acid precursor relative to **2**, a mixture of [1-^{14}C]-D-erythrose (5.5 μCi), **13a**, and [1-^{13}C]-D-erythrose (336 mg), **13b**,[18] was administered and afforded 8 mg of **2c**. From the ^{13}C NMR spectrum it was clear that **2c** was only labeled at C-1 (5.13%), rather than at C-5. Thus, the nitrogen substituent of **2** had been introduced at C-6 of the shikimate intermediate, rather than at C-2. This is consistent with the involvement of either **4**, **10**,[19] or anthranilic acid, **14**.[20]

S. LL-C10037 was next fermented (2 × 200 mL broths) in the presence of ^{18}O molecular oxygen,[21] yielding 10 mg of **2d**. The ^{13}C NMR spectrum exhibited ^{18}O-induced isotope shifts[22] for C-4 (2.0 Hz), C-5 (3.5 Hz), and C-6 (3.0 Hz), revealing that the secondary alcohol and epoxide oxygens had been introduced by oxidative processes rather than having been retained from the organic precursor. The origin of the C-4 hydroxyl from molecular oxygen ruled out **4** as an intermediate.

[5-^{2}H]-3-Hydroxyanthranilic acid **15**[23] was then fed (200 mg to 200 mL of broth) yielding[24] 5.0 mg of **2e** that was analyzed by ^{2}H NMR.[25] A 67% enrichment of the signal at δ 3.8 (H-5) was observed, representing a 2.2% incorporation of **15** (50 mg was recovered).

The biosynthesis of **2** represents a novel aromatic amino acid metabolism. In view of our work, metabolites such as paulomycin A,[26] the enaminomycins,[27] MM14201,[28] and G7063-2[29] are most likely also derived from the shikimate pathway. We are currently investigating the biosynthesis of the latter two.

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(20) Reference 16, pp 138-139, 160-164.
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(23) Obtained by exchange (86%) with deuteriotrifluoroacetic acid at 80 °C in a sealed tube for 48 h. In the ^{1}H NMR, the dd at δ 7.44 was nearly gone and the signals at δ 7.33 and 7.82, previously doublets, were now singlets.
(24) The fermentation was harvested 135 h after innoculation.
(25) Spectrum obtained at 61.4 MHz (sweep width = 952 Hz, 4 K data points zero filled to 8 K, pulse width = 90°, acquisition time = 21.5 s, no. scans = 3000).
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Mechanism-Based Isocoumarin Inhibitors for Trypsin-like Serine Proteases Involved in Blood Coagulation†

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Human plasma contains a number of proteins (zymogens) which are precursors of serine proteases with trypsin-like specificity. The interaction of activated serine proteases with these zymogens and with natural plasma protease inhibitors in a cascade of enzymatic reactions forms the basis of the blood coagulation pathway. Although intravascular clotting is a major health problem in the United States, almost no new anticoagulant drugs have been developed in recent years. A number of heterocyclic compounds have previously been shown to be suicide substrates of serine proteases.[1-6] Substituted isocoumarins have been reported to be mechanism-based inhibitors for elastases and a variety of chymotrypsin-like enzymes.[4,5] Here we report the synthesis of three new isocourmarins with basic functional groups (guanidino or isothiureidoethoxy) attached to the isocoumarin ring system. These compounds are potent inhibitors of coagulation enzymes and are the first effective mechanism-based inhibitors of clotting in human plasma.

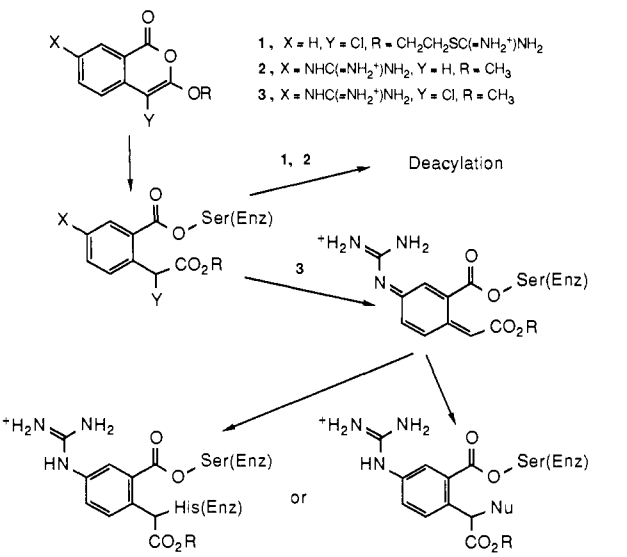

Figure 1. Proposed mechanism of inactivation of serine proteases by substituted isocoumarins.

Incubation of 4-chloro-3-(2-isothiureidoethoxy)isocoumarin (**1**), 7-guanidino-3-methoxyisocoumarin (**2**), and 4-chloro-7-guanidino-3-methoxyisocoumarin (**3**)[7] with several coagulation enzymes and trypsin results in time-dependent loss of enzymatic activity (Table I). Human protein D, human leukocyte elastase, human leukocyte cathepsin G, porcine pancreatic elastase, chymotrypsin, and human plasma plasmin were also inactivated by compound **3** with $k_{obsd}/[I]$ values of 120, 6400, 11000, 860, 7200, and 3600 $M^{-1}s^{-1}$, respectively. Compound **3** was quite selective among serine proteases and has its most potent inhibitory activity toward trypsin, thrombin, and human plasma kallikrein with $k_{obsd}/[I]$ values of 2-3 × 10^5 M^{-1} s^{-1}. It was an order of magnitude

† Dedicated to Professor George Büchi on the occasion of his 65th birthday.

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Table I. Rates of Inhibition of Trypsin-Like Serine Proteases by Substituted Isocoumarins[a]

	$k_{obsd}/[I]$ (M^{-1} s^{-1})						
inhibitor	bovine thrombin	bovine factor Xa	porcine pancreatic kallikrein	human plasma kallikrein	human factor XIa	human factor XIIa	bovine trypsin
1	4700	5600	12000	280000[b]	44000	39000	32000
2	4900	460	1900	13000	1400	520	3300
3	290000[c]	3100	45000[c]	240000[c]	36000	20000	310000[c]

[a] Enzyme (0.06–2.3 μM) was incubated with inhibitor (0.4–11 μM) in 0.25–0.6 mL of 0.1 M Hepes, 0.01 M $CaCl_2$, pH 7.5, and 8–12% Me_2SO at 25 °C. Aliquots (50 μL) were withdrawn at various intervals, and the residual enzymatic activity was measured as previously described.[4] Residual activity was measured with Z-Phe-Phe-Arg-NA (26 μM) as a substrate for trypsin, Boc-Phe-Phe-Arg-SBzl (18 μM) for thrombin, Z-Gly-Arg-SBu-*i* (73 μM) for factor Xa and porcine pancreatic kallikrein, Z-Trp-Arg-SBzl (89 μM) for factor XIIa and human plasma kallikrein, Z-Phe-Arg-SBu-*i* (83 μM) for factor XIa. The k_{obsd} values were calculated from plots of ln v/v_0 vs. time with $r > 0.98$. [b] Inactivation was extremely rapid, and the $k_{obsd}/[I]$ values were based on the residual enzymatic activity at 0.2 min. [c] Inactivation rate was measured by using the progress curve method.[12]

less reactive toward porcine pancreatic kallikrein, human factor XIa, and human factor XIIa, while the other serine proteases tested were 30–2000-fold less reactive. Compound **1** is equally potent as **3** toward human plasma kallikrein, human factor XIa, and human factor XIIa. Loss of the isocoumarin chromophore of **1** (ϵ_{350} = 3620 M^{-1} cm^{-1}) and **3** (ϵ_{360} = 3650 M^{-1} cm^{-1}) was complete within 0.3 min upon the addition of 1.2 equiv of trypsin. In the case of **2** (ϵ_{350} = 3400 M^{-1} cm^{-1}), complete loss of the isocoumarin absorbance occurred in 10 min. The inactivation of trypsin by compounds **1**, **2**, and **3** occurred concurrently with the loss of the isocoumarin absorbance.

Trypsin (0.9 μM) inactivated by **3** (4.3 μM, $t_{1/2}$ = 44 min for spontaneous hydrolysis) regained only 35% activity after standing for 68 h; however, trypsin (0.9–1.3 μM), inactivated by compounds **1** and **2** (9.8 and 8.6 μM, $t_{1/2}$ = 83 and 252 min for spontaneous hydrolysis, respectively), regained 70–100% activity after 6–68 h. Addition of buffered hydroxylamine (0.29 M) to trypsin inactivated by **3** results in the regeneration of 51% activity after 68 h, while addition of hydroxylamine to trypsin inactivated by **1** and **2** resulted in the recovery of 100% activity ($t_{1/2}$ = 7–9 min). Bovine thrombin (1.8 μM) inactivated by **3** (4.4 μM) regained 11% activity after standing for 47 h, while thrombin inactivated by **2** (8.9 μM) regained 85% activity after standing for 47 h. Addition of buffered hydroxylamine (0.29 M) to thrombin inactivated by **3** results in only 27% recovery of activity after 25 h, even though thrombin maintained 63% of its activity under the same conditions.

These results are consistent with mechanism shown in Figure 1 where serine proteases inactivated by isocoumarins **1** and **2** form acyl enzymes which deacylate slowly at varying rates or quickly upon the addition of hydroxylamine. However, the acyl enzyme formed from **3** decomposes further probably through a quinone imine methide[8] to give an irreversibly inactivated enzyme by reaction with an active site nucleophile such as His-57. The quinone imine methide intermediate could also react with a solvent nucleophile to give an acyl enzyme which can be reactivated by hydroxylamine.[5,9]

Compound **3** is an effective anticoagulant in human plasma. The prothrombin time[10] was prolonged from 12 s (first appearance of clotting) to 2.8 min in the presence of 33 μM **3**. The general serine protease inhibitor 3,4-dichloroisocoumarin prolonged the prothrombin time to 2 min at 330 μM. The isocoumarin **3** decomposes quite rapidly in human plasma ($t_{1/2}$ = 7 min), and preincubation of the inhibitor in plasma for 3 min resulted only in an 80% increase in prothrombin time.

The isocoumarins **1** and **3** developed in this study are the most potent inactivators yet reported for several of the coagulation enzymes studied, and **3** is the first mechanism-based serine protease inactivator which is active as an anticoagulant in human plasma.[11] Our future efforts will be directed at improving the selectivity and stability of the inhibitors in plasma and further clarifying the mechanism by structural studies of inhibitor–protease complexes.

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Supplementary Material Available: Experimental details for the synthesis of the new isocoumarins (3 pages). Ordering information is given on any current masthead page.

(8) Direct alkylation of the benzylic chloride would yield the same result and cannot be distinguished from a mechanism involving a quinone imine methide. A quinone imine methide mechanism has been postulated to explain the high reactivity of aminobenzyl halides, which decompose rapidly to 4-quinone imine methides and alkylate nucleophiles such as imidazole: (a) Wakselman, M.; Dome, M. *Bull. Soc. Chim. Fr.* **1975**, 571–575. (b) Dome, M.; Wakselman, M. *Bull. Soc. Chim. Fr.* **1975**, 576–582.

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Membrane-Spanning Steroidal Metalloporphyrins as Site-Selective Catalysts in Synthetic Vesicles

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Substrate recognition and binding have long been recognized as a means to induce regioselectivity and stereoselectivity in enzyme-mediated reactions. We have sought to develop synthetic biomimetic approaches to similar selectivity by using the organization intrinsic to phospholipid bilayers.[1] It has been shown by Tsuchida et al.[2] that amphiphilic metalloporphyrins can be encapsulated in synthetic liposomes. More recently, Nolte et al.[3] have demonstrated that hydrophobic membrane-bound metallo-

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