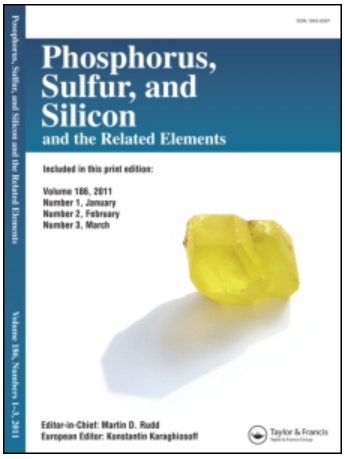

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Synthesis and Characterization of O,S-Dimethylphosphoramidothioate and N-Acetyl O,S-Dimethylphosphoramidothioate

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SYNTHESIS AND CHARACTERIZATION OF O,S-DIMETHYLPHOSPHORAMIDOTHIOATE AND N-ACETYL O,S-DIMETHYLPHOSPHORAMIDOTHIOATE

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O,S-Dimethylphosphoramidothioate (methamidophos) and N-acetyl O,S-dimethylphosphoramidothioate (acephate) were synthesized by new methods to investigate the structure–activity study of acetyl cholinesterase (AChE) inhibition through the parameters of log**P**, δ ^{31}P, and IC_{50}. After their characterization by NMR (^{31}P, ^{31}P{^{1}H}, ^{13}C, and ^{1}H), IR, and mass spectroscopy, log**P** and δ ^{31}P (^{31}P chemical shift in NMR) were used to evaluate lipophilicity and electronical properties. The log**P** values for methamidophos and acephate were experimentally determined by the GC-shake-flask method, and the ability of the compounds to inhibit human AChE was evaluated by a modified Ellman's assay.

Supplemental materials are available for this article. Go to the publisher's online edition of* Phosphorus, Sulfur, and Silicon and the Related Elements *to view the free supplemental file.

Keywords Acephate; acetyl cholinesterase; IC_{50}; lipophilicity; methamidophos; reversibility

INTRODUCTION

Organophosphate insecticides belong to a group of insecticides that act by inhibiting acetyl cholinesterase (AChE) activity in insects and mammals.[1] Phosphoramidothioates are an important class of water soluble organophosphorous insecticides mainly used in agricultural industry to control pests (insects) on fruits, vegetables, and cotton by inhibiting AChE and ChE, resulting in an accumulation of acetylcholine in neural and non-neural tissues,[2] leading to development of cholinergic hyperactivity. Therefore, use of many excellent insecticides has been prohibited due to their high nontarget toxicity.[1] Acephate (Ace) and methamidophos (Met) are water soluble organophosphate (OP) insecticides used in eradicating agricultural and forestry pests. They are known to inhibit cholinesterase (AChE and plasma cholinesterase) in the nervous tissue.[3] Their function in mammals, human beings, and insects are to decrease the activity of AChE.[2] Met and Ace have more applications among the phosphoramidothioate insecticides (Figure 1). While Ace and Met

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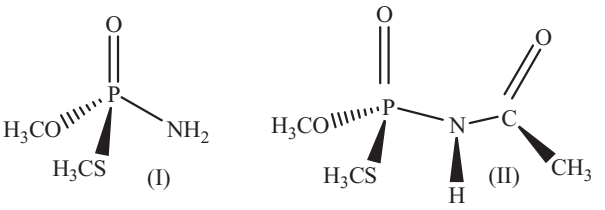


Figure 1 Methamidophos (I) and acephate (II).

are comparable inhibitors of insect AChE, Met is 75 to 100 times more potent an inhibitor of mammalian AChE (mAChE) than Ace.[4]

Two major hypotheses to explain the toxicity of Ace have been developed: i) the toxicity of Ace is simply related to its conversion to Met (unconverted Ace is essentially nontoxic).[4] Insects quickly convert Ace into Met. ii) Mammals poorly convert Ace to Met. Accumulation of Met in mammals inhibits the carboxyamidase enzyme that converts Ace to Met. Thus bioactivation of Ace turns off immediately.[2] The mammalian toxicity of Ace and Met is not only dependent on the rate of metabolic conversion, but also on the molecular composition and structure of mammalian AChE compared to insect AChE.[1]

AChE inhibition by OPs is governed by molecular properties including the following: i) Intermolecular properties such as lipophilic, electronic, refractory, steric, and topological properties that steer the insecticide through the gorge and participate to their binding to the active center, and ii) the three-dimensional structure of OPs that provide positioning of the leaving group in the enzymes' active center, thus facilitating phosphorylation.[2] In other words, the biological activity of organophosphorus compounds is related to their phosphorylating abilities, which is dependent on the electrophilicity of the phosphorus atom, determined by its substituent groups. Steric factors of substituent also play a major role in determining the biological activity of these chemicals. Lipid solubility is important because it enhances the ability of these compounds to cross biological membranes and the blood-brain barrier, leading to increased biological activity.[5]

To develop insect-selective insecticides, many investigators have synthesized a series of phosphoramidothioate analogs and have tested their insecticidal potency and mammalian toxicity. O,O-dimethyl phosphoramidothioate (DMPAT) is a key component in the production of Met. Different methods have been reported for the synthesis of DMPAT and Met in the literature.[6–10] To our knowledge, three methods have been reported for the synthesis of Ace: i) rearrangement of S-Sodium O-methyl N-acetyl phosphoramidothioate, ii) acylation of Met, and iii) rearrangement of O,O-dimethyl N-acetylphosphoramidothioate. Unfortunately, the sodium salt of the corresponding N-acylated phosphoramidothioate is degraded even under mild conditions of reaction before the formation of the product; therefore, this way is not economical for the synthesis of Ace.[11–13]

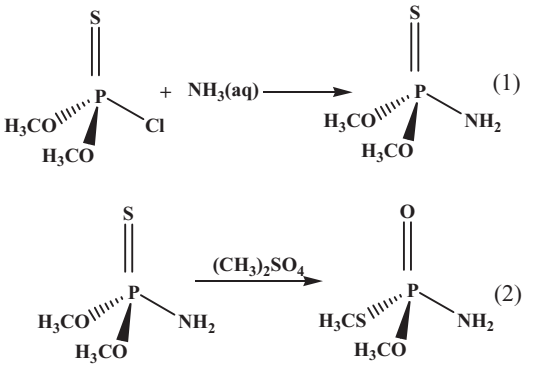
In our research, we synthesized DMPAT, Met (0.03 M scale), and Ace by new methods. After investigating the influence of different factors such as temperature, time, solvent, and mole ratio of the reactants, the optimized conditions for the production of the compounds in the bench scale (0.1 M scale) were determined. After their characterization by ^{31}P, $^{31}P\{^{1}H\}$, ^{13}C, and ^{1}H NMR, IR, and GC−MS, PASS software (version 1.917) was used to predict carboxylesterase inhibitor, and carcinogenic and mutagenic properties of the mentioned compounds. The log*P* values for the target compounds were experimentally

determined by the GC-shake-flask method. Their ability to inhibit human AChE was also evaluated by a modified Ellman's assay.

RESULTS AND DISCUSSION

Synthesis

Met is mainly produced by the reaction of O,O-dimethylphosphoramidothioate (DMPAT) and dimethyl sulfate. DMPAT is also produced through a reaction between ammonium hydroxide with O,O-dimethyl phosphorochloridothioate. In the present research, we successfully produced these compounds with the overall yield of 80%, using the synthetic methodology shown in Scheme 1.



Scheme 1 Procedure for the synthesis of DMPAT (**1**) and Met (**2**).

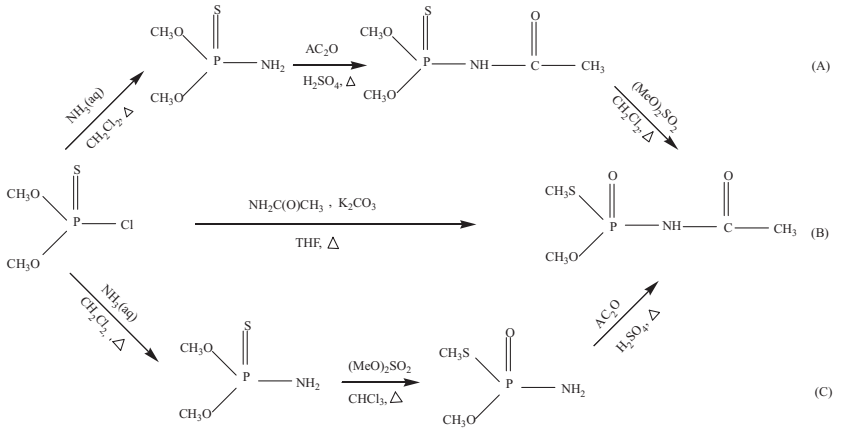
We attempted to reconsider the factors leading to completion of the reactions and to define the optimized conditions for yield and purity of the products in bench scale (0.1 M). To follow the reaction, ^{31}P NMR was chosen as the most appropriate technique to analyze the compounds present in the organic phase.

In 0.03 M scale, the reactions **1** and **2**, performed in a three-necked 25 mL flask equipped with a magnetic stirrer, lasted 3 h. However, in 0.1 M scale, the complete conversion in a 500 mL reactor equipped with a mechanical stirrer lasted for 4 h, after which the mixture became completely clear as a sign of the end of reaction. We used a mechanical stirrer with a four-blade turbine and a rate of 1500 rpm.

Therefore, the reaction time and temperature must be at minimum in the presence of ammonium hydroxide and dimethyl sulfate to prevent the formation of ammonium chloride and N-methyl methamidophos.

Table I The results of different methods on the synthesis

Method	Time (h)	Temperature (°C)	Yield (%)
A	8	5–55	40
B	12	60	63
C	5	5–60	84



Scheme 2 Preparation routes of Ace.

In this study, the one-pot synthesis of Ace was done by three different methods as shown in Scheme 2. After investigation of the influence of different factors such as temperature, time, solvent, and mole ratio of the reactants, the optimized conditions for the production of Ace in one-pot synthesis were achieved (Table I).

Comparison of the data in Table I shows that method A has the lowest yield. In method B, the reaction is done in only one step, and the yield is relatively high compared to the method A, but the alkaline pH of the reaction leads to the hydrolysis of the product. Accordingly, compared to the other two methods, method C seems to be the optimum route for the one-pot synthesis of Ace, due to higher yield and shorter reaction time.

Correlation Between Some Physicochemical Properties of Met and Ace with IC_{50}

Considering that the ^{31}P chemical shift is an index to investigate the phosphorus electron density,[14] and since its value varies from 29.18 for Ace to 37.40 for Met, it may therefore be used to introduce the electronic effect parameter. Physicochemical properties (δ of $^{31}P\{^{1}H\}$ NMR and measured log*P*) and anti-AChE activity of Met and Ace are summarized in Table II (available online in the Supplemental Materials), which shows the reverse correlation between IC_{50} and δ (^{31}P) as well as between IC_{50} and lipophilicity (log*P*) in both Met and Ace.

Mass Study

Ionization of the nitrogen atom in the Met molecule provided a powerful α-cleavage leading to formation of ion $[M\text{-}SCH_3]^+$ (*m/z* 94). This ionization has the lowest potential energy among the four charge sites of the Met molecule: nitrogen, sulfur, and the two oxygen atoms.[15] Regarding the Ace molecule, ionization also led to the formation of the ion $[M\text{-}SCH_3]^+$ (*m/z* 136).[16] This suggests that the $CH_3S{-}P$ bond is the weakest bond and probably $-SCH_3$ is the leaving group on the interaction of both Met and Ace with AChE (Scheme 3, available online in the Supplemental Materials).

EXPERIMENTAL

Materials and Methods

AChE (of human erythrocyte), acetylthiocolin iodide (ATChI), and 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB) were obtained from Sigma. Na_2HPO_4 and NaH_2PO_4 (99%), O,O-dimethyl phosphorochloridothioate (97%), ammonium hydroxide (25% aqueous solution), dimethyl sulfate (99%), methylene chloride (99.5%), chloroform (99.5%), THF (99.5%), potassium carbonate (99.5%), and acetamide (98%) were used as supplied. ^1H, ^{13}C, and ^{31}P NMR spectra were recorded on a Bruker (Avance DRS) 250 MHz spectrometer. ^1H and ^{13}C chemical shifts were determined relative to TMS as internal standard and ^{31}P chemical shift relative to 85% H_3PO_4 as external standard. IR spectra were obtained using KBr pellets on a Perkin-Elmer 783 model spectrometer. Mass spectroscopy (Varian Star 3400 CX) and UV spectrophotometer were performed using a Cecil 8000.

Synthesis

DMPAT, $P(S)NH_2(OCH_3)_2$ (1). In 0.03 M scale, ammonium hydroxide (4.08 g, 69.36 mmol) was slowly added to a solution of O,O-dimethyl phosphorochloridothioate (4.82 g, 30 mmol) in methylene chloride (10 mL), and the obtained mixture was stirred at 0°C. The mixture was heated to 44°C by gentle reflux and cooled down to 21°C. The mixture was stirred for an additional 35 min to form two separate phases. The organic phase was washed with water (3 mL), and the aqueous phase was washed with methylene chloride (2 × 5 mL). The aqueous phase was removed, the organic phase was dried with a little sodium sulfate, and the solvent was left to evaporate in vacuum.

In 0.1 M scale, ammonium hydroxide (16.92 g, 287.6 mmol) was slowly added to a solution of O,O-dimethyl phosphorochloridothioate (20 g, 124.5 mmol) in methylene chloride (50 mL), and the obtained mixture was stirred at 0°C. The mixture was heated to 44°C by gentle reflux and cooled to 25°C. The mixture was stirred for an additional 1 h, leaving two separated phases. The organic phase was removed by addition of anhydrous sodium sulfate. Then the solvent was stripped to obtain a colorless oily product:

Colorless oil, Yield: 99%. TLC (*n*-hexane/ethyl acetate (2:1, v/v)): R_f, 0.40. ^1H NMR ($CDCl_3$), δ_H: 3.23 (brs, 2H, NH_2), 3.74 (d, $^3J_{P\text{-}H}$ = 13.8 Hz, 3H, OCH_3). ^{13}C NMR ($CDCl_3$), δ_C: 53.10 (d, $^2J_{P\text{-}C}$ = 5.0 Hz, 1C, OCH_3). $^{31}P\{^1H\}$ NMR ($CDCl_3$), δ_P: 76.26 (s). ^{31}P NMR, δ_P: 76.24(m). IR (KBr) ($\tilde{\upsilon}$, Cm^{-1}): 3380, 3250, 2920, 2820, 1530, 1440, 1260, 950 (P—OCH_3), 790 (P=S), and 780 (P—NH_2). GC—MS (20 ev): retention time = 5 min; m/z (intensity (%)):142 (12) $(M+1)^+$, 141 (87) M^+, 110 (37) $[M—OCH_3]^+$, 111 (38) $[M—OCH_2]^+$, 78 (100) $[CH_3O—P—NH_2]^+$, 64 (16) $[NH_2POH]^+$, 47 (39) $[SCH_3]^+$, and 46 (42) $[SCH_2]^+$.

Met, $P(O)(NH_2)(SCH_3)(OCH_3)$ (2). In 0.03 M scale, dimethyl sulfate (0.63 g, 5.02 mmol) was slowly added to a solution of DMPAT (**1**) (3 g, 21.23 mmol) in chloroform (8 mL), and the obtained mixture was stirred at 60°C for 3 h. The solvent was left to evaporate in vacuum, and the remaining oily product was purified by flash chromatography (silica gel, ethyl acetate:acetone 1:9) to obtain colorless crystals.

In 0.1 M scale, dimethyl sulfate (3.15 g, 25.1 mmol) was slowly added to a solution of O,O-dimethyl phosphoramidothioate (**1**) (15 g, 106.15 mmol) in chloroform (30 mL), and the obtained mixture was stirred at 56°C. The solvent was left to evaporate in vacuum:

Colorless crystals, mp 43°C; Yield: 90%. TLC (*n*-hexane:ethyl acetate:acetone (6:3:1, v/v/v)): R_f, 0.20. ^1H NMR ($CDCl_3$), δ_H: 2.46 (d, $^3J_{P\text{-}H}$ = 14.8 Hz, 3H, SCH_3), 3.23 (brs,

2H, NH_2), 3.76 (d, $^3J_{P\text{-}H} = 12.8$ Hz, 3H, OCH_3). ^{13}C NMR ($CDCl_3$), δ_C: 12.73 (d, $^2J_{P\text{-}C} = 3.8$ Hz, 1C, SCH_3), 52.80 (d, $^2J_{P\text{-}C} = 5.7$ Hz, 1C, OCH_3). $^{31}P\{^1H\}$ NMR ($CDCl_3$), δ_P: 37.40 (s). ^{31}P NMR, δ_P: 37.40 (m). IR (KBr) ($\tilde{\upsilon}$, Cm^{-1}): 3300, 2860, 2840, 2540, 1550, 1430, 1200 (P=O), 1030, 950 (P−OCH_3), and 780 (P−NH_2). GC−MS (20 ev): retention time = 8.95 min; m/z (intensity (%)): 142 (100) $(M+1)^+$, 141 (43) M^+, 126 (18) $[M-CH_3]^+$, 111 (8) $[M-OCH_2]^+$, 110 (10) $[M-OCH_3]^+$, 95 (32) $[M-SCH_2]^+$, 94 (53) $[M-SCH_3]^+$, 79 (8) $[NH_2PO_2]^+$, 64 (25) $[NH_2POH]^+$, 47 (48) $[SCH_3]^+$, and 46 (27) $[SCH_2]^+$.

Ace, P(O)(NHCOCH$_3$)(SCH$_3$)(OCH$_3$) (3). The solution containing acetamide (5 g, 0.071 mol) and THF (57 mL) was charged in a flask. Then potassium carbonate (12.28 g, 0.085 mol) was added. The obtained mixture was stirred for 30 min. Next, O,O-dimethyl chlorothiophosphate was slowly added over a period of 15 min, and the mixture was then refluxed at 60°C for 12 h until the end of the reaction. It was then stirred for additional 35 min. The organic phase was separated, and the remaining product was purified with flash chromatography (silica gel, *n*-hexane:ethyl acetate 8:2) to obtain colorless crystals:

Mp 86°C; Yield: 63%. TLC (*n*-hexane:ethyl acetate (8:2, v/v)): R_f, 0.19. ^1H NMR ($CDCl_3$), δ_H: 2.16 (d, $^4J_{P\text{-}H} = 1.5$ Hz, 3H, CH_3), 3.85 (d, $^3J_{P\text{-}H} = 12.5$ Hz, 3H, OCH_3), 2.38 (d, $^3J_{P\text{-}H} = 17.5$ Hz, 3H, SCH_3), 9.21 (brs, H, NH). ^{13}C NMR ($CDCl_3$), δ_C: 12.35 (d, $^3J_{P\text{-}C} = 6.3$ Hz, 1C, CH_3), 53.65 (d, $^2J_{P\text{-}C} = 6.3$ Hz, 1C, OCH_3), 24.05 (d, $^2J_{P\text{-}C} = 6.3$ Hz, 1C, SCH_3), 172.00 (s) (C, C(O)).$^{31}P\{^1H\}$ NMR ($CDCl_3$), δ_P: 29.18 (s). ^{31}P NMR, δ_P: 29.80 (m). IR (KBr) ($\tilde{\upsilon}$, Cm^{-1}) 3200, 1680, 1050 (P−O−C), 950 (P−N−C), and 1350 (P=O). GC−MS (20 ev): retention time = 6.41 min; m/z (intensity (%)):184 (10) $(M+1)^+$, 64 (13) $[P(O)OH]^+$, 136 (59) $[C_3H_7NO_2]^+$, 94 (42) $[P(O)(OCH_3)NH_2]^+$, 142 (17) $[(SCH_3)(OCH_3)P(O)NH_2]^+$, 79 (28) $[(CH_3)P(O)]^+$, and 125 (23) $[M-NHC(O)CH_3]^+$.

Computational Evaluation of Biological Activity

The biological activity spectra of DMPAT, Met, and Ace were obtained by PASS (prediction of activity spectra for substances) software.[17] This software is able to predict 900 synchronized biological activities based on the molecular structure of various compounds. The biological activity spectra predicted by the PASS software are capable of distinguishing pharmaceutical effects, side effects, biochemical reaction mechanism, genetic mutation, carcinogenesis, toxicity fetus deficiency, and other biological activities of chemical compounds.[18] A portion of the predicted biological activity spectra for Met and Ace is given in Table III (available online in the Supplemental Materials).

Lipophilicity Study

The rate of transport of a toxicant across cell membranes is directly proportional to its lipid solubility. Water soluble compounds exhibit poor diffusion across lipophilic cell membranes. Small hydrophilic compounds, however, may penetrate membranes through aqueous pores. However, the total surface area of aqueous pores is small compared to the overall lipid–domain surface area.[4] In our research, log*P* values for the target compounds were experimentally determined by the shake-flask method. Calculation of log*P* values were performed as follows:[19]

$$\log P = \log\{(y - x/x)(V_{buffer}/V_{oct})\} \quad (1)$$

where, P is partition coefficient, $(y - x)$ is the mass of compound in the n-octanol phase after partitioning (mg), x is the mass of compound in the buffer phase (mg), V_{buffer} is the volume of buffer (mL), and V_{oc} is the volume of *n*-octanol (mL).

A calibration graph was plotted using different concentrations of the compounds. Then 5, 10, and 15 mL of buffer were added to two solutions of the compounds (0.5 M), respectively. The phases were shaken on a mechanical shaker for 30 min. After complete phase separation, the buffer phase was removed. Concentration of the compounds was determined in n-octanol phase by the calibration graph using a gas chromatograph spectrometer. The log$P$ values were determined by the Equation (1) as mentioned above (see Table IV, available online in the Supplemental Materials).

In Vitro Evaluation of Acetyl Cholinesterase Inhibition

Ellman's method was used to determine AChE activity. For the inhibitory experiments, the enzyme samples (2 μL) were incubated for 1 min at room temperature in different concentrations of the inhibitor, phosphate buffer (pH = 8), and Ellman's reagent (1500 μL). Then the concentration of ATChI (substrate) was optimized as 0.4 mM. Next, the IC_{50} values of Met and Ace were determined as $0.064 \pm (0.002)$ and $4.3 \pm (0.02)$ (see Figure 2, available online in the Supplemental Materials).

Effect of Met & Ace on the Kinetic Parameters of AChE Obtained from Human RBC

The activity of AChE was determined at various substrate concentrations in the presence and absence of Met and Ace. The enzyme was inhibited by 58% and 57% with 0.07 mM of the Met and 3.75 mM of Ace, respectively. Inhibition of AChE by both the compounds was reversible, since removal of the inhibitors by dilution of the enzyme preparation containing inhibitors restored the enzyme activity to the control level. In the absence of Ace with substrate concentration varying from 0.27 to 0.51 mM, the enzyme activity increased from 0.69 to 0.80 μmol/min/mg protein. However increasing the substrate concentration in the reaction mixture did not affect the degree of inhibition of the enzyme activity by Ace; the data were employed to obtain Lineweaver and Burke plots. Apparent K_m for the enzyme was 0.16 mM for both in presence and absence of Ace, while the maximum velocity was reduced from 1.05 in the absence of Ace to 0.68 in the presence of 3.78 mM of Ace, which indicates a noncompetitive type of inhibition. Kinetic analysis of the AChE inhibition by Met revealed that in the presence of Met the apparent K_m was increased from 0.15 to 0.49 mM, while there was no change in the V_{max}, which is the indication for the competitive type of inhibition. Similar results have been reported by Singh et al.[4] They have mentioned that the different pattern of inhibition of AChE by Met and the Ace can be due to the differences in electronic charge distribution of the two compounds.

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