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MECHANISM-BASED INACTIVATION OF SERINE PROTEASES BY DICHLOROCYCLOPROPANE FUSED LACTONE DERIVATIVES

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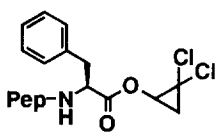
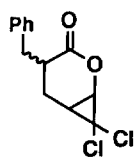
Abstract A dichlorocyclopropane fused lactone derivative was prepared as a novel mechanism-based inactivator of serine protease, α-chymotrypsin. The lactone derivative showed transient irreversible inhibitory activity toward α-chymotrypsin with the value of $k_{obsd}/[I]$ = 54 $M^{-1}s^{-1}$ and the enzyme activity recovered perfectly after 6 hours.

Mechanism-based enzyme inactivators are chemically stable compounds with latent reactivity and, therefore, they are useful for selective inactivation of enzymes in both *in vitro* and *in vivo* systems.[1-3] A number of compounds, such as haloenol lactones and ynenol lactones, have been reported as the mechanism-based inactivators of serine proteases.[4-8]

Recently, we have reported that the peptidic 2,2-dichlorocyclopropyl ester derivatives **1** and **2** are mechanism-based inactivators of serine protease, α-chymotrypsin.[9] They act as masked reactive α-chloroacrolein.[10] The reactive enal appears only by the enzymatic ester cleavage and then reacts with active-site nucleophiles to inactivate α-chymotrypsin irreversibly. However, the inhibitory activity of the ester **1** was very weak and significant improvement in the inhibitory activity was not observed even when the tripeptidic ester derivative **2** was used. We thought this is because most of the reactive enal might be released from active-site before the reaction with active-site nucleophiles. In addition, the steric bulk of dichlorocyclopropane ring moiety would decrease the initial E•I complex formation rate significantly. Therefore, we designed the non-peptidic dichlorocyclopropane fused lactone derivative **3** as a novel mechanism-based inactivator of α-chymotrypsin.



1: Pep=Suc
2: Pep=Suc-Ala-Ala



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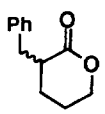

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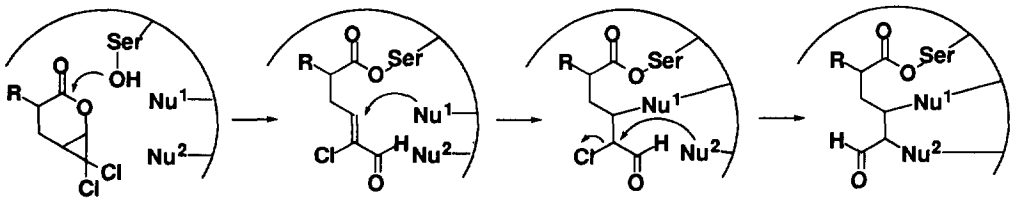


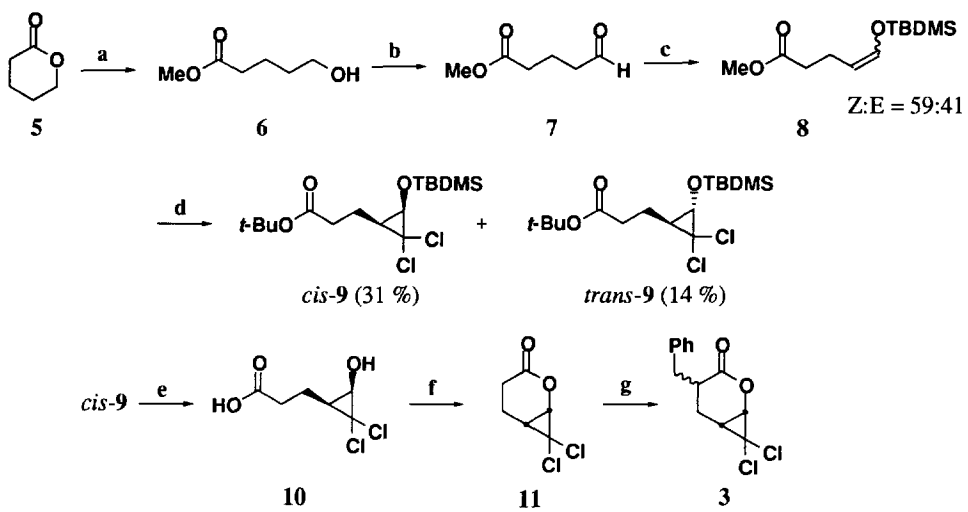
R = $C_6H_5CH_2$
Nu : active-site nucleophile

Figure 1. Postulated inactivation mechanism of α-chymotrypsin by the lactone 3

This lactone derivative **3** is also masked electrophilic α-chloro-α,β-unsaturated aldehyde. However, such reactive species is tethered to the enzyme as a part of acyl enzyme in this case, so that the reactive functional group would react with active-site nucleophiles rapidly prior to its release from active-site and more efficient inactivation would be observed than using the peptidic 2,2-dichlorocyclopropyl ester derivatives (Figure 1).

The lactone **3**[11] was prepared as shown in Scheme 1[12-14] and assayed toward both α-chymotrypsin and porcine pancreatic elastase (PPE) according to the reported methods.[15,16] To investigate the interaction between the benzyl group of the lactone **3** and the S1 subsite of α-chymotrypsin, the lactone **11** possessing no benzyl group at α-position was also assayed toward the both serine proteases. 2-Benzyl-δ–valerolactone (**4**)[17] was also prepared and assayed toward α-chymotrypsin as a reference compound of the lactone **3**.

Scheme 1



a) $Mg(OMe)_2$, MeOH, reflux, quant ; b) $(COCl)_2$, DMSO, NEt_3, CH_2Cl_2 (67 %) ;
c) TBDMSCl, DBU, DMF, r.t. (67 %) ; d) $CHCl_3$, *t*-BuOK, Hexane , r.t.;
e) TFA-H_2O (9:1), CH_2Cl_2, r.t. ; f) EDC•HCl, CH_2Cl_2, r.t. (50 % : 2 steps) ;
g) 1) LDA, THF, -78 °C 2) BnBr, HMPA, -78 to -40 °C, (4 %)

Incubation of the lactone **3** with α-chymotrypsin (0.53 μM) resulted in a time-dependent loss of enzyme activity with $k_{obsd}/[I]$ = 54 $M^{-1}s^{-1}$ ([I] = 7.5 μM).[18] The lactone **3** showed significant improvement in the inhibitory activity compared to the peptidic ester derivatives (1000-fold improvement on the compound **1** and about 160-fold improvement on the compound **2**, respectively). On the other hand, the lactone **11** did not show any irreversible inhibitory activity toward either α-chymotrypsin or PPE[19], and 2-benzyl-δ–valerolactone (**4**, [I] = 500 μM) did not show any irreversible inhibitory activity toward α-chymotrypsin, either. These results indicate that the benzyl group moiety of the lactone **3** is indispensable for the inactivation and the lactone **3** is a masked reactive aldehyde. In addition, the lactone **3** is an active-site directed inhibitor of α-chymotrypsin since the inactivation rate significantly decreased when the substrate (Suc-Ala-Ala-Pro-Phe-4-nitroanilide) which is hydrolyzed within active-site was present in the assay conditions. Furthermore, the lactone **3** did not show any irreversible inhibitory activity toward PPE (Table 1).

Although these experimental results indicate that the lactone **3** is an active-site directed irreversible inhibitor of α-chymotrypsin as well as the peptidic ester derivatives **1** and **2**, it was found that the irreverssible inactivation is transitory since the enzyme activity of α-chymotrypsin inactivated by the lactone **3** was restored perfectly by dialysis of the assay mixture at 4 °C for 24 hours. After further investigation for the inactivation in long-range, it was found that the enzyme activity of α-chymotrypsin recovers perfectly after 6 hours as shown in Figure 2.

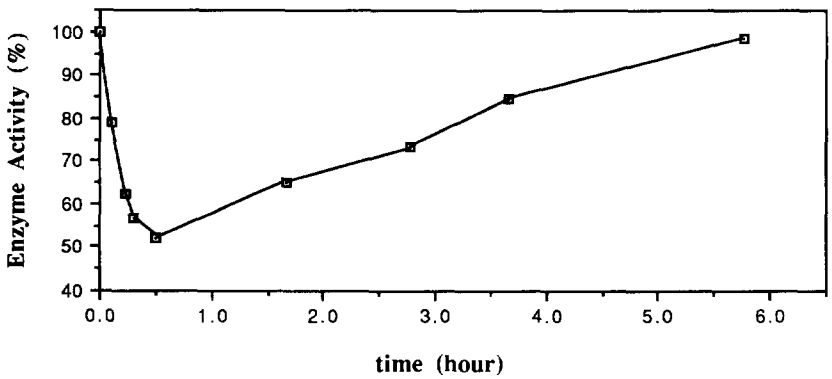


Figure 2. The recovering of the enzyme activity of α-chymotrypsin inactivated by the lactone **3**

Alternate inactivation mechanism for the postulated mechanism (Figure 1) has been proposed for the inactivation as shown in Figure 3. It is likely that the lactone **3** is cleaved by the catalysis of α-chymotrypsin and the reactive α-chloro-α,β-unsaturated aldehyde appears in active-site since neither the lactone **11** nor 2-benzyl-δ–valerolactone (**4**) showed any irreversible inhibitory activity toward α-chymotrypsin. Then, the reactive enal would react with active-site nucleophiles not by 1,4-addition reaction but 1,2-addition one, contrary to our speculation, to form hemiacetal in this case. Therefore, the recovering of the enzyme activity can be accounted for by both the cleavage of transient covalent bond of the hemiacetal and the deacylation step as shown in Figure 3. Although transient irreversible inactivation of serine proteases is observed when stable

acyl enzyme is formed in active-site and such type of inhibitors are referred to as alternate substrate inhibitors.[20] However, it is difficult to consider the formation of stable acyl enzyme in the case of the lactone **3** since 2-benzyl-δ–valerolactone (**4**) did not show any irreversible inhibitory activity toward α-chymotrypsin. Therefore, we are thinking the lactone **3** should be regarded as not an alternate substrate inhibitor but a mechanism-based inactivator of α-chymotrypsin.

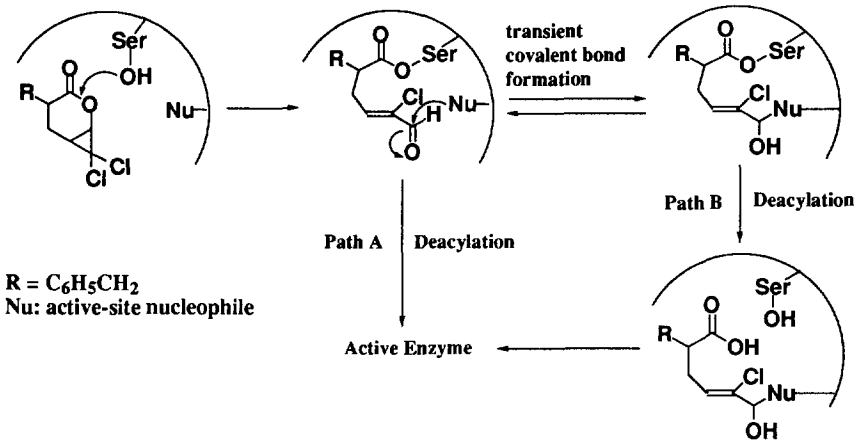


Figure 3. Proposed mechanism for the inactivation of α-chymotrypsin by the lactone **3**

Table 1. Inactivation of serine proteases by the peptidic 2,2-dichlorocyclopropyl ester derivatives and the dichlorocyclopropane fused lactone derivatives

	α-Chymotrypsin[a]		PP elastase[b]	
inhibitor	[I] ; inhibitor concentration (M)	k_{obsd}/[I] ($M^{-1}s^{-1}$)	[I] ; inhibitor concentration (M)	k_{obsd}/[I] ($M^{-1}s^{-1}$)
1	6.0×10^{-3}	0.054	—	—
2	5.0×10^{-4}	0.337	1.2×10^{-3}	N. I.[c]
3	7.5×10^{-6}	54	2.0×10^{-5}	N. I.
4	5.0×10^{-4}	N. I.	—	—
11	1.95×10^{-2}	N. I.	1.95×10^{-2}	N. I.

[a]Conditions were as follows: 0.1 M potassium phosphate buffer, 0.5 M NaCl, pH 7.8, 5 % Me_2SO, 25 °C, enzyme concentration 1.6 μM (**1**, **2** and **4**) or 0.53 μM (**3** and **11**).
[b]Conditions were as follows: 0.1 M potassium phosphate buffer, 0.5 M NaCl, pH 7.8, 5 % Me_2SO, 25 °C, enzyme concentration 4.8 μM (**2**) or 9.7 μM (**3** and **11**). [c]No inactivation.

In conclusion, the non-peptidic dichlorocyclopropane fused lactone derivative **3** was found to show transient irreversible inhibitory activity toward α-chymotrypsin and significant improvement in the inhibitory activity was observed compared to the peptidic 2,2-dichlorocyclopropyl ester derivatives **1** and **2**. Probably, the enhancement is caused by the rapid reaction between the reactive species tethered to the enzyme and active-site nucleophiles. While some of other mechanism-based inactivators of serine proteases are unstable under physiological conditions, the lactone derivatives reported in this paper are quite stable under such conditions. Therefore, they would be useful tools for both the studies of serine proteases and the design of novel serine protease inhibitors as drugs, in future. In addition, since the lactone **11** did not show any irreversible inhibitory activity toward either α-chymotrypsin or PPE, and the lactone **3** did not show any irreversible inhibitory activity toward PPE, the dichlorocyclopropane fused lactone derivatives possessing the substituent at α-position which can interact with the S1 subsite of the target enzyme might be selective inhibitors of serine proteases.

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11. ^{1}H-NMR (270 MHz, $CDCl_3$) δ 1.93–2.11 (m, 2H), 2.23-2.38 (m, 1H), 2.71-2.92 (m, 2H), 3.00 -3.18 (m, 1H), 4.32 (d, J = 7.9 Hz, 1H), 7.15-7.44 (m, 5H) ^{13}C NMR (68 MHz, $CDCl_3$) δ 21.80, 23.76, 37.09, 39.01, 61.37, 62.05, 127.33, 128.95, 136.68, 170.66
The synthetic yield of the lactone **3** has not been optimized.

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17. 2-Benzyl-δ–valerolactone (**4**) was synthesized from δ–valerolactone using same procedure for the synthesis of the lactone **3** as shown in Scheme 1.
18. α-Chymotrypsin was incubated in 500 μl of buffer containing inhibitors. At various time intervals, 10 μl aliquots were withdrawned and assayed with 1500 μl of Suc-Ala-Ala-Pro-Phe-NA (0.5 mM) as a substrate. The production of 4-nitroaniline was monitored at 410 nm.
19. PPE was incubated in 500 μl of buffer containing inhibitors. At various time intervals, 50 μl aliquots were withdrawned and assayed with 1950 μl of Suc-Ala-Ala-Ala-NA (0.7 mM) as a substrate. The production of 4-nitroaniline was monitored at 410 nm.
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