The Protein Journal, Vol. 25, No. 1, January 2006 (© 2006)
DOI: 10.1007/s10930-006-0013-5

Probing Structure–Function Relationships of Serine Hydrolases and Proteases with Carbamate and Thiocarbamate Inhibitors

G. Lin,[1,4] S.-Y. Chiou,[2] B.-C. Hwu,[1] and C.-W. Hsieh[3]

Received June 8, 2005

Benzene-1,3-di-*N*-*n*-octylcarbamate (**1**), benzene-1-hydroxyl-3-*N*-*n*-octylcarbamate (**2**), benzene-1,3-di-*N*-*n*-ocztylthiocarbamate (**3**), and benzene-1-hydroxyl-3-*N*-*n*-octylthiocarbamate (**4**) are synthesized from 1,3-benzene-diol and are characterized as the pseudo-substrate inhibitors of acetylcholinesterase, butyrylcholinesterase, cholesterol esterase, lipase, trypsin, and chymotrypsin. For these six enzyme inhibitions by **1–4**, the pK_i values are linearly correlated with their log k_i values – Brønsted plots. Therefore, **1–4** inhibit these enzymes through a common mechanism. Moreover, both pK_i and log k_i values for the inhibitions by **1**,**3**, and **4** are linearly correlated with both pK_i and log k_i values for the inhibitions by **2**, respectively. Thus, the pK_i values for the inhibitions by **2** are defined as the nucleophilicity constants of these enzymes (n_{enzyme}). The log k_2 values for the inhibitions by **1–4** are also linearly correlated with the n_{enzyme} values. Therefore, the nucleophilicity for serine hydrolases and proteases toward **1–4** also applies the Swain–Scott correlations.

KEY WORDS: Structure–function relationship; serine hydrolase; serine protease; enzyme inhibition; carbamate and thiocarbamate.

1. INTRODUCTION

Serine hydrolases such as acetylcholinesterase (AChE, EC 3.1.1.7) (Bar-on *et al*., 2002; Bartolucci *et al*., 1999; Quinn, 1987; Sussman *et al*., 1991), butyrylcholinesterase (BChE, EC 3.1.1.8) (Loudwig *et al*., 2003; Masson *et al*., 2002; Savini *et al*., 2003), cholesterol esterase (CEase, EC 3.1.1.13) (Chen *et al*., 1998; Wang *et al*., 1997), *Pseudomonas* species lipase (PSL, EC 3.1.1.3) (Lang *et al*., 1998), trypsin (EC 3.4.21.4) and chymotrypsin (CT, EC 3.4.21.1) (Hedstrom *et al*., 1992; Kossiakoff and Spencer, 1981; Kraut, 1977; Phillips and Fletterick, 1992; Seitz and Shulman, 1982; Walsh, 1979) have a common catalytic triad, Ser-His-Asp (or Glu). The active site serines of these enzymes are located at the bottom of a cavity and act as nucleophiles to attack the carbonyl groups of substrates or pseudo-substrate inhibitors. Two forms of cholinesterase coexist ubiquitously throughout the body, AChE and BChE, and although highly homologous, >65%, they are products of different genes on chromosomes 7 and 3 in humans, respectively (Quinn, 1987; Sussman *et al*., 1991). Both subtype unselective cholinesterase and AChE-selective inhibitors have been used in Alzheimer's disease to amplify the action of acetylcholine at remaining cholinergic synapses within the Alzheimer's disease brain. Rivastigmine (Exelon) (Fig. 1) is a carbamate inhibitor of AChE and is used in the

[1] Department of Chemistry, National Chung-Hsing University, Taichung, 402, Taiwan.
[2] Department of Neurosurgery, Institute of Medicine, Chung Shan Medical University, Taichung, 402, Taiwan.
[3] Department of Applied Chemistry, Chung-Cheng Institute of Technology, Tahsi, Taoyuan, 335, Taiwan.
[4] To whom correspondence should be addressed. E-mail: gilin@dragon.nchu.edu.tw

Abbreviations: AChE, acetylcholinesterase; BChE, butyrylcholinesterase; CEase, cholesterol esterase; CT, α-chymotrypsin; LFER, linear free energy relationship; n_{enzyme}, the enzyme nucleophilicity constant; PCL, *Pseudomonas cepacia* lipase; PSL, *Pseudomonas* species lipase; QSAR, quantitative structure activity relationship; QSFR, quantitative structure function relationship.

treatment for patients with mild to moderately severe Alzheimer's disease (Bar-on *et al*., 2002). The X-ray crystal structures of AChE have revealed that AChE contains a catalytic triad similar to that present in other serine hydrolases. It has also revealed that this triad is located near the bottom of a deep and narrow gorge about 20 Å in depth (Sussman *et al*., 1991). The X-ray crystal structure of BChE has been recently reported (Loudwig *et al*., 2003; Masson *et al*., 2002). Cholesterol esterase (CEase), also known as bile salt-activated lipase, catalyzes the hydrolysis of dietary cholesterol esters, triacylglycerols, phospholipids, and vitamin esters. Although different bile salt-activation mechanisms have been proposed, the active sites of CEase from X-ray crystal structures are similar to that of lipase (Chen *et al*., 1998; Wang *et al*., 1997). The X-ray crystal structures of CEase have revealed that the enzyme contains a catalytic triad similar to that present in other serine hydrolases and an acyl chain binding site. The X-ray crystal structures of *Pseudomonas cepacia* lipase (PCL) have revealed that the enzyme contains a catalytic triad similar to that present in other serine hydrolases and three acyl chain binding sites (Lang *et al*., 1998). CT and trypsin are strikingly similar. The primary structures of both enzymes are about 40% identical (Hedstrom *et al*., 1992; Kossiakoff and Spencer, 1981; Kraut, 1977; Phillips and Fletterick, 1992; Seitz and Shulman, 1982; Walsh, 1979). Furthermore, both enzymes have a nucleophilic Ser in their catalytic triads. CT has a hydrophobic pocket that binds the side chains of aromatic or bulky hydrophobic amino residues of substrates. Trypsin has a negatively charged Asp residue at the bottom of the binding pocket, which allows the enzyme to bind the positively charged side chains residues of substrates.

Quantitative structure activity relationships (QSARs) represent an attempt to correlate structural

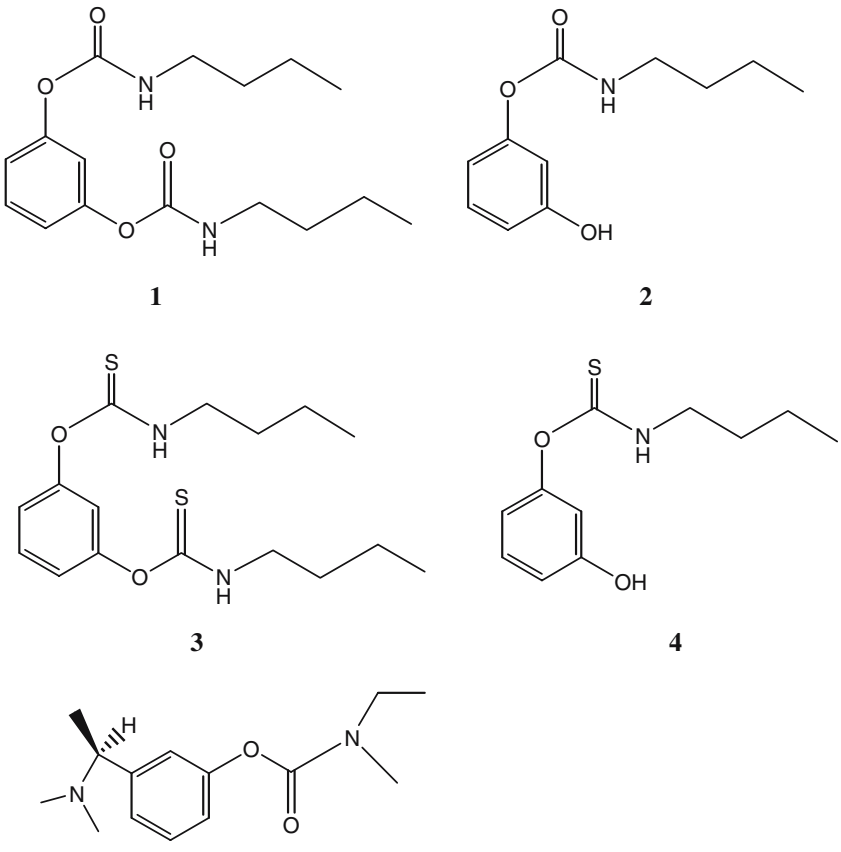

Fig. 1. Structures of inhibitors **1–4** and rivastigmine (Exelon).

properties of compounds with activities (Isaacs, 1995; Lowry and Richardson, 1992; March, 1992; Thomas, 2000). These chemical descriptors, which include parameters to account for hydrophobicity, electronic, inductive, or polar properties, and steric effects, are determined empirically or by calculations (Thomas, 2000). Many biological activities and chemical reactivities are correlated with the Hammett equation (Eq. (1)) (Isaacs, 1995; Lowry and Richardson, 1992; March, 1992; Thomas, 2000).

$$\log k \text{ or } \log K = h + \rho\sigma \tag{1}$$

In Eq. (1), h, ρ, and σ are the log k_0 or log K_0 values, intensity factor for electronic (polar) effect, and Hammett substituent constant, respectively. A traditional means of doing QSAR in organic chemistry is the use of linear free energy relationships (LFERs) that links changes in rate constant for a reaction to changes in its equilibrium constant as the structure of the substrates or inhibitors is altered – Brønsted or Hammett plots (Eq. (2)) (Isaacs, 1995; Lowry and Richardson, 1992; March, 1992).

$$\log k = h + \beta \log K \tag{2}$$

Fersht *et al*. (1986) have found that Brønsted plots may be applied to enzymatic reactions for variation of the structure of an enzyme with mutation. Therefore, quantitative structure–function relationships (QSFRs) represent an attempt to correlate structural properties of enzymes (or proteins) with enzymatic reactivities toward a common substrate (or inhibitor). In this paper, we report that Brønsted plots may be also applied to enzymatic reactions for variation of the structure of enzymes, serine hydrolases and serine proteases.

Carbamate and thiocarbamate inhibitors, such as rivastigmine (Fig. 1), aryl carbamates, and aryl thiocarbamates, are characterized as the pseudo-substrate inhibitors of AChE, BChE, CEase, and PSL (Bar-on *et al*., 2002; Hosie *et al*., 1987; Lin and Chouhwang, 2001; Lin and Lai, 1995, 1996; Lin *et al*., 1999a, b, 2000). In the presence of substrate, the kinetic scheme for pseudo-substrate inhibitions of serine hydrolases by carbamate or thiocarbamate inhibitors is proposed (Fig. 2). Since this inhibition follows first-order kinetics over observed time period for steady-state kinetics, hydrolysis rate of carbamyl (or thiocarbamyl) enzyme EI' must be significantly slower than EI' formation rate ($k_2 \gg k_3$). Therefore, values of K_i and k_2 can be calculated from Eq. (3) (Hosie *et al*., 1987). In Eq. (3), the k_{app} values are first-order rate constants which are obtained by Hosie's method. The bimolecular rate constant, $k_i = k_2/K_i$, is related to overall inhibitory potency.

$$k_{app} = k_2[\mathrm{I}]/(K_i(1 + [\mathrm{S}]/k_m) + [\mathrm{I}]) \tag{3}$$

The nucleophilicity of nucleophiles for S_N2 reactions in organic chemistry is linearly correlated against the Swain–Scott equation (Eq. (4)), where k_0 refers to a standard nucleophile (water), and s is the susceptibility constant expressing sensitivity of the rate to nucleophilicity (Isaacs, 1995; Lowry and Richardson, 1992; March, 1992). Analogies with the Hammett equation (Eq. (1)) are obvious.

$$\log k_{Nu} = \log k_0 + sn \tag{4}$$

The value of s is defined for methyl bromide substitution in water as $s = 1$, with other systems relative to this. Although all serine hydrolase and protease-catalyzed reactions use a common nucleophile, Ser, yet the nucleophilicity for these enzymes is quite different. Therefore, we report here that the necleophilicity for serine hydrolases and protease, such as AChE, BChE, CEase, PSL, CT, and trypsin, is linearly

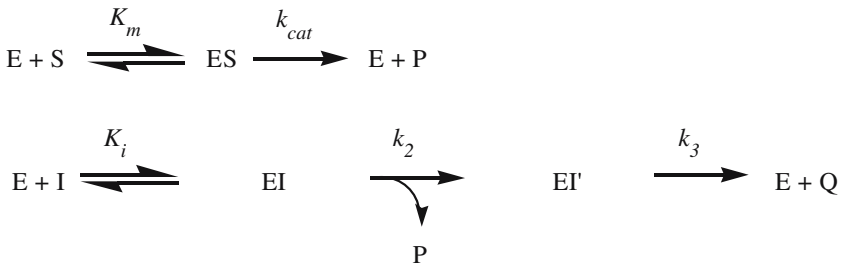

Fig. 2. Kinetic scheme for the pseudo-substrate inhibition of carbamates or thiocarbamates in the presence of substrate. E, enzyme; S, substrate; ES, acylenzyme intermediate; I, carbamate or thiocarbamate, EI, enzyme-inhibitor tetrahedral intermediate; EI', carbamyl (or thiocarbamyl) enzyme intermediate; P, the first product; Q, the second product.

correlated against Eq. (4) toward carbamates and thiocarbamates since these compounds inhibit all six enzymes through the same pseudo-substrate inhibition mechanism (Fig. 2) (Bar-on *et al*., 2002; Hosie *et al*., 1987; Lin and Chouhwang, 2001; Lin and Lai, 1995, 1996; Lin *et al*., 1999a, b, 2000). Thus, benzene-1,3-di-*N*-n-octylcarbamate (**1**) (Fig. 1), benzene-1-hydroxy-3-*N*-*n*-octylcarbamate (**2**), benzene-1,3-di-*N*-n-octylthio- carbamate (**3**), and benzene-1-hydroxy-3-*N*-*n*-octylthiocarbamate (**4**) may be used as standard electrophiles or pseudo-substrates to probe the nucleophilicity of serine hydrolases and proteases.

2. MATERIALS AND METHODS

2.1. Materials

All chemicals were of the highest grade available. Silica gel used in liquid chromatography and thin-layer chromatography plates were obtained from Merk. *Electrophorus electricus* AChE, horse serum BChE, Porcine pancreatic CEase, trypsin (Type II-S, ca. 1800 units/mg solid), PSL (Type XIII, ca. 25 units/mg solid), bovine pancreatic CT (Type II, ca. 50 units/mg protein) were obtained from Sigma. Acetylthiocholine, butyrylthiocholine 5,5′-dithio-bis-2- nitrobenzoate, *p*-nitrophenylbutyrate were also obtained from Sigma.

2.2. Instrumental Methods

^{1}H and ^{13}C NMR spectra were recorded at 400 and 100 MHz, respectively, on a Varian-Gemini 400 spectrometer. All steady state kinetic data were obtained from a UV–visible spectrometer (Agilent 8453) with a cell holder circulated with a water bath.

2.3. Synthesis of Inhibitors 1–4

Benzene-1,3-di-*N*-*n*-octylcarbamate (**1**) and benzene-1-hydroxy-3-*N*-*n*-octylcarbamate (**2**) (Fig. 1) were synthesized from condensation of 1,3-benzene-diol with 2 eqs. of *n*-octyl isocyanate in the presence of 2 eqs. of triethylamine in tetrahydrofuran at 25°C for 24 h yielding a mixture of **1** (35–45%) and **2** (25–35%). **1** and **2** were then separated by liquid chromatography. Benzene-1,3-di-*N*-*n*-octylthiocarbamate (**3**), and benzene-1-hydroxy-3-*N*-*n*-octylthiocarbamate (**4**) were synthesized from condensation of 1,3-benzene-diol with 2 eqs. of *n*-octyl isothiocyanate in the presence of 2 eqs. of triethylamine in tetrahydrofuran at reflux temperature for 24 h yielding a mixture of **3** (30–40%) and **4** (20–35%). **3** and **4** were then separated by liquid chromatography (silica gel, hexane-ethyl acetate).

Benzene-1,3-di-*N*-*n*-octylcarbamate (**1**): ^{1}H NMR ($CDCl_3$, 400 MHz) δ/ppm 0.86 (t, 6H, ω-CH_3), 1.26–1.30 (m, 20H, γ to ω-1-CH_2), 1.52–1.55 (m, 4H, β-CH_2), 3.20 (q, 4H, α-CH_2), 4.97 (br. s, 2H, N*H*), 6.94–7.28 (m, 4H, aromatic *H*'s); ^{13}C NMR ($CDCl_3$, 100 MHz) 14.03 (ω-*C*H_3), 22.58, 26.69, 29.14, 29.17, and 29.73 (γ to ω-1-*C*H_2), 31.74 (β-*C*H_2), 41.24 (α-*C*H_2), 115.33, 118.22, 129.24, 151.48 and 154.16 (aromatic *C*'s and carbamate *C*'s).

Benzene-1-hydroxy-3-*N*-*n*-octylcarbamate (**2**): ^{1}H NMR ($CDCl_3$, 400 MHz) δ/ppm 0.90 (t, 3H, ω-CH_3), 1.28–1.30 (m, 10H, γ to ω-1-CH_2), 1.52–1.55 (m, 2H, β-CH_2), 3.22 (q, 2H, α-CH_2), 5.05 (br. s, 1H, O*H*), 5.23 (br. s, 1H, N*H*), 6.56–7.10 (m, 4H, aromatic *H*'s); ^{13}C NMR ($CDCl_3$, 100 MHz) 13.99 (ω-*C*H_3), 22.54, 26.65, 29.10, 29.13, and 29.57 (γ to ω-1-*C*H_2), 31.69 (β-*C*H_2), 41.24 (α-*C*H_2), 109.35, 112.89, 112.97, 129.62, 151.44, 155.32, and 157.16 (aromatic *C*'s and carbamate *C*).

Benzene-1,3-di-*N*-n-octylthio- carbamate (**3**): ^{1}H NMR ($CDCl_3$, 400 MHz) δ/ppm 0.80 (t, 6H, ω-CH_3), 1.32–1.34 (m, 20H, γ to ω-1-CH_2), 1.52–1.68 (m, 4H, β-CH_2), 3.39 (q, 4H, α-CH_2), 5.11 (br. s, 2H, N*H*), 6.68–7.17 (m, 4H, aromatic *H*'s); ^{13}C NMR ($CDCl_3$, 100 MHz) 14.03 (ω-*C*H_3), 22.59, 26.70, 29.13, 29.17, and 29.65 (γ to ω-1-*C*H_2), 31.73 (β-*C*H_2), 45.91 (α-*C*H_2), 110.42, 113.36, 114.93, 129.76, 153.94, and 156.43 (aromatic *C*'s and carbamate *C*'s).

Benzene-1-hydroxy-3-*N*-*n*-octylthiocarbamate (**4**): ^{1}H NMR ($CDCl_3$, 400 MHz) δ/ppm 0.85 (t, 3H, ω-CH_3), 1.26–1.30 (m, 10H, γ to ω-1-CH_2), 1.50–1.55 (m, 2H, β-CH_2), 3.23 (q, 2H, α-CH_2), 4.96 (br. s, 1H, O*H*), 5.11 (br. s, 1H, N*H*), 6.59–7.11 (m, 4H, aromatic *H*'s); ^{13}C NMR ($CDCl_3$, 100 MHz) 14.05 (ω-*C*H_3), 22.61, 26.72, 29.16, 29.67, and 29.72 (γ to ω-1-*C*H_2), 31.76 (β-*C*H_2), 41.34 (α-*C*H_2), 109.44, 112.97, 113.10, 129.77, 151.62, 155.11, and 157.07 (aromatic *C*'s and carbamate *C*).

2.4. Data Reduction

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

2.5. Inhibitions of Serine Hydrolases and Protease by 1–4

The inhibition reactions of AChE were determined by the Ellman assay (Ellman *et al.*, 1961) as described before (Lin *et al.*, 1999a). The AChE-catalyzed hydrolysis of acetylthiocholine (0.1 m*M*) in the presence of 5,5′-dithio-bis-2-nitrobenzoate (0.1 m*M*) and inhibitors **1–4** were followed continuously at 410 nm on a UV–visible spectrometer at 25°C, pH 7.1. The inhibition reactions of BChE-catalyzed hydrolysis of butyrylthiocholine (0.1 m*M*) by inhibitors **1–4** were also determined by the Ellman assay as described before (Lin *et al.*, 1998). All the other procedures were the same as described in the AChE inhibitions. The inhibition reactions of CEase were determined by the Hosie assay Hosie *et al.*, 1987) as described before (Lin and Lai, 1995, 1996, 1999b, 2000). Porcine pancreatic CEase-catalyzed hydrolysis of *p*-nitrophenylbutyrate (0.5 m*M*) and inhibitors **1–4** in the presence of triton X-100 (0.5% w/w, ca. 7.7 m*M*) were followed continuously at 410 nm on a UV–visible spectrometer (Agilent 8453) at 25°C, pH 7.1. The inhibition reactions of PSL-catalyzed hydrolysis of *p*-nitrophenylbutyrate (0.5 m*M*) by inhibitors **1–4** were also determined by the Hosie assay as described before (Lin and Chouhwang, 2001; Lin *et al.*, 1999b). All the other procedures were the same as described in the CEase inhibitions. The inhibitions of trypsin- and CT-catalyzed hydrolysis of *p*-nitrophenylbutyrate (0.5 m*M*) by inhibitors **1–4** were also determined by the Hosie assay. All the other procedures were the same as described in the CEase inhibitions. The K_i (Table 1) and k_2 (Table 2) values were obtained from the nonlinear least-squares of curve fittings of the k_{app} values versus inhibition concentration ([I]) plot against Eq. (2).

3. RESULTS

Inhibitors **1–4** (Fig. 1) were synthesized from condensation of 1,3-benzene-diol with butyl isocyanate or butyl isothiocyanate in the presence of triethylamine. Inhibitors **1–4** were all characterized as the pseudo-substrate inhibitors of AChE, BChE, CEase, PSL, trypsin, and CT (Tables 1–3). For these enzyme inhibitions by **1–4**, the pK_i values were linearly correlated with their log k_i values – Brønsted or Hammett plots (Isaacs, 1995; Lowry and Richardson, 1992; March, 1992) (Fig. 3 and Table 4). Therefore, **1–4** inhibited these enzymes through a common mechanism. Moreover, both pK_i and log k_i values for the inhibitions by **1**,**3**, and **4** were linearly correlated with both pK_i and log k_i values for the inhibitions by **2**, respectively (Figs. 4 and 5 and Tables 5 and 6). Thus, the pK_i values for

Table 1. The K_i values (in μM)[a] for the inhibitions of AChE, BChE, CEase, PSL, trypsin, and CT by **1–4**

	1	2	3	4
AChE	2.3 ± 0.3	2.2 ± 0.3	1.8 ± 0.1	1.06 ± 0.09
BChE	1.6 ± 0.3	1.7 ± 0.3	2.5 ± 0.2	1.1 ± 0.3
CEase	1.2 ± 0.1	1.5 ± 0.3	1.30 ± 0.08	0.82 ± 0.06
PSL	2.7 ± 0.3	2.2 ± 0.4	4.55 ± 0.03	0.9 ± 0.1
Trypsin	18 ± 9	15 ± 2	40 ± 1	11 ± 2
CT	10.1 ± 0.6	8 ± 4	28 ± 4	6.0 ± 0.6

[a] Obtained from the nonlinear least-squares curve fittings of k_{app} versus [I] plot against Eq. (2) by Hosie's method (Hosie *et al.*, 1987).

Table 2. The k_2 values (in 10^{-3} s^{-1})[a] for the inhibitions of AChE, BChE, CEase, PSL, trypsin, and CT by **1–4**

	1	2	3	4
AChE	10.4 ± 0.2	5.29 ± 0.09	2.76 ± 0.03	18.54 ± 0.02
BChE	8.5 ± 0.7	10 ± 1	16.0 ± 0.3	29 ± 1
CEase	6.4 ± 0.5	7.0 ± 0.2	6.18 ± 0.02	5.68 ± 0.07
PSL	16.6 ± 0.4	6.6 ± 0.4	9.76 ± 0.07	11.4 ± 0.3
Trypsin	0.37 ± 0.05	0.93 ± 0.03	1.1 ± 0.2	0.58 ± 0.02
CT	0.55 ± 0.01	0.57 ± 0.06	1.05 ± 0.05	0.40 ± 0.04

[a] Obtained from the nonlinear least-squares curve fittings of k_{app} versus [I] plot against Eq. (2) by Hosie's method (Hosie *et al.*, 1987).

Table 3. The K_i values (in M^{-1} s^{-1})[a] for the inhibitions of AChE, BChE, CEase, PSL, trypsin, and CT by **1–4**

	1	2	3	4
AChE	4500 ± 600	2400 ± 300	1530 ± 90	17000 ± 1000
BChE	5000 ± 1000	5900 ± 200	6400 ± 500	26400 ± 300
CEase	5300 ± 600	4700 ± 900	4800 ± 300	6900 ± 400
PSL	6100 ± 700	3000 ± 600	2150 ± 20	12700 ± 300
Trypsin	20 ± 10	62 ± 8	28 ± 5	53 ± 2
CT	54 ± 3	70 ± 40	38 ± 6	70 ± 10

[a] $k_i = k_2/K_i$.

the inhibitions by **2** were defined as the enzyme nucleophilicity constants (n_{enzyme}) (Fig. 6). The log k_2 values for the inhibitions by **1–4** were also linearly correlated with the n_{enzyme} values (Fig. 7 and Table 7). Therefore, the nucleophilicity for serine hydrolases and proteases toward **1–4** also applied the Swain–Scott correlations (Eq. (4)). Thus, the pk_i values of inhibitors **1**,**3**, and **4** were linearly correlated with n_{enzyme} (Fig. 6) against Eq. (5) (Fig. 5 and Table 6),

$$pK_i(\log k_i, \text{ or } \log k_2) = h + sn_{enzyme} \qquad (5)$$

where the n_{enzyme} values referred to the pK_i values for the enzyme inhibitions by inhibitor **2** and represented the nucleophilicity constants of the enzyme, and s was the susceptibility constant expressing the sensitivity for nucleophilicity of the enzyme. The value of s was defined for the enzyme inhibitions by inhibitor **2** as $s = 1$, with other inhibition systems relative to this (Table 6). Thus, the order of the n_{enzyme} values were defined as follows: CEase > BChE > AChE ~ PSL > CT > trypsin (Fig. 6).

4. DISCUSSION

4.1. Inhibitory Potencies of 1–4

Inhibitors **1–4** (Fig. 1) are all characterized as the pseudo-substrate inhibitors (Fig. 2) of AChE, BChE, CEase, PSL, trypsin, and CT (Tables 1–3). Among inhibitors **1–4**, thiocarbamate **4** is the most potent inhibitors of these six enzymes when compared their k_i (Table 1), k_2 (Table 2), and k_i (Table 3) values. The possible reason for this is because the thiocarbamate sulfur of inhibitor **4**

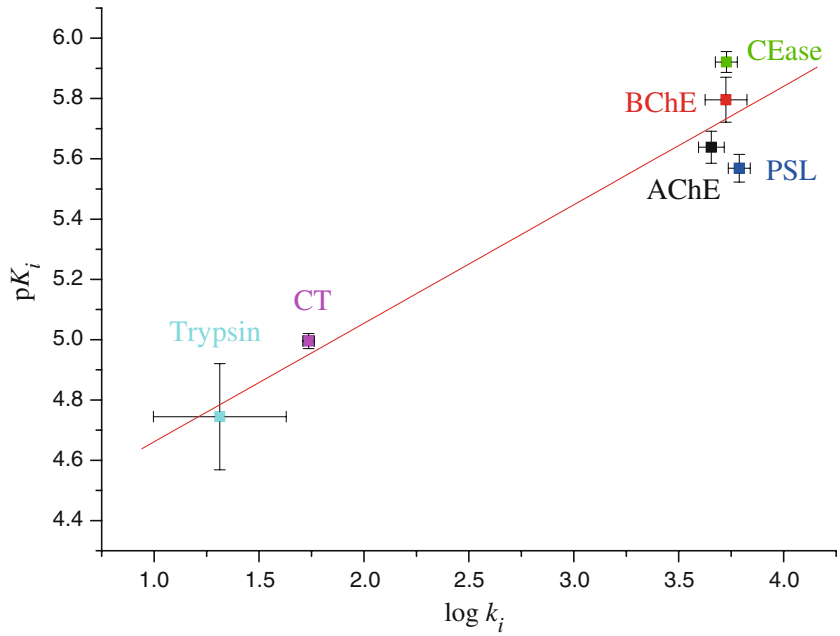

Fig. 3. Plot of pK_i against log k_i for Eq. (2) for the inhibitions of AChE, BChE, CEase, PSL, trypsin, and CT by inhibitor **1**.

Table 4. Correlations of the pK_i values with the log K_i values against Eq. (2)[a] for the inhibitions of AChE, BChE, CEase, PSL, trypsin, and CT inhibitions by **1–4**

Inhibitors	β	h	R^b
1	0.39 ± 0.06	4.3 ± 0.2	0.961
2	0.44 ± 0.05	4.2 ± 0.1	0.978
3	0.56 ± 0.09	3.6 ± 0.3	0.951
4	0.37 ± 0.06	4.5 ± 0.2	0.952

[a] $pK_i = h + \beta \log k_i$.
[b] Correlation coefficient.

more stabilizes the oxyanion hole of the enzyme than the carbamate carbonyl oxygens of inhibitors **1** and **2** and the thiocarbamate sulfur of inhibitor **3**. When the inhibitor binds tightly in the oxyanion hole of the enzyme, distances for the two- or three-pronged hydrogen bonds (Harel *et al.*, 1996; Zhan *et al.*, 2003; Zhang *et al.*, 2002) between the sulfur atom of the inhibitor and the peptidic NH groups in the oxyanion hole are shorter than those between the oxygen atom of the inhibitor and the peptidic NH groups in the oxyanion hole due to a relatively larger size of the sulfur atom. On the other hand, the least potent thiocarbamate **3** may be due to the fact that an oversize effect from the second sulfur atom of the inhibitor reduces the tightness for the inhibitor binding in the oxyanion hole of the enzyme.

4.2. Brønsted or Hammett Correlations of the Inhibitions

For these enzyme inhibitions by **1–4**, the pK_i values are linearly correlated with their log k_i values – Brønsted or Hammett plots (Fig. 3 and Table 4). The β values about 0.4 (Table 4) indicate that about 40% of the change in binding energy on forming the carbamyl or thiocarbamyl enzyme (EI' in Fig. 2) occurs at the stage of formation of the tetrahedral intermediate (EI in Fig. 2) (Fersht *et al.*, 1986). Therefore, **1–4** inhibit these enzymes through a common mechanism (Fig. 2).

4.3. Swain–Scott Correlations of the Inhibitions

Both pK_i and log k_i values for the inhibitions by **1**, **3**, and **4** are linearly correlated with both pK_i and log k_i values for the inhibitions by **2**, respectively (Figs. 5 and 6 and Tables 5 and 6). Thus, pK_i for the inhibitions by **2** is then defined as the nucleophilicity for the serine hydrolase or protease (n_{enzyme}) (Fig. 6). The log k_2 values for the inhibitions by **1–4**

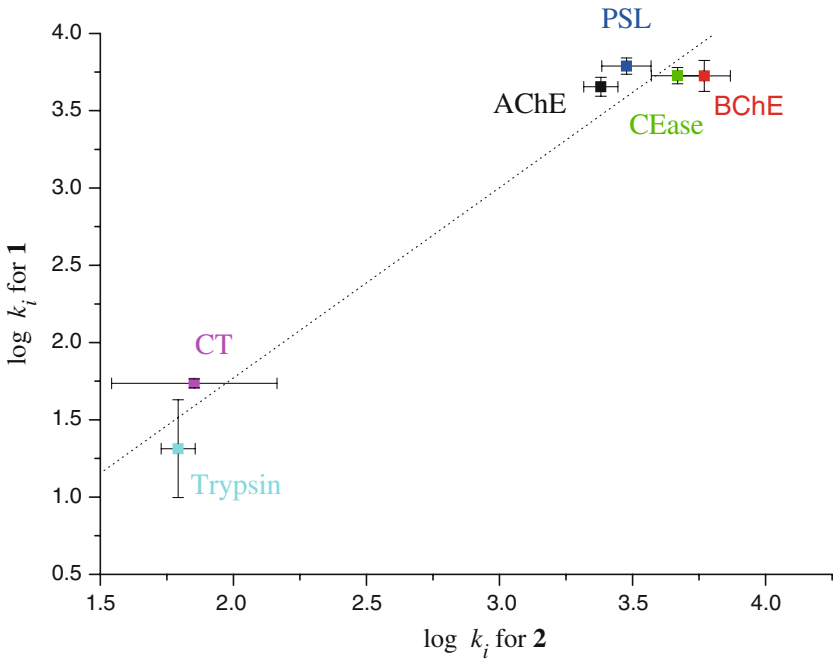

Fig. 4. Plot of log k_i for the enzyme inhibitions by inhibitor **1** against those by inhibitor **1**.

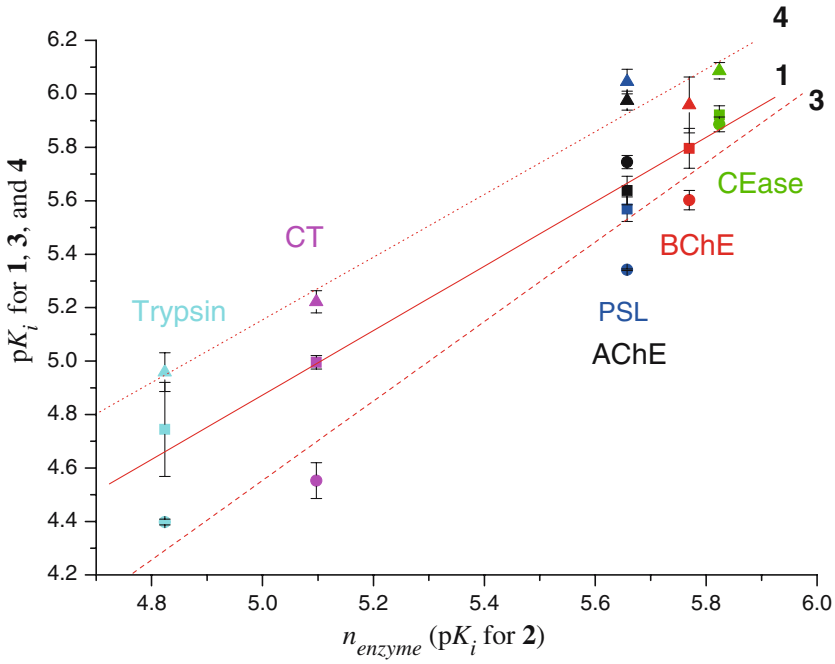

Fig. 5. Plots of pK_i for the enzyme inhibitions by **1**(squares), **3** (circles) and **4** (triangles) against n_{enzyme} (Fig. 7). Results for these linear correlations were summarized in Table 6.

are also linearly correlated with the n_{enzyme} values (Table 7). Therefore, the nucleophilicity for serine hydrolases and proteases toward **1–4** applies the Swain–Scott correlations (Eq. (5)).

4.4. Nucleophilicity Constants (n_{enzyme}) of the Enzymes

All six enzymes in this study belong to the serine hydrolase or protease family and the enzymes use the Ser residue as a common nucleophile in catalysis. Therefore, the nucleophilicity for the enzymes shall be about the same if the nucleophilicity of the Ser residues of the enzymes is the only factor for the enzymatic reactions. Since the n_{enzyme} values (Fig. 6) are varied with enzymes, the n_{enzyme} values may also depend on other factors such as active site shapes and oxyanion holes. The higher n_{enzyme} value (Fig. 6) for an enzyme indicates that this enzyme acts as a better nucleophile than the others. The n_{enzyme} value of 5.77 for BChE is larger than that for AChE (5.66). The possible reasons are as follows. The peptidic NH groups in the oxyanion hole of BChE more stabilizes the negatively charged tetrahedral intermediate **6** (Fig. 8) than the peptidic NH groups in the oxyanion hole of AChE does. The more open acyl pocket for BChE specificity toward bulky substrates or inhibitors is also responsible for stabilization of

Table 5. Correlations of the log k_i values for the inhibitions of AChE, BChE, CEase, PSL, trypsin, and CT inhibitions by **1**, **3**, and **4** with those by **2** against Eq. (6)[a]

Inhibitors	s	h	R[b]
1	1.2 ± 0.1	−0.7 ± 0.3	0.985
2	1	0	1
3	1.14 ± 0.04	−0.6 ± 0.1	0.998
4	1.3 ± 0.1	−0.6 ± 0.4	0.979

[a] $(\log k_i)_{1,\ 3,\ 4} = h + s(\log k_i)_{2.}$ (6)
[b] Correlation coefficient.

Table 6. Correlations of the pK_i values for the inhibitions of AChE, BChE, CEase, PSL, trypsin, and CT inhibitions by **1–4** with n_{enzyme}[a] against Eq. (5)[b]

Inhibitors	s	h	R[c]
1	1.21 ± 0.07	−1.2 ± 0.4	0.993
2	1[d]	0	1
3	1.5 ± 0.2	−3 ± 1	0.966
4	1.17 ± 0.09	−0.7 ± 0.5	0.987

[a] Defined as the pK_i values for the inhibitions of AChE, BChE, CEase, PSL, trypsin, and CT inhibitions by **2** (Fig. 7).
[b] Correlation against $pK_i = h + sn_{enzyme}$.
[c] Correlation coefficient.
[d] Defined as unity.

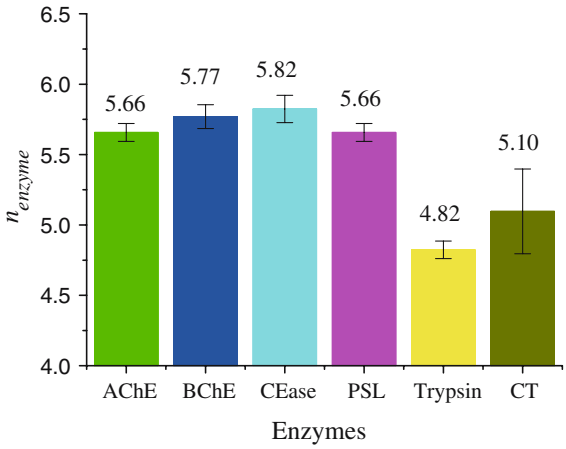

Fig. 6. Enzyme nucleophilicity constant (n_{enzyme}). The n_{enzyme} values were defined as the pK_i values for the enzyme inhibitions by inhibitor **2**.

the tetrahedral intermediate **6** (Fig. 8) (Zhan *et al.*, 2003). It seems that the inhibitor is more easy to enter the active gorge of BChE than to enter the active gorge of AChE (Bar-on *et al.*, 2002; Bartolucci *et al.*, 1999; Quinn, 1987; Sussman *et al.*, 1991) due to a relatively larger active site gorge of the former (Loudwig *et al.*, 2003; Masson *et al.*, 2002; Savini *et al.*, 2003; Zhan *et al.*, 2003).

Like BChE, CEase has a larger binding pocket for substrates (Chen *et al.*, 1998; Wang *et al.*, 1997).

Table 7. Correlations of the log k_2 values for the inhibitions of AChE, BChE, CEase, PSL, trypsin, and CT inhibitions by **1–4** with n_{enzyme}[a] against Eq. (5)[b]

Inhibitors	*s*	*h*	*R*[c]
1	1.6 ± 0.3	−11 ± 2	0.934
2	1.2 ± 0.2	−9 ± 1	0.931
3	1.0 ± 0.3	−8 ± 2	0.856
4	1.7 ± 0.4	−12 ± 2	0.889

[a] Defined as the pK_i values for the inhibitions of AChE, BChE, CEase, PSL, trypsin, and CT inhibitions by **2** (Fig. 7).
[b] Correlation against log $k_2 = h + s\ n_{enzyme}$.
[c] Correlation coefficient.

Although PSL has three acyl chain binding sites, only one of them is the catalytic site (Lang *et al.*, 1998). Therefore, inhibitors **1–4** are more difficult to bind to the catalytic site of PSL than to bind to that of CEase. On the other hand, small n_{enzyme} values for trypsin and CT (Fig. 6) are probably due to lack of any acyl chain binding site in both enzymes (Hedstrom *et al.*, 1992; Kossiakoff and Spencer, 1981; Kraut, 1977; Phillips and Fletterick, 1992; Seitz and Shulman, 1982; Walsh, 1979). All *s* values about unity (Table 6) confirm that all inhibition reactions by inhibitors **1–4** proceed with a common mechanism in the K_i step (Figs. 2 and 8).

The log k_2 values for the enzyme inhibitions by **1–4** are further correlated with n_{enzyme} for the

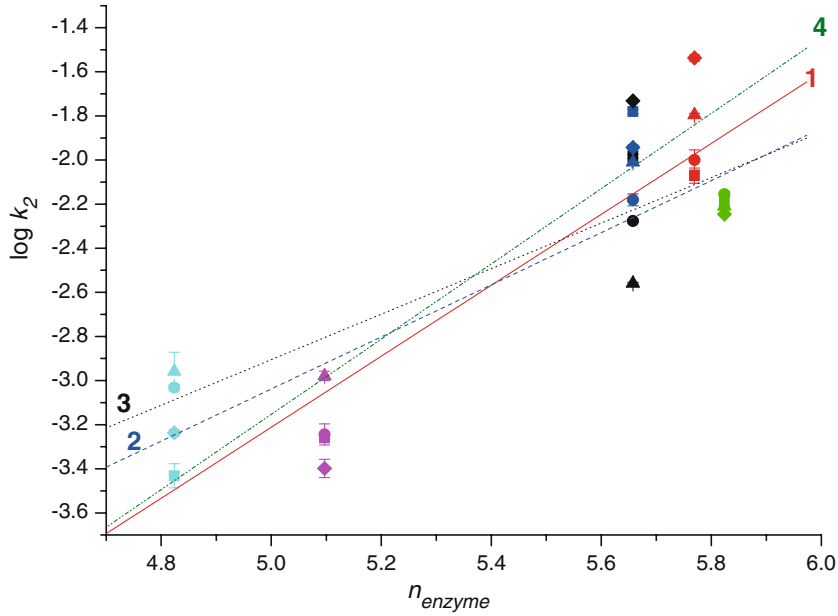

Fig. 7. Plots of log k_2 for the enzyme inhibitions by **1** (squares), **2** (circles), **3** (triangles), and **4** (diamonds) against n_{enzyme} (Fig. 7). Results for these linear correlations were summarized in Table 7.

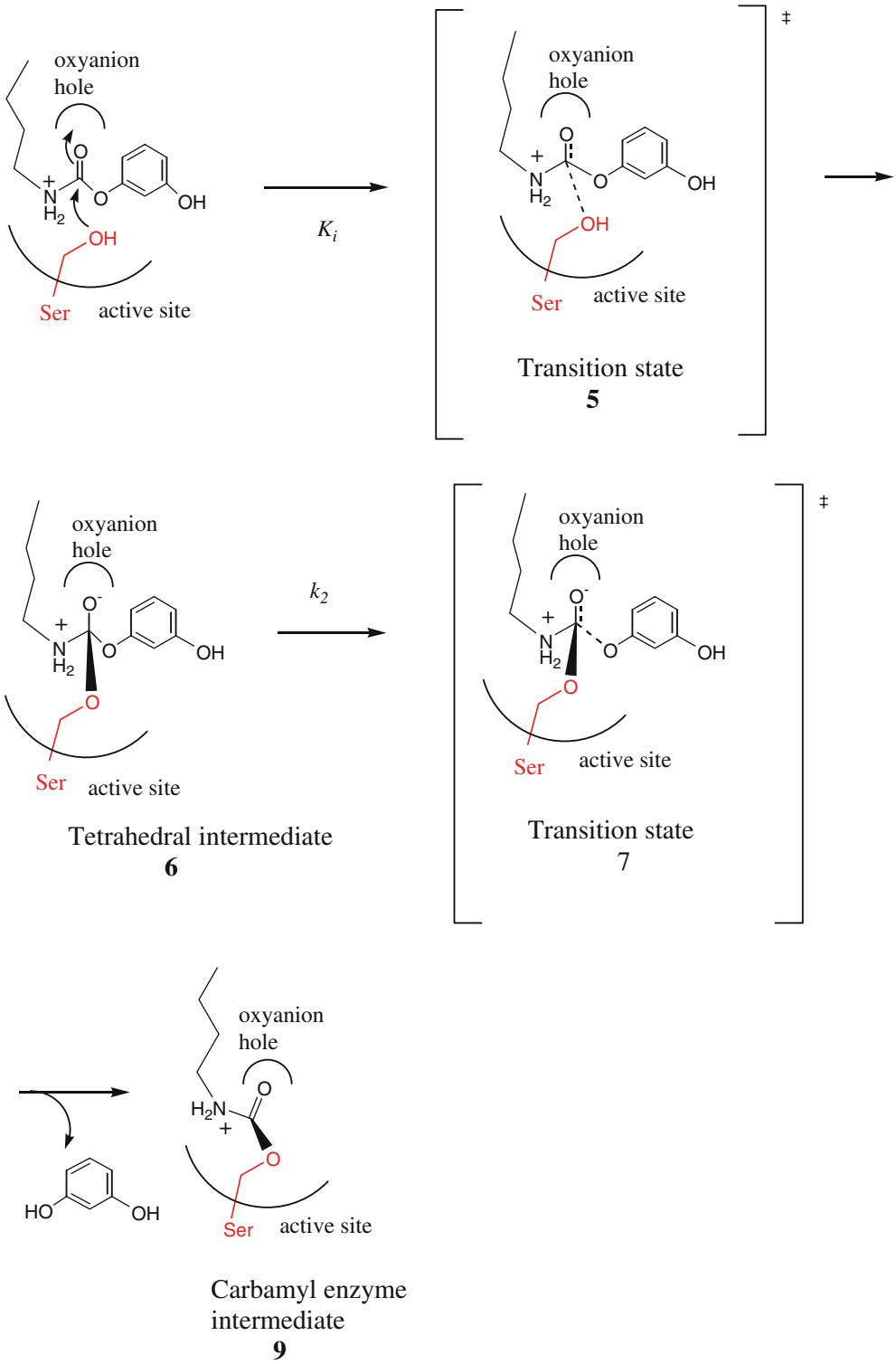

Fig. 8. The proposed mechanism for inhibitions of serine hydrolases or proteases by inhibitor **2**.

enzyme inhibitions by **2** (Fig. 7 and Table 7). These linear correlations indicate similar interactions between the enzymes and inhibitors **1–4** in the transition states of both K_i and k_2 steps (transition states **5** and **7** in Fig. 8). In other words, the k_2 steps involve early transition states; therefore, the transition state **7** resembles the tetrahedral intermediate **6** (Fig. 8). That all s values are about unity (Fig. 7 and Table 7) confirms the same mechanism for all k_2 steps (Figs. 2 and 8).

ACKNOWLEDGMENTS

We thank the National Science Council of Taiwan for financial support.

REFERENCES

Bar-on, P., Millard, C. B., Harel, M., Dvir, H., Enz, A., Sussman, J. L., and Silman, I. (2002). *Biochemistry* **41:** 3555–3564.
Bartolucci, C., Perola, E., Cellai, L., Brufani, M., and Lamba, D. (1999). *Biochemistry* **38:** 5714–5719.
Chen, J. C.-H., Miercke, L. J. W., Krucinski, J., Starr, J. R., Saenz, G., Wang, X., Spillburg, C. A., Lange, L. G., Ellsworth, J. L., and Stroud, R. M. (1998). *Biochemistry* **37:** 5107–5117.
Ellman, C. L., Courtney, K. D., Andres, V. J., and Featherstone, R. M. (1961). *Biochem. Pharmacol.* **7:** 88–95.
Fersht, A. R., Leatherbarrow, R. J., and Wells, T. N. C. (1986). *Nature* **322:** 284–286.
Harel, M., Quinn, D. M., Nair, H. K., Silman, I., and Sussman, J. L. (1996). *J. Am. Chem. Soc.* **118:** 2340–2346.
Hedstrom, L., Szilagyi, L., and Rutter, W. J. (1992). *Science* **255:** 1249–1253.
Hosie, L., Sutton, L. D., and Quinn, D. M. (1987). *J. Biol. Chem.* **262:** 260–264.
Isaacs, N. (1995) Physical Organic Chemistry, 2nd ed., U.K: Longman.
Kossiakoff, A. A., and Spencer, S. A. (1981). *Biochemistry* **20:** 6462–6474.
Kraut, J. (1977), *Ann. Rev. Biochem.* **46:** 331–358.
Lang, D. A., Mannesse, M. L. M., De Haas, G. H., Verheij, H. M., and Dijkstra, B. W. (1998). *Eur. J. Biochem.* **254:** 333–340.
Lin, G., and Lai, C.-Y. (1995). *Tetrahedron Lett.* **36:** 6117–6120.
Lin, G., and Lai, C.-Y. (1996). *Tetrahedron Lett.* **37:** 193–196.
Lin, G., Chen, G.-H., and Ho, H.-C. (1998). *Bioorg. Med. Chem. Lett.* **8:** 2747–2750.
Lin, G., Lai, C.-Y., and Liao, W.-C. (1999a). *Bioorg. Med. Chem.* **7:** 2683–2689.
Lin, G., Shieh, C.-T., Ho, H.-C., Chouhwang, J.-Y., Lin, W.-Y., and Lu, C.-P. (1999b). *Biochemistry* **38:** 9971–9981.
Lin, G., Lai, C.-Y., Liao, W.-C., Kuo, B.-H., and Lu, C.-P. (2000). *J. Chin. Chem. Soc.* **47:** 489–500.
Lin, G., and Chouhwang, J.-Y. (2001). *J. Biochem. Mol. Biol. Biophys.* **5:** 301–308.
Loudwig, S., Nicolet, Y., Masson, P., Fontecilla-camps, J. C., Bon, S., Nachon, F., and Goeldner, M. (2003). *ChemBioChem* **4:** 762–767.
Lowry, T. H., and Richardson, K. S. (1992) Mechanism and Theory in Organic Chemistry. 3rd ed., New York: Harper & Row.
March, J. (1992) Advanced Organic Chemistry, 4th ed., New York: John Wiley.
Masson, P., Froment, M.-T., Fort, S., Ribes, F., Bec, N., Balny, C., and Schopfer, L. M. (2002). *Biochim. Biophys. Acta* **1597:** 229–243.
Phillips, M. A., and Fletterick, R. J. (1992). *Curr. Opin. Struct. Biol.* **2:** 713–720.
Quinn, D. M. (1987). *Chem. Rev.* **87:** 955–979.
Savini, L., Gaeta, A., Fattorusso, C., Catalanotti, B., Campiani, C., Chiasserini, L., Pellerano, C., Novellino, E., McKissic, D., and Saxena, A. (2003). *J. Med. Chem.* **46:** 1–4.
Seitz, T. A., and Shulman, R. G. (1982). *Ann. Rev. Biophys. Bioeng.* **11:** 419–444.
Sussman, J. L., Harel, M., Frolow, F., Oefner, C., Goldman, A., Toker, L., and Silman, I. (1991). *Science* **253:** 872–879.
Thomas, G. (2000) Medicinal Chemistry, New York: John Wiley.
Walsh, C. (1979) Enzymatic Reaction Mechanism, N. Y.: Freeman.
Wang, X., Wang, C.-S., Tang, J., Dyda, F., and Zhang, X. C. (1997). *Structure* **5:** 1209–1218.
Zhan, C.-g., Zheng, F., and Landry, D. W. (2003). *J. Am. Chem. Soc.* **125:** 2462–2474.
Zhang, Y., Kua, J., and McCammon, J. A. (2002). *J. Am. Chem. Soc.* **124:** 10572–10577.