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Structure-activity study of phosphoramido acid esters as acetylcholinesterasf inhibitors

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Abstract

Phosphoramido acid esters $(CH_3)_2NP(O)X(p\text{-}OC_6H_4\text{-}CH_3)$ (containing P-Cl (**1**), P-O (**2**), P-F (**3**), P-CN (**5**), and P-N (**4**,**6**) bonds, X for **2**, **4** and **6** is OCH_3, $(C_2H_5)_2N$ and morpholin) have been synthesized to investigate the structure-activity study of AChE enzyme inhibition, through the parameters log*P*, $\delta^{31}P$ and IC_{50}. After their characterization by ^{31}P, $^{31}P\{^1H\}$, ^{13}C, ^1H NMR, IR and mass spectroscopy, the parameters log*P* and $\delta^{31}P$ (^{31}P chemical shift in NMR) were used to evaluated the lipophilicity and electronical properties. The ability of compounds to inhibit human AChE was predicted by PASS software (version 1.193), and experimentally evaluated by a modified Ellman's assay.

Keywords: *Structure-activity study, phosphoramido acid ester, X-ray crystallography, acetylcholinesterase, IC_{50}, lipophilicity, inhibition*

Introduction

Organophosphorus compounds (OPs) comprise a diverse class of chemicals with extensively usage as insecticides in agriculture. They are known to inhibit a number of esterases such as acetylcholinesterase (AChE), the enzyme responsible for the degradation of the neurotransmitter acethylcholine [1]. Their inhibitory effect is based on phosphorylation of serine hydroxyl group at the esteratic site of the enzyme active site [2]. Substrates and reversible ligands protect cholinesterases from phosphorylation by OP compounds. Even when reversible ligands bind only to the anionic site of the enzyme, the catalytic site can be protected from phosphorylation. Binding sites of reversible ligands and their affinities for cholinesterases can be evaluated by different approaches [3]. Inhibition of AChE by OPs generally is governed by the following molecular properties:

(1) Intramolecular properties such as lipophilicity, electronic, refractory, steric and topological properties [4], that steer the insecticide through the 'gorge' and participate in their binding to the active center.
(2) Three-dimensional structure of OPs that provide positioning of the leaving group in the enzyme's active center [5], thus facilitating phosphorylation.

Hansch and co-workers have proposed a general equation [6] describing important roles of hydrophobic (log*P*), electronic (σ), and steric (Es) interactions in AChE inhibition by OPs. Their equation, however, does not elucidate mechanism of action of phosphoramides or consider possible role of other molecular properties such as topology [7], steric effects of substitutions (sterimol properties), and polarization in enzyme inhibition.

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Many literatures indicated that some biochemical, pharmacological, and environmental processes are depended on the hydrophobicity of the molecules involved, and therefore the parameterization of the hydrophobicity of a compound is important in quantitative structure-activity relationship (QSAR) studies [8]. The equilibrium or pseudoequilibrium exists in biological systems (biophases), modeling by log*P*, can be pictured as

$$\text{octanol phase} \underset{}{\overset{P_{\text{octanol}}}{\rightleftharpoons}} \text{water phase}$$

$$\text{biophase} \underset{}{\overset{P_{\text{bio}}}{\rightleftharpoons}} \text{water phase}$$

The Hammett-like postulate for the correlation equation is as (1), where *P* is partition coefficient [9].

$$\log P_{\text{bio}} = a(\log P_{\text{octanol}}) + b \qquad (1)$$

In previous work [3], lipophilicity and AChE inhibition was studied for some rodenticides $(CH_3)_2$-$NP(O)(p\text{-}OC_6H_4\text{-}X)_2$, where X = H, CH_3, Cl. To extend this study we synthesized $(CH_3)_2$-$NP(O)X(p\text{-}OC_6H_4\text{-}CH_3)$ compounds (X = Cl (**1**), OCH_3 (**2**), F (**3**), $(C_2H_5)_2N$ (**4**), CN (**5**) and C_4H_8NO (**6**)) which in addition to P-N and P-O bonds in $(CH_3)_2NP(O)(p\text{-}OC_6H_4\text{-}CH_3)$ moiety, contain another different bond with P atoms (P-F, P-Cl, P-C, P-O and P-N). Here, the compounds biological activity potential has been predicted by PASS software (version 1.193), and their ability to inhibit human acethylcholinesterase has been evaluated by a modified Ellman's assay.

Materials and methods

The enzyme AChE (of human erythrocyte) was procured from Sigma (Cat. No. C0663), acetylthiocholine iodide (ATChI), 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB) (Sigma), Na_2HPO_4 and NaH_2PO_4 (99%), acetonitrile (99%), KCN (96%), KF (99%), diethylamine, metanol and morpholine (Merck) were used as supplied. ^1H, ^{13}C and ^{31}P NMR spectra were recorded on a Bruker (Advance DRS) 250 MHz spectrometer. ^1H and ^{13}C chemical shifts were determined relative to internal TMS, ^{31}P chemical shifts relative to 85% H_3PO_4 as external standards. IR spectra were obtained using KBr pallets on a PERKIN ELMER 783 model spectrometer. Mass spectroscopy (Varian Star 3400 CX) and UV spectrophotometer were performed using a PERKIN-ELMER Lambda 5 and CECIL 8000 (SER: 35).

Synthesis

*N,N-Dimethyl phosphoramidocholoridic acid, **4**-methyl phenyl ester, $(CH_3)_2NP(O)Cl(p\text{-}OC_6H_4\text{-}CH_3)$ (**1**)*. The compound was prepared as the literature method [10,11]. Yield: 85%.

*N,N-Dimethyl O-methyl phosphoramidic acid, 4-methyl phenyl ester, $(CH_3)_2NP(O)OCH_3(p\text{-}OC_6H_4\text{-}CH_3)$ (**2**)*. To a solution of N,N-dimethyl phosphoramidochloridic acid 4-methyl phenyl ester (0.82 g, 3.5 mmol) in 30 mL dry benzene (CARE Carcinogen), methanol (0.11 g, 3.5 mmol) and then slowly pyridine (0.28 g, 3.5 mmol) was added and stirred at 25°C (12 h). The solvent evaporated in vacuum. The flash gradient chromatography method was used for the purification of the oily product (silicagel, hexane-ethyl acetate 9:1).

Colorless liquid, ^1H NMR ($CDCl_3$), δ (ppm): 2.31 (s, 3 H, *p*-CH_3), 2.74 (d, $^3\mathcal{J}_{PNCH} = 10.1$ Hz, 6 H, $N(CH_3)_2$), 3.78 (d, $^3\mathcal{J}_{POCH} = 11.3$ Hz, 3 H, OCH_3), 7.08–7.13 (m, 4 H, Ar-H); ^{13}C NMR ($CDCl_3$), δ (ppm): 20.62 (s, 1 C, *p*-CH_3), 36.64 (d, $^2\mathcal{J}_{P\text{-}C} = 3.9$ Hz, 2 C, $N(CH_3)_2$), 53.22 (d, $^2\mathcal{J}_{P\text{-}C} = 5.8$ Hz, 1 C, OCH_3), 119.72 (d, $^3\mathcal{J}_{P\text{-}C} = 4.8$ Hz, 2 C, C_{ortho}), 130.03 (s, 2 C, C_{meta}), 134.09 (s, 1 C, C_{para}), 148.76 (d, $^2\mathcal{J}_{P\text{-}C} = 6.8$ Hz, 1 C, C_{ipso}); $^{31}P\{^1H\}$ NMR ($CDCl_3$), δ (ppm): 8.93 (s); ^{31}P NMR, δ(ppm): 8.75–9.12 (m). IR (KBr): $\tilde{\nu} = 2990, 2900, 2890, 1582, 1484, 1435, 1295, 1245$ (P = O), 1200, 1190, 1350, 995, 730. MS (20 ev) *m/z* (%): 229 (64) M^+, 198 (16) $[M\text{-}OCH_3]^+$, 122 (69) $[M\text{-}OC_7H_7^+]$, 107 (67) $[C_7H_7O]^+$, 44 (100) $[C_2H_6N]^+$. Yield: 65%.

*N,N-Dimethyl phosphoramidofluoridic acid, 4-methyl phenyl ester, $(CH_3)_2NP(O)F(p\text{-}OC_6H_4\text{-}CH_3)$ (**3**)*. This compound was prepared similar to the literature method [10] from the reaction of N,N-dimethyl phosphoramidochloridic acid 4-methyl phenyl ester and KF in the presence of 18-crown-6 in dry benzene under reflux condition. Yield: 92%.

*N,N-Diethyl N′,N′-dimethyl phosphoramidic, 4-methyl phenyl ester, $(CH_3)_2NP(O)((C_2H_5)_2N)(p\text{-}OC_6H_4\text{-}CH_3)$ (**4**)*. To a solution of N,N-dimethyl phosphoramidochloridic acid 4-methyl phenyl ester (0.82 g, 3.5 mmol) in 30 mL dry acetonitrile, diethylamine (0.52 g, 7.10 mmol) was slowly added and stirred at 0°C (12 h). The solvent was evaporated in vacuum and, the product purified by water.

Red liquid, ^1H NMR ($CDCl_3$), δ (ppm): 1.08 (t, 6 H, diethylamine-CH_3), 2.28 (s, 3 H, *p*-CH_3), 2.70 (d, $^3\mathcal{J}_{PNCH} = 10.0$ Hz, 6 H, $N(CH_3)_2$), 3.10 (m, 4 H, diethylamine-CH_2), 7.08 (m, 4 H, Ar-H); ^{13}C NMR ($CDCl_3$), δ (ppm): 14.20 (d, $^3\mathcal{J}_{P\text{-}C} = 1.9$ Hz, 2 C, diethylamine-CH_3), 20.67 (s, 1 C, *p*-CH_3), 36.87 (d, $^2\mathcal{J}_{P\text{-}C} = 3.8$ Hz, 2 C, $N(CH_3)_2$), 39.86 (d, $^2\mathcal{J}_{P\text{-}C} = 4.4$ Hz, 2 C, diethylamine-CH_2), 120.00 (d, $^3\mathcal{J}_{P\text{-}C} = 4.8$ Hz, 2 C, C_{ortho}), 129.90 (s, 2 C, C_{meta}), 133.40 (s, 1 C, C_{para}), 149.25 (d, $^2\mathcal{J}_{P\text{-}}$

$_C$ = 6.3 Hz, 1 C, C_{ipso}); $^{31}P\{^1H\}$ NMR ($CDCl_3$), δ (ppm): 15.55 (s); ^{31}P NMR, δ(ppm): 15.18–15.80 (m). IR (KBr): $\tilde{\upsilon}$ = 3050, 2980, 2905, 2880, 1600, 1500, 1450, 1300, 1230 (P = O), 1195, 1050, 1035, 995, 910, 830, 745, 705. MS (20 ev) *m/z* (%): 271 (10) $(M + 1)^+$, 270 (1) M^+, 198 (8) $[M-(C_2H_5)_2N]^+$, 163 (28) $[M-C_7H_7O]^+$, 135 (57) $[(C_2H_5)_2NO_2P]^+$, 107 (89) $[C_7H_7O]^+$, 44 (100) $[C_2H_6N]^+$. Yield: 78%.

*N,N-Dimethy O-p-tolyl phosphoramidocyanidate, $(CH_3)_2NP(O)CN(p\text{-}OC_6H_4\text{-}CH_3)$ (**5**)*. The compound was prepared as the literature method [12]. Yield: 87%.

*N,N-Dimethyl N'-morpholin phosphoramido acid, **4**-methyl phenyl ester, $(CH_3)_2NP(O)(C_4H_8NO)(p\text{-}OC_6H_4\text{-}CH_3)$ (**6**)*. This compound was synthesized similar to the preparation method for compound **4**. Yellow liquid, ^1H NMR ($CDCl_3$), δ(ppm): 2.31 (s, 3 H, *p*-CH_3), 2.74 (d, $^3\mathcal{J}_{PNCH}$ = 10.1 Hz, 6 H, $N(CH_3)_2$), 3.16 (m, 4 H, morpholin-CH_2), 3.62 (t, $^3\mathcal{J}_{H\text{-}H}$ = 4.2 Hz, 4 H, morpholin-CH_2), 7.09 (m, 4 H, Ar-H); ^{13}C NMR ($CDCl_3$), δ(ppm): 20.70 (s, 1 C, *p*-CH_3), 36.86 (d, $^2\mathcal{J}_{P\text{-}C}$ = 3.8 Hz, 2 C, $N(CH_3)_2$), 44.72 (d, $^2\mathcal{J}_{P\text{-}C}$ = 0.6 Hz, 2 C, morpholin-CH_2), 67.08 (d, $^3\mathcal{J}_{P\text{-}C}$ = 6.3 Hz, 2 C, morpholin-CH_2), 119.90 (d, $^3\mathcal{J}_{P\text{-}C}$ = 4.8 Hz, 2 C, C_{ortho}), 130.10 (s, 2 C, C_{meta}), 133.90 (s, 1 C, C_{para}), 148.95 (d, $^2\mathcal{J}_{P\text{-}C}$ = 6.3 Hz, 1 C, C_{ipso}); $^{31}P\{^1H\}$ NMR δ(ppm): 13.40 (s); ^{31}P NMR ($CDCl_3$), δ(ppm): 13.40 (m). IR (KBr): $\tilde{\upsilon}$ = 3000, 2985, 2925, 2825, 1595, 1495, 1445, 1290, 1250 (P = O), 1210, 1110, 995, 970, 915, 825, 750. MS (20 ev) *m/z* (%): 285 (25) $(M + 1)^+$, 284 (3) M^+, 198 (10) $[M-C_4H_8NO]^+$, 177 (48) $[M-C_7H_7O]^+$, 107 (87) $[C_7H_7O]^+$, 44 (100) $[C_2H_6N]^+$. Yield: 70%.

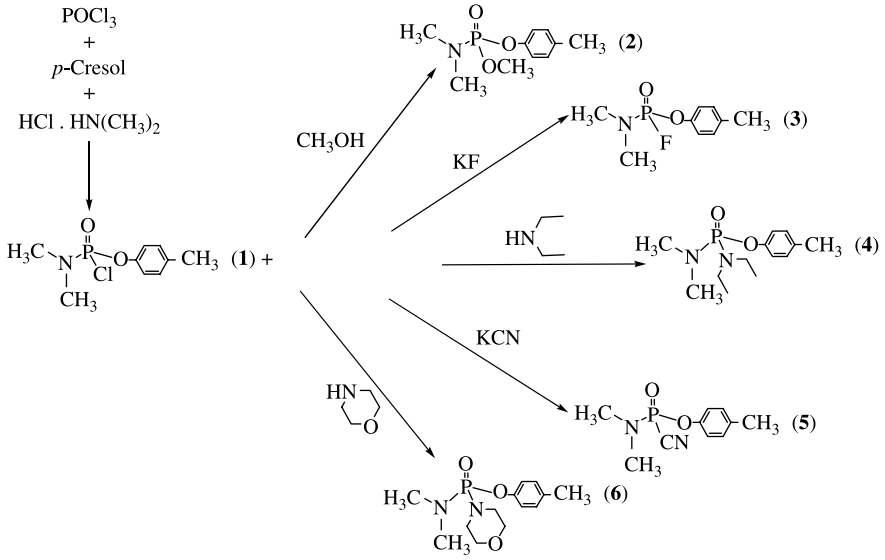
Results and discussion

Synthesis and spectral data

Compounds **2**–**6** were synthesized from the reaction of N,N-dimethyl phosphoramidocholoridic acid, 4-methyl phenyl ester (**1**) and suitable salts of entering groups (for **3** and **5**) or suitable alcohol or amine, in the presence of pyridine as an HCl scavenger (for **2**) or an excess amount of amines (diethylamine and morpholine for **4** and **6**) (Scheme I).

NMR study

The ^{31}P chemical shift ($\delta^{31}P$) varied from −11.82 (for **5**) to 15.55 ppm (for **4**). Due to the coupling with the H atoms of $N(CH_3)_2$ moiety, phosphorus atom's peak are as heptet in compounds **1**, **3** and **5**. In molecules **2**, **4** and **6**, two different kinds $^3\mathcal{J}_{P\text{-}H}$ [sourced from the protons of $N(CH_3)_2$ and then OCH_3 (**2**), $N(C_2H_5)_2$ (**4**) and NC_4H_8O (**6**) moieties] cause the multiplet peaks for their phosphorus atoms. The hydrogen atoms of $N(CH_3)_2$ group in ^1H NMR spectra revealed as doublet peaks with $^3\mathcal{J}_{PNCH}$ at the range of 10.1 Hz (for **2**, **6**) to 13.8 Hz (for **1**). In compound **2**, the hydrogen atoms of OCH_3 group appear as a doublet peak with $^3\mathcal{J}_{POCH}$ = 11.3 Hz. The phosphorus-carbon coupling constant ($^2\mathcal{J}_{PNC}$) revealed for the carbon atoms of $N(CH_3)_2$ moieties in ^{13}C NMR spectra at the range of 3.8 Hz (for **4**, **6**) to 5.1 (for **5**), furthermore $^2\mathcal{J}_{POC}$ = 5.8 Hz for OCH_3 group of



Scheme 1. Preparation of Compounds **1**–**6**.

Table I. Part of the predicted biological activity spectra for the compounds **1**–**6**.

Biological activity	1	2	3	4	5	6
Acetylcholinesterase inhibitor	**0.484**	**0.772**	**0.727**	**0.852**	**0.403**	**0.805**
Insecticide	0.398	0.702	0.477	0.393	0.369	0.385
Cholinergic	0.361	0.675	0.676	0.782	0.274	0.754
Carcinogenic	0.366	0.459	0.617	0.485	0.255	0.592
Carboxylesterase inhibitor	0.557	0.724	0.920	0.529	0.460	0.476
Mutagenic	0.246	0.307	0.377	0.653	0.177	0.319
Embryotoxic	0.381	0.546	0.748	0.505	0.257	0.532

compound **2**. In addition to $^{2}\mathcal{J}_{P\text{-}C}$ coupling constant, compounds **4** and **6** show $^{3}\mathcal{J}_{P\text{-}C}$ coupling constants in $N(CH_2CH_3)_2$ and $N(CH_2)_4O$ moieties which in acyclic group $N(CH_2CH_3)_2$, $^{3}\mathcal{J}_{P\text{-}C} < {}^{2}\mathcal{J}_{P\text{-}C}$ but in cyclic group $N(CH_2)_4O$, $^{2}\mathcal{J}_{P\text{-}C} < {}^{3}\mathcal{J}_{P\text{-}C}$ which is in agreement with the previous results about cyclic and acyclic amines linked to phosphorus atom [13].

Computational evaluation of biological activity. The biological activity spectra of the target compounds were obtained by *PASS (Prediction of activity spectra for substances)* software [14]. This version of software is capable of predicting 900 synchronized biological activities, based on molecular formula of various compounds. The biological activity spectra predicted by PASS software is capable to distinguish pharmaceutical effects, side effects, biochemical reaction mechanism, genetic mutation, carcinogenesis, toxicity fetus deficiency and other biological activities of chemical compounds. Characterizations of about 4500 compounds with different functional groups are stored in the memory of this software and the accuracy of prediction for thousands of chemical compounds is about 85% [15]. A portion of the predicted biological activity spectra for the compounds (anti-AChE activity, insecticide, cholinergic, ...) are given in Table I (P_a for anti-AChE activity is represented as bold font).

Lipophilicity study

The log*P* values were experimentally determined by the shake-flask method [3], and the results are shown in Table II.

Table II. Log*P* values for compounds **1**–**6**.

Compound	λ_{max} (nm)	Mean log*P* value ± S.D
1	292	1.60 ± (0.10)
2	332	1.72 ± (0.10)
3	280	1.61 ± (0.06)
4	274	2.24 ± (0.03)
5	272	2.20 ± (0.01)
6	352	2.69 ± (0.08)

In vitro *evaluation of acetylcholinesterase inhibition*

The activity of AChE was be measured according to a method developed by Ellman *et al.* The colorimetric Ellman method employs acetylthiocholine iodide (ATChI) as a synthetic substrate for AChE. ATChI is broken down to thiocholine and acetate by AChE, and then the thiocholine is reacted with dithiobisnitrobenzoate (DTNB) to produce a yellow color. The quantity of yellow color which develops over time is a measure of the activity of AChE and can be measured using a spectrophotometer in 412 nm. Inhibition potency of organophosphorus compounds is commonly assayed by measuring the decrease in acetylcholinesterase activity [16].

At room temperature, the enzyme samples, 3μL (8u) were incubated with different concentrations of inhibitor in the phosphate buffer (for instance 42.8 mM (for **1**), pH 7.4) and Ellman's reagent (1500 μL). After 2 min incubation, the enzyme activity was determined by using 0.5 mM ATChI as the substrate (the concentration of ATChI is optimized according to the enzyme activity). Compound **1** is a reversible ligand in the range of 0.80–1.50 mM concentration with $IC_{50} = 1.37 \pm (0.06)$ mM. The IC_{50} values for other compounds were determined in the same way and the inhibition graph (Figure 1) was obtained for different concentrations of phosphoramido acid esters. IC_{50} values are given in Table III.

The reversibility was demonstrated with the increasing in absorption intensity (by using reverse dialysis bag technique) in phosphate buffer (pH: 7.4), the enzyme: 5μL (13.3u); substrate: 1 mM, inhibitor: 2.14 mM, Ellman's reagent: 1500 μL, initial absorption: 0.125, time: 24 h, final absorption: 0.398. Furthermore, to confirm the reversibility (competitive inhibitor [17]) of compound **1**, the plot 1/A vs. 1/[S] was obtained in the absence and in the presence of **1** (Figure 2), the enzyme sample, 5μL (13.3u); Ellman's reagent (1500 μL); concentrations of substrate: 0.15, 0.25, 0.40 and 0.50 mM and **1**: 2.14 mM,.

Considering the P atom chemical shift as the index to investigate the phosphorus electron density [9], it may be used to introduce the electronic effect parameter. Equation (2) (obtained by the SPSS

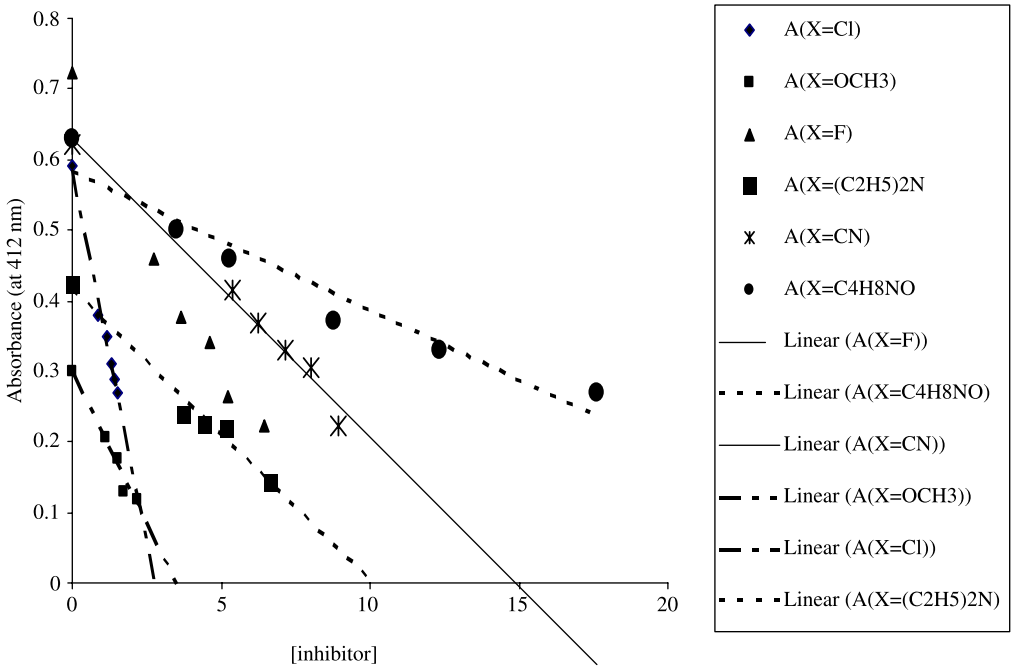


Figure 1. Inhibition calibration for $(CH_3)_2NP(O)X(p\text{-}OC_6H_4\text{-}CH_3)$ derivatives (**1-6**).

software) indicates the correlation between $\log(1/IC_{50})$, log*P* and $\delta(^{31}P)$ (for compounds **1–2** and **4–6**). The values of these parameters are given in Table III.

$$\log(1/IC_{50}) = -0.929(\pm 0.28)\log P + 0.008\,(\pm 0.001)\,\delta(^{31}P) + 1.264\,(\pm 0.60) \quad (2)$$

$$n = 5,\ r = 0.998,\ s = 0.245,\ F = 591.57$$

There is a linear correlation between $\log(1/IC_{50})$ and log*P* with slope near 1, which means that increasing lipophilicity causes a decrease of IC_{50} (compound **3** does not obey this equation possibly due to the high electronic effect of the fluorine group). The $\delta(^{31}P)$ coefficient in Equation (2) demonstrates that the electronic effect parameter has no significant influence on the IC_{50}. Furthermore, it is expected that the decrease in the volume of the phosphoramide analogues increases inhibitory activity [15], although, the steric effect may occasionally play a little role in anti-AChE activity. The $N(C_2H_5)_2$ and morpholine moieties form P-N bonds with their P atoms in compounds **4** and **6** (the other groups linked to their P atoms are similar), so that the anti-AChE activity for **4** is greater than **6**. The molecules **1** and **5** (we reported their structures in previous works [11,12]) are different in only one substitutents linked to the P atom. Figure 3 (a_1 and b_1) indicates the tetrahedral configuration of the phosphorus atoms where the similar moieties linked to the P atoms are omitted for clarity (only the direct atoms linked to P atoms are shown). The spatial distance between P and N in molecule **5** is longer than that of the P—Cl bond length in molecule **1** (2.952 Å and 2.038 Å). Figure 3 (a_2 and b_2) show the space filled representation of the molecules and the spatial distances between the farthest atoms (the two atoms at the greatest distance from each other) are shown (9.356 Å and 9.311 Å for **1** and **5**). Considering Figure 3, it can be concluded that the volume of the molecules are close to each other, but show considerable differences in inhibitory potency (1.37 ± (0.06), 7.53 ± (0.07) mM, respectively).

Table III. Physicochemical properties and anti-AChE activity experimental values for compounds **1**–**6**.

Compound	log*P*	$\delta\ ^{31}P$(ppm)	IC_{50} (mM)
1	1.60	+13.81	1.37 ± (0.06)
2	1.72	+8.934	1.73 ± (0.03)
3	1.61	+0.29 (−4.52 to 5.07)	4.29 ± (0.20)
4	2.24	+15.55	4.93 ± (0.02)
5	2.20	−11.824	7.53 ± (0.07)
6	2.69	+13.40	13.55 ± (0.05)

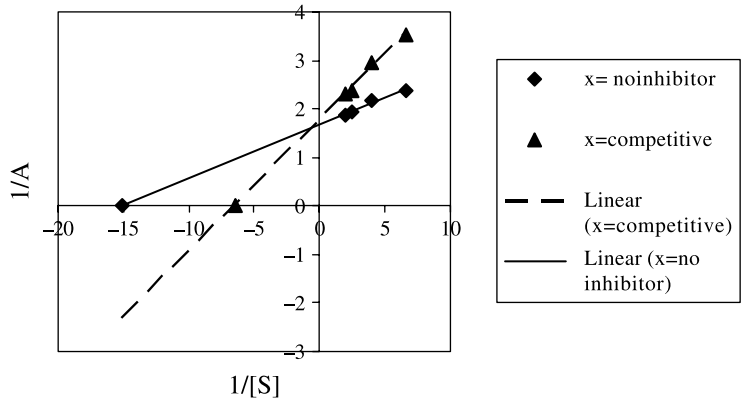


Figure 2. The plot 1/A vs. 1/[S] in the absence (solid line) and in the presence (dashed line) of compound **1**; A: absorption, [S]: substrate concentration.

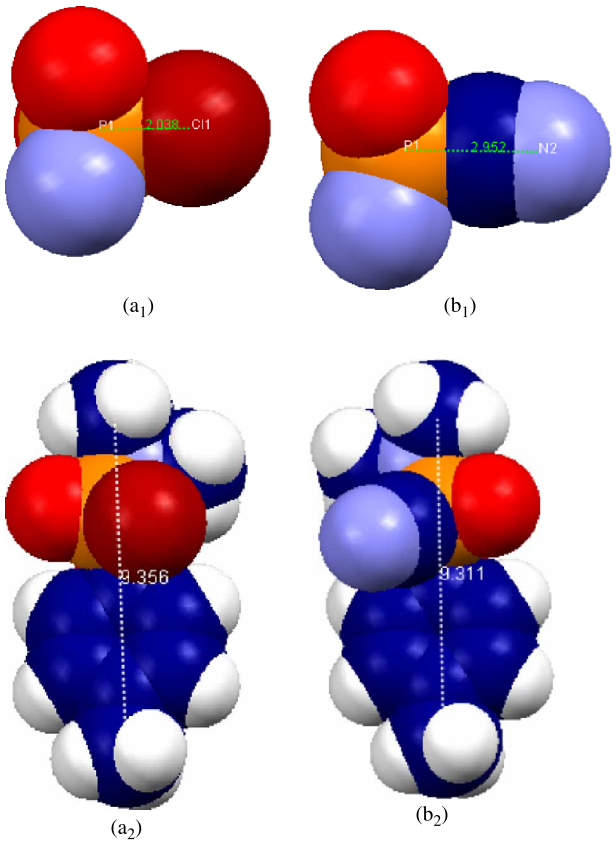


Figure 3. Space filling forms of molecules **1** (a_1 and a_2) and **5** (b_1 and b_2); in a_1 and b_1 only the direct atoms linked to P atoms are shown.

Conclusion

Inhibitory potency of the target compounds vary in the series as **1** > **2** > **3** > **4** > **5** > **6**. The target compounds are reversible, competitive inhibitors. Considering the three parameters electronic, steric and lipophilicity, liphophilicity has the main influence on IC_{50}, this result is in agreement with the QSAR equations in the literatures.

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