6-Substituted 2-Oxo-2*H*-1-benzopyran-3-carboxylic Acid as a Core Structure for Specific Inhibitors of Human Leukocyte Elastase[†]

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Pyridyl esters of 6-substituted 2-oxo-2*H*-1-benzopyran-3-carboxylic acid were designed as mechanism-based inhibitors of human leukocyte elastase. Compounds of series **4** specifically inhibited this enzyme. Several of the tested compounds (series **2** and **3**) acted as powerful time-dependent inhibitors of both human leukocyte elastase and α-chymotrypsin; some compounds of these series inhibited thrombin. Trypsin was not inhibited. A transient inactivation was observed for human leukocyte elastase (k_i/K_I = 107 000 $M^{-1}\cdot s^{-1}$ for **4c**) and thrombin (k_i/K_I = 7 200 $M^{-1}\cdot s^{-1}$ for **3b**) as demonstrated by spontaneous or hydroxylamine-accelerated reactivation, irrespective of the nature of the substituent at the 6-position. Conversely, α-chymotrypsin was irreversibly inhibited by 6-chloromethyl derivatives (k_i/K_I = 107 400 $M^{-1}\cdot s^{-1}$ for **3b**). The presence of a latent alkylating function at the 6-position (chloromethyl group) was required for leading to this inactivation. In the absence of such an alkylating function (series **4**), human leukocyte elastase was specifically inhibited suggesting that this new series of human leukocyte elastase inhibitors may be of potential therapeutic interest in degradative and degenerative processes involving this enzyme.

Introduction

Serine proteases are involved in numerous biological processes such as regulation of hemostasis and fibrinolysis (thrombin, plasmin, factor Xa), digestion (chymotrypsin, trypsin, pancreatic elastase), and phagocytosis (leukocyte elastase, cathepsin G). When the control of proteolysis is deficient, diseases may occur. For example, low concentrations of α_1-proteinase inhibitor, the main plasmatic natural inhibitor of human leukocyte elastase (HLE, EC 3.4.21.37), have been found to be involved in the pathogenesis of pulmonary emphysema, chronic bronchitis, and rheumatoid arthritis.[1] Lack of inhibition of human thrombin (THR, EC 3.4.21.5) may also lead to thrombosis.[2] Therefore, agents able to modulate these uncontrolled proteolytic activities are of therapeutic interest. Inhibitors from different chemical classes and acting through various mechanisms have been described: affinity labels,[3−5] transition-state analogues,[6−8] and mechanism-based inhibitors. In this last category, alternate substrates and suicide substrates include benzoxazinones,[9,10] haloenol lactones,[11,12] isocoumarins,[13] dihydrocoumarins,[14] saccharin derivatives,[15−18] monocyclic[19−24] or bicyclic[25−27] β-lactams, and thiadiazolidinones.[28]

In an earlier work, we designed a series of alkyl or aryl esters and amides of 6-chloromethyl-2-oxo-2*H*-1-benzopyran-3-carboxylic acid that behaved as suicide substrates of α-CT (**1**, Chart 1).[29] These molecules displayed a latent electrophilic function at the 6-position (methylene quinone). In this paper, we present biochemical and mechanistic studies related to the inhibition of HLE, α-CT, and THR by pyridyl esters of the same acid (**2** and **3**, Chart 1) and by related structures displaying various substituents at the 6-position (**4**, Chart 1). We have now demonstrated that, according to the nature of these substituents, the coumarin derivatives may act as general inhibitors of serine proteases or as specific inhibitors of HLE.

Chemistry

In general, esterification of the appropriate coumarin-3-carboxylic acid derivative is obtained by treatment with thionyl chloride leading to the corresponding acid chloride. The latter is then converted into the ester by reacting with the appropriate hydroxy-substituted pyridine derivative. The synthesis of compounds listed in Table 1 was described using a previously reported method.[29]

The acyloxycoumarin-3-carboxylic acid derivatives were obtained by reaction of **5** with the appropriate acid chloride or with the appropriate acid anhydride (Scheme 1, **6**, pathways A and B).

The synthesis of compound **4h** was accomplished using a Delepine-type reaction (Scheme 2). Treatment of **2c** with hexamethylenetetramine led to the corresponding hexamethylenetetrammonium salt **7**, which in

[†] Abbreviations: HLE, human leukocyte elastase; α-CT, bovine α-chymotrypsin; THR, human thrombin; MeO-Suc-AAPV-*p*-NA, methoxysuccinyl-alanyl-alanyl-prolyl-valyl-*p*-nitroanilide; S-2238, H-D-phenylalanyl-pipecolyl-arginyl-*p*-nitroanilide; Suc-AAPF-*p*-NA, succinyl-alanyl-alanyl-prolyl-phenylalanyl-*p*-nitroanilide; BA-*p*-NA, benzoyl-arginyl-*p*-nitroanilide.

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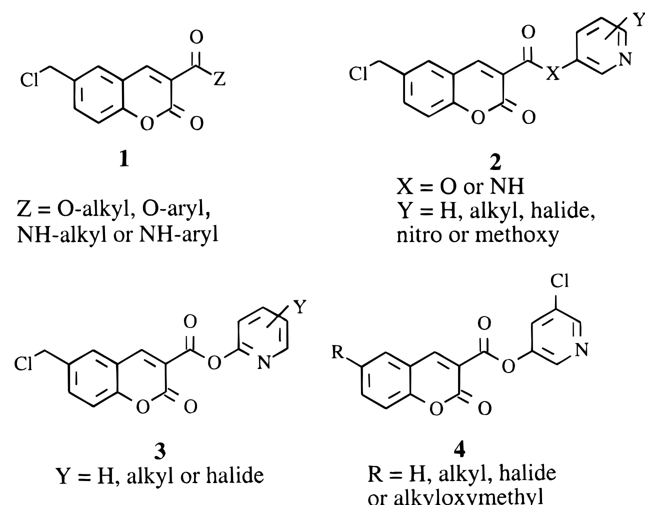
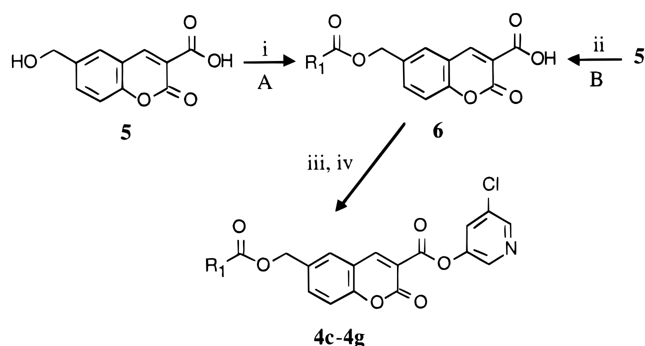
Chart 1. General Structure of Coumarin Derivatives



Scheme 1[a]



[a] Reagents: (i) $(R_1CO)_2O$; (ii) R_1COCl/dioxane/pyridine; (iii) $SOCl_2$; (iv) 5-chloro-3-pyridinol/pyridine/dioxane.

turn was converted into the 6-aminomethyl-substituted compound **4h.**

Results and Discussion

The inhibitory potency of the newly synthesized compounds **2a**–**2k**, **3a**–**3e**, and **4a**–**4i** toward HLE, THR, trypsin, and α-CT (Tables 1–3) was evaluated. Compounds **2a**–**2h** and **3a**–**3e** behaved as time-dependent inhibitors of α-CT and HLE, except for **2b** and **2f**. HLE was specifically inhibited by compounds **4a**–**4i**. Compounds **2c**, **3a**, **3b**, and **3e** led to time-dependent inhibition of THR. Amide-type coumarins (**2i**–**2k**) were devoid of inactivatory efficiency. Trypsin was not inhibited by the tested compounds. No spontaneous reactivation of inactivated α-CT was observed, whereas HLE and THR reactivated slowly.

6-Chloromethyl-2-oxo-*2H*-1-benzopyran-3-carboxylic Acid Derivatives (Series 2 and 3). 1. Inactivation Kinetics. The kinetic parameters k_i/K_I, k_i, and K_I were determined either by the progress curve method (Figure 1) or by the preincubation method as listed in Tables 1 and 2 for pyrid-3′-yl and pyrid-2′-yl derivatives, respectively. For HLE, the determined kinetic parameters correspond to the first step of the enzyme–inhibitor reaction, i.e., the inactivation process (this step was followed by the slow reactivation of the enzyme).

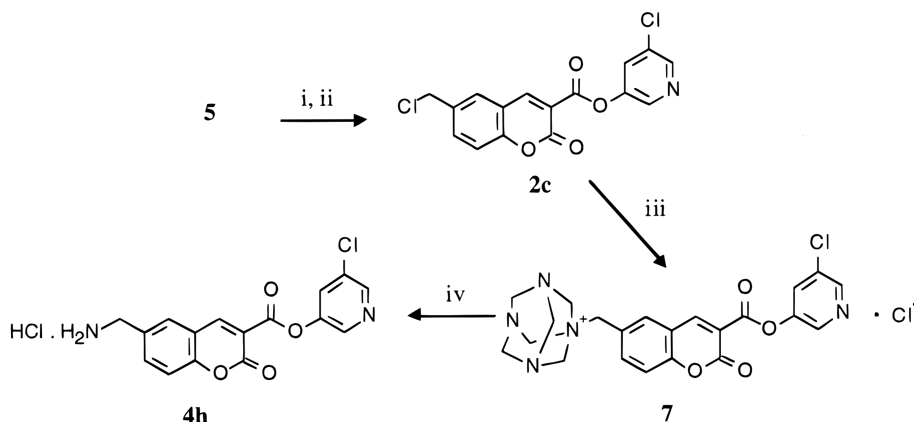
It was noticeable that the presence of a pyridine group as substituent of the exocyclic ester function was essential for leading to HLE inhibitors in this series of 6-chloromethyl-2-oxo-*2H*-1-benzopyran-3-carboxylic acid derivatives. In a previous work, we found that alkyl and phenyl esters of 6-chloromethyl-2-oxo-*2H*-1-benzopyran-3-carboxylic acid failed to efficiently inhibit HLE,[29] whereas the phenyl esters acted as powerful inactivators of α-CT (compound **1a**, Table 1). As observed with alkyl and phenyl esters against α-CT, the corresponding amides (**2i**–**2k**) did not lead to any significant inhibition of either HLE or α-CT. Some of the pyridyl esters were also good inactivators of α-CT (k_i/K_I = 107 400 $M^{-1}\cdot s^{-1}$ for **3b**), and four compounds moderately inactivated THR (**2c**, **3a**, **3b**, and **3e**). Within these two series, compounds **2c** and **3b** were the most powerful compounds toward α-CT and HLE. The partition ratio *r* was determined for the inhibition of α-CT by these molecules and was found to be equal to 6.1 (**2c**) and 5.2 (**3b**). Compounds **2c** and **3b** were also tested against cathepsin G which has the same primary specificity as α-CT, but no inhibition was observed.

2. Substituent Effects. For α-CT and HLE, the inhibitory potency was lower when pyridine was substituted in the ′*para*′ position relative to the oxygen atom of the exocyclic ester function (compounds **2b**, **2f**, and **3c**). A same ′*para*′ substituent effect was already noted for phenyl esters of 6-chloromethyl-2-oxo-*2H*-1-benzopyran-3-carboxylic acid.[29]

Substitution by a chlorine atom of the ′*meta*′ position relative to the ester oxygen atom for 6′-chloropyrid-2′-yl and 5′-chloropyrid-3′-yl esters led to good inhibitory efficiency toward α-CT, HLE, and THR as was observed for *m*-chlorophenyl ester (**1b**, Z = *O*-(*m*-ClC_6H_4), Chart 1).[29] With this substituent, the aryl preference was for α-CT: phenyl (**1b,** 762 700 $M^{-1}\cdot s^{-1}$)[29] > pyrid-2′-yl (**3b**, 104 700 $M^{-1}\cdot s^{-1}$) > pyrid-3′-yl (**2c**, 30 970 $M^{-1}\cdot s^{-1}$); for HLE: pyrid-3′-yl (**2c**, 64 600 $M^{-1}\cdot s^{-1}$) > pyrid-2′-yl (**3b**, 26 000 $M^{-1}\cdot s^{-1}$) ≫ phenyl (**1b**, 630 $M^{-1}\cdot s^{-1}$);[29] for THR: phenyl (**1b**, 21 000 $M^{-1}\cdot s^{-1}$, unpublished result) > pyrid-2′-yl (**3b**, 7 200 $M^{-1}\cdot s^{-1}$) > pyrid-3′-yl (**2c**, 200 $M^{-1}\cdot s^{-1}$) (same order as for α-CT). Substitution of the ′*meta*′ position by a methyl group (**2h** in the pyrid-3′-yl series and **3d** in the pyrid-2′-yl series) led to a noticeable decrease of the inhibitory potency on α-CT as well as on HLE (≈65-fold for **2h** versus **2c** and 130-fold for **3d** versus **3b** in the case of HLE). The efficiency was comparable to that observed in the absence of any substituent (**2a** and **3a**). As a result, the presence of the lipophilic and electron-withdrawing chlorine in the ′*meta*′ position seems to be highly favorable for the inhibition of serine proteases. According to the nature of the aromatic ring (phenyl, pyrid-3′-yl, and pyrid-2′-yl), a relative but not absolute discrimination between the different classes of proteases was achieved, the pyrid-3′-yl substituent being the best choice for targeting HLE.

Inactivation of Serine Proteases by 5′-Chloropyrid-3′-yl Esters of 6-Substituted 2-Oxo-*2H*-1-benzopyran-3-carboxylic Acid (Series 4). Since the inactivation of HLE was transient, the presence of a latent electrophilic function (methylene quinone) at the 6-position did not appear to be essential for the inhibitory activity. The 5′-chloropyrid-3′-yl substituent being favorable, we have synthesized 5′-chloropyrid-3′-yl ester

Scheme 2[a]



[a] Reagents: (i) $SOCl_2$; (ii) 5-chloro-3-pyridinol/pyridine/dioxane; (iii) hexamethylenetetramine/$CHCl_3$; (iv) C_2H_5OH/HCl.

Table 1. Inhibition of HLE, α-CT, and THR by Pyrid-3′-yl Derivatives[a]

1a 2

no.	X	Y	mp (°C)	k_i/K_I ($M^{-1}\cdot s^{-1}$) HLE	α-CT	THR
1a			187−191	23	100000	200
2a	O	H	210[b]	770	14800	NI[c]
				(0.017 s^{-1}; 22 μM)	(0.055 s^{-1}; 3.7 μM)	
2b	O	6′-CH_3	205−208	NI	≈1000	NI
2c	O	5′-Cl	195−198	64600	30970	≈200
				(0.0172 s^{-1}; 0.266 μM)	(0.024 s^{-1}; 0.775 μM)	
2d	O	2′-Cl	198−200	3310	4300	
				(0.0096 s^{-1}; 2.9 μM)	(0.034 s^{-1}; 7.9 μM)	
2e	O	2′-Br	197−199	3000	2700	
				(0.045 s^{-1}; 15 μM)	(0.018 s^{-1}; 6.7 μM)	
2f	O	2′-NO_2-6′-CH_3	209−211	NI	≈1500	
2g	O	2′-NO_2	190−193	≈4000	≈4500	
2h	O	5′-CH_3	211[b]	≈1000	≈2500	NI
2i	NH	H	>270	NI	*d*	
2j	NH	2′-Cl	211[b]	NI	NI	
2k	NH	6′-OCH_3	225[b]	NI	NI	

[a] In parentheses: k_i (s^{-1}) and K_I (μM); standard errors are less than 15%. [b] Decomposition temperature. [c] No inactivation at 10 μM or at maximum solubility of the inhibitor in the buffer. [d] 30% inhibition at 10 μM and 10 min.

derivatives of 2-oxo-*2H*-1-benzopyran-3-carboxylic acid containing different substituents at the 6-position of the coumarin ring (Table 3).

1. Efficiency toward HLE. As predicted, these molecules (**4a**−**4i**) behaved as transient inactivators of HLE. Their inhibitory potency (except for **4h**) was similar to that of **2c**. Therefore, whatever the length and the bulk of the R substituent, the efficiency was not modified. Compound **4h** which displays an aralkylamine base is expected to mainly exist at pH 8 in its protonated ammonium form. Such a positively charged group may explain the low activity of **4h**.

2. Selectivity of Inhibition toward HLE. It should be noted that, in the absence of a latent alkylating function, 5′-chloropyrid-3′-ylcoumarinic derivatives were specific inhibitors of HLE. Indeed, compounds **4a**−**4i** did not inactivate THR or α-CT. It was found that coumarins **4a**−**4h** behaved as substrates of α-CT (estimated value of k_{cat}/K_m = 17 500 $M^{-1}\cdot s^{-1}$ for **4b**) with no accumulation of acyl-enzyme indicating that neither compound behaved as an acylating agent. The Michaelis

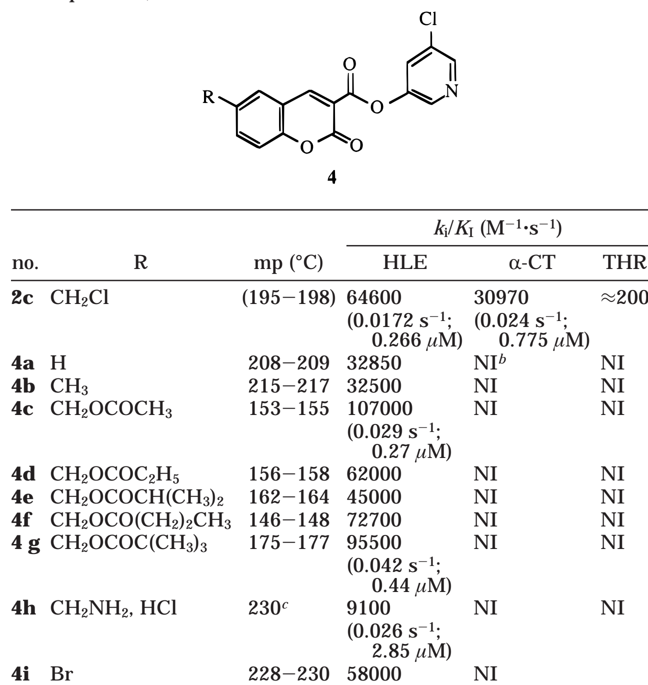
Table 2. Inhibition of HLE, α-CT, and THR by Pyrid-2′-yl Derivatives[a]

3

no.	Y	mp (°C)	k_i/K_I ($M^{-1}\cdot s^{-1}$) HLE	α-CT	THR
3a	H	125−129	≈420	1670	≈240
3b	6′-Cl	172−173	26000	107400	7200
				(0.029 s^{-1}; 0.27 μM)	(0.044 s^{-1}; 6.1 μM)
3c	5′-Cl	171−172	110	≈2500	NI[b]
3d	6′-CH_3	160−162	200	3300	NI
3e	4′-CH_3	152−154	≈300	5950	3900
					(0.012 s^{-1}; 3.1 μM)

[a] In parentheses: k_i (s^{-1}) and K_I (μM); standard errors are less than 15%. [b] No inactivation at 10 μM or at maximum solubility of the inhibitor in the buffer.

Table 3. Inhibition of HLE, α-CT, and THR by 5′-Chloropyrid-3′-yl Derivatives (variation of the substituent at the 6-position)[a]



no.	R	mp (°C)	k_i/K_I ($M^{-1}\cdot s^{-1}$) HLE	α-CT	THR
2c	CH_2Cl	(195−198)	64600 (0.0172 s^{-1}; 0.266 μM)	30970 (0.024 s^{-1}; 0.775 μM)	≈200
4a	H	208−209	32850	NI[b]	NI
4b	CH_3	215−217	32500	NI	NI
4c	CH_2OCOCH_3	153−155	107000 (0.029 s^{-1}; 0.27 μM)	NI	NI
4d	$CH_2OCOC_2H_5$	156−158	62000	NI	NI
4e	$CH_2OCOCH(CH_3)_2$	162−164	45000	NI	NI
4f	$CH_2OCO(CH_2)_2CH_3$	146−148	72700	NI	NI
4 g	$CH_2OCOC(CH_3)_3$	175−177	95500 (0.042 s^{-1}; 0.44 μM)	NI	NI
4h	CH_2NH_2, HCl	230[c]	9100 (0.026 s^{-1}; 2.85 μM)	NI	NI
4i	Br	228−230	58000	NI	

[a] In parentheses: k_i (s^{-1}) and K_I (μM); standard errors are less than 15%. [b] No inactivation at 10 μM or at maximum solubility of the inhibitor in the buffer. [c] Decomposition temperature.

constants K_m were then established by evaluating the competitive effect of **4a**−**4h** on the α-CT hydrolysis of the chromogenic substrate (in this case the inhibition constant K_i is equal to K_m).[30] This constant K_i was obtained for **4c** from a Dixon plot that confirmed the competitive inhibition. For **4a**, **4b**, and **4d**−**4h**, K_i was obtained by determination of the IC_{50} values using the Cheng−Prusoff relation.[31] It was found that $K_i > 20$ μM for **4b** and K_i = 17, 3.8, 2.6, 1.6, 2.1, 1.6, and 15 μM for **4a** and **4c**−**4h**, respectively.

Irreversible or Transient Inactivation Induced by Compounds of Series 2−4. The reversible or irreversible character of the inhibition toward HLE, α-CT, and THR was analyzed. After reaction with compounds **2c** and **3b**, no reactivation of α-CT was observed, either spontaneously after a 46-h incubation or after 1−2-h treatment with NH_2OH (pH 7.5, 25 °C) of the inhibited enzyme. This was in agreement with an irreversible process since an inactivation due to a simple formation of a stable acyl-enzyme was excluded. Moreover, increasing amounts of chromogenic substrate protected the enzyme against inactivation. Conversely, HLE inhibited by **2c** underwent a slow spontaneous reactivation and a more rapid one after treatment with NH_2OH, leading to an enzymic material displaying the same activity as the noninactivated HLE treated in the same experimental conditions. These results are consistent with the formation of a stable acyl-enzyme leading to a transient inactivation. Deacylation kinetics were performed. On standing in buffer, HLE treated with a coumarin inhibitor (**2c**−**e** and **4a**−**d**) remained inactivated for 50−80 min before a slow reactivation occurred (Figure 2). This deacylation was a first-order process characterized by rate constants k ranging from

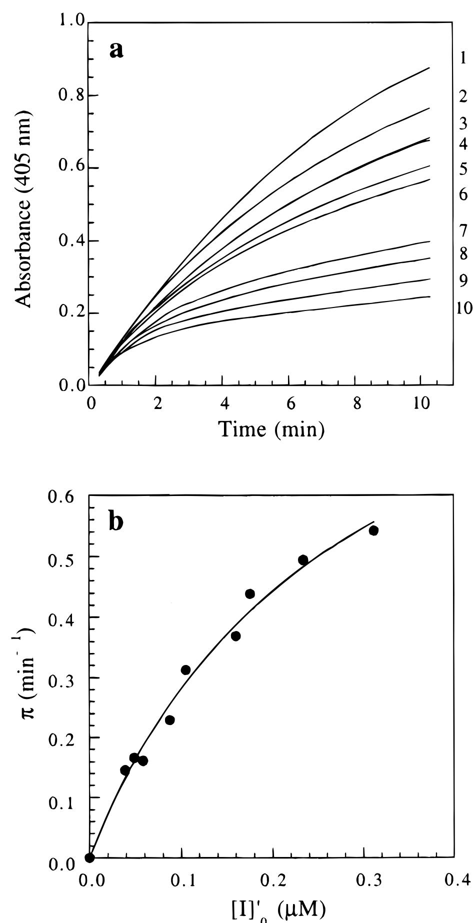


Figure 1. Inactivation of HLE (30 nM) by **2c** at pH 8.0 and 25 °C. The enzyme was added in 0.1 M Hepes buffer (0.5 M NaCl, 0.1% Tween 80, 10% v/v DMSO) containing the chromogenic substrate MeO-Suc-AAPV-*p*-NA (100 μM) and different concentrations of **2c** (0.11−0.89 μM). (a) Absorbance variation at 405 nm as a function of time in the absence (curve 1) or presence of increasing concentrations of **2c** (curves 2−10). (b) Determination of the kinetic parameters k_i and K_I by fitting the experimental data to the equation: $\pi = k_i[I]'_0/(K_I + [I]'_0)$, with $[I]'_0 = [I]_0/(1 + [S]_0/K_m)$.

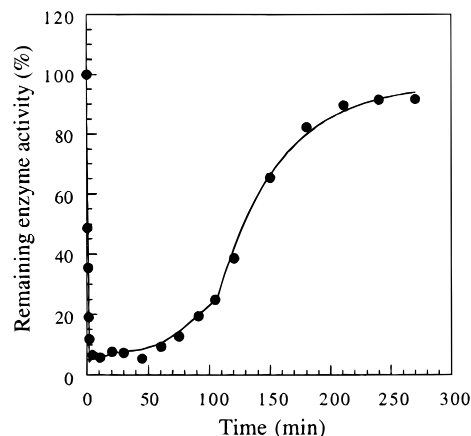


Figure 2. Inactivation and spontaneous reactivation of HLE inhibited by **4b** at pH 8.0 and 25 °C. $[HLE]_0$ = 100 nM; $[\mathbf{4b}]_0$ = 750 nM.

1.2×10^{-4} to 5.2×10^{-4} s^{-1}. The inactivation of THR may also be described by an analogous mechanism: after inactivation by **3b** or **3e** and further treatment with NH_2OH, this enzyme displayed the same activity as a control.

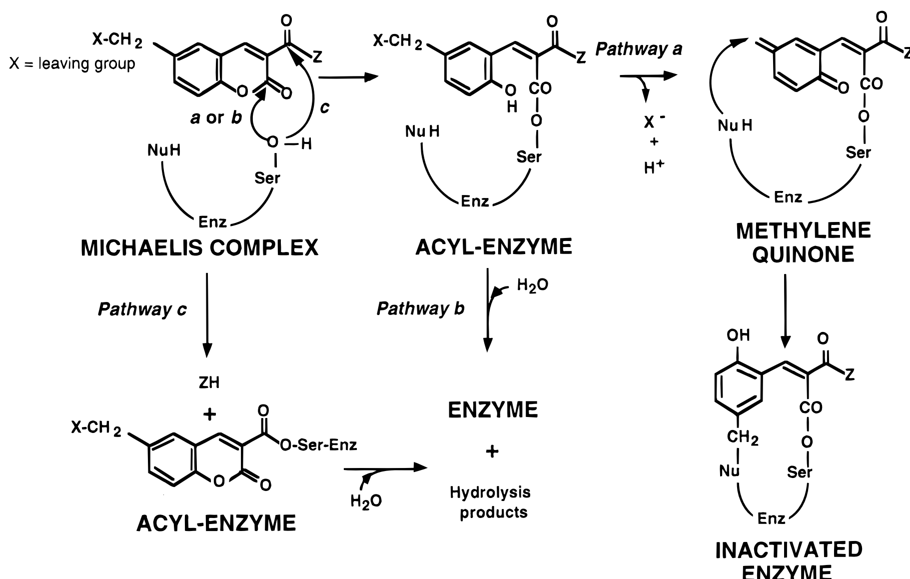


Figure 3. Postulated mechanisms for the inhibition of serine proteases by coumarin derivatives. NuH, nucleophile.

HPLC Analysis of the HLE Reaction Mixture with 2c. After incubation of compound **2c** with HLE during 15 min, the HPLC analysis of the reaction medium showed that **2c** had completely disappeared while a quantitative apparition of 5-chloro-3-pyridinol was detected accompanied by a peak corresponding to 6-chloromethyl-2-oxo-*2H*-1-benzopyran-3-carboxylic acid. Their identification was made by comparison with authentic compounds (retention times and UV−visible spectra). In the absence of enzyme, these two compounds were detected in smaller amounts.

Mechanisms of Inhibition. The analysis of the differential interaction with α-CT, HLE, and THR of coumarin derivatives **2**−**4** points out major differences in the characteristics of the enzyme-mediated process of inhibition which depend on the nature of the targeted enzyme. In the presence of α-CT, a time- and concentration-dependent inhibition by various compounds **2** and **3** was observed, with no significant spontaneous (or hydroxylamine-induced) recovery of activity. This excludes an inactivation process through formation of a stable acyl-enzyme and favors an alkylation reaction. First-order kinetics were observed fitting to the minimal kinetic scheme described in eq 2 (Materials and Methods). Moreover, increasing amounts of chromogenic substrate protected the enzyme against inactivation demonstrating that the inactivation process involved the active site. Consequently, criteria expected for suicide-type inactivation[32] are met in agreement with the postulated mechanism described in Figure 3 (pathway a). Nucleophilic attack of the coumarin carbonyl group by active serine leads to an acyl-enzyme and simultaneously unmasks a *p*-hydroxybenzyl halide. Nucleophilic substitution by an active-site amino acid residue may occur by 1,6-addition−elimination involving a powerful electrophilic methylene quinone intermediate. Alternatively, the enzyme activity may be restored after water-mediated deacylation of the acyl-enzyme (partition ratios of 6.1 and 5.2 were obtained for **2c** and **3b**, respectively). The inactivatory efficiency toward α-CT was usually lowered for pyridyl esters when compared to phenyl ones.[29]

A time-dependent interaction of coumarin derivatives was also found with HLE and THR, but in these cases, spontaneous (or hydroxylamine-induced) complete recovery of enzymatic activity was observed, supporting the formation of a stable acyl-enzyme leading to transient inactivation (Figure 3, pathway b). Conversely, a dihydrocoumarin-related compound (3-benzyl-6-chloromethyl-3,4-dihydrocoumarin) acted as an irreversible inhibitor of elastases (porcine pancreatic elastase and HLE),[14] urokinase,[33] and α-CT.[34] This was due to the formation of a covalent bond with catalytic histidine (His57) as previously demonstrated.[33,34] The surprising lack of alkylating activity of coumarin derivatives toward HLE may be due to the more restricted conformational mobility in the acyl-enzyme with coumarin derivatives if compared to the 3,4-dihydrocoumarin one (unsaturated 3−4 covalent bond in coumarins versus saturated 3−4 covalent bond allowing free rotation in the dihydrocoumarin derivative). An adequate proximity of the methylene quinone group with the catalytic histidine enabling the formation of a covalent linkage may be prevented in the active site of HLE and THR but not in that of α-CT. This latter enzyme is known to accommodate larger groups than HLE in its S1 subsite; THR is reported as an enzyme of restricted specificity.

An alternative explanation for the differential behavior of coumarin derivatives toward the tested enzymes may be the reaction of the active serine of HLE and THR with the carbonyl group of the exocyclic ester function (Figure 3, pathway c) instead of the lactone carbonyl group (Figure 3, pathway b). This possibility was explored by analyzing the reaction mixtures of HLE inhibited with coumarin derivatives. The results support a reaction of the enzyme with the exocyclic ester since the low-molecular-weight products formed from the reaction of HLE with **2c** were found to be 6-chloromethyl-2-oxo-*2H*-1-benzopyran-3-carboxylic acid and 5-chloro-3-pyridinol. These results suggest that pathway c is the major route to describe the transient inactivation of HLE. Nevertheless, an attack of the lactone by the active serine cannot be totally excluded: the low aqueous solubility of **2c** did not allow the preparation of

highly concentrated solutions of this compound, thus products generated in low quantities may be undetectable in HPLC experiments. Moreover, the hydrolysis product generated in the latter case results from ring opening of the lactone nucleus of the coumarin moiety. We may expect that a spontaneous and immediate ring closure will regenerate the starting coumarin inhibitor which in turn will react again with the enzyme according to pathway b or c. Since pathway c leads to the irreversible formation of hydrolysis products, the final outcome after a period of time will correspond to the total conversion of the inhibitor into the pyridinol and the 3-carboxy-substituted coumarin derivative.

Conclusion

In summary, it appears that the incorporation of a pyridyl substituent into the exocyclic ester function of 6-substituted 2-oxo-*2H*-1-benzopyran-3-carboxylic acid has provided an effective means for targeting HLE. In the presence of a latent akylating function at the 6-position (chloromethyl group), α-CT was also inactivated. Conversely, HLE was specifically inhibited in the absence of such an alkylating function. Potency is achieved by rapid acylation combined with slow deacylation. Coumarin derivatives belong to the class of alternate substrate inhibitors of HLE. Their acylation efficiency was better than that observed with saccharin derivatives,[18] in the range of that observed with sulfone derivatives of 1,2,5-thiadiazolidin-3-one 1,1-dioxide[28] and benzisothiazolones,[16] and lower than that observed with *4H*-3,1-benzoxazin-4-ones.[9] 6-Substituted 2-oxo-*2H*-1-benzopyran-3-carboxylic acid appears to be a core structure suitable for the design of selective inhibitors of serine protease acting through different mechanisms according to the nature of the target enzyme. By combination of steric and electronic effects, improvements in acyl-enzyme stability may be reached.

Materials and Methods

Materials. Melting points were determined with a Büchi-Tottoli apparatus in open capillary tubes and are uncorrected. Analyses (C, H, N) were within ±0.4% of the theoretical values. The IR spectra were recorded in KBr on a Perkin-Elmer 1750 spectrophotometer. The ^{1}H NMR spectra were recorded on a Bruker AW 80 (80 MHz) in $CDCl_3$ (or DMSO-d_6) with TMS as an internal standard; chemical shifts are reported in δ values (ppm) relative to internal TMS.

HLE and THR were purchased from Elastin Products Co. (Owensville, MO) and Boehringer-Ingelheim, respectively; bovine α-CT and trypsin were from Sigma. Enzyme concentrations were determined by active-site titrations as described in ref 35 (HLE), ref 36 (α-CT), and ref 37 (THR, trypsin). The enzymes were assayed spectrophotometrically with the appropriate *p*-nitroanilide substrate: MeO-Suc-AAPV-*p*-NA (Sigma) for HLE, S-2238 (KABI) for THR, Suc-AAPF-*p*-NA (Sigma) for α-CT, and BA-*p*-NA (Sigma) for trypsin. The enzymatic reactions were followed in 0.1 M Hepes, 0.5 M NaCl, 0.1% (v/v) Tween 80, pH 8.0, for HLE; 0.01 M Hepes, 0.01 M Tris, 0.1 M NaCl, 0.1% (v/v) PEG_{6000}, pH 7.5, for THR; 0.025 M sodium phosphate, 0.05 M KCl, pH 7.5, for α-CT; 0.1 M Tris, 0.01 M $CaCl_2$, pH 7.5, for trypsin. All assays contained 10% (v/v) DMSO and were run at 25 °C in a Perkin-Elmer Lambda 5 or Kontron Uvikon 941 spectrophotometer equipped with a thermostated cell holder. Characterization of the hydrolysis products of the coumarins was performed by reverse-phase HPLC (C8 column, 5 μm, 250 × 4.6 mm; Interchrom) using Waters 600–996 photodiode array system equipped with Millenium 2100 software.

Pyrid-3-yl 6-Chloromethyl-2-oxo-2*H*-1-benzopyran-3-carboxylate (2a). The title compound was obtained as previously described[28] after the reaction of the acid chloride of 6-chloromethyl-2-oxo-*2H*-1-benzopyran-3-carboxylic acid (1.17 g, 4.54 mmol) with 3-pyridinol (0.47 g, 5 mmol) and pyridine (0.4 mL, 5 mmol). The crude product was crystallized in ethyl acetate:petroleum ether, 40–60 °C (0.51 g, 36%): mp 210 °C dec; IR 3067 (C–H arom), 1774 (C=O ester), 1757 (C=O, lactone), 1620, 1573, 1215 cm^{-1}; ^{1}H NMR (80 MHz, $CDCl_3$, TMS) 4.60 (s, 2H, CH_2Cl), 7.10–7.80 (m, 5H, 5-H, 7-H, 8-H, 4′-H, 5′-H), 8.40–8.60 (m, 2′-H, 6′-H). Anal. ($C_{16}H_{10}NO_4Cl$) C, H, N.

6-Methylpyrid-3-yl 6-Chloromethyl-2-oxo-2*H*-1-benzopyran-3-carboxylate (2b). The title compound was obtained as previously described[28] after the reaction of the acyl chloride of 6-chloromethyl-2-oxo-*2H*-1-benzopyran-3-carboxylic acid (1.17 g, 4.54 mmol) with 6-methyl-3-pyridinol (0.57 g, 5 mmol) and pyridine (0.4 mL, 5 mmol). The crude product was crystallized in chloroform (0.91 g, 61%): mp 205–208 °C; IR 3065 (C–H arom), 1759 (C=O ester), 1712 (C=O lactone), 1621, 1572, 1487, 1380 cm^{-1}; ^{1}H NMR (80 MHz, $CDCl_3$, TMS) 2.55 (s, 3H, CH_3), 4.60 (s, 2H, CH_2Cl), 7.10–7.80 (m, 5H, 5-H, 7-H, 8-H, 4′-H, 5′-H), 8.40 (bm, 1H, 2′-H), 8.60 (s, 1H, 4-H). Anal. ($C_{17}H_{12}NO_4Cl$) C, H, N.

5-Chloropyrid-3-yl 6-Chloromethyl-2-oxo-2*H*-1-benzopyran-3-carboxylate (2c). The title compound was obtained as previously described[28] after the reaction of the acyl chloride of 6-chloromethyl-2-oxo-*2H*-1-benzopyran-3-carboxylic acid (1.17 g, 4.54 mmol) with 5-choro-3-pyridinol (0.65 g, 5 mmol) and pyridine (0.4 mL, 5 mmol). The crude product was crystallized in ethyl acetate:petroleum ether, 40–60 °C (0.94 g, 59%): mp 195–198 °C; IR 3073 (C–H arom), 1777 (C=O ester), 1726 (C=O lactone), 1622, 1576, 1377, 1246, 1221 cm^{-1}; ^{1}H NMR (80 MHz, $CDCl_3$, TMS) 4.60 (s, 2H, CH_2Cl), 7.10–7.80 (m, 4H, 5-H, 7-H, 8-H, 4′-H), 8.45 (bs, 2H, 2′-H, 6′-H), 8.65 (s, 1H, 4-H). Anal. ($C_{16}H_9NO_4Cl_2$) C, H, N.

2-Chloropyrid-3-yl 6-Chloromethyl-2-oxo-2*H*-1-benzopyran-3-carboxylate (2d). The title compound was obtained as previously described[28] after the reaction of the acyl chloride of 6-chloromethyl-2-oxo-*2H*-1-benzopyran-3-carboxylic acid (1.17 g, 4.54 mmol) with 2-choro-3-pyridinol (0.65 g, 5 mmol) and pyridine (0.4 mL, 5 mmol). The crude product was crystallized in chloroform:petroleum ether, 40–60 °C (0.60 g, 38%): mp 198–200 °C; IR 3083 (C–H arom), 1774 (C=O ester), 1732 (C=O lactone), 1619, 1573, 1418, 1372 cm^{-1}; ^{1}H NMR (80 MHz, $CDCl_3$, TMS) 4.65 (s, 2H, CH_2Cl), 7.10–7.80 (m, 5H, 5-H, 7-H, 8-H, 4′-H, 5′-H), 8.55 (d, 1H, 6′-H), 8.75 (s, 1H, 4-H). Anal. ($C_{16}H_9NO_4Cl_2$) C, H, N.

2-Bromopyrid-3-yl 6-Chloromethyl-2-oxo-2*H*-1-benzopyran-3-carboxylate (2e). The title compound was obtained as previously described[28] after the reaction of the acyl chloride of 6-chloromethyl-2-oxo-*2H*-1-benzopyran-3-carboxylic acid (1.17 g, 4.54 mmol) with 2-bromo-3-pyridinol (0.87 g, 5 mmol) and pyridine (0.4 mL, 5 mmol). The crude product was crystallized in chloroform:petroleum ether, 40–60 °C (1.05 g, 59%): mp 197–199 °C; IR 3080 (C–H arom), 1778 (C=O ester), 1732 (C=O lactone), 1619, 1572, 1411, 1372 cm^{-1}; ^{1}H NMR (80 MHz, $CDCl_3$, TMS) 4.65 (s, 2H,CH_2Cl), 7.20–7.80 (m, 5H, 5-H, 7-H, 8-H, 4′-H, 5′-H), 8.35 (d, 1H, 6′-H), 8.85 (s, 1H, 4-H). Anal. ($C_{16}H_9NO_4BrCl$) C, H, N.

6-Methyl-2-nitropyrid-3-yl 6-Chloromethyl-2-oxo-2*H*-1-benzopyran-3-carboxylate (2f). The title compound was obtained as previously described[28] after the reaction of the acyl chloride of 6-chloromethyl-2-oxo-*2H*-1-benzopyran-3-carboxylic acid (1.17 g, 4.54 mmol) with 6-methyl-2-nitro-3-pyridinol (0.77 g, 5 mmol) and pyridine (0.4 mL, 5 mmol). The crude product was crystallized in ethyl acetate:petroleum ether, 40–60 °C (1.15 g, 68%): mp 203–208 °C; IR 3079 (C–H arom), 1778 (C=O ester), 1724 (C=O lactone), 1624, 1574, 1554, 1375 cm^{-1}; ^{1}H NMR (80 MHz, $CDCl_3$, TMS) 2.70 (s, 3H, CH_3), 4.60 (s, 2H, CH_2Cl), 7.10–7.95 (m, 5H, 5-H, 7-H, 8-H, 4′-H, 5′-H), 8.70 (s, 1H, 4-H). Anal. ($C_{17}H_{11}N_2O_6Cl$) C, H, N.

2-Nitropyrid-3-yl 6-Chloromethyl-2-oxo-2*H*-1-benzopyran-3-carboxylate (2g). The title compound was obtained as

previously described[28] after the reaction of the acyl chloride of 6-chloromethyl-2-oxo-*2H*-1-benzopyran-3-carboxylic acid (1.17 g, 4.54 mmol) with 2-nitro-3-pyridinol (0.70 g, 5 mmol) and pyridine (0.4 mL, 5 mmol). The crude product was crystallized in ethyl acetate:petroleum ether, 40−60 °C (1.25 g, 76%): mp 190−193 °C; IR 3079 (C−H arom), 1778 (C═O ester), 1722 (C═O lactone), 1622, 1572, 1560, 1423, 1377 cm^{-1}; ^{1}H NMR (80 MHz, $CDCl_3$, TMS) 4.60 (s, 2H, C*H*$_2$Cl), 7.05−8.00 (m, 5H, 5-H, 7-H, 8-H, 4′-H, 5′-H), 8.55 (d, 1H, 6′-H), 8.75 (s, 1H, 4-H). Anal. ($C_{16}H_9N_2O_6Cl$) C, H, N.

5-Methylpyrid-3-yl 6-Chloromethyl-2-oxo-2*H*-1-benzopyran-3-carboxylate (2h). The title compound was obtained as previously described[28] after the reaction of the acyl chloride of 6-chloromethyl-2-oxo-2H-1-benzopyran-3-carboxylic acid (1.17 g, 4.54 mmol) with 5-methyl-3-pyridinol (0.58 g, 5 mmol) and pyridine (0.4 mL, 5 mmol). The crude product was crystallized in chloroform:petroleum ether, 40−60 °C (0.56 g, 37%): mp 211 °C dec; IR 3058, 3039 (C−H arom), 1760 (C═O ester), 1713 (C═O, lactone), 1621, 1572,1376 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$, TMS) 2.41 (s, 3H, C*H*$_3$), 4.67 (s, 2H, C*H*$_2$-Cl), 7.42 (d, 1H, 8-H), 7.46 (s, 1H, 4′-H), 7.72 (s, 1H, 5-H), 7.74 (d, 1H, 7-H), 8.39 (s, 2H, 2′-H, 6′-H), 8.74 (s, 1H, 4-H). Anal. ($C_{17}H_{12}NO_4Cl$) C, H, N.

***N*-(Pyrid-3-yl)-6-chloromethyl-2-oxo-2*H*-1-benzopyran-3-carboxamide (2i).** The title compound was obtained as previously described[28] after the reaction of the acyl chloride of 6-chloromethyl-2-oxo-*2H*-1-benzopyran-3-carboxylic acid (1.17 g, 4.54 mmol) with 3-aminopyridine (0.47 g, 5 mmol) and pyridine (0.4 mL, 5 mmol). The crude product was crystallized in ethyl acetate:petroleum ether, 40−60 °C (0.98 g, 69%): mp 270 °C dec; IR 3207 (N−H), 3037 (C−H arom), 1707 (C═O lactone), 1664 (C═O amide), 1592, 1573, 1548, 1423 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$, TMS) 4.68 (s, 2H, C*H*$_2$Cl), 7.34 (dd, 1H, 5′-H), 7.48 (d, 1H, 8-H), 7.76 (d, 1H, 7-H), 7.77 (s, 1H, 5-H), 8.27 (d, 1H, 4′-H), 8.43 (d, 1H, 2′-H), 8.86 (d, 1H, 6′-H), 9.02 (s, 1H, 4-H), 10.90 (s, 1H, N*H*). Anal. ($C_{16}H_{11}N_2O_3Cl$) C, H, N.

***N*-(2-Chloropyrid-3-yl)-6-chloromethyl-2-oxo-2*H*-1-benzopyran-3-carboxamide (2j).** The title compound was obtained as previously described[28] after the reaction of the acyl chloride of 6-chloromethyl-2-oxo-*2H*-1-benzopyran-3-carboxylic acid (1.17 g, 4.54 mmol) with 3-amino-2-chloropyridine (0.64 g, 5 mmol) and pyridine (0.4 mL, 5 mmol). The crude product was washed with methanol (1.4 g, 88%): mp 211 °C dec; IR 3177 (N−H), 3079 (C−H arom), 1732 (C═O lactone), 1660 (C═O amide), 1621, 1584, 1574, 1536, 1392 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$, TMS) 4.68 (s, 2H, C*H*$_2$Cl), 7.32 (dd, 1H, 5′-H), 7.48 (d, 1H, 8-H), 7.76 (d, 1H, 7-H), 7.77 (s, 1H, 5-H), 8.18 (d, 1H, 4′-H), 8.92 (d, 1H, 6′-H), 9.00 (s, 1H, 4-H), 11.41 (s, 1H, N*H*). Anal. ($C_{16}H_{10}N_2O_3Cl_2$) C, H, N.

***N*-(6-Methoxypyrid-3-yl)-6-chloromethyl-2-oxo-2*H*-1-benzopyran-3-carboxamide (2k).** The title compound was obtained as previously described[28] after the reaction of the acyl chloride of 6-chloromethyl-2-oxo-*2H*-1-benzopyran-3-carboxylic acid (1.17 g, 4.54 mmol) with 3-amino-6-methoxypyridine (0.62 g, 5 mmol) and pyridine (0.4 mL, 5 mmol). The crude product was washed with methanol (0.95 g, 60%): mp 225 °C dec; IR 3190 (N−H), 3039 (C−H arom), 1704 (C═O lactone), 1660 (C═O amide), 1617, 1594, 1572, 1553, 1491, 1387 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$, TMS) 3.95 (s, 3H, C*H*$_3$), 4.68 (s, 2H, C*H*$_2$-Cl), 6.79 (d, 1H, 5′-H), 7.47 (d, 1H, 8-H), 7.75 (d, 1H, 7-H), 7.76 (s, 1H, 5-H), 8.05 (dd, 1H, 4′-H), 8.47 (d, 1H, 2′-H), 9.00 (s, 1H, 4-H), 10.69 (s, 1H, N*H*). Anal. ($C_{17}H_{14}N_2O_4Cl$) C, H, N.

Pyrid-2-yl 6-Chloromethyl-2-oxo-2*H*-1-benzopyran-3-carboxylate (3a). The title compound was obtained as previously described[28] after the reaction of the acyl chloride of 6-chloromethyl-2-oxo-*2H*-1-benzopyran-3-carboxylic acid (1.17 g, 4.54 mmol) with 2-pyridinol (0.47 g, 5 mmol) and pyridine (0.4 mL, 5 mmol). The crude product was crystallized in ethyl acetate:petroleum ether, 40−60 °C (0.45 g, 31%): mp 125−129 °C; IR 3051 (C−H arom), 1736 (C═O, ester and lactone), 1621, 1577, 1245, 1198 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$, TMS) 4.68 (s, 2H, C*H*$_2$Cl), 6.31−6.35 (m, 1H, 5′-H), 6.63 (d, 1H, 3′-H), 7.38−7.41 (m, 1H, 4′-H), 7.47−7.54 (m, 2H, 8-H, 6′-H), 7.77 (s, 1H, 5-H), 7.80 (d, 1H, 7-H), 8.92 (s, 1H, 4-H). Anal. ($C_{16}H_{10}NO_4Cl$) C, H, N.

6-Chloropyrid-2-yl 6-Chloromethyl-2-oxo-2*H*-1-benzopyran-3-carboxylate (3b). The title compound was obtained as previously described[28] after the reaction of the acyl chloride of 6-chloromethyl-2-oxo-2H-1-benzopyran-3-carboxylic acid (1.17 g, 4.54 mmol) with 6-chloro-2-pyridinol (0.65 g, 5 mmol) and pyridine (0.4 mL, 5 mmol). The crude product was crystallized in ethyl acetate:petroleum ether, 40−60 °C (1.07 g, 67%): mp 172−173 °C; IR 3103 (C−H arom), 1774 (C═O, ester), 1754 (C═O lactone), 1721, 1624, 1573, 1246 cm^{-1}; ^{1}H NMR (80 MHz, $CDCl_3$, TMS) 4.60 (s, 2H, C*H*$_2$Cl), 7.10−8.00 (m, 6H, 5-H, 7-H, 8-H, 3′-H, 4′-H, 5′-H), 8.75 (s, 1H, 4-H). Anal. ($C_{16}H_9NO_4Cl_2$) C, H, N.

5-Chloropyrid-2-yl 6-Chloromethyl-2-oxo-2*H*-1-benzopyran-3-carboxylate (3c). The title compound was obtained as previously described[28] after the reaction of the acyl chloride of 6-chloromethyl-2-oxo-*2H*-1-benzopyran-3-carboxylic acid (1.17 g, 4.54 mmol) with 5-chloro-2-pyridinol (0.65 g, 5 mmol) and pyridine (0.4 mL, 5 mmol). The crude product was crystallized in ethyl acetate:petroleum ether, 40−60 °C (0.85 g, 53%): mp 171−172 °C; IR 3092 (C−H arom), 1774 (C═O, ester and lactone), 1622, 1577, 1466, 1369 cm^{-1}; ^{1}H NMR (80 MHz, $CDCl_3$, TMS) 4.60 (s, 2H, C*H*$_2$Cl), 7.10−7.95 (m, 5H, 5-H, 7-H, 8-H, 3′-H, 6′-H), 8.35 (d, 1H, 4′-H), 8.75 (s, 1H, 4-H). Anal. ($C_{16}H_9NO_4Cl_2$) C, H, N.

6-Methylpyrid-2-yl 6-Chloromethyl-2-oxo-2*H*-1-benzopyran-3-carboxylate (3d). The title compound was obtained as previously described[28] after the reaction of the acyl chloride of 6-hydroxymethyl-2-oxo-2*H*-1-benzopyran-3-carboxylic acid (1.17 g, 4.54 mmol) with 6-methyl-2-pyridinol (0.56 g, 5 mmol) and pyridine (0.4 mL, 5 mmol). The crude product was crystallized in ethyl acetate:petroleum ether, 40−60 °C (0.48 g, 32%): mp 160−162 °C; IR 3055 (C−H arom), 1763 (C═O, ester), 1706 (C═O lactone), 1621, 1576, 1454, 1376 cm^{-1}; ^{1}H NMR (80 MHz, $CDCl_3$, TMS) 2.60 (s, 3H, C*H*$_3$), 4.60 (s, 2H, C*H*$_2$Cl), 6.90−7.90 (m, 6H, 5-H, 7-H, 8-H, 3′-H, 4′-H, 5′-H), 8.75 (s, 1H, 4-H). Anal. ($C_{17}H_{12}NO_4Cl$) C, H, N.

4-Methylpyrid-2-yl 6-Chloromethyl-2-oxo-2*H*-1-benzopyran-3-carboxylate (3e). The title compound was obtained as previously described[28] after the reaction of the acyl chloride of 6-chloromethyl-2-oxo-*2H*-1-benzopyran-3-carboxylic acid (1.17 g, 4.54 mmol) with 4-methyl-2-pyridinol (0.56 g, 5 mmol) and pyridine (0.4 mL, 5 mmol). The crude product was crystallized in ethyl acetate:petroleum ether, 40−60 °C (0.55 g, 37%): mp 152−154 °C; IR 3046 (C−H arom), 1736 (C═O, ester and lactone), 1622, 1576, 1310, 1237 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$, TMS) 2.44 (s, 3H, C*H*$_3$), 4.66 (s, 2H, C*H*$_2$Cl), 7.10 (s, 1H, 3′-H), 7.12 (d, 1H, 5′-H), 7.41 (d, 1H, 8-H), 7.68 (s, 1H, 5-H), 7.77 (d, 1H, 7-H), 8.30 (d, 1H, 6′-H), 8.79 (s, 1H, 4-H). Anal. ($C_{17}H_{12}NO_4Cl$) C, H, N.

5-Chloropyrid-3-yl 2-Oxo-2*H*-1-benzopyran-3-carboxylate (4a). The title compound was obtained as previously described[28] after the reaction of the acyl chloride of 2-oxo-2*H*-1-benzopyran-3-carboxylic acid (1 g, 5.26 mmol) with 5-chloro-3-pyridinol (0.74 g, 5.78 mmol) and pyridine (0.46 mL, 5.78 mmol). The crude product was crystallized in ethyl acetate (0.66 g, 42%): mp 208−209 °C; IR 3070 (C−H arom), 1756 (C═O, ester), 1712 (C═O lactone), 1612, 1566, 1376 cm^{-1}; ^{1}H NMR (80 MHz, $CDCl_3$, TMS) 7.10−7.80 (m, 6H, 5-H, 6-H, 7-H, 8-H, 4′-H), 8.50 (bs, 2H, 2′-H, 6′-H), 8.70 (s, 1H, 4-H). Anal. ($C_{15}H_8NO_4Cl$) C, H, N.

5-Chloropyrid-3-yl 6-Methyl-2-oxo-2*H*-1-benzopyran-3-carboxylate (4b). The title compound was obtained as previously described[28] after the reaction of the acyl chloride of 6-methyl-2-oxo-2*H*-1-benzopyran-3-carboxylic acid[28](1 g, 4.90 mmol) with 5-chloro-3-pyridinol (0.70 g, 5.38 mmol) and pyridine (0.43 mL, 5.38 mmol). The crude product was crystallized in ethyl acetate (1.08 g, 63%): mp 215−217 °C; IR 3060 (C−H arom), 1736 (C═O, ester and lactone), 1623, 1572, 1423 cm^{-1}; ^{1}H NMR (80 MHz, $CDCl_3$, TMS) 2.20 (s, 3H, C*H*$_3$), 7.20−7.80 (m, 4H, 5-H, 7-H, 8-H, 4′-H), 8.45 (bs, 2H, 2′-H, 6′-H), 8.70 (s, 1H, 4-H). Anal. ($C_{16}H_{10}NO_4Cl$) C, H, N.

6-Acetoxymethyl-2-oxo-2*H*-1-benzopyran-3-carboxylic Acid (6a). The suspension of 6-hydroxymethyl-2*H*-1-benzopyran-3-carboxylic acid (3 g, 13.62 mmol) in acetic anhydride (20 mL) was refluxed for 2 h. After cooling, water (200 mL) was added and the resulting mixture was stirred at room temperature for 1 h. The precipitate so obtained was collected by filtration and washed with water. The residue of the crude compound was crystallized in $CHCl_3$:petroleum ether, 40−60 °C (2.2 g, 62% yield): mp 180−182 °C; IR 3058 (C−H arom), 1745 (C=O carboxylic acid and ester), 1677 (C=O lactone), 1620, 1576, 1414, 1371, 1233 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, TMS) 2.15 (s, 3H, CH_3), 5.20 (s, 2H, CH_2), 7.50 (d, 1H, 8-H), 7.76 (s, 1H, 5-H), 7.79 (d, 1H, 7-H), 8.95 (s, 1H, 4-H). Anal. ($C_{13}H_{10}O_6$) C, H.

5-Chloropyrid-3-yl 6-Acetoxymethyl-2-oxo-2*H*-1-benzopyran-3-carboxylate (4c). The title compound was obtained as previously described[28] after the reaction of the acyl chloride of 6-acetoxymethyl-2-oxo-2*H*-1-benzopyran-3-carboxylic acid (0.96 g, 3.43 mmol) with 5-choro-3-pyridinol (0.51 g, 3.98 mmol) and pyridine (0.30 mL, 3.77 mmol). The crude product was crystallized in ethyl acetate (0.7 g, 55% yield): mp 153−155 °C; IR 3075 (C−H arom), 1783, 1773 (C=O esters), 1728 (C=O lactone), 1624, 1557, 1229, 1217 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, TMS) 2.14 (s, 3H, CH_3), 5.18 (s, 2H, CH_2), 7.43 (d, 1H, 8-H), 7.70−7.74 (m, 3H, 5-H, 7-H, 4′-H), 8.51 (bs, 1H, 2′-H), 8.54 (bs, 1H, 6′-H), 8.76 (s, 1H, 4-H). Anal. ($C_{18}H_{12}NO_6Cl$) C, H, N.

6-Propionoxymethyl-2-oxo-2*H*-1-benzopyran-3-carboxylic Acid (6b). A mixture of 6-hydroxymethyl-2*H*-1-benzopyran-3-carboxylic acid (3 g, 13.62 mmol), propionyl chloride (2.52 g, 27.24 mmol), and pyridine (1.21 mL,14.98 mmol) in dioxane (30 mL) was stirred at room temperature for 1 h. The solvent was removed by evaporation under reduced pressure. The residue was dissolved in $CHCl_3$ (50 mL). The solution was washed with 0.1 N HCl (3 × 50 mL). The organic layer was dried over anhydrous sodium sulfate and the solvent was removed under reduced pressure. The residue was crystallized in acetonitrile (2.67 g, 71%): mp 179−181 °C; IR 3071 (C−H arom), 1754, 1734 (C=O carboxylic acid and ester), 1685 (C=O lactone), 1621, 1575, 1414, 1376, 1206 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, TMS) 1.18 (t, 3H, CH_3), 2.42 (q, 2H, CH_2CH_3), 5.21 (s, 2H, $CH_2OCOC_2H_5$), 7.50 (d, 1H, 8-H), 7.75 (s, 1H, 5-H), 7.77 (d, 1H, 7-H), 8.94 (s, 1H, 4-H). Anal. ($C_{14}H_{12}O_6$) C, H.

5-Chloropyrid-3-yl 6-Propionoxymethyl-2-oxo-2*H*-1-benzopyran-3-carboxylate (4d). The title compound was obtained as previously described[28] after the reaction of the acyl chloride of 6-propionoxymethyl-2-oxo-2*H*-1-benzopyran-3-carboxylic acid (1.07 g, 3.62 mmol) with 5-choro-3-pyridinol (0.51 g, 3.98 mmol) and pyridine (0.32 mL, 3.98 mmol). The crude product was crystallized in ethyl acetate (1.06 g, 75%): mp 156−158 °C; IR 3059 (C−H arom), 1779, 1761 (C=O esters), 1732 (C=O lactone), 1620, 1570, 1421, 1377, 1241, 1211 cm^{-1}; ^1H NMR ($CDCl_3$, TMS) 1.18 (t, 3H, CH_3), 2.41 (q, 2H, CH_2-CH_3), 5.19 (s, 2H, $CH_2OCOC_2H_5$), 7.42 (d, 1H, 8-H), 7.70−7.74 (m, 3H, 5-H, 7-H, 4′-H), 8.50 (d, 1H, 2′-H), 8.53 (d, 1H, 6′-H), 8.76 (s, 1H, 4-H). Anal. ($C_{19}H_{14}NO_6Cl$) C, H, N.

6-Dimethylacetoxymethyl-2-oxo-2*H*-1-benzopyran-3-carboxylic Acid (6c). The title compound was obtained as describe above for **6a** after the reaction of 6-hydroxymethyl-2*H*-1-benzopyran-3-carboxylic acid (3 g, 13.62 mmol) with isobutyric anhydride (20 mL) and was crystallized in acetonitrile (3.35 g, 85%): mp 136−137 °C; IR 3049 (C−H arom), 1752, 1725 (C=O carboxylic acid and ester), 1684 (C=O lactone), 1620, 1575, 1416, 1222 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, TMS), 1.20 (d, 6H, $(CH_3)_2$), 2.63 (q, 1H, *CH*), 5.20 (s, 2H, CH_2), 7.49 (d, 1H, 8-H), 7.74 (s, 1H, 5-H), 7.76 (d, 1H, 7-H), 8.94 (s, 1H, 4-H). Anal. ($C_{15}H_{14}O_6$) C, H.

5-Chloropyrid-3-yl 6-Dimethylacetoxymethyl-2-oxo-2*H*-1-benzopyran-3-carboxylate (4e). The title compound was obtained as previously described[28] after the reaction of the acyl chloride of 6-dimethylacetoxymethyl-2-oxo-2*H*-1-benzopyran-3-carboxylic acid (1.06 g, 3.44 mmol) with 5-choro-3-pyridinol (0.49 g, 3.77 mmol) and pyridine (0.30 mL, 3.77 mmol). The crude product was crystallized in acetonitrile (0.50 g, 36%): mp 162−164 °C; IR 3078, 3024 (C−H arom), 2974 (C−H aliph), 1783, 1774 (C=O esters), 1727 (C=O lactone), 1625, 1577, 1229, 1221 cm^{-1}; ^1H NMR 1.20 (d, 6H, $(CH_3)_2$), 2.63 (q, 1H, *CH*), 5.19 (s, 2H, CH_2), 7.42 (d, 1H, 8-H), 7.66−7.73 (m, 3H, 5-H, 7-H, 4′-H), 8.50 (d, 1H, 2′-H), 8.53 (d, 1H, 6′-H), 8.76 (s, 1H, 4-H). Anal. ($C_{20}H_{16}NO_6Cl$) C, H, N.

6-Butyryloxymethyl-2-oxo-2*H*-1-benzopyran-3-carboxylic Acid (6d). The title compound was obtained as describe above for **6a** after the reaction of 6-hydroxymethyl-2*H*-1-benzopyran-3-carboxylic acid (3 g,13.62 mmol) with butyric anhydride (20 mL) and was crystallized in acetonitrile (3.05 g, 77%): mp 149−151 °C; IR 3052 (C−H arom), 1738 (C=O carboxylic acid and ester), 1679 (C=O lactone), 1619, 1572, 1263 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, TMS) 0.96 (t, 3H, CH_3), 1.68 (m, 2H, $CH_2CH_2CH_3$), 2.37 (t, 2H, $CH_2CH_2CH_3$), 5.19 (s, 2H, $CH_2OCO(CH_2)_2CH_3$), 7.49 (d, 1H, 8-H), 7.75 (s, 1H, 5-H), 7.71 (d, 1H, 7-H), 8.94 (s, 1H, 4-H). Anal. ($C_{15}H_{14}O_6$) C, H.

5-Chloropyrid-3-yl 6-Butyryloxymethyl-2-oxo-2*H*-1-benzopyran-3-carboxylate (4f). The title compound was obtained as previously described[28] after the reaction of the acyl chloride of 6-butyryloxymethyl-2-oxo-2*H*-1-benzopyran-3-carboxylic acid (1.06 g, 3.44 mmol) with 5-choro-3-pyridinol (0.49 g, 3.77 mmol) and pyridine (0.30 mL, 3.77 mmol). The crude product was crystallized in acetonitrile (0.6 g, 43%): mp 146−148 C; IR 3066, 3037 (C−H arom), 2965 (C−H aliph), 1780, 1763 (C=O esters), 1736 (C=O lactone), 1621, 1572, 1215, 1168 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, TMS) 0.96 (t, 3H, CH_3), 1.68 (m, 2H, $CH_2CH_2CH_3$), 2.37 (t, 2H, $CH_2CH_2CH_3$), 5.19 (s, 2H, $CH_2OCO(CH_2)_2CH_3$), 7.42 (d, 1H, 8-H), 7.67−7.74 (m, 3H, 5-H, 7-H, 4′-H), 8.50 (d, 1H, 2′-H), 8.53 (d, 1H, 6′-H), 8.76 (s, 1H, 4-H). Anal. ($C_{20}H_{16}NO_6Cl$) C, H, N.

6-Trimethylacetoxymethyl-2-oxo-2*H*-1-benzopyran-3-carboxylic Acid (6e). The title compound was obtained as describe above for **6b** after the reaction of 6-hydroxymethyl-2*H*-1-benzopyran-3-carboxylic acid (3 g, 13.62 mmol) with the acyl chloride of trimethylacetic acid (3.35 mL, 27.24 mmol) and pyridine (1.21 mL,14.98 mmol). The crude product was crystallized in acetonitrile (3 g, 72%): mp 138−140 °C; IR 3044 (C−H arom), 2968 (C−H aliph), 1742 (C=O carboxylic acid and ester), 1674 (C=O lactone), 1619, 1575, 1417, 1157 cm^{-1} ; ^1H NMR (400 MHz, $CDCl_3$, TMS) 1.24 (s, 9H, $(CH_3)_3$), 5.20 (s, 2H, CH_2), 7.50 (d, 1H, 8-H), 7.74 (s, 1H, 5-H), 7.76 (d, 1H, 7-H), 8.95 (s, 1H, 4-H). Anal. ($C_{16}H_{16}O_6$) C, H.

5-Chloropyrid-3-yl 6-Trimethylacetoxymethyl-2-oxo-2*H*-1-benzopyran-3-carboxylate (4g). The title compound was obtained as previously described[28] after the reaction of the acyl chloride of 6-trimethylacetoxymethyl-2-oxo-2*H*-1-benzopyran-3-carboxylic acid (1.06 g, 3.28 mmol) with 5-choro-3-pyridinol (0.46 g, 3.61 mmol) and was crystallized in acetonitrile (0.2 g, 15%): mp 175−177 °C; IR 3076 (C−H arom), 2976 (C−H aliph), 1783,1771 (C=O esters), 1721 (C=O lactone), 1624, 1576, 1376 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, TMS) 1.24 (s, 9H, $(CH_3)_3$), 5.18 (s, 2H, CH_2), 7.42 (d, 1H, 8-H), 7.67−7.72 (m, 3H, 5-H, 7-H, 4′-H), 8.50 (d, 1H, 2′-H), 8.53 (d, 1H, 6′-H), 8.76 (s, 1H, 4-H). Anal. ($C_{21}H_{18}NO_6Cl$) C, H, N.

5-Chloropyrid-3-yl 6-Aminomethyl-2-oxo-2*H*-1-benzopyran-3-carboxylate Hydrochloride (4h). Hexamethylenetetramine (1.2 g, 8.57 mmol) was added to a solution of **2c** (2 g, 5.71 mmol) in $CHCl_3$ (20 mL). The mixture was refluxed for 3 h. The white precipitate was collected by filtration and washed with chloroform to give 5-chloropyrid-3-yl 6-(hexamethylenetetrammoniomethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylate chloride. Without further purification, ethanol (1.5 mL) and 12 N HCl (0.75 mL) were added to the precipitate and the suspension was heated in a steam bath during 15 min. After cooling, the precipitate was discarded. The solvent was removed under reduced pressure. The residue was suspended in a 1% aqueous solution of sodium hydrogen carbonate (10 mL). The precipitate was collected by filtration and washed with water, methanol (5 mL), and ether (10 mL) (1.69 g, 80%): mp 230 °C dec; IR 2961, 2893 ($NH_3^{+\bullet}$), 1778, 1763 (C=O esters), 1718 (C=O lactone), 1624, 1577, 1493, 1245, 1220 cm^{-1}; ^1H NMR (400 MHz, DMSO, TMS) 4.14 (s, 2H, $CH_2-NH_3^+$), 7.60 (d, 1H, 8-H), 7.94 (d, 1H, 7-H), 8.04 (s,

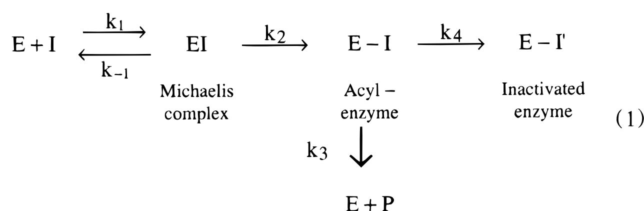
1H, 5-H), 8.13 (bs, 1H, 4′-H), 8.60 (bs, 1H, 2′-H), 8.65 (bs, 1H, 6′-H), 9.10 (s, 1H, 4-H). Anal. ($C_{16}H_{11}N_2O_4Cl{\cdot}HCl$) C, H, N.

5-Chloropyrid-3-yl 6-Bromo-2-oxo-2*H*-1-benzopyran-3-carboxylate (4i). The title compound was obtained as previously described[28] after the reaction of the acyl chloride of 6-bromo-2-oxo-2*H*-1-benzopyran-3-carboxylic acid (1.17 g, 3.71 mmol) with 5-choro-3-pyridinol (0.53 g,4.08 mmol) and pyridine (0.32 mL, 4.08 mmol). The crude product was washed with water (1.23 g, 87%): mp 228−230 °C; IR 3066 (C−H arom), 1774 (C=O ester), 1721 (C=O lactone), 1558, 1372 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$, TMS) 7.32 (d, 1H, 8-H), 7.71 (dd, 1H, 7-H), 7.80−7.83 (m, 2H, 5-H, 4′-H), 8.50 (d, 1H, 2′-H), 8.54 (d, 1H, 6′-H), 8.67 (s, 1H, 4-H). Anal. ($C_{15}H_7NO_4BrCl$) C, H, N.

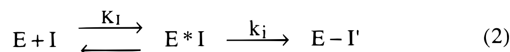
Biochemical Studies. 1. Inhibition Studies. The kinetic parameters for the inhibition were obtained either by the preincubation method or by the progress curve method as described in ref 38. When the inhibition was too fast to be observed by the preincubation method, the progress curve method was used. For the preincubation method, the enzyme and the coumarinic derivative concentrations were $[\alpha\text{-CT}]_0 = 12.5$ nM, $[I]_0 = 0.5{-}10$ μM; $[HLE]_0 = 20$ nM, $[I]_0 = 1{-}10$ μM; $[THR]_0 = 1.45$ nM, $[I]_0 = 1{-}10$ μM; $[trypsin]_0 = 200$ nM, $[I]_0 = 10$ μM. For the progress curve method, the enzyme, substrate, and coumarinic derivative concentrations were for α-CT: $[\alpha\text{-CT}]_0 = 12.5$ nM, $[S]_0 = 40$ or 100 μM, $[I]_0 = 0.04{-}40$ μM; for HLE: $[HLE]_0 = 30$ nM, $[S]_0 = 100$ μM, $[I]_0 = 0.05{-}70$ μM; for THR: $[THR]_0 = 0.58$ nM, $[S]_0 = 10$ μM, $[I]_0 = 1{-}14$ μM.

For competitive inhibitors of α-CT (compounds **4a**−**4i**), IC_{50} values (concentration of inhibitor leading to 50% inhibition) were determined by measuring the initial rates of chromogenic substrate hydrolysis for various inhibitor concentrations ($[I]_0 = 0.5{-}20$ μM, $[\alpha\text{-CT}]_0 = 12.5$ nM, $[S]_0 = 50$ or 100 μM). In the case of compound **4c**, a Dixon plot was used ($[\mathbf{4c}]_0 = 2{-}20$ μM, $[\alpha\text{-CT}]_0 = 12.5$ nM, $[S]_0 = 50{-}150$ μM).

2. Kinetic Analysis. The general kinetic scheme for the inactivation of serine proteases by compounds **2a**−**2k**, **3a**−**3e**, and **4a**−**4i** can be described by eq 1:



When examining the first moments of the reaction, k_3 is usually small enough to be neglected. If the enzyme is inactivated, the acyl-enzyme cannot be kinetically distinguished from the Michaelis complex. Then, the minimum kinetic scheme for the inactivation is described by eq 2:



E*I is a kinetic chimere; K_I and k_i are the constants characterizing the inactivation process. The k_i/K_I ratio is an index of the inhibitory potency.

The analysis of the data obtained by using the incubation method consisted in the determination of the slopes of the semilogarithmic plots of remaining enzyme activity versus time (eq 3) where $[E]/[E]_0$ is the amount of active enzyme remaining at time *t*. The pseudo-first-order constant k_{obs} is related to k_i and K_I by eq 4:[39]

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

$$k_{obs} = \frac{k_i \times [I]_0}{K_I + [I]_0} \qquad (4)$$

At low inhibitor concentrations, the ratio k_i/K_I was obtained as $k_{obs}/[I]$.

For the progress curve method, the rate of change in absorbance at 405 nm *v* was evaluated as the change in absorbance in a defined short time interval. This rate *v* plotted versus time followed eq 5 where the constant π depended on $[I]_0'$, a modified inhibitor concentration, according to eq 6. $[I]_0'$ is defined by eq 7, K_m being the Michaelis constant for the enzymic hydrolysis of the appropriate chromogenic substrate.

$$v = v_0 \times \exp(-\pi \times t) \qquad (5)$$

$$\pi = \frac{k_i \times [I]_0'}{K_I + [I]_0'} \qquad (6)$$

$$[I]_0' = \frac{[I]_0}{1 + [S]_0/K_m} \qquad (7)$$

With both methods, the inactivation constants k_i and K_I were determined by linear or nonlinear fit of the experimental data to the equations developed above using Kaleidagraph version 3.0.1 from Abelbeck Software. When the compounds led to stable acyl-enzymes ($k_4 = 0$; $k_3 \ll k_2$), we used the same kinetic scheme (eq 2), since in the first moments of the reaction, the reactivation process could be considered as negligible.

3. Hydroxylamine Reactivation. Hydroxylamine reactivation was checked by treatment of the inactivated enzyme (>85% inhibition) by hydroxylamine 0.4−0.5 M at 25 °C and pH 7.5 (α-CT and THR) or pH 8.0 (HLE) during 30−120 min. Aliquots were withdrawn, assayed for enzyme activity using the appropriate substrate, and compared to the activity of an enzyme solution containing no inhibitor and treated with hydroxylamine in the same experimental conditions.

4. Reactivation of the HLE−Inhibitor Complexes. The spontaneous reactivation of HLE inactivated by inhibitors **2c**−**2e**, **3b**, and **4a**−**4d** was followed after incubation of HLE (100 or 200 nM) during about 15 min in the presence of inhibitor excess (7.5-fold for **2c**, **3b**, and **4a**−**4d**; 50-fold for **2d** and **2e**) at pH 8.0 and 25 °C. At the end of the incubation time, an aliquot was removed and assayed for remaining enzyme activity. The pH of the reaction mixture dropped to ≈4.5 with 5 N HCl and excess inhibitor was removed by centrifuging at 4000*g* for 1 h at 7 °C using microconcentrators Centricon-10 with regular washing of the enzyme solution with buffer. The pH of the retentates was adjusted to 8.0 with 10 N NaOH and the reactivation was followed by removing aliquots with time and assaying them for enzyme activity. A control (HLE not treated by the inhibitor) was run in the same experimental conditions. In some cases (compounds **4a**−**4d**), the reactivation process had been followed without removal of the excess of inhibitor. It was verified with one compound that identical reactivation rates were obtained with and without removal of this excess.

5. Partition Ratio Determination. α-CT (1 μM) was incubated with various concentrations of compounds **2c** and **3b** (0.5−5 μM). After a 4-h incubation, an aliquot of the enzyme solution was removed, assayed for remaining activity using Suc-AAPF-*p*-NA, and compared to a control (enzyme solution without inhibitor). Percentage of remaining activity ($[E]/[E]_0$) was plotted against excess inhibitor to enzyme ($[I]_0/[E]_0$) to give a straight line which intercepted with the *x* axis at $r+1$, *r* being the partition ratio k_3/k_4.[40]

6. Enzymatic Hydrolysis of Coumarins by α-CT. The enzyme hydrolysis of compound **4b** by α-CT was monitored at 310 nm. The kinetic parameter V_{max}/K_m was obtained as the slope of the linear curve observed when plotting the initial velocity *V* against the concentration of substrate. *V* was obtained from eq 8:

$$V\,(\mathrm{M{\cdot}s^{-1}}) = |\Delta A/\Delta t\,(\mathrm{s})|/|\Delta\epsilon|\ (\mathrm{M^{-1}{\cdot}cm^{-1}}) \qquad (8)$$

where $|\Delta A/\Delta t\,(s)|$ is the absolute value of the rate of absorbance change and $|\Delta\epsilon|$ is the absolute value of the difference between the molar extinction coefficient of the product of hydrolysis and that of the substrate. The former was evaluated as the

molar extinction coefficient of the reaction mixture after 24 h. k_{cat}/K_m was then deduced using the enzyme active-site concentration. The experimental conditions were $[\alpha\text{-CT}]_0 = 100$ nM, $[\mathbf{4b}]_0 = 1-20$ μM, $|\Delta\epsilon| = 5\,800$ $M^{-1}\cdot cm^{-1}$.

Reaction Mixture Analysis. This was investigated using reverse-phase HPLC. The tested inhibitor (10 μM) was incubated in the presence (4 μM HLE) or absence of enzyme (0.1 M Hepes, 0.1% Tween 80, pH 8.0). Aliquots were usually removed at a given time and chromatographed. The experimental conditions were flow rate of 0.75 mL/min, linear gradient during 95 min, 5−100% acetonitrile (0.07% trifluoroacetic acid) in water (0.1% trifluoroacetic acid). The detection of products was monitored at 300 nm. A spectral analysis of each peak was performed.

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