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Quantitative Structure-Activity Relationships for the Pre-Steady State of *Pseudomonas Species* Lipase Inhibitions by *p*-Nirophenyl-*N*-Substituted Carbamates

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The pre-steady states of *Pseudomonas species* lipase inhibitions by *p*-nitrophenyl-*N*-substituted carbamates **(1–6)** are composed of two steps: (1) formation of the non-covalent enzyme–inhibitor complex (E:I) from the inhibitor and the enzyme and (2) formation of the tetrahedral enzyme–inhibitor adduct (E–I) from the E:I complex. From a stopped-flow apparatus, the dissociation constant for the E:I complex, K_S, and the rate constant for formation of the tetrahedral E–I adduct from the E:I complex, k_2 are obtained from the non-linear least-squares of curve fittings of first-order rate constant (k_{obs}) versus inhibition concentration ([I]) plot against $k_{obs}=k_2+k_2[I]/(K_S+[I])$. Values of p$K_S$, and log k_2 are linearly correlated with the σ^* values with the ρ^* values of −2.0 and 0.36, respectively. Therefore, the E:I complexes are more positive charges than the inhibitors due to the ρ^* value of −2.0. The tetrahedral E–I adducts on the other hand are more negative charges than the E:I complexes due to the ρ^* value of 0.36. Formation of the E:I complex from the inhibitor and the enzyme are further divided into two steps: (1) the pre-equilibrium protonation of the inhibitor and (2) formation of the E:I complex from the protonated inhibitor and the enzyme.

KEY WORDS: Carbamate inhibitors; QSAR; *Pseudomonas species* lipase; pre-steady state kinetics.

1. INTRODUCTION

Lipases (EC 3.1.1.3) are lipolytic enzymes and catalyze hydrolysis of the ester bonds of triacylglycerols (Svendsen, 1994). Moreover, lipases may also catalyze the hydrolysis of a wide range of ester substrates. Because of the broad substrate specificity and high substrate enantioselectivity, lipases have found widespread applications in enantioselective synthesis of organic compounds and resolution of racemates (Boland *et al*., 1991; Theil, 1995).

All lipases contain the α/β hydrolase fold, a structural motif common to a wide variety of hydrolases (Ollis *et al*., 1992). From X-ray crystal structures of various lipases (Grochulski *et al*., 1994; Kim *et al*., 1997; Lang *et al*., 1998; Schrag *et al*., 1997; van Tilbeurgh *et al*., 1993), their active

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Abbreviations: ACS, the first acyl chain binding site; CEase, cholesterol esterase; CRL, *Candida rugosa* lipase; CS, the catalytic side; δ, the intensity factor of a reaction to the substituent steric effects; E, enzyme; E_s, Taft steric substituent constant; E–I, tetrahedral enzyme-inhibitor adduct; E:I, non-covalent enzyme-inhibitor complex in the pre-steady state; E–I', carbamyl enzyme; I, inhibitor; k_2, rate constant for formation of E–I from E:I in the pre-steady state; k_{-2}, rate constant for re-dissociation of E–I to E:I in the pre-steady state; k_c, carbamylation constant or rate constant for the formation of E–I' from E–I; k_d, decarbamylation or rate constant for hydrolysis of E–I' to product; K_i, inhibition constant or dissociation constant of the steady state; k_i, bimolecular inhibition constant ($=k_c/k_i$); K_s, dissociation constant of E:I in the pre-steady state ($=k_1/k_{-1}$); OAH, oxyanion hole; PCL, *Pseudomonas cepacia* lipase; PSL, *Pseudomonas species* lipase; PNPB, *p*-nitrophenylbutyrate; QSAR, quantitative structure-activity relationship; R, correlation coefficient; ρ^*, intensity factor of a reaction to the substituent electronic effects; SACS, the second acyl chain binding site; σ, Hammett substituent constant; σ^*, Taft inductive substituent constant; TACS, the third acyl chain binding site.

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sites contain a catalytic triad, Ser-His-Asp/Glu, similar to those serine proteases, acetylcholinesterase (AChE) (Bar-On *et al.*, 2002; Bartolucci *et al.*, 1999; Harel *et al.*, 1996; Sussman *et al.*, 1991), and cholesterol esterase (CEase) (Chen *et al.*, 1998; Wang *et al.*, 1997). 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 from the nucleophilic attack of the active site serine hydroxyl group to the substrate carbonyl group, AChE, serine proteases, CEase, and lipases may well be expected to be inhibited by the same classes of mechanism-based inhibitor. To date, this has been demonstrated for carbamates (Bar-On *et al.*, 2002; Bartolucci *et al.*, 1999; Cavalier *et al.*, 2000; Feaster et al., 1996; Fourneron *et al.*, 1991; Hosie *et al.*, 1987; Lin and Chouhwang, 2001; Lin and Lai, 1995, 1996; Lin *et al.*, 1999a, b, c, 2000a, b, 2003a, b, 2004a, b, c; Simons *et al.*, 1997), chiral phosphonates (Lang *et al.*, 1998), hexadecylsulfonyl fluoride (Grochulski *et al.*, 1994), fluoroketones (Allen and Abeles, 1989; Brady *et al.*, 1989; Harel *et al.*, 1996; Imperiali and Abeles, 1986; Kokotos *et al.*, 2003; Sohl *et al.*, 1988), and bornic acid (Koehler and Hess, 1974; Nakatani *et al.*, 1975a; Seufer-Wasserthal *et al.*, 1994; Simpelkamp and Jones, 1992; Steiner *et al.*, 1994).

Although different activation mechanisms are proposed, the active sites of *Pseudomonas cepacia* lipase (PCL) (Kim *et al.*, 1997; Lang *et al.*, 1998; Schrag *et al.*, 1997), *Candida rugosa* lipase (CRL) (Grochulski *et al.*, 1994), AChE (Bar-On *et al.*, 2002; Bartolucci *et al.*, 1999; Harel *et al.*, 1996; Sussman *et al.*, 1991), and CEase (Chen *et al.*, 1998; Wang *et al.*, 1997) are similar to each other. The active site of PCL consists of five major binding sites (Lang *et al.*, 1998; Kim *et al.*, 1997; Schrag *et al.*, 1997) (Fig. 1): (a) the acyl binding site (ACS), a hydrophobic groove of 0.8×1.0 nm in width, comprised of Pro113, Leu17, Phe119, Leu164, Leu167, Va1266, and Va1267, which binds to the first (*sn*−1 or *sn*−3) acyl chain of triacylglycerol via van der Waals' interactions, (b) the oxyanion hole (OAH), the hydrogen bonding peptide NH function of Leu17 and Gln88, which stabilizes the incipient carbonyl C–O$^-$ of the ester function during turn over, (c) the catalytic site (CS), Ser87–His286–Asp264, comprised of the active site Ser which wound attack the carbonyl carbon of the substrate, (d) the second acyl chain binding site (SACS), Leu287, Leu293, and Thr18, located in a crevice above ES, which may bind to the second (*sn*–2) acyl chain of triacylglycerol via van der Waals' interactions, and (e) the third acyl chain binding site (TACS), Ala247 and Thr251, located at the opposite direction of ACS, which may bind to the third (*sn*–3 or *sn*–1) acyl chain of triacylglycerol via a small number of van der Waals' interactions.

The lipase-catalyzed hydrolysis of lipid substrates via a serine protease mechanism has been proposed (Fourneron *et al.*, 1991; Lin and Chouhwang, 2001; Lin *et al.*, 1999a, 2003a; Simons *et al.*, 1997). In the presence of substrate, the pseudo-substrate of *Pseudomonas species* lipase (PSL) inhibition mechanism by aryl carbamates has been proposed (Scheme 1).

Because the PSL inhibition follows first-order kinetics over the observed time period for the steady-state kinetics, the hydrolysis rate of E–I' (decarbamylation constant, k_d) must be significantly slower than the formation rate of EI' (carbamylation constant, K_c) (Simons *et al.*, 1997). The inhibition constant (K_i) and k_c for the steady-state of the PSL inhibitions by carbamates are obtained from the non-linear least-squares of curve fittings of the first-order rate constant (k_{app}) versus concentration of the inhibitor ([I]) plot against equation 1 (Feaster *et al.*, 1996; Hosie *et al.*, 1987):

$$k_{app} = k_c[\mathrm{I}]/K_i(1 + [\mathrm{S}]/K_m) + [\mathrm{I}] \qquad (1)$$

In Eq. (1), k_{app} can be obtained by Hosie's method (Feaster *et al.*, 1996; Hosie *et al.*, 1987). Bimolecular inhibition constant ($k_i = k_c/K_i$) is related to overall inhibitory potency.

The pre-steady state of an enzyme inhibition mechanism has been proposed (Scheme 2), where k_1,k_{-1},k_2 and k_{-2} are the rate constants for formation of the enzyme–inhibitor complex (E:I), for re-dissociation of E:I, for formation of E–I, and re-dissociation of E–I, respectively (Fersht, 1984; Hart, 1974; Ikeda *et al.*, 1982; Nakatani *et al.*, 1975a, b, 1977, 1978). Moreover, values of $K_S(=k_{-1}/k_1)$, k_2 and k_{-2} are obtained from the non-linear least squares of curve fittings of the first-order rate constant (k_{obs}) versus [I] plot against Eq. (2).

$$k_{obs} = k_{-2} + k_2[\mathrm{I}]/(K_S + [\mathrm{I}]) \qquad (2)$$

In Eq. (2), k_{obs} and K_S are the first-order rate constant and dissociation constant of the E:I complex in the pre-steady state, respectively. Therefore,

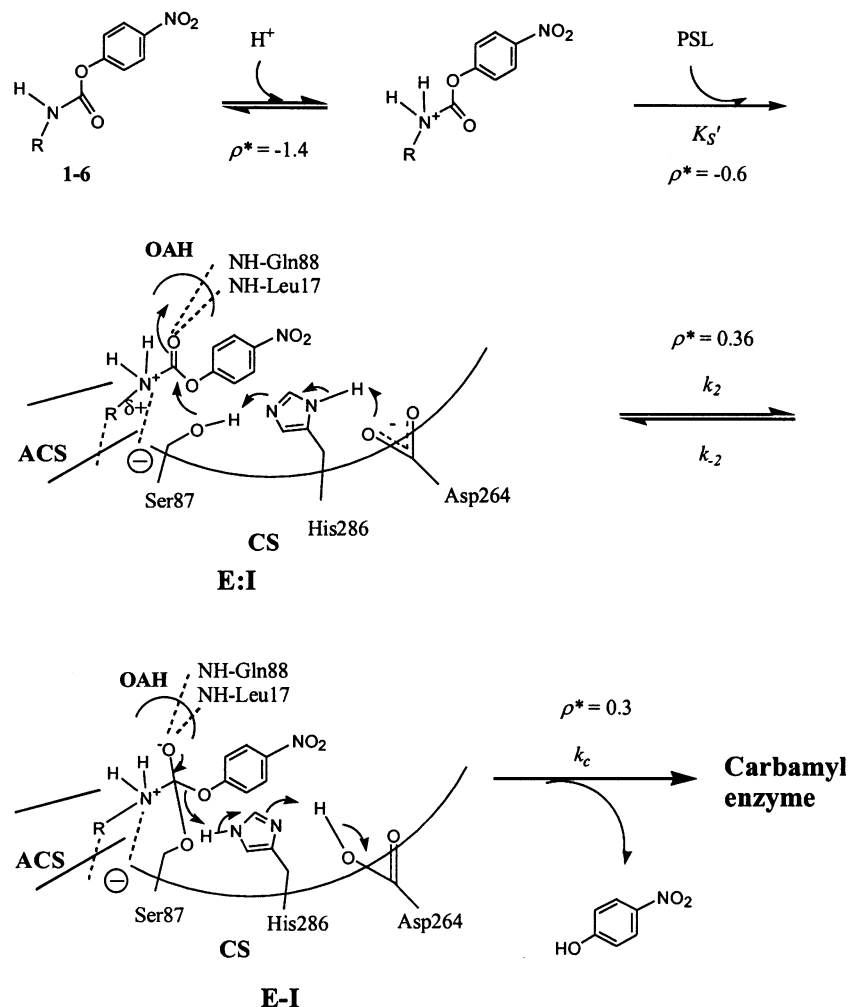
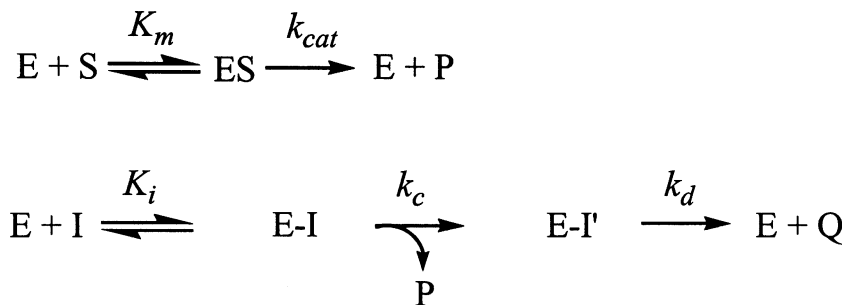


Fig. 1. The proposed mechanism for the PSL inhibitions by carbamates **1–6**. The first step is the pre-equilibrium protonation of the inhibitor. The conformation for both inhibitor and protonated inhibitor is pseudo-*trans* one. The second step is formation of the non-covalent enzyme-inhibitor complex (E:I). The conformation for the inhibitor in this complex is pseudo-*cis* (Lin *et al*., 2003a). The third step is formation of the tetrahedral enzyme–inhibitor adduct (E–I) from the E:I complex. This step involves a nucleophilic attack of the active site Ser87 to the carbamate carbonyl group of the inhibitor. The fourth step is formation of the carbamyl enzyme from the tetrahedral E–I adduct.



Scheme 1. Kinetic scheme for the steady state of pseudo-substrate inhibition of PSL in the presence of substrate[a]. [a]E, enzyme; S, substrate; I, carbamate inhibitor; E–I, tetrahedral enzyme–inhibitor adduct; E–I', carbamyl enzyme; k_c carbamylation constant or formation rate constant of E–I'; k_d, decarbamylation or hydrolysis rate constant of E–I'; K_i, inhibition or dissociation constant.

Scheme 2. Kinetic scheme for the pre-steady state of pseudo-substrate inhibition of PSL[a]. [a]E:I, non-covalent enzyme-inhibitor complex; k_2, rate constant for formation of the E–I adduct from the E:I complex; k_{-2}, rate constant for re-dissociation of the E–I adduct to the E:I complex; k_1, rate constant for formation of the E:I complex; k_{-1}, rate constant for re-dissociation of the E:I complex.

the inhibition constant, K_i equals to $K_S\ k_{-2}/k_2$ (Eq. (3)).

$$1/K_i = (1/K_S)(k_{-2}/k_2) \quad (3)$$

Quantitative structure-activity relationships (QSARs) for the steady states of CEase (Feaster *et al.*, 1996; Lin and Lai, 1995, 1996; Lin *et al.*, 1999a, 2000b, 2004b), AChE (Lin, 2004; Lin *et al.*, 2000b, 2004a), and PSL (Lin and Chouhwang, 2001; Lin *et al.*, 1999a, 2003a; Simons *et al.*, 1997) inhibitions by carbamates have been reported. QSARs for the pre-steady states of CEase (Lin *et al.*, 2000a) and AChE (Lin *et al.*, 2004c) inhibitions by carbamates have been also reported. In this paper, we further report QSARs for the pre-steady state of the PSL inhibitions by carbamates **1–6**. Furthermore, the mechanism for pre-steady state of PSL inhibition by the carbamate inhibitor is proposed.

2. MATERIAL AND METHODS

2.1. Materials

PSL and *p*-nitrophenyl butyrate (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) were obtained from Merck; other chemicals were of the highest quality available commercially. *p*-Nitrophenyl-*N*-substituted carbamates **(1–6)** were prepared from condensation of *p*-nitrophenol and the corresponding isocyanate (Lin and Chouhwang, 2001; Lin *et al.*, 2000b).

2.2. Instrumental Methods

^{1}H and ^{13}C NMR spectra were recorded at 300 and 75.4 MHz, respectively, on a Varian-XL 300 spectrometer. The ^{1}H and ^{13}C chemical shifts were referred to internal tetramethylsilane. Pre-steady state kinetic data were obtained from an UV-visible spectrometer (HP 8452A, Beckman DU-650, or Agilent 8453) equipped with a stopped flow apparatus (Hi-Tech SFA-20 or Applied Photo Physics RX-2000). The p*Ka* values were obtained from the pH-stat-titration (Radiometer PHM 290).

2.3. Data Reduction

Origin (version 6.0) was used for linear and nonlinear least squares of curve fittings.

2.4. Steady State of Enzyme Kinetics

The pseudo-substrate inhibitions of PSL by carbamates **1–6** were reported before (Lin and Chouhwang, 2001). The temperature was maintained at 25.0°C by a refrigerated circulating water bath. All reactions were performed in sodium phosphate buffer (1 ml, 0.1 *M*, pH 7.0) containing NaCl (0.1 *M*), acetonitrile (2% by volume), triton X-100 (0.5% by weight), substrate (0.2 m*M* of PNPB), and varying concentration (from 10^{-8} to 10^{-3} *M*) of inhibitors. Requisite volumes of stock solution of substrate and inhibitors in acetonitrile were injected into reaction buffer via a pipet. Porcine pancreatic PSL was dissolved in sodium phosphate buffer (0.1 *M*, pH 7.0). Reactions were initiated by injecting enzyme {10 μg 0.5 unit (μmole/min) of PSL} and monitored at 410 nm on the UV-visible spectrometer. First-order rate constants (k_{app}) were determined by non-linear least squares of curve fittings on the absorbances versus time plot (Feaster *et al.*, 1996; Hosie *et al.*, 1987). Values of K_i were obtained from non-linear least squares of curve fittings on k_{app} versus [I] plot against Eq. (1) (Hosie *et al.*, 1987). Duplicate or triplicate sets of data were collected for each inhibitor concentration.

2.5. Pre-steady State of Enzyme Kinetics

The PSL-catalyzed hydrolysis of PNPB was followed continuously at 410 nm in presence and absence of an inhibitor on an UV-visible spectrometer equipped with a stopped-flow apparatus. One syringe of the apparatus contained the enzyme (1 μg) in the buffer solution (1 ml, 0.1 *M* NaCl,

0.1 *M* phosphate buffer, pH 7.0) and the other syringe contained PNPB (200 μ*M*) in the buffer solution (1 ml) and varying concentration of carbamates **1–6** (from 10^{-8} to 10^{-10}). The reaction temperature was kept at 25.0°C. All reastion and cycle times were 60 s and 0.1 s, respectively.

First-order rate constants (k_{obs}) for inhibition of CEase by carbamates **1–6** were determined. The K_S, and k_2 values were obtained by non-linear least squares of curve fittings of k_{obs} versus [I] plot against Eq. (2), The k_{-2} values were calculated from Eq. (3). Duplicate or triplicate sets of data were collected for each inhibitor concentration.

3. RESULTS

3.1. Steady State of Inhibition

Carbamates **1–6** are characterized as the pseudo-substrate inhibitors of PSL and the K_i values were obtained from non-linear least squares of curve fitting of Eq. (1) as described before (Lin and Chouhwang, 2001). Further, the linear correlation between pK_i values and σ^* was observed (Eq. 4) (Connors, 1990; Isaacs, 1995; Lowry and Richardson, 1987) and the slope (ρ^*) for this correlation was 1.7 ± 0.2 (Lin and Chouhwang, 2001).

$$\mathrm{p}K_i = \rho^* \sigma^* + \mathrm{h} \qquad (4)$$

3.2. Pre Steady State of Inhibition

The K_S and k_2 values (Table 1) were obtained from the non-linear least squares of curve fittings of the k_{obs} (or 1/T) values versus [I] plot against Eq. (2) (Lin *et al.*, 2000a, 2004c). The K_S values for *p*-nitrophenyl-*N*-*n*-alkylcarbamates (**1–3**) are much lower than those for carbamates **3–6** (Table 1). Similarly to Eq. (4), the linear correlation between pK_S and σ^* was also observed and the ρ^* value for this correlation was -2.0 ± 0.1 (Table 2). The k_2 values were about the same; moreover, the k_2 steps were the rate determining steps for the pre-steady states of formations of the tetrahedral E–I adducts (Table 1). The log k_2 values were linearly correlated with σ^* and the ρ^* value for this correlation was 0.36 ± 0.03 (Table 2). The k_{-2} values were also about the same but much less (< 1/20) than the k_2 values (Table 1). The log k_{-2} values were not correlated with σ^* at all (Table 2). The log (k_2/k_{-2}) values were fairly correlated with σ^* and the ρ^* value for this correlation was 0.3 ± 0.2 (Table 2).

4. DISCUSSION

4.1. Steady State of Inhibition

Carbamates **1–6** are characterized as the pseudo-substrate inhibitors of PSL (Lin and Chouhwang, 2001). The ρ^* value of -1.7 ± 0.2 for the pK_i–σ^* correlation (Table 2) indicates that the tetrahedral E–I adducts (Scheme 1) are more positive charges than reactions (CEase and inhibitor). However, the tetrahedral E–I adducts (Fig. 1) are more negative charges than carbamates **1–6**. Therefore, the K_i step composes at least two steps in the pre-steady state.

4.2. Pre-steady State Inhibition

4.2.1. K_S Step

The ρ^* value of -2.0 ± 0.1 for the pK_S–σ^* correlation indicates that the non-covalent E:I complexes (Scheme 2) are more positive charges than carbamates **1–6**, Carbamates **1–6** are protonated in pre-equilibrium at pH 7 (Fig. 1) with the ρ^* value of -1.4 ± 0.2 for the pK_a–σ^* correlation (Lin *et al.*, 2000b, 2003b). Therefore, formation of the E:I complexes from PSL and carbamates **1–6** is composed of two steps (Fig. 1): (1) the pre-equilibrium protonation and (2) formation of the E:I complexes from PSL and protonated carbamates **1–6** with the virtual dissociation constant of the E:I complex, K_S'. Thus, the ρ^* value for the pK_S–σ^* correlation is calculated to be -0.6 ± 0.1 (Fig. 1). The ρ^* value of -0.6 suggests that the E:I complexes are more positive charges than the protonated inhibitors (Fig. 1). Electrostatic potential calculations show that the region at Glu208 and Asp452, near the mouth of tunnel has one of the most negative potentials in CRL (Grochulski *et al.*, 1994). Therefore, the positive charges increase in the E:I complex may be due to the ionic interaction between the region of the negative charges and the protonated inhibitor (Fig. 1).

The K_S values for *p*-nitrophenyl-*N*-*n*-alkylcarbamates **(1–3)** are much lower than those for carbamates **3–6** (Table 1). In other words, carbamates **1–3** are easier to enter the ACS tunnel (Fig. 1) than carbamates **4–6**. It is reasonable that the chlorine

Table 1. Kinetic Constants for Pre-steady States of the PSL Inhibitions by Carbamates **1–6**

Inhibitor	R =	σ*	K_S(μ M)[a]	$k_2(10^{-3}s^{-1})$[a]	$k_{-2}(10^{-4}s^{-1})$[b]	K_i(μM)[c]
1	*n*-Bu	−0.13	10 ± 1	3.3 ± 0.3	1.0 ± 0.2	3.1 ± 0.5
2	*n*-Hex	−0.15	8 ± 1	3.2 ± 0.2	1.6 ± 0.2	4.1 ± 0.3
3	*n*-Oct	−0.13	11 ± 1	3.4 ± 0.3	1.0 ± 0.2	3.1 ± 0.2
4	C_2H_4C1	0.39	120 ± 10	5.0 ± 0.5	1.3 ± 0.2	30 ± 2
5	CH_2Ph	0.22	45 ± 6	4.5 ± 0.4	1.1 ± 0.2	11 ± 1
6	Allyl	0.1	35 ± 5	4.2 ± 0.4	1.1 ± 0.2	8.9 ± 0.5

[a]Obtained from non-linear least squares of curve fittings of the k_{obs} versus. [I] plot against Eq. (2).
[b]Calculated from on Eq. (3).
[c]Taken from (Lin and Chouhwang, 2001).

Table 2. Correlation Results for the PSL Inhibitions by Carbamates **1–6**

	pK_i	pK_s	$\log k_2$	$\log k_{-2}$	$\log (k_2/k_{-2})$
ρ*[a]	−1.7 ± 0.2	−2.1 ± 0.1	0.36 ± 0.03	0.0 ± 0.2	0.3 ± 0.2
h[b]	5.28 ± 0.04	3.92 ± 0.01	−2.429 ± 0.005	−2.94 ± 0.04	0.5 1 ± 0.04
R[c]	0.982	0.993	0.990	0.002	0.650
SEM[d]	0.034	0.025	0.0052	0.038	0.039

[a]Correlations of the pK_i, pK_S, $\log k_2$, $-\log k_{-2}$, and $\log (k_2/k_{-2})$ values with the σ* values of carbamates **1–6** on Eq. (4).
[b]Calculated pK_i, pK_S, $\log k_2$, $-\log k_{-2}$, and $\log (k_2/k_{-2})$ values for the PSL inhibition by 4-nitrophenyl-*N*-methyl carbamate.
[c]Correlation coefficient.
[d]Standard error of the mean, $n = 6$.

atom, phenyl group, or allyl group in carbamates **4–6** repulses the negative region of the enzyme and this repulsion make the carbamate moieties of carbamates **4–6** difficult to pass the ACS tunnel.

4.2.2. k_2 Step

The ρ^* value of 0.36 ± 0.03 for the $\log k_2–\sigma^*$ correlation (Table 2) indicates that the tetrahedral E–I adducts are more negative charges than the E:I complexes, Therefore, the k_2 step involves a nucleophilic attack of the active site serine to the carbamate carbonyl group of the inhibitor (Fig. 1) and is the rate determining steps for the pre-steady states of formations of the tetrahedral E–I adducts. The ρ^* value of the $\log k_2–\sigma^*$ correlation for PSL inhibitions by carbamates **1–6** is less than that for CEase inhibitions by carbamates **1–6** (0.9) (Lin *et al.*, 2000a). The conformation of the carbamate inhibitor changes from pseudo-*trans* to pseudo-*cis* one during the PSL-catalyzed reaction (Lin *et al.*, 2003a) (Fig. 1) but the conformation of the carbamate inhibitor retains in pseudo-*trans* one during the CEase-catalyzed reaction (Lin *et al.*, 2004a). It is possible that the pseudo-*cis* conformation of the inhibitor is less reactive to the catalytic serine than the pseudo-*trans* one.

4.2.3. k_{-2} Step

That the $\log k_{-2}$ values are not correlated with σ^* (Table 2) may due to two reasons: (1) the k_{-2} values are calculated from too many mathematic transformations and (2) the ρ^* value for the $\log k_{-2}–\sigma^*$ correlation is null (Table 2). Lack of the $\log k_{-2}–\sigma^*$ correlation also lowers the $\log (k_2/k_{-2}–\sigma^*$ correlation.

Overall, both pre-steady state and steady state of the PSL inhibition mechanisms by carbamates **1–6** are proposed (Fig. 1).

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