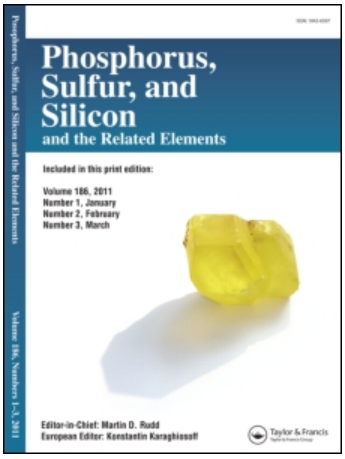

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SYNTHESIS, CHARACTERIZATION, MOLECULAR MODELING AND BIOLOGICAL ACTIVITY AGAINST *ARTEMIA SALINA* OF NEW SYMMETRICAL BISPHOSPHORAMIDATES

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SYNTHESIS, CHARACTERIZATION, MOLECULAR MODELING AND BIOLOGICAL ACTIVITY AGAINST *ARTEMIA SALINA* OF NEW SYMMETRICAL BISPHOSPHORAMIDATES

Viviane Martins Rebello dos Santos, João Batista Neves DaCosta,[1] *Carlos Mauricio R. Sant'Anna and Marcia Cristina Campos de Oliveira*
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A series of N,N′-bis(dialkylphosphoryl)diamines were prepared by Todd-Atherton reaction of dialkylphosphites with symmetrical diamines in a biphasic system. They were characterized by IR, ^{1}*H-NMR,* ^{13}*C-NMR and mass spectroscopy. Compounds with butoxy groups, isobutoxy groups and isopropoxy groups on the phosphorus atoms showed the lowest* LD_{50} *values when tested against* Artemia salina. *All other compounds that showed* LD_{50} *values higher than 363 ppm are considered non toxic. The results of a molecular modeling study suggest that the biological activity of the compounds may be related to AChE inhibition. Contrary to classical organophophorus AChE inhibitors, the compounds synthestized in this study do not possess a good leaving group, which suggests that they may act only as reversible inhibitors.*

Keywords: Artemia salina; biological activity; bisphosphoramidates; organophosphorus compounds

Various organophosphorus compounds have been widely used as pesticides,[1] herbicides, nematicides and fungicides.[2] Their mode of action is well known and involves inhibition of acetylcholinesterase (AChE).[3–6]

Organophosphorus compounds in general have low residual action, low stability in the environment and limited accumulation in living

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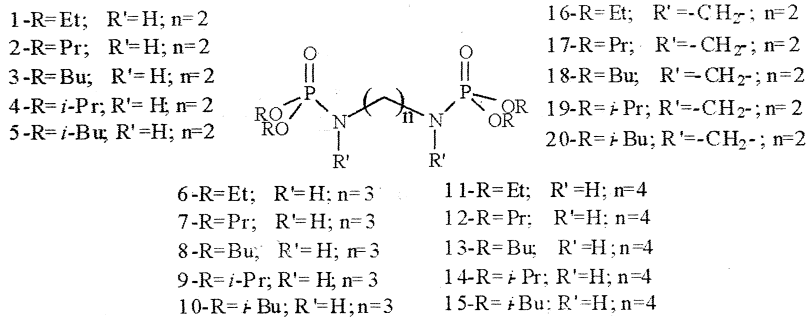


FIGURE 1 The new synthesized bisphosphoramidates.

organisms such that 80% to 90% of these compounds are eliminated after 48 h of contact. An increasing number of research projects have been reported which aim at developing weaker mammal AChE inhibitors and such is the case of the phosphoramidates synthesized by Mavrommatis et al.[7,8]

The mechanism of inhibition of AChE by organophosphorus compounds generally involves the replacement of a good leaving group bound to P atom by nucleophilic adition of a serine residue in the enzyme active site. The aim of this work is to investigate alternative structures which do not follow this mechanism of action.

This work describes the synthesis and characterization of twenty phosphoramidates (Figure 1) along with the evaluation of their biological activity employing the *Artemia salina*. lethality assay. The sensitivity of simple organisms such as *Artemia salina* Leach provides as fast and simple way to monitor biological response.[9–11] The results may be easily analyzed statistically. This assay allows the evaluation of the general toxicity and for this reason it is essential as a preliminary bioassay for the investigation of compounds with potential pesticide activity.[12,13]

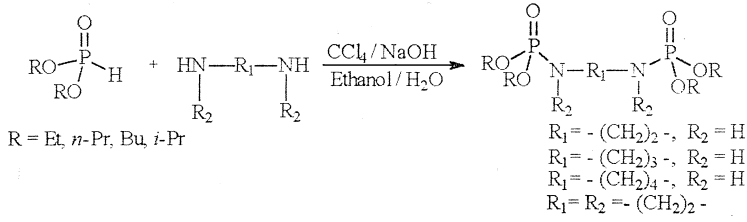
In order to investigate the possible action of the bisphosphoramidates as AChE inhibitors, the enthalpy of interaction between compound **3** (the most active) and a model of the active site of the enzyme[14–16] was calculated employing a semiempirical molecular modeling method. The choice of this method was based on the fact that it allows the evaluation of systems containing a high number of atoms, in the order of 10^2, within the quantum approximation.

RESULTS AND DISCUSSION

Bisphosphoramidates are compounds derived from phosphoramidic acid ($(HO)_2P(O)NH_2$). Their preparation can be achieved in two steps.

The first one involves the synthesis of dialkylphosphites, which is carried out by the reaction of (3 mols) of the appropriate alcohol with (1 mol) of PCl_3 to form the trialkyl phosphite which in turn suffers protonation and is finally attacked by a chloride ion furnishing the corresponding phosphites.[17–19]

The second step involves the reaction of (2 mols) of the dialkylphoposphites with (1 mol) of the symmetrical diamines in a biphasic system with carbon tetrachloride and basic water/ethanol, following the Todd-Atherton[20] reaction (Scheme 1). Water and ethanol work as solvents while carbon tetrachloride is the electrophile.[21]



SCHEME 1 Synthesis of N,N′-bis(dialkylphopshoryl)diamines.

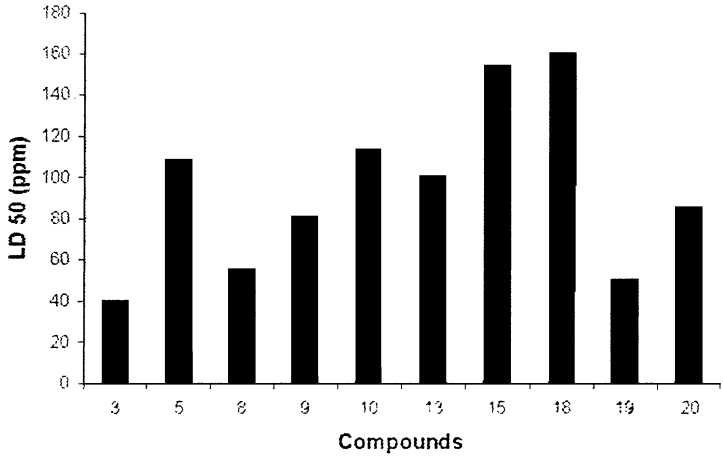
Following the syntheses and characterization of the N,N′-bis(dialkylphosphoryl)diamines, the biological activity towards *Artemia salina* was evaluated and the corresponding LD_{50} values are shown in (Table I).

The LD_{50} is lower for compounds with a butoxy group (**3**, **8**, **13** and **18**) followed by compounds with a isobutoxy group (**5**, **10**, **15** and **20**) and finally those compounds with an isopropoxy group (**4**, **9** and **19**).

TABLE I Values of LD_{50} of N,N′-Bis (dialkylphosphoryl)diamines

Compounds	[a]LD_{50}	Compounds	[a]LD_{50}
1	>363	**11**	>363
2	>363	**12**	>363
3	40	**13**	154
4	108	**14**	>363
5	55.3	**15**	160
6	>363	**16**	>363
7	>363	**17**	>363
8	80	**18**	50
9	113	**19**	111
10	100	**20**	85

[a]LD50 in ppm.



GRAPH 1 Compounds with the lowest LD_{50} values.

The remaining compounds showed LD_{50} values higher than 363 ppm and are considered non toxic. Graph 1 above shows only compounds with the lowest LD_{50} values.

Molecular modeling can help better understand the higher toxicity of compound **3**. According to the mechanism described in the literature, the H atom of Ser238 hydroxy group has been transferred to one of the N atoms of the His480 side chain, so as to increase serine nucleophilicity. The initial geometry of the adduct was built locating the phophoroamidate P atom in the vicinity of the negatively charged O atom of Ser 238. After optimization, the adduct's structure is shown to be 20 kcal/mol more stable than those of compound **3** and the active site separated but also optimized (Table II), which indicates that this bisphosphoramidate may effectively bind to the active site of the enzyme.

Besides the expected participation of the residues of the oxyanion hole, hydrophobic interactions inside the active site of the enzyme with butyl groups of compound **3** must also be important, along with

TABLE II Calculated ΔH_f (PM_3 Method) for Bisphosphoramidate **3,** for the Enzyme Active Site and for the Interaction of Compound **3** with the Active Site

Structure	ΔH_f (Kcal/mol)
3	−401,96
DmAChE	−961,06
DmAChE/**3** adduct	−1383,05

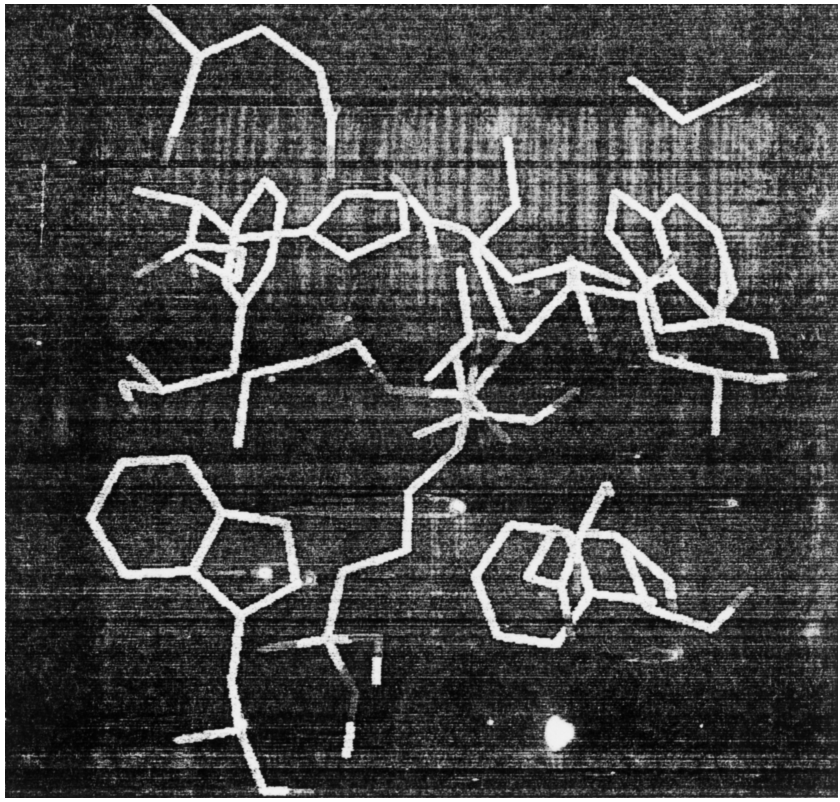


FIGURE 2 Representation of the Dm AchE/3 adduct after optimization (PM3 method). H atoms attached to carbons can be omitted for clarity.

a hydrogen bond between the Gly150 N atom and the NH group of the phosphoroamidate moiety which undergoes nucleophilic addition of Ser238 in Figure 2.

EXPERIMENTAL

1. Syntheses of Dialkyl Phosphites

1.1 Dialkyl phophites were prepared by treating tricholride phosphorus with three molar equivalents of the apropriate alcohol.[17–19]

2. Syntheses of N,N′-bis(dialkylphosphoryl)diamines

General procedure: In a 200 ml round flask equipped with an addition funnel, were placed diamine and NaOH in a 1:2 ratio dissolved

TABLE III Mass (g) and Volume (ml) of Reagents

	Phosphites		Diamines				
Compostos	R	V (ml)	R_1	R_1	V (ml)	NaOH (g)	CCl_4 (ml)
1	Et	2.3	$-CH_2CH_2-$	H	0.6	0.7	2.0
2	Pr	2.5	$-CH_2CH_2-$	H	0.5	0.6	1.8
3	Bu	3.5	$-CH_2CH_2-$	H	0.6	0.6	2.0
4	*i*-Pr	1.1*	$-CH_2CH_2-$	H	0.3	0.4	2.0
5	*i*-Bu	0.9*	$-CH_2CH_2-$	H	0.2	0.2	0.5
6	Et	4.5	$-CH_2CH_2-CH_2-$	H	1.5	1.4	4.0
7	Pr	2.4	$-CH_2CH_2-CH_2-$	H	0.6	0.6	1.7
8	Bu	2.6	$-CH_2CH_2-CH_2-$	H	0.6	0.5	1.5
9	*i*-Pr	2.4*	$-CH_2CH_2-CH_2-$	H	0.6	0.6	1.7
10	*i*-Bu	4.6*	$-CH_2CH_2-CH_2-$	H	0.9	0.9	2.5
11	Et	1.0	$-CH_2CH_2CH_2CH_2-$	H	0.4	0.3	0.9
12	Pr	1.6	$-CH_2CH_2CH_2CH_2-$	H	0.4	0.4	1.1
13	Bu	4.6	$-CH_2CH_2CH_2CH_2-$	H	0.9	0.9	2.5
14	*i*-Pr	2.0*	$-CH_2CH_2CH_2CH_2-$	H	1.0	0.8	2.5
15	*i*-Bu	4.1*	$-CH_2CH_2CH_2CH_2-$	H	1.1	0.9	2.5
16	Et	2.2	$-CH_2CH_2-$	$-CH_2CH_2-$	0.7*	0.7	2.0
17	Pr	1.6	$-CH_2CH_2-$	$-CH_2CH_2-$	0.4*	0.4	1.1
18	Bu	2.5	$-CH_2CH_2-$	$-CH_2CH_2-$	0.5*	0.5	1.5
19	*i*-Pr	1.1*	$-CH_2CH_2-$	$-CH_2CH_2-$	0.4*	0.4	1.2
20	*i*-Bu	4.1*	$-CH_2CH_2-$	$-CH_2CH_2-$	0.9*	0.8	2.0

*Expressed in grams.

in the same volume of distilled water and distilled ethanol (5.0 ml). The corresponding dialkylphosphite, dissolved in carbon tetrachloride, was placed in the addition funnel which was 20% excess to ensure complete chlorination of the phosphite. The flask was immersed in an ice bath and the dropwise addition of the phosphite was carried out with stirring. The reaction mixture was then left at room temperature for 5 h. Dichloromethane (5.0 ml) and water in equal amounts were then added to the reaction mixture and the organic layer was separated and the aqueous layer was washed 3 more times with dichloromethane (30 ml). Finally, the organic extract was dried with magnesium sulfate and the solvent was evaporated under reduced pressure.

Synthesis of N,N′-bis(diethylphosphoryl)ethylenediamine **(1)**: (2.0 g, 67%), m.p. 80–82°C; MS (m/z): 333 (M^{+1}); δ_H (DMSO) 4.9 (N-**H**), 3.9 (-C**H**$_2$-O, q/d, J_{POCH2} 7.0 Hz), 1.2 (C**H**$_3$CH$_2$-O, t, $J_{POCH2CH3}$ 7.0 Hz) and 2.8 (-HN-C**H**$_2$-, m); δ_C ($CDCl_3$) 61.3 (-**C**H$_2$-O, d), 16.1 (**C**H$_3$CH$_2$-O, d) and 42.2 (-HN-**C**H$_2$-, d); ν_{max}/cm^{-1} 3187 (N-H), 1243 (P=O), 1037 (P-O), 971 (P-N).

Synthesis of N,N′-bis(dipropylphosphoryl)ethylenediamine **(2)**: a yellowish oil (2.0 g, 67%); MS (m/z) 389 (M^{+1}); δ_H ($CDCl_3$) 3.5 (N-**H**), 4.0

(-C**H**$_2$-O, t/d, J_{POCH2} 6.7 Hz), 1.7(-C**H**2CH2-O, m, $J_{POCH2CH2}$ 7.0 Hz), 1.0 (C**H**$_3$CH$_2$CH$_2$-O, t, $J_{POCH2CH2CH3}$ 7.0 Hz) and 3.0 (-HN-C**H**$_2$-, m); δ_C ($CDCl_3$) 67.0 (-**C**H$_2$-O, d), 23.0(-C**H**$_2$CH$_2$-O, d), 9.4 (C**H**$_3$CH$_2$CH$_2$-O, d) and 42.1 (-HN-**C**H$_2$-, d); ν_{max}/cm^{-1} 3191 (N-H), 1240 (P=O), 1064 (P-O), 1003 (P-N).

Synthesis of N,N′-bis(dibutylphosphoryl)ethyelenediamine **(3)**: a colorless oil (1.7 g, 43%); MS (m/z) 445 (M^{+1}); δ_H ($CDCl_3$) 3.8 (N-**H**), 4.1 (-C**H**$_2$-O, t/d,J_{POCH2} 6.0 Hz), 1.5 (-C**H**$_2$CH$_2$-O, m, $J_{POCH2CH2}$ 6.4 Hz), 1.3 (-C**H**$_2$CH$_2$CH$_2$-O, m, $J_{POCH2CH2CH2}$ 7.3 Hz), 0.8 (C**H**$_3$CH$_2$CH$_2$CH$_2$-O, t, $J_{POCH2CH2CH2CH3}$ 7.2 Hz) and 2.8 (-HN-C**H**$_2$-); δ_C ($CDCl_3$) 65.4 (-**C**H$_2$-O, d), 31.9 (-**C**H$_2$CH$_2$-O, d), 18.4 (-**C**H$_2$CH$_2$CH$_2$-O, d), 13.2 (**C**H$_3$CH$_2$CH$_2$CH$_2$-O, d) and 42.3 (-HN-**C**H$_2$-, d); ν_{max}/cm^{-1} 3243 (N-H), 1234 (P=O), 1066 (P-O), 984 (P-N).

Synthesis of N,N′-bis(diisopropylphosphoryl)ethylenediamine **(4)**: a colorless oil (1.0 g, 79%); MS (m/z) 389 (M^{+1}); δ_H (DMSO) 4.8 (N-**H**), 4.4 (-C**H**-O, m, J_{POCH} 6.2 Hz), 1.2 ((C**H**$_3$)$_2$CH-O, d, $J_{POCH(CH3)2}$ 6.1 Hz) and 2.7 (-HN-C**H**$_2$-, m); δ_C ($CDCl_3$) 70.2 (-**C**H-O, d), 23.7 ((**C**H$_3$)$_2$CH-O, d) and 44.4 (-HN-**C**H$_2$-, d); ν_{max}/cm^{-1} 3242 (N-H), 1236 (P=O), 1109 (P-O), 991 (P-N).

Synthesis of N,N′-bis(diisobutylphosphoryl)ethylenediamine **(5)**: a yellowish oil (0.9 g, 85%); MS (m/z) 445 (M^{+1}); δ_H (DMSO) 4.9 (N-**H**), 3.6 (-C**H**$_2$-O, d/d, J_{POCH2} 6.4 Hz), 1.9 ((CH$_3$)$_2$C**H**CH$_2$-O, m, $J_{POCH2CH}$ 6.5 Hz), 0.9 ((C**H**$_3$)$_2$CHCH$_2$-O), d, $J_{POCH2CH(CH3)2}$ 6.5 Hz) and 2.8 (-HN-C**H**$_2$-, m); δ_C ($CDCl_3$) 71.3 (-**C**H$_2$-O, d), 28.2 ((**C**H$_3$)$_2$**C**HCH$_2$-O, d), 17.9 ((**C**H$_3$)$_2$CHCH$_2$-O, d) and 42.1 (-HN-**C**H$_2$-, d); ν_{max}/cm^{-1} 3237 (N-H), 1232 (P=O), 1020 (P-O), 961 (P-N).

Synthesis of N,N′-bis(diethylphosphoryl)1,3-propylenediamine **(6)**: a yellowish oil (2.2 g, 36%); MS (m/z) 347 (M^{+1}); δ_H ($CDCl_3$) 4.0 (N-**H**), 3.3 (-C**H**$_2$-O, q/d, J_{POCH2} 7.3 Hz), 1.2 (C**H**$_3$CH$_2$-O, t, $J_{POCH2CH3}$ 6.3 Hz), 2.8 (-HN-C**H**$_2$-, m) and 1.6 (-HN-CH$_2$-C**H**$_2$-, m); δ_C ($CDCl_3$) 66.5 (-**C**H$_2$-O, d), 15.9 (**C**H$_3$CH$_2$-O, d), 38.3 (-HN-**C**H$_2$-, d) and 32.5 (-HN-CH$_2$-**C**H$_2$-, d); ν_{max}/cm^{-1} 3240 (N-H), 1232 (P=O), 1020 (P-O), 966 (P-N).

Synthesis of N,N′-bis(dipropylphosphoryl)1,3-propylenediamine **(7)**: a colorless oil (1.8 g, 60%); MS (m/z) 402 (M^{+1}); δ_H (DMSO) 4.8 (N-**H**), 3.8 (-C**H**$_2$-O, t/d, J_{POCH2} 6.8 Hz), 1.6 (-C**H**$_2$CH$_2$-O, m, $J_{POCH2CH2}$ 7.0 Hz), 0.9 (C**H**$_3$CH$_2$CH$_2$-O, t, $J_{POCH2CH2CH3}$ 6.7 Hz), 2.8 (-HN-C**H**$_2$-, m) and 1.6 (-HN-CH$_2$-C**H**$_2$-, m); δ_C ($CDCl_3$) 66.5 (-**C**H$_2$-O, d), 33.2 (-C**H**$_2$CH$_2$-O, d), 9.9 (C**H**$_3$CH$_2$CH$_2$-O, d), 38.3 (-HN-**C**H$_2$-, d) and 23.1 (-HN-CH$_2$-**C**H$_2$, d); ν_{max}/cm^{-1} 3239 (N-H), 1234 (P=O), 1108 (P-O), 990 (P-N).

Synthesis of N,N′-bis(dibutylphosphoryl)1,3-propylenediamine **(8)**: a colorless oil (2.4 g, 80%); MS (m/z) 459 (M^{+1}); δ_H ($CDCl_3$) 3.5 (N-**H**), 3.9 (-C**H**$_2$-O, t/d, J_{POCH2} 6.2 Hz), 1.5 (-C**H**$_2$CH$_2$-O, m, $J_{POCH2CH2}$ 6.2 Hz), 1.3 (-C**H**$_2$CH$_2$CH$_2$-O, m, $J_{POCH2CH2CH2}$ 7.5 Hz), 0.8 (C**H**$_3$CH$_2$CH$_2$CH$_2$-O, t,

$J_{POCH2CH2CH2CH3}$ 7.5 Hz), 2.9 (HN-C**H**$_2$-, m) and 1.5 (HN-CH$_2$-C**H**$_2$-, m); δ_C ($CDCl_3$) 65.1 (-**C**H$_2$-O, d), 31.5(-**C**H$_2$CH$_2$-O, d), 17.9 (-**C**H$_2$CH$_2$CH$_2$-O), 12.7 (**C**H$_3$CH$_2$CH$_2$CH$_2$-O, d), 37.0 (-HN-**C**H$_2$-, d) and 31.5 (-HN-CH$_2$-**C**H$_2$, d); ν_{max}/cm^{-1} 3243 (N-H), 1233 (P=O), 1067 (P-O), 984 (P-N).

Synthesis of N,N′-bis(diisopropylphosphoryl)1,3-propylenediamine **(9)**: a colorless oil (2.0 g, 70%); MS (m/z) 404 (M^{+1}); δ_H ($CDCl_3$) 3.2 (N-**H**), 4.6 (-C**H**-O, m, J_{POCH} 6.1 Hz), 1.3 ((C**H**$_3$)$_2$CH-O, d, $J_{POCH(CH3)2}$ 6.3 Hz), 3.0 (-HN-C**H**$_2$-, m) and 1.6 (-HN-CH$_2$-C**H**$_2$-, m); δ_C ($CDCl_3$) 70.5 (-**C**H-O, d), 23.7 ((**C**H$_3$)$_2$CH-O, d), 32.7 (-HN-**C**H$_2$-, d) and 34.5 (-HN-CH$_2$-**C**H$_2$-, d); ν_{max}/cm^{-1} 3238 (N-H), 1233 (P=O), 1063 (P-O), 999 (P-N).

Synthesis of N,N′-bis(diisobutylphosphoryl)1,3-propylenediamine **(10)**: a colorless oil (4.7 g, 87%); MS (m/z) 459 (M^{+1}); δ_H (DMSO) 4.8 (N-**H**), 3.6 (-C**H**$_2$-O, d/d, J_{POCH2} 6.4 Hz), 1.8 ((CH$_3$)$_2$C**H**CH$_2$-O, m, $J_{POCH2CH(CH3)2}$ 6.6 Hz), 0.9 ((C**H**$_3$)$_2$CHCH$_2$-O, d, $J_{POCH2CH(CH3)2}$ 5.5 Hz), 2.8 (-HN-C**H**$_2$-, m) and 1.5 (-HN-CH$_2$-C**H**$_2$-, m); δ_C ($CDCl_3$) 72.0 (-**C**H-O, d), 28.8 ((CH$_3$)$_2$**C**HCH$_2$-O, d), 19.8 ((**C**H$_3$)$_2$CHCH$_2$-O, d), 37.8 (-HN-**C**H$_2$-, d) and 32.8 (-HN-CH$_2$-**C**H$_2$-,d); ν_{max}/cm^{-1} 3237 (N-H), 1235 (P=O), 1019 (P-O), 960 (P-N).

Synthesis of N,N′-bis(diethylphosphoryl)1,4-butylenediamine **(11)**: a yellowish oil (0.4 g, 28%); MS (m/z) 362 (M^{+2}); δ_H ($CDCl_3$) 2.6 (N-**H**), 4.0 (-C**H**$_2$-O, q/d, J_{POCH2} 7.3 Hz), 1.3 (C**H**$_3$CH$_2$-O, t, $J_{POCH2CH3}$ 7.1 Hz), 2.9 (-HN-C**H**$_2$-, m) and 1.6 (-HN-CH$_2$-C**H**$_2$-, m); δ_C ($CDCl_3$) 61.6 (-**C**H$_2$-O, d), 15.6 (**C**H$_3$CH$_2$-O, d), 40.4 (-HN-**C**H$_2$-, d) and 28.1 (-HN-CH$_2$-**C**H$_2$-, d); ν_{max}/cm^{-1} 3260 (N-H), 1228 (P=O), 1032 (P-O), 967 (P-N).

Synthesis of N,N′-bis(dipropylphosphoryl)1,4-butylenediamine **(12)**: a yellowish oil (0.5 g, 25%); MS (m/z) 417 (M^{+1}); δ_H ($CDCl_3$) 2.4 (N-**H**), 4.5 (-C**H**$_2$-O, t/d, J_{POCH2} 7.1 Hz), 1.2 (-C**H**$_2$CH$_2$-O, m, $J_{POCH2CH2}$ 6.5 Hz), 0.9 (C**H**$_3$CH$_2$CH$_2$-O, t, $J_{POCH2CH2CH3}$ 6.5 Hz), 2.8 (-HN-C**H**$_2$-, m) and 1.5 (-HN-CH$_2$-C**H**$_2$-, m); δ_C ($CDCl_3$) 70.8 (-**C**H$_2$-O, d), 30.6 (-C**H**$_2$CH$_2$-O, d), 20.4 (C**H**$_3$CH$_2$CH$_2$-O, d), 39.6 (-HN-**C**H$_2$-, d) and 34.6 (-HN-CH$_2$-**C**H$_2$, d); ν_{max}/cm^{-1} 3425 (N-H), 1220 (P=O), 1040 (P-O), 970 (P-N).

Synthesis of N,N′-bis(dibutylphosphoryl)1,4-butylenediamine **(13)**: a colorless oil (3.2 g, 57%); MS (m/z) 474 (M^{+2}); δ_H ($CDCl_3$) 3.2 (N-**H**), 3.9 (-C**H**$_2$-O, t/d, J_{POCH2} 6.1 Hz), 1.5 (-C**H**$_2$CH$_2$-O, m, $J_{POCH2CH2}$ 6.4 Hz), 1.4 (-C**H**$_2$CH$_2$CH$_2$-O, m, $J_{POCH2CH2CH2}$ 6.3 Hz), 0.9 (C**H**$_3$CH$_2$CH$_2$CH$_2$-O, t, $J_{POCH2CH2CH2CH3}$ 6.0 Hz), 2.8 (-HN-C**H**$_2$-, m) and 1.4 (HN-CH$_2$-C**H**$_2$-, m); δ_C ($CDCl_3$) 66.0 (-**C**H$_2$-O, d), 28.9 (-**C**H$_2$CH$_2$-O, d), 17.7 (-**C**H$_2$CH$_2$CH$_2$-O), 13.8 (**C**H$_3$CH$_2$CH$_2$CH$_2$-O, d), 41.1 (-HN-**C**H$_2$-, d) and 32.5 (-HN-CH$_2$-**C**H$_2$, d); ν_{max}/cm^{-1} 3342 (N-H), 1232 (P=O), 1067 (P-O), 983 (P-N).

Synthesis of N,N′-bis(diisopropylphosphoryl)1,4-butylenediamine **(14)**: (1.4 g, 57%), m.p. 75–77°C; MS (m/z) 417 (M^{+1}); δ_H ($CDCl_3$) 2.5

(N-**H**), 4.6 (-C**H**-O, m, J_{POCH} 6.2 Hz), 1.3 (($C\mathbf{H}_3)_2$CH-O, d, $J_{POCH(CH3)2}$ 6.2 Hz), 2.9 (-HN-C$\mathbf{H}_2$-) and 1.5 (-HN-CH_2-C$\mathbf{H}_2$-); δ_C ($CDCl_3$) 70.2 (-**C**H-O, d), 23.6 (($\mathbf{C}H_3)_2$CH-O, d), 40.9 (-HN-**C**H_2-, d) and 28.5 (-HN-CH_2-**C**H_2-, d); ν_{max}/cm^{-1} 3227 (N-H), 1234 (P=O), 1107 (P-O), 1007 (P-N).

Synthesis of N,N′-bis(diisobutylphosphoryl)1,4-butylenediamine **(15)**: a colorless oil (2.9 g, 58%); MS (m/z) 471 (M^{+1}); δ_H (DMSO) 4.8 (N-**H**), 3.6 (-C$\mathbf{H}_2$-O, d/d, J_{POCH2} 6.0 Hz), 1.8 (($CH_3)_2$C**H**CH_2-O, m, $J_{POCH2CH(CH3)2}$ 6.5 Hz), 0.9 (($C\mathbf{H}_3)_2$$CHCH_2$-O, d, $J_{POCH2CH(CH3)2}$ 6.5 Hz), 2.7 (-HN-C$\mathbf{H}_2$-, m) and 1.4 (-HN-CH_2-C$\mathbf{H}_2$-,m); δ_C 71.7 (-**C**H-O, d), 29.2 (($CH_3)_2$**C**HCH_2-O, d), 19.2 (($\mathbf{C}H_3)_2$$CHCH_2$-O, d), 40.6 (-HN-**C**$H_2$-, d) and 29.2 (-HN-$CH_2$-**C**$H_2$-); ν_{max}/cm^{-1} 3400 (N-H), 1232 (P=O), 1019 (P-O), 960 (P-N).

Synthesis of N,N′-bis(diethylphosphoryl)piperazine **(16)**: a yellowish oil, (2.5 g, 82%); MS (m/z) 360 (M^{+1}); δ_H ($CDCl_3$) 3.5 (-C$\mathbf{H}_2$-O, q/d, J_{POCH2} 7.4 Hz), 0.8 (C$\mathbf{H}_3CH_2$-O, t, $J_{POCH2CH3}$ 6.4 Hz) and 2.8 (-HN-C$\mathbf{H}_2$-, m); δ_C 60.2 (-**C**H_2-O, d), 13.1 (**C**H_3CH_2-O, d) and 41.2 (-HN-**C**H_2-, d); ν_{max}/cm^{-1} 1248 (P=O), 1030 (P-O), 974 (P-N).

Synthesis of N,N′-bis(dipropylphosphoryl)piperazine **(17)**: a yellowish oil, (1.0 g, 50%); MS (m/z) 415 (M^{+1}); δ_H ($CDCl_3$) 3.8 (-C$\mathbf{H}_2$-O, q/d, J_{POCH2} 6.5 Hz), 0.9 (C$\mathbf{H}_3CH_2$-O, t, $J_{POCH2CH2}$ 6.9 Hz), 1.6 (-C$\mathbf{H}_2CH_2$-O, m, $J_{POCH2CH2CH3}$ 6.8 Hz), 3.0 (-HN-C$\mathbf{H}_2$-, m); δ_C ($CDCl_3$) 68.4 (-**C**H_2-O, d), 24.1 (-C$\mathbf{H}_2CH_2$-O, d), 10.5 (C$\mathbf{H}_3CH_2CH_2$-O, d) and 45.0 (-HN-**C**H_2-, d); ν_{max}/cm^{-1} 1250 (P=O), 1062 (P-O), 989 (P-N).

Synthesis of N,N′-bis(dibutylphosphoryl)piperazine **(18)**: a colorless oil, (1.2 g, 40%); MS (m/z) 472 (M^{+2}); δ_H ($CDCl_3$) 3.9 (-C$\mathbf{H}_2$-O, t/d, J_{POCH2} 6.6 Hz), 1.6 (-C$\mathbf{H}_2CH_2$-O, m, $J_{POCH2CH2}$ 6.3 Hz), 1.3 (-C$\mathbf{H}_2CH_2CH_2$-O, m, $J_{POCH2CH2CH2}$ 6.9 Hz), 0.9 (C$\mathbf{H}_3CH_2CH_2CH_2$-O, t, $J_{POCH2CH2CH2CH3}$ 6.6 Hz) and 3.1 (-HN-C$\mathbf{H}_2$-, m); δ_C ($CDCl_3$) 65.4 (-**C**H_2-O, d), 31.7 (-**C**H_2CH_2-O,d), 18.2 (-**C**$H_2CH_2CH_2$-O,d), 13.1 (**C**$H_3CH_2CH_2CH_2$-O, d) and 45.3 (-HN-**C**H_2-, m); ν_{max}/cm^{-1} 1249 (P=O), 1026 (P-O), 979 (P-N).

Synthesis of N,N′-bis(diisopropylphosphoryl)piperazine **(19)**: a colorless oil, (1.1 g, 78%); MS (m/z) 415 (M^{+1}); δ_H ($CDCl_3$) 4.5 (-C**H**-O, m, J_{POCH} 6.3 Hz), 1.2 (($C\mathbf{H}_3)_2$CH-O, d, $J_{POCH(CH3)2}$ 6.1 Hz) and 3.0 (-HN-C$\mathbf{H}_2$-, m); δ_C ($CDCl_3$) 71.9 (-**C**H-O, d), 25.1 (($\mathbf{C}H_3)_2$CH-O, d) and 46.1 (-HN-**C**H_2-,d); ν_{max}/cm^{-1} 1250 (P=O), 1106 (P-O), 977 (P-N).

Synthesis of N,N′-bis(diisobutylphosphoryl)piperazine **(20)**: (2.9 g, 58%), mp 45°C; MS (m/z) 471 (M^{+1}); δ_H ($CDCl_3$) 3.7 (-C$\mathbf{H}_2$-O, d/d, J_{POCH2} 6.4 Hz), 1.9 (($CH_3)_2$C**H**CH_2-O, m, $J_{POCH2CH(CH3)2}$ 6.4 Hz), 0.9 (($C\mathbf{H}_3)_2$$CHCH_2$-O, d, $J_{POCH2CH(CH3)2}$ 6.4 Hz) and 3.1 (-HN-C$\mathbf{H}_2$-, m); δ_C ($CDCl_3$) 71.6 (-**C**H-O, d), 28.8 (($\mathbf{C}H_3)_2$CH-O, d) 18.7 (($C\mathbf{H}_3)_2$$CHCH_2$-O, d) and 44.4 (-HN-**C**H_2-, d); ν_{max}/cm^{-1} 1252 (P=O), 1052 (P-O), 980 (P-N).

Lethality Assay with Larvae of *Artemia salina* Leach[22–25]

General Procedure: The *Artemia salina* eggs were left in artificial sea water for 48 h in order for them to eclode.[26–29] The larvae were collected with a Pasteur pipette in such a way that 10 larvae were present in test tubes already containing saline solution (5 ml). A stock solution was prepared from the various N,N′-bis(dialkylphosphoryl)diamines (20 mg) dissolved in DMSO (2 ml) and the volume was made up to 10 ml with water. Each assay was carried out in quadruplicate by adding volumes of the stock solution (50, 100, 200, 300 and 500 μl) to the test tubes.

Blank assays were carried out as controls. The tubes were kept under light and the live and dead organisms counted after 24 h. Employing the Origin 6.0 software, the LD_{50} of the different phosphoroamidates was determined by plotting the percentage of live animals against *log* dose.

Compounds with butyl, isopropyl and isobutyl groups have LD_{50} values in the 40–160 ppm range while the others showed LD_{50} values higher than 360 ppm. A new assay with the most active compounds was carried out with volumes of the stock solution (10, 20, 50, 100 and 200 μl), in order to calculate the more accurate LD_{50} values. Compounds with LD_{50} values equal or above 360 ppm were considered non toxic to *Artemia salina*.

Molecular Modelling[30,31]

The model used in this study (DmAChE) was built by selecting 16 aminoacid residues (together with 5 molecules of water) from the active site of the only AChE crystalline structure available in the Protein Data Bank (PDB) from the fruit fly, *Drosophila melanogaster*. Details on the building of this model will be published elsewhere. All H and side chain atoms were kept free during the structural optimization, while the peptide bond atoms were fixed in space to avoid great movements during the optimization process. We assumed that only one of the phosphoroamidate groups of **3** interacts with Ser238 of the active site (which corresponds to Ser200 of *Torpedo californica* AChE). Thus, the butyl groups of the other phosphoroamidate moiety were substituted for methyls to simplify calculations. These were made by employing the PM3 Hamiltonian from the Mopac 6.0 software on either a Pentium II 333 MHz or a Pentium III 1.1 GHz. There are some recent 16 examples of the usage of PM3 in the calculation of reaction mechanisms involving hypervalent phosphorus.[32,33]

CONCLUSION

The phosphorylation of diamines with alkyl phosphites is a simple and efficient reaction which affords products in moderate yields. The use of the *Artemia salina* Leach bioassay as a preliminary test for the general toxicity of these compounds showed interesting results and warrants further toxicity assays against *Musca domestica* and *Stomoxys calcitrans*, especially with those compounds with lower LD_{50} values. The results of the molecular modelling experiments suggest that the toxicity of compound **3** may be related to AChE inhibition. Contrary to organophosphorus compounds which act as classical AChE inhibitors, the structures in this study do not possess a good leaving group associated with the P atom, which in turn suggests that they may act only as reversible inhibitors of the enzyme. However, the LD_{50} for compound **3**, in the nanomolar range, suggests that it may bind to the active site for a longer period, acting as an inhibitor which slowly dissociates from AChE. The modeling results indicate that a hydrogen bond between the N atom of AChE Gly150 and the NH group of the fosforamidate moiety, together with hydrophobic interactions with the butyl groups of compound **3** may contribute to the adduct stability.

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