Irreversible Inactivation of Serine Proteases by Peptidyl α,β-Unsaturated Nitro Compounds

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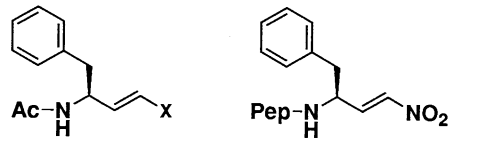
Peptidyl α,β-unsaturated nitro compounds were found to be active-site directed irreversible inhibitors of serine protease, α-chymotrypsin. The inactivation was selective because tripeptidyl derivative **4** did not show any irreversible inhibitory activity toward another serine protease, porcine pancreatic elastase.

Serine proteases play important roles in biological processes and, therefore, selective irreversible inhibitors of serine proteases are useful tools to characterize the catalytic functional groups, recognition specificity and catalytic mechanism of serine proteases.[1]

Since the catalysis by serine proteases involves attack by active-site serine residue on the carbonyl group of the scissile peptide bond, the compound which can trap the active-site serine residue of target enzyme, specifically, would be selective inhibitor of serine proteases. Previous work by Hanzlik and co-workers[2,3,4] demonstrates that peptide Michael acceptors are specific irreversible inactivators of cysteine protease, papain, and compounds **1** and **2** designed as irreversible inhibitors for serine protease, α-chymotrypsin, were not irreversible inhibitors but reversible inhibitors of α-chymotrypsin due to the low chemical reactivity. However, we thought that such concept is also useful for the development of selective irreversible inhibitors of serine proteases and synthesized peptidyl α,β-unsaturated nitro compounds **3** and **4** as new irreversible inhibitors of α-chymotrypsin (Figure 1). Although α,β-unsaturated nitro compounds are stronger Michael acceptor than α,β-unsaturated esters and sulfonates, they are not so electrophilic species to react with nucleophiles non-specifically. In addition, recently, we have reported that peptidyl allylic bromide and chloride derivatives are selective irreversible inhibitors of serine proteases and demonstrated that such derivatives possessing carbon-carbon double bond in place of scissile peptide bond of substrates are applicable to selective irreversible inhibitors of serine proteases.[5] Therefore, we thought peptidyl α,β-unsaturated nitro compounds would be also selective inhibitors of serine proteases which can trap the active-site serine residue of target serine protease, specifically.

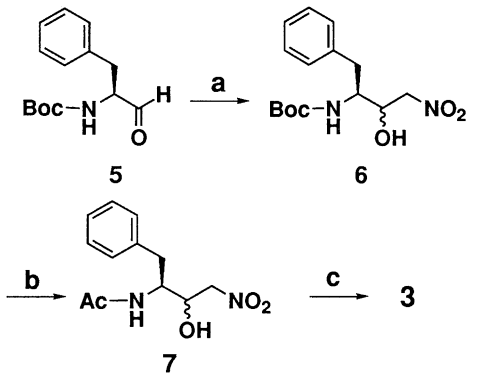
Compounds **3**[6] and **4**[7] were synthesized as shown in Scheme 1. N-Boc-L-phenylalaninal (**5**) was prepared from N-Boc-phenylalanine according to the method reported by Fehrentz and Castro.[8] Addition reaction of nitromethane to aldehyde **5** was carried out according to the method reported by Shuto and co-workers.[9] The irreversible inhibitory activity of each compound toward α-chymotrypsin was evaluated according to the method reported by Kitz and Wilson.[10]



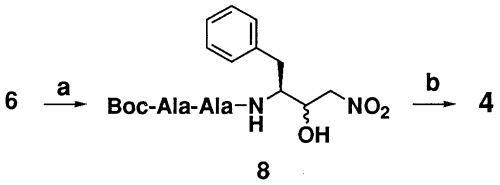
1: X=CO_2Me **3: Pep=Ac-**
2: X=SO_2Me **4: Pep=Ms-Ala-Ala-**

Figure 1.

Incubation of compound **3** ([I] = 2.04-5.10 mM) with α-chymotrypsin(1.6 μM) resulted in a time-dependent loss of enzyme activity.[11] Dialysis of the assay solution containing the inactivated enzyme against phosphate buffer (pH 7.5, 24 h) at 4 °C did not restore any enzyme activity, indicating that the inactivation was irreversible. The K_I and the k_{inact} values for



(a) CH_3NO_2, NaH, THF, 0 °C; (b) 1) 4 M HCl-dioxane 2)Ac_2O, CH_2Cl_2, NEt_3; (c) MsCl, NEt_3, CH_2Cl_2



(a) 1) 4 M HCl-dioxane 2) Boc-Ala-Ala-OH, EDC•HCl, HOBt, NEt_3, CH_2Cl_2 ; (b) 1) 4 M HCl-dioxane 2)MsCl, NEt_3, CH_2Cl_2

Scheme 1.

compound **3** were obtained, 1.93 x 10^{-2} M and 3.74 x 10^{-3} s^{-1}, respectively (Table 1). Next, we prepared tripeptidyl derivative **4** as more potent irreversible inhibitors of α-chymotrypsin and evaluated the inhibitory activity. Compound **4** showed irreversible inhibitory activity toward α-chymotrypsin at low concentration([I] = 21-150 μM) compared to the result of compound **3**. Furthermore, the inactivation assay of α-chymotrypsin by compound **4** (37.5 μM) in the presence of the substrate (Suc-Ala-Ala-Pro-Phe-4-nitroanilide, 0.5 mM) resulted in a significant decrease in the inactivation rate(Figure 2). These experimental results indicate that compound **4** is active-site directed irreversible inhibitor of α-chymotrypsin. In addition, compound **4** is selective inhibitor of α-chymotrypsin because compound **4** (300 μM) exhibited no irreversible inhibitory activity toward another serine protease, porcine pancreatic

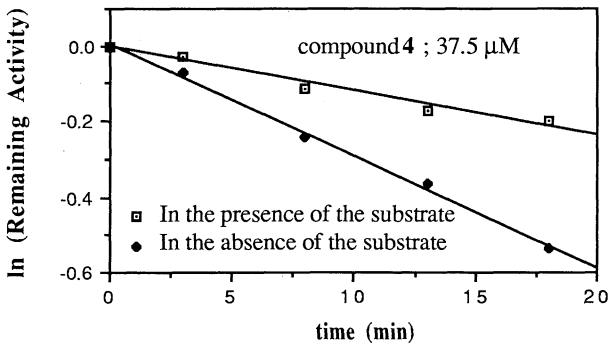


Figure 2. Substrate protection.

elastase (PPE, 5 μM).[12] The K_I and the k_{inact} values for compound **4** were obtained , 1.52 x 10 $^{-4}$ M and 2.62 x 10^{-3} s^{-1}, respectively (Table 1).

Table 1. Inactivation of α-chymotrypsin by compounds **3** and **4**

compound	k_{inact} (s^{-1})	K_I (M)	k_{inact}/K_I ($M^{-1}s^{-1}$)
3	3.74 x 10^{-3}	1.93 x 10^{-2}	0.19
4	2.62 x 10^{-3}	1.52 x 10^{-4}	17.24

While mono amino acid derivative **3** showed weak irreversible inhibitory activity toward α-chymotrypsin(k_{inact}/K_I = 0.19 $M^{-1}s^{-1}$), tripeptidyl derivative **4** showed significant improvement on the inhibitory activity(k_{inact}/K_I = 17.24 $M^{-1}s^{-1}$, about 90-fold improvement compared with compound **3**). From these experimental results, it can be said that the irreversible inhibitory activity of peptidyl α,β-unsaturated nitro compounds depends, mainly, on the affinity toward serine proteases. Therefore, peptidyl α,β-unsaturated nitro compounds, weak Michael acceptor, would be also potent and selective inhibitors by considering the affinity toward serine proteases.

In conclusion, peptidyl α,β-unsaturated nitro compounds were found to be active-site directed and selective irreversible inhibitors of α-chymotrypsin and it is clear that peptide Michael acceptors are applicable to not only irreversible inhibitors of cysteine proteases but also those of serine proteases. In view of both the mechanism of the enzymatic hydrolysis and the structure of the inhibitor, the inactivation should be caused as the result of trapping the active-site serine residue of α-chymotrypsin. Further investigations for other serine proteases or cysteine proteases are now in progress in our laboratory.

References and Notes

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6 ^{1}H NMR (270 MHz) 1.98 (s, 3H), 2.99 (d , 2H, J = 6.93 Hz), 5.02 (m, 1H), 5.40 (d, 1H), 6.93 (dd , 1H, J = 1.65 Hz and 14.02 Hz) , 7.14 - 7.35 (m, 6H) ; ^{13}C NMR (68 MHz) 23.18 , 40.00 , 48.43 , 127.58 , 129.05 , 129.14 , 135.04 , 140.27 , 140.63 , 169.56.

7 ^{1}H NMR (270 MHz) 1.26 (d, 3H, J = 6.93 Hz), 1.49 (d, 3H, J = 6.93 Hz), 2.93 (m, 2H), 3.07 (s, 3H), 3.97 (m, 1H), 4.35 (m, 1H), 4.49 - 5.02 (m, 1H), 5.11 (d, 1H, J = 3.38 Hz), 6.69 (d , 1H, J = 6.93 Hz), 6.96 (d, 1H, J = 9 Hz), 6.99 (dd, 1H, J = 1.65 Hz and 13.20 Hz), 7.14 - 7.34 (6H, m) ; ^{13}C NMR (68 MHz) 17.50, 19.01, 39.66, 39.89, 48.86, 49.72, 53.75, 127.19, 128.75, 129.18, 135.97, 140.29 , 140.72 , 171.43 , 171.71.

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11 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-4-nitroanilide (0.5 mM, buffered as above) as a substrate. The production of 4-nitroaniline was monitored at 410 nm.

12 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-4-nitroanilide (0.7 mM , buffered as above) as a substrate. The production of 4-nitroaniline was monitored at 410 nm.