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Quantitative Structure–Activity Relationships for the Pre-Steady-State Inhibition of Cholesterol Esterase by 4-Nitrophenyl-*N*-substituted Carbamates

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Abstract—4-Nitrophenyl-*N*-substituted carbamates (**1**–**6**) are the pseudo-substrate inhibitors of porcine pancreatic cholesterol esterase. Thus, the first step of the inhibition (K_i step) is the formation of the enzyme–inhibitor tetrahedral adduct and the second step of the inhibition (k_c) is the formation of the carbamyl enzyme. The formation of the enzyme–inhibitor tetrahedral adduct is further divided into two steps, the formation of the enzyme–inhibitor complex with the dissociation constant, K_S, at the first step and the formation of the enzyme–inhibitor tetrahedral adduct from the complex at the second step. The two-step mechanism for the formation of the enzyme–inhibitor tetrahedral adduct is confirmed by the pre-steady-state kinetics. The results of quantitative structure–activity relationships for the pre-steady-state inhibitions of cholesterol esterase by carbamates **1**–**6** indicate that values of $-\log K_S$ and $\log k_2/k_{-2}$ are correlated with the Taft substituent constant, σ^*, and the ρ^* values from these correlations are -0.33 and 0.1, respectively. The negative ρ^* value for the $-\log K_S-\sigma^*$-correlation indicates that the first step of the two-step formation of the enzyme–inhibitor tetrahedral adduct (K_S step) is the formation of the positive enzyme–inhibitor complex. The positive ρ^* value for the $\log k_2/k_{-2}-\sigma^*$-correlation indicates that the enzyme–inhibitor tetrahedral adduct is more negative than the enzyme–inhibitor complex. Finally, the two-step mechanism for the formation of the enzyme–inhibitor tetrahedral adduct is proposed according to these results. Thus, the partially positive charge is developed at nitrogen of carbamates **1**–**6** in the enzyme–inhibitor complex probably due to the hydrogen bonding between the lone pair of nitrogen of carbamates **1**–**6** and the amide hydrogen of the oxyanion hole of the enzyme. The second step of the two-step formation of the enzyme–inhibitor tetrahedral adduct is the nucleophilic attack of the serine of the enzyme to the carbonyl group of carbamates **1**–**6** in the enzyme–inhibitor complex and develops the negative-charged oxygen in the adduct.

Introduction

Recently there has been increased interest in pancreatic cholesterol esterase (CEase, EC 3.1.1.13, also known as bile salt-activated lipase) due to the correlation between enzymatic activity in vivo and absorption of dietary cholesterol.[1,2] Results of a recent study showed that CEase is responsible for mediating intestinal absorption of cholesteryl esters but does not play a primary role in free cholesterol absorption.[3] Physiological substrates of CEase include cholesteryl esters, retinyl esters, acylglycerols, vitamin esters, and phospholipids.[4–7] CEase plays a role in digestive lipid absorption in the upper intestinal tract, though its role in cholesterol absorption in particular is controversial.[1,2,8] A recent report indicates that CEase is directly involved in lipoprotein metabolism, in that the enzyme catalyzes the conversion of large low-density liproprotein (LDL) to smaller, denser, more cholesteryl ester-rich lipoproteins, and that the enzyme may regulate serum cholesterol levels.[9] A major cause of atherosclerosis is an excess of plasma LDL, a phenomenon that is particularly evident in individuals with familial hypercholesterolemia, who lack functional LDL receptors. The enzyme may function in these roles by acting as a cholesterol transfer protein.[10] CEase shares the same catalytic machinery as serine proteases in that they have an active site serine residue which, with a histidine and an aspartic or glutamic acid, forms a catalytic triad.[11] The conservation of this catalytic triad suggests that as well as sharing a common mechanism for substrate hydrolysis, that is, formation of a discrete acyl enzyme intermediate via the serine hydroxyl group, serine proteases, CEase, serine phospholipase A2, and lipases may well be expected to be inhibited by the same classes of mechanism-based inhibitors. To date, this has been demonstrated for diethyl-*p*-nitrophenol phosphate,[12]

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hexadecylsulfonyl fluoride,[13] fluoroketones,[14,15] boronic acid,[16] chloroisocoumarin,[17] β-lactones,[18,19] β-lactams,[20] and carbamates.[21–28]

Two different X-ray crystal structures of bovine pancreatic CEase have been reported recently.[29,30] Although different bile salt-activation mechanisms for CEase are proposed, the active site of CEase is similar to those of *Pseudomonas cepacia* lipase[31,32] and *Candida rugosa* lipase (CRL).[33] The active site of CEase may consist of at least five major binding sites (Fig. 1): (a) an alkyl chain binding site (ACS) that binds to the acyl chain of cholesteryl ester and the first alkyl chain of triacylglycerol, (b) an oxyanion hole (OH), the H-bonding peptide NH functions of Gly107, Ala108, and Ala195, that stabilizes the incipient carbonyl C−O$^-$ of the ester function during turnover, (c) an esteratic site (ES), comprised of the Ser194-His435-Asp320 active site triad that is involved in nucleophilic and general acid–base catalysis, (d) a leaving group hydrophobic binding site (LHOS) that binds to the hydrophobic part of the leaving group, (e) the second alkyl chain binding site (SACS) that binds to the second alkyl chain of triacylglycerol and is in a crevice above the catalytic site, and (f) a leaving group hydrophilic binding site (LHIS) that binds to the hydrophilic part of the leaving group and is located at the opposite direction of ACS.

In the presence of substrate, the mechanism of transient or pseudo-substrate inhibitions of CEase has been proposed in Scheme 1.[21–28]

Because the inhibition of CEase follows first-order kinetics over the observed time period for the steady-state kinetics, the rate of hydrolysis of EI′ must be significantly slower than the rate of formation of EI′ ($k_c \gg k_3$).[34] Therefore, values of K_i and k_c can be calculated from eqn (1):[21–28]

$$k_{app} = \frac{k_c[I]}{K_i\left(1 + \frac{[S]}{K_m}\right) + [I]} \quad (1)$$

In eqn (1), k_{app} values are the first-order rate constants which are obtained according to Hosie's method.[21] Bimolecular rate constant, $k_i = k_c/K_i$, is related to overall inhibitory potency.

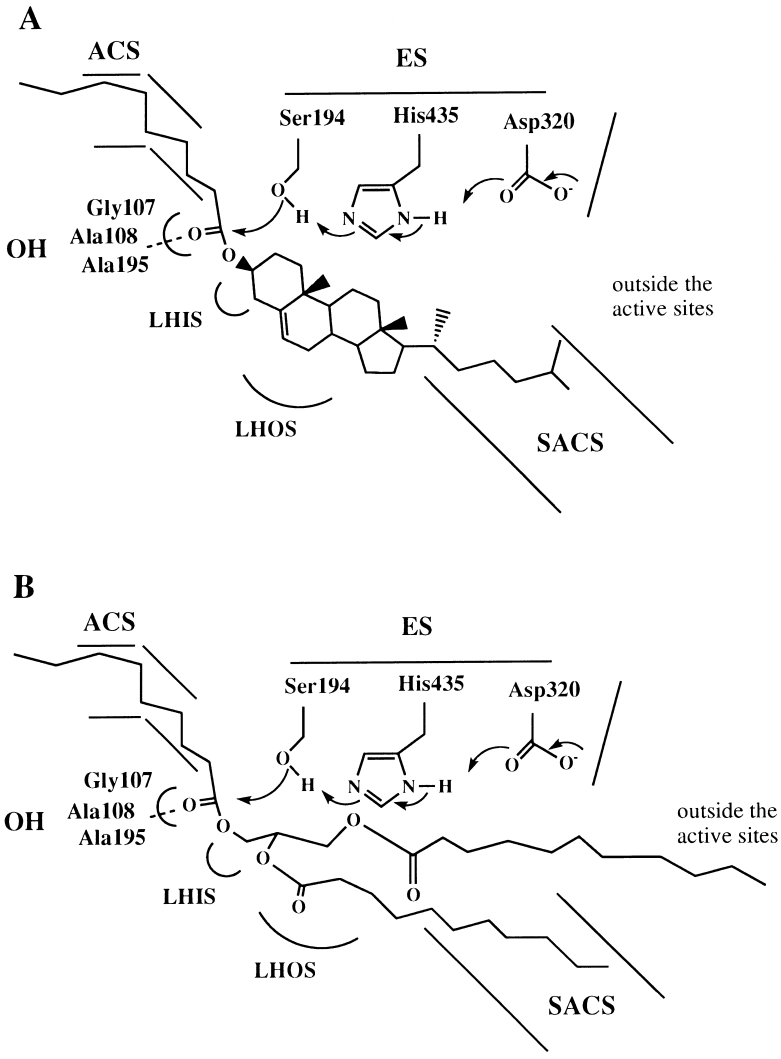
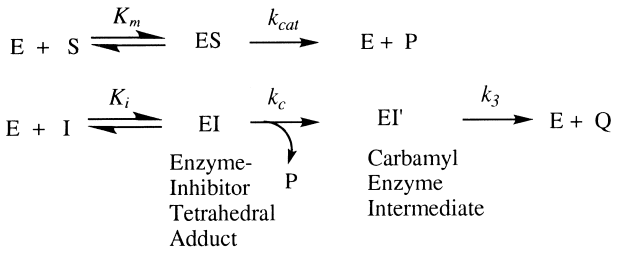


Figure 1. (A) Interactions between cholesteryl ester and the active sites of CEase. (B) Interactions between triacylglycerol and the active sites of CEase.



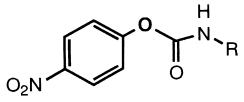
Scheme 1. Kinetic scheme for pseudo-substrate inhibition of CEase in the presence of substrate.

Quantitative structure–activity relationships (QSARs) for the serine protease- and acetylcholinesterase-catalyzed hydrolyses have been reported.[35–37] QSARs for the inhibitions of CEase by aryl carbamates have been reported by us[23,24,28] and Quinn et al.[21,22] There exist the Hammett type of QSAR (eqn (2))[38–41] for the inhibition of CEase by substituted phenyl-*N*-butyl carbamates[21–23] and the Taft–Ingold type of QSAR (eqn (3))[38–41] for the inhibition of CEase by 4-nitrophenyl-*N*-alkyl carbamates.[24] In eqn (2), values of k_0, ρ^*, and σ^* are the k value for the non-substituted phenyl parent compound, the reaction constant (the intensity factor of the inductive effect or the slope for the log$k$ versus σ plot), and the Hammett substituent constant, respectively. In eqn (3), values of k_0, ρ^*, and σ^* are the k value for the non-substituted methyl parent compound, the reaction constant (the slope for the log$k$ versus σ^* plot), and the Taft substituent constant, respectively. In this paper, we further draw attention to QSARs for the pre-steady-state inhibitions of CEase by carbamates **1**–**6** (Fig. 2).

$$\log k = \log k_0 + \rho\sigma \quad (2)$$

$$\log k = \log k_0 + \rho^*\sigma^* \quad (3)$$

According to the results of the pre-steady-state kinetic data, more detailed reaction mechanism for the inhibition of CEase by carbamates **1**–**6** may be proposed.



R=

1 $CH_3CH_2CH_2CH_2$

2 $CH_3CH_2CH_2CH_2CH_2CH_2$

3 $CH_3(CH_2)_6CH_2$

4 $ClCH_2CH_2$

5 $CH_2C_6H_5$

6 CH_2CHCH_2

Figure 2. Structures of carbamates **1**–**6**.

Results

The K_i values for the inhibitions of CEase by carbamates **1**–**6** are obtained from non-linear least squares curve fitting of eqn (1) (Table 1).[21–28] The results of QSARs for the steady-state inhibition of CEase by carbamates **1**–**6** (Table 2) indicate that values of $-\log K_i$ are only correlated with σ^* and the ρ^* value for the correlation is −0.50, which is close to the values obtained previously.[24] Since the negative ρ^* value (should form a positive intermediate) for the $-\log K_i$-σ^* -correlation and the negative enzyme–inhibitor tetrahedral adduct are contradictory to each other, the K_i step is further divided into two steps, the formation of the enzyme–inhibitor complex (E.I) with the dissociation constant, K_S $(= k_1/k_{-1})$, and then the formation of EI from E.I (Scheme 2), where k_1, k_{-1}, k_2, and k_{-2} are the rate constants for the formation and redissociation of E.I, the formation and redissociation of EI from E.I.[35,36,42–46] Values of K_S, k_2, and k_{-2} are obtained from the non-linear least squares curve fitting of eqn (4) (Table 1).

Table 2. Correlation results for the pre-steady-state inhibitions of CEase by carbamates **1**–**6**

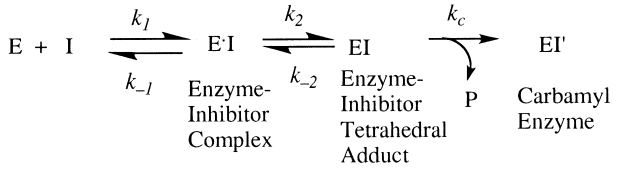
	$-\log K_i$	$-\log K_s$	$\log k_2$	$-\log k_{-2}$
ρ^*[a]	−0.5±0.1	−0.33±0.02	0.9±0.1	−0.83±0.09
h[b]	5.45±0.02	4.58±0.01	−2.97±0.03	3.81±0.02
R[c]	0.920	0.993	0.966	0.976

[a]Correlations of $-\log K_i$, $-\log K_S$, $\log k_2$, and $-\log k_{-2}$ with σ^* (eqn (3)) for carbamates **1**–**6.**
[b]The $-\log k_i$, $-\log K_S$, $\log k_2$, and $-\log k_2$ values for the inhibition of CEase by the parent compound, 4-nitrophenyl-*N*-methyl carbamate (the $\log k_0$ values of eqn (3)).
[c]Correlation coefficient.

Table 1. Pre-steady-state kinetic data for the inhibitions of CEase by carbamates **1**–**6**[a]

Inhibitor	R =	σ^*	E_s	K_i (μM)[b]	K_S (μM)[c]	k_2 (10^{-3} s^{-1})[c]	k_{-2} (10^{-4} s^{-1})[d]
1	*n*-Bu	−0.13	−0.39	2.6±0.2	24±2	0.82±0.06	1.2±0.1
2	*n*-Hex	−0.15	−0.40	3.2±0.4	23±3	0.82±0.07	1.12±0.05
3	*n*-Oct	−0.13	−0.33	3.6±0.4	23±2	0.73±0.04	1.1±0.1
4	C_2H_4Cl	0.39	−0.43	5.8±0.7	35±3	2.2±0.2	2.9±0.1
5	CH_2Ph	0.22	−0.38	4.4±0.5	31±3	2.2±0.2	2.7±0.1
6	Allyl	0.1	−0.39	3.8±0.5	27±5	1.3±0.1	1.80±0.06

[a]Carbamates **1**–**6** are the ACS–ES–OH-directed pseudo-substrate inhibitors.
[b]Calculated from eqn (1).
[c]Obtained from non-linear least squares curve fitting of eqn (4).
[d]Calculated from eqn (5).



Scheme 2. Pre-steady-state kinetic scheme for pseudo-substrate inhibition of CEase.

$$k_{obs} = k_{-2} + \frac{k_2[I]}{K_s + [I]} \quad (4)$$

In eqn (4), k_{obs} and K_s are the first-order rate constant and the dissociation constant of E.I for the pre-steady-state inhibition of CEase, respectively. The k_{-2} values can also be obtained from eqn (5).

$$k_{-2} = \frac{k_2 K_i}{K_s} \quad (5)$$

The pre-steady-state kinetic data for the inhibition of CEase by carbamates **1**–**6** are summarized in Table 1. In principle, the k_{-2} values can be obtained from the non-linear least squares curve fitting of eqn (4). However, the k_{-2} values obtained by this method vary too much. Therefore, the k_{-2} values (Table 1) are calculated from eqn (5).

QSARs for these pre-steady-state kinetic data are summarized (Table 2). All these pre-steady-state kinetic data are correlated with σ^* (Fig. 3). Therefore, the pre-steady-state inhibition of CEase by carbamates **1**–**6** shows the Taft–Ingold type of QSAR without any steric effect. Due to the negative ρ^* value (−0.33) for the K_S step, E.I is more positive than E and I. EI is more negative than E.I because of the positive ρ^* value (0.1) for the $\log k_2/k_{-2}$ $-\sigma^*$-correlation. Therefore, the two-step formation mechanism for EI is confirmed by QSARs for the pre-steady-state inhibitions of CEase (Table 2).

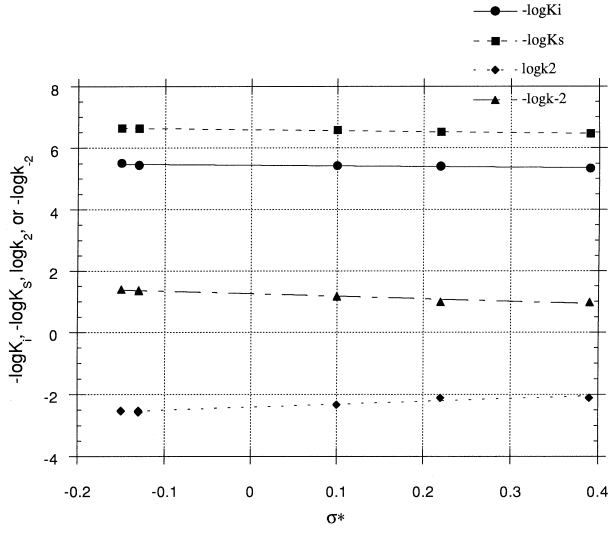


Figure 3. Plot of $-\log k_i$, $-\log k_S$, $\log k_2$, and $-\log k_{-2}$ for the pre-steady-state inhibition of CEase by carbamates **1**–**6** against σ^*. The correlation results are summarized in Table 2.

Discussion

Like all aryl-*N*-alkyl carbamates,[21–28] carbamates **1**–**6** are also characterized as the pseudo-substrate inhibitors (Scheme 1) of CEase because these inhibitors meet three criteria proposed by Abeles and Maycock.[47] Both enzymes can be protected from inhibition by carbamates **1**–**6** in the presence of a competitive inhibitor, trifluoroacetophenone.[15] Therefore, carbamates **1**–**6** are characterized as the ACS–ES–OH-directed inhibitors of CEase.[26] Moreover, the mechanism for the steady-state inhibition of CEase by carbamates **1**–**6** is common.

Multiple linear regression analyses of kinetic data in Table 1 by the Taft–Ingold–Hansch–Järv type of QSAR (eqn (6))[37,48,49] do not improve the correlation (data not shown). In eqn (6), values of δ, E_s, Ψ, and π are the intensity factor of the steric effect, the Taft substituent steric constant, the intensity factors of the hydrophobicity, and Hansch hydrophobicity constant, respectively. The polar (electronic) effect of the substituent plays a major role in these QSARs; however, the steric effect and the hydrophobicity of the substituent do not. The results of QSARs for the pre-steady-state inhibitions of CEase (Table 2) indicate all these pre-steady-state kinetic data are only correlated with σ* (Fig. 3) and the pre-steady-state inhibition of CEase by carbamates **1**–**6** shares a common mechanism.

$$\log k = \log k_0 + \rho^*\sigma^* + \delta E_s + \psi\pi \quad (6)$$

The formation of EI (K_i step) is further divided into two steps, the formation of E.I and the formation of EI from E.I (Scheme 2). The mechanism for the two-step formation of EI is proposed in Figure 4. The first step of the two-step mechanism for the formation of EI (K_S step) is the formation of E.I and the second step of the two-step mechanism (k_2/k_{-2} step) is the formation of EI from E.I. Due to the negative ρ^* value (−0.33) (Table 2) for the K_S step, E.I is more positive than E and I. Therefore, the partially positive charge at nitrogen of the inhibitors is developed probably due to the hydrogen bonding between the lone pair of nitrogen of the inhibitors and the amide hydrogen of OH of CEase (Fig. 4). The second step of the formation of EI is the k_2/k_{-2} step (Scheme 2 and Figure 4). EI is more negative than E.I because of the positive ρ^* value (0.1) (Table 2) for the $\log k_2/k_{-2}$ $-\sigma^*$-correlation. Therefore, the k_2/k_{-2} step is the formation of the relatively negative EI via the nucleophilic attack of Ser194 of CEase to the carbonyl function of the inhibitor (Fig. 4).

According to eqn (5), the ρ^* value for $-\log K_i - \sigma^*$-correlation should be the sum of those for $-\log K_S - \sigma^*$, $\log k_2 - \sigma^*$, and $-\log k_{-2} - \sigma^*$-correlations. The observed ρ^* value for the $-\log K_i - \sigma^*$-correlation (0.5±0.1) is less than the sum of ρ^*s for the $-\log K_S - \sigma^*$, $\log k_2 - \sigma^*$, and $-\log k_{-2} - \sigma^*$-correlations (−0.2±0.1) (Table 2). The former ρ^* value is more reliable than the latter ρ^* value due to the fact that the former ρ^* value is confirmed by the previous study.[24] Therefore, the ρ^*

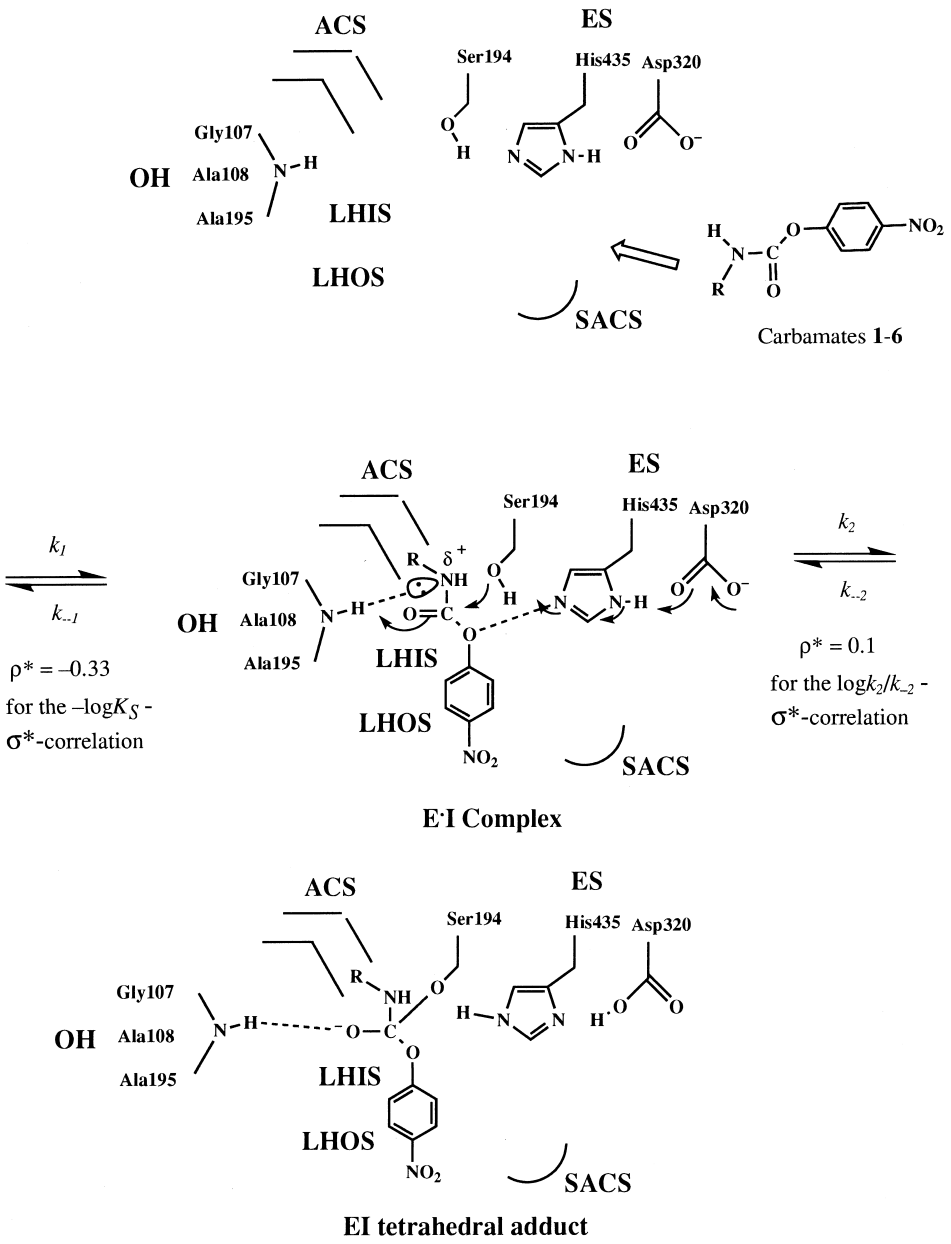


Figure 4. The proposed mechanism for inhibitions of CEase by carbamates **1**–**6**. The first step is the formation of the E.I complex via the hydrogen bonding between the lone pair of nitrogen of the inhibitors and the amide hydrogen of OH, which results in the development of the partially positive charge at nitrogen of the inhibitor in the complex. The second step is the formation of the EI tetrahedral adduct from the E.I complex via the nucleophilic attack of Ser194 to the carbonyl group of the inhibitor, which results in the development of the negative charge on the carbonyl oxygen.

value observed for the $-\log K_S-\sigma^*$-correlation (-0.33 ± 0.02) is greater than the true value since the ρ^* value for the $\log k_2/k_{-2}-\sigma^*$-correlation is positive (0.1 ± 0.1) and the ρ^* value is -0.5 ± 0.1 for the $-\log K_i-\sigma^*$-correlation. Thus, the true ρ^* value for the $-\log K_S-\sigma^*$-correlation should be about -0.6. The inaccuracy for the observed ρ^* value for the $-\log K_S-\sigma^*$-correlation (-0.33 ± 0.02) may be due to experimental errors from the stopped-flow apparatus such as the air peak.

Overall, QSARs for the pre-steady-state inhibition of CEase by carbamates **1**–**6** allow us to understand more the mechanism of CEase catalysis than before.

Experimental

Materials. All chemicals were of the highest grade available. CEase from porcine pancreas, *Pseudomonas* species lipase (PSL), and *p*-nitrophenylbutyrate (PNPB) were obtained from Sigma; other chemicals were obtained from Aldrich; silica gel used in liquid chromatography (Licorpre Silica 60, 200-400 mesh) and thin-layer chromatography plates (60 F254) was obtained from Merck; other chemicals and biochemicals were of the highest quality available commercially. Carbamates **1**–**6** were synthesized according to the procedures reported previously.[24,50]

Instrumental methods. Steady-state kinetic data were obtained from a UV–visible spectrophotometer (HP 8452 or Beckman DU-650) with a cell holder circulated with a water bath. Pre-steady-state kinetic data were obtained from the UV–visible spectrophotometer equipped with a stopped-flow apparatus (HI-TECH SFA-20).

Data reduction. Kaleida Graph™ (version 2.0) and Origin (version 4.0) were used for both linear and non-linear least squares curve fittings. Stat Work™ and Origin were used for multiple linear least squares regression analyses.

Steady-state enzyme kinetics. The K_i values (Table 1) were obtained from non-linear least squares curve fitting of k_{app} and inhibitor concentration to eqn (1) according to Hosie's method.[21–28]

Pre-steady-state enzyme kinetics. The CEase-catalyzed hydrolysis of PNPB was followed continuously at 410 nm in the presence and absence of inhibitor on the UV–visible spectrophotometer that was equipped with a stopped-flow apparatus. One syringe of the apparatus contained the enzyme (5 μg of CEase) in 0.1 M phosphate buffer solution (1 mL, pH 7.0, 0.1 M NaCl) and the other syringe contained PNPB (20 μM) in the same buffer solution (1 mL) and varying concentration of carbamates **1**–**6** (from 10^{-8} to 10^{-3} M). The reaction temperature was kept at 25.0±0.1 °C. All reaction and cycle times were 60 s and 0.1 s, respectively. First-order rate constants (k_{obs}s) for inhibition of CEase by carbamates **1**–**6** were determined. Values of K_S and k_2 were obtained by fitting the data of k_{obs} and [I] to eqn (4) by non-linear least squares regression analyses. The k_{-2} values were obtained from eqn (5). Duplicate or triplicate sets of data were collected for each inhibitor concentration.

Acknowledgements

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