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INACTIVATION OF SERINE PROTEASE, α-CHYMOTRYPSIN BY FLUORINATED PHENYLALANINE ANALOGUES

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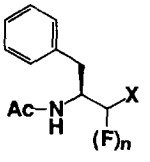
Abstract Fluorinated phenylalanine analogues were found to be slow-binding or reversible competitive inhibitors of α-chymotrypsin. A series of these compounds were designed to inactivate α-chymotrypsin as a result of the formation of hydrogen-bonding between fluorine atom of the inhibitors and the amide protons known as oxy-anion hole in the active-site of serine and cysteine proteases.

Since serine proteases are concerned in numerous reactions *in vivo*, selective inhibitors are useful tools for the studies of serine proteases. Particularly, selective inhibitors of uncontrolled serine proteases which cause pathological states would be used for clinical application as drugs.[1,2]

The catalysis of serine and cysteine proteases proceeds *via* tetrahedral intermediate which is stabilized by the amide protons known as oxy-anion hole in the active-site.[3-5] Therefore, peptide analogues which can interact with oxy-anion hole of the target enzyme specifically are thought to show selective inhibitory activity for serine and cysteine proteases, and a series of fluorinated phenylalanine analogues **1-6** were designed as novel selective inhibitors of serine protease, α-chymotrypsin.



1: n = 2, X = CH_3
2: n = 1, X = $CH{=}CH_2$
3: n = 2, X = $CH{=}CH_2$
4: n = 1(syn), X = CO_2Me
5: n = 1(anti), X = CO_2Me
6: n = 2, X = CO_2Me

Fluorine atoms were introduced to all compounds in place of the oxygen atom of the scissile peptide bond in the structure of substrates. These compounds were thought to inactivate α-chymotrypsin, reversibly or irreversibly, as a result of the formation of hydrogen bonding between fluorine atom and the amide protons of oxy-anion hole in the active-site (Figure 1). Although fluorides are not susceptible to substitution reaction with nucleophiles compared to other halides, the above hydrogen bonding might assist the displacement of the fluorine atom of these inhibitors by nucleophilic attack of the active-site serine residue(Ser-195). Actually, the allylic fluorides[6,7] and the peptidyl monofluoromethyl ketone inhibitor[8] have been known as irreversible inhibitors of isopentenyl-diphosphate isomerase and α-chymotrypsin, respectively.

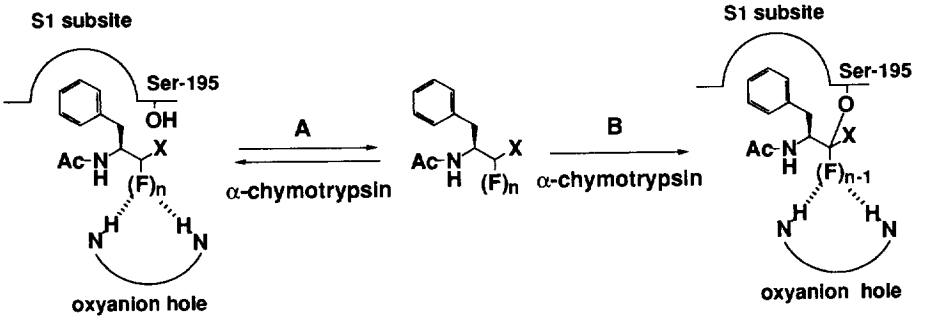
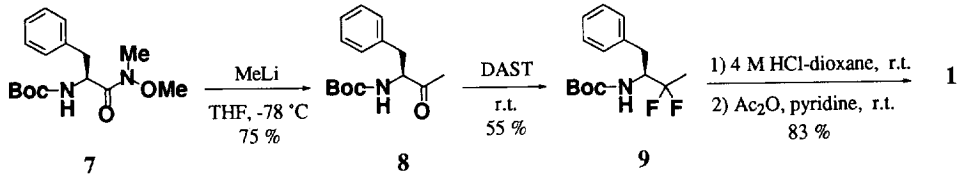
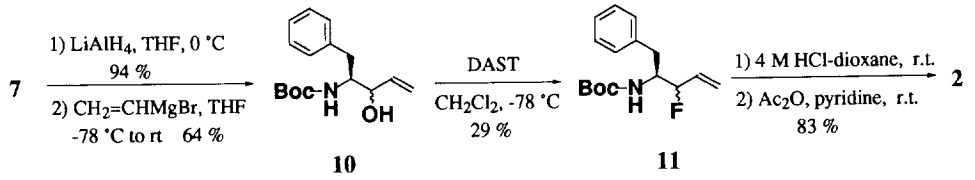


Figure 1. Postulated inactivation mechanism of α-chymotrypsin by a series of fluorinated phenylalanine analogues (A or B) A; reversible competitive inactivation B; irreversible inactivation

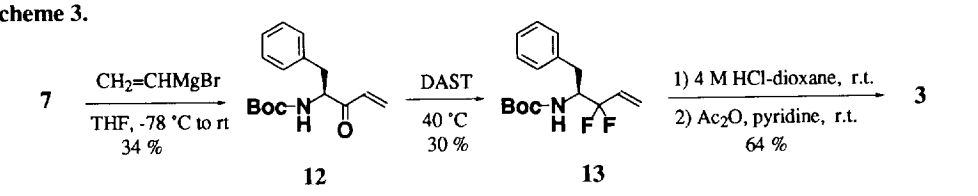
Scheme 1.



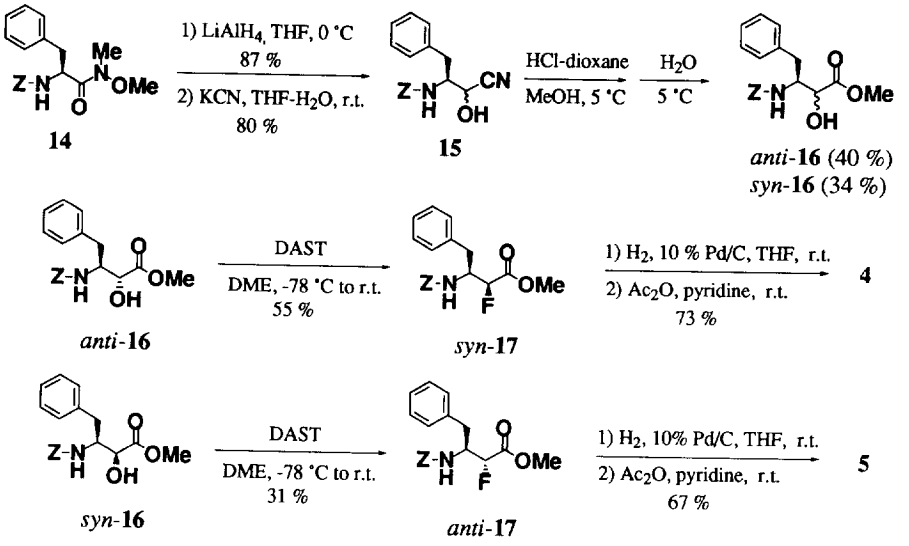
Scheme 2.



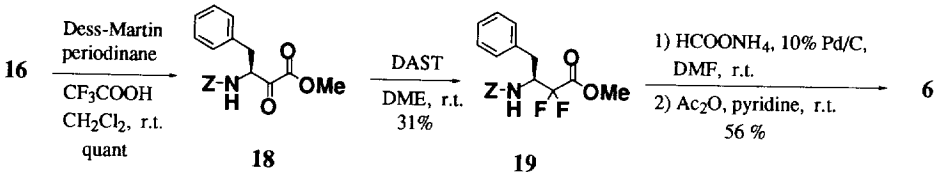
Scheme 3.



Scheme 4.



Scheme 5.



All compounds were synthesized as shown in Scheme 1-5.[9-13] The absolute configuration of the esters **16** (*syn* and *anti*) were assigned on the basis of the reported data.[14] Fluorination of the intermediates were carried out with diethylaminosulfur trifluoride(DAST).[15,16] Since fluorination of alcohols was reported to proceed with inversion of configuration at chiral center,[17] the structures of compounds **4** and **5** were tentatively assigned as shown in Scheme 4.

First, we investigated the irreversible inhibitory activity of each compound toward α-chymotrypsin by the incubation method.[18,19] Time-dependent loss of enzyme activity was observed only when the compound **3** was incubated with α–chymotrypsin and this result indicates that the compound **3** is irreversible inhibitor or slow-binding inhibitor[20] of α–chymotrypsin. The experimental result that the enzyme activity of α-chymotrypsin inactivated by the compound **3** was restored perfectly after dialysis of the assay solution at 4 °C implies that the compound **3** is not irreversible inhibitor but slow-binding inhibitor of α-chymotrypsin. In addition, the progress curve of the hydrolysis by α-chymotrypsin in the presence of the compound **3** also supports that the compound **3** is slow-binding inhibitor of α-chymotrypsin. The values of k_{on}, k_{off} and K_i of the slow-binding inhibitor **3** were obtained by the method of Cha.[21] (See Table 1)

Next we evaluated the reversible inhibitory activity of other compounds. Although compounds **1** and **2** did not show any reversible inhibitory activity toward α-chymotrypsin, a series of ester derivatives **4-6** were found to be reversible competitive inhibitors of α-chymotrypsin. The K_i values of the ester compounds were obtained from Dixon-plot[22] and listed in Table 1.

Table 1. Inactivation of α-chymotrypsin by compounds **1 - 6**

inhibitor	incubation method[a]		progress curve method[b]	type of inactivation[c]
	[I] (mM)	k_{obsd}/[I] ($M^{-1}s^{-1}$)	K_i (mM)	
1	4.85	N.I.[d]	N.I.	
2	9.73	N.I.	N.I.	
3	8.70	*e*	0.46	B
syn-**4**	4.58	N.I.	19.1	A
anti-**5**	6.25	N.I.	6.44	A
6	6.86	N.I.	0.034	A

[a]Conditions were as follows : 0.1 M potassium phosphate , 0.5 M NaCl , pH 7.8, 5 % Me_2SO, 25 °C, enzyme concentration 1.6 μM.

[b]Conditions were as follows : 0.1 M potassium phosphate , 0.5 M NaCl , pH 7.8, 5% Me_2SO, 25 °C, substrate concentration 0.025 - 0.3 mM (Suc-Ala-Ala-Pro-Phe-4-nitroanilide), enzyme concentration 5 nM.

[c]A: reversible competitive inactivation, B: slow-binding inactivation.

[d]No inactivation. [e]Not determined. [f]k_{on} = 7.34 $M^{-1}s^{-1}$, k_{off} = 3.34 x 10^{-3} $M^{-1}s^{-1}$

anti-Mono fluorinated ester compound **5** showed lower K_i value than the corresponding *syn*-ester compound **4**. Furthermore, α,α'-difluorinated ester **6** showed significant decrease in the K_i value compared to the both α-mono fluorinated ester derivatives and the K_i value was 34 μM. Its inhibitory activity is comparable to that of N-acetyl phenylalanine trifluoromethyl ketone derivative[8] known as good reversible competitive inhibitor of α-chymotrypsin(K_i = 40 μM).

These experimental results suggest that the fluorine atoms are concerned in the inactivations and the formation of the postulated hydrogen bonding as shown in Figure 1 might result in the inactivation. In the case of esters, both the number of fluorine atoms and the configuration of fluorinated carbon of the inhibitors are important for the inhibitory activity toward α–chymotrypsin and α,α'-difluorinated ester **6** showed the most potent inhibitory activity.

In conclusion, fluorinated phenylalanine analogues were found to be slow-binding inhibitor or reversible competitive inhibitors of serine protease, α-chymotrypsin. If the postulated hydrogen bonding is formed only in the active-site of the target enzyme and responsible for the inactivations, our concept would be useful for the development of selective inhibitors of serine and cysteine proteases, in future. Studies leading to the proof of the precise inactivation mechanism and the application of our concept for other serine proteases and cysteine proteases are now in progress in our laboratory.

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