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MECHANISM-BASED INACTIVATION OF α-CHYMOTRYPSIN

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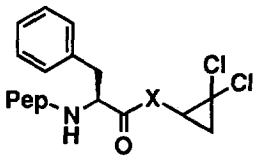
Abstract: The peptidyl ester derivatives of 2,2-dichlorocyclopropanol and the amide derivative of 2,2-dichlorocyclopropylamine were prepred as novel mechanism-based inactivators of α-chymotrypsin. The esters inactivated α-chymotrypsin irreversibly but the amide did not show any irreversible inhibitory activity toward α-chymotrypsin.

Mechanism-based enzyme inactivators are useful tools in the study of enzyme mechanisms and in the design of highly specific, low-toxicity drugs[1]. They are chemically stable compounds and substrates of the target enzyme. However, the catalysis of the target enzyme converted them to be reactive species which proceed to inactivate the enzyme by covalent bond formation with the residue at or near the active-site[2].

It has been known that cyclopropanols and cyclopropylamines possessing leaving group at 2-position are unstable under basic or, in some cases, neutral conditions to convert reactive α,β-unsaturated carbonyl or aldimine compounds[3]. Therefore, their acyl or amide derivatives are masked reactive species and we thought that the peptidyl ester derivatives of 2,2-dichlorocyclopropanol and the amide derivatives of 2,2-dichlorocyclopropylamine would be applicable to mechanism-based inactivators of proteases.



1: Pep=Suc, X=O
2: Pep=Suc, X=NH
3: Pep=Suc-Ala-Ala, X=O

We chose α-chymotrypsin (α-Cht)[4], which favors peptide substrates possessing Trp, Tyr, and Phe side chains at position P_1, as our target enzyme and synthesized the peptidyl ester derivatives of 2,2-dichlorocyclopropanol **1**, **3** and the amide derivative of 2,2-dichlorocyclopropylamine **2** . We thought that they would be converted to reactive α-chloroacrolein or α-chloro-α,β-unsaturated aldimine compound by the catalysis of α-Cht and, then, such reactive species would react with active-site nucleophiles irreversibly, before releasing from the active-site, to inactivate α-Cht (Figure 1).

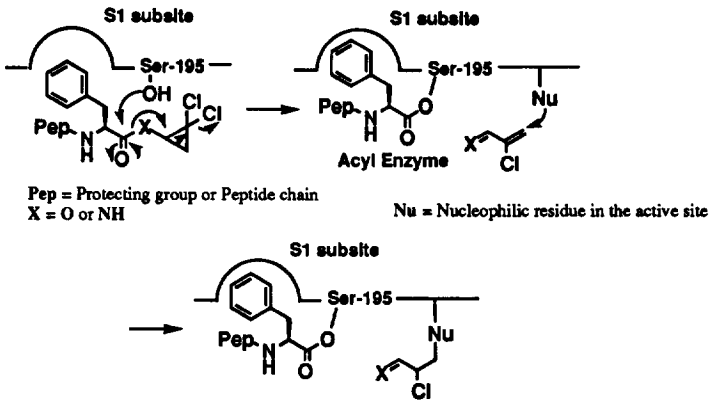
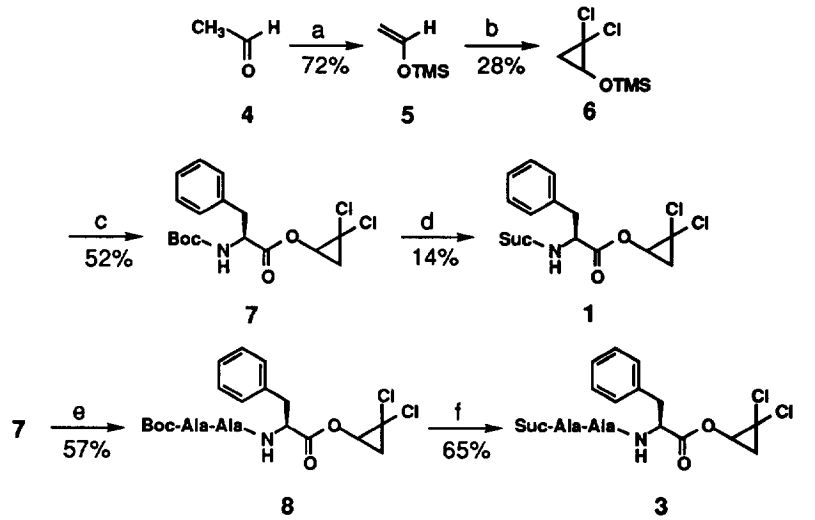


Figure 1. Posturated Inactivation Mechanism of α-Chymotrypsin by Compound 1-3

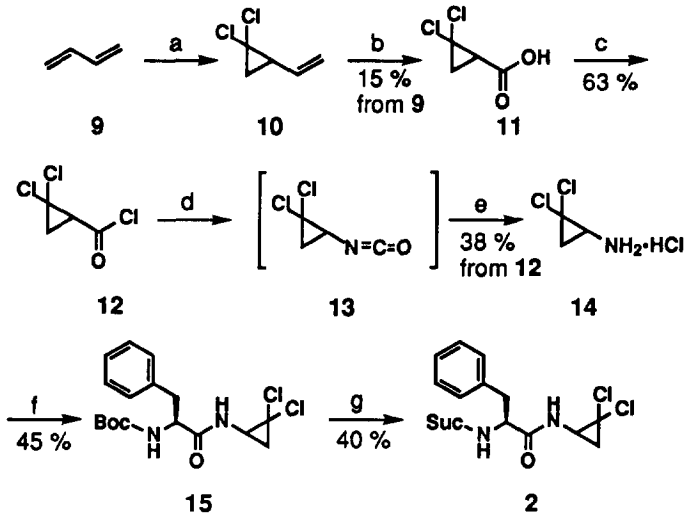
The ester derivatives were synthesized from acetaldehyde (4) as shown in Scheme 1. Silyl ether **6** was desilylated with 2 N-HCl in THF. After careful removal of solvent under reduced pressure, the crude racemic alcohol was used for the following coupling reaction with Boc-Phe-OH. The amide derivative was synthesized as shown in Scheme 2. The racemic cyclopropylamine hydrochloride **14** was prepared from 1,3-butadiene (**9**) according to the method reported by Gassen and Baasner[5,6] and purified on a Dowex-50 (H^+) column.

Scheme 1



(a) TMSCl, DBU, DMF; (b) $CHCl_3$, n-BuLi, n-C_5H_{12}; (c) 1) 2 N-HCl, THF 2) Boc-Phe-OH, EDC•HCl, DMAP (cat.), CH_2Cl_2; (d) 1) 4 N-HCl/dioxane 2)Succinic anhydride, NEt_3, CH_2Cl_2; (e) 1) 4 N-HCl/dioxane 2) Boc-Ala-Ala-OH, EDC•HCl, HOBt, CH_2Cl_2; (f) 1) 4 N-HCl/dioxane 2)Succinic anhydride, NEt_3, CH_2Cl_2

Scheme 2



(a) $CHCl_3$, Bu_4NCl, CH_2Cl_2, 50 % NaOHaq.; (b) $KMnO_4$, $BnEt_3NBr$, CH_2Cl_2, H_2O; (c) $SOCl_2$, reflux; (d) $TMSN_3$, toluene, reflux; (e) conc.HCl; (f) Boc-Phe-OH, EDC•HCl, HOBt, NEt_3, CH_2Cl_2; (g) 1) 4 N-HCl/dioxane 2)Succinic anhydride, NEt_3, CH_2Cl_2

First, we prepared and evaluated the irreversible inhibitory activities of mono amino acid derivatives **1** and **2**, respectively, and obtained the second-order rate constants (k_{obsd}/[I]) according to the literature procedures[7]. Incubation of the ester **1** with α-Cht[8] resulted in a time-dependent loss of enzyme activity with k_{obsd}/[I] = 3.24 $M^{-1}min^{-1}$ ([I]= 6 mM). Dialysis of assay solution against phosphate buffer solution (pH 7.5, 24 h) at 4 °C did not restore any enzyme activity, indicating that the inactivation is irreversible. However, such irreversible inactivation required high inactivator concentration toward α-Cht (1.6 μM) and the ester **1** did not show any irreversible inhibitory activity toward α-Cht at low concentration (< 1 mM), indicating the low affinity of the ester **1**. On the other hand, the amide derivative **2** did not show any irreversible inhibitory activity toward α-Cht even at high concentration of the amide **2** (14.5 mM).

Next, we prepared tripeptidyl ester derivative **3** to improve the low affinity of the ester **1** toward α-Cht and investigated the inhibitory activity. The ester **3** exhibited irreversible inhibitory activity toward α-Cht at low concentration (500 μM) compared to mono amino acid derivative **1** and the k_{obsd}/[I] value was 20.23 $M^{-1}min^{-1}$ (5.6-fold improvement to the ester **1**). The inactivation assay with the ester **3** (500 μM) in the presence of the substrate (Suc-Ala-Ala-Pro-Phe-NA, 500 μM) resulted in a decrease on inactivation rate (k_{obsd}/[I] = 6.97 $M^{-1}min^{-1}$) and dialysis of assay solution against phosphate buffer solution (pH 7.5, 24 h) at 4 °C did not restore any enzyme activity, indicating that the ester **3** is active-site directed. In addition, the inactivation was selective because the ester **3** (1.2 mM) did not show any irreversible inhibitory activity toward another serine protease, porcine pancreatic elastase[9] (PPE, 4.8 μM).

In conclusion, the ester derivatives of 2,2-dichlorocyclopropanol **1** and **3** were found to be active-site directed irreversible inhibitors of α-Cht. From the experimental results, we are thinking that they are regarded as mechanism-based inactivators of α-Cht. The selectivity of the ester **3** was also observed. However, the steric bulk of dichlorocyclopropane ring moiety would decrease the rate of initial E•I complex

formation rate since mono amino acid derivative **1** did not show any irreversible inhibitory activity toward α-Cht at low concentration. Therefore, for instance, the replacement of chloride for fluorine would decrease such a steric bulk of dichlorocyclopropane ring moiety and our compounds might be more potent inhibitors of proteases. On the other hand, the amide derivative **2** did not show any irreversible inhibitory activity toward α-Cht. For this reason, we are thinking that the amide was not hydrolyzed by α-Cht because of both the low reactivity of the amide and more rigid structure of amide bond than that of ester bond in addition to the steric bulk of dichlorocyclopropane ring moiety.

In any event, we could confirm that such ester derivatives are applicable to mechanism-based inactivators of proteases. Since our compounds are quite stable under physiological conditions, they would be useful for future studies of proteases and in the designing of new protease inhibitors. In addition, amide derivatives, which did not show any irreversible inhibitory activity toward α-Cht in this experiment, might be also mechanism-based inactivators of other proteases. Exploitation of our concept for developing more potent and selective protease inactivators is now in progress.

References and Notes

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4. Abbreviations: Boc, *tert.*-butyloxycarbonyl; DBU, 1,8-diazabicyclo [5.4.0.] undec-7-ene; DMF, dimethylformamide; DMAP, 4-dimethylaminopyridine; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; HOBt, 1-hydroxybenzotriazole; NA, 4-nitroanilide; Suc, succinyl; THF,tetrahydrofuran; TMS, trimethylsilyl
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8. α-Chymotrypsin (1.6 μM) was incubated in 500 μl of buffer (0.1 M sodium phosphate buffer, 0.5 M NaCl, 5 % Me_2SO, pH 7.8 at 25 °C) 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, buffered as above) as a substrate. The production of 4-nitroaniline was monitored at 410 nm.
9. PPE (4.8 μM) was incubated in 500 μl of buffer (0.1 M sodium phosphate buffer, 0.5 M NaCl, 5 % Me_2SO, pH 7.8 at 25 °C) containing inhibitor **3** . At various time intervals, 50 μl aliquots were withdrawned and assayed with 1950 μl of Suc-Ala-Ala-Ala-NA (0.7 mM, buffered as above) as a substrate. The production of 4-nitroaniline was monitored at 410 nm.

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