, is found within 5 Å of essentially all of the atoms of Leu-41, most of the Lys-60F side chain atoms, and at least four atoms of the Phe-60H phenyl ring. Other residues with three or more contiguous atoms within a 5-Å distance of some modeled productive complexes are Arg-35, Cys-42, Cys-58, Gly-193, Ser-195, Glu-39, and occassionally Trp-60D and His-57. A typical modeled complex structure is shown in Figure 3.

There is no correlation between inhibitor potency and the energies listed in Table 4 or the orientation of the inhibitors relative to the Asp-189 carboxylate in the modeled structures. The crystal structures of thrombin complexed with benzamidine derivatives support the concept that this relative orientation does not control the

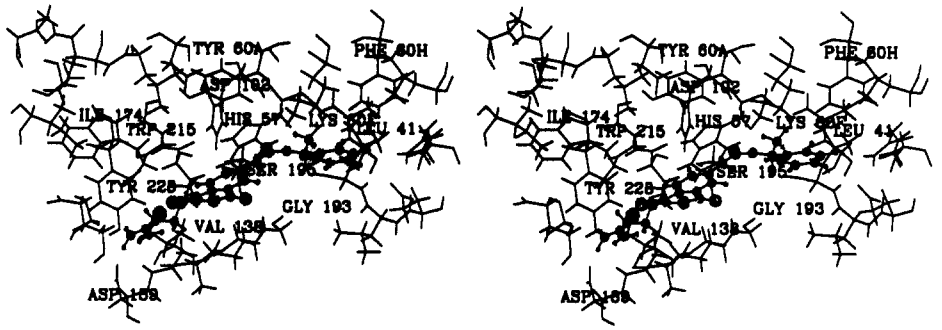


Figure 3. Stereoview of a representative modeled productive structure of human α-thrombin active site noncovalently complexed with the inhibitor (*S*)-Ph(CH_3)CHNHCONH-CiTEtOIC (**25**). The structure was obtained as described in the Experimental Section. Atoms of the inhibitor are represented by spheres filled with dots.

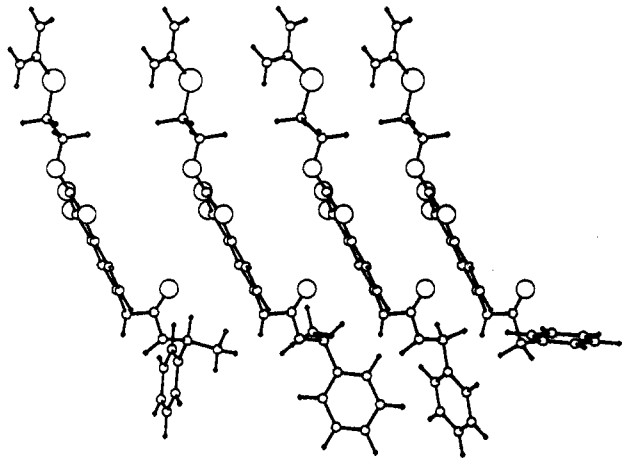


Figure 4. Ball-and-stick drawings of isocoumarin structures which has been geometry optimized *in vacuo*. The structures were generated as described in Experimental Section. The structures depicted from left to right are (*S*)-Ph(CH_3)CHNH-CONH-CiTEtOIC (**25**), (*R*)-Ph(CH_3)CHNHCONH-CiTEtOIC (**26**), PhCH_2NHCONH-CiTEtOIC (**15**), and PhCH_2CONH-CiTEtOIC (**16**).

docking geometry of the inhibitor.[32] The only structural feature of the modeled productive complexes which does correlate with inhibitor potency is H-bonding between the Lys-60F NH_3^+ and the polar region of the inhibitor's 7-substituent. The H-bonding sometimes involves an intermediate H_2O molecule. No good H-bonds are observed for complexes involving compounds **16** and **27**, which have the lowest $k_{obs}/[I]$ values (6700 and 7100 M^{-1} s^{-1}) of the compounds modeled. For compounds **15** and **26** with $k_{obs}/[I]$ values of 12 000 M^{-1} s^{-1}, a majority (50–67%) of the complexes possess these H-bonds. All of the modeling complexes with the most potent of the inhibitors (**25**) ($k_{obs}/[I]$ = 21,000 $M^{-1}s^{-1}$) exhibit the H-bonds.

Another feature which might affect inhibitor potency is the geometry of the phenyl group in the various 7-substituents (Figure 4). The more potent inhibitors have extended conformations, whereas the least potent inhibitor has a compact structure. The phenyl group might exert its effect by interfering with the H-bonding between the inhibitor and the Lys-60F NH_3^+. For example, the phenyl group could alter the geometry of the Lys-60F side chain, preventing formation of a hydrogen bond with the polar region of the inhibitor. The majority of the complexes involving the weakest inhibitor, PhCH_2CONH-CiTETOIC (**16**), show an altered orientation for the Lys-60F NH_3^+. We also predicted that **27** would alter the geometry of Lys-60F, and when this compound was synthesized, it proved to have an inhibitory potency similar to that of **16** (Table 1).

Antocoagulant Activity. NH_2-CiTPrOIC (**4**) has a large anticoagulant activity on APTT and a small effect on PT with human plasma.[17] Like NH_2-CiTPrOIC (**4**), compounds **14**, **15**, **25**, **26**, **28**, and **29** show a large anticoagulant effect in the APTT assay. Compounds **14** and **28** also have a profound effect on PT. Apparently, these isocoumarins are more effective than **4** *in vitro*. However, the isocoumarins with large hydrophobic groups at the 7-position are less soluble than **4** in plasma or in the buffer which contained 10% Me_2SO. Thus, these isocoumarins need to be modified to improve their solubility for *in vivo* tests.

Summary

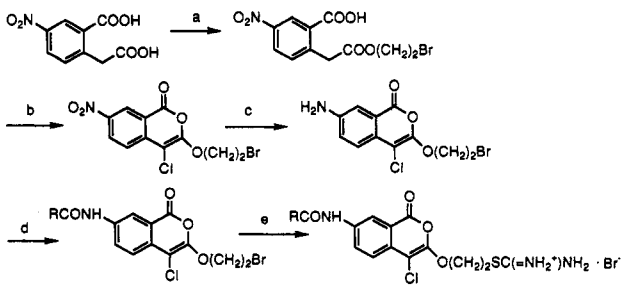
A series of NH_2-CiTPrOIC (**4**) derivatives with various substituents at the 7- and 3-positions are potent inhibitors of several blood coagulation enzymes. Substituted isocoumarins with the shorter isothioureidoethoxy group at the 3 position and a large hydrophobic group at the 7 position are better inhibitors of thrombin, factor VIIa, factor Xa, factor XIa, factor XIIa, and factor IXa than the parent compound, NH_2-CiTPrOIC (**4**). Modeled structures of several isocoumarins noncovalently complexed with human α-thrombin suggest that H-bonding between the 7-substituent and the Lys-60F NH_3^+ relates to the inhibitory potency. Thrombin inhibitor complexes formed by reaction with **14**, **25**, or **26** are quite stable and should be suitable for X-ray studies. Several isocoumarins prolong the PT and APTT in human and pig plasma and thus are effective anticoagulants *in vitro*.

Experimental Section

Synthesis. Homophthalic acid, 2-bromoethanol, 3-bromopropanol, and phosphorus pentachloride were obtained from Aldrich Chemical Co., Milwaukee, WI. Thiourea was obtained from Aldrich or Fisher Scientific, Norcross, GA. All chemicals and solvents are of reagent grade. Each new compound was checked by NMR, mass spectroscopy, TLC, and elemental analysis, and the results are consistent with proposed structures. The solvent system used for TLC of the final product was CH_3CN–acetic acid–H_2O, 8:1:1. The NMR spectra were recorded on Varian T-60 or Gemini 300-MHz instruments. The mass spectra were recorded on a Varian MAT Model 112S mass spectrometer. Elemental analyses were performed by Atlantic Microlabs, Norcross, GA.

The synthesis of 7-substituted 4-chloro-3-(isothioureidoalkoxy)-isocoumarins is described in Scheme 1. All of the 7-substituted 4-chloro-3-(isothioureidoalkoxy)isocoumarins were prepared from the reaction of the corresponding 7-substituted 3-(bromoalkoxy)-4-chloroisocoumarins with thiourea as previously described[16] in yields of 20–80%. The detailed synthesis of compound **14** given below is representative of the general synthetic procedure for these isothioureidoalkoxy isocoumarins. Generally, 1.1 equiv of thiourea was utilized, and overnight refluxing was required for

Scheme 1[a]



[a] Reagents: (a) $Br(CH_2)_2OH$, cat. H_2SO_4; (b) 2.5 equiv of PCl_5 in benzene, reflux; (c) Pd/C, H_2 in EtOAc–MeOH; (d) appropriate acid chloride, anhydride, or isocyanate; (e) 1.1 equiv of thiourea, reflux in THF.

all compounds except **27**, **28**, and **29** which were synthesized with 2–3 equiv of thiourea and 2 days of refluxing. The syntheses of the corresponding precursors, 7-substituted 3-(bromoalkoxy)-4-chloroisocoumarins are described elsewhere.[33] Generally, 7-substituted 3-(bromoalkoxy)-4-chloroisocoumarins were prepared by coupling of 7-amino-3-(bromoalkoxy)-4-chloroisocoumarins with appropriate acid chloride, anhydride, or isocyanate (Scheme 1). L-Phe-NH-CiTPrOIC (**11**), D-Phe-NH-CiTPrOIC (**13**), L-Phe-NH-CiTEtOIC (**20**), D-Phe-NH-CiTEtOIC (**18**), and Ala-Ala-NH-CiTEtOIC (**22**) were obtained by deblocking of the corresponding Boc derivatives (0.06–0.08 mmol) with 2 mL of CF_3COOH at 0 °C for 5 min. After CF_3COOH was evaporated, the residue was solidified by addition of anhydrous ether. Most of the (isothioureidoalkoxy)isocoumarins which precipitated out in the final step of reaction were pure products and were used directly for kinetic studies. The few derivatives which contained impurities were further washed with hot THF to remove thiourea and recrystallized from MeOH–ether.

7-[(Benzylcarbamoyl)amino]-4-chloro-3-(3-isothioureidopropoxy)isocoumarin hydrobromide (PhCH$_2$NHCONH-CiTPrOIC, 5): yellow solid (74%); mp 165–166 °C dec; TLC R_f 0.66; MS (FAB) *m/e* 461 (M – Br)$^+$. Anal. ($C_{21}H_{22}N_4O_4$ClBrS·0.75THF) C, H, N, Cl.

4-Chloro-3-(3-isothioureidopropoxy)-7-[(phenylcarbamoyl)amino]isocoumarin hydrobromide (PhNHCONH-CiTPrOIC, 6): yellow solid (70%); mp 194–195 °C dec; TLC R_f 0.66; MS (FAB) *m/e* 447 (M – Br)$^+$. Anal. ($C_{20}H_{20}N_4O_4$ClBrS) C, H, N, Cl.

7-(Acetylamino)-4-chloro-3-(3-isothioureidopropoxy)isocoumarin hydrobromide (CH$_3$CONH-CiTPrOIC, 7): yellow solid (50%); mp 180–181 °C; TLC R_f 0.47; MS (FAB) *m/e* 370 (M – Br)$^+$. Anal. ($C_{15}H_{17}N_3O_4$ClBrS) C, H, N, Cl.

4-Chloro-3-(3-isothioureidopropoxy)-7-[(3-phenylpropionylamino]isocoumarin hydrobromide (PhCH$_2$CH$_2$CONH-CiTPrOIC, 8): yellow solid (78%); mp 184–185 °C; TLC R_f 0.66; MS (FAB) *m/e* 460 (M – Br)$^+$. Anal. ($C_{22}H_{23}N_3O_4$ClBrS) C, H, N, Cl.

4-Chloro-3-(3-isothioureidopropoxy)-7-[(phenylacetyl)amino]isocoumarin hydrobromide (PhCH$_2$CONH-CiTPrOIC, 9): yellow solid (40%); mp 135–136 °C; TLC R_f 0.66; MS (FAB) *m/e* 446 (M – Br)$^+$. Anal. ($C_{21}H_{21}N_3O_4$ClBrS·0.75H_2O) C, H, N, Cl.

7-[[(*tert*-Butyloxycarbonyl)-L-phenylalanyl]amino]-4-chloro-3-(3-isothioureidopropoxy)isocoumarin hydrobromide (Boc-L-Phe-NH-CiTPrOIC, 10): yellow solid (65%); mp 180–185 °C; TLC R_f 0.70; MS (FAB) *m/e* 575 (M – Br)$^+$. Anal. ($C_{27}H_{32}N_4O_6$ClBrS) C, H, N.

4-Chloro-3-(3-isothioureidopropoxy)-7-(L-phenylalanylamino)isocoumarin trifluoroacetate (L-Phe-NH-CiTPrOIC, 11): pale yellow solid (90%); mp 150–170 °C; TLC R_f 0.15; MS (FAB) *m/e* 475 (M – Br – CF_3COOH)$^+$. Anal. ($C_{24}H_{25}N_4O_6$ClBrSF_3) C, H, N.

7-[[(*tert*-Butyloxycarbonyl)-D-phenylalanyl]amino]-4-chloro-3-(3-isothioureidopropoxy)isocoumarin hydrobromide (Boc-D-Phe-NH-CiTPrOIC, 12): yellow solid (40%); mp 180–185 °C; TLC R_f 0.72; MS (FAB) *m/e* 575 (M – Br)$^+$. Anal. ($C_{27}H_{32}N_4O_6$ClBrS) C, H, N.

4-Chloro-3-(3-isothioureidopropoxy)-7-(D-phenylalanylamino)isocoumarin trifluoroacetate (D-Phe-NH-CiTPrOIC, 13): pale yellow solid (90%); mp 167–175 °C; TLC R_f 0.15; MS (FAB) *m/e* 475 (M – Br – CF_3COOH)$^+$. Anal. ($C_{24}H_{25}N_4O_6$ClBrSF_3) C, H, N.

4-Chloro-3-(2-isothioureidoethoxy)-7-[(phenylcarbamoyl)amino]isocoumarin Hydrobromide (PHNHCONH-CiTEtOIC, 14). 3-(2-Bromoethoxy)-4-chloro-7-[(phenylcarbamoyl)amino]isocoumarin (**14a**) was prepared by reaction of 7-amino-3-(2-bromoethoxy)-4-chloroisocoumarin (0.32 g, 1 mmol) and phenyl isocyanate (0.12 g, 1 mmol) in 10 mL of THF. The reaction mixture was stirred at room temperature for 1 day, and during this time a yellow solid slowly precipitated. The product was recrystallized from THF–pentane in 41% yield: mp 215–217 °C; TLC, R_f 0.68 ($CHCl_3$–MeOH, 9:1); MS *m/e* 437 (M + 1)$^+$. Anal. ($C_{18}H_{14}N_2O_4$ClBr) C, H, N, Cl. The final product **14** was prepared by refluxing compound **14a** (0.1 g, 0.23 mmol) with thiourea (0.02 g, 0.26 mmol) in 10 mL of THF overnight to give a yellow solid in 40% yield: mp 161–163 °C dec; TLC, R_f 0.60; MS (FAB) *m/e* 433 (M – Br)$^+$. Anal. ($C_{19}H_{18}N_4O_4$ClBrS·0.25THF) C, H, N, Cl.

7-[(Benzylcarbamoyl)amino]-4-chloro-3-(2-isothioureidoethoxy)isocoumarin hydrobromide (PhCH$_2$NHCONH-CiTEtOIC, 15): yellow solid (40%); mp 122–125 °C dec; TLC R_f 0.57; MS (FAB) *m/e* 447 (M – Br)$^+$. Anal. ($C_{20}H_{20}N_4O_4$ClBrS·0.75THF) C, H, N, Cl.

4-Chloro-3-(2-isothioureidoethoxy)-7-[(phenylacetyl)amino]isocoumarin hydrobromide (PhCH$_2$CONH-CiTEtOIC, 16): yellow solid (36%); mp 115–120 °C dec; TLC R_f 0.60; MS (FAB) *m/e* 432 (M – Br)$^+$. Anal. ($C_{20}H_{19}N_3O_4$ClBrS·0.5H_2O) C, H, N, Cl.

7-[[(*tert*-Butyloxycarbonyl)-D-phenylalanyl]amino]-4-chloro-3-(2-isothioureidoethoxy)isocoumarin hydrobromide (Boc-D-Phe-NH-CiTEtOIC, 17): yellow solid (62%); mp 179–182 °C; TLC R_f 0.72; MS (FAB) *m/e* 561 (M – Br)$^+$. Anal. ($C_{26}H_{30}N_4O_6$ClBrS·0.75H_2O) C, H, N.

4-Chloro-3-(2-isothioureidoethoxy)-7-[(D-phenylalanyl)amino]isocoumarin trifluoroacetate (D-Phe-NH-CiTEtOIC, 18): pale yellow solid (90%); mp 155–163 °C; TLC R_f 0.11; MS (FAB) *m/e* 461 (M – Br – CF_3COOH)$^+$. Anal. ($C_{23}H_{23}N_4O_6$ClBrSF_3·0.5H_2O) C, H, N.

7-[[(*tert*-Butyloxycarbonyl)-L-phenylalanyl]amino]-4-chloro-3-(2-isothioureidoethoxy)isocoumarin hydrobromide (Boc-D-Phe-NH-CiTEtOIC, 19): yellow solid (20%); mp 178–182 °C; TLC R_f 0.72; MS (FAB) *m/e* 561 (M – Br)$^+$. Anal. ($C_{26}H_{30}N_4O_6$ClBrS·H_2O) C, H, N.

4-Chloro-3-(2-isothioureidoethoxy)-7-(L-phenylalanylamino)isocoumarin trifluoroacetate (L-Phe-NH-CiTEtOIC, 20): pale yellow solid (90%); mp 130–145 °C; TLC R_f 0.17; MS (FAB) *m/e* 461 (M – CF_3COOH – Br)$^+$. Anal. ($C_{23}H_{23}N_4O_6$ClBrSF_3·0.5H_2O) C, H, N.

7-[[(*tert*-Butyloxycarbonyl)-L-alanylalanyl]amino]-4-chloro-3-(2-isothioureidoethoxy)isocoumarin hydrobromide (Boc-Ala-Ala-NH-CiTEtOIC, 21): pale yellow solid (32%); TLC R_f 0.58; MS (FAB) *m/e* 556 (M – Br)$^+$.

7-(L-Alanyl-L-alanylamino)-4-chloro-3-(2-isothioureidoethoxy)isocoumarin trifluoroacetate (Ala-Ala-NH-CiTEtOIC, 22): pale yellow solid (90%); TLC R_f 0.17; MS (FAB) *m/e* 456 (M – Br – CF_3COOH)$^+$.

4-Chloro-3-(2-isothioureidoethoxy)-7-[(isopropylcarbamoyl)amino]isocoumarin hydrobromide ((CH$_3$)$_2$CHNHCONH-CiTEtOIC, 23): yellow solid (60%); mp 145–150 °C; TLC R_f 0.72; MS (FAB) *m/e* 399 (M – Br)$^+$. Anal. ($C_{16}H_{20}N_4O_4$ClBrS·0.5H_2O) C, H, N.

4-Chloro-3-(2-isothioureidoethoxy)-7-[(1-naphthylcarbamoyl)amino]isocoumarin hydrobromide (1-NaphthylNHCONH-CiTEtOIC, 24): yellow solid (30%); TLC R_f 0.68; MS (FAB) *m/e* 483 (M – Br)$^+$.

4-Chloro-3-(2-isothioureidoethoxy)-7-[[((*S*)-methylbenzyl)carbamoyl]amino]isocoumarin hydrobromide ((*S*)-Ph(CH$_3$)CHNHCONH-CiTEtOIC, 25): pale yellow solid (80%); mp 143–146 °C; TLC R_f 0.66; MS (FAB) *m/e* 461 (M – Br)$^+$. Anal. ($C_{21}H_{22}N_4O_4$ClBrS·0.75THF) C, H, N, Cl.

4-Chloro-3-(2-isothioureidoethoxy)-7-[[((*R*)-methylbenzyl)carbamoyl]amino]isocoumarin hydrobromide ((*R*)-Ph(CH$_3$)CHNHCONH-CiTEtOIC, 26): pale yellow solid (68%); mp 143–150 °C; TLC R_f 0.66; MS (FAB) *m/e* 461 (M – Br)$^+$. Anal. ($C_{21}H_{22}N_4O_4$ClBrS·H_2O) C, H, N, Cl.

4-Chloro-7-[[(diphenylmethyl)carbamoyl]amino]-3-(2-isothioureidoethoxy)isocoumarin hydrobromide ((Ph)$_2$-

CHNHCONH-CiTEtOIC, 27): yellow powder (42%); mp 196 °C dec; MS (FAB) *m/e* 523 (M - Br)$^+$; HRMS calcd for $C_{26}H_{24}N_4O_4ClS$ *m/e* 523.1207, found 523.1213.

4-Chloro-3-(2-isothioureidoethoxy)-7-[(3-oxopentanoyl)-amino]isocoumarin hydrobromide ($CH_3COCH_2CH_2CONH$-CiTEtOIC, 28): yellow powder (19%); mp 156 °C dec; MS (FAB) *m/e* 412 (M-Br)$^+$; HRMS calcd for $C_{17}H_{19}N_3O_5ClS$ *m/e* 412.0734, found 412.0745.

4-Chloro-7-(hydrocinnamoylamino)-3-(2-isothioureidoethoxy)isocoumarin hydrobromide ($PhCH_2CH_2CONH$-CiTEtOIC, 29): light yellow powder (62%); mp 158 °C dec; MS (FAB) *m/e* 446 (M - Br)$^+$. Anal. ($C_{21}H_{21}N_3O_4BrClS$·0.2thiourea·0.3H_2O) C, H, N.

Biochemistry. Bovine and human thrombin were obtained from Sigma Chemical Co., St. Louis, MO. Human factor Xa, factor XIa, and factor XIIa were kindly supplied by Dr. Kazuo Fujikawa, Department of Biochemistry, University of Washington. Human factor VIIa was a generous gift of Dr. Georgia Vlasuk at Merck, Sharp, & Dohme Research Laboratory. Porcine factor IXa was prepared as previously described,[19] and the concentration of active enzyme was determined by active-site titration with NPGB.[34] A stable preparation of porcine factor VIIIa was obtained as previously described.[35] Phosphatidylcholine–phosphatidylserine (75%/25%, w/w) small unilamellar vesicles (PCPS) were prepared by modification of a previously described method.[36,37] Hepes was purchased from Research Organics Inc., Cleveland, OH. 4,4'-Dithiodipyridine was purchased from Aldrich Chemical Co., Milwaukee, WI. Activated thrombofax and thrombosil 1 were obtained from Ortho Diagnostic Systems Inc., Raritan, NJ. Dade thromboplastin reagent, Actin FS reagent, human plasma Ci-Trol, and calcium chloride solution (0.02 M) were obtained from American Scientific Products, Stone Mountain, GA. Pig plasma was kindly provided by the Surgery Department of Emory University Medical School. Z-Arg-SBzl, Z-Trp-Arg-SBzl, and Dns-Glu-Gly-Arg-CH_2Cl were synthesized previously.[9,23] DCI (1), 4-Cl-3-EtO-7-GuaIC (2), CiTPrOIC (3), and NH_2-CiTPrOIC (4) were synthesized as previously described.[15,16] The PT and APTT coagulant assays were performed on a MLA Electra 750 coagulation timer.

Enzyme Inactivation: Incubation Method. An aliquot of inhibitor (25–50 μL) in Me_2SO was added to 0.275–0.55 mL of a buffered enzyme solution (0.03–4.3 μM) to initiate the inactivation. Aliquots (50–500 μL) were withdrawn at various intervals, and the residual enzymatic activity was measured as described below. Enzymatic activities of bovine thrombin, human thrombin, human factor Xa, human factor XIa, human factor XIIa, and porcine factor IXa were measured in 0.1 M Hepes, 0.01 M $CaCl_2$, pH 7.5 buffer. Enzymatic activity of human factor VIIa was measured in 0.05 M Hepes, 0.15 M NaCl, 0.005 M $CaCl_2$, pH 7.5 buffer. The Me_2SO concentration in the reaction mixtures was 8–12% (v/v). The inhibitor concentrations are shown in the appropriate table. Porcine factor IXa was assayed with Z-Trp-Arg-SBzl (0.157 mM), and other coagulation enzymes were assayed with Z-Arg-SBzl (0.07–0.133 mM). All peptide thioester hydrolysis rates were measured with assay mixtures containing 4,4'-dithiodipyridine (ϵ_{324} = 19 800 M^{-1} cm^{-1}).[38] Inhibition rates were measured with two or three determinations at each inhibitor concnetration. Pseudo-first-order inactivation rate constants (k_{obs}) were obtained from plots of ln v_t/v_o vs time, and the correlation coefficients were greater than 0.98.

Determination of Spontaneous Hydrolysis Rates of Inhibitors in Buffer. An aliquot of isocoumarin derivatives in Me_2SO was added to 0.1 M Hepes, 0.01 M $CaCl_2$, pH 7.5 buffer such that the inhibitor concentration was 0.01–0.07 mM and the Me_2SO concentration was 10% v/v. The spontaneous hydrolysis rates were monitored by following the decrease in absorbance at wavelengths in the range of 355–366 nm. The hydrolysis products gave negligible absorbance at the wavelength utilized. Hydrolysis rates were measured with two determinations for each inhibitor. First-order rate constants were obtained from plots of ln (A_t - A_f) vs time, where A_t and A_f are the absorbance of the mixture at time *t* and that of the final hydrolysis product. All the plots gave correlation coefficients of 0.99 or greater.

Effect of Factor VIIIa and Phospholipid Vesicles on the Inhibition of Factor IXa by Three Inhibitors. The inhibition of factor IXa-catalyzed hydrolysis of Z-Lys-SBzl was studied with three inhibitors 4-Cl-3-EtO-7-GuaIC (2), NH_2-CiTPrOIC (4), and Dns-Glu-Gly-Arg-CH_2Cl. The enzymatic hydrolysis of Z-Lys-SBzl (0.18 mM) by factor IXa (200 nM) was measured continuously at 412 nm in a 0.15 M NaCl, 0.02 M Hepes, 5 mM $CaCl_2$, 0.1% PEG-8000, pH 7.4 buffer at 22 °C in the presence of DTNB (0.21 mM).[39] Various inhibitor solution was added to the assay mixture (2, 3 μM; 4, 60 μM; Dns-Glu-Gly-Arg-CH_2Cl, 10 μM), and the inhibition data was analyzed by the method of Tsou and Tian.[40] To test whether factor VIIIa affected the inhibition rates, identical reactions were also performed in the presence of 200 nM factor VIIIa and 40 μM PCPS. The possible effect of the inhibitors on factor VIIIa was evaluated by a previously described factor VIIIa assay.[41] It was found that neither the inhibitors nor Me_2SO had any effect on factor VIIIa, and factor VIIIa was stable under the conditions of the assay.

Coagulant Assays. The prothrombin time (PT) and activated partial thromboplastin time (APTT) were used to measure the coagulant activity of pig or human plasma in the presence of inhibitors. For PT and APTT determinations, the inhibitor solution was first prepared in Me_2SO and further diluted with 0.1 M Hepes, 0.01 M $CaCl_2$, pH 7.5 buffer such that the diluted stock inhibitor solution had 10% Me_2SO. Control experiments showed that the addition of 10% Me_2SO to assay mixture did not prolong the PT and APTT. In the PT assay, citrated pig plasma or human plasma Ci-Trol (0.1 mL) was incubated with 0.02 mL of inhibitor solution at 37 °C for 1 min, and then 0.2 mL of Dade thromboplastin reagent was added and the clotting time was determined. In the APTT assay, citrated pig or human plasma (0.1 mL) was incubated with 0.02 mL of inhibitor solution and 0.1 mL of Ortho activated thrombofax, thrombosil 1, or Dade Actin FS reagent at 37 °C for 4 min, followed by the addition of 0.1 mL of 0.02 M $CaCl_2$ (precinbated at 37 °C), and the clotting time was determined.

Molecular Modeling. Coordinates for human α-thrombin were generously provided by Dr. Bode.[42] The active site contained a covalently bound D-Phe-Pro-Arg-CH_2Cl and 423 crystallographic waters. The chloromethyl ketone was removed, and CHARMm (version 21, release 1.7b)[43] obtained from Molecular Simulations, Inc. (Burlington, MA) was used to add both polar and nonpolar hydrogens to the appropriate atoms. The hydrogen positions were energy minimized using the procedure previously described for PPE.[44] The Adopted-Basis Newton Raphson (ABNR) method with an energy tolerance of 0.001 kcal/mol was utilized.

In our previous work,[44] it was found that atomic charges obtained from a self-consistent field (SCF) calculation on a serine protease active site are very different from those provided with CHARMm. Results consistent with inhibitor potency were only obtained using the SCF-calculated charges. Quantum mechanical calculations using the MNDO Hamiltonian originally formulated by Dewar and Thiel[45] were employed to calculate the partial atomic point charges (SCF charges) for atoms of the human α-thrombin active site and the inhibitors. One SCF calculation was performed on each portion of the active site (see Table 5) due to memory limitations. The command line for the calculation consisted of the keywords: CHARGE=* NOINTER NOMM 1SCF T=600M. The value for CHARGE is shown in Table 5. SCF charges for the inhibitors were those resulting from a complete geometry optimization of the structures. The command line for the calculation consisted of the keywords: CHARGE=1 NOINTER NOMM T=600M GRADIENTS PRECISE. Geometry optimizations were complete when either Herbert's or Peter's test was satisfied. Initial coordinates for the geometry optimizations were obtained using QUANTA (version 3.2) from Molecular Simulations, Inc. A scaling factor of 1.39 was applied to the resulting charges.[44,46]

Initially, the atoms utilized for a quantum mechanical calculation on the human α-thrombin active site were selected on the basis of the thrombin modeled complex structure with 4-Cl-3-EtO-7-GuaIC (2) (described later). Initially, the largest active site fragment for which one SCF calculation completed successfully consists of 129 atoms and contains the residues shown as fragment I of Table 5. It is clear that many other residues considered important for the interaction of human α-thrombin with inhibitors need to be included.[28,42] To accomplish this, several additional fragments of the active site were utilized for quantum mechanical calculations. A successful SCF calculation was completed for four additional portions containing 133–150

Table 5. Fragments of the Human α-Thrombin Active Site Used in Quantum Mechanical Calculations[a]

fragment	residues	net charge
I	His-57 Lys-60F Asp-102 Asp-189 Gly-193 Asp-194 Ser-195 Ser-214	−1
II	Asp-189 Ala-190 Asp-194 Val-213 Ser-214 Gly-216 Gly-217 Gly-219 Cys-220 Gly-226	−3
partial residues[b]	Cys-191 Tyr-225 Phe-227	
III	His-57 Leu-99 Asp-102 Glu-192 Ser-195 Val-213 Ser-214 Trp-215	−1
IV	Cys-42 His-57 Cys-58 Tyr-60A Trp-60D Lys-60F Ser-195	+2
V	Tyr-60A Trp-60D Lys-60F Phe-60H	+1
partial residues[c]	Cys-58 Leu-59 Leu-60	

[a] One SCF (self-consistent field) calculation was performed on each fragment using the MNDO Hamiltonian[45] of MOPAC.[47] [b] The peptide carbonyl group of Cys-191 was replaced with H; the phenol group of Tyr-225 was replaced with H; the phenyl group of Phe-227 was replaced with H. [c] The S of Cys-58 was replaced with H; the Leu-59 and Leu-60 side chains were each replaced with CH_3.

atoms each. The residues for each fragment are listed in Table 5 (fragments II–V). Each fragment contains some residues which overlap with one of the other fragments. This enabled us to make sure that each fragment resulted in SCF charges consistent with those obtained from the other fragments. All peptide chains and isolated residues (those not part of a chain of consecutive residues) begin and end with NH_2. Only portions of some residues were included, and these are listed as partial residues in Table 5. Since the successful prediction of the potency of an isocoumarin inhibitor of PPE was obtained using a positive charged His-57,[46] a positive charged His-57 was used in the present study, and the total net charge for each fragment is shown in Table 5. Wherever possible the SCF charges selected for a residue were those obtained from a fragment where that residue was part of a chain of at least two consecutive residues. All cysteines utilized were connected to the corresponding cysteines via disulfide bonds. No waters were included in the calculations.

To obtain the initial modeled structures of human α-thrombin complexed with the inhibitor 4-Cl-3-EtO-7-GuaIC (**2**), the isocoumarin benzoyl ester carbonyl group was oriented at an angle (α) of 100.43° relative to the Ser-195 O_γ with the carbonyl C–O_γ distance (d) set at 2.2 Å. The benzoyl ester carbonyl O of compound **2** was located at distances of 1.96 (d_1) and 2.01 Å (d_2), respectively, from the Ser-195 and Gly-193 >NH hydrogens. Only the hydrogens in one of the guanidinium NH_2 groups were within 5 Å of the Asp-189 carboxylate oxygens. One hydrogen was 2.86 Å from the Asp-189 O_{δ_2}. For $PhCH_2CONH$-CiTEtOIC (**16**), the benzoyl ester carbonyl group was oriented with α = 105.32° and d = 2.45 Å. The oxyanion hole distances d_1 and d_2 were set at 2.41 and 2.23 Å, respectively. The terminal hydrogens of the positive charged isocoumarin substituent were closer to the Asp-189 O_{δ_2} as for 4-Cl-3-EtO-7-GuaIC (**2**). The distances for the Asp-189 O_{δ_2}–terminal hydrogen ranged from 3.37 to 4.73 Å. The orientations of the isocoumarins **2** and **16** were similar to those used previously with PPE.[29] Each of the remaining isocoumarins were docked into the human α-thrombin active site by a pairwise, least-squares superimposition of their benzene atoms on the corresponding atoms of compound **16** complexed with human α-thrombin. Each isocoumarin was then translated to 64 different positions within the thrombin active site, and the resulting inhibitor–enzyme complex structures were energy minimized as described previously.[46] The default value of 1.0 for the dielectric of the Coulombic potential was used for the reasons discussed earlier.[44,46] To minimize the computational time, no further waters were added to the 423 crystallographic waters included with the thrombin structures. Complexes with minimized energies ≤ −1490 kcal/mol and isocoumarin benzoyl ester carbonyl C–Ser-195 O_γ distance (d) < 2.85 Å were selected as modeled productive complexes. An energy cutoff of −1490 kcal/mol was selected on the basis of an examination of the total energy distribution of the complex structures as described previously.[44] The smallest d possible was chosen such that more than one low-energy complex structure for each isocoumarin would be selected as productive.

The quantum mechanical calculations were performed on a Digital Equipment Corp. VAX 6440 using MOPAC 6.0.[47] The inhibitor translations, energy minimizations, viewing of the various structures, H-bond calculations, and stereoview generation were conducted using the software and hardware described previously.[46] Insight II version 2.1.0 from Biosym Technologies, Inc. (San Diego, CA) was used to generate the stereoview. The criteria for the formation of good H-bonds are those described earlier.[48]

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(2) Abbreviations used: ACITIC (NH_2-CiTPrOIC), 7-amino-4-chloro-3-(3-isothioureidopropoxy)isocoumarin; BABCH, 2,7-bis(4-amidinobenzylidene)cycloheptanone; Boc, *tert*-butyloxycarbonyl; DABE, 1,2-diamidino-2-benzofuranylethane; CiTPrOIC, 4-chloro-3-(3-isothioureidopropoxy)isocoumarin; 4-Cl-3-EtO-7-GuaIC, 4-chloro-3-ethoxy-7-guanidinoisocoumarin; DCI, 3,4-dichloroisocoumarin; Dns, [5-(dimethylamino)-1-naphthylsulfonyl; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); Hepes, 4-(2-hydroxyethyl)1-piperazineethanesulfonic acid; MQPA, 4-methyl-1-[N^α-[(3-methyl-1,2,3,4-tetrahydro-8-quinolinyl)sulfonyl]-L-arginyl]-2-piperidinecarboxylic acid; NAPAP, 2-(naphthylsulfonyl)-Gly-4-amidinophenylalaninepiperidine amide; NPGB, *p*-nitrophenyl *p*′-guanidinobenzoate; PCPS, phosphatidylcholine–phophatidylserine; PPE, porcine pancreatic elastase; SCF, self-consistent field; SBzl, thiobenzyl ester; TAPAM, N^α-(tosylglycyl)-3-amidinophenylalanine methyl ester; Z, benzyloxycarbonyl.

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