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A novel type of structurally simple nonpeptide inhibitors for α-chymotrypsin. Induced-fit binding of methyl 2-allyl-3-benzenepropanoate to the S_2 subsite pocket

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Abstract

Unexpectedly, methyl and benzyl esters of 2-allyl-3-benzenepropanoic acid were found to be not substrates but potent competitive inhibitors for α-chymotrypsin. The inhibitory property of the structurally simple nonpeptidic compounds is ascribed to their high binding affinity to the enzyme at the S_2 rather than S_1 subsite pocket. These inhibitors exist in a flexible form in solution, but as they bind to the enzyme bulky contrained conformers present in a minute concentration play an important role, forming tighter enzyme-inhibitor complexes by binding to the large hydrophobic S_2 pocket. The contrained conformers are thought to be resulted from intramolecular CH/π interactions between a vinylic proton and the aromatic π-electron cloud in the inhibitor molecules. These compounds constitute novel examples of the induced-fit binding inhibitor of possibly simplest structure. © 1998 Elsevier Science Ltd. All rights reserved.

Keywords: α-Chymotrypsin, methyl 2-allyl-3-benzenepropanoate, competitive inhibitors, induced-fit binding, CH/π interaction.

1. Introduction

α-Chymotrypsin (EC 3. 4. 21. 1) is a prototypic serine protease which catalyzes the hydrolysis of protein at the carboxyl side of amino acid residue having an aromatic side chain [1]. Hydrolysis of esters of such amino acids is also accelerated by the enzyme, rate of which is even greater than that for amide hydrolysis, and thus this enzyme is useful for kinetic resolution of esters of racemic acids having a hydrophobic substituent at the α-position [2]. Indeed, optically active 2-vinyl-3-benzenepropanoic acid which we needed in connection with preparing an optically active carboxypeptidase A inhibitor could be obtained readily by the enzymic kinetic resolution of racemic methyl 2-vinyl-3-benzenepropanoate (**1**) using α-chymotrypsin (Scheme 1) [3]. However, when we subjected methyl ester of its homologous acid, methyl 2-allyl-3-benzenepropanoate (**2a**) to the enzymic hydrolysis, the ester resisted to be hydrolyzed. We found that **2a** is an inhibitor rather than a substrate for the enzyme. This communication addresses the nature and the origin of the enzyme inhibitory property exhibited by **2a** towards α-chymotrypsin.

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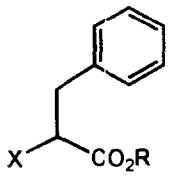
2. Results

2.1 Synthesis of inhibitors

Racemic **2a** was prepared by allylation of 3-benzenepropanoic acid using allyl bromide in the presence of LDA followed by esterification of the product with a large excess of methanol (Scheme 2). Similarly was prepared **4** (Scheme 2). Compound **2b** was obtained by allowing **9** to react with benzyl bromide in the presence of K_2CO_3 in DMF. In order to gain knowledge on the stereospecificity of the α-chymotrypsin inhibition, we needed to prepare optically active **2a**. The requisite compounds were synthesized using an Evans chiral auxilliary as depicted in Scheme 3. Synthesis of **3** is illustrated in Scheme 4, in which **17** was separated from a mixture of **17** and **18** and purified by column chromatography.



	X	R
2a	$CH_2CH{=}CH_2$	CH_3
2b	$CH_2CH{=}CH_2$	CH_2Ph
3	$CH_2CF{=}CH_2$	CH_3
4	$CH_2C{\equiv}CH$	CH_3
5	$CH_2CH_2CH_3$	CH_3
6	$CH_2CH_2CH_2CH_3$	CH_3
7	$CH_2CH_2CH_2CH_2CH_3$	CH_3

Table 1
Inhibitory potency against α-chymotrypsin

Compound no.	K_i,μM
(*R*)-**2a**	736
(*S*)-**2a**	731
2b	56
3	3870
4	—[a]
5	—[b]
6	—[b]
7	—[b]

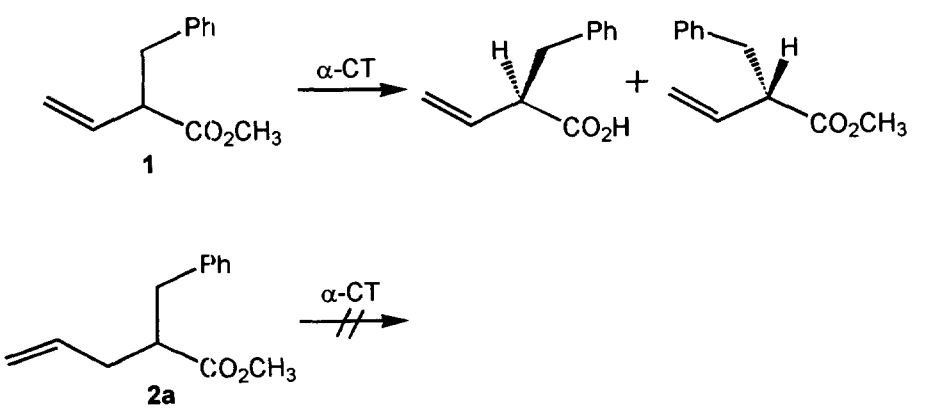
[a]Compound **4** is a substrate for α-chymotrypsin.
[b]Kinetic parameters cannot be obtained due to their extreme insolubility.

2.2 α-Chymotrypsin inhibition

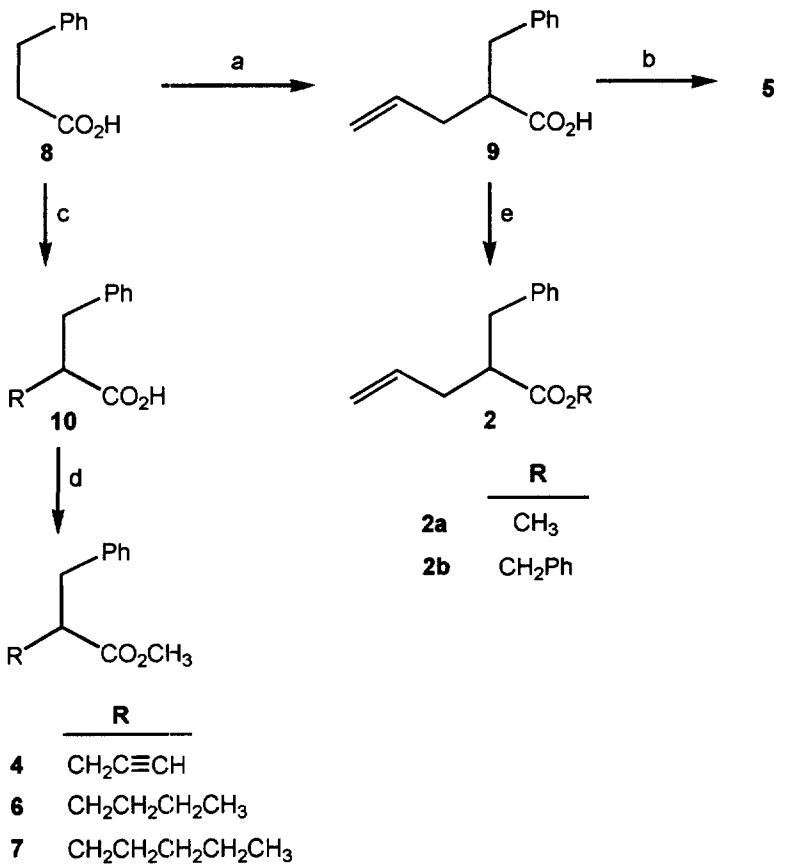
All of the compounds synthesized were tested for substrate and as inhibitors for α-chymotrypsin. The inhibitory activity assays were carried out using succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Suc-AAPFpNA) as substrate at pH 7.5. Semireciprocal plots of the initial velocity of the enzymic action for the substrate in the presence of each inhibitor at different concentrations against concentrations of the inhibitors were constructed according to the method of Dixon [4], from which inhibitory constants (K_i) were estimated. The Dixon plots represented by Fig. 1 suggest strongly that the enzyme is inhibited in a reversible competitive fashion. The inhibitory constants thus obtained are listed in Table 1. Compound **4** was hydrolyzed by the enzyme, yielding the corresponding acid having the (*R*)-configuration with (*S*)-**4** being intact. Compound **3** was hydrolyzed very slowly and only in partial. On the basis of the amount of 0.02 M NaOH solution consumed the hydrolysis of **3** was estimated to proceed only up to 21% in 10 days. Compounds **5**–**7** resisted the enzymic hydrolysis. No kinetic experiments could be performed with these compounds because of their extreme insolubility in water.

3. Discussion

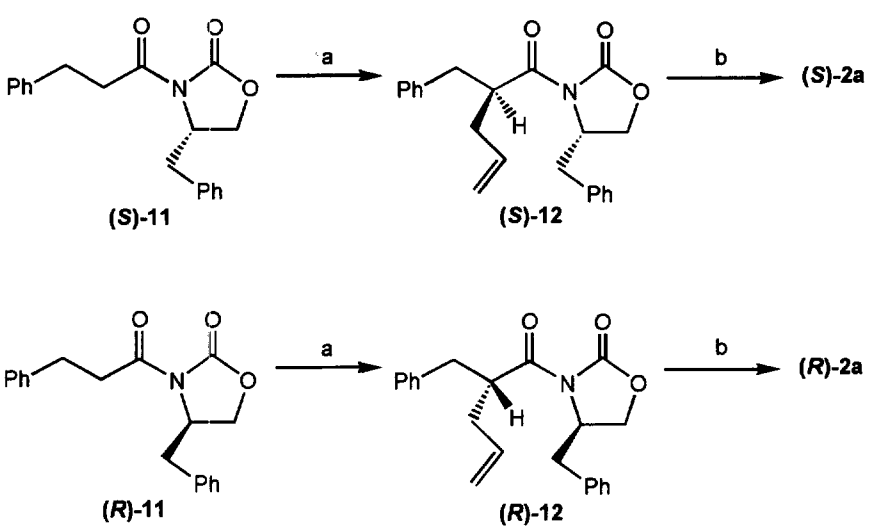
We were surprised to observe that **2a** which is a homolog of α-chymotrypsin substrate **1** resists to be hydrolyzed by α-chymotrypsin. When the compound was then tested for any inhibitory activity against the enzyme, we found that it inhibits α-chymotrypsin competitively with the K_i value of 730 μM. It is remarkable that such an abrupt change from a substrate to an inhibitor results upon the increment of one methylene unit. The observation suggests that **2a** binds the active site of the enzyme but not in a productive mode, i.e., the phenyl ring and the ester group of **2a** are not accommodated in the S_1 subsite pocket and the catalytic site, respectively [5]. When both enantiomers of **2a** were evaluated as



Scheme 1.

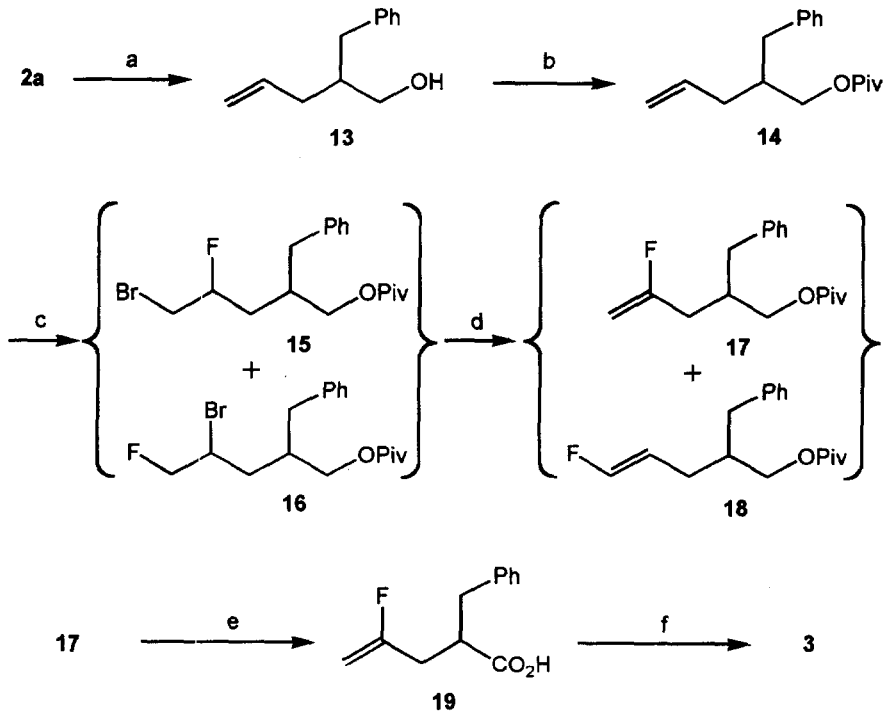


Scheme 2. Reagents, conditions, and (yields): (a) LDA (2.05 eq.), allyl bromide (1.05 eq.), 0°C, THF (82%); (b) H_2, Pd/C, MeOH, 3 h (96%); (c) LDA (2.05 eq.), RX (1.05 eq.), 0°C, THF; (d) MeOH, H^+; (e) MeOH, H^+ (for **2a**, 95%) or K_2CO_3 (1.2 eq.), benzyl bromide (1.0 eq.), DMF (for **2b**, 87%).



Scheme 3. Reagents, conditions, and (yields): (a) LDA (1.0 eq.), allyl bromide (1.0 eq.), −78°C, THF (79%); (b) MeONa (1.05 eq.), 0°C, THF (96%).

inhibitors for α-chymotrypsin, they were found to be equally potent (Fig. 1), demonstrating that the inhibition is non-stereospecific. These results taken together suggest strongly that the aromatic ring of **2a** is not accommodated in the primary substrate recognition pocket (S_1 subsite pocket) of the enzyme [6]. Since the S_1 subsite pocket exhibits stereospecificity for the 'L' isomer in its interaction with ligands, if **2a** anchors in the S_1 pocket, the stereospecificity should be manifested in the inhibition. The lack of stereospecificity shown by optically active **2a** tends to exclude also a possible explanation that the inhibition may be arisen as a result of reverse binding of **2a** to the enzyme, i.e., the binding in which the allyl moiety anchors in the hydrolytic subsite, because if it would, there is expected some differences in the inhibitory potency between the enantiomeric pair. Anchoring of the inhibitor in an auxiliary binding subsite most likely in the S_2 subsite



Scheme 4. Reagents, conditions, and (yields): (a) $LiAlH_4$ (0.6 eq.), 0°C, THF (95%); (b) PivCl, pyridine, CH_2Cl_2 (88%); (c) *N*-bromosuccinimide (1.2 eq.), pyridinium poly(hydrogen fluoride), ether (65%); (d) *tert*-BuOK (1.2 eq.), −78°C, THF (40%); (e) DIBAL (2 eq.), −78°C, CH_2Cl_2 (94%) and Jones' reagent, acetone:water = 1:1 (78%); (f) MeOH, H^+ (84%).

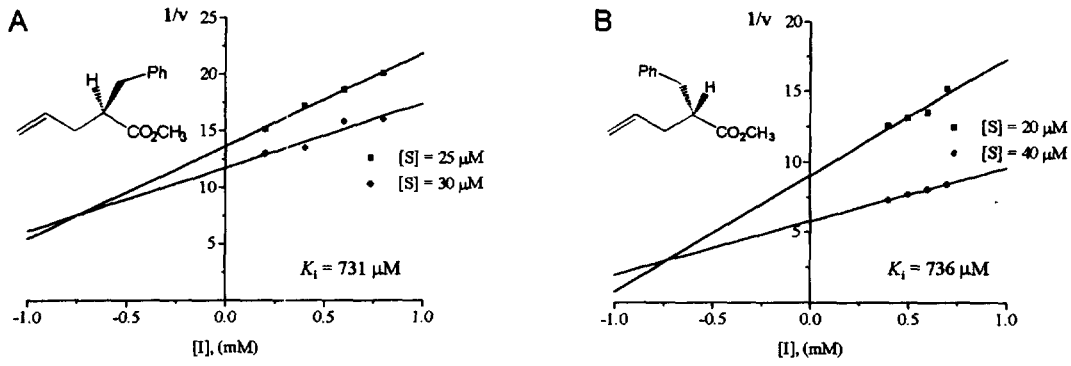


Fig. 1. Dixon plots for inhibition of α-chymotrypsin-catalyzed hydrolysis of Suc-AAPFpNA by (*S*)-**2a** (A) and (*R*)-**2a** (B): 0.036 M Tris buffer, pH 7.8; 0.045 M in $CaCl_2$; $[E] = 1.0 \times 10^{-8}$ M; Temperature 25°C.

pocket is conjectured. Figure 2 depicts the possible binding mode of **2a**. The S_2 subsite pocket is known to be a large hydrophobic pocket consisting of His-57, Ile-99, and Trp-219, and prefers a bulky residue [7]. The proposed binding mode of **2a** is supported by the observation that the benzyl ester **2b** is an inhibitor having binding affinity ($K_i = 56.2\,\mu M$) 13 times that of **2a**. It may be envisioned that in the binding of **2b** to α-chymotrypsin, the S_1 subsite pocket, dimensions of which is known to be 10–12Å in depth and (3.5–4.0Å)×(5.5–6.5Å) in cross section [8] is also occupied by the phenyl ring in the ester portion of **2b** to strengthen the binding (Fig. 3).

The observation that **4** in which the double bond in **2a** is replaced with an acetylenic bond is not an inhibitor but a substrate for α-chymotrypsin suggests that the critical structural feature that is responsible for the unusual mode of binding in the case of **2a** is the terminal double bond. We propose that there may operate non-bonded interactions, mostly of delocalization (charge transfer from π to σ^*) and dispersion forces, between a vinylic proton of the double bond and the π-electron cloud of the phenyl ring in the case of **2a**, forming a hydrophobic core that binds the S_2 pocket. Noncovalent attractive interactions between **CH** and π electron cloud have recently been proposed by Nishio et al. as an

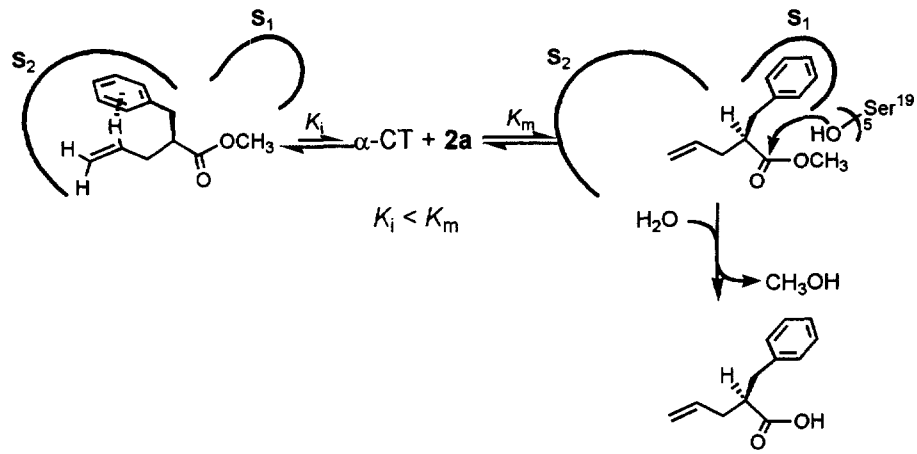


Fig. 2. Methyl 2-allyl-3-benzenepropanoate (**2a**) binds α-chymotrypsin at the S_2 subsite pocket: Since the S_2 subsite pocket is larger than the primary substrate recognition pocket (S_1 pocket), the binding to the S_2 pocket is expectedly much tighter than the binding to the S_1 pocket due to increased hydrophobic interactions. Consequently, the equilibrium shifts in favor of **2a** binding to the S_2 subsite pocket.

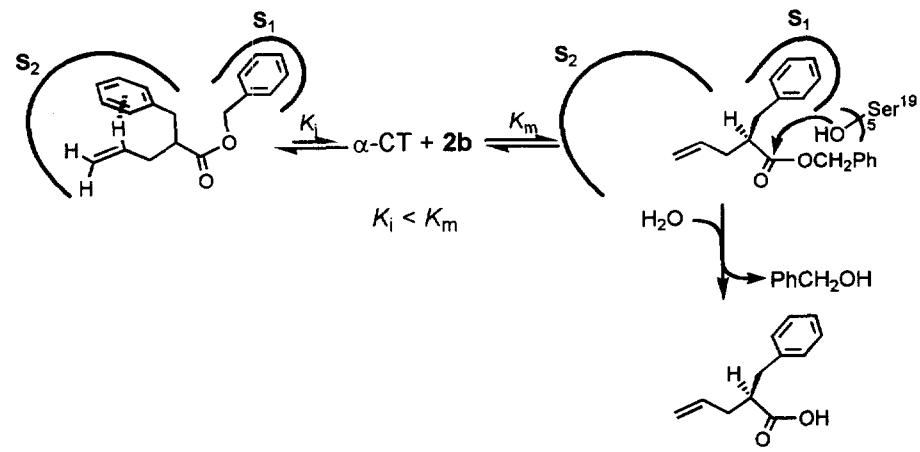


Fig. 3. Schematic representation that shows the binding mode of benzyl 2-allyl-3-benzenepropanoate (**2b**). Compound **2b** binds α-chymotrypsin with much higher binding affinity than **2a** does as a result of the additional hydrophobic interactions that arises from anchoring the phenyl ring of the ester portion in the S_1 subsite pocket.

important binding force in the biological system [9]. Such constrained conformer in the case of **2a** was suggested from the PM3 calculations [10]: The distance between the center of the phenyl ring and the internal vinylic proton in one of energy minimized conformers of **2a** is 2.99Å which falls within the range of noncovalent interactions (Fig. 5(A)) [9,11]. The finding that **4** is not an inhibitor but a substrate for the enzyme can now be envisaged on the following ground that because of the linear nature of the triple bond, the intramolecular CH/π interaction observed in **2a** is not feasible in **4**, and no bulky constrained conformer postulated for **2a** can be formed. Accordingly, **4** binds the enzyme in a productive mode for the catalytic hydrolysis with its aromatic ring being fitted into the S_1 pocket as shown in Fig. 4.

It was thought that the binding mode proposed for **2a** would become impossible if the internal vinylic proton is replaced with a fluorine and such a compound should be hydrolyzed by the enzyme. Indeed, **3** was a substrate for the enzyme although the rate of the hydrolysis was extremely slow. However, unexpectedly, **3** also exhibited an inhibitory activity for the enzyme albeit very weak with K_i value of 3.87 mM. We thought initially that the external vinylic proton may also interact with the π electron cloud of the phenyl ring, leading to form another constrained conformer such as that depicted in Fig. 5(B), but the PM3 calculations revealed that the distance between the external vinylic proton and the center of the aromatic ring is 4.24Å which is too far for the CH/π interactions. The molecular origin of the α-chymotrypsin inhibitory action of **3** is not apparent to us.

Recently, Shimohigashi et al. reported that H-D-Leu-Phe-O-CH_2Ph is a potent inhibitor for α-chymotrypsin having the K_i value of 3.6 μM [12]. Its inhibitory property was attributed to the bulky constrained conformation (hydrophobic core) resulted from intramolecular CH/π interactions between the isobutyl group of D-Leu and the phenyl ring of Phe. The bulky hydrophobic core thus formed was proposed to anchor in the S_2 subsite with the phenyl ring in the ester portion being fitted in the S_1 subsite pocket. The formation of the bulky hydrophobic core was substantiated by the ^{1}H NMR spectrum in which the protons of the isobutyl side chain of D-Leu experience a substantial upfield chemical shifts. In comparison, no such upfield chemical shift was

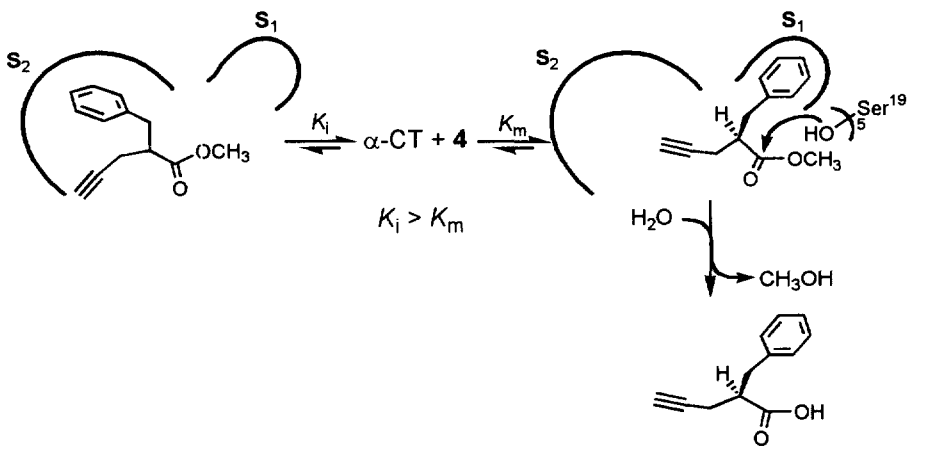


Fig. 4. Schematic representation that shows methyl 3-benzene-2-propagylpropanoate (**4**) binds α-chymotrypsin in a productive mode resulting in the hydrolysis of the ester.

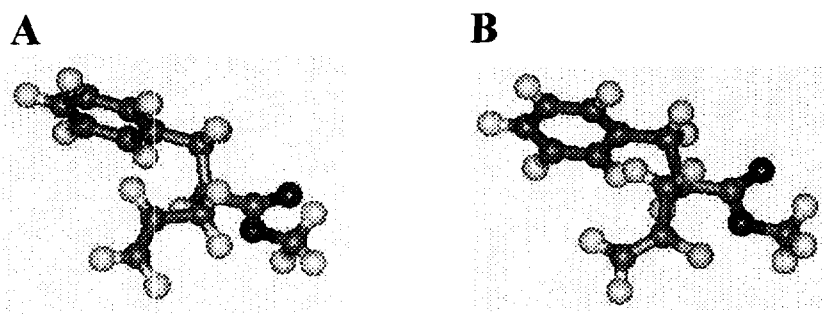


Fig. 5. Stick and ball drawing of constrained conformers of **2a** generated by MOPAC using PM3 program.

noted in the ^{1}H NMR spectrum of **2a**, demonstrating that in the case of **2a** the hydrophobic core such as that observed with H-D-Leu-Phe-O-CH_2Ph is not formed to any meaningful concentration, which suggests that the non-bonded interactions of the vinylic proton with the aromatic π-electron cloud in **2a** become to play an important role only when **2a** binds the enzyme. Thus, in the case of **2a** which exists in a flexible form in solution, the hydrophobic constrained conformer may develop as it binds to the S_2 subsite pocket. Presumably, there may be in effect hydrophobic and van der Waals interactions between the S_2 subsite pocket and the constrained conformer upon binding. The hydrophobic interactions are known to increase in proportion to the contact area of the interacting moieties both at molecular and macromolecular levels [13]. Thus, it may be plausible that since the S_2 pocket is larger than the S_1 pocket, the binding of **2a** to the S_2 pocket is expectedly much tighter compared with that resulting from the anchoring of just the phenyl ring alone in the S_1 pocket as illustrated schematically in Fig. 2. Therefore, the K_i value is much smaller than the K_m value in the case of binding **2a** to α-chymotrypsin, and as a consequence **2a** behaves as a competitive inhibitor (Fig. 2). Thus, the inhibition of α-chymotrypsin by **2a** may be referred to the binding of **2a** to the S_2 subsite pocket in an induced-fit manner, but double-induced fit binding, in which the enzyme as well as the inhibitor change their conformations upon forming complex may not be excluded.

The induced-fit theory proposed by Koshland has provided a plausible explanation for the catalytic action of enzymes since its inception in 1958 [14]. According to the theory, binding of a substrate to an enzyme induces conformational changes of the enzyme and these conformational changes would result in the catalytic functional groups to orient themselves for the ensuing catalytic process. Thus, the theory focuses the conformational changes of the enzyme in explaining the enzymic action, but recent works have drawn attention on the possibility of conformational changes in ligands [15]. The inhibition of the enzymic activity of α-chymotrypsin by **2a** may constitute a novel example of such induced-fit binding of a ligand to enzyme and **2a** may possibly be a minimum structural unit for such binding.

4. Conclusion

Methyl 2-allyl-3-benzenepropanoate **2a** which is a homolog of methyl 2-vinyl-3-benzenepropanoate, a substrate of α-chymotrypsin resists the hydrolysis by α-chymotrypsin. Rather, it inhibited the catalytic activity of the enzyme. This unexpected inhibitory property shown by **2a** and its analogs towards α-chymotrypsin is attributed to their high binding affinity to the enzyme at the large hydrophobic S_2 subsite pocket. Such a mode of binding in the case of **2a** and analogs is possibly ascribed to the unique property of these molecules to form bulky constrained conformers resulting from the intramolecular **CH**/π interactions between the internal vinylic proton and the phenyl aromatic ring. Although such constrained conformers exist in a minute quantity, they are thought to bind preferentially to the large hydrophobic pocket of the S_2 subsite in the active site of α-chymotrypsin because the hydrophobic and van der Waals forces involved in the binding are stronger than those resulting from the normal binding of **2a** and its analogs to the S_1 subsite. Inhibitors **2a** and its analogs exemplify the induced-fit binding of a flexible ligand to an enzyme.

5. Experimental

Infrared (IR) spectra were obtained with a Bruker Equinox 55 FT-IR spectrometer. ^{1}H and ^{19}F NMR spectra were recorded on a Bruker AM 300 NMR spectrometer in $CDCl_3$ using tetramethylsilane (TMS) as the internal reference. Chemical shifts of ^{19}F NMR spectra are reported in δ values upfield from the ^{19}F signal of CF_3CO_2H. Low and high resolution mass spectral analyses were performed at the Inter-University Center for Natural Science Research Facilities, Seoul National University. Optical rotations were measured on Rudolph Research Autopol III digital polarimeter. Flash chromatography was performed using silica gel 60 (230~400 mesh). Thin layer chromatography (TLC) was carried out on silica coated glass sheets (Merck silica gel 60 F-254). The pH-stat used in enzymic kinetic resolution is a Cole-Parmer pH/mV controller type 5997-20 which is coupled with a Orion gel-filled combination pH electrode and Pharmacia Peristaltic Pump P-1. Enzyme assays were carried out using Hewlett-Packard 8452a UV spectrometer fitted with a cell temperature controller. α-Chymotrypsin and its substrate, succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (sucAAPFpNA) were purchased from Sigma Chemical Co.

5.1 2-Allyl-3-bezenepropanoic acid (9)

To an ice-chilled solution of diisopropylamine (5.9 ml, 42.0 mmole) in dried THF (30 ml) was added slowly n-butyllithium (16.8 ml of 2.5 M solution in n-hexane, 42.0 mmole) under nitrogen atmosphere. After the mixture was stirred for 30 min, hydrocinnamic acid (**8**, 3.0 g, 20.0 mmole) in dried THF (20 ml) was added dropwise over a period of 20 min. The resulting yellowish solution was stirred for 40 min, and then allyl bromide (1.8 ml, 21.0 mmole) was added dropwise. The reaction mixture was stirred for 3 h to give a colorless solution. The pH of the solution was adjusted to 2.5 with 3 N HCl solution. The organic layer was separated and extracted with

saturated sodium bicarbonate solution. The aqueous layer was acidified with 3 N HCl solution to pH 2, saturated with sodium chloride, and extracted with ethyl acetate (50 ml×3). The combined extracts were dried over anhydrous $MgSO_4$, filtered, and evaporated under reduced pressure. The residue was purified by column chromatography, eluting with a solution of ethyl acetate and n-hexane (25:75) to give **9** (3.12 g, 82% yield) as a colorless oil: IR (neat) 1712 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.13–7.35 (m, 5H), 5.65–5.74 (m, 1H), 4.98–5.06 (m, 2H), 2.91–2.97 (m, 1H), 2.70–2.81 (m, 2H), 2.26–2.39 (m, 2H).

*5.2 Methyl 2-allyl-3-bezenepropanoate (**2a**)*

To an ice-chilled solution of **9** (2.83 g, 15 mmole) in anhydrous methanol (50 ml) was added 2 ml of acetyl chloride. The resulting mixture was stirred for 12 h at room temperature, then evaporated under reduced pressure. The residue was dissolved in ether, and the ether solution was washed with water (30 ml), 5% sodium bicarbonate solution (30 ml×3), and brine (30 ml). The organic solution was dried over anhydrous $MgSO_4$, filtered, and evaporated under reduced pressure. The residue was purified by column chromatography, eluting with a solution of ethyl acetate and n-hexane (10:90) to give **2a** (3.20 g, 95%) as a colorless oil: IR (neat) 1722 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.13–7.29 (m, 5H), 5.70–5.80 (m, 1H), 5.01–5.09 (m, 2H), 3.59 (s, 3H), 2.91–2.99 (m, 1H), 2.73–2.81 (m, 2H), 2.26–2.40 (m, 2H); MS (*m/z*) 204, 172, 163, 144, 131, 103, 91.

*5.3 Benzyl 2-allyl-3-benzenepropanoate (**2b**)*

To a stirred mixture of **9** (380 mg, 2 mmole) and K_2CO_3 (332 mg, 2.4 mmole) in 7 ml of DMF was added slowly benzyl bromide (360 mg, 2 mmole) at 0°C. The stirring was continued for about 2 h, and then the reaction mixture was diluted with ethyl acetate. The solution thus obtained was washed with sodium thiosulfate (20 ml), 5% sodium bicarbonate solution (20 ml), 10% citric acid (20 ml), and brine (20 ml), successively, then dried over anhydrous $MgSO_4$, and evaporated under reduced pressure. The oily residue was purified by column chromatography, eluting with a solution of ethyl acetate and hexane (5:95) to give **2b** (485 mg, 87%): IR (neat) 3067, 3031, 1733 (C=O), 1496, 1449, 1181 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.15–7.35 (m, 5H), 5.77 (m, 1H), 5.02–5.11 (m, 2H), 5.05 (s, 2H), 2.98 (m, 1H), 2.79–2.87 (m, 2H), 2.44 (m, 1H), 2.30 (m, 1H); MS (*m/z*) 281 ($M^+ + 1$) 280, 239, 228, 190, 181, 144, 127, 111.

*5.4 Methyl (S)-2-allyl-3-benzenepropanoate ((S)-**2a**)*

To an ice-chilled solution of diisopropylamine (3.5 ml, 25 mmole) in dried THF (100 ml) was added slowly n-butyllithium (10 ml of 2.5 M solution in n-hexane, 25 mmole) under nitrogen atmosphere. After the mixture was stirred for 30 min and cooled to −78°C, (4*S*)-3-(3-phenylpropanoyl)-4-benzyl-1,3-oxazolidin-2-one [16] ((*S*)-**11**, 7.73 g, 25 mmole) in dried THF (20 ml) was added dropwise over a period of 20 min. The resulting yellowish solution was stirred for 1.5 h, and then allyl bromide (2.2 ml, 25 mmole) was added dropwise. The reaction mixture was allowed to warm to room temperature over 7 h, then quenched with saturated ammonium chloride solution and partitioned between water and ether. The combined ether extracts were washed with saturated sodium bicarbonate solution and brine, then evaporated under reduced pressure. The oily residue was subjected to flash chromatography, eluting with a solution of methylene chloride and petroleum ether (50:50) to give (*S*)-**12** [17] (6.9 g, 79%): ^1H NMR ($CDCl_3$) δ 7.18–7.36 (m, 10H), 5.82–5.93 (m, 1H), 5.08–5.18 (m, 2H), 4.32–4.51 (m, 2H), 4.01–4.05 (dd, 1H), 3.84 (t, 1H), 3.25 (dd, 1H), 2.85–3.02 (m, 2H), 2.67 (dd, 1H), 2.30–2.62 (m, 2H).

A methanol solution of sodium methoxide (7.3 mmole) was added dropwise to the stirred solution of (*S*)-**12** (2.44 g, 7 mmole) in 20 ml of anhydrous methanol at 0°C under nitrogen atmosphere, then stirred for 2 h. The reaction mixture was quenched with saturated ammonium chloride solution and the remaining methanol was evaporated under reduced pressure. The residue was diluted with ethyl acetate, and washed with water and brine, then dried over anhydrous $MgSO_4$, filtered, and evaporated under reduced pressure. The residue was subjected to flash column chromatography, eluting with a solution of ethyl acetate and hexane (20:80) to give (*S*)-**2a** (1.37 g, 96%): IR (neat) 3018, 2961, 1722 (C=O), 1212 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.13–7.29 (m, 5H), 5.70–5.80 (m, 1H), 5.10–5.09 (m, 2H), 3.59 (s, 3H), 2.91–2.99 (m, 1H), 2.73–2.81 (m, 2H), 2.26–2.40 (m, 2H); MS (*m/z*) 204, 172, 163, 144, 131, 113, 103, 91; $[\alpha]_D = +30.0°$(c = 1.0, EtOH).

*5.5 Methyl (R)-2-allyl-3-benzenepropanoate ((R)-**2a**)*

This was prepared in a fashion similar to that used for the preparation of (*S*)-**12** using (*R*)-**11**: ^1H NMR ($CDCl_3$) δ 7.18–7.36 (m, 10H), 5.82–5.93 (m, 1H), 5.08–5.18 (m, 2H), 4.32–4.51 (m, 2H), 4.01–4.05 (dd, 1H), 3.84 (t, 1H), 3.25 (dd, 1H), 2.85–3.02 (m, 2H), 2.67 (dd, 1H), 2.30–2.62 (m, 2H). (*R*)-**12** was then treated with a methanol solution of sodium methoxide in a similar fashion to that used for preparation of (*S*)-**2a** to give (*R*)-**2a**: IR (neat) 3015, 2958, 1721 (C=O), 1220 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.11–7.31 (m, 5H), 5.68–5.76 (m, 1H), 5.07–5.10 (m, 2H), 3.55 (s, 3H), 2.89–2.96 (m, 1H), 2.72–2.79 (m, 2H), 2.24–2.37 (m, 2H); MS (*m/z*) 204, 172, 163, 144, 131, 113, 103, 91; $[\alpha]_D = -28.5°$(c = 1.0, EtOH).

5.6 Methyl 2-propagyl-3-benzenepropanoate (4)

3-Benzene-2-propagylpropanoic acid (**10**) was prepared in a similar fashion to that used for the preparation of **9** using propagyl bromide instead of allyl bromide: IR (neat) 1720 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.13–7.26 (m, 5H), 3.02 (dd, 1H), 2.82–2.95 (m, 2H), 2.37–2.42 (m, 2H), 2.00 (t, 1H). Compound **10** was then converted into methyl ester in a fashion similar to that used for the preparation of **2a** to give **4**: IR (neat) 2349, 1737 (C=O), 1442, 1370 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.18–7.32 (m, 5H), 3.67 (s, 3H), 3.06 (dd, 1H), 2.85–2.98 (m, 2H), 2.42–2.46 (m, 2H), 2.06 (t, 1H); MS (*m/z*) 203 ($M^+ + 1$), 171, 163, 141, 131, 115, 103, 91, 79.

5.7 Methyl 2-benzylpentanoate (5)

To a solution of **2a** (200 mg, 0.98 mmole) in anhydrous methanol (10 ml) was added a catalytic amount of 10% Pd/C and the resulting solution was stirred under hydrogen gas (1 atm) for 3 h. The catalyst was removed by filtration and the filtrate was evaporated under reduced pressure to give **5** (195 mg, 96%): IR (neat) 1730 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.13–7.29 (m, 5H), 3.60 (s, 3H), 2.68–2.96 (dq, 2H), 2.66–2.70 (m, 1H), 1.45–1.65 (m, 2H), 1.23–1.35 (m, 2H), 0.88 (t, 3H).

5.8 Methyl 2-benzylhexanoate (6)

This was prepared in a fashion similar to that used for the preparation of **4** using 1-iodobutane and subsequent esterification with methanol: IR (neat) 1731 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.13–7.30 (m, 5H), 3.60 (s, 3H), 2.70–2.96 (dq, 2H), 2.62–2.68 (m, 1H), 1.49–1.63 (m, 2H), 1.25–1.31 (m, 4H), 0.87 (t, 3H).

5.9 Methyl 2-benzylheptanoate (7)

This was prepared in a fashion similar to that used for the preparation of **4** using 1-bromopentane and subsequent esterification with methanol: IR (neat) 1728 (C=O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.14–7.30 (m, 5H), 3.58 (s, 3H), 2.71–2.97 (dq, 2H), 2.62–2.68 (m, 1H), 1.49–1.65 (m, 2H), 1.26–1.32 (m, 6H), 0.86 (t, 3H).

5.10 2-Benzyl-4-pentenol (13)

To an ice-chilled solution of **2a** (4.62 g, 22.7 mmole) in 150 ml of dried THF was added $LiAlH_4$ (0.52 g, 13.6 mmole) slowly and stirred for 3 h at 0°C. The reaction mixture was quenched with 3 ml of water, dried over anhydrous $MgSO_4$, and filtered. The filtrate was concentrated under reduced pressure and chromatographed to give **13** (3.79 g, 95%): IR (neat) 3064, 2976, 1480, 1285, 1163 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.18–7.32 (m, 5H), 5.80–5.89 (m, 1H), 5.04–5.13 (m, 2H), 3.50–3.60 (m, 2H), 2.63–2.66 (m, 2H), 2.12–2.17 (m, 2H), 1.90–1.94 (m, 1H), 1.26 (br, 1H).

5.11 1-Benzyl-3-butenyl pivaloate (14)

To a solution of **13** (4 g, 22.7 mmole) in 20 ml of dry CH_2Cl_2 was added 10 ml of dry pyridine. The resulting mixture was chilled in an ice-bath. To the ice-chilled solution was added dropwise pivaloyl chloride (3.08 ml, 25 mmole) over a period of 30 min under argon atmosphere. The reaction mixture was allowed to warm to room temperature, stirred for 14 h, then evaporated under reduced pressure, and the residue was purified by column chromatography, eluting with a solution of ether and petroleum ether (50:50) to give **14** (5.2 g, 88%) as a colorless oil: IR (neat) 2976 (s, C-H), 1738 (s, C=O), 1653, 1640 (w, C=C), 1161 (s, C-O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.14–7.30 (m, 5H), 5.72–5.86 (m, 1H), 5.02–5.08 (m, 2H), 3.94–3.98 (d, 2H), 2.64–2.66 (d, 2H), 2.06–2.15 (m, 3H), 1.21–1.26 (m, 9H).

5.12 1-Benzyl-3-fluoro-4-bromobutyl pivaloate (15) and 1-benzyl-3-bromo-4-fluorobutyl pivaloate (16)

To a stirred solution of *N*-bromosuccinimide (3.79 g, 21.3 mmole) obtained by dissolving it in ether (20 ml) in a plastic bottle was added pyridinium poly(hydrogen fluoride) solution (20 ml) under nitrogen atmosphere. The mixture was stirred in an ice bath for 15 min, at which time **14** (5.03 g, 19.3 mmole) dissolved in ether (10 ml) was added slowly. The reaction mixture was allowed to warm to room temperature over 30 min. After being stirred at room temperature for 2 h, the reaction mixture was poured into saturated sodium bicarbonate solution (50 ml), and extracted each with ether (100 ml×4), dried over anhydrous Na_2SO_4, and evaporated under reduced pressure. The resulting yellowish oil was chromatographed eluting with a solution of ethyl acetate and hexane (10:90) to give a mixture (4.94 g, 65% yield) of **15** and **16** (1:1). A small amount of **15** could be isolated and purified by chromatography for identification: IR (neat) 2972 (s, C-H), 1733 (s, C=O), 1161 (s, C-O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.14–7.32 (m, 5H), 4.65–4.86 (m, 1H), 3.90–4.10 (m, 2H), 3.38–3.48 (m, 2H), 2.67–2.74 (m, 2H), 2.17–32 (m, 1H), 1.68–1.88 (m, 2H), 1.21 (s, 9H); ^{19}F NMR ($CDCl_3$) δ −103.60, −103.66. The mixture of **15** and **16** was used for the following reaction without separation.

5.13 1-Benzyl-3-fluoro-3-butenyl pivaloate (17)

To a stirred and chilled (−78°C) solution of the mixture of **15** and **16** (3.94 g, 11 mmole) in 20 ml of dried THF was added slowly 15 ml of 1.0 M potassium *tert*-butoxide in THF. The reaction mixture was allowed to warm to −30°C and kept between −30 and −20°C for 2 h. The

temperature of the reaction mixture was lowered to −78°C, and 15 ml of ether and 0.5 ml of acetic acid were added successively. The organic solvents were evaporated under reduced pressure to give a mixture of **17** and 1-benzyl-4-fluorobutenyl pivaloate (**18**). Compound **17** (1.23 g, 40%) was separated from the mixture by column chromatography, eluting with a solution of ethyl acetate and hexane (20:80): IR (neat) 2975 (s, C-H), 1732 (s, C=O), 1674 (m, C=C), 1284, 1160 (s, C-O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.15–7.32 (m, 5H), 4.58–4.65 (dd, 1H), 4.19–4.36 (dd, 1H), 3.92–4.07 (m, 2H), 2.63–2.72 (m, 2H), 2.22–2.32 (m, 3H), 1.24 (s, 9H); ^{19}F NMR ($CDCl_3$) δ −16.0.

5.14 2-Benzyl-4-fluoro-4-pentenoic acid (19)

To a stirred and chilled (−78°C) solution of **17** (628 mg, 2.26 mmol) in 20 ml of dry CH_2Cl_2 was added dropwise diisobutylaluminium hydride (5.6 ml, 1.0 molar solution in hexane) under nitrogen atmosphere. After 30 min stirring, the reaction mixture was transferred to a separatory funnel containing 50 ml of saturated solution of sodium tartarate and 100 ml of ethyl acetate. The organic layer was separated after vigorous shaking, dried over anhydrous $MgSO_4$, and evaporated under reduced pressure. The residue was column chromatographed, eluting with a solution of ethyl acetate and hexane (15:85) to give 2-benzyl-4-fluoro-4-pentenol (410 mg, 94%) as a colorless oil: IR (neat) 3361 (br, O-H), 2923 (m, C-H), 1674 (s, C=C), 1030 (m, C-O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.17–7.33 (m, 5H), 4.57–4.64 (dd, 1H), 4.21–4.38 (dd, 1H), 3.52–3.64 (m, 2H), 2.68–2.71 (d, 2H), 2.22–2.36 (m, 2H), 2.10–2.17 (m, 1H), 1.39 (br, 1H); ^{19}F NMR ($CDCl_3$) δ −17.8.

To an ice-chilled solution of 2-benzyl-4-fluoro-4-pentenol (380 mg, 1.96 mmole) in 10 ml of acetone was added slowly the Jones' reagent until the brownish color of the solution remains over 20 min, then 2-propanol was added until the solution became clear. The precipitate that separated was filtered using a celite pad and the filtrate was evaporated under reduced pressure. The residue was diluted with 5 ml of 1 N HCl then extracted with ethyl acetate (30 ml×3). The extract was dried over anhydrous $MgSO_4$ and evaporated under reduced pressure to give a yellowish syrup (320 mg, 78%) of **19** which was used in the next reaction without purification: IR (neat) 3031 (br, COOH), 1713 (s, C=O), 1683 (s, C=CF), 1252 (m, C-O) cm^{-1}; ^1H NMR ($CDCl_3$) δ 10.84 (br, 1H), 7.15–7.34 (m, 5H), 4.59–4.66 (dd, 1H), 4.24–4.42 (dd, 1H), 2.94–3.06 (m, 2H), 2.84–2.89 (m, 1H), 2.46–2.60 (m, 1H), 2.39–2.45 (m, 1H); ^{19}F NMR ($CDCl_3$) δ −22.1.

5.15 Methyl 2-benzyl-4-fluoro-4-pentenoate (3)

To an ice-chilled solution of **19** (250 mg, 1.2 mmole) in anhydrous methanol (20 ml) was added 1 ml of acetyl chloride. The resulting mixture was stirred for 12 h at room temperature, then evaporated under reduced pressure. The residue was dissolved in ether, and the ether solution was washed with water (30 ml), 5% sodium bicarbonate solution (30 ml×3), and brine (30 ml). The organic solution was dried over anhydrous $MgSO_4$, filtered, and evaporated under reduced pressure. The residue was purified by column chromatography, eluting with a solution of ethyl acetate and n-hexane (10:90) to give **3** (220 mg, 84%) as a colorless oil: IR (neat) 1739 (s, C=O), 1684 (m, C=C) cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.14–7.31 (m, 5H), 4.55–4.61 (dd, 1H), 4.20–4.37 (dd, 1H), 3.61 (s, 3H), 2.91–2.98 (m, 2H), 2.81–2.87 (m, 1H), 2.49–2.63 (m, 1H), 2.33–2.43 (m, 1H); ^{19}F NMR ($CDCl_3$) δ−19.4; MS (*m/z*) 222, 202, 191, 162, 131, 103, 91, 77, 65; HRMS (EI+) obsd. 222.1057, calc. 222.1056 ($C_{13}H_{15}O_2F$).

5.16 Enzyme-catalyzed hydrolysis of racemic methyl 2-propagyl-3-benzenepropanoate (4)

Racemic **4** (202 mg, 1.0 mmole) was suspended in 10 ml of 0.01 M phosphate buffer solution. To the mixture was added α-chymotrypsin (40 mg), and stirred slowly. The pH of the reaction mixture was maintained at 7.8 by addition of sodium hydroxide solution (0.1 N) using a pH-stat. When 3.5 ml of the alkaline solution was consumed after 24 h, the reaction mixture was acidified with 1N HCl solution and saturated with sodium chloride, then extracted with ethyl acetate. The extract was dried over anhydrous $MgSO_4$, filtered, and evaporated under reduced pressure. The residue was purified by column chromatography, eluting with a solution of ethyl acetate and n-hexane (20:80) to give (*R*)-3-benzene-2-propagylpropanoic acid and methyl (*S*)-3-benzene-2-ethynylpropanoate. The spectral data of these compounds were identical to those of respective racemic compounds, i.e., **10** and **4**.

5.17 Enzyme-catalyzed hydrolysis of Rracemic methyl 2-benzyl-4-fluoro-4-pentenoate (3)

Racemic **3** (111 mg, 0.5 mmole) was suspended in 20 ml of 0.01 M phosphate buffer solution. To the mixture was added α-chymotrypsin (10 mg), and stirred slowly. The pH of the reaction mixture was maintained at 7.8 by addition of sodium hydroxide solution (0.02 N) using a pH-stat. The alkaline solution was consumed slowly up to 5.2 ml in 2 days. No further consumption was noticed in the following 7 days. The reaction mixture was acidified with 1N HCl solution and saturated with sodium chloride, then extracted with ethyl acetate. The extract was dried over anhydrous $MgSO_4$, filtered, and evaporated under reduced pressure. The residue was purified by column chromatography, eluting with a solution of ethyl acetate and n-hexane (20:80) to give (*R*)-2-benzyl-4-fluoro-4-pentenoic acid ($[\alpha]_D = +11.2°$

(c = 1.0, $CHCl_3$)) and (*S*)-2-benzyl-4-fluoro-4-pentenoic acid methyl ester. The spectral data of these compounds were identical to those of respective racemic compounds, i.e., **19** and **3**.

5.18 Enzyme inhibition assay

All solutions were prepared using deionized water. The stock solutions of α-chymotrypsin and substrate (sucAAPFpNA) were prepared in Tris buffer (0.036 M Tris-0.045 $CaCl_2$, pH 7.8 at 25°C). The stock solution of inhibitors were prepared in DMSO. The stock solutions were filtered prior to use. The enzyme reaction was inhibited by adding an aliquot of the α-chymotrypsin stock solution to a mixture of inhibitor and substrate in a 1 ml cuvett at 25°C. Final concentration of α-chymotrypsin was 1.0 μg/ml. Initial rates of the hydrolysis of the substrate, in the absence and presence of different concentrations of inhibitors, were determined from the change in absorbance at 400 nm. The reversible inhibitory constants (K_i) were calculated from plots obtained by the method of Dixon [4].

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