Acetylcholinesterase(AChE)-Catalyzed Hydrolysis of Long-Chain Thiocholine Esters: Shift to a New Chemical Mechanism

Dai Il Jung, Young Ju Shin, Eun Seok Lee,† Taesung Moon,‡ Chang No Yoon,‡ and Bong Ho Lee†,*

Department of Chemistry, Dong-A University, Busan 604-714, Korea
†Department of Chemical Technology, Hanbat National University, Daejon 305-719, Korea
‡Bioanalysis and Biotransformation Research Center, Korea Institute of Science and Technology, Seoul 136-791, Korea
Received October 23, 2002

The kinetic and chemical mechanisms of AChE-catalyzed hydrolysis of short-chain thiocholine esters are relatively well documented. Up to propanoylthiocholine (PrTCh) the chemical mechanism is general acid-base catalysis by the active site catalytic triad. The chemical mechanism for the enzyme-catalyzed butyrylthiocholine(BuTCh) hydrolysis shifts to a parallel mechanism in which general base catalysis by E199 of direct water attack to the carbonyl carbon of the substrate. [Selwood, T., *et al. J. Am. Chem. Soc.* **1993**, *115*, 10477-10482] The long chain thiocholine esters such as hexanoylthiocholine (HexTCh), heptanoylthiocholine (HepTCh), and octanoylthiocholine (OcTCh) are hydrolyzed by electric eel acetylcholinesterase (AChE). The kinetic parameters are determined to show that these compounds have a lower Michaelis constant than BuTCh and the pH-rate profile showed that the mechanism is similar to that of BuTCh hydrolysis. The solvent isotope effect and proton inventory of AChE-catalyzed hydrolysis of HexTCh showed that one proton transfer is involved in the transition state of the acylation stage. The relationship between the dipole moment and the Michaelis constant of the long chain thiocholine esters showed that the dipole moment is the most important factor for the binding of a substrate to the enzyme active site.

Key Words : Acetylcholinesterase, Hydrolysis, Thiocholine esters, Kinetic studies, Chemical mechanism

Introduction

Acetylcholinesterase (AChE, EC 3.1.1.7) is a serine hydrolase that catalyzes the hydrolysis of the neurotransmitter, acetylcholine (ACh) to acetate and choline at neuromuscular synapses.[1,2] The elucidation of the kinetic and chemical mechanisms of AChE-catalyzed hydrolysis of acetylcholine(ACh) is of importance for numerous academic and clinical reasons. For example, organophosphate inhibitors of AChE can be used as chemical warfare agents and insecticides and specific inhibitors of this enzyme, such as tacrine, donepezil, rivastigmine and huperzine A are used as anti-Alzheimer's Disease drugs.[3-5] The X-ray crystallographic studies have shown that AChE has a deep and narrow active site gorge that contains a catalytic triad.[6,7] This catalytic triad is composed of a serine, histidine, and glutamate.[8,9] Kinetic studies have shown that the bimolecular rate constant for ACh hydrolysis by AChE is diffusion-controlled.[8]

To elucidate the mechanism underlying AChE binding and action, less reactive long chain thiocholine esters are used for kinetic studies. These substrates allow the involved chemical steps to be rate determining. Studies of the pH-rate profile, solvent isotope effect, and proton inventory have shown that the chemical mechanism for ATCh and PrTCh hydrolysis operate under conventional general acid-base catalysis in which S200 attacks the carbonyl carbon of the substrate and H440 and E327 act as general bases.[8,9] As the length of acyl chain of the substrate moves to butanoyl the chemical mechanism changes to general base catalysis by E199 of direct water attack on the scissile carbonyl group.[8,10] The pH-V_{max}(V) profile for electric eel AChE-catalyzed hydrolysis of benzoylcholine(BzCh) gave a pK_a of 4.77. The pH-V_{max}/K_m(V/K) profile for electric eel AChE-catalyzed hydrolysis of BuTCh gave a pK_a value of 4.72. These results indicate that E199 is involved in general base catalysis.[8] With this in mind, we therefore undertook kinetic studies, pH-rate profile, and solvent isotope effect studies to characterize the transition state and chemical mechanism underlying AChE-catalyzed hydrolysis of long chain thiocholine esters.

Materials and Methods

Materials. Electric eel AChE, type V-S lyophilized powder, and horse serum BuChE were purchased from Sigma Chemical Co. and were used as received. Prior to use it was dissolved in 0.1 M, pH 7.3 sodium phosphate buffer, containing 0.1 M NaCl. ATCh, BuTCh, 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), and buffer components were also purchased from Sigma Chemical Co. Deuterium oxide (99.8% D) was purchased from Aldrich Chemical Co. and was used as received. Water used in experiments was distilled and deionized by passage through a mixed bead ion-exchange column.

Synthesis of Thiocholine Esters

Hexanoylthiocholine

2-Choroethyl thiohexanoate: 0.61 g (0.01 mol) of ethyl-

*Correspondence: Phone: +82-42-821-1542; Fax: +82-42-822-1562; e-mail: lbh011@hanbat.ac.kr

ene sulfide was dissolved in 3 mL of dry carbon tetrachloride, and 1.45 g (0.011 mol) of hexanoyl chloride was added. The small formed precipitate was removed, and any remaining carbon tetrachloride was distilled off at ordinary pressure. The residue was chromatographed on silica gel (eluent: *n*-hexane) to yield 2-chloroethyl thiohexanoate (0.82 g, 42%) as a colorless liquid. ^{1}H NMR ($CDCl_3$, 200 MHz): δ 0.86-0.93 (m, 3H), 1.27-1.37 (m, 4H), 1.60-1.71 (m, 2H), 2.53-2.60 (t, 2H), 3.19-3.26 (t, 2H), 3.56-3.64 (t, 2H) Mass, *m/z* (rel. int, %): 195, 165, 151, 138, 131 (6), 115, 99 (100), 71(30), 55(16), 51.

Hexanoylthiocholine: 0.59 g (0.01 mol) of anhydrous trimethylamine was added to a mixture of 1.95 g (0.01 mol) of 2-chloroethyl thiohexanoate and 10 mL of dry acetone, cooled to -20 °C. After a week at room temperature, 10 mL of dry ether was added to precipitate the ester of thiocholine chloride still in solution. The precipitate was filtered off and the residue was dried under reduced pressure to yield hexanoylthiocholine (2.54 g, 100%) as a hygroscopic white solid. ^{1}H NMR ($CDCl_3$, 200 MHz): δ 0.84-0.95 (m, 3H), 1.34-1.36 (m, 4H), 1.60-1.76 (m, 2H), 2.62-2.69 (t, 2H), 3.45-3.52 (m, 2H), 3.56 (s, 9H), 3.76-3.90 (m, 2H) ^{13}C NMR ($CDCl_3$, 200 MHz): δ 1.38 (C-6'), 22.1, 22.2 (-S-CH_2CH_2-), 25.0 (C-5'), 31.0 (C-4'), 43.9 (C-3'), 53.4, 53.5, 53.6 (-N(CH_3)$_3$), 65.2 (C-2'), 199.0 (C=O). Mass, *m/z* (rel. int, %): 203, 159, 143, 114, 99(1), 87, 71(7), 58 (100), 51.

Other thiocholine esters were synthesized by the same procedures.

Methods

General: ^{1}H NMR and ^{13}C NMR spectra were recorded in $CDCl_3$ at 25 °C on a Bruker AC 200 FT NMR spectrometer. GC-Mass spectra were obtained by using HP 5890 II GC/MSD spectrometer.

Enzyme assays: AChE-catalyzed hydrolysis of the thiocholine esters was monitored by following production of the thio anion of nitrobenzoic acid at 412 nm by the Ellman's coupled assay.[11] Assays were conducted on HP8452A or HP8453A diode array UV-visible spectrophotometers and the cell compartments were thermostatted by circulating water or Peltier temperature controller, respectively. The kinetic parameters, V, K, and V/K were determined, as described previously.[12] The pH values of buffers were measured on an Orion Model 420A pH meter that was equipped with a glass combination electrode. To determine pD values of the deuterated buffer, a value of 0.4 was added to the measured pH values.[13]

Solvent Isotope Effect and Proton Inventory: Solvent isotope effects were determined by measuring the kinetic parameters in H_2O and D_2O buffers.[14] Proton inventories were determined by measuring rates in buffers of different atom fraction of deuterium. The theory and experimental procedure are well documented.[15-17]

Ligand-Receptor Docking: For the construction of AChE-ligand complexes, the crystal structure of AChE-acetylcholine complex[6] was used. The conformations and locations of ligand molecules in the active sites were determined by using flexible docking. The ligand molecules were docked into the active sites by Monte Carlo method, which considers the interaction energy between the ligands and the active sites. After docking the AChE-ligand complexes were subjected to molecular dynamics simulation to get optimum conformations. Prior to molecular dynamics, the ligand molecules were minimized using steepest descents for 200 steps and conjugate gradients 300 steps. The minimized structures were then used as a starting point for 10-ps molecular dynamics simulations at 300 K with a distance-dependent dielectric constant. The initial velocities were taken from a Maxwell-Boltzmann distribution for target temperature. The leap-frog algorithm was used to integrate the equations of motion with an integration time step of 1 fs. The minimum energy conformations during molecular dynamics were sampled and minimized for QSAR (Quantitative Structure-Activity Relationships) analysis. All molecular mechanics and molecular dynamics calculations were carried out using Discover2.97 program (Msi Inc.) with CVFF (Consistent Valence Force Field) using nonbonded cutoff of 13.0 Å.

Results

The Kinetic Parameters for Thiocholine Esters. The long-chain thiocholine esters were hydrolyzed by electric eel AChE and the associated kinetic parameters are shown in Table 1. These are compared to those of short chain thiocholine esters such as ATCh and PrTCh. As shown in Table 1, the Michaelis constant, K_m, increases with acyl length up to BuTCh and, thereafter, decreases as acyl group length continues to increase. The trend for k_{cat} is to decline with increasing acyl group length in the order ATCh > PrTCh >> BuTCh > HexTCh > HepTCh > OcTCh. Although the reactivity of long chain thiocholine esters is much less than ATCh, AChE is still needed to hydrolyze the substrates. We confirm this as in the presence of 0.1 mM edrophonium these substrates were not hydrolyzed to any capacity. Edrophonium is an active site directed inhibitor of AChE with a K_i in the submicromolar range.[9] These substrates are also hydrolyzed by 25 μM NaOH with an initial rate equivalent to that of AChE-catalyzed hydrolysis.

pH-Rate Profile. The pH-rate profile for electric eel

Table 1. Kinetic Parameters for AChE-Catalyzed Hydrolysis of Thiocholine Esters

Enzyme	Substrate	K_m (mM)	k_{cat} (s^{-1})
EE-AChE	ATCh[a]	0.11 ± 0.009	8800 ± 200
	PrTCh[a]	0.15 ± 0.01	3660 ± 70
	BuTCh[a]	1.20 ± 0.10	95 ± 3
	BzCh[a]	0.18 ± 0.04	6.0 ± 0.7
	HexTCh	0.83 ± 0.09	53 ± 3
	HepTCh	0.65 ± 0.08	44 ± 3
	OcTCh	0.48 ± 0.09	38 ± 2
Horse serum BuChE	BuTCh	0.29 ± 0.015	791 ± 12
	HexTCh	0.12 ± 0.003	482 ± 14
	OcTCh	0.11 ± 0.008	387 ± 13

[a]Data taken from reference 12.

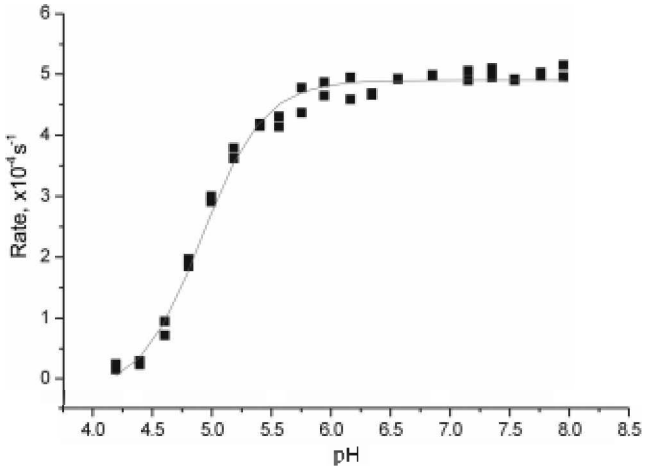

Figure 1. pH-V/K profile for EE-AChE-Catalyzed Hydrolysis of HexTCh. V/K values were determined by first order kinetics. The initial HexTCh concentration was 0.04 mM.

Table 2. The kinetic parameters in H_2O and D_2O buffers

Enzyme	Substrate	$^{D2O}K_m$	$^{D2O}V_{max}$	$^{D2O}V/K$
EE-AChE	HexTCh	1.62 ± 0.08	1.79 ± 0.05	1.10 ± 0.09
	OcTCh	1.28 ± 0.01	1.39 ± 0.05	1.08 ± 0.06
Human AChE	HexTCh	2.04 ± 0.03	1.71 ± 0.05	0.87 ± 0.07
Horse BuChE	HexTCh	2.04 ± 0.05	2.52 ± 0.05	1.24 ± 0.08

AChE-catalyzed hydrolysis of the more reactive substrates gives a typical sigmoidal curve. In contrast, the pH-V/K profile for electric eel AChE-catalyzed hydrolysis of BuTCh provided a complex sigmoidal curve. The pH-rate profile of electric eel AChE-catalyzed hydrolysis of HexTCh under V/K condition in which the substrate concentration is much lower than the K_m value proved to be different from that of short chain thiocholine esters, as shown in Figure 1. A nonlinear least-square fit to the known equation gives a pK_a value of 4.87 ± 0.06.[19] This value is similar to one of the pK_a

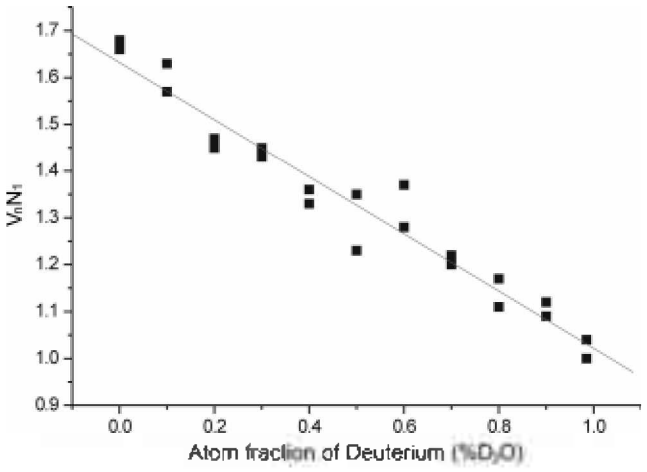

Figure 2. Proton Inventory of AChE-Catalyzed Hydrolysis of HexTCh. V values were determined by measuring initial rates of HexTCh hydrolysis under V_{max} condition, [HexTCh] = 8 mM (=10 K_m).

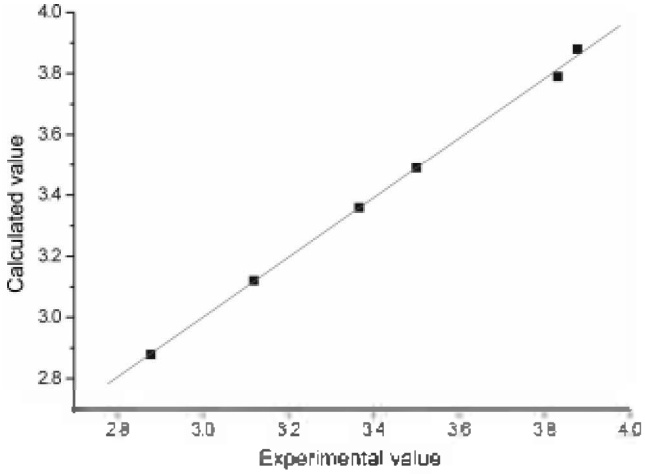

Figure 3. Comparison of calculated and experimental-logK_m values of AChE-Catalyzed Hydrolysis of the long chain thiocholine esters. The detailed procedure is in the Materials and Methods section.

values for the electric eel AChE-catalyzed hydroysis of BuTCh, 4.72 ± 0.02.[8]

Solvent Isotope Effect and Proton Inventory. The kinetic parameters are measured in H_2O and D_2O buffers and the isotope effects are calculated and shown in Table 2.

Discussion

Although the studied long chain thiocholine ester substrates proved not very reactive towards AChE, they were, nevertheless, hydrolyzed by the enzyme. Without the presence of enzyme, these compounds are not hydrolyzed at a detectable rate and, coadministration of active site directed inhibitors blocked their enzyme-catalyzed hydrolysis. A simple comparison of the initial rates of the OH^--catalyzed and AChE-catalyzed hydrolysis of HexTCh shows that the enzyme catalyzed reaction is about 10^7 times faster than the base-catalyzed reaction. This result is consistent with the rate acceleration by the enzyme for AChE-catalyzed hydrolysis of BzCh.[8] The affinity of long chain thiocholine esters to AChE is greater than that of short chain thiocholine esters. The active site of BuChE is bigger than that of AChE and it is therefore easier for BuChE to accommodate bigger substrates than AChE.[9]

The K_m values of the thiocholine esters are well correlated to the inhibition of AChE by the thiocarbonate derivatives of choline.[20] As the length of alkyl group increases up to hexyl, the inhibition potency increases. The IC_{50} of the choline ethylthiocarbonate is greater than 10 mM, while the IC_{50} of choline hexylthiocarbonate is in the submicromolar range. Most of the inhibitors are competitive inhibitors. Thus, these compounds compete with substrates for the enzyme active site. Cho *et al*. measured the IC_{50} of choline esters for the AChE-catalyzed hydrolysis of acetylthiocholine.[21] Their results show that as the length of the acyl group increases, from pentanoyl to decyl, the IC_{50} value decreases. Based on this Cho *et al*. suggested the presence of a hydrophobic acyl

pocket, separate from an acetyl-binding site, is in the active site.

The pH-profile for the more reactive substrates, such as ATCh or PrTCh, gives a pK_a value of 6.30 which indicates that the active catalytic triad S200-H440-E327 functions in catalysis. The pH-rate profile for the electric eel AChE-catalyzed hydrolysis of BuTCh gave two pK_a values of 4.72 and 6.30. Quinn *et al.* interpreted this result to mean that parallel mechanisms may operate in the AChE-catalyzed hydrolysis of choline esters.[8,10] The pK_a value of 4.87 determined from pH-V/K profile study with HexTCh is comparable to the lower pK_a value of BuTCh. The pK_a value of 4.77 determined from pH-V profile for the AChE-catalyzed hydrolysis of BzCh is also comparable to the value.

The kinetic solvent isotope effects show that proton transfer is involved in the deacylation step. The trend is the same for human AChE and horse BuChE. The kinetic solvent isotope effect on the Michaelis constant, K_m, and on V_{max} are approximately the same. Consequently, there is only small kinetic solvent isotope effect on V/K. These results imply that either the acylation step is not a rate determining step or that there is no proton transfer involved in the transition state in the acylation step. Proton inventories on V and V/K of the AChE-catalyzed hydrolysis of HexTCh provide information to study protonic interactions in the transition state. As shown in Figure 2, the proton inventories on both V and V/K (data not shown) are straight.

Ligand-receptor binding studies have been investigated with AChE and E2020 and related inhibitors. Specifically, a receptor-independent 3D QSAR was developed for a set of 15 compounds identical to E2020 (Aricept), except for substitution onto the aromatic unit of the indanone ring. The following equation was obtained from the studies for the interaction between AChE and E2020.[22-24]

$$-\log IC_{50} = 2.21C_4 - 6.65U_T + 1.18U_T^2 - 162.99\ (\text{HOMO}) - 8.58\ (\text{HOMO})^2 - 757.2$$

$N = 15$, $R = 0.94$, SD = 0.25, $F = 14.8$, U_T (max) = 2.8 Debye, HOMO (max) = -9.49 eV

Where C_4 is the HOMO out-of-plane π orbital coefficient of ring carbon four. U_T is the dipole moment of the substituted indanone ring, and HOMO, the highest occupied molecular orbital of the indanone ring. We undertook a similar study with AChE and thiocholine substrates and obtained the following equation,

$$-\log K_m = -0.530\,U_T + 0.107U_T^2 + 2.758[\text{HOMO}] + 30.786$$

where, $N = 6$, $R^2 = 0.99981$, $F = 348.846$

As shown in Figure 3, the experimental values correlate well with the calculated ones. Thus, the dipole moment plays an important role for the binding between the substrate and the enzyme active site. In the previous described study on E2020 and its derivatives, the charge-π interaction plays critical role for the binding of the inhibitors to the enzyme active site. Ripoll *et al.* have reported that AChE has a remarkably large dipole moment along the axis defining the center of the aromatic gorge.[18] Thus, dipole-dipole interactions may play an essential role in the long range molecular recognition of AChE ligands. The hydrophobic region of long chain thiocholine substrates likely interacts with the enzyme much in the same way that the indanone group of E2020 interacts with the enzyme. The primary feature of substrate binding to the enzyme can be characterized by a long substrate that possesses a dipole running through the axis of the compound which slides down the narrow gorge to interact with the enzyme active site.

In summary, taken together, our results indicate that long chain thiocholine esters are substrates for AChE, and the chemical procedure involved in their hydrolysis is somewhat different from the catalytic triad mechanism. Rather, the procedure resembles a parallel mechanism in which a water molecule attacks the carbonyl carbon of the substrate and the acylation step does not contribute to the rate determination. The dipole interaction plays a pivotal role to allow the binding of the substrate to the enzyme active site to initiate the process.

Acknowledgment. The authors thank much Dr. Quinn of the University of Iowa for helpful discussion and comments. This work was supported by the Korea Science and Engineering Foundation (KOSEF) through the Advanced Material Research Center for Better Environment at Hanbat National University.

References

1. Rosenberry, T. L. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1975**, *43*, 103-218.
2. Quinn, D. M. *Chem. Rev.* **1987**, *87*, 955-979.
3. Finkelstein, B. L.; Benner, E. A.; Hendrixson, M. C.; Kranis, K. T.; Rauh, J. J.; Sethuraman, M. R.; McCann, S. F. *Bioorg. Med. Chem.* **2002**, *10*, 599-613.
4. Rocca, P.; Cocuzza, E.; Marchiaro, L.; Bogetto, F. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **2002**, *26*, 369-373.
5. Potkin, S. G.; Anand, R.; Fleming, K.; Alva, G.; Keator, D.; Carreon, D.; Messina, J.; Wu, J. C.; Hartman, R.; Fallon, J. H. *Int. J. Neuropsychopharmacol.* **2001**, *4*, 223-230.
6. Sussman, J. L.; Harel, M.; Frolow, F.; Oefner, C.; Goldman, A.; Toker, L.; Silman, I. *Science* **1991**, *253*, 872-879.
7. Bourne, Y.; Taylor, P.; Bougis, P. E.; Marchot, P. *J. Biol. Chem.* **1999**, *274*(5), 2963-2970.
8. Selwood, T.; Feaster, S. R.; States, M. J.; Pryor, A. N.; Quinn, D. M. *J. Am. Chem. Soc.* **1993**, *115*, 10477-10482.
9. Saxena, A.; Redman, A. M. G.; Jiang, X.; Lockridge, O.; Doctor, B. P. *Biochem.* **1997**, *36*, 14642-14651.
10. Quinn, D. M.; Selwood, T.; Pryor, A. N.; Lee, B. H.; Leu, L. S.; Acheson, S. A.; Silman, I.; Doctor, B. P.; Rosenberry, T. L. In *Multidisciplinary Approaches to Cholinesterase Functions*; Shafferman, A., Velan, B., Eds.; Plenum: New York, 1992; pp 141-148.
11. Ellman, G. L.; Coutney, K. D.; Andres, V., Jr.; Featherstone, R. M. *Biochem. Pharmacol.* **1961**, *7*, 88-95.
12. Pryor, A. N.; Selwood, T.; Leu, L. S.; Andracki, M. A.; Lee, B. H.; Rao, M.; Rosenberry, T.; Doctor, B. P.; Silman, I.; Quinn, D. M. *J. Am. Chem. Soc.* **1992**, *114*, 3896-3900.
13. Salomma, P.; Schaleger, L. L.; Long, F. A. *J. Am. Chem. Soc.* **1964**, *86*, 1-7.
14. Schowen, K. B. J. In *Transition States of Biochemical Processes*;

Gandour, R. D., Schowen, R. L., Eds.; Plenum: New York, 1978; pp 225-283.
15. Schowen, K. B.; Schowen, R. L. *Methods Enzymol.* **1982**, *87*, 551-606.
16. Venkatasubban, K. S.; Schowen, R. L. *CRC Crit. Rev. Biochem.* **1985**, *17*, 1-44.
17. Quinn, D. M.; Sutton, L. D. In *Enzyme Mechanism from Isotope Effects*; Cook, P. F., Ed.; CRC Press: Boca Raton, FL, 1991; pp 73-126.
18. Ripoll, D. R.; Faerman, C. H.; Axelsen, P. H.; Silman, I.; Sussman, J. L. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 5128-5139.
19. Wentworth, W. E. *J. Chem. Edu.* **1965**, *42*, 96-103.
20. Boyle, N. A. J.; Talesa, V.; Giovannini, E.; Rosi, G.; Norton, S. J. *J. Med. Chem.* **1997**, *40*, 3009-3013.
21. Cho, Y.; Cha, S. H.; Sok, D. E. *Neurochem. Res.* **1994**, *19*, 799-803.
22. Cardozo, M. G.; Iimura, Y.; Sugimoto, H.; Yamanishi, Y.; Hopfinger, A. J. *J. Med. Chem.* **1992**, *35*, 584-593.
23. Inoue, A.; Kawai, T.; Wakita, M.; Iimura, Y.; Sugimoto, H.; Kawakami, Y. *J. Med. Chem.* **1996**, *39*, 4460-4470.
24. Camps, P.; Achab, R. E.; Görbig, D. M.; Morral, J.; Muñoz-Torreo, D.; Badia, A.; Baños, J. E.; Vivas, N. M.; Barril, X.; Orozco, M.; Luque, F. J. *J. Med. Chem.* **1999**, *42*, 3227-3242.