Esters and Amides of 6-(Chloromethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylic Acid as Inhibitors of α-Chymotrypsin: Significance of the "Aromatic" Nature of the Novel Ester-Type Coumarin for Strong Inhibitory Activity

Lionel Pochet,[†] Caroline Doucet,[‡] Marc Schynts,[†] Nicole Thierry,[‡] Nicole Boggetto,[‡] Bernard Pirotte,*[,†] Kai Y. Jiang,[‡] Bernard Masereel,[†] Pascal de Tullio,[†] Jacques Delarge,[†] and Michèle Reboud-Ravaux[‡]

Laboratoire de Chimie Pharmaceutique, Université de Liège, 3, rue Fusch, B-4000 Liège, Belgium, and Laboratoire d'Enzymologie Moléculaire et Fonctionnelle, Département de Biologie Supramoléculaire et Cellulaire, Institut Jacques Monod, Université de Paris VII, tour 43, 2, place Jussieu, F-75251 Paris Cedex 05, France

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A series of esters and amides of 6-(chloromethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylic acid were synthesized and evaluated *in vitro* for their inhibitory activity toward bovine α-chymotrypsin and human leukocyte elastase. Both series behaved as time-dependent inhibitors of α-chymotrypsin, but ester-type coumarins were clearly more efficient than the corresponding amides in inactivating the serine proteinase. The best inactivations were observed with "aromatic" esters, in particular with *meta*-substituted phenyl esters such as *m*-chlorophenyl 6-(chloromethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylate, which appears to be one of the most powerful inactivators of α-chymotrypsin yet reported (k_{inact}/K_I = 760 000 M^{-1} s^{-1} at pH 7.5 and 25 °C). Usually, the coumarin derivatives failed to inhibit significantly human leukocyte elastase. As a result, the reported series of aromatic coumarinic esters behaves as a new chemical family of selective α-chymotrypsin inhibitors.

Introduction

Serine proteinases have been the focus of extensive study in terms of their vital roles in biological processes, their involvement in numerous diseases, and the development of suitable therapeutic inhibitors.[1,2] Among them, chymotrypsin-like proteinases are implicated in a wide variety of pathological states including inflammation for cathepsin G, allergic responses, and psoriasis for mast cell chymases.[1] The multicatalytic proteinase complex (proteasome) responsible for much of the proteolysis within cells possesses a chymotrypsin-like activity besides a trypsin-like and a peptidylglutamyl-peptide hydrolase activity.[3] Bovine α-chymotrypsin (α-CT; EC 3.4.21.1) constitutes a model for the evaluation of new synthetic inhibitors directed against chymotrypsin-like proteinases and for the comprehensive approach of the biological role of the targeted protease. Human leukocyte elastase (HLE; EC 3.4.21.37) has been implicated in the pathogenesis of numerous disease states such as pulmonary emphysema and rheumatoid arthritis.[4]

Various types of synthetic inhibitors of chymotrypsin- and elastase-like proteinases were reported including substrate and transition-state analogs, affinity labels, and mechanism-based inhibitors.[1,2,4,5] The inactivation by mechanism-based inhibitors (also called suicide substrates) is assumed to result from the unmasking of a reactive latent group after catalytic action by the enzyme, which then reacts with an active site residue leading to enzyme inactivation.[6] These inhibitors are expected to display a maximum selectivity *in vitro* and *in vivo* since their inhibitory activity requires discrimination in the binding steps, the catalytic activation by the enzyme, and the irreversible modification of the active center.

A variety of chemical structures have been described as suicide substrates. They are usually activated by an esterolytic reaction, as for halo enol and ynenol lactones,[7−9] isocoumarins,[10,11] and 3,4-dihydro-6-halomethylcoumarins,[12,13] or by an amidolytic reaction, as for functionalized cyclopeptides[14,15] and β-lactams.[16−19] Halomethylated 3,4-dihydrocoumarins (Figure 1, **1**) were the first suicide substrates described for serine proteinases. They act as efficient inactivators of serine proteinases lacking selectivity since bovine α-CT,[12,20] porcine pancreatic elastase,[21] HLE,[22] human urokinase,[23] human plasmin, t-PA, and thrombin[13] are inhibited. The enzymatic hydrolysis of the ester bond of the lactone ring leads to the unmasking of a *p*-hydroxybenzyl halide. After elimination of the halogen ion, a very reactive quinone methide is formed[24] which alkylates the active site histidine.[20,23] In order to develop a new class of inactivators of serine proteinases displaying simpler synthetic pathways, a higher efficiency and hopefully better selectivity, we prepared a series of esters (Figure 1, **3**) and amides (Figure 1, **4**) of 6-(chloromethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylic acid and evaluated their potency as inactivators of α-CT and HLE. These novel coumarins differ from 6-(bromomethyl)coumarin (Figure 1, **2**) previously described as a very poor inactivator of α-CT ($k_{obs}/[I]$ = 12.5 M^{-1} s^{-1} at 20 °C[12]) by the presence of a strong electron-withdrawing group at the 3-position, expected to increase the electrophilicity of the lactonic carbonyl group, thus facilitating the nucleophilic attack by the serine hydroxyl residue. Moreover, modification of the nature of R is assumed to facilitate discrimination between different classes of serine proteinases.

In order to evaluate the importance of a latent alkylating moiety in the 6-position, the 6-methyl-substituted coumarinic ester (Scheme 2, **14**) was prepared.

* Author to whom correspondence should be addressed.
[†] Université de Liège.
[‡] Université de Paris VII.
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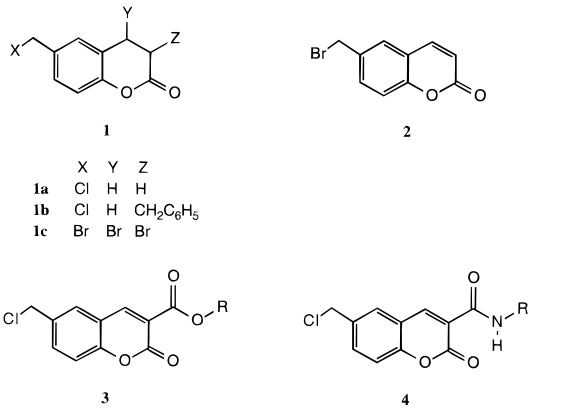
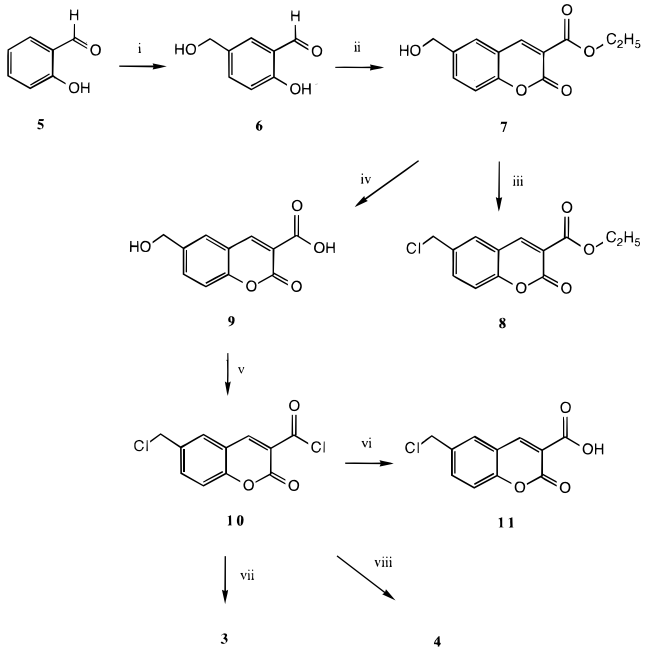


Figure 1. Chemical structures of 6-(halomethyl)dihydrocoumarins (**1**), 6-(bromomethyl)coumarin (**2**), and newly designed 6-(halomethyl)coumarin esters (**3**) and amides (**4**).

Scheme 1[a]



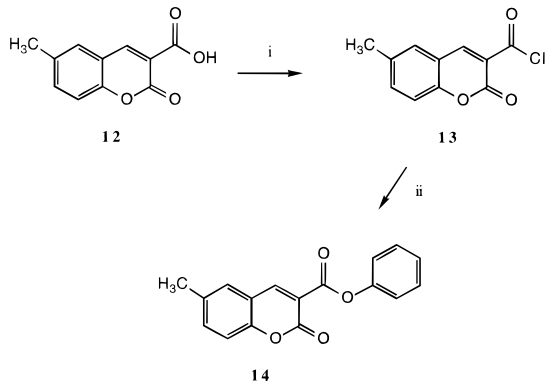
[a] Reagents: (i) HCHO/HCl; (ii) diethyl malonate/piperidine/EtOH/HOAc; (iii) $SOCl_2$; (iv) HCl/H_2O/EtOH; (v) $SOCl_2$; (vi) H_2O; (vii) ROH/pyridine/dioxane; (viii) RNH_2/pyridine/dioxane.

Chemistry

5-(Hydroxymethyl)salicyl aldehyde (**6**) obtained from salicyl aldehyde (**5**) following the Stoerner and Behn process[25] was engaged in a Knoevenagel-type reaction with diethyl malonate and piperidine as described for the preparation of ethyl 2-oxo-2*H*-1-benzopyran-3-carboxylate[26] to give the corresponding 6-(hydroxymethyl)-coumarin homologue **7** (Scheme 1). Treatment with thionyl chloride provided the 6-chloromethyl ethyl ester derivative **8**. Hydrolysis of the ester function of **7** in acidic hydroalcoholic solution led to the corresponding carboxylic acid **9**, which was converted with thionyl chloride to the 6-chloromethyl acid chloride **10**, the starting material for the preparation of the coumarinic esters **3** and amides **4**. The 6-chloromethyl carboxylic acid **11** was obtained by treatment of the acid chloride **10** with water.

Starting from 6-methyl-2-oxo-2*H*-1-benzopyran-3-carboxylic acid (Scheme 2, **12**)[27] treatment with thionyl chloride led to the corresponding acid chloride **13**, which in turn was converted to the phenyl ester **14**.

Scheme 2[a]



[a] Reagents: (i) $SOCl_2$; (ii) C_6H_5OH/pyridine/dioxane.

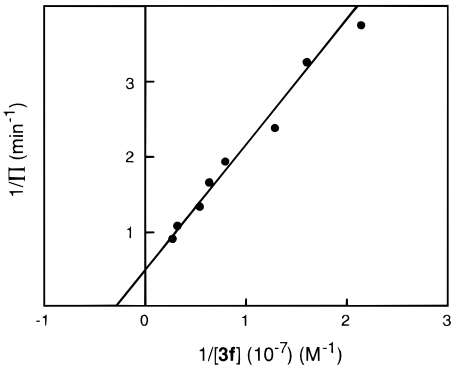
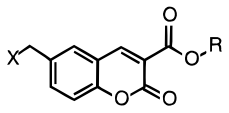


Figure 2. Inactivation of α-chymotrypsin by **3f** at pH 7.5 and 25 °C. The enzyme (12.5 nM) was incubated in 0.025 M sodium phosphate buffer (0.05 M KCl, 10% v/v DMSO) with the chromogenic substrate Suc-A_2-P-F-*p*NA (40 μM) in the presence of different concentrations of coumarin **3f** ranging from 75 to 600 nM. The changing slopes $\Delta A/\Delta t$ (or velocities v) of the progress curves for the hydrolysis of the chromogenic substrate were obtained from computer-assisted spectrophotometer (A was the absorbance at 405 nm). The kinetic parameters K_I and k_{inact} were determined by fitting the experimental data to the equation $\pi = k_{inact}[I](1 - \alpha)/[K_I + [I](1 - \alpha)]$ where $-\pi$ is the slope of the linear plot of ln v versus time at a given inhibitor concentration, α the ratio $[S]/[K_M + [S]]$, [S] the substrate concentration, [I] the inhibitor concentration, and K_M the Michaelis constant for the substrate. $-1/K_I$ and $1/k_{inact}$ are also the x- and y-intercept, respectively, of the plot of $1/\pi$ versus $1/[I](1 - \alpha)$.

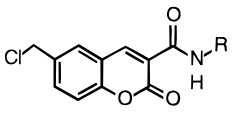
Results and Discussion

α-CT and HLE were exposed to the novel coumarins using either the preincubation method, in which the enzyme and the inhibitor were incubated before the determination of remaining activity at intervals of time, or a method in which the reagent competed with the chromogenic substrate (Figure 2). Time-dependent inhibitions of α-CT were observed for all compounds except for **4a**−**c** and **11** (Tables 1 and 2). After 24 h of incubation at 4 °C, the enzyme inactivated by **3f** regained less than 2% of its initial activity. In the same conditions, the spontaneous loss of activity of control samples was less than 5%. No noticeable reactivation resulted from the treatment of the inactivated enzyme by 0.66 M hydroxylamine (pH 7.5; 30 min; 25 °C). This excluded inactivation simply due to the formation of a stable acyl enzyme. Increasing amounts of the chromogenic substrate (Suc-A_2-P-F-*p*NA) at a fixed coumarin **3f** concentration protected α-CT against inactivation, suggesting that a chemical modification occurred in the active site.

Table 1. Kinetic Parameters for the Inactivation of α-CT (pH 7.5 and 25 °C) and HLE (pH 8.0 and 25 °C) by Esters and Amides of 6-(Chloromethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylic Acid[a]



3, 8, 11, 14



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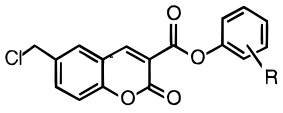
compd no.	R	X	α-CT k_{inact} (s^{-1})	α-CT K_I (M)	α-CT k_{inact}/K_I (M^{-1} s^{-1})	HLE k_{obs}/[I] (M^{-1} s^{-1})
11	H	Cl			ni[b]	ni
3a	CH_3	Cl			110[c]	ni
8	CH_2CH_3	Cl	0.013	5.9×10^{-5}	220	ni
3b	$CH(CH_3)_2$	Cl	0.011	1.6×10^{-5}	687	ni
3c	$CH_2(CH_2)_3CH_3$	Cl			12[c]	ni
3d	$CH_2(CH_2)_6CH_3$	Cl			190[c]	ni
3e	$CH_2CH{=}CH_2$	Cl	0.014	3.3×10^{-5}	424	ni
3f	C_6H_5	Cl	0.034	3.4×10^{-7}	100 000	23
3g	$CH_2C_6H_5$	Cl			186[c]	ni
14	C_6H_5	H			ni	330
4a	CH_3				ni	ni
4b	$CH(CH_3)_2$				ni	ni
4c	$CH_2(CH_2)_3CH_3$				ni	ni
4d	$CH_2(CH_2)_8CH_3$				36[c]	ni
4e	$CH_2CH{=}CH_2$				80[c]	ni
4f	C_6H_5				32[c]	ni
4g	$CH_2C_6H_5$				24[c]	58
4h	$CH_2CH_2C_6H_5$				6[c]	ni
4i	$CH_2CO_2CH_3$				12[c]	ni
4j	$CH(CH_2C_6H_5)CO_2CH_3$				80[c]	ni

[a] Standard errors are less than 15%. [b] No inhibition. [c] Obtained as k_{obs}/[I] at low inhibitor concentration.

Table 2. Kinetic Parameters for the Inactivation of α-CT (pH 7.5 and 25 °C) and HLE (pH 8.0 and 25 °C) by "Phenolic" Esters of 6-(Chloromethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylic Acid[a]



compd no.	R	α-CT k_{inact}/K_I (M^{-1} s^{-1})	HLE k_{obs}/[I] (M^{-1} s^{-1})
3h	*o*-CH_3	114 000[b]	ni[c]
3i	*m*-CH_3	71 750	ni
3j	*p*-CH_3	685[d]	ni
3k	*o*-Cl	48 100[b]	23
3l	*m*-Cl	762 700	630
3m	*p*-Cl	330[d]	ni
3n	*o*-I	80 000[b]	85
3o	*m*-I	72 120	85
3p	*p*-I	1 775[d]	ni
3q	*m*-NO_2	11 300[d]	500
3r	*m*-CH_3-*p*-Cl	7 550[d]	90

[a] Standard errors are less than 15%. [b] **3h**: $k_{inact} = 0.043$ s^{-1}; $K_I = 3.8 \times 10^{-7}$ M. **3k**: $k_{inact} = 0.038$ s^{-1}; $K_I = 7.9 \times 10^{-7}$ M. **3n**: $k_{inact} = 0.020$ s^{-1}; $K_I = 2.5 \times 10^{-7}$ M. [c] No inhibition. [d] Obtained as k_{obs}/[I] at low inhibitor concentration.

Noticeable differences in the inactivation potency towards α-CT were obtained (Tables 1 and 2). The potency dramatically depended upon the nature of the substituent at the 3-position. Better inactivations were observed with the esters than with the corresponding amides (table 1). In both series, the presence of an aromatic group enhanced the inhibition potency when the aryl side chain was attached directly to the oxy and aminocarbonyl moiety. As expected from the known primary specificity of α-CT (hydrophobic pocket), no inactivation was detected with **11** (charged carboxylate). Conversely, small (**3a**, **8**, **3b**) or large (**3f**−**r**) hydrophobic groups favored the inactivation. However, a too long aliphatic chain (**3c** and **3d** *versus* **3b** or **8**) or a benzylic chain (**3g** *versus* **3f**) led to a decrease of k_{inact}/K_I. For **3f** *versus* **3b**, k_{inact} was increased by a factor of ∼3 whereas K_I was lowered by a factor of ∼50, suggesting that for these compounds the inactivation potency appeared to be rather governed by the recognition step (K_I) than by the inactivation step (k_{inact}).

A peptide mimic at the 3-position in an aliphatic (**4i**) or an aromatic (**4j**) amide did not enhance the inhibitory activity (Table 1).

The influence of the nature and the position of substituents on the aromatic ring is reported in Table 2. Interestingly, *ortho* and *meta* substitutions were always found to be more favorable than *para* ones. Among those, the *meta*-substituted derivative **3l** (*m*-chlorophenyl 6-(chloromethyl)-2*H*-1-benzopyran-3-carboxylate) appeared to be the most powerful α-CT inactivator of the series (k_{inact}/K_I = 762 700 M^{-1} s^{-1}).

Phenyl 6-methyl-2-oxo-2*H*-1-benzopyran-3-carboxylate (**14**), which corresponded to the nonhalogenated analog of **3f** and therefore was devoid of latent alkylating function, was found to be essentially inactive on α-CT. These results indicated that the halomethyl moiety in the 6-position was required to lead to an irreversible inhibition probably through the formation of a covalent bond between the benzylic carbon atom and a nucleophilic residue located within the enzyme recognition site, i.e. a histidine residue. Nevertheless, an alkylation of the Met-192 residue, which caps the primary specificity pocket, could not be excluded.

When human leukocyte elastase was used as the target serine proteinase, no significant inhibition of the serine enzyme was observed except with **3l** and **3q** (Table 2). Interestingly, compound **14** also inhibited HLE. As for **3l** and **3q**, this inactivation was transient and the enzyme slowly recovered its activity, suggesting the formation of a stable acyl enzyme for the three compounds. The reported values of k_{obs}/[I] in Tables 1 and 2 correspond to the acylation step.

The present results led to the identification of aryl 6-(chloromethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylate esters as a new generic class of α-CT inactivators. Structure−activity relationships strongly suggest that the position of the substituent on the phenyl ring dramatically influenced the inhibitory potency on the serine proteinase since the *meta* or the *ortho* position appear to be the most favorable. This core coumarinic structure may be of interest in the design of inactivators targeting serine proteinases of other specificities by modifying the nature of the substituent at the 3-position.

Experimental Section

Chemistry. Melting points were determined with a Büchi-Tottoli apparatus in open capillary tubes and are uncorrected. Analysis (C, H, N, S) 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 Brucker AW 80 (80 MHz) in $CDCl_3$ (or DMSO-d_6) with TMS (or HMDS) as an internal standard; chemical shifts are reported in δ values (ppm) relative to internal TMS (or HMDS).

Ethyl 6-(Hydroxymethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylate Hemihydrate (7). A mixture of (hydroxy-

methyl)salicyl aldehyde[25] (30.0 g, 197 mmol), diethyl malonate (34.8 g, 217 mmol), piperidine (2.5 mL), and acetic acid (1.0 mL) in absolute ethanol (100 mL) was heated under reflux for 17 h. Boiling distilled water (250 mL) was then added with stirring to the hot mixture, and the resulting suspension was placed at +4 °C overnight. The crystalline precipitate so obtained was collected by filtration, washed 2-fold with ethanol:water (6:4, v/v, 200 mL), and dried. Recrystallization in boiling ethanol (4 mL/g) gave **7** (43,1 g, 85%): mp 109−110 °C; IR (KBr) 3548, 3508, 3392, 3239 (O−H), 3057 (C−H arom), 2981, 2914 (C−H aliph), 1746 (C═O ester), 1698 (C═O lactone), 1625, 1578, 1259 cm^{-1}; ^1H NMR ($CDCl_3$, TMS) δ 1.35 (t, 3H, OCH_2CH_3), 2.20 (s, 2H, CH_2OH, HO*H*), 4.35 (q, 2H, OCH_2CH_3), 4.75 (s, 2H, CH_2OH), 7.25 (d, 1H, 8-H), 7.60 (s, 1H, 5-H), 7.65 (d, 1H, 7-H), 8.45 (s, 1H, 4-H). Anal. ($C_{13}H_{12}O_5 \cdot 0.5H_2O$) C, H.

Ethyl 6-(Chloromethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylate (8). Ethyl 6-(hydroxymethyl)-2*H*-1-benzopyran-3-carboxylate (**7**) (0.5 g, 1.94 mmol) was heated in thionyl chloride (10 mL) for 3 h. Most of the excess reagent was evaporated under reduced pressure, and the residue was suspended in water (30 mL) and extracted 2-fold with $CHCl_3$ (30 mL). The combined organic layers were dried over MgSO4, filtered, and evaporated to dryness under reduced pressure. The residue of crude **8** was recrystallized in $CHCl_3$:petroleum ether, 40−60 °C (1:3, v/v), to give the title compound (0.42 g, 81%): mp 182−186 °C; IR 3061 (C−H arom), 2978 (C−H aliph), 1777 (C═O ester), 1757 (C═O lactone), 1622, 1574, 1380, 1252 cm^{-1}; ^1H NMR ($CDCl_3$, TMS) δ 1.35 (t, 3H, OCH_2CH_3), 4.35 (q, 2H, OCH_2CH_3), 4.65 (s, 2H, CH_2Cl), 7.30 (d, 1H, 8-H), 7.60 (s, 1H, 5-H), 7.65 (d, 1H, 7-H), 8.45 (s, 1H, 4-H). Anal. ($C_{13}H_{11}O_4Cl$) C, H.

6-(Hydroxymethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylic Acid (9). Ethyl 6-(hydroxymethyl)-2*H*-1-benzopyran-3-carboxylate (**7**) (20.0 g, 78 mmol) was dissolved in boiling ethanol (350 mL). Ebullition was maintained during the slow and progressive addition of 3 N HCl (2000 mL). After heating for 3 h in an open vessel, the mixture was cooled and the white precipitate of crude **9** was collected by filtration, washed with water and recrystallized in ethanol:water (1:5, v/v) (14.5 g, 85%), mp 238−240 °C; IR 3388 (O−H), 1735 (C═O carboxylic acid), 1703 (C═O lactone), 1620, 1575, 1247 cm^{-1}; ^1H NMR (CDCl3, DMSO-d_6, TMS) δ 4.65 (s, 2H, CH_2OH), 7.35 (d, 1H, 8-H), 7.65 (d, 1H, 7-H), 7.70 (s, 1H, 5-H), 8.65 (s, 1H, 4-H). Anal. ($C_{11}H_8O_5$) C, H.

6-(Chloromethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylic Acid (11). The suspension of 6-(hydroxymethyl)-2*H*-1-benzopyran-3-carboxylic acid (**9**) (1.0 g, 4.54 mmol) in thionyl chloride (10 mL) was refluxed for 3 h. The resulting solution was evaporated to dryness under reduced pressure, and the residue of crude **10** was dispersed in dry toluene (10 mL). After elimination of the solvent under reduced pressure, the residue was suspended in distilled water (10 mL) and supplemented with pyridine (0.76 mL, 9.40 mmol). After 10 min of stirring at room temperature, the suspension was ajusted to pH 3 and the precipitate of crude **11** was collected by filtration, washed with water, and recrystallized in ethanol (0.89 g, 82%): mp 158−162 °C; IR 3046 (C−H arom), 1741 (C═O carboxylic acid), 1694 (C═O lactone), 1623, 1576 cm^{-1}; ^1H NMR ($CDCl_3$, TMS) δ 4.65 (s, 2H, CH_2Cl), 7.50 (d, 1H, 8-H), 7.75 (s, 1H, 5-H), 7.80 (d, 1H, 7-H), 8.90 (s, 1H, 4-H). Anal. ($C_{11}H_7O_4Cl$) C, H.

General Procedure for the Preparation of the Esters (3) and the Amides (4) of 6-(Hydroxymethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylic Acid. The suspension of 6-(hydroxymethyl)-(2*H*-1-benzopyran-3-carboxylic acid (**9**) (1.0 g, 4.54 mmol) in thionyl chloride (10 mL) was refluxed for 3 h. The resulting solution was evaporated to dryness under reduced pressure, and the residue of crude **10** was dispersed in dry toluene (10 mL). The solvent was eliminated under reduced pressure. Dispersion in dry toluene and solvent elimination was repeated twice. The residue was dissolved in anhydrous dioxane (3 mL) and added with stirring to a solution of the appropriate alcohol (5.0 mmol) [or amine (5.0 mmol)] and of anhydrous pyridine (5.0 mmol) in anhydrous dioxane (7 mL). After 30−90 min at room temperature, depending upon the nature of the alcohol or the amine, the solvent was removed by evaporation under reduced pressure. The residue was partitioned between $CHCl_3$ (30−60 mL) and 0.1 N HCl (30 mL), and the decanted organic layer was dried over $MgSO_4$, filtered, and evaporated to dryness under reduced pressure. Crude **3** or **4** was then recrystallized in the appropriate solvent. Yields were 40−80%.

Methyl 6-(Chloromethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylate (3a). The title compound was obtained as described above after the reaction of **10** with methanol and was recrystallized in ethyl acetate:petroleum ether, 40−60 °C: mp 152−155 °C; IR 3068 (C−H arom), 2947 (C−H aliph), 1758 (C═O ester), 1714 (C═O lactone), 1622, 1576, 1378, 1251 cm^{-1}; ^1H NMR ($CDCl_3$, TMS) δ 3.95 (s, 3H, OCH_3), 4.65 (s,2H, CH_2Cl), 7.35 (d, 1H, 8-H), 7.60 (s, 1H, 5-H), 7.65 (d, 1H, 7-H), 8.45 (s, 1H, 4-H). Anal. ($C_{12}H_9O_4Cl$) C, H.

Isopropyl 6-(Chloromethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylate (3b). The title compound was obtained as described above after the reaction of **10** with 2-propanol and was recrystallized in ethyl acetate:petroleum ether, 40−60 °C: mp 185−189 °C; IR 3063 (C−H arom), 2980 (C−H aliph), 1746 (C═O ester), 1709 (C═O lactone), 1628, 1580, 1254 cm^{-1}; ^1H NMR ($CDCl_3$, TMS) δ 1.30 (d, 6H, $CH(CH_3)_2$), 4.60 (s, 2H, CH_2Cl), 5.25 (m, 1H, $CH(CH_3)_2$), 7.30 (d, 1H, 8-H), 7.55 (s, 1H, 5-H), 7.65 (d, 1H, 7-H), 8.40 (s, 1H, 4-H). Anal. ($C_{14}H_{13}O_4Cl$) C, H.

***n*-Pentyl 6-(Chloromethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylate (3c)**. The title compound was obtained as described above after the reaction of **10** with 1-pentanol and was recrystallized in ethyl acetate:petroleum ether, 40−60 °C: mp 120−124 °C; IR 3060 (C−H arom), 2957, 2929, 2859 (C−H aliph), 1751 (C═O ester), 1703 (C═O lactone), 1627, 1579, 1253 cm^{-1}; ^1H NMR ($CDCl_3$, TMS) δ 0.95 (t, 3H, $CH_2(CH_2)_3CH_3$), 1.25−1.85 (m, 6H, $CH_2(CH_3)_2CH_3$), 4.30 (t, 2H, $CH_2(CH_3)_2CH_3$), 4.65 (s, 2H, CH_2Cl), 7.30 (d, 1H, 8-H), 7.60 (s, 1H, 5-H), 7.65 (d, 1H, 7-H), 8.45 (s, 1H, 4-H). Anal. ($C_{16}H_{17}O_4Cl$) C, H.

***n*-Octyl 6-(Chloromethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylate (3d)**. The title compound was obtained as described above after the reaction of **10** with 1-octanol and was recrystallized in ethyl acetate:petroleum ether, 40−60 °C: mp 118−121 °C; IR 3062 (C−H arom), 2955, 2921, 2852 (C−H aliph), 1748 (C═O ester), 1703 (C═O lactone), 1626, 1579, 1308, 1252 cm^{-1}; ^1H NMR ($CDCl_3$, TMS) δ 0.90 (t, 3H, $CH_2(CH_2)_6CH_3$), 1.25−1.95 (m, 12H, $CH_2(CH_2)_6CH_3$), 4.25 (t, 2H, $CH_2(CH_2)_6CH_3$), 4.60 (s, 2H, CH_2Cl), 7.30 (d, 1H, 8-H), 7.60 (s, 1H, 5-H), 7.65 (d, 1H, 7-H), 8.50 (s, 1H, 4-H). Anal. ($C_{19}H_{23}O_4Cl$) C, H.

Allyl 6-(Chloromethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylate (3e). The title compound was obtained as described above after the reaction of **10** with allyl alcohol and was recrystallized in ethyl acetate:petroleum ether, 40-60 °C: mp 135−139 °C; IR 3050 (C−H arom), 1746 (C═O ester), 1708 (C═O lactone), 1623, 1575, 1253 cm^{-1}; ^1H NMR ($CDCl_3$, TMS) δ 4.60 (s, 2H, CH_2Cl), 4.85 (d, 2H, $CH_2CH{=}CH_2$), 5.30−5.65 (m, 2H, $CH_2CH{=}CH_2$), 6.00 (m, 1H, $CH_2CH{=}CH_2$), 7.30 (d, 1H, 8-H), 7.60 (s, 1H, 5-H), 7.65 (d, 1H, 7-H), 8.50 (s, 1H, 4-H). Anal. ($C_{14}H_{11}O_4Cl$) C, H.

Phenyl 6-(Chloromethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylate (3f). The title compound was obtained as described above after the reaction of **10** with phenol and was recrystallized in ethyl acetate:petroleum ether, 40−60 °C: mp 187−191 °C; IR 3068 (C−H arom), 1772 (C═O ester), 1733 (C═O lactone), 1624, 1577, 1245 cm^{-1}; ^1H NMR ($CDCl_3$, TMS) δ 4.65 (s, 2H, CH_2Cl), 7.10−7.50 (m, 6H, 8-H, C_6H_5), 7.60 (s, 1H, 5-H), 7.65 (d, 1H, 7-H), 8.65 (s, 1H, 4-H). Anal. ($C_{17}H_{11}O_4Cl$) C, H.

Benzyl 6-(Chloromethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylate (3g). The title compound was obtained as described above after the reaction of **10** with benzyl alcohol and was recrystallized in ethyl acetate:petroleum ether, 40-60 °C: mp 164−166 °C; IR 3057 (C−H arom), 1746 (C═O ester), 1709 (C═O lactone), 1629, 1581, 1252 cm^{-1}; ^1H NMR ($CDCl_3$, TMS) δ 4.60 (s, 2H, CH_2Cl), 5.40 (s, 2H, $CH_2C_6H_5$), 7.30 (d, 1H, 8-H), 7.35 (s, 5H, $CH_2C_6H_5$), 7.60 (s, 1H, 5-H), 7.65 (d, 1H, 7-H), 8.45 (s, 1H, 4-H). Anal. ($C_{18}H_{13}O_4Cl$) C, H.

***o*-Tolyl 6-(Chloromethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylate (3h)**. The title compound was obtained as described above after the reaction of **10** with *o*-methylphenol, and was recrystallized in ethyl acetate:petroleum ether: mp 179−182 °C; IR 3069 (C−H arom), 1732 (C═O ester and lactone), 1625, 1578, 1489, 1246 cm^{-1}; ^1H NMR ($CDCl_3$, TMS) δ 2.25 (s, 3H, CH_3), 4.60 (s, 2H, CH_2Cl), 7.00−7.35 (m, 4H, C_6H_4), 7.40 (d, 1H, 8-H), 7.70 (s + d, 2H, 5-H, 7-H), 8.65 (s, 1H, 4-H). Anal. ($C_{18}H_{13}O_4Cl$) C, H.

***m*-Tolyl 6-(Chloromethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylate (3i)**. The title compound was obtained as described above after the reaction of **10** with *m*-methylphenol and was recrystallized in ethyl acetate:petroleum ether: mp 163−167 °C; IR 3065 (C−H arom), 2955 (C−H aliph), 1729 (C═O ester and lactone), 1626, 1578, 1487, 1250 cm^{-1}; ^1H NMR ($CDCl_3$, TMS) δ 2.40 (s, 3H, CH_3), 4.60 (s, 2H, CH_2Cl), 7.00−7.30 (m, 4H, C_6H_4), 7.35 (d, 1H, 8-H), 7.70 (s + d, 2H, 5-H, 7-H), 8.65 (s, 1H, 4-H). Anal. ($C_{18}H_{13}O_4Cl$) C, H.

***p*-Tolyl 6-(Chloromethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylate (3j)**. The title compound was obtained as described above after the reaction of **10** with *p*-methylphenol and was recrystallized in chloroform:petroleum ether: mp 191−193 °C; IR 3065 (C−H arom), 1776 (C═O ester), 1734 (C═O lactone), 1627, 1579, 1507, 1247 cm^{-1}; ^1H NMR ($CDCl_3$, HMDS) δ 2.30 (s, 3H, CH_3), 4.50 (s, 2H, CH_2Cl), 6.90−7.30 (m, 4H, C_6H_4), 7.35 (d, 1H, 8-H), 7.70 (s + d, 2H, 5-H, 7-H), 8.45 (s, 1H, 4-H). Anal. ($C_{18}H_{13}O_4Cl$) C, H.

***o*-Chlorophenyl 6-(Chloromethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylate (3k)**. The title compound was obtained as described above after the reaction of **10** with *o*-chlorophenol and was recrystallized in ethyl acetate:petroleum ether: mp 153−156 °C; IR 3063 (C−H arom), 1735 (C═O ester and lactone), 1623, 1576, 1475, 1244, 1209 cm^{-1}; ^1H NMR ($CDCl_3$, TMS) δ 4.65 (s, 2H, CH_2Cl), 7.00−7.60 (m, 5H, C_6H_4, 8-H), 7.70 (s + d, 2H, 5-H, 7-H), 8.75 (s, 1H, 4-H). Anal. ($C_{17}H_{10}O_4Cl_2$) C, H.

***m*-Chlorophenyl 6-(Chloromethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylate (3l)**. The title compound was obtained as described above after the reaction of **10** with *m*-chlorophenol and was recrystallized in ethyl acetate:petroleum ether: mp 176−179 °C; IR 3087 (C−H arom), 1772 (C═O ester), 1727 (C═O lactone), 1621, 1591, 1576, 1472, 1244, 1224, 1206 cm^{-1}; ^1H NMR ($CDCl_3$, HMDS) δ 4.60 (s, 2H, CH_2Cl), 7.00−7.40 (m, 5H, C_6H_4, 8-H), 7.60 (s + d, 2H, 5-H, 7-H), 8.60 (s, 1H, 4-H). Anal. ($C_{17}H_{10}O_4Cl_2$) C, H.

***p*-Chlorophenyl 6-(Chloromethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylate (3m)**. The title compound was obtained as described above after the reaction of **10** with *p*-chlorophenol and was recrystallized in chloroform:petroleum ether: mp 223−226 °C; IR 3091, 3067 (C−H arom), 1768 (C═O ester), 1718 (C═O lactone), 1623, 1577, 1485, 1250, 1223, 1200 cm^{-1}; ^1H NMR ($CDCl_3$ + DMSO-d_6, HMDS) δ 4.65 (s, 2H, CH_2Cl), 7.05−7.50 (m, 5H, C_6H_4, 8-H), 7.65−7.95 (m, 2H, 5-H, 7-H), 8.90 (s, 1H, 4-H). Anal. ($C_{17}H_{10}O_4Cl_2$) C, H.

***o*-Iodophenyl 6-(Chloromethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylate (3n)**. The title compound was obtained as described above after the reaction of **10** with *o*-iodophenol and was recrystallized in ethyl acetate:petroleum ether: mp 162−164 °C; IR 3060 (C−H arom), 1772 (C═O ester), 1733 (C═O lactone), 1623, 1576, 1465, 1241, 1221, 1199 cm^{-1}; ^1H NMR ($CDCl_3$, TMS) δ 4.60 (s, 2H, CH_2Cl), 6.80−7.50 (m, 4H, 3H of C_6H_4, 8-H), 7.60−7.95 (m, 3H, 1H of C_6H_4, 5-H, 7-H), 8.85 (s, 1H, 4-H). Anal. ($C_{17}H_{10}O_4ClI$) C, H.

***m*-Iodophenyl 6-(Chloromethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylate (3o)**. The title compound was obtained as described above after the reaction of **10** with *m*-iodophenol and was recrystallized in ethyl acetate:petroleum ether: mp 161−163 °C; IR 3082, 3048 (C−H arom), 1771 (C═O ester), 1720 (C═O lactone), 1619, 1573, 1466, 1246, 1224, 1183 cm^{-1}; ^1H NMR ($CDCl_3$, TMS) δ 4.65 (s, 2H, CH_2Cl), 7.05−7.80 (m, 7H, C_6H_4, 8-H, 5-H, 7-H), 8.65 (s, 1H, 4-H). Anal. ($C_{17}H_{10}O_4ClI$) C, H.

***p*-Iodophenyl 6-(Chloromethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylate (3p)**. The title compound was obtained as described above after the reaction of **10** with *p*-iodophenol and was recrystallized in ethyl acetate: mp 262−263 °C; IR 3082, 3062 (C−H arom), 1773 (C═O ester), 1717 (C═O lactone), 1624, 1577, 1478, 1249, 1223, 1199 cm^{-1}; ^1H NMR (DMSO-d_6, HMDS) δ 4.80 (s, 2H, CH_2Cl), 6.90−8.20 (m, 7H, C_6H_4, 8-H, 5-H, 7-H), 9.00 (s, 1H, 4-H). Anal. ($C_{17}H_{10}O_4ClI$) C, H.

***m*-Nitrophenyl 6-(Chloromethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylate (3q)**. The title compound was obtained as described above after the reaction of **10** with *m*-nitrophenol and was recrystallized in ethyl acetate: mp 208−210 °C; IR 3113, 3086, 3055 (C−H arom), 1772 (C═O ester), 1723 (C═O lactone), 1620, 1573, 1530, 1241, 1223, 1212 cm^{-1}; ^1H NMR ($CDCl_3$, HMDS) δ 4.60 (s, 2H, CH_2Cl), 7.20−7.80 (m, 5H, 2H of C_6H_4, 8-H, 5-H, 7-H), 8.05 (m, 2H, 2H of C_6H_4), 8.65 (s, 1H, 4-H). Anal. ($C_{17}H_{10}NO_6Cl$) C, H.

4-Chloro-3-methylphenyl 6-(Chloromethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylate (3r). The title compound was obtained as described above after the reaction of **10** with 4-chloro-3-methylphenol and was recrystallized in ethyl acetate: mp 188−192 °C; IR 3065 (C−H arom), 1774 (C═O ester), 1757 (C═O lactone), 1620, 1573, 1479, 1245, 1221 cm^{-1}; ^1H NMR ($CDCl_3$, TMS) δ 2.35 (s, 3H, CH_3), 4.60 (s, 2H, CH_2-Cl), 6.80−7.20 (m, 3H, C6H3), 7.40 (d, 1H, 8-H), 7.65 (s + d, 2H, 5-H, 7-H), 8.60 (s, 1H, 4-H). Anal. ($C_{18}H_{12}O_4Cl_2$) C, H.

***N*-Methyl-6-(chloromethyl)-2-oxo-2*H*-1-benzopyran-3-carboxamide Hemihydrate (4a)**. The title compound was obtained as described above after the reaction of **10** with methylamine and was recrystallized in ethyl acetate: mp 215−218 °C; IR 3360 (N−H), 3068 (C−H arom), 1702 (C═O lactone), 1657 (C═O amide), 1618, 1577, 1536 cm^{-1}; ^1H NMR ($CDCl_3$, TMS) δ 3.00 (d, 3H, NHCH_3), 4.60 (s, 2H, CH_2Cl), 7.35 (d, 1H, 8-H), 7.65 (d, 1H, 7-H), 7.70 (s, 1H, 5-H), 8.70 (m, 1H, NHCH_3), 8.90 (s, 1H, 4-H). Anal. ($C_{12}H_{10}NO_3Cl\cdot0.5H_2O$) C, H, N.

***N*-Isopropyl-6-(chloromethyl)-2-oxo-2*H*-1-benzopyran-3-carboxamide (4b)**. The title compound was obtained as described above after the reaction of **10** with isopropylamine and was recrystallized in ethyl acetate: mp 202−208 °C; IR 3321 (N−H), 2970, 2917, 2849 (C−H aliph), 1731 (C═O lactone), 1649 (C═O amide), 1618, 1580, 1545 cm^{-1}; ^1H NMR ($CDCl_3$, TMS) δ 1.25 (d, 6H, CH(CH_3)$_2$), 4.30 (m, 1H, CH(CH$_3$)$_2$), 4.65 (s, 2H, CH_2Cl), 7.30 (d, 1H, 8-H), 7.60−7.75 (m, 2H, 5-H, 7-H), 8.55 (m, 1H, NH), 8.85 (s, 1H, 4-H). Anal. ($C_{14}H_{14}NO_3Cl$) C, H, N.

***N*-Pentyl-6-(chloromethyl)-2-oxo-2*H*-1-benzopyran-3-carboxamide (4c)**. The title compound was obtained as described above after the reaction of **10** with *n*-pentylamine and was recrystallized in ethyl acetate: mp 167−170 °C; IR 3322 (N−H), 3047 (C−H arom), 2935, 2856 (C−H aliph), 1707 (C═O lactone), 1656 (C═O amide), 1618, 1575, 1540 cm^{-1}; ^1H NMR ($CDCl_3$, TMS) δ 0.95 (t, 3H, $CH_2(CH_2)_3CH_3$), 1.10−1.80 (m, 6H, $CH_2(CH_2)_3CH_3$), 3.45 (m, 2H, $CH_2(CH_2)_3CH_3$), 4.60 (s, 2H, CH_2Cl), 7.35 (d, 1H, 8-H), 7.65 (d, 1H, 7-H), 7.75 (s, 1H, 5-H), 8.80 (m, 1H, NH), 8.90 (s, 1H, 4-H). Anal. ($C_{16}H_{18}NO_3Cl$) C, H, N.

***N*-Dodecyl-6-(chloromethyl)-2-oxo-2*H*-1-benzopyran-3-carboxamide (4d)**. The title compound was obtained as described above after the reaction of **10** with *n*-dodecylamine and was recrystallized in ethyl acetate: mp 142−145 °C; IR 3312 (N−H), 3046 (C−H arom), 2919, 2852 (C−H aliph), 1704 (C═O lactone), 1654 (C═O amide), 1618, 1575, 1533 cm^{-1}; ^1H NMR ($CDCl_3$, TMS) δ 0.85 (t, 3H, $CH_2(CH_2)_8CH_3$), 1.20−1.80 (m, 16H, $CH_2(CH_2)_8CH_3$), 3.45 (m, 2H, $CH_2(CH_2)_8CH_3$), 4.60 (s, 2H, CH_2Cl), 7.35 (d, 1H, 8-H), 7.65 (d, 1H, 7-H), 7.75 (s, 1H, 5-H), 8.75 (m, 1H, NH), 8.85 (s, 1H, 4-H). Anal. ($C_{21}H_{28}NO_3Cl$) C, H, N.

***N*-Allyl-6-(chloromethyl)-2-oxo-2*H*-1-benzopyran-3-carboxamide (4e)**. The title compound was obtained as described above after the reaction of **10** with allylamine and was recrystallized in ethyl acetate: mp 216−219 °C; IR 3332 (N−H), 3050 (C−H arom), 1704 (C═O lactone), 1658 (C═O amide), 1618, 1576, 1530 cm^{-1}; ^1H NMR ($CDCl_3$, TMS) δ 4.05 (dd, 2H, CH_2CH═CH$_2$), 4.60 (s, 2H, CH_2Cl), 5.05−5.55 (m, 2H, CH$_2$-CH═CH_2), 5.95 (m, 1H, CH$_2$CH═CH$_2$), 7.30 (d, 1H, 8-H), 7.65 (d, 1H, 7-H), 7.70 (s, 1H, 5-H), 8.80 (m, 1H, NH), 8.90 (s, 1H, 4-H). Anal. ($C_{14}H_{12}NO_3Cl$) C, H, N.

***N*-Phenyl-6-(chloromethyl)-2-oxo-2*H*-1-benzopyran-3-carboxamide (4f)**. The title compound was obtained as

described above after the reaction of **10** with aniline and was recrystallized in ethyl acetate: mp 213−216 °C; IR 1704 (C═O lactone), 1664 (C═O amide), 1615, 1597, 1574, 1557 cm^{-1}; ^1H NMR ($CDCl_3$, TMS) δ 4.65 (s, 2H, CH_2Cl), 6.95 (m, 1H, NH), 7.25−7.85 (m, 8H, 5-H, 7-H, 8-H, C_6H_5), 9.05 (s, 1H, 4-H). Anal. ($C_{17}H_{12}NO_3Cl$) C, H, N.

***N*-Benzyl-6-(chloromethyl)-2-oxo-2*H*-1-benzopyran-3-carboxamide (4g)**. The title compound was obtained as described above after the reaction of **10** with benzylamine, and was recrystallized in ethyl acetate: mp 186−189 °C; IR 3339 (N−H), 3049 (C−H arom), 2917 (C−H aliph), 1707 (C═O lactone), 1658 (C═O amide), 1621, 1577, 1536 cm^{-1}; ^1H NMR ($CDCl_3$, TMS) δ 4.65 (s, 2H, CH_2Cl), 4.70 (s, 2H, $CH_2C_6H_5$), 7.25−7.50 (m, 6H, 8-H, C_6H_5), 7.60−7.80 (m, 2H, 5-H, 7-H), 8.90 (s, 1H, 4-H), 9.15 (bs, 1H, NH). Anal. ($C_{18}H_{14}NO_3Cl$) C, H, N.

***N*-(Phenylethyl)-6-(chloromethyl)-2-oxo-2*H*-1-benzopyran-3-carboxamide (4h)**. The title compound was obtained as described above after the reaction of **10** with phenethylamine and was recrystallized in ethyl acetate: mp 190−193 °C; IR 3341 (N−H), 3047 (C−H arom), 2943 (C−H aliph), 1704 (C═O lactone), 1657 (C═O amide), 1620, 1576, 1543 cm^{-1}; ^1H NMR ($CDCl_3$, TMS) δ 2.90 (t, 2H, $CH_2CH_2C_6H_5$), 3.75 (m, 2H, $CH_2CH_2C_6H_5$), 4.60 (s, 2H, CH_2Cl), 7.20−7.45 (m, 6H, 8-H, C_6H_5), 7.60−7.80 (m, 2H, 5-H, 7-H), 8.80 (m, 1H, NH), 8.85 (s, 1H, 4-H). Anal. ($C_{19}H_{16}NO_3Cl$) C, H, N.

***N*-[(Methoxycarbonyl)methyl]-6-(chloromethyl)-2-oxo-2*H*-1-benzopyran-3-carboxamide (4i)**. The title compound was obtained as described above after the reaction of **10** with glycine methyl ester and was recrystallized in ethyl acetate: mp 200−205 °C; IR 3302 (N−H), 3046 (C−H arom), 1741 (C═O ester), 1708 (C═O lactone), 1656 (C═O amide), 1617, 1575, 1530 cm^{-1}; ^1H NMR ($CDCl_3$, HMDS) δ 3.70 (s, 3H, CH_3), 4.15 (d, 2H, CH_2NH), 4.55 (s, 2H, CH_2Cl), 7.25 (d, 1H, 8-H), 7.40 (s, 1H, 5-H), 7.60 (m, 1H, 7-H), 8.80 (s, 1H, 4-H), 9.10 (bt, 1H, NH). Anal. ($C_{14}H_{12}NO_5Cl$) C, H, N.

***N*-[1-Benzyl-1-(methoxycarbonyl)methyl]-6-(chloromethyl)-2-oxo-2*H*-1-benzopyran-3-carboxamide (4j)**. The title compound was obtained as described above after the reaction of **10** with phenylalanine methyl ester and was recrystallized in ethyl acetate: mp 122−126 °C; IR 3327, 3290 (N−H), 3030 (C−H arom), 2952 (C−H aliph), 1742 (C═O ester), 1731 (C═O lactone), 1652 (C═O amide), 1618, 1577, 1528 cm^{-1}; ^1H NMR ($CDCl_3$, HMDS) δ 3.15 (d, 2H, $CH_2C_6H_5$), 3.65 (s, 3H, CH_3), 4.55 (s, 2H, CH_2Cl), 4.90 (q, 1H, CHNH), 7.20 (s, 5H, C_6H_5), 7.30 (d, 1H, 8-H), 7.55 (s, 1H, 5-H), 7.60 (d, 1H, 7-H), 8.70 (s, 1H, 4-H), 9.10 (bd, 1H, NH). Anal. ($C_{21}H_{18}NO_5Cl$) C, H, N.

Phenyl 6-Methyl-2-oxo-2*H*-1-benzopyran-3-carboxylate (14). 6-Methyl-2-oxo-2*H*-1-benzopyran-3-carboxylic acid (**12**)[27] was converted into the corresponding acid chloride **13** and then into the title ester **14** following the general procedure described for the preparation of the esters **3** of 6-(hydroxymethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylic acid. The title compound **14** was recrystallized in ethyl acetate:petroleum ether, 40−60 °C: mp 178−183 °C; IR 3066 (C−H arom), 1734 (C═O ester and lactone), 1627, 1577, 1491, 1243, 1195 cm^{-1}; ^1H NMR ($CDCl_3$, HMDS) δ 2.35 (s, 3H, CH_3), 7.05−7.60 (m, 8H, C_6H_5, 8-H, 5-H, 7-H), 8.55 (s, 1H, 4-H). Anal. ($C_{17}H_{12}O_4$) C, H.

Enzymatic Studies. Bovine α-CT and HLE were purchased from Sigma and Elastase Products Co., respectively. Enzyme concentrations were determined by active-site titrations as described in ref 19. The enzymes were assayed spectrophotometrically with the appropriate *p*-nitroanilide substrate (Sigma): succinylalanylalanylprolylphenylalanyl *p*-nitroanilide for α-CT and methoxysuccinylalanylalanylprolylvalyl *p*-nitroanilide for HLE. The enzymatic reactions were followed in 0.025 M sodium phosphate, 0.05 M KCl, pH 7.5, 10% (v/v) DMSO for α-CT and in 0.1 M Hepes, 0.5 M NaCl, 0.01% (v/v) Tween 80, pH 8.0, 10% (v/v) DMSO for HLE. Assays were run at 25 °C in a Perkin-Elmer Lambda 5 spectrophotometer equipped with a thermostated cell holder.

Enzyme inhibitions were analyzed either by the preincubation method or by the progress curve method as described in ref 15. The progress curve method was used when the inhibition was too fast to be observed by the preincubation method. In both cases, the inactivation constants k_{obs}, k_{inact}, and K_I were obtained by linear or nonlinear regression to the equations developed in ref 15 (see also legend to Figure 2) using Kaleidagraph version 2.1.3 from Abelbeck Software. At low inhibitor concentrations, the ratio k_{inact}/K_I was obtained as $k_{obs}/[I]$. For the preincubation method, enzyme and coumarinic derivative concentrations were $[\alpha\text{-CT}]_0 = 12.5$ nM or 7 nM (**3r**), [inhibitor concentrations] = 0.5−100 μM; $[HLE]_0$ = 20 or 30 nM, [inhibitor concentrations] = 5−100 μM. For the progress curve method, enzyme, substrate, and coumarinic derivative concentrations were $[S]_0 = 40$ μM, $[\alpha\text{-CT}]_0 = 12.5$ nM, [**8**] = 60−170 μM, [**3b**] = 10−90 μM, [**3e**] = 10−150 μM, [**3f**] = 0.05−0.6 μM, [**3h**] = 0.08−0.5 μM, [**3i**] = 0.04−1 μM, [**3k**] = 0.15−1 μM, [**3l**] = 0.01−0.08 μM, [**3n**] = 0.01−0.065 μM, [**3o**] = 0.1−0.6 μM.

Hydroxylamine reactivation assays were performed by treatment of inactivated α-CT solutions with the nucleophile hydroxylamine (0.66 M) at pH 7.5 and 25 °C during 30 min. Enzyme activity of aliquots versus a control was monitored.

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