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Bioorganic Chemistry 35 (2007) 68–81
BIOORGANIC CHEMISTRY
www.elsevier.com/locate/bioorg

New bisphosphorothioates and bisphosphoroamidates: Synthesis, molecular modeling and determination of insecticide and toxicological profile

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Received 26 July 2006
Available online 18 October 2006

Abstract

A series of new compounds, *N*,*N*′-bis(dialkylphosphoryl)diamines and *S*,*S*′-bis(dialkylphosphoryl)-1,3-propanedithiols were prepared by a Todd–Atherton like reaction of dialkylphosphites with symmetrical diamines and 1,3-propanedithiols in a biphasic system [F.R. Athertoon, H.T. Howard, A.R. Todd, J. Chem. Soc. (1948) 1106–1111; F.R. Athertoon, H.T. Openshaw, A.R. Todd, J. Chem. Soc. (1945) 660–663]. The structures were characterized by IR, ^{1}H NMR, ^{13}C NMR and mass spectrometry. Compounds with butoxy, isobutoxy and isopropoxy groups linked in the phosphorus atom showed the lowest LD_{50} values when tested against *Musca domestica* and *Stomoxys calcitrans*. The pharmacological and toxicological evaluation of *N*,*N*′-bis(diisobutylphosphoryl)-1,3-propylenediamine and *S*,*S*′-bis(diisobutylphosphoryl)-1,3-propanedithiol, which were very active against *M. domestica* and *S. calcitrans*, demonstrated that these compounds present no toxicological effects against mice in a concentration of 200 mg/kg. An explanation for the observed activity profile is

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0045-2068/$ - see front matter
doi:10.1016/j.bioorg.2006.08.006

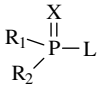
presented based on results obtained in a molecular modeling study with insect and mammalian acetylcholinesterase models.

Keywords: Bisphosphorothioates; Bisphosphoramidates; Biological activity

1. Introduction

The main classes of compounds employed in the control of pests are organophosphorus (OPs), carbamates, organochlorine, cyclodienes and pyrethroids. All these classes have a common target, which is the arthropod nervous system. OPs [1] and carbamates act as classical acetylcholinesterase (AChE) inhibitors. Cyclodienes act on the γ-aminobutyric acid (GABA) dependent chlorine channels. Finally, pyrethroids and organochlorine compounds act on the voltage dependent sodium channels [2,3].

OP compounds remain as one of the most interesting classes of insecticides from both the commercial and toxicological point of view. The relative low risk OP insecticides offer to mammals is due to their selectivity, which is attributable to structural differences in the molecular targets associated to amino acid changes, differential metabolic activation and detoxification, and/or to their biodegradability [4]. They are esters or thioesters derived from phosphoric, phosphonic, phosphinic and phosphoramidic acids. The basic structure of these compounds is shown in Fig. 1. Usually, R_1 and R_2 are alkyl or aryl groups which are either directly bound to the P atom to form phosphinates, or they are connected to it through an O or S atom, forming phosphates or phosphorothioates, respectively. In other instances, R_1 is directly bound to the P atom and R_2 is connected to it through an O or S atom forming phosphonates or thiophosphonates, respectively. Phosphoramidates carry at least one amino group in the molecule, which may have substituents. Oxygen, sulfur or selenium atoms may form double bonds with the P atom. The leaving group **L**, liberated when the OP is either hydrolyzed by phosphotriesterase (PTE) or by AChE [5,6], is a halogen atom or an alkyl, aryl or heterocyclic group bound to the P atom through O or S. The AChE-promoted hydrolysis mechanism involves a serine residue (Ser200 in *Torpedo californica* AChE) present in the active site located at the end of a narrow gorge, which is phosphorylated after the **L** group is displaced [7]. The reaction became essentially irreversible after a process called "aging", where the bond between R_1 or R_2 and the O atom is heterolytically cleaved, producing an alcoxide anion that forms a strong hydrogen bond with a protonated histidine residue of the active site (His440). ^{31}P NMR spectroscopy results clearly show the formation of a $P–O^-$ bond into the active site of OP–AChE adducts [8].



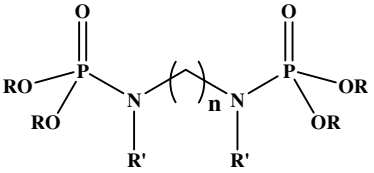
X = O, S e Se

Fig. 1. Basic structure of organophosphorous insecticides.

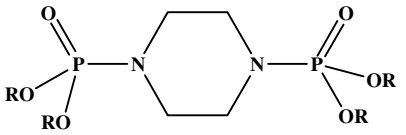
The OP insecticide class is characterized by the ease of preparation of derivatives, to the possibility of synthesis of pro-insecticides, which undergo preferential activation in insects and not in mammals, and finally to their higher biodegradability when compared to other classes, such as organochlorine insecticides. OP compounds possess low residual action, low stability in the environment and limited accumulation in living organisms, where 80–90% of the compounds are eliminated 48 h after contact.

There is a continuing need for new, safe, effective, and economical insecticides for crop and livestock protection, and public health programs. One of the greatest challenges faced by researchers investigating insecticides is the continuous development of new structures as a response to the resistance that insect populations develop to known insecticides after their prolonged and indiscriminate use. *Musca domestica* and *Stomoxys calcitrans* (both *Diptera Muscidae*) are examples of fly species that present permanent threats to human health and livestock production [9–12] In continuing the work of our research group on new insecticide leads, we investigated the activity of some phosphoramidates and two new bisphosphorothioates (Fig. 2 and Scheme 1) against both fly species. The structural design of these compounds was proposed with the expectation that they could establish additional interactions with the residues of the gorge conducting to the enzyme active site, which we anticipated to result in more stable AChE–OP complexes. Previous results showed that these compounds were toxic when tested against *Artemia salina* [13].

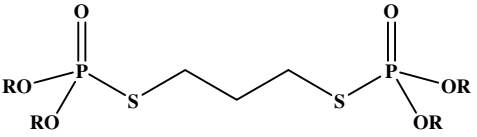
Since a useful insecticide should possess a low degree of toxicity towards mammals, we also determined the activity of the most active compounds against mice, which serves as a preliminary evaluation of the toxic properties to mammals of a potential insecticide [14] Acute toxicity is the malign effect which is produced in a short period of time and which results from the administration of a single or multiple doses of a substance in a 24 h period. This bioassay makes it possible to obtain preliminary information on the mode of action of the toxic substance [15]. Finally, we present a rationalization of the observed



(1) R=Et; R'=H; n=2
(2) R=Pr; R'=H; n=2
(3) R=Bu; R'=H; n=2
(4) R=*i*-Pr; R'=H; n=2
(5) R=*i*-Bu; R'=H; n=2
(11) R=Et; R'=H; n=3
(12) R=Pr; R'=H; n=3
(13) R=Bu; R'=H; n=3
(14) R=*i*-Pr; R'=H; n=3
(15) R=*i*-Bu; R'=H; n=3
(16) R=Et; R'=H; n=4
(17) R=Pr; R'=H; n=4
(18) R=Bu; R'=H; n=4
(19) R=*i*-Pr; R'=H; n=4
(20) R=*i*-Bu; R'=H; n=4

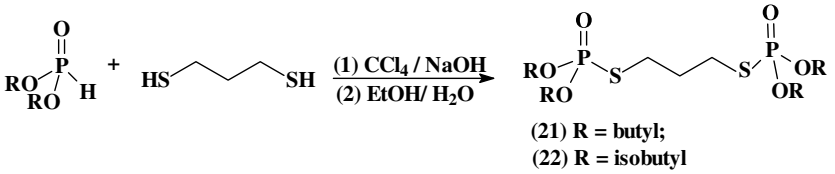


(6) R=Et
(7) R=Pr
(8) R=Bu
(9) R=*i*-Pr
(10) R=*i*-Bu



(21) R=Bu
(22) R=*i*-Bu

Fig. 2. Bisphosphoroamidates and bisphosphorodithiols tested against *M. domestica* and *S. calcitrans*.



Scheme 1.

activity results based on enthalpy profiles obtained by semiempirical molecular orbital calculations of phosphorylation reactions of AChE models involving the most promising compounds of our series.

2. Results and discussion

2.1. Synthesis and biological activity

The *S*,*S*′-bis(dialkylphosphororyl)-1,3-propanedithiols were synthesized by the reaction of 2 moles of the suitable dialkylphosphites with 1 mol of propanedithiol. The reaction proceeds via a nucleophylic substitution on the P atom, which is very reactive.

Both *S*,*S*′-bis-(dibutylphosphoryl)-1,3-propanedithiols and *S*,*S*′-bis-(diisobutylphosphoryl)-1,3-propanedithiols are white solids with low melting points which were obtained with 80–90% yields. The products were used without further purification since no impurities were observed either by TLC eluting with ethyl acetate or by IR and ^{1}H and ^{13}C NMR spectroscopies. The preparation of phosphorothioates from thiols and diethyl and diisopropylphosphites is not favored, most probably because the S atom prefers the attack on the C from the ester alkyl group instead of the P atom. The same behavior is found for dibutyl and diisobutylphosphites, this time steric hindrance being the most probable reason.

The results of the insecticide bioassays are described in Table 1. The LD_{50} of the commercial insecticide crufomate ((*R*,*S*)-4-*tert*-butyl-2-chlorophenyl methyl methylphosphoramidate) was determined by the same procedure and is presented as a reference.

Bisphosphorothioates (**21** and **22**) are in general more active as insecticides than bisphosphoroamidates and the most active compound is **22**. As can be seen in Table 1, *S. calcitrans* is more susceptible than *M. domestica* to the compounds tested, but it is interesting to observe that the order of insecticide activity is roughly the same against *M. domestica* and *S. calcitrans*. Compounds **1**, **2**, **6**, **7**, **11**, **12**, **16** and **17** are the least toxic and, when compared to crufomate, cannot be considered active. There is no significant difference in activity of compounds with the ethoxide and propoxide groups both for *M. domestica* and *S. calcitrans*. Among the bisphosphoroamidates, the compounds with butoxide groups (**3**, **8**, **13** and **18**) are the most toxic for both species, with LD_{50} values near that of crufomate. These compounds are followed by the ones with isobutoxide groups (**5**, **10**, **15** and **20**) and isopropoxide groups (**4**, **9**, **14** and **19**). Table 1 also shows that the species are generally susceptible to the same bisphosphoroamidates, the ethylenediamine series being more active than the 1,4-butylene series. Both species are very sensitive to compound **3**, and especially to the bisphosphorothioates **21** and **22**, which are even

Table 1
LD_{50} obtained from insecticide bioassays against *M. domestica* and *S. calcitrans*

Compound	LD_{50} *M. domestica* (mM)	LD_{50} *S. calcitrans* (mM)
1	26.2	8.0
2	22.4	6.3
3	2.5	0.8
4	3.8	2.0
5	2.9	1.4
6	35.6	8.4
7	28.4	7.1
8	3.1	1.7
9	4.9	2.6
10	3.2	1.9
11	41.6	9.0
12	35.0	7.6
13	4.0	2.7
14	8.0	4.9
15	4.7	2.9
16	47.0	1.5
17	39.7	1.2
18	9.5	5.2
19	16.2	7.0
20	12.2	6.1
21	1.9	0.7
22	0.7	0.2

Crufomate LD_{50} values, obtained by the same procedure, are 2.2 mM for *M. domestica*, and 1.0 mM for *S. calcitrans*.

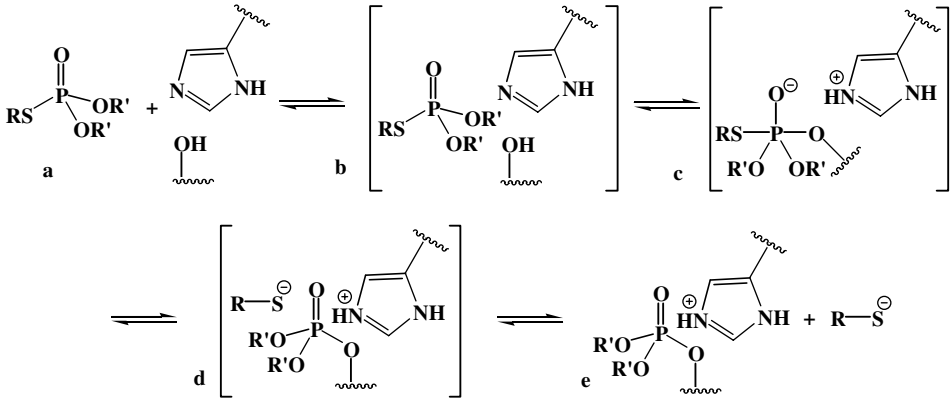
more active than the reference compound, crufomate. This fact shows that **21** and **22** are promising lead compounds and points to further tests with them.

A possible explanation for the greater activity of bisphosphoroamidates with the butoxide and isobutoxide groups is the formation of favorable interactions between the longer alkyl groups of these OPs and the side chains of hydrophobic amino acids, which may contribute to the adducts stability [13].

A representative compound of the bisphosphoramidate class (**15**) and a representative compound of the bisphosphorothioate class (**22**) were tested against mice, with the purpose of evaluating the acute toxicity of these compounds to mammals. None of the symptoms associated to toxicity, as described in Section 3.5, were observed in animals treated up to a dose of 200 mg/kg at the following time after treatment: 5, 10, 15, 30 and 60 min; 4, 8, 24 and 48 h; 4 and 7 days. Both compounds were found to be non-toxic to mice since even a dose of 200 mg/kg produced no mortality or side effects were observed. This result is very satisfactory because it suggests a relative selectivity for the target species.

2.2. Molecular modeling

The presence of phosphoroamidate or phosphorothioate groups in the molecules evaluated in this work is indicative that they may act as AChE inhibitors, but it is not clear how they interact with the enzyme active site and gorge, and how these interactions may influence the selectivity observed for the selected compounds. In order to clarify these



Scheme 2.

points, we implemented a molecular modeling study describing some representative compounds interacting with insect and mammalian AChE models. Scheme 2 describes the reaction steps evaluated in the molecular modeling study, which was initially implemented on compounds **21** and **22**, the most active compounds identified in the insecticide assay, inside the insect AChE model, DmAChE.

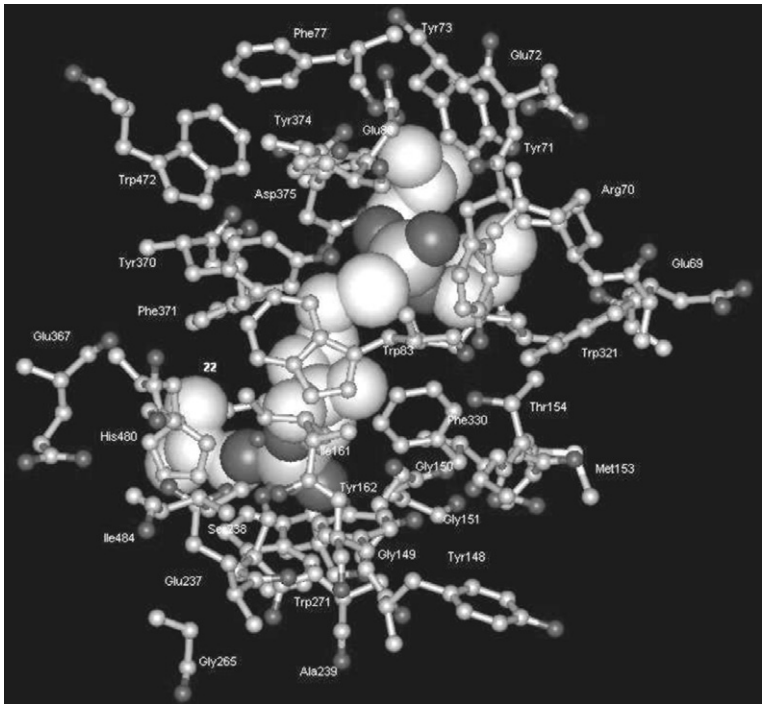


Fig. 3. 3D representation of the Michaelis complex between compound 22 and DmAChE-G. Hydrogen atoms were omitted for clarity.

Both compounds are able to completely occupy the active-site gorge, extending from the catalytic triad up to the outer peripheral binding site, as is exemplified for compound **22** in Fig. 3. In both Michaelis complexes (system **b**), it is observed that a number of hydrophobic amino acid side chains are interacting with different portions of the bisphosphorothioates molecules. Near the bottom of the gorge, for example, one of the butyl (compound **21**) or isobutyl (compound **22**) groups is inserted in a cavity delimited by the side chains of Trp271, Leu328, and His480, whereas the other is partially enclosed by Trp83 and Tyr370 side chains. In the peripheral binding site, the side chains of the aromatic residues Tyr73, Trp321, Tyr324, and the alpha carbon of Asp375, which forms a H-bond with the hydroxyl group of Tyr324, surround the alkyl groups located at the other end of both molecules. The propane "spacer" is positioned between the side chains of residues Leu328, Phe330 and Tyr370. It is important to note that the phenyl ring of the acyl pocket residue Phe330 is very close to the propane "spacer" of both compounds. Consequently, compounds **21** and **22** are expected to be susceptible to the natural mutation Phe330Tyr that is known to confer insecticide resistance to insects.

The same kind of hydrophobic interactions, with small variations, is observed in system **c**. This system, however, is calculated to become more stable than the previous one (**b**) by 11.5 kcal/mol for compound **21** and by 13.9 kcal/mol for compound **22**, as a consequence of the formation of the covalent bond between the P atom of the most internal phosphorodithioate group and the Ser238 residue.

The selectivity of insecticidal OP agents is attributable to structural differences in enzyme binding subsites and to selective bioactivation by insects or improved detoxification by mammals [4]. In order to evaluate the possible involvement of the first mechanism in the observed different activities of the bisphosphorothioates **22** against mice and flies, we calculated the enthalpy profile associated to the reaction steps described in Scheme 2 for a mammalian (HuAChE-G) and an insect (DmAChE-G) AChE models. The calculated enthalpy values are presented on Table 2.

Assuming that solvent effects, which were not considered in our calculations, are similar for both enzymes, the quantity $\Delta\Delta H_f$ on Table 2 can be regarded as a measure of the relative enthalpic advantage/disadvantage on going from mammalian to insect model AChEs for each reaction step. System **e** corresponds to the phosphorylated enzyme together with a non-interacting leaving group [$(i\text{-BuO})_2P(O)S(CH_2)_3S^-$]. The corresponding $\Delta\Delta H_f$ value is indicative that the group $(i\text{-BuO})_2P{=}O$ covalently bound to the catalytic serine residue is in a much more comfortable situation in DmAChE-G. As can be seen in Fig. 4, the

Table 2
Calculated enthalpy profiles associated to interaction of compound **22** with AChE models, PM3 method

System[a]	Relative ΔH_f[b]		$\Delta\Delta H_f$[b,c]
	DmAChE-G	HuAChE-G	
a	0	0	0
b	37.82	35.96	1.86
c	23.91	21.69	2.22
d	13.79	−2.87	16.66
e	−28.22	−0.26	−27.96

[a] See Scheme 2.
[b] kcal/mol.
[c] $\Delta\Delta H_f = \Delta H_f$(human) − ΔH_f(*D. melanogaster*).

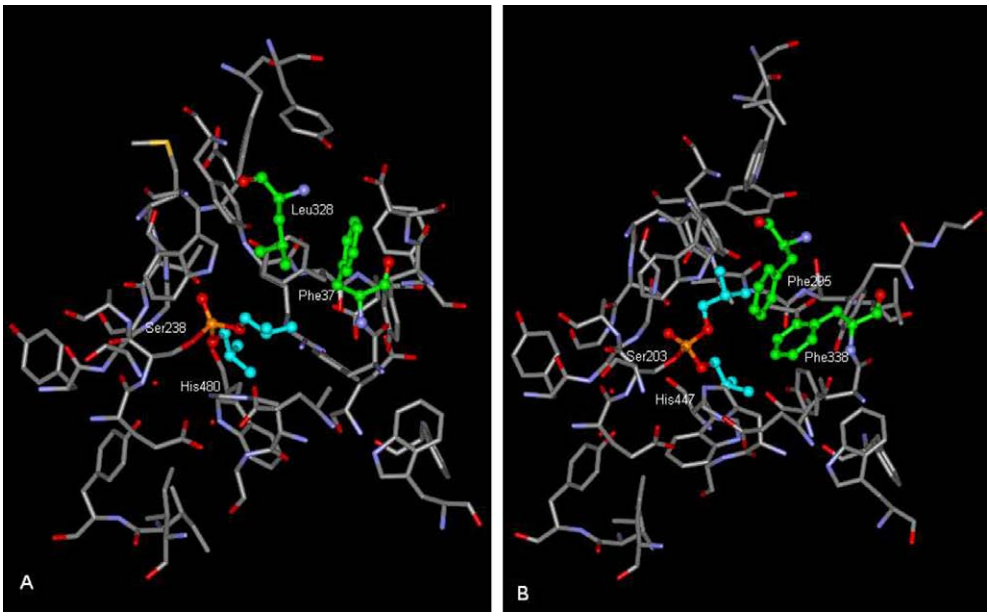


Fig. 4. 3D representation of DmAChE-G (A) and HuAChE-G (B) phosphorylated by compound **22**. $(i\text{-BuO})_2$P═O groups, Phe295 and Phe338 (in HuAChE-G), and Leu328 and Phe371 (in DmAChE-G) are presented in ball-and-stick. Color code: grey, C; green, C (Phe295, Phe338, Leu328, Phe371); cyan, C ($(i\text{-BuO})_2$P═O); red, O; blue, N; orange, P; yellow, S. Hydrogen atoms were omitted for clarity. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

$(i\text{-BuO})_2$P═O group is rotated ca. 35° in the mammalian model relative to its insect counterpart. The rigid stacking between Phe295 and Phe338 in HuAChE-G causes this rotation; in DmAChE, residue Phe295 is a leucine (Leu328), and the stacking is lost. In accordance to a proposal of Harel et al. [25], the loss of rigid stacking in DmAChE renders Phe371, the residue that corresponds to Phe338, more mobile, thus enabling the insect acyl binding pocket to accommodate larger moieties, such as one of the isobutyl groups of compound **22**.

The situation is reversed when the leaving group is still inside the gorge (systems **b**, **c** and **d**), as indicated by the values of $\Delta\Delta H_f$ (Table 2). The interactions of compound **22** are now more unfavorable inside the insect than in the mammalian AChE model. Harel and coworkers have determined that the active site gorge is wider in TcAChE than in DmAChE: the volume of the lower part of the active site gorge is about 50% of that of the equivalent portion of the gorge in TcAChE [24]. There is also a shift in the trajectory of the gorge. Thus, the less unfavorable calculated enthalpy values of compound **22** inside HuAChE-G in systems **b**, **c** and **d** are expected, as the HuAChE has almost the same amino acid residues in the gorge as TcAChE (Table 3).

3. Experimental

3.1. Synthesis of N,N′-bis(dialkylphosphoryl)diamines

Compounds **1**–**20** were prepared by a Todd–Atherton reaction of dialkylphosphites with symmetrical diamines in a biphasic system, as described previously [13,16–18].

Table 3
Selected residues used in the models, including the corresponding *Torpedo californica* AChE (TcAChE) residues as a reference

DmAChE	HuAChE	TcAChE[a]
Glu69	Tyr72	Tyr70
Arg70	Val73	Val71
Tyr71	Asp74	Asp72
Glu72	Thr75	Glu73
Tyr73	Leu76	Gln74
Phe77[b]	*Phe80*	*Phe78*
Glu80	Thr83	Ser81
Trp83	Trp86	Trp84
Tyr148	Tyr119	Tyr116
Gly149	Gly120	Gly117
Gly150	Gly121	Gly118
Gly151	Gly122	Gly119
Met153	Tyr124	Tyr121
Thr154	Ser125	Ser122
Gly155	Gly126	Gly123
Ile161	*Val132*	*Val129*
Tyr162	Tyr133	Tyr130
Glu237	Glu202	Glu199
Ser238	Ser203	Ser200
Ala239	*Ala204*	*Ala201*
Gly265	*Gly230*	*Gly227*
Trp271	Trp236	Trp233
Trp321	Trp286	Trp279
Tyr324	Leu289	Leu282
Leu328	Phe295	Phe288
Phe330	Phe297	Phe290
Glu367	Glu334	Glu327
Tyr370	Tyr337	Phe330
Phe371	Phe338	Phe331
Tyr374	Tyr341	Tyr334
Asp375	Gly342	Gly335
Trp472	Trp439	Trp432
His480	His447	His440
Gly481	Gly448	Gly441
Ile484	Ile451	Ile444

[a] Amino acids and numbers of the corresponding analogous residues in TcAChE according to the recommended nomenclature [34].
[b] Residues in italics were included because they are associated to mutations that confer resistance to OP insecticides.

3.2. Synthesis of S,S′-bis(dialkylphosphoryl)dithiols

The general preparation was carried out as follows: to a 200 mL round flask, equipped with an addition funnel, were added stoichiometric amounts of 1,3-propanedithiol and sodium hydroxide dissolved in equal volumes of distilled water and ethanol. The suitable dialkylphosphite (20% excess), dissolved in carbon tetrachloride, was poured in the funnel and added dropwise at 0 °C. When addition was completed, the reaction mixture was left stirring at room temperature for 12 h. Equal volumes of water and CH_2Cl_2 were then

added and the contents of the flask were transferred to a separation funnel to isolate the organic layer. The aqueous layer was extracted three more times with CH_2Cl_2. The combined organic extracts were dried with magnesium sulphate and the solvent was eliminated at under reduced pressure.

*3.2.1. Synthesis of S,S′-bis(dibutylphosphoryl)-1,3-propanedithiol (**21**)*

Following the above procedure, 0.6 mL (6.3 mmol) of 1,3-propanedithiol and a solution containing 0.5 g (13 mmol) of NaOH in 5.0 mL of water and 5 mL of ethanol were allowed to react with 2.5 mL (12 mmol) of diisobutylphosphite dissolved in 1.5 mL of CCl_4. This reaction furnished 1.8 g (80% yield) of a white solid. IR (NaCl pellet): $\nu = 1266\ cm^{-1}$ (P=O), $1030\ cm^{-1}$ (P–O), $1392\ cm^{-1}$ (C–S), $813\ cm^{-1}$ (P–S); ^1H NMR ($CDCl_3$): 4.0 [d/t($CH_3CH_2CH_2CH_2O$–, $J_{HH} = 6.5$ Hz, $J_{PH} = 6.5$ Hz, 8H)]; 1.5 [m($CH_3CH_2CH_2CH_2O$–, $J_{HH} = 6.2$ Hz, 8H)]; 1.3 [m($CH_3CH_2CH_2CH_2O$–, $J_{HH} = 6.2$ Hz, 8H)]; 0.9 [t($CH_3CH_2CH_2CH_2O$–, $J_{HH} = 6.2$ Hz, 12H)]; 4.1 [m(–CH_2CH_2S–, 4H)]; 1.3 [m(–CH_2CH_2S–, 2H)]; ^{13}C NMR ($CDCl_3$): 66.6 [d($CH_3CH_2CH_2CH_2O$–, $J_{PC} = 6.1$ Hz, 4C)]; 17.9 [d($CH_3CH_2CH_2CH_2O$–, $J_{PC} = 6.1$ Hz, 4C)]; 15.4 [d($CH_3CH_2CH_2CH_2O$–, 4C)]; 12.8 [s($CH_3CH_2CH_2CH_2O$–, 12C)] 62.9 [d(–CH_2CH_2S–, 2C)]; 31.5 [m(–CH_2CH_2S–, 1C)]; *m/e* (%): 55 (1); 81 (1); 99 (20); 127 (20); 183 (5); 239 (100).

*3.2.2. Synthesis of S,S′-bis(diisobutylphosphoryl)-1,3-propanedithiol (**22**)*

Reaction of 0.8 mL (8.5 mmol) of 1,3-propanedithiol and 0,7 g NaOH (18 mmol) in 5.0 mL of water and 5 mL of ethanol with 3.2 g (16 mmol) of diisobutylphosphite in 1.8 mL of CCl_4 furnished 3.6 g (90% yield) of a white solid. IR (NaCl pellet): 1242 (νP=O), 1031 (νP–O); 1396 (νC–S); 874 (νP–S); ^1H RMN ($CDCl_3$): 3.7 [d/d($(CH_3)_2CHCH_2O$–, $J_{HH} = 6.3$ Hz, $J_{PH} = 6.3$, 8H)]; 1.9 [m($(CH_3)_2CHCH_2O$–, $J_{HH} = 6.3$, 4H)]; 0.9 [d($(CH_3)_2CHCH_2O$–, 24H)]; 3.8 [t(–CH_2CH_2S–, 4H)]; 1.4 [m(–CH_2CH_2S–, 2H)]; ^{13}C RMN ($CDCl_3$): 72.5 [d($(CH_3)_2CHCH_2O$, $J_{PC} = 6.1$ Hz, 4C)]; 28.7 [d($(CH_3)_2CHCH_2O$–, $J_{PH} = 6.1$ Hz, 8H)]; 18.5 [d($(CH_3)_2CHCH_2S$, 4C)]; 73.6 [d(–CH_2CH_2S– 2C)]; 28.9 [d(–CH_2CH_2S–, 1C)]; *m/e* (%): 57 (10); 82 (10); 99 (60) 127 (100); 183 (15); 239 (30).

3.3. Insect rearing

A colony of *M. domestica* was established in the Veterinary Entomology Laboratory of the Animal Parasitology Department at the Rural Federal University of Rio de Janeiro. The adult flies were collected with entomological nets and placed in wooden cages of $30 \times 30 \times 30$ cm covert with plastic screens. The adults were fed daily with powdered milk and sugar (1:1 ratio) and water was provided *ad libitum* in small Petri dish. The medium used for oviposition and larval development was a 1:1 mixture of moistened bovine meat and wheat flours placed in a Petri dish. The eggs were collected and transferred to small vials 12 cm high × 7 cm diameter containing the same medium mentioned above. Pupae were collected using entomological forceps and placed in wooden cages.

Other colony of *S. calcitrans* was established in the same laboratory. Adult flies of this species were captured from parasitized bovines and equines at the Experimental Station for Animal Parasitology and placed in similar cages used for *M. domestica.* Adults of *S. calcitrans* were fed in small piece of gauze saturated with citrated bovine blood. The same medium was used for oviposition. Eggs were collected by decantation and placed in vials of 12 cm high × 7 cm diameter containing the following larval growth medium: ground

sugar cane bagasse (330 g), wheat flower (125 g), bovine meat flower (40 g), sodium bicarbonate (5 g) and distilled water (125 mL). Carbon dioxide was used to anesthetize the flies for the application of the compounds.

3.4. Insecticide activity assay

Stock solutions for the study with *M. domestica* were prepared by dissolving suitable amounts of the test compounds: 130–160 mg for the bisphosphoramidates with butyl, isopropyl and isobutyl groups; 170–200 mg for the others bisphophoramidates and 30 mg for the bisphosphorothioates in a 5 mL of a solution of water/ethanol 7:1. Stock solutions for the investigation of activity against *S. calcitrans* were prepared by dissolving 54–65 mg for the bisphosphoramidates with butyl, isopropyl and isobutyl groups; 160–200 mg for the others bisphophoramidates, 2–8 mg for the bisphosphorothioates in the same water/ethanol solution as above. For the reference compound, crufomate, the stock solutions for *M. domestica* and *S. calcitrans* tests were prepared by dissolving 1.8 and 0.3 mg, respectively, in 5 mL of the same solvent mixture. The stock solutions were then diluted so as to give five different concentrations for each compound. Flies were treated with 1.0 μL of the different solutions, using twenty individuals and four repetitions per dose. Three to six days old male and female *M. domestica* individuals from the third generation (F3) and 3–6 days old male and female *S. calcitrans* individuals from the fourth (F4) and fifth generation (F5) were employed.

Flies were collected with an industrial vacuum cleaner fitted with a suction tube equipped with a tulle cloth. The flies were immobilized with CO_2 for the maximum period of 50 min. Both handling of flies and the topical application of the compounds on the insect's backs were carried out with fine tweezers and a Hamilton type syringe. The flies were then put in wide mouth flasks and fed a solution of honey in water (*M. domestica*) and citrated blood (*S. calcitrans*). The bisphosphoroamidates and bisphosphorothioates were tested against adult *M. domestica* and *S. calcitrans* individuals with five different concentrations, used to determine the corresponding LD_{50}. In all tests, controls were employed in which only the diluting solvents were used. The observation of mortality was carried out 24 h after the application of the products. Lethal doses were calculated by the probit regression method from four independent experiments.

3.5. Mice (*Mus musculus*) lethality assay

Adult male and female SW-55 mice (*M. musculus*) weighing between 20 and 30 g were employed. Animals that received oral treatment were fasting for 8 h before the experiments, which were carried out at 8:00 and 12:00 h. The animals were then sacrificed with inhalation of diethyl ether. All experiments were carried out following ethical regulations for tests involving pain [19–21]. Drugs and reagents used were: saline (0.9% NaCl); DMSO 2% (drug medium); *N*,*N*′-bis-(diisobutylphosphoryl)-1,3-propylenediamine, *S*-*S*′-bis-(diisobutylphosphoryl)-1,3-propanedithiol.

Acute LD_{50} was obtained through screening of the acute toxicity. This method uses only three mice of each gender for dosages of the substances [22]. Animals fasting for 12 h were treated with the dosages of 2, 20 and 200 mg/kg and were then observed for morbidity and mortality. If no animals die with these dosages, tests are stopped and the substance is considered to have an LD_{50} above 200 mg/kg.

The pharmacological tests were carried out with the same animals employed for the LD_{50} tests. The general pharmacological activity test [23] involved the observation of possible action on central nervous system (CNS) as well as on the other systems at the following time after treatment: 5, 10, 15, 30 and 60 min; 4, 8, 24 and 48 h; 4 and 7 days. Possible activity is evaluated through observation of the following parameters: (a) CNS stimulation: increase in mobility, tachypnea (increase in respiratory frequency), piloerection, exophthalmy, stereotyped movements, paw licking, grooming, tail biting, clonic seizures, tonic seizures, fine tremors, rough tremors, syalorrea (increase in salivation), fasciculations, mydriasis, tail erection and tail tremors. (b) CNS depression: decrease in mobility, bradycardia (decrease in heart frequency), catatony, eyelid paralysis, analgesia, anesthesia, loss of cornean reflex, ataxy, dyspnea (large increase in respiratory frequency), environment alienation, exophthalmy, paralysis of posterior paws, sedation and miosis. (c) Circulatory system (observation through the ear): paleness, cyanosis, hyperemia (increase of blood flow). (d) Urinary system (observation of the micturition): increased, decreased and color. (e) Other actions: diarrhea, writhing, escape reactions, passivity, aggressivity and yelps.

3.6. Computational details

The structures of the models were constructed on the basis of the crystalline structures of a mammalian (human AChE complexed with fasciculin-II, entry 1B41 in the Protein Data Bank [24]) and of an insect (*Drosophila melanogaster*, entry 1QO9 in the Protein Data Bank [25]) enzymes. The final models (HuAChE-G and DmAChE-G) were composed by all residues of the active-site gorge, which spans from the outer peripheral binding site, ca. 20 Å toward the catalytic triad [25] (Table 3), selected with the Rasmol 2.6 program [26]. Two water molecules located in the active site (35 and 37) and four additional residues [Phe77 (Phe80), Ile161 (Val132), Ala239 (Ala204), and Gly265 (Gly230)] were also included. These residues, together with Phe330, are associated to mutations that confer resistance to insecticides [27–29]. The truncated peptide bonds were saturated with hydrogen atoms. Hydrogen atoms were added with the PC Spartan Plus package [Wavefunction, Inc., Irvine, CA], considering glutamic acid and aspartic acid residues in carboxylate form. The coordinates of the peptide atoms were held fixed during energy minimization, accomplished with the default options of the Mopac2002 program [Fujitsu Limited]. All calculations were carried out with the PM3 method [30,31] using the linear scaling approach [32], which enables fast quantum calculations on systems composed of many hundreds of atoms. We have recently used the same procedure for the investigation of the enzymatic mechanism of acetylcholine hydrolysis catalyzed by *M. domestica* AChE [33].

4. Conclusion

The LD_{50} values of the compounds synthesized by our group, obtained with bioassays using *M. domestica* and *S. calcitrans*, show that the bisphosphorothioates are more active than the bisphosphoroamidates. Compound **22**, *S*,*S*′-bis-(diisobutylphosphoryl)-1,3-propanedithiol, is particularly active, possessing an LD_{50} lower than that of the commercial insecticide crufomate. Even more interesting is the fact that two of the most active compounds, **15** and **22**, do not bring about any significant toxic effects on mice when tested

at concentrations of up to 200 mg/kg. These results are very promising and require further experiments, such as the determination of effects associated to long-term exposure (i.e., cancer, birth defects or reproductive toxicity).

The molecular modeling results show that the new bisphosphorothioates are able to interact with hydrophobic residues of the whole active site gorge. Based on calculations with mammalian and insect AChE models, we propose as a possible explanation for the observed toxicity of this bisphosphorothioates against *M. domestica* and *S. calcitrans* and the absence of toxicity toward mice the occurrence of a steric-oriented mechanism during inhibition of AChE. The combination of a more unfavorable situation of the leaving group and a more favorable situation of the phosphorylated active-site serine residue inside the insect AChE is suggested. Steric effects are known to determine insecticide selectivity; the classical example is that of methyl paraoxon and the much more selective 3-methyl analog fenitroxon where addition of the methyl substituent increases the affinity for insect and decreases for mammalian AChEs [4]. Work is under development to further explore this mechanism. It must be remembered, however, that alternative mechanisms could also influence the differential activity profile, such as selective bioactivation by insects or improved detoxification by mammals.

Acknowledgments

We thank the Brazilian agencies CAPES, CNPq, and Faperj for financial support.

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