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STUDIES ON IRREVERSIBLE INHIBITION OF SERINE PROTEASES BY α-SULFONYLOXYKETONE DERIVATIVES

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Abstract α-Sulfonyloxyketone derivatives were found to be active-site directed irreversible inhibitors of serine proteases.

Irreversible enzyme inhibitors are useful tools for enzymology and classified as affinity labels or mechanism-based enzyme inactivators according to the inactivation mechanisms[1]. Affinity labels are reactive species possessing analogous structures to substrates and react with the active-site amino acid residues. On the other hand, mechanism-based enzyme inactivators are masked reactive species and transformed to reactive form by normal enzymatic reactions. We are thinking that even a reactive compound should be also regarded as a mechanism-based enzyme inactivator if it is converted to another reactive species by the catalysis of the target enzyme and then only such reactive one can react with the active site nucleophiles to inactivate the enzyme.

By the way, chloromethylketones have been known as classic affinity labels of serine proteases and alkylate the histidine residue of active site specifically. N-Tosyl-L-phenylalanine chloromethylketone (**1**)[2], for example, is a specific reagent for α-chymotrypsin[3]. However, X-ray crystallographic as well as ^{13}C-NMR studies of several protease-inhibitor complexes have revealed another covalent bond formation between the enzyme active site serine hydroxyl group and the inhibitor carbonyl carbon[4-8]. Based on these experimental results, Powers et al. proposed alternate mechanisms as shown in Figure 1 which suggest that chloromethylketones should be classified as mechanism-based inactivators of serine proteases[9,10]. In addition, recently, Prorok et al. reported that hydrolysis of chiral chloroethylketone by the chymotrypsin methylated at N-3 of the active-site histidine proceeds with retention of configuration at chiral center[11] and Kreutter et al. reported that the inactivation of γ-chymotrypsin with chiral chloroethylketone proceeds with retention of configuration at chiral center (Figure 2)[12]. These data supports mechanism B(Figure 1) which involves an intermediate, strongly.

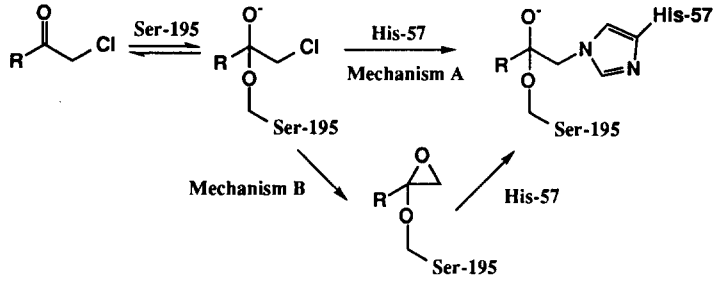


Figure 1. Possible mechanisms for the inactivation of serine proteases by chloroketones

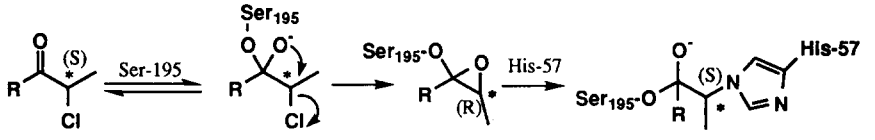
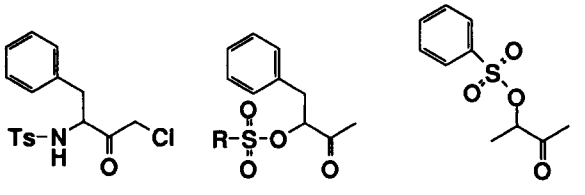


Figure 2. Proposed inactivation mechanism of γ-chymotrypsin by chiral chloroethylketone

We designed α-sulfonyloxyketone derivatives **2**-**4**, as novel mechanism-based inactivators of α-chymotrypsin. Since, generally, sulfonates of secondary alcohols are not susceptible to direct substitution reaction by nucleophiles compared to those of primary alcohols, our designed compounds were thought to inactivate α-chymotrypsin not by direct substitution reaction but by two step mechanism as shown in Figure 3. In addition, they would show high affinity toward α-chymotrypsin similarly to compound **1** since compound **2** and **3** can be regarded as analogous molecule of compound **1** replaced the sulfonamide moiety for sulfonates. As extension of our concept, compound **4** was also prepared. The benezenesulfonyloxy moiety in the structure of compound **4** was thought to play the role of the side-chain of phenylalanine as well as the role of leaving group and interact with S_1 subsite[13] of α-chymotrypsin. Although these α-sulfonyloxyketone derivatives are highly electrophilic species itself, they should be regarded as not affinity labels but mechanism-based enzyme inactivators if the inactivation proceeds only by the mechanism *via* epoxy ether intermediate as shown in Figure 3.



1 **2**: R = *p*-$CH_3C_6H_4$ **3**: R = CH_3 **4**

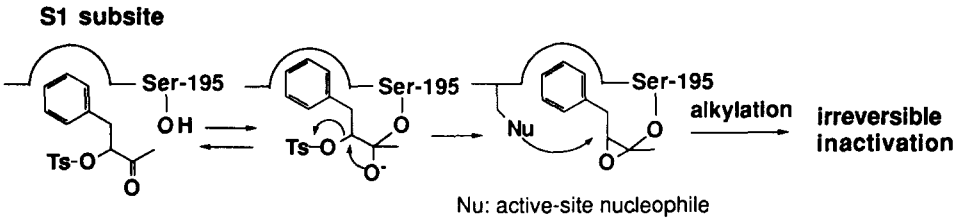
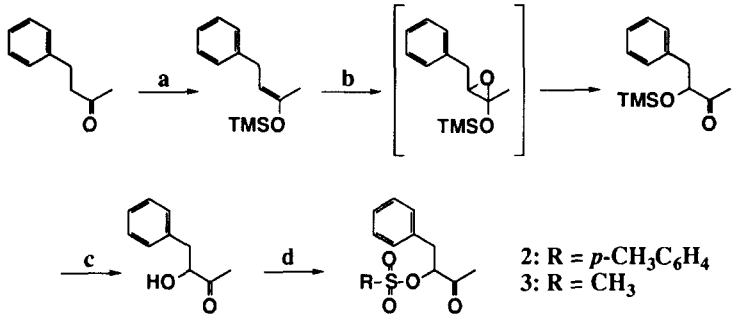


Figure 3. Postulated inactivation mechanism by compound **2**



Scheme 1.
(a)TMSCl, NEt_3, DMF; (b)MCPBA, $NaHCO_3$, CH_2Cl_2 (a,b: 27 %); (c)2 N-HCl (58 %); (d)for 2: TsCl, NEt_3, CH_2Cl_2 (50 %), for 3: MsCl, NEt_3, CH_2Cl_2 (50 %)

Scheme 1 outlines our synthetic route for the preparation of compound **2** and **3**. Hydroxylation of benzylacetone was accomplished *via* silyl enol ether using the methodology described by Hassner et al.[14]. Compound **4** was synthesized by sulfonylation of commercially available acetoin in usual manner.

The irreversible inhibitory activities of all compounds toward serine proteases were evaluated according to the literature procedures[15] and the second-order rate constants (k_{obsd}/[I]) were obtained. Incubation of compound **2** (96 μM) with α-chymotrypsin (1.6 μM) resulted in a time-dependent loss of enzyme activity with k_{obsd}/[I] value = 10.5 $M^{-1}s^{-1}$. On the other hand, the inactivation assay of α-chymotrypsin by compound **2** (96 μM) in the presence of the substrate (Suc-Ala-Ala-Pro-Phe-NA, 0.5 mM) resulted in a significant decrease in the inactivation rate (k_{obsd}/[I] = 3.75 $M^{-1}s^{-1}$, Figure 4). In addition, 4 °C dialysis of the assay solution toward phosphate buffer at pH 7.5 did not restore any enzyme activity. These experimental results indicate that compound **2** is active-site directed irreversible inhibitor. Furthermore, compound **2** was selective inhibitor of α-chymotrypsin because compound **2** (103 μM) did not exhibit any irreversible inhibitory activity toward another serine protease, porcine pancreatic elastase (PPE, 5 μM).

Compound **3** and **4** also showed irreversible inhibitory activity toward α-chymotrypsin as we expected. Compound **4** was also active-site directed irreversible inhibitors of α-chymotrypsin because compound **4** did not show any irreversible inhibitory activity toward α-chymotrypsin in the presence of the substrate (Suc-Phe-NA, 14.5 mM) and no enzyme activity was recovered after dialysis of the assay solution. The second-order rate constants of compounds **1-4** are listed in Table 1.

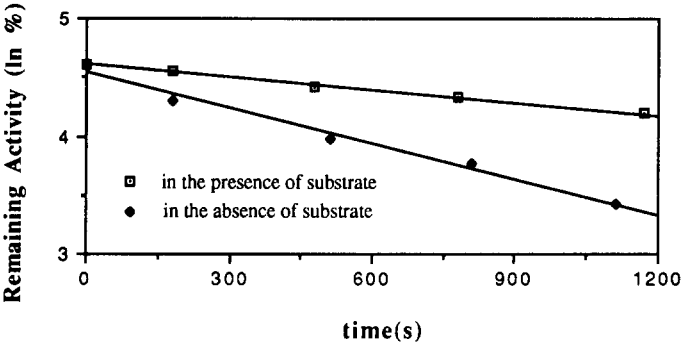


Figure 4. Substrate protection.

Table 1. Inactivation of serine proteases by α-sulfonyloxyketone derivatives

	enzyme			
	α-Chymotrypsin[a]		PP elastase[b]	
inhibitor	[I] ; inhibitor concentration (M)	*kobsd* /[I] ($M^{-1}s^{-1}$)	[I] ; inhibitor concentration (M)	*kobsd* /[I] ($M^{-1}s^{-1}$)
1	5.70×10^{-5}	7.7[c]	—	—
2	9.60×10^{-5}	10.5	1.03×10^{-4}	N. I.[d]
3	2.31×10^{-3}	0.33	—	—
4	3.28×10^{-3}	0.01	2.01×10^{-3}	0.19

[a]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.;
[b]PP elastase (5 μM) was incubated in 500 μl of the above 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, buffered as the above) as a substrate; [c]see ref. 3;
[d]No inactivation

Here, we noticed that compound **4** inactivated both α-chymotrypsin and PPE. However, this result does not imply that compound **4** is non-selective inhibitor for serine proteases. Compound **4** can be regarded as analogous molecule of alanine as well as analogous one of phenylalanine and PPE prefer peptide substrate possessing Ala at P1[16,17]. Therefore, compound **4** was recognized as alanine analog by PPE to show irreversible inhibitory activity toward PPE. That is to say, this result suggests that our concept is applicable to not only α-chymotrypsin but other serine proteases. Furthermore, since compound **2** showed potent irreversible inhibitory activity toward α-chymotrypsin as much as that of compound **1**, our compounds would be more selective and potent inhibitors of serine proteases by replacing the *p*-toluene sulfonyl group for others such as N-peptidyl tauryl group.

In conclusion, α-sulfonyloxyketone derivatives were found to be irreversible inhibitors of serine proteases. Since they are active-site directed and selective inhibitors, they would be useful tools for the future studies of serine proteases even though they are classified as affinity labels or mechanism-based enzyme inactivators. Studies leading to the proof of the precise inactivation mechanism and the exploitation of our concept for developing more selective and potent inhibitors of serine proteases are now in progress.

References and Notes

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2. Abbreviations used: MCPBA, *m*-chloroperoxybenzoic acid; Ms, methanesulfonyl; TMSCl, chlorotrimethylsilane; Ts, *p*-toluenesulfonyl; DMF, dimethylformamide; NA, *p*-nitroanilide
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