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ENOL LACTONE DERIVATIVES AS INHIBITORS OF HUMAN NEUTROPHIL ELASTASE AND TRYPSIN-LIKE PROTEASES

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Abstract. We report on the development of substituted valero enol lactones as powerful inhibitors of human neutrophil elastase (HNE) by valine mimic enol lactones and trypsin-like enzymes (trypsin, plasmin, urokinase, t-PA, and thrombin) by guanidino-aryl substituted enol lactones.

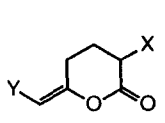
Serine proteases have attracted a growing interest due to the vital role they play in a number of biological processes. The design of mechanism-based inhibitors is relevant to the elucidation of their mechanism of action and ultimately, to the regulation of these enzymes in vivo for therapeutic purposes.[1] While mammalian serine proteases share similar tertiary structures and utilize equivalent mechanisms, the differences in their selectivity for peptide bond cleavage stem from differences in their primary specificity pocket, which is open and hydrophobic for chymotrypsin (Phe and Tyr selective), sterically congested for elastase (Ala and Val selective), and negatively charged for trypsin (Lys and Arg selective). Where primary site specificity is not great, interactions at secondary sites can play an important role in defining hydrolytic selectivity.

Lactone Systems Investigated — A number of heterocyclic compounds have been shown to act as mechanism-based inactivators of serine proteases, e.g., isatoic anhydrides,[2] isocoumarins,[3] ynenol lactones,[4] 6-chloro-2-pyrones,[5] etc. as well as halo enol lactones developed in our group (lactones **1-8**). Enol lactones utilize the normal catalytic machinery of the enzyme by initially acylating the active-site serine residue. Protio enol lactones can act simply as alternate substrate inhibitors, i.e., as transient inactivators which form very stable acyl-enzymes. By contrast, with halo enol lactones, the formation of the acyl-enzyme reveals a reactive halomethyl ketone group, which can alkylate a suitably positioned active-site residue and become permanently tethered to the enzyme. Our early studies with α-chymotrypsin have shown that α- and β-aryl (phenyl and naphthyl) substituted enol lactones (**1-8**) are potent inhibitors.[6] More recently, some members of this series have shown marked enantioselectivity in substrate binding and in deacylation rates.[7]

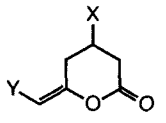
Recently our success with enol lactones as alternate substrate inhibitors and suicide substrates of α-chymotrypsin had been extended, by the development of specific inhibitors of elastase and trypsin-like enzymes. We expected that enol lactone-based compounds with α-isopropyl groups, and their analogs, or those in which the isopropyl group is part of the ring structure (**9-14**), could act as valine mimic elastase inhibitors. Compounds **15-22** are guanidino substituted analogs of α-chymotrypsin inhibitors **1**, **3**, **5** and **7**, and are designed as potential inhibitors of trypsin-like enzymes. The methylene bridge between the phenyl ring and the guanidino group of lactones **17** and **18** provides greater conformational mobility to the proposed acyl enzyme intermediate; compounds **21** and **22** have an α-benzamido substituent, mimicing the structure of α-amino acids. These lactones **9-22** were synthesized in our laboratory by established methods (to be reported elsewhere). Their enzyme inhibitory activity is described below.

Assay Strategy — Preliminary kinetic analysis of lactones **9-22** as inhibitors of elastase or trypsin-like enzymes involved a simple incubation assay to determine how rapidly, how completely, and for what period, were the enzymes inhibited and how permanent was the inhibition (hydrazine reactivation). The potential elastase inhibitors **9-14** were tested with human neutrophil elastase (HNE) and porcine pancreatic elastase (PPE), as well as, in some cases, α-chymotrypsin and trypsin, and the guanidino substituted compounds (**15-22**), with trypsin, urokinase, t-PA, plasmin and thrombin, as well as α-chymotrypsin. Following the preliminary screen, we undertook an in-depth analysis of the inhibition process (see equation 1) for selected enzymes and inhibitors by a competitive substrate assay to determine the binding constant ($K_{s/i}$) and the rate constant of acylation (k_a); the rate constant of deacylation (k_d) was determined in a separate assay. Tight binding (low $K_{s/i}$ value) and a fast rate of acylation (high k_a value), both contribute to a high specificity (high $k_a/K_{s/i}$ value) in a good inhibitor. The potency of an alternate substrate inhibitor is further enhanced by a slow rate of deacylation (low k_d value). A good suicide substrate would show efficient partitioning between alkylation and deacylation, such that permanent and complete inactivation could be achieved with only a few equivalents of inhibitor.

Enol Lactones as α-Chymotrypsin Inhibitors

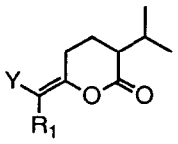


	X	Y
1	Ph	I
2	1-Np	I
3	Ph	H
4	1-Np	H

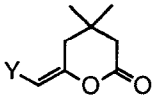


	X	Y
5	Ph	I
6	1-Np	I
7	Ph	H
8	1-Np	H

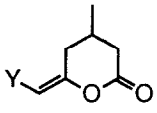
Enol Lactones as HNE Inhibitors



	Y	R_1
9	H	H
10a	Br	H
10b	H	Br
10c	Br	CH_3

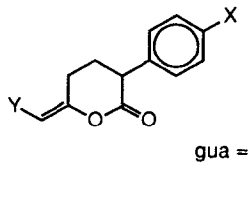


	Y
11	H
12	Br



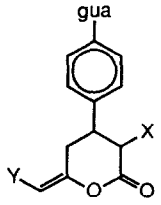
	Y
13	H
14	Br

Enol Lactones as Trypsin-like Protease Inhibitors



	Y	X
15	H	-gua
16	I	-gua
17	H	$-CH_2$-gua
18	I	$-CH_2$-gua

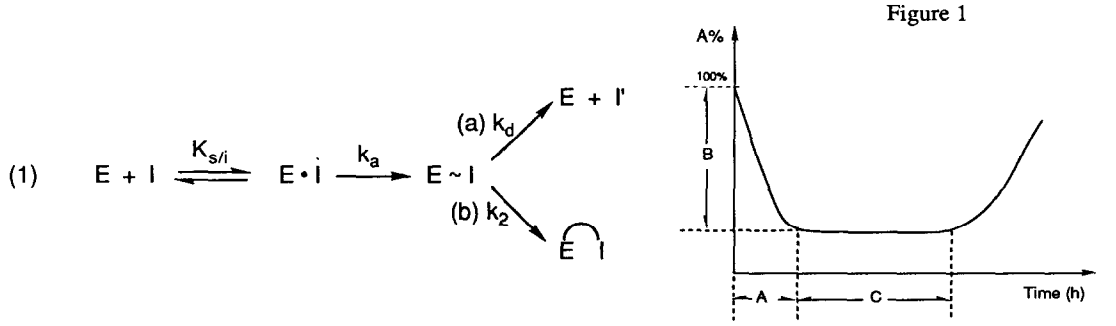
gua = $-NH-C(=NH_2^+)-NH_2$



	Y	X
19	H	H
20	I	H
21	H	NHC(O)Ph (3R*,4R*)
22	I	NHC(O)Ph (3R*,4R*)

Lactones Targeted to Elastase — The results of the incubation assays of potential elastase inhibitors **9-14** are summarized in Table I and are presented in a format that indicates conveniently the maximum percent of inhibition (B), the time in minutes to reach maximum extent of inhibition (A), and the persistence of this inhibition in minutes (C) (Fig. 1). This qualitative assay shows that all valine mimic enol lactones inhibit HNE and PPE with maximum inhibition being in all but a few cases in the 75-99% range. We found, as well (results not shown, W. Dai, in preparation), that systems such as α-isopropyl, α-propyl, α-sec-butyl and α-isobutyl display better inhibition than β-substituted ones.

Figure 1



The binding constant ($K_{i/s}$) and the acylation rate constant (k_a, and together the specificity constant k_a/K_i) were also determined (Table I). These results show that among α-alkyl substituted enol lactones, lactone **9** shows good selectivity to HNE compared to PPE, α-chymotrypsin, and trypsin. Lactone **10a** and **10b** selectively inhibit HNE as well as α-chymotrypsin. The hydrazine reactivation assay shows that lactones **10a-c** act as mechanism-based inhibitors with formation of greater than 62% alkylated HNE. These three lactones also inactivate HNE nearly stoichiometrically. These lactones should be useful tools to study elastases and related enzymes both in vitro and in vivo.

Lactones Targeted to Trypsin-Like Enzymes — The parameters for inhibition of selected proteases by the guanidino-aryl-substituted lactones **15-22** are outlined in Tables II and III. (Only those that showed good inhibitory potency in the preliminary incubation assay are reported here.) From Table II, it is immediately apparent that the α-guanidino-aryl substituted protio lactones **15** and **17** are selective for their target, the trypsin-like enzymes. The incubation assays indicated that the other enzymes tested in each case, were not effectively inhibited (results not shown). The guanidinomethyl-phenyl substituted protio lactone **17** was a potent alternate substrate inhibitor of urokinase and thrombin, as reflected by the stability of the acyl enzymes formed (low k_d values). The guanidino-phenyl substituted iodo lactone **16** was a suicide substrate of urokinase, plasmin, t-PA, thrombin and α-chymotrypsin, with an exceptionally high specificity for the former two enzymes. The increased conformational mobility in the guanidinomethyl-phenyl substituted iodo lactone **18** resulted in a different selectivity pattern; it was a suicide substrate of most of the trypsin like enzymes tested, exhibiting an exceptionally high specificity in its inhibition of trypsin and urokinase.

Table I. Parameters for the Inhibition of Elastases by Lactones **9-14**.

Lactone Protease	Incubation Assay A/B/C[a]	$K_{s/i}$ (μM)	k_a (min^{-1})	k_a/K_i (μM^{-1} min^{-1})
Lactone 9				
HNE	30/80/∞[b]	6.25	13	2.08
PPE	30/99/∞	88	0.42	0.005
α-Chy	10/98/∞	89	3.2	0.04
Try	10/83/∞	24	0.45	0.02
Lactone 10a				
HNE	90/93/∞	0.77	0.67	0.87
PPE	30/99/∞	26.9	0.52	0.02
α-Chy	10/99/∞	9.8	6	0.6
Try	10/95/∞	44	1.8	0.04
Lactone 10b				
HNE	20/99/∞	0.08	0.18	2.25
PPE	30/99/∞	58.8	0.28	0.005
α-Chy	--	2.3	1.6	0.7
Try	--	65	2.4	0.04
Lactone 10c				
HNE	20/95/∞	3.0	0.81	0.27
PPE	15/35/0	48.7	1.12	0.02
Lactone 11				
HNE	48/60/0	43.3	0.77	0.02
PPE	42/55/∞	104	0.51	0.005
Lactone 12				
HNE	18/26/∞	43.5	1.8	0.04
PPE	60/30/∞	284	0.43	0.002
Lactone 13				
HNE	30/10/0	22.7	0.6	0.03
PPE	10/73/∞	393	4.47	0.01
Lactone 14				
HNE	30/35/0	22.6	0.48	0.02
PPE	10/78/∞	216	0.33	0.001

[a]For a definition of A, B, and C, see Fig. 1. [b]∞ indicates that no enzyme activity was recovered over the 2 h-time course of the assay.

Table III shows the parameters for inhibition of selected proteases by the β-guanidino-aryl substituted enol lactones **19-22**. No new suicide substrates for the trypsin-like enzymes were found, as evidenced by the results of the hydrazine reactivation study. The α-benzamido substituted iodo lactone **22** was a suicide substrate of α-chymotrypsin. The β-guanidino-aryl protio and iodo enol lactones **19** and **20** were both alternate substrate inhibitors, selective for the trypsin-like enzymes. In particular, the protio lactone **19** formed very stable acyl-enzymes with trypsin and urokinase, exhibiting low rates of deacylation (k_d values were 0.002 and 0.009 min^{-1} respectively - Table III). The α-benzamido substituted protio and iodo lactones **21** and **22** were designed with the hope of improving the inactivation parameters of the β-guanidino-aryl substituted systems. The α-benzamido substituted iodo lactone **22** was again a transient inactivator of trypsin and urokinase, but with greatly increased potency. The acyl enzymes formed were stable, and deacylated very slowly, even upon treatment with hydrazine. In addition, our investigation revealed the iodo lactone **22** to be a very selective and effective suicide substrate of α-chymotrypsin.

Table II. Parameters for Inhibition of Selected Proteases by the α-Aryl Substituted Enol Lactones **15-18.**

Lactone	Incubation Assay		Competitive Substrate Assay			Deacylation
Proteases	A/B/C[a]	D[b]	$K_{s/i}$ (μM)	k_a (min^{-1})	k_a/K_s (μM^{-1} min^{-1})	k_d (min^{-1})
Lactone 15:						
Trypsin	0/97/45	--	0.23	14.3	62	fast[c]
Urokinase	0/97/∞	--	0.33	391	1185	fast[c]
Lactone 16:						
Urokinase	0/98/∞	0	0.16	18.65	117	--
t-PA	15/48/∞	0	0.67	--[c]	--	--
Plasmin	0/95/∞	14	0.03	13.6	453	--
Thrombin	0/93/∞	85	0.93	12.9	14	--
α-Chymotrypsin	15/78/∞	32	2.4	7.22	3	--
Lactone 17:						
Urokinase	0/91/∞	--	0.35	9.8	28	0.044
Plasmin	30/82/∞	--	466	57	0.12	fast[c]
Thrombin	15/94/∞	--	37.87	5.58	0.15	0.006
Lactone 18:						
Trypsin	0/99/∞	33	0.08	18.4	230	--
Urokinase	0/91/∞	20	0.01	7.56	756	--
t-PA	15/90∞	0	0.12	--[c]	--	--
Plasmin	0/98/∞	40	1.19	10.2	9	--
Thrombin	15/98/∞	100	14.84	27.25	2	0.013

[a]For a definition of A, B, and C, see Fig. 1. ∞ indicates that no enzyme activity was recovered over the 2 h-time course of the assay. [b]N_2H_4 treatment - percent activity regained. [c]Could not be measured by the method used.

Table III. Parameters for Inhibition of Selected Proteases by the β-Aryl Substituted Enol Lactones **19-22.**

Lactone	Incubation Assay		Competitive Substrate Assay			Deacylation
Proteases	A/B/C[a]	D[b]	$K_{s/i}$ (μM)	k_a (min^{-1})	k_a/K_s (μM^{-1} min^{-1})	k_d (min^{-1})
Lactone **19**:						
Trypsin	20/98/∞	--	9.4	91.4	10	0.002
Urokinase	0/99/∞	--	0.12	0.24	2	0.009
Plasmin	0/79/∞	--	63.7	97.2	1.5	fast[c]
Lactone **20**:						
Trypsin	0/99/∞	100	4.28	0.54	0.13	0.02
Urokinase	0/98/∞	100	7.83	2.61	0.33	fast[a]
Plasmin	60/97/∞	100	11	10.75	0.9	fast[a]
Lactone **21**:						
Trypsin	0/95/∞	100	10	10.2	1.02	0.0015
Urokinase	0/86/∞	100	6	4.83	0.8	--
α-Chymotrypsin	0/98/∞	100	0.46	9.51	21	fast[c]
Lactone **22**:						
Trypsin	15/96/∞	100	0.67	2.76	4	0.005
Urokinase	45/85/∞	100	18.73	--[c]	--	0.0008
α-Chymotrypsin	45/96/∞	20	0.17	--[c]	--	--

[a]For a definition of A, B, and C, see Fig. 1. ∞ indicates that no enzyme activity was recovered over the 2 h-time course of the assay. [b]N_2H_4 treatment - percent activity regained. [c]Could not be measured by the method used.

Our studies indicated that, in general, the α-guanidino-aryl substituted iodo lactones are potent suicide substrates of some trypsin-like enzymes. The inhibition was characterized by exceptionally high specificities in some cases (the high k_a/K_i values were in the range of 117-760 min^{-1} μM^{-1}). An amino-substituted isatoic anhydride[2] and guanidino substituted isocoumarins[3] are the only other examples of mechanism-based inhibitors designed for the trypsin-like enzymes, but the latter have specificities in the range of 30 min^{-1} μM^{-1}.[3] In addition, by comparing the specificity constants of lactones **15-22** with those obtained for the inactivation of α-chymotrypsin by the lactones **1-8** (2.4-17.5 min^{-1} μM^{-1}),[6] we find that placing a guanidino group on some α-chymotrypsin inhibitors had provided us with compounds that are not only selective in inhibiting the trypsin-like enzymes, but are also more specific in their interaction with the target enzymes, than were the corresponding α-chymotrypsin inhibitors.

Conclusions - We have developed enol lactone-based inhibitors for the three types of serine protease primary specificities. Earlier, we showed that aryl-substituted enol lactones are good inhibitors of α-chymotrypsin. In this report, we describe that elastase and trypsin-like enzyme can be inactivated by valine mimic and guanidino-aryl substituted enol lactones, respectively. Some α-alkyl substituted enol lactones are potent HNE inhibitors, lactone **9** inhibiting HNE by formation of a stable acyl-enzyme intermediate and lactones **10a-10c** acting as mechanism-based inhibitors by formation of permanently inactivated (alkylated) enzyme. The moderate selectivity of these lactones is most likely the result of the weak primary selectivity of HNE and could probably be enhanced by additional structural features that interact with known sites of secondary selectivity in this enzyme. In the case of the guanidino-aryl substituted enol lactones, our endeavor to prepare compounds which exhibited a higher selectivity in inhibiting trypsin-like serine proteases over α-chymotrypsin and HNE was very successful. Our studies show that an α-substitution on valero enol lactones is a good system for the design of potent suicide substrates. This reiterates the similarity in tertiary structure at the active-site of these enzymes.

ACKNOWLEDGEMENTS

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