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Synthesis, lipophilicity study and *in vitro* evaluation of some rodenticides as acetylcholinesterase reversible inhibitors

SAIED GHADIMI[1], SEYED LATIF MOUSAVI[2] & ZAHRA JAVANI[1]

[1]*Department of chemistry, Imam Hossein University, Tehran, Iran, and* [2]*Department of biology, Shahed University, Tehran, Iran*

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Abstract
The anti-AChE activity of phosphoramidates has been noticed for many years. Because of the wide application of phosphoramidates in recent years, there has been a continuing research for synthesis, purification and identification of effective and safe derivatives. In this study some rodenticides with the general formula $Me_2NP(O)(p\text{-}OC_6H_4\text{-}X)_2$, where X = H, CH_3, Cl, have been synthesized in water (without organic solvent) and characterized by ^{31}P, $^{31}P\{^1H\}$, ^{13}C and ^1H NMR spectroscopy. Since lipophilicity has been recognized for its importance in QSAR studies, efforts have been made to determine the log*P* values. The ability of these rodenticides to inhibit human acetylcholinesterase (hAChE) has been predicted with *PASS* (Prediction of Activity Spectra for Substances) software (version 1.917) and then has been evaluated by a modified Ellman's assay and spectrophotometric measurements.

Keywords: *Phosphoramidate, rodenticide, lipophilicity, acetylcholinesterase, inhibition*

Introduction

Cholinesterases are targets for phosphoramidates which have wide applications in medicine (treatment of cancer, AIDS and Alzheimer's disease), agriculture (insecticides, herbicides, rodenticides and fungicides), industry (stabilizers, oxidants) and other interesting scientific fields. Correlation between the *in vitro* inhibition of AChE and the acute LD_{50} measured *in vivo* provide further evidence confirming the central role of the sensivity of AChE as the principal determinate of acute toxicity of phosphoramidates[1]. The physiological role of acetylcholinesterase (AChE) is to hydrolyze the neurotransmitter acetylcholine (ACh) released in the process of cholinergic transmission of nerve impulses[2]. Phosphoramidates rodenticides that usually act by inhibiting AChE and ChE activity, resulting in an accumulation of acetylcholine in neural and non-neural tissues. Accumulation of ACh in neuromuscular junction and synaptic cleft is belived to be the major cause of death in the target species [3]. AChE active center, according to classical theory, consists of an anionic site that binds with the inhibitor and an catalytic site that participates in the enzymes phosphorylation. Substrates such as acetylcholine react at the catalytic site. Phosphoramidates usually phosphylate the catalytic site of AChE, and that reaction inhibits the enzyme[4]. Substrates and reversible ligands protect cholinesterases from phosphylation by OP compounds. Even when reversible ligands bind only to the anionic site of the enzyme, the catalytic site can be protected from phosphylation[5]. Binding sites of reversible ligands and their affinities for cholinesterases can be evaluated by different approaches. For details concerning the evaluation of binding sites and the mechanism of action of cholinesterase inhibitors, the reader is referred to extensive publications[4]. Screening of cholinesterase inhibition has been done by determining the IC_{50} value[6,7].

The basic philosophy in Structure-Activity relationships is that the structural changes that affect the

Correspondence. Fax: + 98-21-77104030. E-mail: Ghadimi_Saied@yahoo.com.

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biological activities of a set of congeners are of three major types: electronic, steric, and hydrophobic. Other factors, such as hydrogen bonding, polarizability, and dipole moment, appear to play less important roles[8]. Thus, the biological activity of chemical compounds can be related to their physicochemical properties by several functions:

$$Biological\ \ activity = f\ \ (physicochemical\ \ properties)$$

$$Log\ \ (1/IC_{50}) = a\ \ LogP + b\sigma + cEs$$

Log*P*: hydrophobic parameter, σ: Hammet electronic constant, Es:Taft steric constant.

Since lipophilicity (the affinity of a molecule or a moiety for a lipophilic environment) has been recognized for its importance in QSAR (**Q**uantitative **S**tructure-**A**ctivity **R**elationship) studies, efforts have been made to determine the log*P* (logarithm of partition coefficient in biphasic systems, such as n-octanol/water) value. This parameter is closely related to the transport properties of rodenticides and their interaction with receptors. This parameter can be either determined experimentally or calculated[9].

Rodenticides such as warfarin, bromodiolone and difenacoum, are defined as any substance that is used to kill rats, mice, and other rodent pests. It has been found that certain esters of amidophosphoric acids or of amidothiophosphoric acids especially those shown with the following general formula (1) can be used with great advantage as rodenticides in the surface spraying process[10].

$$(R_2R_1N)P(X)(O-aryl)_2 \qquad (1)$$

Where X represents a member selected from the group consisting of oxygen and sulfur, aryl preferably represents phenyl radicals, which furthermore may be substituted with halogen or nitro groups, especially with chlorine groups; R_1, R_2 represents hydrogen, a lower alkyl or alkenyl radical or halogen-substituted lower alkyl radical or aryl group.

In this research three kinds of phosphoramidates with the general formula $Me_2NP(O)(p\text{—}OC_6H_4\text{—}X)_2$, with X = H,$CH_3$,Cl that have application in agriculture as rodenticides, have been selected. The basis of this selection was the Fujita hydrophobic constants (π parameter). After synthesis and characterization, Log*P* values for the target compounds have been experimentally determined by the "shake-flask" method. *PASS* software (version 1.917) was utilized for prediction of anti-AChE and other biological activity. Inhibitory potency were experimentally determined by Ellman's method.

Methods

NMR spectra were recorded on a Bruker DOX-250 instrument. $CDCl_3$ was used as solvent. ^1H and ^{13}C chemical shifts were determined relative to internal TMS and ^{31}P chemical shifts relative to 85% H_3PO_4 as external standard. Absorbances were measured with PERKIN-ELMER Lambda5 UV spectro photometer.

Synthesis

The target rodenticides were prepared by treatment of $POCl_3$ with the N,N-dimethylamine salt and then phenolic derivatives.

N,N-Dimethyl phosphoramidic acid di,phenyl ester, $Me_2NP(O)(p\text{-}OC_6H_5)_2$. Phenol (15.06 g, 160 mmol) was dissolved in a 10% aqueous solution containing 6.40 g of sodium hydroxide (160 mmol). This solution was cooled at 10°C; then 6.48 g (40 mmol) dimethyl amidophosphoric acid dichloride (from reaction between $P(O)Cl_3$ and $(CH_3)_2NH.HCl$ in reflux system) was added slowly into this solution. By cooling the reaction temperature was kept at about 4°C. After the addition had been completed the mixture was stirred for a further 4 h. Then the two layers were separated and the lower oily layer washed twice with 500 mL of water. The remaining water was removed by distillation in vacuum[10]. Flash gradient chromatography method was used for the purification of the product (silicagel, hexane-ethyl acetate 4:1).

Red liquide, υ_{max}/cm^{-1}(KBr): 1250(P═O), 1108(P—O—C), 740(P—N), ^1H NMR ($CDCl_3$), δ(ppm): 2.70 (6 H, d, $^3J_{P-H} = 2.5$ Hz, 2 CH_3), 6.76–7.22 (10 H, m, ArH); ^{13}C NMR ($CDCl_3$), δ(ppm): 152.00 (d), 129.70 (s), 124.90 (s), 120.00 (s), 36.70 (s); $^{31}P\{^1H\}$ NMR δ(ppm): 1.60 (s); ^{31}P NMR, δ(ppm): 1.29–1.89 (hept., $^3J_{P-H} = 10.1$ Hz), yield:80%.

Lipophilicity study

Hydrophobicity is the affinity of a molecule or a moiety for a lipophilic environment. It is commonly measured by its distribution in a biphasic system, either liquid-liquid (e.g., partition coefficient in 1-octanol/water) or solid/solid (retention on reversed-phase high performance liquid chromatography (RP-HPLC) or thin-layer chromatography (TLC) system). Partition coefficient in octanol/water (log*P*) has long been used to parameterize hydrophobic character in QSAR studies. In our research log*P* values for the target compounds were experimentally determined by the shake-flask method. Calculation of log*P* values was performed as

follows[11]:

$$\log P = \log\{(y - x/x)\ (V_{buffer}/V_{oct})\} \qquad (2)$$

where: P – partition coefficient, y – total mass of compound (mg), x – mass of compound in the buffer phase after partitioning (mg), V_{buffer} – volume of buffer (ml), V_{oct} – volume of *n*-octanol (ml).

With different concentrations of compound in buffer, a calibration graph was plotted. Then 0.047 g of compound was dissolved in 5 mL of n-octanol and 10, 20, 30 mL of buffer was added. The phases were shaken together on a mechanical shaker for 30 min, centrifuged for 20 min to afford complete phase separation, and the n-octanol phase was removed. Absorbance of the buffer phase was measured using a UV/VIS spectrophotometer at 272 nm. The concentration was then calculated from the calibration graph and log*P* value was determined by formula (2) (Table I).

In vitro *evaluation of Acetylcholinesterase inhibition:*

ATCh(acetylthiocholine) is a suitable substrate for AChE. The enzyme used in this work was of human erythrocyte AChE from Sigma (Cat. No. C0663). The colorimetric Ellman method is the most commonly used assay for the determination of AChE activity through acetylthiocholine hydrolysis to acetic acid and thiocholine. The assay makes use of the thiocholine-mediated cleavage of the chromogenic disulfide DTNB[10] and activity is measured by following the increase in absorbance at 412 nm.

Absorption of organophosphorus compounds *in vivo* is commonly assessed by measuring the decrease in acetylcholinesterase activity. Inhibition curves were obtained with acetylthiocholine in the different concentrations of rodenticide compounds[13].

The enzyme samples, 3μL (8 u) were incubed at room temperature in different concentrations of rodenticide in the phosphate buffer (70 mM). Then the enzyme activity was determined using ATCh as the substrate[14], (final ATCh conc. during enzyme assay = 0.5 mM). At concentrations ranging from 10 to 60 mM the phosphoramidic acid showed a reversible inhibition with an IC_{50} of 31.3 mM, (Figure 1).

Table I. Log P value for $(Me_2N)P(O)(O{-}C_6H_5)_2$ by the shake-flask method.

n-octanol/buffer (v/v)	C_1	Ā(n=3)	C_2	log *P*
1:2	3.48×10^{-2}	0.276	2.7×10^{-3}	1.037
1:4	3.48×10^{-2}	0.245	2.4×10^{-3}	1.035
1:6	3.48×10^{-2}	0.214	2.1×10^{-3}	1.010

Overall mean log *P* value ± SD: 1.027 ± 0.015

C_1 – *concentration of stock solution in n-octanol before partitioning (mol* L^{-1}*);* Ā –absorbance in buffer solution after partitioning (λ = 272 nm); C_2 – concentration of sample in buffer solution after partitioning (*mol* L^{-1}); log *P* – logarithm of the partition coefficient

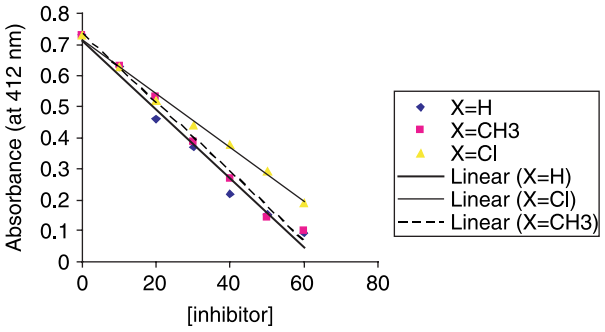


Figure 1. Inhibition graph for target rodenticides.

The two following compounds were prepared and their log*P* and IC_{50} values were determined similarly.

N,N-Dimethyl phosphoramidic acid bis-(4-methyl-phenyl) ester, $Me_2NP(O)(p\text{-}OC_6H_4\text{-}CH_3)_2$. Yellow liquid, υmax/$cm^{-1}$(KBr): 1235(P═O), 1090 (P—O—C), 650(P—N), ^{1}H NMR ($CDCl_3$), δ(ppm): 2.85 (3 H, s, *p*—CH_3), 3.32 (6H, d, $^3J_{P-H} = 7.5$ HZ, 2 CH_3), 7.66 (8 H, s, ArH); ^{13}C NMR ($CDCl_3$), δ(ppm): 152.00 (d), 134.20 (s), 130.00 (s), 119.70 (s), 36.75 (s), 20.60 (s); $^{31}P\{^1H\}$ NMR δ(ppm): 2.15 (s); ^{31}P NMR, δ(ppm): 1.95–2.35 (hept., $^3J_{P-H} = 10.1$ HZ). yield:74%.

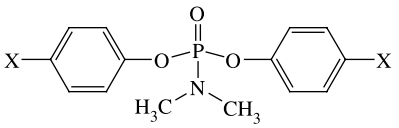
$$\log P = 1.63 \pm 0.027,\ IC_{50} = 35.4\ mM.$$

N,N-Dimethyl phosphoramidic acid bis-(4-chloro-phenyl) ester, $Me_2NP(O)(p\text{-}OC_6H_4\text{-}Cl)_2$. Yellow liquid, υmax/$cm^{-1}$(KBr): 1310(P═O), 1090(P—O—C), 810(P—N), ^{1}H NMR($CDCl_3$), δ(ppm): 2.78 (6 H, d, $^3J_{P-H} = 35.0$ HZ, 2 CH_3), 6.65–7.30 (8 H, m, ArH); ^{13}C NMR ($CDCl_3$), δ(ppm): 149.20 (d), 129.80 (s), 121.00 (s), 116.70 (s), 36.60 (s); $^{31}P\{^1H\}$ NMR δ(ppm): δ = 1.68 (s); ^{31}P NMR, δ(ppm):

Table II. *PASS* output for N,N-Dimethyl phosphoramidic acid di,phenyl ester.

Biological activity	p_a
CYP2A6 human substrate	0.824
Acetylcholinesterase inhibit	0.816
Dynein ATPase inhibitor	0.796
GTP diphosphokinase inhibitor	0.786
CYP2 substrate	0.777
Granzyme B inhibitor	0.731
Cholinergic	0.725
Carboxylesterase inhibitor	0.709

Table III. Physicochemical properties and anti-AChE activity experimental and predictive values for target rodenticides.



X	Δ	π	log*P*	*miLogP*	IC_{50}(mm)	pa(anti-AChE)
H	1.6	0	1.027	1.95	31.3	0.816
CH_3	2.153	0.56	1.63	2.53	35.4	0.722
Cl	1.676	0.71	2.75	3.14	40.9	0.718

1.38–1.99(hept., $^3J_{P-H} = 10.1$ HZ), yield:89%.

$$\log P = 2.75 \pm 0.05, \quad IC_{50} = 40.9 \text{ mM}$$

Computational evaluation of lipophilicity and biological activity:

Because experimental measurements are time consuming and sometimes difficult, computational methods are very valuable tools for calculation of log*P* values. A number of different computer programs for prediction of lipophilicity have been recently developed[9]. In our work, *miLogP 1.2.,* computer program[15] for predicting log*P* have been compared with experimental data. *PASS* software[16] for computational evaluation of biological activity has been used, too.

miLogP 1.2. – The *miLogP 1.2.* program calculates log *P* values as a sum of group contributions and correction factors. The group contributions were obtained by fitting calculated log*P* values with experimental log*P* values for a training set of several thousands of drug-like molecules[9].

PASS (Prediction of activity spectra for substances). This is a software product for predicting the biological activity spectrum for chemical substances on the basis of their structural formulas[17]. Biological activity spectra for our target compounds have been predicted as probable with pa > 0.7 (pa: probabilities to be active), (version 1.917-July 2005). Biological activity spectrum for N,N-Dimethyl phosphoramidic acid di,phenyl ester are shown in Table II.

Results and discussion

Physicochemical properties (δ of $^{31}P\{^1H\}$ NMR, measured and calculated log*P*) and anti-AChE activity experimental and predictive values for target rodenticides are summarized in Table III. There are many various methods for synthesis of phosphoramidates, and the utilization of non-safe organic solvents such as benzene and pyridine is incidental to all of them. long time and high temperature are disadvantages for these. Target compounds have been synthesized previously by different methods, but here we have synthesized them, in water (without organic solvents), in high yield and at room temperature.

Reproducibility and low standard deviation for hydrophobicity results confirm that the shake-flask method in its simplicity, is a suitable method for log*P* measurements. Qualitative comparison between measured and calculated log*P* results show that Cl- and CH_3- substituents increase the affinity of a molecule for lipophilic environment in concordance with Fujita constants (π). One should not lose sight of the fact that, even at the present stage of development, computer calculation of log*P* has often prompted remeasurement of a solute with the result that the more carefully measured value agrees well with the calculated.

Conformity between computational and *in vitro* evaluation of anti-AChE activity denote reliable prediction by *PASS* software. Medium hAChE inhibition activity for the rodenticides under study show that these compounds can be used widely in the agricultural industry.

The most important result in our research is a decrease in inhibitory activity in lieu of an increase in lipophilicity that can assist to the preparation of new, safe rodenticides. Although the electronic parameter has an important role in QSAR studies no correlation was observed between the electronic parameter (δ) and IC_{50}, so showing that the hydrophobic parameter has a more important role.

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