

## Structure-Reactivity Relationships as Probes for the Inhibition Mechanism of Cholesterol Esterase by Aryl Carbamates. I. Steady-State Kinetics<sup>a</sup>

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For substituted phenyl-*N*-butyl carbamates (**1**) and 4-nitrophenyl-*N*-substituted carbamates (**2**), linear relationships between values of NH proton chemical shift ( $\delta_{\text{NH}}$ ),  $pK_a$ , and  $\log k_{[\text{OH}]}$  and Hammett substituent constant ( $\sigma$ ) or Taft substituent constant ( $\sigma^*$ ) are observed. Carbamates **1** and **2** are pseudo-substrate inhibitors of porcine pancreatic cholesterol esterase. Thus, the mechanism of the reaction necessitates that the inhibitor molecule and the enzyme form the enzyme-inhibitor tetrahedral species at the  $K_i$  step of the reaction and then form the carbamyl enzyme at the  $k_c$  step of the reaction. Linear relationships between the logarithms of  $K_i$  and  $k_c$  for cholesterol esterase by carbamates **1** and  $\sigma$  are observed, and the reaction constants ( $\rho$ s) are -3.4 and -0.13, respectively. Therefore, the above reaction forms the negative-charge tetrahedral species and follows the formation of the relatively neutral carbamyl enzymes. For the inhibition of cholesterol esterase by carbamates **2** except 4-nitrophenyl-*N*-phenyl carbamate and 4-nitrophenyl-*N*-*t*-butyl carbamate, linear relationships of  $-\log K_i$  and  $\log k_c$  with  $\sigma^*$  are observed and the  $\rho^*$  values are -0.50 and 1.03, respectively. Since the above reaction also forms the negative-charge tetrahedral intermediate, it is possible that the  $K_i$  step of this reaction is further divided into two steps. The first  $K_i$  step is the development of the positive-charge at the carbamate nitrogen from the protonation of the carbamate nitrogen. The second  $K_i$  step is the formation of the tetrahedral intermediate with the negative-charge at the carbonyl oxygen. From Arrhenius plots of a series of inhibition reactions by carbamates **1** and **2**, the isokinetic and isoequilibrium temperatures are different from the reaction temperature (25 °C). Therefore, the observed  $\rho$  and  $\rho^*$  values only depend upon the electronic effects of the substituents. Taken together, the cholesterol esterase inhibition mechanism by carbamates **1** and **2** is proposed.

### INTRODUCTION

Recently there has been increased interest in pancreatic cholesterol esterase (CEase,<sup>b</sup> 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.<sup>3,4</sup> 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.<sup>5</sup> Physiological substrates of CEase include cholesteryl esters, retinyl esters, acylglycerols, vitamin esters, and phospholipids.<sup>6-9</sup> CEase plays a role in digestive lipid absorption in the upper intestinal tract, though its role in cholesterol absorption in particular is controversial.<sup>3,4,10</sup> A recent report indicates that

CEase is directly involved in lipoprotein metabolism, in that the enzyme catalyzes the conversion of large LDL to smaller, denser, more cholesteryl ester-rich lipoproteins, and that the enzyme may regulate serum cholesterol levels.<sup>11</sup> The enzyme may function in these roles by acting as a cholesterol transfer protein.<sup>12</sup> Serine lipases and CEase share the same catalytic machinery as serine proteases<sup>13</sup> in that they have an active site serine residue which, with a histidine and an aspartic or glutamic acid, forms a catalytic triad. 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

<sup>a</sup> Two preliminary accounts of part of the results have been published.<sup>1,2</sup>

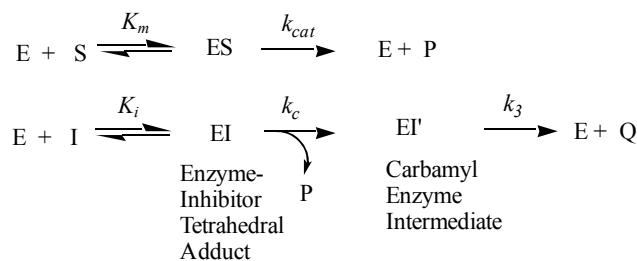
<sup>b</sup> Abbreviations: ACS, the first alkyl chain binding site;  $\beta$ , the isoequilibrium or isokinetic temperature; CEase, cholesterol esterase; CRL, *Candida rugosa* lipase;  $\delta$ , the <sup>1</sup>H NMR chemical shifts or the intensity factor of the reaction to steric factors; EA, elemental analysis; ES, the esteratic site; HRMS, high resolution mass spectra;  $k_c$ , the carbamylation constant;  $K_i$ , the inhibition constant;  $k_i$ , the bimolecular inhibition constant; LHIS, the leaving group hydrophilic binding site; LHOS, leaving group hydrophobic binding site; OH, oxyanion hole; PNPB, *p*-nitrophenylbutyrate; R, the correlation coefficient;  $\rho$  or  $\rho^*$ , the reaction constant; SACS, the second alkyl chain or group binding site;  $\sigma$ , the Hammett substituent constant;  $\sigma^*$ , the Taft substituent constant; TFA, trifluoroacetic acid; TMS, tetramethyl silane.

classes of mechanism-based inhibitors. To date, this has been demonstrated for diethyl-*p*-nitrophenol phosphate,<sup>14</sup> hexadecylsulfonyl fluoride,<sup>15</sup> fluoroketones,<sup>16,17</sup> boronic acid,<sup>18</sup> chloroisocoumarin,<sup>19</sup>  $\beta$ -lactones,<sup>20,21</sup>  $\beta$ -lactams,<sup>22</sup> and carbamates.<sup>1,2,23-26</sup> CEase has also been used in the resolution of binaphthols and spirobiindanols.<sup>27</sup>

Two different X-ray crystal structures of bovine pancreatic CEase have been reported recently.<sup>28,29</sup> Although different bile salt-activation mechanisms for CEase are proposed, the shape of the active site is similar to that of CRL. CEase, like other lipases, possesses a Ser-His-Asp active site triad that is involved in nucleophilic and general acid-base catalysis, and a neighboring oxyanion hole (OH), the H-bonding peptide NH functions of Gly 107, Ala 108, and Ala 195, that stabilizes the incipient carbonyl C=O of the ester function during turnover. Therefore, 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 the substrate, (b) an OH that stabilizes the tetrahedral intermediate, (c) an esteratic site (ES), comprised of the active site serine which would attack the *re* face of the ester carbonyl, (d) a leaving group hydrophobic binding site (LHOS) that binds to the hydrophobic part of the leaving group and is in a crevice above the catalytic site, (e) a leaving group hydrophilic binding site (LHIS) that binds to the hydrophilic part of the leaving group and is located in the opposite direction of ACS, and (f) the second alkyl chain or group binding site (SACS) that binds to the second alkyl chain or group of the lipid substrate or inhibitor.<sup>30</sup>

The CEase-catalyzed hydrolysis of lipid substrates *via* a serine protease mechanism has been proposed by Quinn.<sup>31</sup> In the presence of substrate, the mechanism of transient or pseudo-substrate inhibitions of CEase has been proposed (Scheme I and Fig. 1).<sup>1,2,23-26,30,31</sup>

**Scheme I** Kinetic scheme for the pseudo-substrate inhibition of CEase in the presence of sub-



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' ( $k_c \gg k_3$ ).<sup>32</sup> Therefore, values of  $K_i$  and  $k_c$  can be calculated from Equation 1.<sup>23,24</sup>

In Equation 1,  $k_{app}$  values are the first-order rate con-

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

stants which can be obtained according to Hosie's method.<sup>23</sup> Bimolecular rate constant,  $k_i = k_c/K_i$ , is related to overall inhibitory potency.

Structure-activity relationships in serine protease catalysis<sup>33-38</sup> and acetylcholinesterase<sup>39</sup> have been reported. For enzyme inhibition, less attention has been paid to the correlation with the structure of inhibitor.<sup>34</sup> We and Quinn's group have reported that there exist the linear free energy relationships for the inhibitions of CEase by substituted phenyl-*N*-butyl carbamates (**1**),<sup>1,24</sup> 4-nitrophenyl-*N*-substituted carbamates (**2**),<sup>2</sup> biphenyl-*N*-substituted carbamates, alkyl-*N*-phenyl carbamates, and alkyl-*N*-phenyl thiocarbamates.<sup>30</sup> In this paper, we further draw attention to the comparison of the linear free energy relationships on the inhibitions of CEase by both carbamates **1** and **2**. According to these data, the inhibition mechanisms for CEase by aryl carbamates are proposed.

## RESULTS

### Chemical properties of carbamates **1** and **2**

Carbamates **1** and **2** are prepared by condensation of

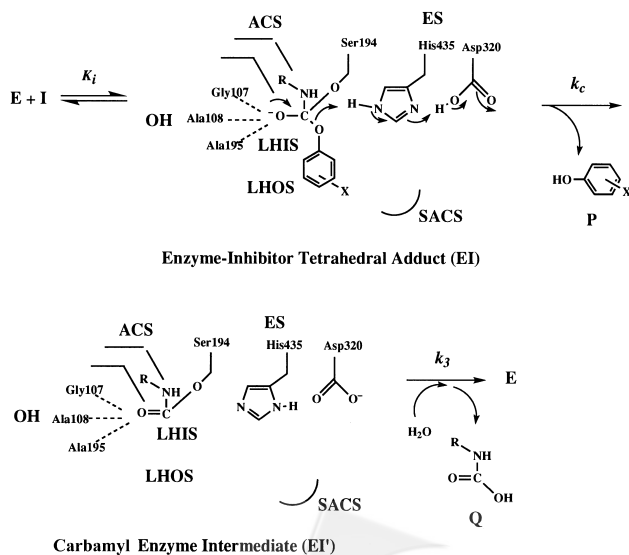


Fig. 1. The proposed mechanism for the inhibition of CEase by carbamates **1** and **2**. Numbers are referred to residue positions in CEase.<sup>28,29</sup>

Table 1. Some Linear Free Energy Relationships for Carbamates **1** and **2**

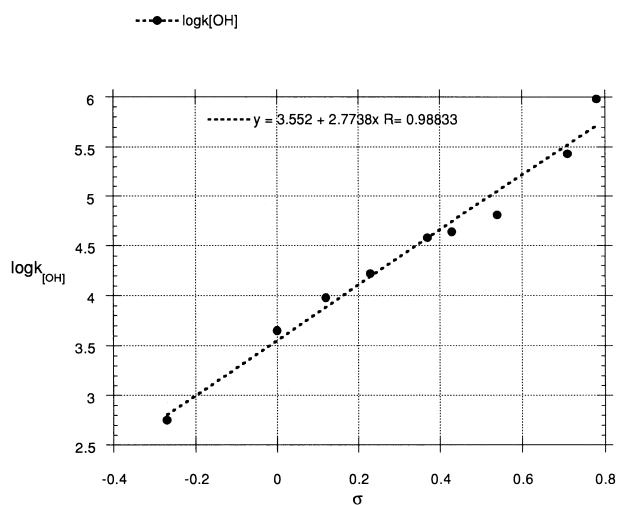
Compound	<b>1</b> <sup>a</sup>			<b>2</b> <sup>b</sup>		
	$\delta_{\text{NH}}^{\text{c}}$	$pK_a$	$\log k_{[\text{OH}]}$	$\delta_{\text{NH}}^{\text{c,d}}$	$pK_a$	$\log k_{[\text{OH}]}$
$\rho$ or $\rho^*$	$0.15 \pm 0.02$	$-2.5 \pm 0.1$	$2.77 \pm 0.16$	$0.97 \pm 0.06$	$-1.4 \pm 0.2$	$0.75 \pm 0.05$
$h$	$4.97 \pm 0.01$	$9.97 \pm 0.06$	$3.55 \pm 0.07$	$5.26 \pm 0.01$	$7.64 \pm 0.04$	$6.09 \pm 0.01$
$R$	0.94	0.99	0.99	0.99	0.96	0.99

<sup>a</sup> For carbamates **1**, the Hammett equation ( $\delta$ ,  $\log k_{[\text{OH}]}$ , or  $pK_a = h + \rho\sigma$ ) was used in correlation. <sup>b</sup> For carbamates **2**, the Taft equation without steric effects ( $\delta$ ,  $\log k_{[\text{OH}]}$ , or  $pK_a = h + \rho^*\sigma^*$ ) was used in correlation. <sup>c</sup> All <sup>1</sup>H chemical shifts ( $\delta$ /ppm) are referenced to internal TMS (300 MHz, CDCl<sub>3</sub>). <sup>d</sup> Correlation without the data from those of 4-nitrophenyl-*N*-phenylcarbamate.

substituted phenols with isocyanates in the presence of pyridine. All compounds are characterized by <sup>1</sup>H (300 MHz) NMR, <sup>13</sup>C (75.4 MHz) NMR, mass spectrum, and elemental analysis. Table 1 summarizes some linear free energy relationships for these compounds. All NH proton chemical shifts ( $\delta$ ) of carbamates **1** and **2** are correlated linearly with  $\sigma$  (or  $\sigma^*$ ) except that of 4-nitrophenyl-*N*-phenyl carbamate (**2b**). All  $pK_a$  and  $\log k_{[\text{OH}]}$  (Fig. 2) values are correlated linearly with  $\sigma$  (or  $\sigma^*$ ) without steric constants (Table 1). Multiple linear regression analyses of these data by the Taft-Ingold equation ( $\log(k/k_0) = \rho^*\sigma^* + \delta E_s$ )<sup>44-47</sup> or the Järv-Hansch equation ( $\log(k/k_0) = \rho^*\sigma^* + \delta E_s + \psi\pi$ ,<sup>39</sup> where  $\pi$  are the hydrophobicity constants<sup>48,49</sup>) do not improve the correlation (data not shown) based on the  $R$  values.

### Inhibition of CEase

Like all aryl-*N*-alkyl carbamates,<sup>23,24,26</sup> carbamates **1** and **2** are characterized as pseudo-substrate inhibitors of both CEase and meet three criteria proposed by Abeles and Maycock.<sup>43</sup> First, the inhibition is time-dependent and fol-

Fig. 2. Plot of  $\log k_{[\text{OH}]}$  of carbamates **1** against  $\sigma$ .

lows first-order kinetics (Fig. 4); second, with increasing concentration of inhibitor the enzyme displays saturation kinetics (Fig. 4); third, the enzyme can be protected from inhibition by carbamates **1** and **2** in the presence of a competitive inhibitor, TFA (see Return of activity and protection by TFA in EXPER-

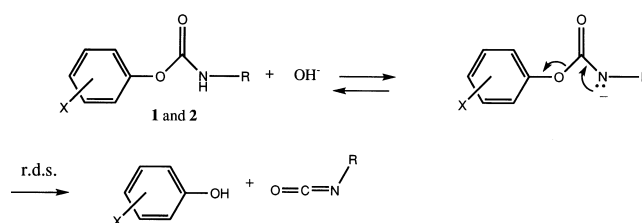
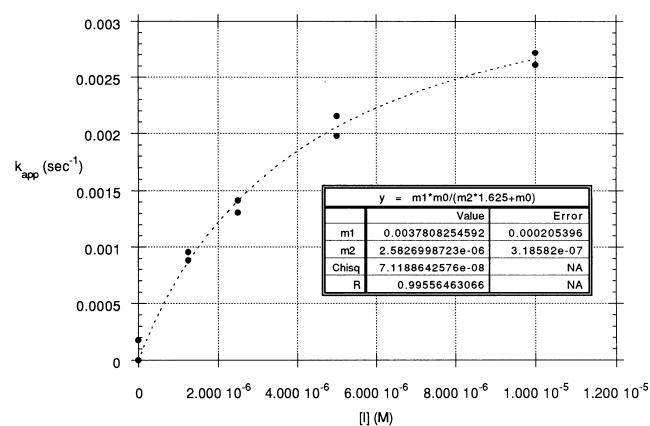
Fig. 3. The alkaline-catalyzed hydrolysis of carbamates **1** and **2** via the E1cB mechanism.

Fig. 4. Michaelis-Menten plot for inhibition of the CEase-catalyzed hydrolysis of PNPB by carbamate **1i** (Ar = 4-NO<sub>2</sub>-Ph). First-order rate constants ( $k_{app}$  values) were calculated as described by Quinn et al.<sup>23</sup> 50  $\mu\text{M}$  of substrate PNPB was used. Nonlinear least squares fitting to Equation 1 of the text gave  $K_i = 2.6 \pm 0.3 \mu\text{M}$  and  $k_c = 0.0038 \pm 0.0002 \text{ sec}^{-1}$ .

Table 2. Substituent Constants, Kinetic Data, and Correlation Results for Inhibitions of CEase by Carbamates **1**

X=	$\sigma$	$K_i(\mu\text{M})$	$k_c(10^{-3} \text{ s}^{-1})$	$k_i(\text{M}^{-1} \text{ s}^{-1})$
<i>p</i> -OMe	-0.27	$(7\pm 1)\times 10^3$	$5.4\pm 0.4$	$0.8\pm 0.1$
H	0	$740\pm 70$	$5.0\pm 0.3$	$6.7\pm 0.8$
<i>m</i> -OMe	0.12	$390\pm 30$	$4.5\pm 0.3$	$12\pm 1$
<i>p</i> -Cl	0.23	$82\pm 8$	$4.4\pm 0.2$	$54\pm 6$
<i>m</i> -Cl	0.37	$120\pm 10$	$4.3\pm 0.2$	$36\pm 3$
<i>m</i> -CF <sub>3</sub>	0.43	$35\pm 5$	$4.4\pm 0.2$	$120\pm 20$
<i>p</i> -CF <sub>3</sub>	0.54	$4.4\pm 0.3$	$4.2\pm 0.2$	$960\pm 80$
<i>m</i> -NO <sub>2</sub>	0.71	$3.6\pm 0.3$	$4.0\pm 0.2$	$1,100\pm 100$
<i>p</i> -NO <sub>2</sub>	0.78	$2.6\pm 0.3$	$3.8\pm 0.2$	$1,500\pm 100$
$\rho^a$		$-\log K_i$	$\text{Log} k_c$	$\text{Log} k_i$
		$3.4\pm 0.3$	$-0.13\pm 0.02$	$3.2\pm 0.3$
$h^a$		$3.1\pm 0.1$	$-2.32\pm 0.01$	$0.8\pm 0.1$
$R^a$		0.98	0.94	0.97

<sup>a</sup> Correlations of  $-\log K_i$ ,  $\log k_c$ , and  $\log k_i$  with  $\sigma$  the Hammett equation ( $\log k = h + \rho\sigma$ ).

IMENTAL SECTION).<sup>17</sup> Thus, the mechanism of the reaction requires that the inhibitor molecule and the enzyme form the enzyme-inhibitor tetrahedral species at the first step ( $K_i$ ) of the reaction and then form the carbamyl enzyme at the second step ( $k_c$ ) of the reaction (Fig. 1 and Scheme 1).

The inhibition data  $K_i$ ,  $k_c$ , and  $k_i$  for CEase by carbamates **1** and **2** are obtained from nonlinear least squares curve fitting of Equation 1 as described by Hosie et al.<sup>23</sup> (Fig. 4, Tables 2 and 3). After linear regression analyses of these logarithmic data (Figs. 5 and 6), the results are also shown in Tables 2 and 3. Linear relationships between  $-\log K_i$  and  $\log k_c$  for CEase by carbamates **1** and  $\sigma$  are observed, and the  $\rho$  values are 3.4 and  $-0.13$ , respectively (Fig. 5 and Table 2). Linear relationships between  $-\log K_i$  and  $\log k_c$  for CEase by carbamates

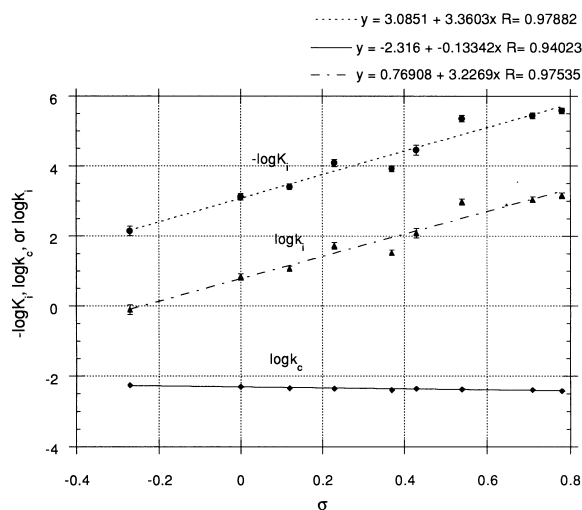


Fig. 5. Plot of  $-\log K_i$ ,  $\log k_c$ , and  $\log k_i$  for the inhibition of CEase by carbamates **1** against  $\sigma$ .

**2** (except **2b** and **2c**) and  $\sigma^*$  are observed, and the  $\rho^*$  values are  $-0.5$  and  $0.5$ , respectively (Fig. 6 and Table 3). Carbamate **2b** and **2c** like other aryl carbamates are also characterized as pseudo-substrate inhibitors of CEase from the experiment of the return of activity and protection by TFA. However, the inhibition data for these two carbamates deviate from the linearity (Fig. 6). Multiple linear regression analyses of kinetic data in Table 3, except those for carbamate **2b** and **2c** by using the Taft-Ingold equation<sup>44-47</sup> or Järv-Hansch equation,<sup>39</sup> do not improve the correlation based on the  $R$  values (data not shown). However, multiple linear regression analyses of all kinetic data in Table 3 by using the Taft-Ingold equation (data not shown)<sup>44-47</sup> or Järv-Hansch equation<sup>39</sup> do improve the correlation based on the  $R$  values (Table 3). The  $\rho^*$  values for  $-\log K_i$  and  $\log k_i$  are  $-0.3$  and  $0.4$ , respectively. The  $\delta$  values for  $-\log K_i$  and  $\log k_i$  are  $0.14$  and  $0.39$ , respectively.

The isokinetic relationships for the inhibition of CEase by carbamates **1** and **2** are further studied by Arrhenius plots (Fig. 7). Table 4 summarizes all isokinetic and isoequilibrium temperatures ( $\beta$ ).<sup>45-47</sup> All these  $\beta$  values are away from the reaction temperature,  $25^\circ\text{C}$ ; therefore, some low  $\rho$  (or  $\rho^*$ ) values we obtained are not due to isokinetic but structural effects.

## DISCUSSION

### Chemical properties of carbamates **1** and **2**

Table 1 summarizes some linear free energy relationships for these compounds. All NH proton chemical shifts ( $\delta$ ) of carbamates **1** and **2** are correlated linearly with  $\sigma$  (or  $\sigma^*$ ) ex-

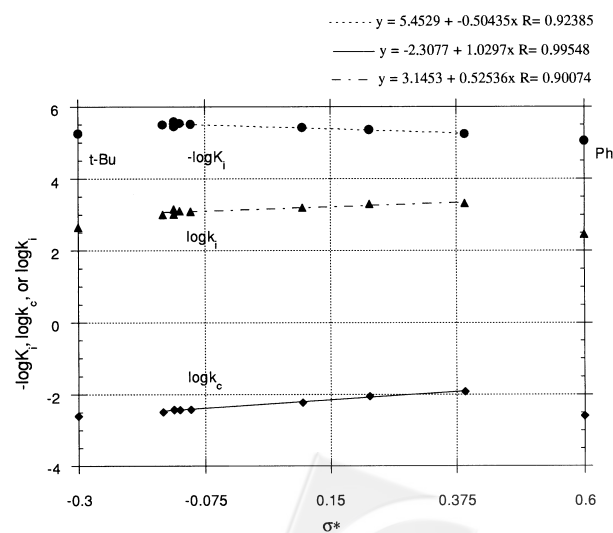


Fig. 6. Plot of  $-\log K_i$ ,  $\log k_c$ , and  $\log k_i$  for the inhibition of CEase by carbamates **2** against  $\sigma^*$ .

Table 3. Enzyme Kinetic Data and Correlation Results for the Inhibitions of CEase by Carbamates **2**

R=	$\sigma^*$	Es	$K_i$ ( $\mu\text{M}$ )	$k_c$ ( $10^{-3}\text{s}^{-1}$ )	$k_i$ ( $\text{M}^{-1}\text{s}^{-1}$ )
<i>t</i> -Bu	-0.3	-1.54	$5.6 \pm 0.5$	$2.5 \pm 0.1$	$450 \pm 40$
Et	-0.1	-0.07	$3.1 \pm 0.3$	$3.85 \pm 0.03$	$1,200 \pm 100$
<i>n</i> -Pr	-0.12	-0.36	$2.9 \pm 0.3$	$3.74 \pm 0.04$	$1,300 \pm 100$
<i>n</i> -Bu	-0.13	-0.39	$2.6 \pm 0.2$	$3.78 \pm 0.04$	$1,500 \pm 100$
<i>n</i> -Hex	-0.15	-0.40	$3.2 \pm 0.4$	$3.00 \pm 0.03$	$900 \pm 100$
<i>n</i> -Oct	-0.13	-0.33	$3.6 \pm 0.4$	$3.75 \pm 0.04$	$1,000 \pm 100$
$\text{C}_2\text{H}_4\text{Cl}$	0.39	-0.43	$5.8 \pm 0.7$	$1.21 \pm 0.04$	$210 \pm 30$
$\text{CH}_2\text{Ph}$	0.22	-0.38	$4.4 \pm 0.5$	$8.81 \pm 0.09$	$2,000 \pm 200$
Allyl	0.1	-0.39	$3.8 \pm 0.5$	$6.00 \pm 0.05$	$1,600 \pm 200$
Ph	0.6	-2.55	$9 \pm 1$	$2.6 \pm 0.1$	$290 \pm 30$
$\rho^{*a}$			$-\log K_i$	$\text{Log} k_c$	$\text{Log} k_i$
$h^a$			$-0.50 \pm 0.1$	$1.03 \pm 0.04$	$0.5 \pm 0.1$
$R^a$			$5.45 \pm 0.02$	$-2.31 \pm 0.01$	$3.15 \pm 0.02$
$\rho^{*b,c}$			0.920	0.995	0.900
$\delta^{b,c}$			$-0.3 \pm 0.1$	$0.6 \pm 0.2$	$0.4 \pm 0.1$
$h^{b,c}$			$0.14 \pm 0.04$	$0.25 \pm 0.06$	$0.39 \pm 0.04$
$R^{b,c}$			$5.49 \pm 0.04$	$-2.21 \pm 0.06$	$3.28 \pm 0.02$
			0.910	0.862	0.956

<sup>a</sup> Correlations of  $-\log K_i$ ,  $\log k_c$ , and  $\log k_i$  with  $\sigma^*$  ( $\log k = h + \rho^*\sigma^*$ ) for all data except those of R = Ph and *t*-Bu. <sup>b</sup> Correlations of  $-\log K_i$ ,  $\log k_c$ , and  $\log k_i$  with  $\sigma^*$  and *Es* ( $\log k = h + \rho^*\sigma^* + \delta E_s$ ) for all data. <sup>c</sup> Correlations of  $-\log K_i$ ,  $\log k_c$ , and  $\log k_i$  with  $\sigma^*$ , *Es*, and  $\pi$  ( $\log k = h + \rho^*\sigma^* + \delta E_s + \psi\pi$ )<sup>39</sup> for all data.

cept that of 4-nitrophenyl-*N*-phenyl carbamate (**2b**). The exception may be due to the fact that the NH lone pair electrons in carbamate **2b** hyperconjugate to the phenyl ring. All  $pK_a$  and  $\log k_{[\text{OH}]}$  (Fig. 2) values are correlated linearly with  $\sigma$  (or  $\sigma^*$ ) or without steric constants (Table 1). Multiple linear regression analyses of these data by the Taft-Ingold equation<sup>44-47</sup> or the Järv-Hansch equation<sup>39</sup> do not improve the correlation. Therefore, the structural effect for alkaline-

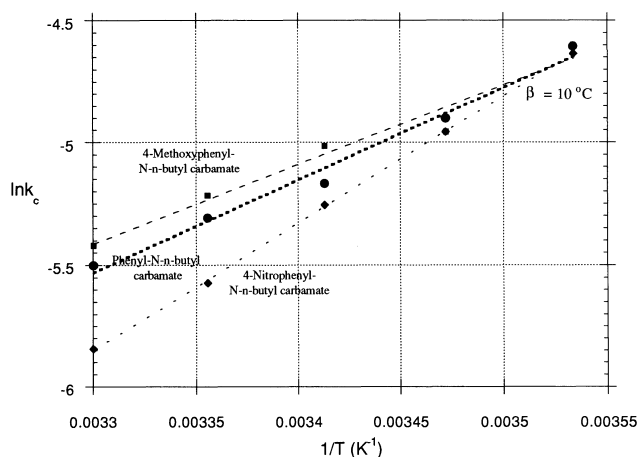


Fig. 7. Arrhenius plot of  $\ln k_c$  for inhibitions of CEase by carbamates **1a**, **1b**, and **1i** against  $1/T$ . The isokinetic temperature  $\beta = 10^\circ\text{C}$ .

catalyzed hydrolysis of these carbamates, except for **2b**, is dependent only upon the electronic effect but independent of both steric and hydrophobic effects. The fact that the alkaline-catalyzed hydrolysis of these carbamates is independent of steric effects indicated that the hydroxy anion is relatively small in size compared to carbamates and easily deprotonates the carbamate NH proton without any steric hindrance from carbamates. Moreover, the alkaline-catalyzed hydrolysis of these carbamates proceeds *via* a common mechanism-E1cB<sup>40-42</sup> (Fig. 3) based on the linear correlation between  $\log k_{[\text{OH}]}$  values and  $\sigma$  (or  $\sigma^*$ ). Overall, the fact that some structure-reactivity relationships for carbamates **1** and **2**, except for **2b**, are linear indicates that these carbamates have a structure, conformation, and mechanism similar to alkaline-catalyzed hydrolysis. The deviation for carbamate **2b** can be due to steric, hydrophobic, and hyperconjugated effects in the structure.

Table 4. Isokinetic and Isoequilibrium Temperatures for the Inhibition of CEase by Carbamates **1** and **2**

	1	2
$\beta(K_i)$	$140 \pm 2^\circ\text{C}$	$35 \pm 3^\circ\text{C}$
$\beta(k_c)$	$11 \pm 1^\circ\text{C}$	$13 \pm 1^\circ\text{C}$
$\beta(k_i)$	$106 \pm 3^\circ\text{C}$	$36 \pm 4^\circ\text{C}$

### Inhibition of CEase

The inhibition data  $K_i$ ,  $k_c$ , and  $k_f$  for CEase are obtained from nonlinear least squares curve fitting of Equation 1 as described by Hosie et al.<sup>23</sup> (Tables 2 and 3). The formation constant of E-I (Fig. 1 and Scheme 1) is  $1/K_i$  and the  $\rho$  value for this reaction is 3.4 (Fig. 5). This suggests that the transition state for this step is more negative than reactants (CEase and inhibitor) and the tetrahedral species is proposed to be E-I (Scheme I and Fig. 1). Like B<sub>AC</sub>2 of alkaline hydrolysis of substituted phenyl-*N*-methyl carbamates ( $\rho = 2.7 \pm 0.2$ ),<sup>40</sup> the formations of covalent E-I intermediate ( $\rho = 3.4$ ) and E-I' carbamyl enzyme ( $\rho = 3.2$ ) are very sensitive to structure and rates are strongly accelerated by electron-withdrawing groups. For  $K_i$  the  $\rho$  value we obtained of 3.4 is in the range of that obtained from the inhibition of subtilisin Carsberg by boronic acid ( $\rho = 3.75 \pm 0.76$ ).<sup>34</sup> Similar results of Hammett analysis have been reported by Feaster et al.<sup>24</sup> for inhibition of porcine and rat CEases by aryl-*N*-alkyl carbamates ( $\rho = 1.8 \pm 0.4$ ). That the  $\rho$  values in  $-\log K_i$ - $\sigma$ -correlation for CEase-inhibition by carbamates **1** (Fig. 5) are greater than one indicates that the carbamylation transition state is more sensitive to aryl substituents than the reference processes, the ionizations of *p*-substituted phenols and anilinium ions, that are used to define the substituent constants,  $\sigma$ .<sup>44-47</sup> This likely reflects the fact that the phenolate anion in the carbamylation transition state is made in a relatively hydrophobic active site, whereas upon phenol ionization the charge on the phenolate anion is partially masked by the hydrogen bonding with water.<sup>24</sup> The greater  $\rho$  values observed also reflects the greater charge separation in E-I intermediate, involving a positively charged histidine imidazolium group and the hydroxy group, than in the corresponding carbamates-hydroxide ion adduct, which is stabilized by water only.

Multiple linear regression analyses of kinetic data except carbamates **2b** and **2c** in Table 3 do not improve the correlation based on the R values. The electronic effect of the substituent plays a major role in these structure-reactivity correlations; however, the steric effect and the hydrophobicity of the substituent do not.

The  $\rho^*$  values for  $-\log K_i$ - $\sigma^*$ -correlation for CEase-inhibition are negative (-0.5) (Table 3); therefore, a concerted mechanism (Scheme I and Fig. 1) for the  $K_i$  step is less possible, but a two-step mechanism for the  $K_i$  step may occur. Since the E-I tetrahedral species carry the negative charge, the concerted mechanism ( $\rho^* > 0$ ) has been ruled out due to the fact that the negative  $\rho^*$  values are observed. Like A<sub>AC</sub>2 mechanism,<sup>45</sup> the  $K_i$  step for the CEase-inhibition by carbamates **2** is insensitive to electronic perturbation ( $\rho^* = -0.5$ ) (Table 3). The possible reason for this is the composite nature of the observed rate constant. In other words, electronic de-

mands on the first step with a negative  $\rho^*$  value and the second step with a positive  $\rho^*$  value tend to cancel each other. Since the above reaction also forms the negative-charge tetrahedral intermediate, it is possible that the first step of the  $K_i$  step is the formation of the hydrogen bonding between the amide protons of the OH of CEase and the lone pair of the carbamate nitrogen and the development of the partial positive-charge at the carbamate nitrogen (Fig. 8). Therefore, the  $\rho^*$  value for

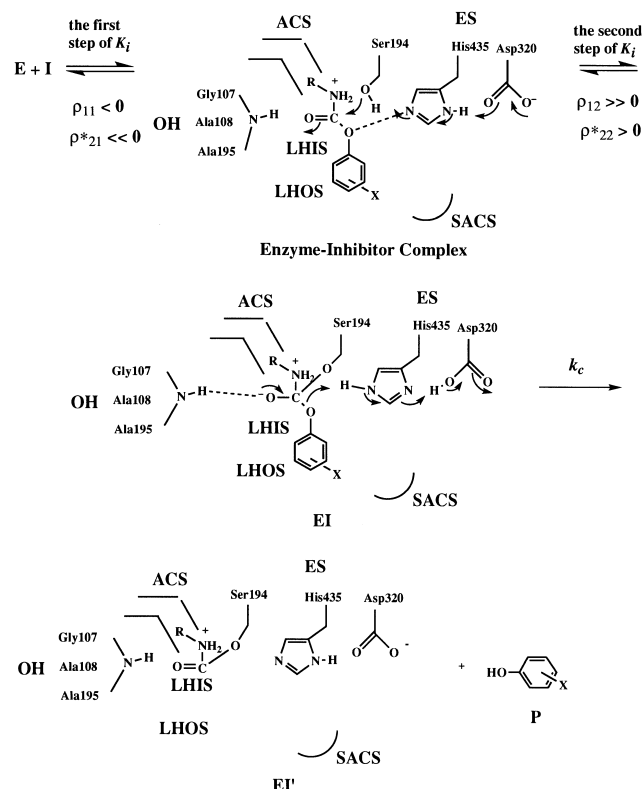


Fig. 8. The proposed two-step- $K_i$ -mechanism for the inhibition of CEase by carbamates **1** and **2**. The first step of  $K_i$  is the protonation of carbamate nitrogen. The reaction constants of the first step of  $K_i$  for carbamates **1** and **2** are defined as  $\rho_{11}$  and  $\rho^*_{21}$ , respectively. The  $\rho^*_{21}$  value is more negative than the  $\rho_{11}$  value because the R substituent of carbamates **2** is much closer to the positive charge ( $\text{RNH}_2^+$ ) than the X substituent of carbamates **1**. The reaction constants of the second step of  $K_i$  for carbamates **1** and **2** are defined as  $\rho_{12}$  and  $\rho^*_{22}$ , respectively. The  $\rho_{12}$  value is more positive than the  $\rho^*_{22}$  value because the X substituent of carbamates **1** is much closer to the negative charge ( $\text{C-O}^-$ ) of the EI complex than the R substituent of carbamates **2**. Therefore, the overall reaction constant of  $-\log K_i$  for carbamates **1** is positive ( $\rho = \rho_{11} + \rho_{12} = 3.4$ ) but that for carbamates **2** is negative ( $\rho^* = \rho^*_{21} + \rho^*_{22} = -0.5$ ).

this step should be negative. Since the partial positive nitrogen is away from the substituted phenyl group, the  $\rho$  value for the CEase-inhibition by carbamates **1** should not be affected by the first step of the  $K_i$  step ( $\rho \sim 0$ ). The second step of the  $K_i$  step is the formation of the tetrahedral intermediate with the negative-charge at the carbonyl oxygen and should have positive  $\rho^*$  and  $\rho$  values. Overall, the  $\rho^*$  value of the  $K_i$  step for the CEase-inhibition by carbamates **2** is negative ( $\rho^* = -0.5$ ) (Table 3).

Carbamates **2b** and **2c** are also characterized as pseudo-substrate inhibitors of CEase; however, the inhibition data for these two carbamates deviate from the linearity (Fig. 6). Therefore, the mechanism for inhibitions of CEase by these two carbamates may be different. There are three possibilities for these deviations: i) the change of mechanism that means the binding site is SACS instead of ACS, ii) the change in rate limited step, and iii) further dependence upon both steric and hydrophobic effects. The first and second possibilities are ruled out since these two carbamates like other aryl-*N*-alkyl carbamates are all pseudo-substrate inhibitors of CEase and follow the same mechanism (Scheme I and Fig. 1). Multiple linear regression analysis of kinetic data in Tables 3 and 5 by using Taft-Ingold and Järv-Hansch equations improves the correlation compared to simple linear regression analysis by using the Taft-Ingold equation without the steric constant,  $E_s$ , based on the  $R$  values. Therefore, deviations for inhibitions of CEase by carbamates **2b** and **2c** may come from both steric and hydrophobic effects of the substituent. Thus, these two carbamates are still accommodated to the ACS-ES-OH-active site of CEase (Fig. 1) nevertheless it is created a prohibitively steric hindrance and an unfavorably hydrophobic environment for the reaction.

Some  $\rho$  (or  $\rho^*$ ) values in Tables 2 and 3 are very close to zero; therefore, isokinetic relationships for the inhibition of CEase by carbamates **1** and **2** are further studied by Arrhenius plots (Fig. 7). Table 4 summarizes all isokinetic and iso-equilibrium temperatures ( $\beta$ s).<sup>45-47</sup> All these  $\beta$  values are away from the reaction temperature, 25 °C. Therefore, the low  $\rho$  (or  $\rho^*$ ) values we obtained are due to the structural effect.

In conclusion, the common CEase-inhibition mechanism by carbamates **1** and **2** is proposed (Fig. 8). The first step of the mechanism is the development of the positive-charge species at the carbamate nitrogen from the protonation of the carbamate nitrogen and the formation of the enzyme-inhibitor complex. The second step of the mechanism is the formation of the tetrahedral intermediate (EI) with the negative-charge at the carbonyl oxygen. The third step of the mechanism is the formation of the carbamyl enzyme (EI') from the tetrahedral

intermediate. There are two possible reasons for the formation of the positive charge at the carbamate nitrogen instead of the carbamate carbonyl oxygen (C=O) or the carbamate ether oxygen (Ar-O-C(O)-). First, the carbamate NH is the most basic group among the carbamate carbonyl oxygen, the carbamate ether oxygen, and the carbamate NH; therefore, the carbamate NH is protonated but two oxygen positions are not at pH 7.0 (both carbonyl and ether oxygens may be protonated in strongly acidic conditions). Second, the reaction constants of  $-\log K_i$  for carbamates **1** ( $\rho = 3.4$ ) and carbamates **2** ( $\rho^* = -0.5$ ) are more likely from the positive carbamate nitrogen than from the positive carbamate oxygens. The reaction constants of the first step of  $K_i$  for carbamates **1** and **2** are defined as  $\rho_{11}$  and  $\rho^*_{21}$ , respectively (Fig. 8). The  $\rho^*_{21}$  value is more negative than the  $\rho_{11}$  value because the R substituent of carbamates **2** is much closer to the positive charge (RNH<sub>2</sub><sup>+</sup>) than the X substituent of carbamates **1**. The reaction constants of the second step of  $K_i$  for carbamates **1** and **2** are defined as  $\rho_{12}$  and  $\rho^*_{22}$ , respectively. The  $\rho_{12}$  value is more positive than the  $\rho^*_{22}$  value because the X substituent of carbamates **1** is much closer to the negative charge (C-O<sup>-</sup>) of the EI complex than the R substituent of carbamates **2**. Therefore, the overall reaction constant of  $-\log K_i$  for carbamates **1** is positive ( $\rho = \rho_{11} + \rho_{12} = 3.4$ ) but that for carbamates **2** is negative ( $\rho^* = \rho^*_{21} + \rho^*_{22} = -0.5$ ). If the positive-charge is developed at the carbamate carbonyl or ether oxygen instead of the carbamate nitrogen,  $\rho_{11}$  would be more negative than  $\rho^*_{21}$  (because the X substituent of carbamate **1** is closer to the positive oxygen than the R substituent of carbamates **2**) and  $\rho^*_{22}$  would be more positive than  $\rho_{12}$ , and  $\rho$  of  $-\log K_i$  for carbamates **1** would be negative and  $\rho^*$  of  $-\log K_i$  for carbamates **2** would be positive. Therefore, the first step of the  $K_i$  step (Fig. 8) protonates the carbamate nitrogen and forms the positive-charge at the carbamate nitrogen instead of either carbamate oxygen. The pre-steady state inhibitions of CEase by carbamates **2** should be further studied to confirm the two-step mechanism for the  $K_i$  step.

## EXPERIMENTAL SECTION

### Materials

All chemicals were of the highest grade available. CEase from porcine pancreas and 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 and biochemicals were of the highest quality available commercially.

### Instrumental Methods

$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were recorded at 300 and 75.4 MHz, respectively, on a Varian-XL 300 spectrometer. The  $^1\text{H}$  and  $^{13}\text{C}$  chemical shifts were referenced to internal TMS. Steady state kinetic data were obtained from UV-visible spectrophotometer (HP 8452 or Beckman DU-650) with a cell holder circulated with a water bath. The  $\text{pK}_a$  values were obtained from the pH-stat titration (Radiometer PHM 290).

### Synthesis of Carbamates 1 and 2

Carbamates **1** were prepared from the condensation of the corresponding phenol with 1.2 mole equivalents of *n*-butyl isocyanate in the presence of a catalytic amount of pyridine in dichloromethane at 25 °C for 48 h (76-85% yield). Carbamates **2** were prepared from the condensation of 4-nitrophenol with 1.2 mole equivalents of the corresponding isocyanate in the presence of a catalytic amount of pyridine in dichloromethane at 25 °C for 48 h (74-90% yield). All compounds were purified by liquid chromatography on silica gel (hexane-ethyl acetate solvent gradient) and characterized by  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra, high resolution mass spectra (HRMS), and elemental analysis (EA).

#### Phenyl-*N-n*-butyl carbamate (**1a**)

$^1\text{H}$  NMR ( $\text{CDCl}_3$ , 300 MHz)  $\delta$ /ppm 0.95 (t,  $J = 7$  Hz, 3H,  $\omega\text{-CH}_3$ ), 1.42 (sextet,  $J = 7$  Hz, 2H,  $\gamma\text{-CH}_2$ ), 1.56 (quintet,  $J = 7$  Hz, 2H,  $\beta\text{-CH}_2$ ), 3.22 and 3.27 (ABq,  $J = 7$  Hz, 2H,  $\alpha\text{-CH}_2$ ), 5.02 (s, 1H, NH), 7.11-7.37 (m, 5H, phenyl-H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 75.4 MHz)  $\delta$ /ppm 13.60 ( $\omega\text{-C}$ ), 19.76 ( $\gamma\text{-C}$ ), 31.76 ( $\beta\text{-C}$ ), 40.81 ( $\alpha\text{-C}$ ), 121.57, 125.13, and 129.20 (phenyl C-2-C-6), 151.01 (phenyl C-1), 154.66 (C=O). HRMS calculated for  $\text{C}_{11}\text{H}_{15}\text{O}_2\text{N}$ : 193.1103, found: 193.1107. EA calculated for  $\text{C}_{11}\text{H}_{15}\text{O}_2\text{N}$ : C, 68.35; H, 7.83; N, 7.25; found: C, 68.27; H, 7.96, N, 7.15.

#### 4-Methoxyphenyl-*N-n*-butyl carbamate (**1b**)

$^1\text{H}$  NMR ( $\text{CDCl}_3$ , 300 MHz)  $\delta$ /ppm 0.95 (t,  $J = 7$  Hz, 3H,  $\omega\text{-CH}_3$ ), 1.38 (sextet,  $J = 7.2$  Hz, 2H,  $\gamma\text{-CH}_2$ ), 1.54 (quintet,  $J = 7.2$  Hz, 2H,  $\beta\text{-CH}_2$ ), 3.22 and 3.28 (ABq,  $J = 6.6$  Hz, 2H,  $\alpha\text{-CH}_2$ ), 3.78 (s, 3H,  $\text{OCH}_3$ ), 5.04 (s, 1H, NH), 6.84-7.06 (m, 4H, phenyl-H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 75.4 MHz)  $\delta$ /ppm 13.51 ( $\omega\text{-C}$ ), 16.79 ( $\gamma\text{-C}$ ), 31.67 ( $\beta\text{-C}$ ), 40.75 ( $\alpha\text{-C}$ ), 55.37 ( $\text{OCH}_3$ ), 114.14 (phenyl C-3,5), 122.37 (phenyl C-2,6), 144.59 (phenyl C-4), 155.06 (phenyl C-1), 156.74 (C=O). HRMS calculated for  $\text{C}_{12}\text{H}_{17}\text{O}_3\text{N}$ : 223.1208, found: 223.1201. EA calculated for  $\text{C}_{12}\text{H}_{17}\text{O}_3\text{N}$ : C, 64.54; H, 7.68; N, 6.28; found: C, 64.47; H, 7.76, N, 6.19.

#### 3-Methoxyphenyl-*N-n*-butyl carbamate (**1c**)

$^1\text{H}$  NMR ( $\text{CDCl}_3$ , 300 MHz)  $\delta$ /ppm 0.95 (t,  $J = 7.5$  Hz, 3H,  $\omega\text{-CH}_3$ ), 1.39 (sextet,  $J = 7.5$  Hz, 2H,  $\gamma\text{-CH}_2$ ), 1.55 (quintet,  $J = 7.5$  Hz, 2H,  $\beta\text{-CH}_2$ ), 3.24 and 3.30 (ABq,  $J = 7.2$  Hz,

2H,  $\alpha\text{-CH}_2$ ), 3.79 (s, 3H,  $\text{OCH}_3$ ), 5.01 (s, 1H, NH), 6.69-7.27 (m, 4H, phenyl-H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 75.4 MHz)  $\delta$ /ppm 13.66 ( $\omega\text{-C}$ ), 19.83 ( $\gamma\text{-C}$ ), 31.82 ( $\beta\text{-C}$ ), 40.91 ( $\alpha\text{-C}$ ), 55.53 ( $\text{OCH}_3$ ), 17.55, 111.22, 113.85, and 129.65 (phenyl C-2,4,5,6), 152.09 (phenyl C-3), 154.52 (phenyl C-1), 160.40 (phenyl C-1), 156.74 (C=O); HRMS calculated for  $\text{C}_{12}\text{H}_{17}\text{O}_3\text{N}$ : 223.1208, found: 223.1196. EA calculated for  $\text{C}_{12}\text{H}_{17}\text{O}_3\text{N}$ : C, 64.54; H, 7.68; N, 6.28; found: C, 64.44; H, 7.77, N, 6.18.

#### 4-Chlorophenyl-*N-n*-butyl carbamate (**1d**)

$^1\text{H}$  NMR ( $\text{CDCl}_3$ , 300 MHz)  $\delta$ /ppm 0.96 (t,  $J = 7.5$  Hz, 3H,  $\omega\text{-CH}_3$ ), 1.40 (sextet,  $J = 6.9$  Hz, 2H,  $\gamma\text{-CH}_2$ ), 1.56 (quintet,  $J = 7.2$  Hz, 2H,  $\beta\text{-CH}_2$ ), 3.24 and 3.30 (ABq,  $J = 6.9$  Hz, 2H,  $\alpha\text{-CH}_2$ ), 5.00 (s, 1H, NH), 7.05-7.32 (m, 4H, phenyl-H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 75.4 MHz)  $\delta$ /ppm 13.62 ( $\omega\text{-C}$ ), 19.80 ( $\gamma\text{-C}$ ), 31.77 ( $\beta\text{-C}$ ), 40.93 ( $\alpha\text{-C}$ ), 122.95 (phenyl C-3,5), 129.27 (phenyl C-2,6), 130.48 (phenyl C-4), 149.63 (phenyl C-1), 154.25 (C=O). HRMS calculated for  $\text{C}_{11}\text{H}_{14}\text{O}_2\text{NCl}$ : 227.7013, found: 227.7026. EA calculated for  $\text{C}_{11}\text{H}_{14}\text{O}_2\text{NCl}$ : C, 58.13; H, 6.21; N, 6.17; found: C, 58.03; H, 6.33, N, 6.07.

#### 3-Chlorophenyl-*N-n*-butyl carbamate (**1e**)

$^1\text{H}$  NMR ( $\text{CDCl}_3$ , 300 MHz)  $\delta$ /ppm 0.96 (t,  $J = 7.5$  Hz, 3H,  $\omega\text{-CH}_3$ ), 1.40 (sextet,  $J = 6.9$  Hz, 2H,  $\gamma\text{-CH}_2$ ), 1.55 (quintet,  $J = 7.2$  Hz, 2H,  $\beta\text{-CH}_2$ ), 3.24 and 3.30 (ABq,  $J = 6.9$  Hz, 2H,  $\alpha\text{-CH}_2$ ), 5.00 (s, 1H, NH), 7.02-7.30 (m, 4H, phenyl-H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 75.4 MHz)  $\delta$ /ppm 13.64 ( $\omega\text{-C}$ ), 19.82 ( $\gamma\text{-C}$ ), 31.78 ( $\beta\text{-C}$ ), 40.97 ( $\alpha\text{-C}$ ), 119.97, 122.26, 125.48, and 129.98 (phenyl C-2,4,5,6), 134.49 (phenyl C-3), 151.64 (phenyl C-1), 154.05 (C=O). HRMS calculated for  $\text{C}_{11}\text{H}_{14}\text{O}_2\text{NCl}$ : 227.7013, found: 227.7026. EA calculated for  $\text{C}_{11}\text{H}_{14}\text{O}_2\text{NCl}$ : C, 58.13; H, 6.21; N, 6.17; found: C, 58.05; H, 6.30, N, 6.08.

#### 4-Trifluorophenyl-*N-n*-butyl carbamate (**1f**)

$^1\text{H}$  NMR ( $\text{CDCl}_3$ , 300 MHz)  $\delta$ /ppm 0.96 (t,  $J = 7.5$  Hz, 3H,  $\omega\text{-CH}_3$ ), 1.40 (sextet,  $J = 7.5$  Hz, 2H,  $\gamma\text{-CH}_2$ ), 1.55 (quintet,  $J = 7.0$  Hz, 2H,  $\beta\text{-CH}_2$ ), 3.26 and 3.32 (ABq,  $J = 6.6$  Hz, 2H,  $\alpha\text{-CH}_2$ ), 5.04 (s, 1H, NH), 7.24-7.63 (m, 4H, phenyl-H);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 75.4 MHz)  $\delta$ /ppm 13.61 ( $\omega\text{-C}$ ), 19.80 ( $\gamma\text{-C}$ ), 31.73 ( $\beta\text{-C}$ ), 40.96 ( $\alpha\text{-C}$ ), 121.87 (phenyl C-3,5), 124.24 (q,  $^1J_{\text{CF}} = 300$  Hz,  $\text{CF}_3$ ), 126.60 (phenyl C-2,6), 126.63 (q,  $^2J_{\text{CF}} = 20$  Hz, phenyl C-4), 153.67 (phenyl C-1), 153.82 (C=O). HRMS calculated for  $\text{C}_{12}\text{H}_{14}\text{O}_2\text{NF}_3$ : 261.0976, found: 261.0988. EA calculated for  $\text{C}_{12}\text{H}_{14}\text{O}_2\text{NF}_3$ : C, 55.15; H, 5.40; N, 5.36; found: C, 55.09; H, 5.48, N, 5.25.

#### 3-Trifluorophenyl-*N-n*-butyl carbamate (**1g**)

$^1\text{H}$  NMR ( $\text{CDCl}_3$ , 300 MHz)  $\delta$ /ppm 0.96 (t,  $J = 7.5$  Hz, 3H,  $\omega\text{-CH}_3$ ), 1.41 (sextet,  $J = 6.9$  Hz, 2H,  $\gamma\text{-CH}_2$ ), 1.58 (quintet,  $J = 7.2$  Hz, 2H,  $\beta\text{-CH}_2$ ), 3.26 and 3.32 (ABq,  $J = 6.6$  Hz, 2H,  $\alpha\text{-CH}_2$ ), 5.06 (s, 1H, NH), 7.31-7.50 (m, 4H, phenyl-H);

$^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 75.4 MHz)  $\delta$ /ppm 13.56 ( $\omega$ -C), 19.77 ( $\gamma$ -C), 31.69 ( $\beta$ -C), 40.94 ( $\alpha$ -C), 118.78, 121.89, 125.17 and 129.77 (phenyl C-2,4,5,6), 123.55 (q,  $^1J_{\text{CF}} = 230$  Hz,  $\text{CF}_3$ ), 131.24 (q,  $^2J_{\text{CF}} = 20$  Hz, phenyl C-3), 151.21 (phenyl C-1), 154.02 (C=O). HRMS calculated for  $\text{C}_{12}\text{H}_{14}\text{O}_2\text{NF}_3$ : 261.0976, found: 261.0985. EA calculated for  $\text{C}_{12}\text{H}_{14}\text{O}_2\text{NF}_3$ : C, 55.15; H, 5.40; N, 5.36; found: C, 55.06; H, 5.49, N, 5.24.

### 3-Nitrophenyl-*N-n*-butyl carbamate (1h)

$^1\text{H}$  NMR ( $\text{CDCl}_3$ , 300 MHz)  $\delta$ /ppm 0.97 (t,  $J = 7.2$  Hz, 3H,  $\omega$ - $\text{CH}_3$ ), 1.41 (sextet,  $J = 6.9$  Hz, 2H,  $\gamma$ - $\text{CH}_2$ ), 1.59 (quintet,  $J = 7.2$  Hz, 2H,  $\beta$ - $\text{CH}_2$ ), 3.26 and 3.32 (ABq,  $J = 6.6$  Hz, 2H,  $\alpha$ - $\text{CH}_2$ ), 5.13 (s, 1H, NH), 7.48-8.09 (m, 4H, phenyl- $H$ );  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 75.4 MHz)  $\delta$ /ppm 13.62 ( $\omega$ -C), 19.80 ( $\gamma$ -C), 31.70 ( $\beta$ -C), 41.07 ( $\alpha$ -C), 117.24, 120.14, 128.08, and 129.81 (phenyl C-2,4,5,6), 148.72 (phenyl C-1), 151.46 (phenyl C-3), 153.61 (C=O). HRMS calculated for  $\text{C}_{11}\text{H}_{14}\text{N}_2\text{O}_4$ : 238.0953, found: 238.0942. EA calculated for  $\text{C}_{11}\text{H}_{14}\text{N}_2\text{O}_4$ : C, 55.44; H, 5.93; N, 11.76; found: C, 55.33; H, 6.02, N, 11.65.

### 4-Nitrophenyl-*N-n*-butyl carbamate (1i or 2a)

$^1\text{H}$  NMR ( $\text{CDCl}_3$ , 300 MHz)  $\delta$ /ppm 0.97 (t,  $J = 6.9$  Hz, 3H,  $\omega$ - $\text{CH}_3$ ), 1.41 (sextet,  $J = 6.9$  Hz, 2H,  $\gamma$ - $\text{CH}_2$ ), 1.58 (m, 2H,  $\beta$ - $\text{CH}_2$ ), 3.26 and 3.32 (ABq,  $J = 6.9$  Hz, 2H,  $\alpha$ - $\text{CH}_2$ ), 5.15 (s, 1H, NH), 7.31 (dd,  $J = 4.5$  and 1.5 Hz, 2H, 2,6-phenyl  $H$ ), 8.24 (dd,  $J = 4.5$  and 1.5 Hz, 2H, 3,5-phenyl  $H$ );  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 75.4 MHz)  $\delta$ /ppm 13.59 ( $\omega$ -C), 19.77 ( $\gamma$ -C), 31.64 ( $\beta$ -C), 41.02 ( $\alpha$ -C), 121.94 (phenyl C-2 and C-6), 125.10 (phenyl C-3 and C-5), 144.66 (phenyl C-1), 153.16 (phenyl C-4), 156.04 (C=O). HRMS calculated for  $\text{C}_{10}\text{H}_{14}\text{O}_4\text{N}_2$ : 238.0953, found: 238.0957. EA calculated for  $\text{C}_{10}\text{H}_{14}\text{O}_4\text{N}_2$ : C, 55.44; H, 5.93; N, 11.76; found: C, 55.36; H, 6.01, N, 11.68.

### 4-Nitrophenyl-*N*-phenyl carbamate (2b)

$^1\text{H}$  NMR ( $\text{CDCl}_3$ , 300 MHz)  $\delta$ /ppm 7.07 (s, 1H, NH), 7.38 (d,  $J = 7$  Hz, 2H, 2,6-phenyl  $H$ ), 8.29 (d,  $J = 7$  Hz, 2H, 3,5-phenyl  $H$ ), 7.15-7.46 (m, 5H, NH-phenyl);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 75.4 MHz)  $\delta$ /ppm 122.13 (phenyl C-2 and C-6), 125.20 (phenyl C-3 and C-5), 145.01 (phenyl C-1), 150.11 (phenyl C-4), 118.94, 124.53, 129.27, and 136.59 (NH-phenyl), 155.32 (C=O). HRMS calculated for  $\text{C}_{13}\text{H}_{10}\text{O}_4\text{N}_2$ : 258.0640, found: 258.0650. EA calculated for  $\text{C}_{13}\text{H}_{10}\text{O}_4\text{N}_2$ : C, 60.45; H, 3.91; N, 10.85; found: C, 60.38; H, 4.05, N, 10.79.

### 4-Nitrophenyl-*N-t*-butyl carbamate (2c)

$^1\text{H}$  NMR ( $\text{CDCl}_3$ , 300 MHz)  $\delta$ /ppm 1.37 and 1.31 (s, 9H,  $\text{CH}_3$ ), 5.12 (s, 1H, NH), 7.30 (d,  $J = 6$  Hz, 2H, 2,6-phenyl  $H$ ), 8.24 (d,  $J = 6$  Hz, 2H, 3,5-phenyl  $H$ );  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 75.4 MHz)  $\delta$ /ppm 28.65 and 29.58 ( $\text{CH}_3$ ), 50.24 and 50.25 ( $\text{C}(\text{CH}_3)_3$ ), 121.00 (phenyl C-2 and C-6), 125.03 (phenyl C-3 and C-5), 144.50 (phenyl C-1), 151.09 (phenyl C-4), 155.94

(C=O). HRMS calculated for  $\text{C}_{10}\text{H}_{14}\text{O}_4\text{N}_2$ : 238.0953, found: 238.0959. EA calculated for  $\text{C}_{10}\text{H}_{14}\text{O}_4\text{N}_2$ : C, 55.44; H, 5.93; N, 11.76; found: C, 55.34; H, 6.02, N, 11.69.

### 4-Nitrophenyl-*N-n*-ethyl carbamate (2d)

$^1\text{H}$  NMR ( $\text{CDCl}_3$ , 300 MHz)  $\delta$ /ppm 1.60 (t,  $J = 7.5$  Hz, 3H,  $\text{CH}_3$ ), 3.27-3.36 (m, 2H,  $\text{CH}_2$ ), 5.15 (s, 1H, NH), 7.44-8.07 (m, 4H, phenyl- $H$ );  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 75.4 MHz)  $\delta$ /ppm 31.65 ( $\text{CH}_3$ ), 41.09 ( $\text{CH}_2$ ), 121.95 (phenyl C-2,6), 125.13 (phenyl C-3,5), 144.68 (phenyl C-1), 153.11 (phenyl C-4), 156.01 (C=O). HRMS calculated for  $\text{C}_9\text{H}_{10}\text{N}_2\text{O}_4$ : 210.0640, found: 210.0653. EA calculated for  $\text{C}_9\text{H}_{10}\text{N}_2\text{O}_4$ : C, 51.41; H, 4.80; N, 13.33; found: C, 51.30; H, 4.87, N, 13.21.

### 4-Nitrophenyl-*N-n*-propyl carbamate (2e)

$^1\text{H}$  NMR ( $\text{CDCl}_3$ , 300 MHz)  $\delta$ /ppm 1.38 (t,  $J = 7.2$  Hz, 3H,  $\omega$ - $\text{CH}_3$ ), 1.62 (quintet,  $J = 7.5$  Hz, 2H,  $\beta$ - $\text{CH}_2$ ), 3.29 and 3.35 (ABq,  $J = 7.0$  Hz, 2H,  $\alpha$ - $\text{CH}_2$ ), 5.16 (s, 1H, NH), 7.46-8.05 (m, 4H, phenyl- $H$ );  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 75.4 MHz)  $\delta$ /ppm 19.67 ( $\theta$ -C), 31.68 ( $\beta$ -C), 41.07 ( $\alpha$ -C), 121.92 (phenyl C-2,6), 125.11 (phenyl C-3,5), 144.69 (phenyl C-1), 153.10 (phenyl C-4), 156.02 (C=O). HRMS calculated for  $\text{C}_{10}\text{H}_{12}\text{N}_2\text{O}_4$ : 224.0797, found: 226.1278. EA calculated for  $\text{C}_{10}\text{H}_{12}\text{N}_2\text{O}_4$ : C, 53.55; H, 5.40; N, 12.50; found: C, 53.43; H, 5.51, N, 12.32.

### 4-Nitrophenyl-*N-n*-hexyl carbamate (2f)

$^1\text{H}$  NMR ( $\text{CDCl}_3$ , 300 MHz)  $\delta$ /ppm 0.91 (t,  $J = 6.6$  Hz, 3H,  $\omega$ - $\text{CH}_3$ ), 1.25-1.40 (quintet,  $J = 6.9$  Hz, 2H,  $\gamma$ - $\text{CH}_2$ ), 1.54-1.61 (m, 6H,  $\beta$ ,  $\delta$ ,  $\epsilon$ - $\text{CH}_2$ ), 3.26 and 3.32 (ABq,  $J = 6.9$  Hz, 2H,  $\alpha$ - $\text{CH}_2$ ), 5.13 (s, 1H, NH), 7.48-8.09 (m, 4H, phenyl- $H$ );  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 75.4 MHz)  $\delta$ /ppm 13.92 ( $\omega$ -C), 22.47 ( $\gamma$ -C), 26.47 ( $\beta$ -C), 26.47 and 29.60 ( $\delta$  and  $\epsilon$ -C), 41.37 ( $\alpha$ -C), 121.95 (phenyl C-2,6), 125.13 (phenyl C-3,5), 144.72 (phenyl C-1), 153.11 (phenyl C-4), 156.09 (C=O). HRMS calculated for  $\text{C}_{13}\text{H}_{18}\text{N}_2\text{O}_4$ : 266.1266, found: 226.1278. EA calculated for  $\text{C}_{13}\text{H}_{18}\text{N}_2\text{O}_4$ : C, 58.62; H, 6.82; N, 10.54; found: C, 58.50; H, 6.95, N, 10.45.

### 4-Nitrophenyl-*N-n*-octyl carbamate (2g)

$^1\text{H}$  NMR ( $\text{CDCl}_3$ , 300 MHz)  $\delta$ /ppm 0.88 (t,  $J = 7$  Hz, 3H,  $\omega$ - $\text{CH}_3$ ), 1.22-1.43 (m,  $J = 6.9$  Hz, 2H,  $\gamma$ - $\text{CH}_2$ ), 1.54-1.61 (m, 10H,  $\beta$  and  $\delta$  to  $\omega$ -1- $\text{CH}_2$ ), 3.26 and 3.32 (ABq,  $J = 6.9$  Hz, 2H,  $\alpha$ - $\text{CH}_2$ ), 5.13 (s, 1H, NH), 7.48-8.09 (m, 4H, phenyl- $H$ );  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 75.4 MHz)  $\delta$ /ppm 14.01 ( $\omega$ -C), 22.56 ( $\gamma$ -C), 26.66 ( $\beta$ -C), 29.12, 29.64, and 31.71 ( $\delta$  to  $\epsilon$ -C), 41.37 ( $\alpha$ -C), 121.94 (phenyl C-2,6), 125.13 (phenyl C-3,5), 144.73 (phenyl C-1), 153.10 (phenyl C-4), 156.08 (C=O). HRMS calculated for  $\text{C}_{15}\text{H}_{22}\text{N}_2\text{O}_4$ : 294.1579, found: 294.1592. EA calculated for  $\text{C}_{15}\text{H}_{22}\text{N}_2\text{O}_4$ : C, 61.19; H, 7.54; N, 9.52; found: C, 61.08; H, 7.63, N, 9.43.

### 4-Nitrophenyl-*N*-2-chloroethyl carbamate (2h)

$^1\text{H}$  NMR ( $\text{CDCl}_3$ , 300 MHz)  $\delta$ /ppm 3.65-3.73 (m, 4H,

ClCH<sub>2</sub>CH<sub>2</sub>), 5.66 (s, 1H, NH), 7.32-8.27 (m, 4H, phenyl-H); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 75.4 MHz) δ/ppm 42.99 and 43.43 (ClCH<sub>2</sub>CH<sub>2</sub>), 122.09 (phenyl C-2,6), 126.21 (phenyl C-3,5), 144.98 (phenyl C-1), 153.51 (phenyl C-4), 155.61 (C=O). HRMS calculated for C<sub>9</sub>H<sub>9</sub>N<sub>2</sub>O<sub>4</sub>Cl: 244.0251, found: 244.0262. EA calculated for C<sub>9</sub>H<sub>9</sub>N<sub>2</sub>O<sub>4</sub>Cl: C, 44.26; H, 3.72; N, 11.48; found: C, 44.12; H, 3.81, N, 11.30.

#### 4-Nitrophenyl-*N*-allyl carbamate (**2i**)

<sup>1</sup>H NMR (CDCl<sub>3</sub>, 300 MHz) δ/ppm 3.93 (m, 2H, CH<sub>2</sub>CH=CH<sub>2</sub>), 5.21-5.27 (m, 2H, CH=CH<sub>2</sub>), 5.91 (m, 1H, CH=CH<sub>2</sub>), 5.32 (s, 1H, NH), 7.30-8.26 (m, 4H, phenyl-H); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 75.4 MHz) δ/ppm 43.69 (CH<sub>2</sub>CH=CH<sub>2</sub>), 117.19 and 133.28 (CH<sub>2</sub>CH=CH<sub>2</sub>), 122.03 (phenyl C-2,6), 126.16 (phenyl C-3,5), 144.89 (phenyl C-1), 153.33 (phenyl C-4), 155.84 (C=O). HRMS calculated for C<sub>10</sub>H<sub>9</sub>N<sub>2</sub>O<sub>4</sub>: 221.0562, found: 221.0574. EA calculated for C<sub>10</sub>H<sub>9</sub>N<sub>2</sub>O<sub>4</sub>: C, 54.28; H, 4.10; N, 12.67; found: C, 54.16; H, 4.17, N, 12.59.

#### 4-Nitrophenyl-*N*-benzyl carbamate (**2j**)

<sup>1</sup>H NMR (CDCl<sub>3</sub>, 300 MHz) δ/ppm 4.48 (m, 2H, CH<sub>2</sub>Ph), 5.50 (s, 1H, NH), 7.27-8.26 (m, 9H, phenyl-H); <sup>13</sup>C NMR (CDCl<sub>3</sub>, 75.4 MHz) δ/ppm 45.37 (CH<sub>2</sub>Ph), 117.19 and 133.28 (CH<sub>2</sub>CH=CH<sub>2</sub>), 122.03 (phenyl C-2,6), 126.13, 121.98, 125.15, 126.13, 127.73, 128.00, 128.91, 144.82, and 153.27 (phenyl), 155.91 (C=O). HRMS calculated for C<sub>14</sub>H<sub>12</sub>N<sub>2</sub>O<sub>4</sub>: 272.0797, found: 272.0809. EA calculated for C<sub>14</sub>H<sub>12</sub>N<sub>2</sub>O<sub>4</sub>: C, 61.75; H, 4.44; N, 10.29; found: C, 61.64; H, 4.52, N, 10.17.

#### Data Reduction

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

#### p*K*<sub>a</sub> and log*k*<sub>[OH]</sub>

The p*K*<sub>a</sub> values were obtained from the pH-stat titration. The values of log*k*<sub>[OH]</sub> were obtained according to the procedures of Fujita et al.<sup>40</sup> The first order rate constant, *k*<sub>hyd</sub>, of the acyl derivatives was obtained from a UV-visible spectrophotometer after calculation. The values of log*k*<sub>[OH]</sub> were determined as the intercept of the plot of log*k*<sub>hyd</sub> versus log[OH]. The reaction temperature was kept at 25.0 ± 0.1 °C. All reactions were performed in sodium phosphate buffer (1 mL, 0.05 M, pH 8.0) containing NaCl (0.2 M), acetonitrile (2.5% by volume), triton X-100 (0.5% by weight), and substrate (5 μmol). Reactions were monitored from 214 to 288 nm according to different absorptions of X-C<sub>6</sub>H<sub>4</sub>-OH.<sup>41</sup>

#### Steady-state Enzyme Kinetics

The steady-state CEase inhibitions were assayed by

Hosie's method.<sup>23</sup> The temperature was maintained at 25.0 ± 0.1 °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 (50 μM of PNPB for CEase), and varying concentrations (from 10<sup>-7</sup> to 10<sup>-2</sup> M for carbamates **1**; from 10<sup>-8</sup> to 10<sup>-3</sup> M for carbamates **2**) of inhibitors. Requisite volumes of stock solution of substrate and inhibitors in acetonitrile were injected into reaction buffers *via* a pipet. Porcine pancreatic CEase was dissolved in sodium phosphate buffer (0.1 M, pH 7.0). Reactions were initiated by injecting enzyme (50 μg or 1 unit (μmol/min) of CEase) and monitored at 410 nm on the UV-visible spectrophotometer. First-order rate constants (*k*<sub>app</sub> values) for inhibition of CEase were determined as described by Hosie et al. (Scheme 1).<sup>23</sup> Values of *K*<sub>*i*</sub> and *k*<sub>*c*</sub> can be obtained by fitting the data of *k*<sub>app</sub> and [I] to Equation 1 by nonlinear least squares regression analyses (Fig. 3). Duplicate or triplicate sets of data were collected for each inhibitor concentration.

#### Return of Activity and Protection by TFA

For the return of activity study, CEase (50 μg) was incubated with a carbamate **1** or **2** (1 μM) in the absence and presence of TFA (2 μM), a known competitive inhibitor of the enzyme<sup>17</sup> before the inhibition reaction. The concentrations of substrate (PNPB) were 0.2 mM for CEase, respectively. All the other procedures followed those of Hosie et al.<sup>23</sup>

#### Isokinetic and Isoequilibrium Temperatures

The β (*K*<sub>*i*</sub>), β (*k*<sub>*c*</sub>), and β (*k*<sub>*i*</sub>) values (Table 4) were obtained from the Arrhenius plots of -ln*K*<sub>*i*</sub>, ln*k*<sub>*c*</sub>, or ln*k*<sub>*i*</sub> by carbamates **1a**, **1b**, **1i** against 1/*T* (Fig. 7) and those by carbamates **2a**, **2g**, and **2i** against 1/*T*. The CEase inhibitions were assayed as described in **Enzyme Kinetics**. The temperature was maintained at 10.0, 15.0, 20.0, 25.0, or 30.0 ± 0.1 °C by a refrigerated and heated circulating water bath.

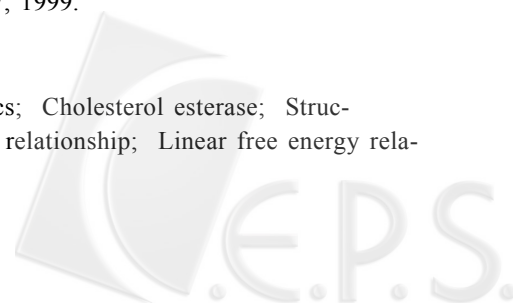
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#### Key Words

Enzyme kinetics; Cholesterol esterase; Structure-Reactivity relationship; Linear free energy relationship.



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