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Ortho Effects in Quantitative Structure-activity Relationships for Acetylcholinesterase Inhibition by Aryl Carbamates

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***Ortho*-substituted phenyl-*N*-butyl carbamates (1-9) are characterized as "pseudo-pseudo-substrate" inhibitors of acetylcholinesterase. Since the inhibitors protonate at pH 7.0 buffer solution, the virtual inhibition constants (K_i's) of the protonated inhibitors are calculated from the equation, $-\log K_i' = -\log K_i - \log K_b$. The logarithms of the inhibition constant (K_i), the carbamylation constant (k_c), and the bimolecular inhibition constant (k_i) for the enzyme inhibitions by carbamates 1-9 are multiply linearly correlated with the Hammett *para*-substituent constant (σ_p), the Taft-Kutter-Hansch *ortho* steric constant (E_S), and the Swan-Lupton *ortho* polar constant (F). Values of ρ, δ, and f for the $-\log K_i$-, $\log k_c$-, and $\log k_i$-correlations are −0.6, −0.16, 0.7; 0.11, 0.03, −0.3; and −0.5, −0.12, 0.4, respectively. The K_i step further divides into two steps: 1) the pre-equilibrium protonation of the inhibitors, K_b step and 2) formation of a negatively charged enzyme-inhibitor Michaelis-Menten complex—virtual inhibition, K_i' step. The K_i step has little *ortho* steric enhancement effect; moreover, the $k_c$step is insensitive to the *ortho* steric effect. The f value of 0.7 for the K_i step indicates that *ortho* electron-withdrawing substituents of the inhibitors accelerate the inhibition reactions from the *ortho* polar effect; however, the f value of −0.3 for the $k_c$step implies that *ortho* electron-withdrawing substituents of the inhibitors lessen the inhibition reactions from the *ortho* polar effect.**

Keywords: Acetylcholinesterase; QSAR; *Ortho* effects; Carbamate inhibitors

[a]*Abbreviations*: ABS, acyl binding site; ACh, acetylcholine; AChE, acetylcholinesterase; AS, anionic substrate binding site; ATCh, acetylthiocholine; δ, intensity factor for Taft equation; DTNB, 5,5′-dithio-bis-2-nitrobenzoate; ES, esteratic site; E_s, Taft-Kutter-Hansch *ortho* steric constant; F, Swain-Lupton-Hansch *ortho* polar constant; f, intensity factor to the *ortho* polar constant; k_c, carbamylation constant; k_d, decarbamylation constant; K_i, inhibition constant; K_i', virtual inhibition constant; k_i, bimolecular inhibition constant; LFER, linear free energy relationship; OAH, oxyanion hole; PAS, peripheral anionic binding sites; QSAR, quantitative structure activity relationship; ρ, Hammett reaction constant; σ_p, Hammett *para*-substituent constant

INTRODUCTION

Acetylcholinesterase (AChE[a], EC 3.1.1.7) plays a vital role in the central and peripheral nervous systems, where it catalyzes the hydrolysis of the neurotransmitter acetylcholine (ACh).[1] Many X-ray structures of AChEs and AChE-inhibitor complexes have been reported recently.[2–5] The active site of AChE contains, (a) an esteratic site (ES) comprised of the catalytic triad Ser200-His440-Glu327, which is located at the bottom of a 20 Å gorge, (b) an oxyanion hole (OAH) composed of Gly118, Gly119, and Ala201, that stabilizes the tetrahedral intermediate, (c) an anionic substrate binding site (AS) composed of Trp84, Glu199, and Phe330, that contains a small number of negative charges but many aromatic residues, where the quaternary ammonium pole of ACh and of various active site ligands binds through a preferential interaction of quaternary nitrogens with the π electrons of aromatic groups, and (d) an acyl binding site (ABS) composed of Phe288 and Phe290, that binds the acetyl group of ACh (Figure 1).[1–5] Besides the active site, AChE also has a peripheral anionic binding site (PAS) composed of Trp279, Tyr70, Tyr121 and Asp72, which is located at the entrance (mouth) of the active site gorge that may bind to tacrine[2–8] and cage amines[30] (Figure 1).

In Alzheimer's disease, a neurological disorder, cholinergic deficiency in the brain has been reported.[9–11] Because of the pivotal role that AChE plays in the nervous system, the enzyme has long

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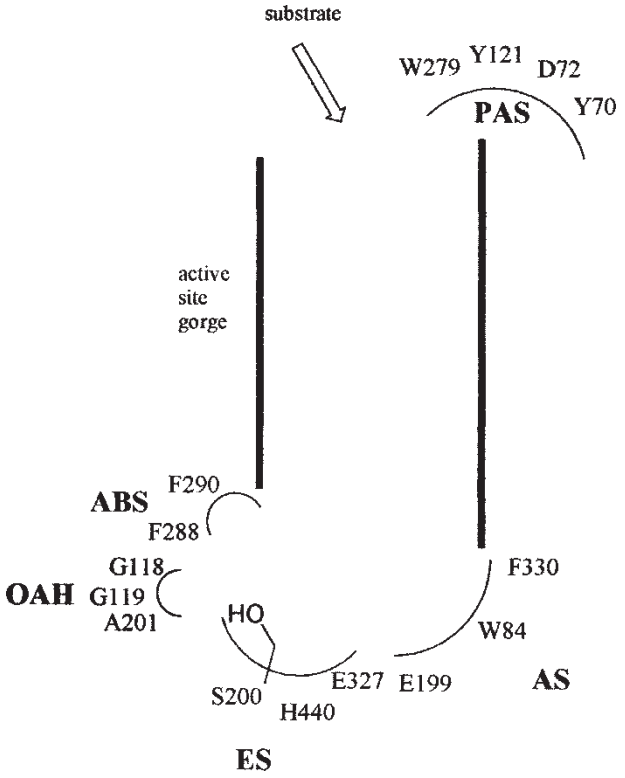


FIGURE 1 The active site and PAS of AChE.

been an attractive target for the rational design of mechanism-based inhibitors as drugs for treatment of Alzheimer's disease. Rivastigmine (Figure 2), trade name of Exelon, is a carbamate inhibitor of AChE already in use for the treatment of Alzheimer's disease.[12]

The mechanism for AChE-catalyzed hydrolysis of substrate is formation of the enzyme-substrate Michaelis-Menten complex via nucleophilic attack of the active site Ser200 on the substrate then formation of the acyl enzyme intermediate from the Michaelis-Menten complex (Scheme 1). In the presence of substrate, the pseudo-substrate AChE inhibitions by

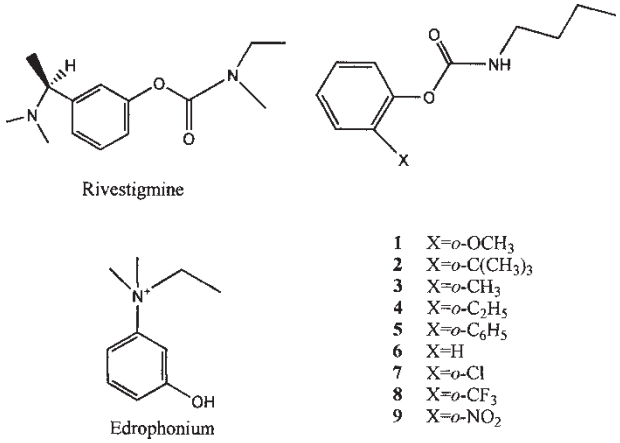
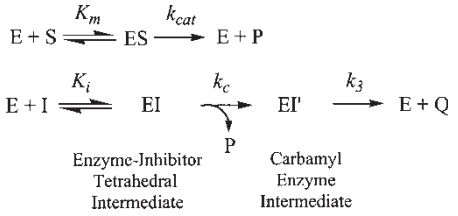


FIGURE 2 Structures of Rivestigmine, Edrophonium, and inhibitors **1**–**9**.



SCHEME 1 Kinetic scheme for pseudo-substrate inhibition of AChE in the presence of substrate.

aryl carbamates have been proposed (Scheme 1).[13] The carbamylation stage is rapid compared to subsequent decarbamylation ($k_c >> k_d$), thus the two stages are easily resolved kinetically.[14–17,19–21] In the presence of a carbamate inhibitor, time courses for hydrolysis of acetylthiocholine are biphasic, and k_{app} values can be calculated as in Equation (1).[16,20,21]

$$A = A_0 + (v_o - v_{ss})(1 - \exp(-k_{app}\, t))/k_{app} + v_{ss}t \quad (1)$$

In Equation (1), A_0, k_{app}, v_o, and v_{ss} are the absorbance at t = 0, the observed first-order inhibition rate constant, the initial velocity, and the steady-state velocity, respectively.[16,20,21] Once k_{app} values have been determined at various inhibitor concentration, the resulting data are fitted to Equation (2) to obtain K_i and k_c values. In other words, K_i and k_c values are obtained from each nonlinear least squares curve fit of k_{app} values against [I] according to Equation (2) (Figure 3).[15–17,19–21]

$$k_{app} = kc[\mathrm{I}]/(K_i(1 + [\mathrm{S}]/K_m) + [\mathrm{I}]) \quad (2)$$

The method we used to obtain K_i and k_c values is called the continuous assay method and is much more rapid than a traditional stopped-time (or dilution) assay method.[21] The bimolecular rate constant, $k_i = k_c/K_i$, is related to overall inhibitory potency. Thus, aryl carbamates are called pseudo-substrate or

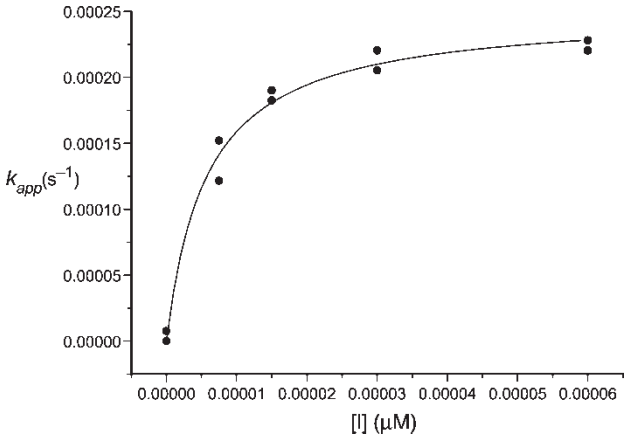


FIGURE 3 Nonlinear least-squares curve fit of k_{app} against [I]. The reactions were run as describe in Materials and Methods. The inhibitor is *o*-nitrophenyl-*N*-butylcarbamate (**9**). The solid curve is a least-squares fit to Equation (3); the parameters of the fit are $K_i = 2.4 \pm 0.4\,\mu M$ and $k_c = (2.5 \pm 0.2) \times 10^{-4} s^{-1}$ (Table I).

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active site-directed irreversible inhibitors of AChE. However, aryl carbamates do not meet the third criterion for the pseudo-substrate inhibitors as proposed by Abeles and Maycock,[18] in that carbamyl enzyme activities are not reactivated in the presence of a reversible inhibitor, Edrophonium (Figure 2).[3,4] Edrophonium cannot enter the active site of the carbamyl enzyme because the carbamyl moiety stretches along the active site gorge and/or due to the conformational change for His440 and Phe330 of the carbamyl enzyme.[5,12] Therefore, like Rivastigmine,[12] aryl carbamates (Figure 2) are characterized as pseudo-substrate inhibitors of AChE.[19]

Quantitative structure-activity relationships (QSARs) represent an attempt to correlate structural properties of compounds with biological activities or chemical reactivities.[22,23] These chemical descriptors, which include parameters to account for hydrophobicity, electronic, inductive, or polar properties, and steric effects, are determined empirically or by calculation. Little additional development of QSAR has occurred since the work of Louis Hammett, who has correlated electronic properties of substituted benzoic acids with their equilibrium constants and reactivities by the Hammett equation (Equation 3).[22,23]

$$\log k = h + \rho\sigma \tag{3}$$

In Equation (3), the h value is the $\log k_0$ value for the standard reaction (unsubstituted benzoic acid) and the parameters ρ and σ are the reaction constant and the Hammett substituents constant, respectively. The investigation also reveals that *meta*- and *para*-substituted compounds generally correlate well but *ortho*-substituted ones do not.[22] *Ortho* problems due to complications from direct steric and polar effects, is not generally applicable.[24] According to Fujita and Nishioka's suggestion, the total *ortho* effect is composed of the ordinary polar effect, the *ortho* steric effect, and the *ortho* polar effect (Equation 4).[22,24]

$$\log k = h + \rho\sigma_p + \delta E_S + fF \tag{4}$$

In Equation (4), the parameters h, ρ, σ, E_S, δ, F, and f are the $\log k_o$ value, the reaction constant for ordinary polar effect, the Hammett substituent constant, the Taft-Kutter-Hansch *ortho* steric constant, the intensity factor to the *ortho* steric constant, the Swain-Lupton-Hansch *ortho* polar constant, and the intensity factor to the *ortho* polar constant, respectively. Once K_i k_c, and k_i values have been determined (Table I), the resulting data are fitted to Equation (4) to obtain h, ρ, δ, and f values. In other words, h, ρ, δ, and f values are obtained from each multiple regression of K_i, k_c, or k_i values against σ, E_S, and F values[22–24] according to Equation (4).

Aryl carbamates, such as *meta*- and *para*-substituted phenyl-*N*-substituted carbamates (**10**), have been characterized as pseudo-substrate inhibitors of AChE and have been correlated with the Hammett equation.[13] In this paper, *ortho*-substituted phenyl-*N*-butyl carbamates (**1-9**) are synthesized to explore the *ortho* effects in QSARs for AChE inhibition.

MATERIALS AND METHODS

Materials

Electrophorus electricus AChE, DTNB, and ATCh 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 F_{254}) were obtained from Merck. All other chemicals were of the highest purity available commercially.

Synthesis of Carbamates

Carbamates **1-9** were prepared from the condensation of the corresponding phenol with *n*-butyl isocyanate in the presence of a catalytic amount of pyridine in toluene (80–95% yield). All compounds were purified by liquid chromatography on silica gel and characterized by ^{1}H and ^{13}C NMR spectra and high resolution mass spectra (HRMS).

***o*-Methoxyphenyl-*N*-butylcarbamate (1)**

^{1}H NMR ($CDCl_3$, 400 MHz) δ/ppm 0.94 (t, J = 7 Hz, 3H, $CH_2CH_2CH_3$), 1.39 (sextet, J = 7 Hz, 2H, $CH_2CH_2CH_3$), 1.57 (quintet, J = 7 Hz, 2H, $CH_2CH_2CH_3$),

TABLE I *Ortho* substituent and inhibition constants for the steady state AChE inhibitions by *o*-substituted phenyl-*N*-butyl carbamates (**1-9**)[a]

Inhibitor	X	σ_p	E_S^{ob}	F	pK_b	K_i(μM)	$k_c(10^{-4}s^{-1})$	$k_i(10^2 M^{-1}s^{-1})$
1	*o*-Ome	−0.27	−0.55	0.26	3.3 ± 0.1	3.0 ± 0.2	3.3 ± 0.2	1.1 ± 0.1
2	*o*-*t*-Bu	−0.2	−2.78	−0.07	3.5 ± 0.1	2.0 ± 0.4	2.9 ± 0.3	1.5 ± 0.3
3	*o*-CH_3	−0.17	−1.24	−0.04	3.6 ± 0.1	4 ± 1	3.7 ± 0.2	0.9 ± 0.2
4	*o*-Et	−0.15	−1.31	−0.05	3.6 ± 0.1	3 ± 1	3.5 ± 0.2	1 ± 0.3
5	*o*-Ph	−0.01	−1.01	0.08	4.0 ± 0.1	3.2 ± 0.8	3.0 ± 0.2	0.9 ± 0.2
6	H	0	0	0	4.0 ± 0.1	3.2 ± 0.6	3.1 ± 0.1	1.0 ± 0.2
7	*o*-Cl	0.23	−0.97	0.41	3.6 ± 0.1	4 ± 1	2.5 ± 0.2	0.6 ± 0.2
8	*o*-CF_3	0.54	−2.40	0.38	5.3 ± 0.1	2.8 ± 0.8	2.6 ± 0.2	1.0 ± 0.3
9	*o*-NO_2	0.78	−2.52[c]	0.67	6.0 ± 0.1	2.4 ± 0.4	2.5 ± 0.2	1.0 ± 0.2

[a] The K_i and k_c are obtained according to Equation (1) and $k_i = k_c/K_i$.[13–17] [b] Hydrogen as a standard.[22] [c] The maximum value for the coplanar orientation.[22]

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3.27 (q, J = 7 Hz, 2H, NHCH_2), 3.85 (s, 3H, OCH_3), 5.05 (br s, 1H, N*H*), 6.91–7.20 (m, 4H, aromatic *H*); ^{13}C NMR ($CDCl_3$, 100 MHz) δ/ppm 13.82 (CH_2CH_2 *C*H_3), 19.93 (CH_2*C*H_2CH_3), 31.93 (*C*$H_2CH_2CH_3$), 41.07 (NH*C*H_2), 55.88 (O*C*H_3), 112.22, 120.53, 123.12, 126.17 (phenyl *C*H), 139.83 (phenyl *C*-2), 151.47 (phenyl *C*-1), 154.17 (*C*═O). HRMS calculated for $C_{12}H_{17}NO_3$: 223.1208. Found: 223.1211.

o-*t*-Butylphenyl-*N*-butylcarbamate (2)

^{1}H NMR ($CDCl_3$, 400 MHz) δ/ppm 0.96 (t, J = 7 Hz, 3H, $CH_2CH_2CH_3$), 1.32 (s, 9H, C(CH_3)$_3$), 1.38 (sextet, J = 7 Hz, 2H, $CH_2CH_2CH_3$), 1.58 (quintet, J = 7 Hz, 2H, $CH_2CH_2CH_3$), 3.31 (q, J = 7 Hz, 2H, NHCH_2), 5.02 (br s, 1H, N*H*), 7.04–7.37 (m, 4H, aromatic *H*); ^{13}C NMR ($CDCl_3$, 100 MHz) δ/ppm 13.84 (CH_2CH_2 *C*H_3), 19.98 (CH_2*C*H_2CH_3), 30.34 (C(*C*H_3)$_3$), 32.09 (*C*H_2CH_2 CH_3), 34.23 (*C*(CH_3)$_3$), 41.06 (NH*C*H_2), 124.05, 125.04, 126.63, 126.76 (phenyl *C*H), 141.08 (phenyl *C*-1), 149.35 (phenyl *C*-2), 154.42 (*C*═O). HRMS calculated for $C_{15}H_{23}NO_2$: 249.1729. Found: 249.1733.

o-Methylphenyl-*N*-butylcarbamate (3)

^{1}H NMR ($CDCl_3$, 400 MHz) δ/ppm 0.94 (t, J = 7 Hz, 3H, $CH_2CH_2CH_3$), 1.37 (sextet, J = 7 Hz, 2H, CH_2 CH_2CH_3), 1.54 (quintet, J = 7 Hz, 2H, $CH_2CH_2CH_3$), 2.21 (s, 3H, *o*-*C*H_3), 3.25 (q, J = 7 Hz, 2H, NHCH_2), 5.09 (br s, 1H, N*H*), 7.05–7.25 (m, 4H, aromatic *H*); ^{13}C NMR ($CDCl_3$, 100 MHz) δ/ppm 13.81 (CH_2CH_2 *C*H_3), 16.16 (*o*-*C*H_3), 19.96 (CH_2*C*H_2CH_3), 32.00 (*C*$H_2CH_2CH_3$), 41.00 (NH*C*H_2), 122.02, 125.34, 126.58, 130.77 (phenyl *C*H), 130.42 (phenyl *C*-2), 149.29 (phenyl *C*-1), 154.24(*C*═O). HRMS calculated for $C_{12}H_{17}NO_2$: 207.1260. Found: 207.1252.

o-Ethylphenyl-*N*-butylcarbamate (4)

^{1}H NMR ($CDCl_3$, 400 MHz) δ/ppm 0.94 (t, J = 7 Hz, 3H, $CH_2CH_2CH_3$), 1.19 (t, 3H, J = 7 Hz, *o*-CH_2CH_3), 1.37 (sextet, J = 7 Hz, 2H, $CH_2CH_2CH_3$), 1.54 (quintet, J = 7 Hz, 2H, $CH_2CH_2CH_3$), 2.59 (q, J = 7 Hz, 2H, *o*-CH_2CH_3), 3.24 (q, J = 7 Hz, 2H, NHCH_2), 5.10 (br s, 1H, N*H*), 7.05–7.25 (m, 4H, aromatic *H*); ^{13}C NMR ($CDCl_3$, 100 MHz) δ/ppm 13.79 (CH_2CH_2*C*H_3), 14.31 (*o*-CH_2*C*H_3), 19.93 (CH_2*C*H_2CH_3), 23.18 (*C*H_2CH_2 CH_3), 31.98 (*o*-*C*H_2CH_3), 41.00 (NH*C*H_2), 122.28, 125.46, 126.49, 129.02 (phenyl *C*H), 136.07 (phenyl *C*-2), 148.81 (phenyl *C*-1), 154.49 (*C*═O). HRMS calculated for $C_{13}H_{19}NO_2$: 221.1416. Found: 221.1407.

o-Biphenyl-*N*-butylcarbamate (5)

^{1}H NMR ($CDCl_3$, 400 MHz) δ/ppm 0.96 (t, J = 7 Hz, 3H, $CH_2CH_2CH_3$), 1.32 (sextet, J = 7 Hz, 2H, CH_2 CH_2CH_3), 1.43 (quintet, J = 7 Hz, 2H, $CH_2CH_2CH_3$), 3.19 (q, J = 7 Hz, 2H, NHCH_2), 5.03 (br s, 1H, N*H*), 7.27–7.51 (m, 9H, aromatic *H*); ^{13}C NMR ($CDCl_3$, 100 MHz) δ/ppm 13.74 (CH_2CH_2*C*H_3), 19.73 (CH_2 *C*H_2CH_3), 31.81 (*C*$H_2CH_2CH_3$), 40.78 (NH*C*H_2), 127.93, 128.13, 129.07, 123.10, 125.57, 127.01, 130.48 (phenyl *C*H), 134.82 (phenyl *C*-1′), 137.62 (phenyl *C*-2), 147.71 (phenyl *C*-1), 154.31 (*C*═O). HRMS calculated for $C_{17}H_{19}NO_2$: 269.1416. Found: 269.1419.

Phenyl-*N*-butylcarbamate (6)[13]

^{1}H NMR ($CDCl_3$, 400 MHz) δ/ppm 0.96 (t, J = 7 Hz, 3H, $CH_2CH_2CH_3$), 1.35 (sextet, J = 7 Hz, 2H, CH_2 CH_2CH_3), 1.56 (quintet, J = 7 Hz, 2H, $CH_2CH_2CH_3$), 3.27 (q, J = 7 Hz, 2H, NHCH_2), 4.99 (br s, 1H, N*H*), 7.12–7.37 (m, 4H, aromatic *H*); ^{13}C NMR ($CDCl_3$, 100 MHz) δ/ppm 13.81(CH_2CH_2*C*H_3), 19.99 (CH_2 *C*H_2CH_3), 31.96 (*C*$H_2CH_2CH_3$), 41.00 (NH*C*H_2), 121.42 (phenyl *C*-3, *C*-5), 125.00 (phenyl *C*-4), 129.05 (phenyl *C*-2, *C*-6), 150.89 (phenyl *C*-1), 154.37 (*C*═O). HRMS calculated for $C_{11}H_{15}N_2O_4$: 193.1103. Found: 193.1104.

o-Chlorophenyl-*N*-butylcarbamate (7)

^{1}H NMR ($CDCl_3$, 400 MHz) δ/ppm 0.92 (t, J = 7 Hz, 3H, $CH_2CH_2CH_3$), 1.37 (sextet, J = 7 Hz, 2H, CH_2 CH_2CH_3), 1.52 (quintet, J = 7 Hz, 2H, $CH_2CH_2CH_3$), 3.23 (q, J = 7 Hz, 2H, NHCH_2), 5.37 (br s, 1H, NH), 7.12–7.41 (m, 4H, aromatic *H*); ^{13}C NMR ($CDCl_3$, 100 MHz) δ/ppm 13.72 (CH_2CH_2*C*H_3), 19.84 (CH_2 *C*H_2CH_3), 31.77 (*C*$H_2CH_2CH_3$), 41.04 (NH*C*H_2), 123.92, 126.23, 127.32, 129.87 (phenyl *C*H), 127.08 (phenyl *C*-2), 146.92 (phenyl *C*-1), 153.35 (*C*═O). HRMS calculated for $C_{11}H_{14}NO_2Cl$: 227.0713. Found: 227.0721.

o-Trifluoromethylphenyl-*N*-butylcarbamate (8)

^{1}H NMR ($CDCl_3$, 400 MHz) δ/ppm 0.93 (t, J = 7 Hz, 3H, $CH_2CH_2CH_3$), 1.38 (sextet, J = 7 Hz, 2H, CH_2CH_2 CH_3), 1.54 (quintet, J = 7 Hz, 2H, $CH_2CH_2CH_3$), 3.26 (q, J = 7 Hz, 2H, NHCH_2), 5.21 (br s, 1H, N*H*), 7.26–7.64 (m, 4H, aromatic *H*); ^{13}C NMR ($CDCl_3$, 100 MHz) δ/ppm 13.75 (CH_2CH_2*C*H_3), 19.86 (CH_2 *C*H_2CH_3), 31.84 (*C*$H_2CH_2CH_3$), 41.11 (NH*C*H_2), 122.10 (q, $^1J_{CF}$ = 180 Hz, *C*F_3), 123.05 (q, $^2J_{CF}$ = 20 Hz, phenyl *C*-2), 125.07, 126.48, 132.65 (phenyl *C*H), d, 148.38 (phenyl *C*-1), 153.42 (*C*═O). HRMS calculated for $C_{12}H_{14}NO_2F_3$: 261.0977. Found: 261.0969.

o-Nitrophenyl-*N*-butylcarbamate (9)

^{1}H NMR ($CDCl_3$, 400 MHz) δ/ppm 0.94 (t, J = 7 Hz, 3H, $CH_2CH_2CH_3$), 1.32 (sextet, J = 7 Hz, 2H, CH_2 CH_2CH_3), 1.51 (quintet, J = 7 Hz, 2H, $CH_2CH_2CH_3$), 3.28 (t, J = 7 Hz, 2H, NHCH_2), 5.28 (br s, 1H, N*H*), 7.22–8.05 (m, 4H, aromatic *H*); ^{13}C NMR ($CDCl_3$, 100 MHz) δ/ppm 13.70 (CH_2CH_2*C*H_3), 19.80 (CH_2 *C*H_2CH_3),

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TABLE II Correlation results for the steady state AChE inhibition by *o*-substituted phenyl-*N*-butyl carbamates (**1–9**)[a]

	$\log K_b$	$-\log K_i'$ [b]	$-\log K_i$	$\log k_c$	$\log k_i$
ρ	-2.52 ± 0.06	1.9 ± 0.6	-0.6 ± 0.2	0.11 ± 0.06	-0.5 ± 0.1
δ	0 ± 0	-0.16 ± 0.05	-0.16 ± 0.05	0.03 ± 0.02	-0.12 ± 0.04
f	0 ± 0	0.7 ± 0.3	0.7 ± 0.3	-0.3 ± 0.1	0.4 ± 0.2
H	4.00 ± 0.02	9.2 ± 0.2	5.21 ± 0.08	-3.34 ± 0.04	1.87 ± 0.06
R [c]	0.998	0.904	0.915	0.934	0.956

[a] Correlation of $\log K_b$, $-\log K_i$, $\log k_c$, and $\log k_i$ (Table I) with $\log k = h + \rho\sigma_p + \delta E_S^o + fF$ (Equation (4)).[22] [b] $-\log K_i' = -\log K_i - \log K_b$ (Equation (5)). [c] Correlation coefficient.

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32.03 ($CH_2CH_2CH_3$), 41.20 ($NHCH_2$), 125.40, 125.70, 125.90, 134.20 (phenyl *C*H), 142.10 (phenyl *C*-2), 144.20 (phenyl *C*-1), 153.10 (*C*=O). HRMS calculated for $C_{11}H_{14}N_2O_4$: 238.0954. Found: 238.0959.

Instrumental Methods

^1H and ^{13}C NMR spectra were recorded at 400 and 100 MHz, respectively, on a Varian-GEMINI 400 spectrometer. HRMS were recorded at 70 eV on a Joel JMS-SX/SX-102A mass spectrometer. All steady state kinetic data were obtained from a UV-VIS spectrometer (HP 8452, Beckman DU-650, Spectronic Genesys 8, Agilent 8453, or Scinco S-3100) with a cell holder circulated with a water bath.

Data Reduction

Origin (version 6.0) was used for linear, nonlinear, and multiple linear regression analyses.

Steady-state Enzyme Kinetics

The AChE inhibitions by carbamates **1-9** were assayed by Ellman's method.[13,19–21,28–30] The temperature was maintained at 25.0 ± 0.1°C by a refrigerated circulating water bath. All inhibition 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% w/v), substrate (50 μM), and varying concentrations of inhibitors. Requisite volumes of stock solution of substrate and inhibitors in acetonitrile were injected into reaction buffer via a pipette. AChE was dissolved in sodium phosphate buffer (0.1 M, pH 7.0). First-order rate constant (k_{app}) for inhibition was determined as described by Hosie *et al.* (Equation (1)).[16,20,21] The K_i and k_c values were obtained by fitting the k_{app} values and [I] to Equation (2) by nonlinear least-squares regression analyses (Figure 3).[13,15–17,19–21,28,29] Duplicate sets of data were collected for each inhibitor concentration.

RESULTS

Like Rivastigmine, carbamates **1-9** (Figure 2) are characterized as pseudo-substrate inhibitors of AChE because all inhibitors were time-dependent and followed first-order kinetics but the enzyme activities were not recovered by a competitive inhibitor, Edrophonium (Figure 2).[19] The Hammett *para*-substituent constant (σ_p), the Taft-Kutter-Hansch *ortho* steric constant (E_s), the Swain-Lupton-Hansch *ortho* polar constant (F), pK_b values, and the K_i, k_c, and k_i values for the AChE inhibitions by carbamates **1-9** are summarized (Table I).

Good multiple regression of $-\log K_I$, $\log k_c$ and $\log k_i$ with σ_p, E_s, and F (Equation 4) are observed (Table II). All k_i data confirm those of K_i and k_c (Table II). Since the K_i step composes the protonation $K_b$step and the K_i' step (discussion below) (Figure 4), the virtual inhibition constant, K_i', is calculated according to Equation (5).

$$-\log K_i' = -\log K_i - \log K_b \quad (5)$$

Meanwhile, good multiple linear correlations of the $-\log K_i'$ values against Equation (4) are also observed (Table II).

Compared to *p*- and *m*-substituted carbamates (**10**),[13] the AChE inhibitions by *o*-substituted carbamates **1–9** are insensitive to substituents (Table I). For substituted phenyl *N*-butylcarbamates, the electron-donating substituent (such as methoxy) at the *para* position is more potent than that at the *ortho* position and the latter is more potent than that at the *meta* position (discussion below).[13] For substituted phenyl *N*-butylcarbamates, the electron-withdrawing substituent such as nitro at the *ortho* position is more potent than that at the *meta* or *para* position (discussion below).[13]

DISCUSSION

At a glance, the ρ value of -0.6 for the $-\log K_i$-correlation against Equation (4) (Table II) does not agree with formation of the negative charged enzyme-carbamate Michaelis-Menten complex, which should have a positive ρ value in this correlation. Therefore, the K_i step should divide into the protonation K_b step and the virtual inhibition K_i' step (discussion below) (Figure 4).[19] The ρ value of -0.6 for the $-\log K_i$-correlation agrees with the ρ value of 1.9 for $-\log K_i'$-correlation plus the ρ value of -2.5 for the $\log K_b$-correlation (Table II).[17,19]

A three-step AChE inhibition mechanism by carbamates **1-9** is proposed (Figure 4).[19] The first

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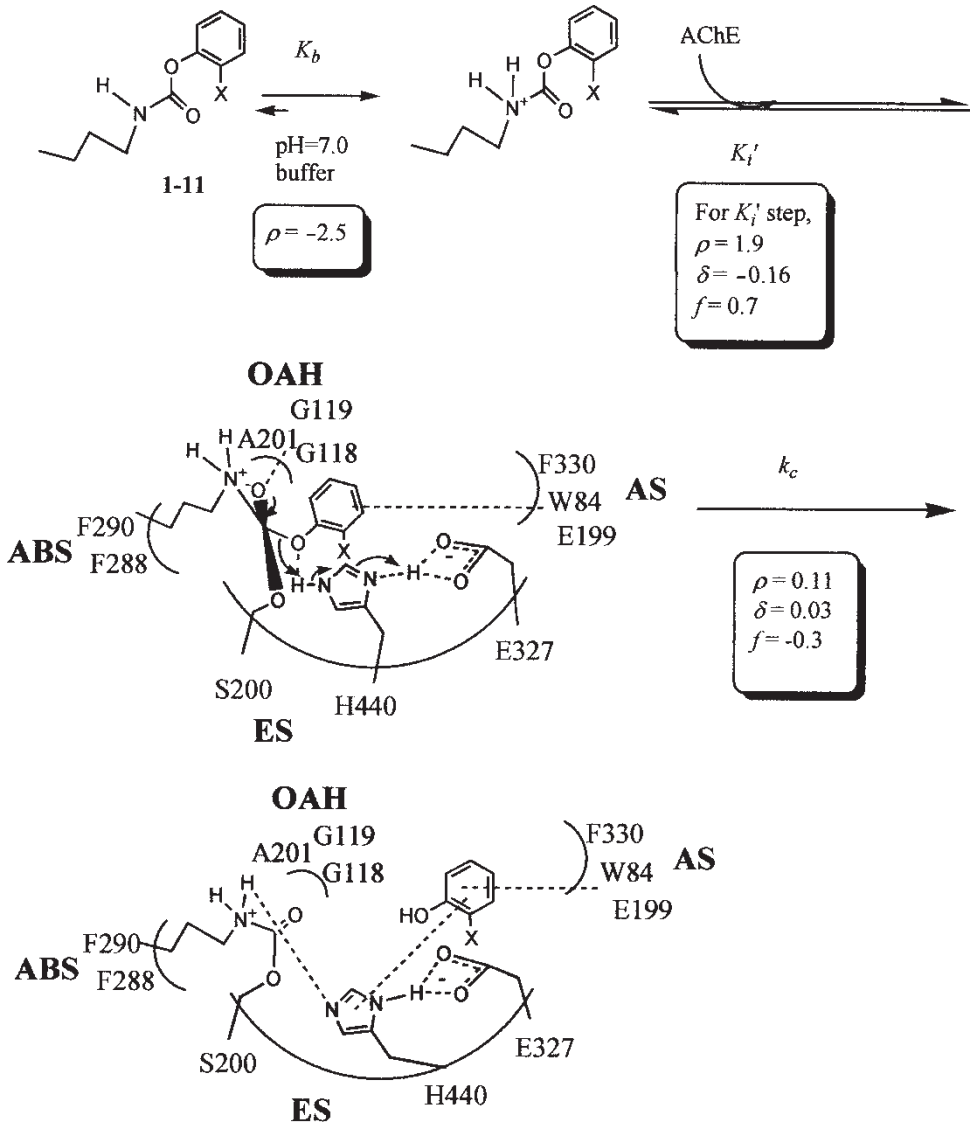

FIGURE 4 The proposed mechanism for the AChE inhibition by carbamates **1-9**.

step (K_b) is protonation of the inhibitors. Since carbamates **1-9** are weak bases (Table I), all carbamates **1-9** are protonated under the enzyme reaction buffer conditions (pH = 7.0). The second step (K_i') is formation of the enzyme-carbamate Michaelis-Menten adduct from nucleophilic attack of Ser200 on the protonated inhibitors. Therefore, K_i' is the virtual inhibition constant. The third step (k_c) is formation of the carbamyl enzyme from this Michaelis-Menten adduct (Scheme 1, Figure 4).

For the K_b step, the multiple linear correlation of the logK_b values of carbamates **1-9** against Equation (4) gives the ρ value of -2.5 and negligible δ and f values (Table II). Therefore, the *ortho* effects do not play an important role in the K_b step because *ortho* substituents are far away from the reaction (protonation) center.

For the K_i' step, the ρ value of 1.9 for the $-\log K_i'$-correlation suggests that the K_i' step is formation of the negative charged enzyme-carbamate Michaelis-Menten complex (Figure 4).[19] The δ value of -0.16 (Table II) indicates little *ortho* steric enhancement for this step probably because bulky *ortho* substituents of the inhibitors fit well into the large active site of the enzyme.[30] The f value of 0.7 suggests that electron-withdrawing substituents accelerate the reaction through *ortho* polar effects. In other words, the through space field effect may play a major role in this step.

For the k_c step, the ρ value of 0.11 for the logk_c-correlation (Table II) indicates that the transition state of this step is slightly more negatively charged than the enzyme-carbamate Michaelis-Menten complex. The reason for this is because negative charges on the carbonyl oxygen redistribute to the phenol oxygen and make them closer to *ortho* substituents. Moreover, *ortho* steric effects in the k_c step are negligible ($\delta = 0.03$) (Table II). Thus, the leaving

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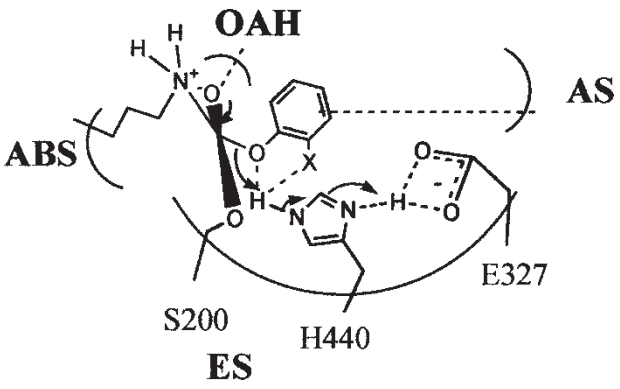

FIGURE 5 The putative interaction between His440 and the ortho substituent of the inhibitor in the enzyme-inhibitor tetrahedral intermediate. Electron-withdrawing *ortho* substituents (such as nitro) attract the His440 acidic proton through the *ortho* field effect and make the His440 proton difficult to protonate the leaving group.

group binding site of the enzyme is large enough to adapt any bulky *ortho* substituted phenol (Figure 4). Interestingly, the f value of -0.3 (Table II) indicates that the *ortho* polar effect is opposite to the ordinary polar effect ($\rho = 0.11$) in this step. In other words, the electron-withdrawing *ortho* substituents lessen the inhibition reaction probably because these substituents attract the His440 acidic proton through the *ortho* field effect and make the His440 proton less acidic and difficult to protonate the leaving group (Figure 5).

For the overall reaction, the f value of 0.4 for the logk_i-correlation (Table II) is close to that for basic hydrolysis of *o*-substituted pH phenyl acetate ($f = 0.54,\ \delta = 0.19$)[22,25–27] but the δ value of -0.12 for the logk_i-correlation is opposite. The reason why the AChE inhibitions by *o*-substituted carbamates **1-9** are insensitive to substituents (Table I) is because the ordinary polar effect ($\rho = -0.5$) and the *ortho* polar effect ($f = 0.4$) cancel each other out (Table II).

For substituted phenyl-*N*-butylcarbamates, the ordinary polar effects for *para*-[13] and *ortho*-methoxy (Table I) substituted carbamates are similar but the latter inhibitors have an extra reverse *ortho* polar effect (discussion above). On the other hand, the electron-withdrawing nitro substituent lessens the inhibition due to the ordinary polar effect but *ortho*-substituted inhibitors increase the inhibition due to the *ortho* polar effect (discussion above). Therefore, *ortho*-carbamates are more rigid in the active site of the enzyme than *para*- and *meta*-ones probably due to the putative interaction between the His440 of the enzyme and the *ortho*-substituent through *ortho* polar effect (Figure 5).

In conclusion, the *ortho* effects for QSARs for the steady-state inhibitions of AChE by aryl carbamates are better understood than before and inhibition mechanisms are proposed (Figure 4).

Acknowledgements

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