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Acetylcholinesterase Inhibition by Diaza- and Dioxophosphole Compounds: Synthesis and Determination of IC_{50} Values

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Cholinesterases are targets for organophosphorus compounds which are used as pesticides, insecticides, chemical warfare agents and drugs for the treatment of disease such as glaucoma or parasitic infections. Most organophosphorus compounds impart their toxic action via inhibition of cholinesterases by reacting at an essential serine hydroxyl group. The inhibition process depends on the leaving group, stereochemistry and reactivity of the organophosphorus compound. In this study, the inhibitory potency of two isoelectronic and isostructural diaza- and dioxophospholes A ($CH_3C_6H_3O_2P(O)Cl$) and B ($CH_3C_6H_3(NH)_2P(O)Cl$) against human acetylcholinesterase (hAChE) was examined by spectrophotometric measurements based on Ellman's method. Results indicated that compounds A and B were irreversible inhibitors with IC_{50} values of 0.48 and 1.54 mM, respectively and inactivation constants (k_i) of 0.0363 and 0.0207 min^{-1}, respectively. The differences in the inhibitory potency of two phosphole compounds is discussed with respect to their structures. In addition, the synthesis and characterization of compound A is discussed.

Keywords: Human acetylcholinesterase; Ellman's method; IC_{50} values; Phospholes; Irreversible inhibitors

INTRODUCTION

Acetylcholinesterase (AChE, EC 3.1.1.7) plays an important role in cholinergic transmission by catalyzing the rapid hydrolysis of the neurotransmitter acetylcholine into acetate and choline.[1,2] The enzyme effectively terminates the chemical impulse at rates that are similar to a diffusion-controlled process leading to a rapid and repetitive response.[2] Inhibitors of AChE, such as organophosphates and carbamates, interfere with this process and may cause a severe impairment of the nervous system or even death. Inhibition of AChE by organophosphorous compounds produced covalent conjugates with the active site serine, located at the bottom of a deep narrow gorge.[3] Many papers have reported the irreversible inhibition of human acetylcholinesterase by organophosphorous compounds.[4–11] while some has considered reversible inhibitors.[12–17] The idea that the biological activities of chemical compounds can be a function of physico-chemical properties was suggested by Frazer and Crum[18] and the biological activities of many compounds on enzymes has been reported. These bioactivities are determined with respect to their structural features as well as lipophilic (π), electronic (σ) and steric (r_v or E_s) parameters.[19,20] While detailed studies on the inhibitory potency of some chemical compounds on acetylcholinesterase have been made in the literature,[4–17] no reports on phosphol derivatives have been made. In this paper we present full experimental procedures for the synthesis and spectroscopic characterization of compound **A** and the relative inhibitory potencies of the two isoelectronic and isostructural dioxophosphol, **A**, and diazaphosphol, **B** and discuss their different inhibitory potencies.

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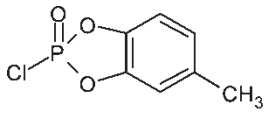
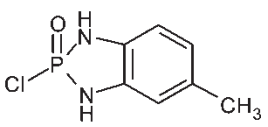


A



B

MATERIALS AND METHODS

All reactions for synthesis of the dioxophosphole, **A**, were carried out under argon atmosphere. Melting points were determined on a Gallenkamp apparatus. ^{1}H, ^{13}C and ^{31}P NMR spectra were recorded on a Bruker (Avance DRS) 500 spectrometer. ^{1}H, ^{13}C and ^{31}P chemical shifts were determined relative to TMS and 85% H_3PO_4, respectively, as external standards. IR spectra (KBr pellets) were obtained with a Shimadzu, IR-60 model spectrometer. Elemental analysis were performed using a Heraeus CHN-O-RAPID instrument. High resolution Mass spectrum was obtained with a Shimadzu model PQIIOOEX spectrometer. UV measurements were performed by Shimadzu UV-2100 spectrophotometer. All chemicals and solvents for syntheses were from Merck.

Synthesis

O,O′-(3-methyl)phenylenediphorylchloride, ($CH_3C_6H_3(O)_2P(O)Cl$), A

A mixture of 3,4-dihydroxytoluene (1.02 g, 5 mmol) and pyridine (1.58 g, 10 mmol) was added dropwise to a stirred suspension of phosphorus pentachloride (1.04 g, 5 mmol) in 30 ml dried benzene at 0°C. The mixture was stirred for 1 h, then cooled and acetic anhydride (0.5 ml, 5 mmol) added dropwise to the mixture. After filtration of the pyrridinium salt, the solvent was removed and the product was purified by column chromatography [silica gel; n-hexane/ethyl acetate (4:1)]. (yield 30%), m.p. = 62°C. Anal. Calc. for $C_{19}H_{17}N_4O_6P$: C, 41.08; H, 2.93. Found: C, 41.01; H, 2.90%. ^{1}H NMR (D_2O), δ (ppm): 2.10 (s, 3 H, CH_3), 6.46–6.95 (m, 3 H, ArH); ^{13}C NMR (D_2O), δ (ppm): 19.87 (s, CH_3), 121.21 (s), 121.34 (s), 124.0 (s), 135.0 (s), 138.1 (d., $^{2}J_{P-C} = 6.8$ Hz), 146.7 (d., $^{3}J_{P-C} = 4.4$ Hz); ^{31}P NMR (D_2O), δ (ppm): −3.06 (s); IR (KBr), ν (cm^{-1}): 3320 (w), 2900 (w), 1588 (s), 1502 (vs), 1230 (s, P═O), 1182 (s), 1108 (w), 1032 (s), 970 (vs), 802 (s), 740 (m), 585 (s), 940 (vs), 464 (vs, P-Cl). Mass spectrum (70 eV) *m/z* (%): 204 (25) M^+, 185 (100) $[CH_3C_6H_3O_2PCl]^+$, 122 (20) $[CH_3C_6H_3O_2]^+$, 66 (25) $[P - Cl]^+$, 43 (25) $[P{=}O]^+$.

N,N′-(3-methyl)phenylenediaminophosphoryl-chloridate, ($CH_3C_6H_3(NH)_2P(O)Cl$), B

N,N′-(3-methyl)phenylenediaminophosphorylchloridate, ($CH_3C_6H_3(NH)_2P(O)Cl$), B was synthesized and characterized by ^{1}H, ^{13}C, ^{31}P NMR, IR spectroscopies and elemental analysis.[21]

Kinetic Experiments

Human acetylcholinesterase (hAChE) from Sigma (50 units/785 μl) was diluted 25 times in a phosphate buffer (Na_2HPO_4/NaH_2PO_4, 70 mM, pH = 7.8). All other reagents for enzymatic experiments were from Fluka.

Activity of the enzyme was measured at 25°C by a modified Ellman's method.[22] The reaction mixture for determination of IC_{50} values consisted of: DTNB solution, 50 μl; Inhibitor, x μl ($5 < x < 400$); acetylthiocholine (ASCh) solution, 15 μl; phosphate buffer, (835 − x) μl; AChE solution, 100 μl. The concentrations of substrate (s_0), DTNB, inhibitors **A** and **B** were 1.35×10^{-4}, 10^{-4}, 0.031 and 0.025 M, respectively and the enzyme concentration in the assay conditions was 33.4×10^{-9} M. The measured K_m value for the enzyme was 108.20×10^{-5} M.

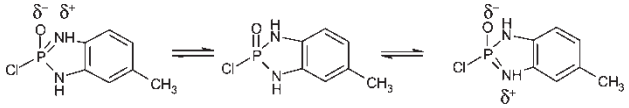
A solution containing all of above materials except the inhibitor was used to determine the activity of enzyme as a control. The reaction mixture for determination of the inhibition mechanism and k_i values was: DTNB solution, 50 μl; acetylthiocholine (ASCh) solution, 15 μl; phosphate buffer, 835 μl; a solution of the enzyme plus inhibitor, 100 μl.

RESULTS

Synthesis and Spectral Data

Compounds **A** and **B** are isoelectronic and isostructural (NH is the isostere of O) and it would be expected that these two compounds would have similar chemical properties and a similar biological behavior.

The ^{1}H NMR spectra of compounds **A** and **B** showed zero $^{7}J_{PH}$. Similar phenomena have also been observed in some phosphoramidates.[21] The reason for lack of $^{7}J_{PH}$ coupling is suggested to be due to the formation of partial multiple bonds between phosphorus and aromatic nitrogens as follows:[21]



^{13}C NMR spectrum of compound **A** indicated the coupling of aromatic carbons with phosphorus as

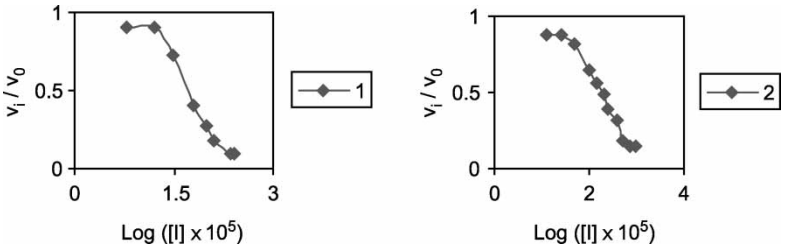


FIGURE 1 A plot of v_i/v_0 aginst log ([I] × 10^5) for inhibitors: 1 − **A** and 2 − **B**. v_i and v_0 are the activity of enzyme in the presence and absence of inhibitor, respectively and [I] is the inhibitor concentration (mol L^{-1}).

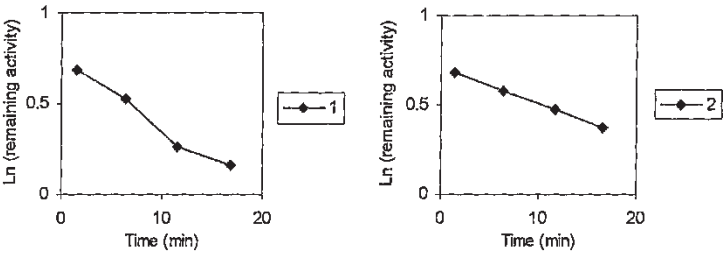


FIGURE 2 A plot of Ln (remaining activity) versus time (min) for: 1-Dioxo and 2-Diaza inhibitors.

$^2J_{P-C} = 6.8$ Hz and $^3J_{P-C} = 4.4$ Hz, while the ^{13}C NMR spectrum of compound **B** showed no coupling with phosphorus. ^{31}P NMR spectra indicated that the phosphorus atom in molecule **A** was more deshielded than in compound **B**. IR spectra showed that $\nu_{P=O}$ in compound **A** was stronger than in compound **B**. Mass spectrum of compound **A** indicated a parent ion (M^+) at 204 and other peaks corresponding to the other parts of this molecule. It can be concluded that ^1H, ^{13}C and ^{31}P NMR, IR and Mass spectra as well as elemental analysis confirmed the structure of compound **A**.

Human Acetylcholinesterase Inhibition

Enzymatic experiments on compounds **A** and **B** were performed to illustrate the inhibition behavior and different biological activity characteristics of these two phospholes. The plot of v_i/v_0 (v_i and v_0 are the activity of the enzyme in the presence and absence of inhibitors, respectively) against log [I], where [I] is the inhibitor concentration, gave the IC_{50} values of compounds **A** and **B** was 0.48 and 1.54 mM, respectively (Figure 1).

The apparent bimolecular phosphorylation rate constants (k_i) were determined from the slopes of the plots of Ln (remaining activity) vs time.[7,23] Results show that these molecules act as time-dependent irreversible inhibitors and the rate constants for inactivation of the enzyme by compounds **A** and **B** were 0.0363 and 0.0207 min^{-1}, respectively (Figure 2).[24,25] For these irreversible inhibitions, and from Figure 2 the K_i values can be obtained. These plots give an intercept at $v_i/v_o < 0$. The K_i values calculated from the equation $v_i/v_o = (1 + s_0/K_m)/(1 + s_0/K_m + i/K_i)$ for inhibitors **A** and **B** were 1.202 and 3.323 mM, respectively.

If we assume tight-binding competitive inhibition for inhibitors **A** and **B**, the K_i values can be obtained from the equation $IC_{50} = K_i(1 + s_0/K_m) + [E]/2$.[26] The K_i values which we calculated for inhibitors **A** and **B** were 0.427 and 1.33 mM, respectively i.e. three times smaller than those obtained from the plots of Figure 2 so that a tight-binding competitive inhibition mechanism for inhibitors **A** and **B** is not supported.

The slope of the plots in Figure 2 is $k_i = k_2/(1 + K_i/i)$. Therefore, by substitution of K_i values, the phosphorylation rate constants (k_2) calculated for molecules **A** and **B** were 0.127 and 0.067 min^{-1}, respectively.

DISCUSSION

Organophosphorus compounds (OP) inhibit human acetylcholinesterase at the active site serine by phosphorylation of the enzyme with formation of a covalent bond at this center.[2–11,26–28] The common mechanism of acetylcholinesterase inactivation by organophosphorus compounds is the ejection of a good leaving group synchronous with formation of a phosphoserine linkage (Scheme 1).[2,27–29] Potent

SCHEME 1 The mechanism of acetylcholinesterase inhibition by organophosphorus compounds (E—OH is the enzyme).

TABLE I The spectral and enzymatic data for compounds **A** and **B**

Compound	δ_{31P} (ppm)	$^{2}J_{PNH}$ (Hz)	$^{7}J_{P-H}$ (Hz)	$^{2}J_{P-C}$ (Hz)	$^{3}J_{P-C}$ (Hz)	$^{n}J_{P-C}$ (Hz), (n = 4–5)	$\nu_{P=O}$ (cm^{-1})	IC_{50} (mM)	k_i (min^{-1})	k_2 (min^{-1})	K_i (mM)
A	−3.06	–	s, 0	4.4	6.8	s, 0	1230	0.48	0.0363	0.127	1.202
B	−11.69	11.3 11.2	s, 0	s, 0	s, 0	s, 0	1217	1.54	0.0207	0.067	30323

organophosphorus inhibitors of acetylcholinesterase usually have a double bond P═O in their structures.[18,19]

The spectral and enzymatic data for compounds **A** and **B** are summarized in Table I. These molecules contain a P—Cl bond which is a labile bond. Therefore, the dissociation of P—Cl bonds in these compounds is responsible for the irreversible inhibition. The IC_{50} values of inhibitors **A** and **B** are 0.48 and 1.54 mM, respectively, which suggest a higher toxicity for compound **A**. The inactivation constants (k_i values) for inhibitors **A** and **B** are 0.0363 and 0.0207 min^{-1}, respectively.

^{31}P NMR spectra showed that the phosphorus atom in molecule **A** was more deshielded than in compound **B**. Also the formation of partial multiple bonds between phosphorus and nitrogen atoms are stronger than these bonds between phosphorus and oxygen atoms.[30] These findings demonstrate that the phosphorus atom has a less negative charge in molecule **A** in comparison with molecule **B** and forms a weaker bond with the chlorine atom so that the chlorine atom in compound **A** is a better leaving group than in molecule **B**. Consequently, in the active site of the enzyme, the dissociation of the P—Cl bond in inhibitor **A** is faster than in inhibitor **B**, which provides a smaller IC_{50} value for **A** in comparison with **B**.

Cholinesterases use a Glu-His-Ser catalytic triad to increase the nucleophilicity of the catalytic serine. ^{1}H NMR spectra were used to indicate the formation of a short and strong hydrogen bond between the Glu-His pair upon binding mechanism which form tetrahedral adducts.[31–33] Therefore, another possibility is the formation of strong hydrogen bonding between the oxygen atom of both inhibitors with the active site of the enzyme. It could be that the oxygen atoms in molecule **A** have more access to the active site than in compound **B** which could account for greater affinity of inhibitor **A** relative to inhibitor **B** to conjugation with the active site via hydrogen bonding.

Finally, it can be concluded that two isoelectronic and isostructural molecules **A** and **B** have similar biological behaviors in the interaction with human acetylcholinesterase and the differences in their spectra and their inhibitory potencies is due to the electronic effects of substituents in these compounds.

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