Novel 3-Carboxamide-coumarins as Potent and Selective FXIIa Inhibitors

Séverine Robert,[†] Carine Bertolla,[†] Bernard Masereel, Jean-Michel Dogné, and Lionel Pochet*

Department of Pharmacy, Drug Design and Discovery Center, FUNDP, University of Namur, 61, Rue de Bruxelles, B-5000 Namur, Belgium

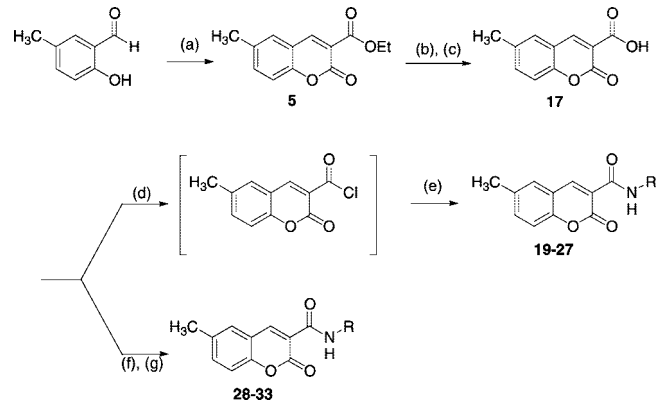
Received March 12, 2008

Abstract: Recently, FXIIa was highlighted as an original attractive target for the development of new anticoagulant drugs with low rates of therapy-related hemorrhages. In this work, we describe the development of a new series of 3-carboxamide-coumarins that are the first potent and selective nonpeptidic inhibitors of FXIIa.

The coagulation cascade takes place through a series of proteolytic reactions involving trypsin-like serine proteases in order to generate sufficient amount of thrombin (THR[a]) to form a fibrin clot. Initiation of fibrin formation through the "extrinsic pathway" occurs when plasmatic activated factor VII (FVIIa) forms a complex with the transmembrane protein tissue factor (TF), which is exposed following a lesion of endothelium. Alternatively, the coagulation cascade may be initiated through the "intrinsic pathway" when factor XII (FXII) is activated on a negatively charged surface by a process called "contact activation". Activation of FXII is followed sequentially by activation of factor XI and factor IX. The intrinsic and extrinsic pathways converge at the level of factor X (FX) activation. Activated factor X (FXa) activates prothrombin to THR in the presence of the cofactor activated factor V, and THR subsequently converts fibrinogen to fibrin.[1]

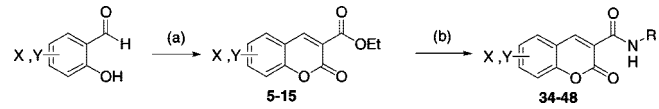
FXII has long been considered to be unnecessary for normal hemostasis because humans with hereditary deficiencies of FXII do not suffer from spontaneous or injury-related abnormal bleedings.[2,3] However, recent studies have highlighted the potential role of FXII in pathological thrombogenesis.[1,4,5] So FXII-deficient mice were found to be protected against arterial thrombosis, collagen- and epinephrine-induced thromboembolism,[6] and ischemic brain stroke.[7] In all these in vivo models, the protection was abolished by infusion of human FXII into FXII-null mice. Wild type mice treated by D-Pro-Phe-Arg chloromethyl ketone (PCK), which is known to inhibit the amidolytic activity of activated factor XII (FXIIa),[8] were also found to be protected from ischemic brain stroke.[7] Moreover, as their human counterparts, the FXII-null mice do not suffer from impaired hemostasis.[9] FXIIa is thus an attractive target for inhibitors designed to treat or prevent thromboembolic disorders. Indeed, FXIIa inhibitors would likely have only limited effects on hemostasis. Consequently, it is anticipated that their use in clinic would be associated with relatively low rates of therapy-related hemorrhages,[10] a well-known adverse drug reaction of the majority of anticoagulant agents.

Scheme 1. Synthesis of the Amide **19**−**27** and **28**−**33** Derivatives[a]



[a] Reagents: (a) diethyl malonate, piperidine, MW ; (b) 10% w/v NaOH; (c) 6 N HCl; (d) $SOCl_2$; (e) R-NH_2; (f) EDC, HOBt, DMF; (g) R-NH_2.

Scheme 2. Synthesis of the Amide **34**−**48** Derivatives[a]



[a] Reagents: (a) diethyl malonate, piperidine, MW; (b) R-NH_2, $BMimPF_6$ ethanol, MW.

Natural anticoagulant proteins displaying FXIIa inhibitory potency were reported from leguminous plants,[11–17] hematophagous insects,[18,19] helminth parasites,[20] and bacteria.[21] However, despite their efficacy, all these proteins were generally not selective over blood coagulation proteases.

In our previous studies, we demonstrated the efficacy of 3,6-disubstituted coumarins for inhibition of THR and FXa, two enzymes implicated in the common pathway of the coagulation cascade.[22] Within this series, we highlighted two important structural features to inhibit both enzymes: the ester link between the coumarin ring and the side chain and the chloromethyl function in the 6-position (which has been demonstrated to play a key role in the inhibition process). The removal of these structural features led to a drastic loss of inhibitory potency toward THR and FXa. Taking into account the similarities between the trypsin-like serine proteases THR, FXa, and FXIIa, we decided to design coumarins as potent and selective inhibitors of FXIIa. In order to afford selectivity toward THR and FXa, we synthesized novel 3-carboxamide-coumarins deprived of a chloromethyl moiety in the 6-position.

These novel coumarins have been synthesized according to Schemes 1 and 2. In the first step, the commercially available salicylaldehydes were converted into the corresponding 2-oxo-2*H*-1-benzopyran-3-carboxylic acid ethyl esters via a Knoevenagel reaction under microwave irradiation (Schemes 1 and 2).[23] This procedure allowed not only drastical reduction of the reaction time (from 6−24 h to 2−30 min) but also improvement in the yields (from 14−21% to 45−64%).

The ester hydrolysis was then easily realized by alkaline hydrolysis followed by acidification to afford the intermediate **17** (Scheme 1). Several routes were investigated to obtain the amide derivatives. For derivatives **19**−**27**, the suitable amine was reacted with the acyl chloride obtained by treatment of **17** with thionyl chloride. Alternatively, derivatives **28**−**33** were

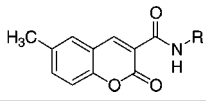
* To whom correspondence should be addressed. Phone: +32 (0)81 72 42 90. Fax: +32(0)81 72 42 99. E-mail: lionel.pochet@fundp.ac.be.

[†] These authors equally contributed to this work.

[a] Abbreviations: FVIIa, activated factor VII; FX, factor X; FXa, activated factor X; FXII, factor XII; FXIIa, activated FXII; Kall, kallikrein; PCK, D-Pro-Phe-Arg chloromethyl ketone; TF, tissue factor; THR, thrombin.

10.1021/jm8002697 CCC: $40.75
Published on Web 05/07/2008

Table 1. Inhibitory Potency of N-Substituted 6-Methyl-2-oxo-2*H*-1-benzopyran-3-carboxamides toward FXIIa, THR, FXa, TF/FVIIa, and Kallikrein



		IC_{50} (μM)[a]				
compd	R	FXIIa	THR	FXa	TF/FVIIa	Kall
19	isopropyl	NI[b]	NI[b]	NI[b]	>50	>50
21	cyclohexyl	NI[b]	NI[b]	NI[b]	NI[b]	NI[b]
22	cyclododecyl	33 (27−39)	NI[b]	NI[b]	NI[b]	NI[b]
23	adamantyl	29 (24−34)	NI[b]	NI[b]	NI[b]	NI[b]
20	phenyl	23 (20−28)	>50	NI[b]	NI[b]	>50
24	benzyl	>50	NI[b]	NI[b]	NI[b]	NI[b]
25	ethylphenyl	>50	NI[b]	NI[b]	NI[b]	NI[b]
26	naphthalen-1-ylmethyl	15 (11−20)	>50	NI[b]	NI[b]	NI[b]
27	quinol-3-yl	8.3 (5.9−11.5)	NI[b]	NI[b]	NI[b]	NI[b]

[a] Calculated by nonlinear regression from dose−response curves. Values in parentheses are 95% confidence intervals. [b] NI, no inhibition at 50 μM.

prepared by reaction between the suitable amine and the carboxylic acid activated by EDC/HOBt.[24]

The 3-carboxamide-coumarins **34**−**48** were obtained in one step by aminolysis of the corresponding 2-oxo-2*H*-1-benzopyran-3-carboxylic acid ethyl ester under microwave irradiation (Scheme 2). This latter route gave high yields (ranging from 50% to 81%) with phenylamine derivatives and reaction times from 20 to 60 min.

The inhibitory potency of the newly 3-carboxamide-coumarins was investigated by measuring the IC_{50} value for inhibition of FXIIa. First, we tested a series of N-substituted-6-methyl-2-oxo-2*H*-1-benzopyran-3-carboxamides (Table 1). Among this series, the compounds bearing a small aliphatic side chain such as isopropyl (**19**) or cyclohexyl (**21**) were inactive toward FXIIa. However, the presence of a bulky aliphatic group such as cyclododecyl (**22**) or adamantyl (**23**) as well as a bulky aromatic group such as naphthalen-1-ylmethyl (**26**) or quinol-3-yl (**27**) led to active compounds. Phenylamide derivative (**20**) was also found to be potent, but the introduction of a spacer between this phenyl side chain and the carboxamide in the 3-position led to the lost of the inhibitory potency (**24**, **25** vs **20**).

Second, we introduced various modifications on the aromatic part of the coumarin ring keeping a 3-phenylcarboxamide side chain (Table 2). We observed that the presence of a methyl group in the 6-position was favorable to the inhibitory potency on FXIIa (**20** vs **18**). Moreover, the replacement of this methyl group in the 6-position by a halogen or a nitro moiety in the same position did not significantly improve the inhibitory potency (**4**, **35**, **36** vs **20**). However, a disubstitution in the 6 and 8 positions by halogens improved the inhibitory potency (**41**, **44** vs **20**). In contrast, the presence of a methoxy group in the 8-position alone (**40**) or in combination with a bromo in the 6-position (**47**) was unfavorable. The substitution in the 5,6-positions by a benzo group (**48**) or in the 7-position by a hydroxyl (**37**), a methoxy (**38**), or a diethylamino (**39**) always led to inactive compounds.

For comparison purposes, we also tested compounds bearing a 3-phenyl ester side chain. These derivatives presented no (**1**, **2**) or lesser (**3**) inhibitory potency on FXIIa compared to their amide counterparts (**18**, **20**, **4**). In conclusion, the best compound in this series was the phenylamide of 2-oxo-2*H*-1-benzopyran-3-carboxylic acid substituted by a 8-bromo-6-chloro group (**44**) with IC_{50} for inhibition of FXIIa of 4.4 μM.

Finally, we investigated the influence of the nature and the position of substituents on the phenyl side chain in the 3-position (Table 3). These substituents strongly affect the inhibitory potency. Indeed, in the 6-methyl series, a methyl group was more favorable than a chloro group especially when this methyl is in the 3′-position (**28**, **29**, **30** vs **31**, **32**, **33**). The introduction of a second methyl group in 5′-position led to **34**, which is as active as the monosubstituted one (**29**). In the 6,8-dichloro series, a single methyl group did not improve potency (**42** vs **41**) whereas the introduction of the second methyl led to a 4-fold activity increase (**43** vs **41**). Surprisingly, the introduction of methyl substituents in the 8-bromo-6-chloro series led to a decrease of inhibitory potency (**45**, **46** vs **44**). The best compounds among the newly designed 3-carboxamide-coumarins were **43** and **44** with IC_{50} values for inhibition of FXIIa of 4.3 and 4.4 μM, respectively.

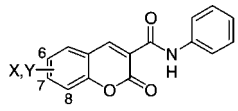
Furthermore, the newly designed 3-carboxamide-coumarins were selective for structurally related enzymes such as THR, FXa, TF/FVIIa, and kallikrein (Kall) (Tables 1–3). The selectivity of these compounds toward THR could be explained by the absence of an ester link between the coumarin and the side chain in the 3-position but also by the lack of a chloromethyl function in the 6-position. Indeed, it has been shown that the chloromethyl group participates in the mechanism-based behavior of 3,6-disubstituted coumarins toward THR.[22] With this latter series, it has been postulated that the lactone carbonyl would be first attacked by the catalytic nucleophilic serine 195. The subsequent lactone ring opening would result in an increase of the leaving properties of the chlorine atom and formation of an electrophilic quinone methide. This latter compound would form a covalent bond with a nucleophilic residue within the THR active site leading to the formation of an alkyl enzyme.

However, with the 3-carboxamide-coumarins presented in this paper, the most potent FXIIa inhibitors were devoid of a latent alkylating function, suggesting that this new series was not likely acting through the same mechanism. We thus investigated the inhibition profile of **44** toward FXIIa. Time dependent inhibition was first observed, indicating a slow binding of **44** to FXIIa (see Supporting Information). Moreover, this inhibition was persistent even after elimination of the excess of inhibitor by ultracentrifugation. These data demonstrate the irreversible nature of the inhibition. The alkylation of FXIIa being impossible, the inactivation is probably due to the formation of a stable acyl enzyme. This acyl enzyme could be formed by the reaction of the FXIIa active serine with the carbonyl group of the lactone ring or with the exocyclic amide function. An acylation of the lactone group has been already observed for the inhibition of α-chymotrypsin by the ester of 3-chlorophenyl-6-methylcoumarin 3-carboxylic acid.[27] Therefore, these newly designed 3-carboxamide-coumarins would act as mechanism-based inhibitors of FXIIa through the formation of an acyl enzyme instead of an alkyl enzyme as observed with thrombin and 6-chloromethyl ester coumarins.

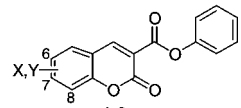
For comparison purposes, we also tested the inhibitory potency of the peptidic compound PCK. It had an IC_{50} for inhibition of FXIIa of 0.18 μM. However, it was nonselective toward THR, FXa, TF/FVIIa, and kallikrein with IC_{50} values of 2.5, 1.9, 1.5, and 0.031 μM, respectively. Moreover, the peptidic structure and the alkylating behavior of the chloromethyl function makes PCK unsuited for oral clinical use as anticoagulant agent.

In this work, we successfully developed original 3-carboxamide-coumarins that selectively inhibit FXIIa, a recent interest-

Table 2. Inhibitory Potency for Phenyl Esters or Amides of Substituted 2-Oxo-2*H*-1-benzopyran-3-carboxylic Acid toward FXIIa, THR, FXa, TF/FVIIa, and Kallikrein



4, 18, 20, 35-41, 44, 47-48

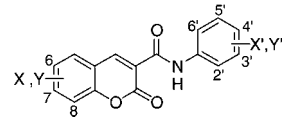


1-3

		IC_{50} (μM)[a]				
compd	X, Y	FXIIa	THR	FXa	TF/FVIIa	Kall
18		42 (27−66)	NI[b]	NI[b]	NI[b]	NI[b]
20	6-CH_3	25 (21−31)	>50	NI[b]	NI[b]	>50
4[c]	6-CH_2Cl	17 (12−23)	>50	>50	NI[b]	NI[b]
35	6-NO_2	22 (16−30)	NI[b]	NI[b]	NI[b]	NI[b]
36	6-Br	24 (18−31)	NI[b]	NI[b]	NI[b]	NI[b]
41	6,8-diCl	19 (12−30)	NI[b]	NI[b]	NI[b]	>50
44	6-Cl, 8-Br	4.4 (3.2−6.2)	NI[b]	NI[b]	NI[b]	NI[b]
47	6-Br, 8-OMe	>50	>50	NI[b]	>50	NI[b]
40	8-OMe	NI[b]	NI[b]	>50	NI[b]	>50
48	5,6-benzo	NI[b]	>50	>50	>50	>50
37	7-OH	NI[b]	>50	>50	NI[b]	>50
38	7-OMe	NI[b]	>50	>50	NI[b]	>50
39	7-$NH(C_2H_5)_2$	NI[b]	NI[b]	NI[b]	>50	>50
1[d]		NI[b]	NI[b]	NI[b]	NI[b]	NI[b]
2[c]	6-CH_3	NI[b]	NI[b]	NI[b]	NI[b]	NI[b]
3[c]	6-CH_2Cl	>50	7.0 (3.7−13.5)	>50	NI[b]	NI[b]

[a] Calculated by nonlinear regression from dose−response curves. Values in parentheses are 95% confidence intervals. [b] NI, no inhibition at 50 μM. [c] From ref 25. [d] From ref 26.

Table 3. Inhibitory Potency for Phenyl Amides of Substituted 2-Oxo-2*H*-1-benzopyran-3-carboxylic Acid toward FXIIa, THR, FXa, TF/FVIIa, and Kallikrein



			IC_{50} (μM)[a]				
compd	X, Y	X′, Y′	FXIIa	THR	FXa	TF/FVIIa	Kall
20	6-CH_3		23 (20−28)	>50	NI[b]	NI[b]	>50
31	6-CH_3	2′-Cl	30 (21−44)	>50	NI[b]	NI[b]	NI[b]
32	6-CH_3	3′-Cl	31 (23−42)	NI[b]	NI[b]	NI[b]	NI[b]
33	6-CH_3	4′-Cl	34 (25−45)	>50	NI[b]	NI[b]	NI[b]
28	6-CH_3	2′-CH_3	32 (21−47)	>50	NI[b]	NI[b]	NI[b]
29	6-CH_3	3′-CH_3	10 (7.2−15)	>50	NI[b]	NI[b]	NI[b]
30	6-CH_3	4′-CH_3	20 (14−27)	>50	NI[b]	>50	NI[b]
34	6-CH_3	3′,5′-diCH_3	9.8 (5.8−16.6)	>50	NI[b]	NI[b]	NI[b]
41	6,8-diCl		19 (12−30)	NI[b]	NI[b]	NI[b]	>50
42	6,8-diCl	3′-CH_3	25 (22−30)	NI[b]	NI[b]	NI[b]	NI[b]
43	6,8-diCl	3′,5′-diCH_3	4.3 (2.5−7.6)	>50	>50	>50	>50
44	6-Cl, 8-Br		4.4 (3.2−6.2)	NI[b]	NI[b]	NI[b]	NI[b]
45	6-Cl, 8-Br	3′-CH_3	10 (8.6−12)	>50	NI[b]	>50	NI[b]
46	6-Cl, 8-Br	3′,5′-diCH_3	32 (23−44)	>50	>50	>50	>50
PCK	D-Pro-Phe-Arg-CH_2Cl		0.18 (0.14−0.23)	2.5 (2.1−3.0)	1.9 (1.4−2.5)	1.5 (1.2−1.9)	0.031 (0.024−0.039)

[a] Calculated by nonlinear regression from dose−response curves. Values in parentheses are 95% confidence intervals. [b] NI, no inhibition at 50 μM.

ing new target for the prevention and the treatment of thromboembolic diseases. Despite a lower inhibitory potency on FXIIa than PCK, these derivatives were selective toward THR, FXa, TF/FVIIa, and kallikrein, contrary to PCK. To our knowledge, these compounds are the first potent and selective nonpeptidic inhibitors of FXIIa described in the literature to date. Their uses as anticoagulant agents are currently evaluated. They also constitute very interesting pharmacological tools to investigate the role of FXIIa in thrombosis and hemostasis. Further pharmacomodulations of the compounds should be investigated in order to increase the inhibitory potency on FXIIa while keeping the selectivity toward the other blood coagulation proteases.

Acknowledgment. This work was partly supported by the Fonds de la Recherche Scientifique Medicale Belge (Grant No. 1.5.066.07).

Supporting Information Available: Experimental procedures and analytical data. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Gailani, D.; Renne, T. Intrinsic pathway of coagulation and arterial thrombosis. *Arterioscler Thromb Vasc Biol* **2007**, *27*, 2507–2513.
(2) Hoffman, M.; Monroe, D. M. A cell-based model of hemostasis. *Thromb Haemostasis* **2001**, *85*, 958–965.
(3) Ratnoff, O. D.; Colopy, J. E. A familial hemorrhagic trait associated with a deficiency of a clot-promoting fraction of plasma. *J. Clin. Invest.* **1955**, *34*, 602–613.

(4) Renne, T.; Nieswandt, B.; Gailani, D. The intrinsic pathway of coagulation is essential for thrombus stability in mice. *Blood Cells, Mol. Dis.* **2006**, *36*, 148–151.

(5) Renne, T.; Gailani, D. Role of factor XII in hemostasis and thrombosis: clinical implications. *Expert Rev. Cardiovasc. Ther.* **2007**, *5*, 733–741.

(6) Renne, T.; Pozgajova, M.; Gruner, S.; Schuh, K.; Pauer, H. U.; Burfeind, P.; Gailani, D.; Nieswandt, B. Defective thrombus formation in mice lacking coagulation factor XII. *J. Exp. Med.* **2005**, *202*, 271–281.

(7) Kleinschnitz, C.; Stoll, G.; Bendszus, M.; Schuh, K.; Pauer, H. U.; Burfeind, P.; Renne, C.; Gailani, D.; Nieswandt, B.; Renne, T. Targeting coagulation factor XII provides protection from pathological thrombosis in cerebral ischemia without interfering with hemostasis. *J. Exp. Med.* **2006**, *203*, 513–518.

(8) Silverberg, M.; Kaplan, A. P. Enzymatic activities of activated and zymogen forms of human Hageman factor (factor XII). *Blood* **1982**, *60*, 64–70.

(9) Pauer, H. U.; Renne, T.; Hemmerlein, B.; Legler, T.; Fritzlar, S.; Adham, I.; Muller-Esterl, W.; Emons, G.; Sancken, U.; Engel, W.; Burfeind, P. Targeted deletion of murine coagulation factor XII gene-a model for contact phase activation in vivo. *Thromb. Haemostasis* **2004**, *92*, 503–508.

(10) Gailani, D.; Renne, T. The intrinsic pathway of coagulation: a target for treating thromboembolic disease. *J. Thromb. Haemostasis* **2007**, *5*, 1106–1112.

(11) Carter, T. H.; Everson, B. A.; Ratnoff, O. D. Cabbage seed protease inhibitor: a slow, tight-binding inhibitor of trypsin with activity toward thrombin, activated Stuart factor (factor Xa), activated Hageman factor (factor XIIa), and plasmin. *Blood* **1990**, *75*, 108–115.

(12) Cruz-Silva, I.; Gozzo, A. J.; Nunes, V. A.; Carmona, A. K.; Faljoni-Alario, A.; Oliva, M. L.; Sampaio, M. U.; Sampaio, C. A.; Araujo, M. S. A proteinase inhibitor from *Caesalpinia echinata* (pau-brasil) seeds for plasma kallikrein, plasmin and factor XIIa. *Biol. Chem.* **2004**, *385*, 1083–1086.

(13) Hayashi, K.; Takehisa, T.; Hamato, N.; Takano, R.; Hara, S.; Miyata, T.; Kato, H. Inhibition of serine proteases of the blood coagulation system by squash family protease inhibitors. *J. Biochem.* **1994**, *116*, 1013–1018.

(14) Hojima, Y.; Pierce, J. V.; Pisano, J. J. Pumpkin seed inhibitor of human factor XIIa (activated Hageman factor) and bovine trypsin. *Biochemistry* **1982**, *21*, 3741–3746.

(15) Krishnamoorthi, R.; Gong, Y. X.; Richardson, M. A new protein inhibitor of trypsin and activated Hageman factor from pumpkin (*Cucurbita maxima*) seeds. *FEBS Lett.* **1990**, *273*, 163–167.

(16) Sampaio, C. A.; Oliva, M. L.; Tanaka, A. S.; Sampaio, M. U. Proteinase inhibitors in Brazilian Leguminosae. *Mem. Inst. Oswaldo Cruz* **1991**, *86* (Suppl. 2), 207–209.

(17) Sampaio, C. A.; Motta, G.; Sampaio, M. U.; Oliva, M. L.; Araujo, M. S.; Stella, R. C.; Tanaka, A. S.; Batista, I. F. Action of plant proteinase inhibitors on enzymes of the kallikrein kinin system. *Agents Actions Suppl.* **1992**, *36*, 191–199.

(18) Campos, I. T.; Tanaka-Azevedo, A. M.; Tanaka, A. S. Identification and characterization of a novel factor XIIa inhibitor in the hematophagous insect, *Triatoma infestans* (Hemiptera: Reduviidae). *FEBS Lett.* **2004**, *577*, 512–516.

(19) Campos, I. T.; Amino, R.; Sampaio, C. A.; Auerswald, E. A.; Friedrich, T.; Lemaire, H. G.; Schenkman, S.; Tanaka, A. S. Infestin, a thrombin inhibitor presents in *Triatoma infestans* midgut, a Chagas' disease vector: gene cloning, expression and characterization of the inhibitor. *Insect Biochem. Mol. Biol.* **2002**, *32*, 991–997.

(20) Foster, C. B.; Flanigan, T. P.; DeStigter, K. K.; Blanton, R.; Dumenco, L. L.; Gallagher, C.; Ratnoff, O. D. Inhibition of the activation of Hageman factor (factor XII) by extracts of *Schistosoma mansoni*. *J. Lab. Clin. Med.* **1992**, *120*, 735–739.

(21) Ulmer, J. S.; Lindquist, R. N.; Dennis, M. S.; Lazarus, R. A. Ecotin is a potent inhibitor of the contact system proteases factor XIIa and plasma kallikrein. *FEBS Lett.* **1995**, *365*, 159–163.

(22) Frederick, R.; Robert, S.; Charlier, C.; de Ruyck, J.; Wouters, J.; Pirotte, B.; Masereel, B.; Pochet, L. 3,6-Disubstituted coumarins as mechanism-based inhibitors of thrombin and factor Xa. *J. Med. Chem.* **2005**, *48*, 7592–7603.

(23) Bogdal, D. Coumarins: fast synthesis by Knoevenagel condensation under microwave irradiation. *J. Chem. Res. (S)* **1998**, 468–469.

(24) Montalbetti, C. A. G. N.; Falque, V. Amide bond formation and peptide coupling. *Tetrahedron* **2005**, *61*, 10827–10852.

(25) Pochet, L.; Doucet, C.; Schynts, M.; Thierry, N.; Boggetto, N.; Pirotte, B.; Jiang, K. Y.; Masereel, B.; de Tullio, P.; Delarge, J.; Reboud-Ravaux, M. Esters and amides of 6-(chloromethyl)-2-oxo-2*H*-1-benzopyran-3-carboxylic acid as inhibitors of alpha-chymotrypsin: significance of the "aromatic" nature of the novel ester-type coumarin for strong inhibitory activity. *J. Med. Chem.* **1996**, *39*, 2579–2585.

(26) Pochet, L.; Doucet, C.; Dive, G.; Wouters, J.; Masereel, B.; Reboud-Ravaux, M.; Pirotte, B. Coumarinic derivatives as mechanism-based inhibitors of alpha-chymotrypsin and human leukocyte elastase. *Bioorg. Med. Chem.* **2000**, *8*, 1489–1501.

(27) Pochet, L.; Dieu, M.; Frédérick, R.; Murray, A. M.; Kempen, I.; Pirotte, B.; Masereel, B. Investigation of the inhibition mechanism of coumarins on chymotrypsin by mass spectrometry. *Tetrahedron* **2003**, *59*, 4557–4561.

JM8002697