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Design, synthesis and biological evaluation of organophosphorous-homodimers as dual binding site acetylcholinesterase inhibitors

Ruliang Xie [a,†], Qianfei Zhao [a,†], Tao Zhang [a], Jing Fang [b], Xiangdong Mei [a], Jun Ning [a,*], Yun Tang [b,*]

[a] State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100193, China
[b] School of Pharmacy, East China University of Science and Technology, Shanghai 200237, China

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ABSTRACT

The cluster effect is an effective strategy to explore new lead compounds, and has been successfully applied in rational drug design and screening. A series of novel organophosphorous-homodimers were designed and synthesized based on the dual-site structure characteristics of acetylcholinesterase (AChE). The compounds were evaluated in vitro for their inhibitory activity to AChE extracted from *Drosophila melanogaster* and *Musca domestic*. Compound **4H** showed an excellent inhibitor activity to both *Drosophila melanogaster* and *Musca domestic* with the corresponding IC_{50} values of 23 and 168 nM, respectively. Meanwhile, its activities against *Drosophila melanogaster* and *Musca domestic* AChE were more than 10,00,000 and 100,000-fold higher compared with the parent compound (MH), and was up to 245 and 107-fold higher than those of the positive control omethoate. The molecular docking study revealed that **4H** possessed an optimal spacer length and can perfectly fit into the central pocket, active gorge, and peripheral site of *Dm*AChE, and consequently exhibited highly improved inhibitor potency to *Dm*AChE. The bioassay tests showed that **4** series compounds showed prominent insecticidal activities against both *Lipaphser erysimi* and *Tetranychus cinnbarinus* at a concentration of 200 mg/L. The insecticide activity of compound **4H** was particularly significant that can cause 96% mortality to *Tetranychus cinnbarinus* after 24 h of treatment.

1. Introduction

The cluster effect, the multivalent ligands simultaneously binding to the multiple receptors of a biological system,[1–3] has been shown being an effective strategy for improving drug potency and selectivity. Particularly, when both the multiple ligands and the multiple receptors are on the same molecular entities, respectively, the homo-dimers can exhibit the excellent affinity binding[4–8] as well as the excellent selectivity[9–11] to the corresponding monomers. The cluster effect has been successfully applied in rational drug design, it is therefore reasonable to expect that the theoretical hypothesis underlying the cluster effect can similarly serve as the guidance for the discovery of new agrochemicals.

Many organophosphorus compounds that serve as nerve agents and insecticides (e.g., parathion and malathion) are acetylcholinesterase (AChE: E. C. 3.1.1.7) inhibitors. The electrophilic phosphorous atom can react with the hydroxyl group of the serine in the AChE through nucleophilic attack. The enzymes are phosphorylated and deactivated due to the attack of these agents, and consequently, these organophosphorus agents are pretty toxic. The inhibition of AChE usually leads to an increase in the amount of neurotransmitter acetylcholine at the central and peripheral sites of the nervous system, and induces the excessive stimulation to muscarinic and nicotinic receptors.[12,13] Organophosphorus insecticides represent a class of classical agrochemicals that have been used over the past several decades around the world. They are one of the major pesticides that target insects. However, organophosphorus insecticides have been excessively used over the past decade, Consequently, insects have developed strong resistances to them. Therefore, it is urgent to develop a new action mechanism by which organophosphorus insecticides can defeat the resistance that insects have been developed over the time.

The molecular configuration of AChE has three key characteristics. First, it has a deep and narrow gorge, referred to as the 'active site gorge', where fourteen aromatic residues occupied a substantial portion of the surface of the gorge (~40%). These residues as well as their flanking sequences are highly conserved in AChEs among different species.[14] Second, at the bottom of the active site gorge located a catalytic anionic site. Finally, there is a second binding site that known as peripheral anion site (PAS) in the entry

* Corresponding authors. Tel./fax: +86 10 62899142 (J.N.); tel./fax: +86 21 64251052 (Y.T.).
E-mail addresses: jning@ippcaas.cn (J. Ning), ytang234@ecust.edu.cn (Y. Tang).
† These authors equally contributed to this work and should be considered co-first authors.

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of the active site gorge.[15] It is located around 18 Å away from the active site.[6] Because AChE has two binding sites (so called the bivalent fashion), many compounds with dual binding site have been selected and tested for AChE as drug candidates to treat AD.[7,8,12,16] They showed the interesting activities toward the corresponding monomers tacrine, huperzine A, huperzine B and normeptazinol, etc., and have been considered as drug candidates for AD due to their high AChE inhibition activities. The high AChE inhibition activities of these compounds are very probably due to their bi-function structure.

In this paper, we report the design and synthesis of a new series of dual binding site AChEIs **4A**–**I**. They all possessed an important pharmacophore, that is, the organophosphorous moiety linked by linear alkanoic acid amides of different lengths, and proved to be endowed with a strong inhibitory activity due to the increased capability to interact with the active sites of the target AChE. One AChEI binding unit binds to the catalytic site via the classical mode of action,[12] while the other unit binds to the active site gorge or the PAS due to the presence of a spacer of appropriate length and flexibility. The synthesized AChEIs were tested in vitro against AChE from the head of *Drosophila melanogaster* AChE (*Dm*AChE) and *Musca domestic* AChE (*Md*AChE).

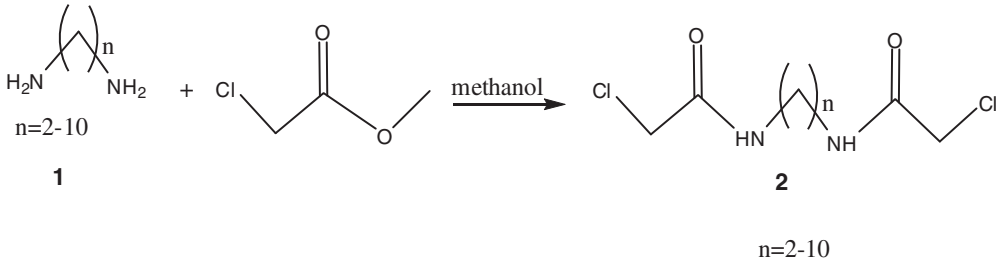
2. Results and discussion

2.1. Synthesis

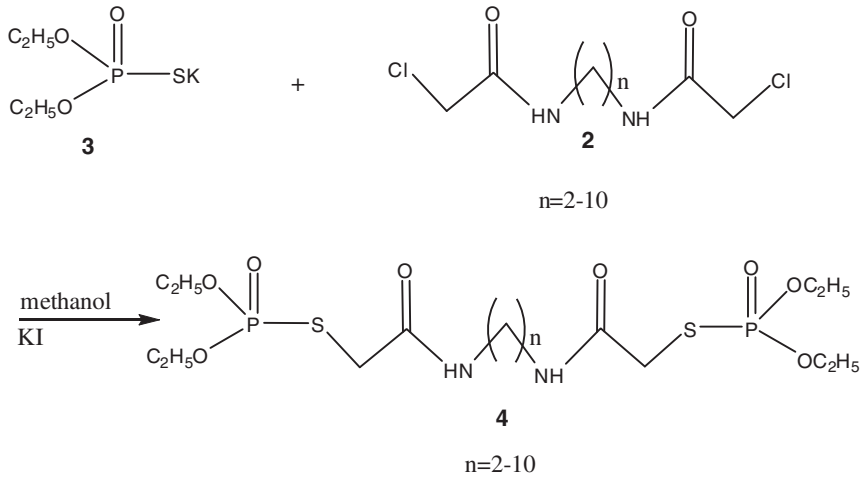
The derivatives were prepared following the synthetic routines outlined in Schemes 1 and 2. As depicted in Scheme 1, the intermediates **2A**–**I** were synthesized by using linear chain di-amines of various lengths and methyl chloroacetate as the starting materials, all can be purchased from most chemical companies. It was easy to obtain the **2** series compounds via a continuing violent stir for 6 h with the temperature below ten centigrade, and it was better to use cold methanol for filtering the **2** series to reduce the product loss by dissolving in the methanol. Moreover, the good yield was achieved by slowly adding the di-amines into the cold methyl chloroformate methanol solution. In order to get the title compounds, it was crucial to choose the optimal ratio, that is, the 2.4 equiv of methyl clhlorofonmate to the diamines. In short, the yield up to 90% was achieved by setting the suitable temperature, selecting the correct chemical addition order, taking the optimal reactant ratio, and reacting over an appropriate time of period. The desired compounds were got through the reaction between the intermediate **2** and the **3** in the presence of potassium iodide in methanol for 24–48 h under refluxing. The yield of 20–40% was achieved after purification by chromatography. The yields were below 20% when the di-amine spacer length was 2–4 carbon atoms. The low yield is very probably due to the steric hindrance between the intermediate **2** and **3**. Besides, 5% of the potassium iodide or potassium bromine was necessary for obtaining the title compounds, indicating that the reaction may be facilitated by the possible catalytic action of the salts of potassium. The optimal ratio for the intermediate **2** and **3** was 1.05:2, by which the title compound **4** can be conveniently produced. The purity of the synthesized compounds was less than 45%. The structures of the target compounds were confirmed by element analysis and ^{1}H NMR.

2.2. AChE inhibitory activity

Newly synthesized compounds were tested in vitro for their AChE inhibitory activity, in which the AChE extracts from *Drosophila melanogaster* and *Musca domestic* brain were adopted. The results are shown in Table 1, Those results indicated that all the compounds except **4A** and **4B** were the potent inhibitors to both *Drosophila melanogaster* and *Musca domestic* AChE. Compound **4H** exhibited the best inhibitor activity to both *Drosophila melanogaster* and *Musca domestic* AChE with the corresponding IC_{50} value of 23 and 168 nM, respectively, which were 1000,000 and 100,000 times larger than their corresponding IC_{50} values to the parent compound (MH) and 245 and 107 time larger than those to the positive control, respectively. The inhibitory activities of other compounds, however, were significantly lower, which is very probably due to the difference in the length of the alkyl linker between these compounds and **4H**. Particularly, the inhibitory activities of compound **4H** to both AChEs were 79- and 56-fold higher than those of the commercial insecticide chlorpyrifos. These observations suggested that both the linker type and linker length can impose significant influences on compound potency. Given the unique structure of AChE, it is reasonable to expect that the linker length is probably the most important factor affecting the inhibitory activity, for the compounds can reach both the catalytic and the peripheral binding sites of AChE only when the linker length is right. Surprisingly, our results revealed that the linker type also imposed significant influences on the compound potency. It can be ascribed to the specific interaction between the linkers and the residues consisting the gorge of the AChE. In addition, it was found that the activities of compound **4I** to *Dm*AChE and *Md*AChE were 62 and 75 times higher than those of omethoate. Despite the only structural difference between compound **4H** and **4I** is that compound **4I** has one more methylene group than **4H** does, the activity of compound **4I** to AChE was remarkably lower compared to compound **4H**. Even though, both **4H** and **4I** exhibited the ideal inhibitory activities with the IC_{50} values of 23 and 91 nM to *Dm*AChE and 168 and 238 nM to *Md*AChE, respectively. In addition, the data in Table 1 revealed a general tendency. The inhibitory activities of the synthesized compounds towards both *Dm*AChE and *Md*AChE increased as the linker length increased, in spite the increase in the activity toward *Md*AChE being more gentle than that toward *Dm*AChE. This is reasonable, for the *Dm*AChE was obviously more susceptible to the synthesized compounds than *Md*AChE did. Nevertheless, other factors such as the temperature, the humidity and other environmental factors during the evolution may also get involved. The activities of compound **4J** were also lower than those of



Scheme 1. Synthesis of intermediate **2A**–**I** n = 2–10 **2A**, **2B**, **2C**, **2D**, **2E**, **2F**, **2G**, **2H**, **2I**.



Scheme 2. Synthesis of target compounds **4A–I** n = 2–10 **4A**, **4B**, **4C**, **4D**, **4E**, **4F**, **4G**, **4H**, **4I**.

Table 1
In vitro inhibition of AChE activities

Entry	Chain length n	*Dm*AChE IC_{50} (nM)	*Md*AChE IC_{50} (nM)
MH		>400,00,000	>250,00,000
4A	2	68,791	6083
4B	3	4575	10,70,000
4C	4	1063	3681
4D	5	427	572
4E	6	1138	722
4F	7	109	564
4G	8	1862	7252
4H	9	23	168
4I	10	91	238
Omethoate		5633	18,028
Chlorpyrifos		1825	9514

Treatments were grouped into statistically distinct classes by applying analysis of variance (ANOVA) at P = 0.05 based on Fisher' s LSD.

4H. Ikuya et al. reported that the optimal configuration of alkyl spacers, although relatively nonspecific, was determined by the regional binding domain involving various stabilization interactions. Amongst all the compounds studied here, compound **4B–C** exhibited the lowest potency, implying that either their linkers may be too short to reach both the catalytic and peripheral anion sites or the fitted residues simultaneously occupy the gorge of the AChE. In contrast, compounds **8D–J** demonstrated moderate potencies, that is, their activities to *Dm*AChE and *Md*AChE were 5 and 240 times larger than those of omethoate to the same AChEs, indicating that their conformation may be more better fit to the structure of *Dm*AChE.

2.3. Molecular docking studies

Molecular docking study has been conducted and the results were reported elsewhere.[5] The purpose was to ascertain at the molecular level that compounds **4H** can bind to both the catalytic and peripheral sites of *Dm*AChE, and meanwhile to provide a molecular explanation for the surprisingly good activity of compound **4H**.

The AChE active sites including the central catalytic pocket, the binding gorge and peripheral pocket were taken as the target in the docking simulations. The docking results showed that compound **4H** demonstrated the highest inhibitory activity (IC_{50}: 23 nM) among all the **4**-series compounds. It can simultaneously bind at both the PAS and the CAS of 1QO9 which was the entry code in protein database of X-ray crystal structure of native *DmAChE*. Meanwhile, **4H** took an extended molecular conformation within the active-site gorge, as shown in Figure 1. The primary interactions between **4H** and 1QO9 were electrostatic interactions, hydrogen bonding interaction and hydrophobic interaction. At the CAS, there was a hydrogen bond between **4H** and the substrate involving the ethoxy oxygen atom of crucial functional phosphate group of **4H** as well as the side chain imidazole ring nitrogen atom of His 480 (O. . .N distance: 3.11 Å). Meanwhile, the nitrogen atom of the lower amide group approached to the PAS and formed a hydrogen bond with hydroxyl oxygen of Tyr71 (N. . .O distance: 3.04 Å). Additionally, due to its long alky spacer (having nine methylene groups), part of **4H** structure extended to the outside of the active site gorge and formed two hydrogen bonds with 1QO9 in the peripheral region of the active site. The first was formed between

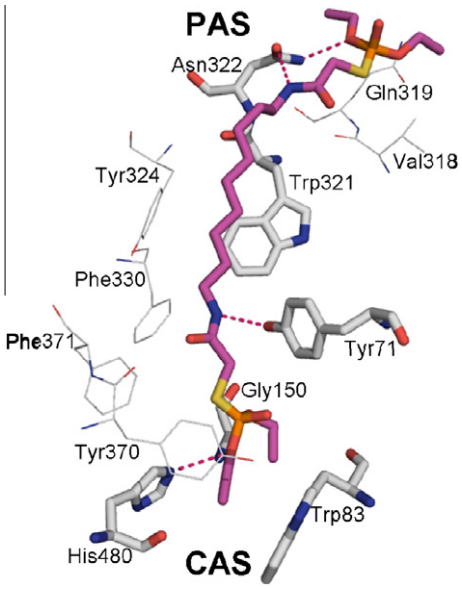


Figure 1. Two representations of **4H** (C atoms colored magentas and green respectively, shown as stick models) docked into the binding sites of DmAChE 1QO9. Interaction details between **4H** and 1QO9, the hot pink dash lines represent H-bonds formed between the two linking atoms. The side chains of active-site residues involved in interactions are all displayed (C atoms colored gray), of which the critical residues shown in stick and others shown in line.

Table 2
In vivo insecticide activities

Entry	Chain length *n*	*Lipaphis erysimi* (Mortality %, 200 mg/L)	*Tetranychus cinnbarinus* (Mortality %, 200 mg/L)
MH		12 i	10 g
4A	2	44 h	80 e
4B	3	83 cd	95 ab
4C	4	85 c	85 d
4D	5	80 de	99 a
4E	6	92 b	69 f
4F	7	84 cd	92 bc
4G	8	90 b	93 b
4H	9	77 ef	96 ab
4I	10	60 g	83 de
Omethoate		75 f	94 ab
Chlorpyrifos		99 a	89 c

Treatments were grouped into statistically distinct classes by applying analysis of variance.

the ethoxy oxygen of **4H** phosphate group and the side chain amino nitrogen atom of Asn322 (O. . .N distance: 2.87 Å), while the second involved the amide nitrogen at the upper part of **4H** and the side chain carbonyl oxygen of Asn322 (N. . .O distance: 3.01 Å). Besides, the binding free energy of **4H** to 1QO9 was found to be −57.04 kcal/mol.

It has been expected that the homo-dimers that linked by distinct types of spacers of different lengths may show potential inhibitory activity to AChE by acting on its CAS and PAS.[4,7] Our docking study have revealed that, compound **4H**, which consists of monomer **3** and the linker ononanediamine, possessed the optimal spacer length that allows it strongly interacting with 1QO9. As a result, compound **4H** is expected to be able to better bind to the central pocket, the active gorge, and peripheral site of *Dm*AChE, and therefore show the improved inhibitory potency to AChE. The homo-dimer **4H** could take an optimal conformation to best fit into the binding sites of AChE.

2.4. Insecticidal activities

The insecticide activities of **4** series compounds against *L. erysimi* and *Tetranychus cinnbarinus* were investigated. The results are shown in Table 2. All the homo-dimers, except **4B** and **4I**, exhibited the excellent insecticide activity against *L. erysimi* at a concentration of 200 mg/L. The compounds **4C**–**H** showed a higher insecticide activity than the positive control omethoate, while **4E**–**G** were at least as active as the commercial insecticide chlorpyrifos. Meanwhile, all the compounds also showed a high insecticidal activity against *Tetranychus cinnbarinus,* and the activities of these compounds were at least as active as or even more active than that of chlorpyrifos at the concentration of 200 mg/L. The in vivo results of insecticide activity of these compounds, as well as their further structure optimization and the detailed structure–activity relationships will be reported in the future.

3. Conclusions

A series of homo-organophosphorous compounds with various spacer lengths were designed on the basis of homo-dimer strategy that has been successfully applied in rational drug design. The compounds were prepared by using a group of inactive organophosphorous compounds as the monomers. The prepared compounds were evaluated for their inhibitory activity against *Dm*AChE and *Md*AChE, and most exhibited a pretty good activity. Particularly, the activities of homo-dimer **4H** were 100,000-fold and 245-fold higher than those of monomer and ometheoate, respectively. In addition, the homo-dimer **4H** exhibited the excellent insecticidal activities against *L. erysimi* and *tetranychus cinnbarinus*. The results present in this work demonstrate the application potential of the homo-dimer strategy in the field of agrochemical.

4. Experimental section

4.1. Chemicals and characterizations

All chemicals were purchased from commercial suppliers. ^{1}H NMR spectra were recorded on a Bruker DPX300 spectrometer in $CDCl_3$ with TMS as an internal standard. Elemental analysis (C, H, N, S, P) was conducted at the Institute of Chemistry, Chinese Academy of Sciences. Analytical thin-layer chromatography (TLC) was carried out on precoated plates (silica gel 60 F 254), and spots were visualized under UV light.

4.2. General synthetic procedure for 2A–I

To a solution of methyl 2-chloroacetate (13.0 g, 120.0 mmol) in methanol (20.0 ml), ethane-1, 2-diamine (**1A**) (3.0 g, 50.0 mmol) of methanol was added dropwise at room temperature, and stirred for 10 h. The white solid was filtered, washed, dried and collected. The solution was condensed and cooled to crystallize the residual

Table 3
^{1}H NMR data of organophosphorus compounds **4A–I**

Compounds	^{1}H NMR ($CDCl_3$/TMS)(δ), *J* (Hz)
4A	1.35–1.39 (m, 12H), 3.40 (d, 4H, *J* = 3.3 Hz), 3.48 (d, 4H, *J* = 18.9 Hz), 4.17–4.23 (m, 8H), 7.57 (s, 2H)
4B	1.35–1.40 (m, 12H), 1.70 (t, 2H, *J* = 6.3 Hz), 3.30–3.36 (m, 4), 3.48(d, 4H, *J* = 19.2 Hz) 4.13–4.24 (m, 8H), 7.50 (s, 2H)
4C	1.26–1.40 (m, 12H), 1.57–1.60 (m, 4H), 3.28 (t, 4H, *J* = 11.1 Hz), 3.43 (d, 4H, *J* = 21.0 Hz) 4.12–4.24 (m, 8H), 7.29 (s, 2H)
4D	1.35–1.40 (m, 14H), 1.50–1.60 (m, 4H), 3.22–3.28 (m, 4H), 3.44 (d, 4H, *J* = 19.5 Hz) 4.11–4.24 (m, 8H), 7.20 (s, 2H)
4E	1.36–1.40 (m, 16H), 1.53 (t, 4H, *J* = 13.2 Hz), 3.20–3.27 (m, 4H), 3.39 (d, 4H, *J* = 1.5 Hz), 4.11–4.24 (m, 8H), 7.17(s, 2H)
4F	1.33–1.40 (m, 18H), 1.52 (t, 4H, *J* = 13.5 Hz), 3.20–3.27 (m, 4H), 3.36 (d, 4H, *J* = 16.8 Hz), 4.1–4.24 (m, 8H), 7.12 (s, 2H)
4G	1.35–1.40(m, 20H), 1.52 (d, 4H, *J* = 6.3 Hz), 3.23–3.31 (m, 4H), 3.43 (d, 4H, *J* = 19.5 Hz), 4.15–4.21 (m, 8H), 7.15 (s, 2H)
4H	1.25–1.40 (m, 22H), 1.49–1.53 (m, 4H), 3.20–3.27 (m, 4H), 3.43 (d, 4H, *J* = 21.0 Hz), 4.11–4.25 (m, 8H), 7.19 (s, 2H)
4I	1.24–1.37 (m, 24H), 1.39 (t, 4H, *J* = 7.2 Hz), 3.22–3.24 (m, 4H), 3.42 (d, 4H, *J* = 19.5 Hz), 4.13–4.22 (m, 8H), 7.19 (s, 2H)

Table 4
Physical properties, yields and elemental analyses of compounds **4A–I**

Compound	Physical properties	Yield (%)	Elemental analyses (found)				
			C	H	N	P	S
4A	Colorless oil	15.3	35.00 (34.87)	6.29 (6.20)	5.83 (5.94)	12.8 9(12.81)	13.3 5(13.44)
4B	Colorless oil	16.5	36.43 (36.32)	6.52 (6.62)	5.66 (5.54)	12.53 (12.60)	12.97 (13.06)
4C	Colorless oil	12.7	37.79 (37.66)	6.74 (6.65)	5.51 (5.64)	12.18 (12.30)	12.61 (12.72)
4D	Colorless oil	24.3	39.07 (39.14)	6.94 (7.04)	5.36 (5.28)	11.85 (1196)	12.27 (12.36)
4E	Colorless oil	27.0	40.29 (40.42)	7.14 (7.08)	5.22 (5.31)	11.54 (11.66)	11.95 (12.04)
4F	Colorless oil	35.8	41.45 (41.59)	7.32 (7.26)	5.09 (5.14)	11.25 (11.32)	11.65 (11.73)
4G	Colorless oil	42.6	42.54 (42.46)	7.50 (7.41)	4.96 (5.04)	10.9 7(11.04)	11.3 6(11.54)
4H	Colorless oil	40.4	43.59 (43.51)	7.66 (7.57)	4.84 (4.94)	10.71 (10.80)	11.08 (11.20)
4I	Colorless oil	38.8	44.58(44.49)	7.82 (7.71)	4.73 (4.82)	10.45 (10.56)	10.82 (10.91)

product. The leading white solid was washed, dried and collected. Two products were combined to get *N*′,*N*′-(ethane-1, 2-diyl) bis(2-chloroacetamide) (**2A**). The same procedure was employed in the preparation of the products **2B–I** by using the corresponding alkane diamine.

To a solution of *O*,*O*,-diethylthiophosphate, potassium (**3**) (2.18 g, 10.5 mmol) in methanol (20.0 ml), *N*′,*N*′-(ethane-1,2-diyl) bis(2-chloroacetamide) (**2A**) (1.06 g, 5.0, mmol) was added, and the mixture was refluxed for 16 h. The reaction mixture was filtered and the filtrate was concentrated under vacuum. The viscous residue was dissolved with 20 ml ethyl acetate, then washed with water and the ethyl acetate layer was dried over anhydrous sodium sulfate. The dried solution was concentrated under reduced pressure, the crude compound was chromatographed on silica gel to give *S*′,*S*′-2,2-(ethane-1,2-diylbis(azanediyl)) bis(2-oxoethane-2,1-diyl) *O*,*O*,*O*′,*O*′-tetraethyl diphosphorodithioate (**4A**). The same procedure was employed in the preparation of the products **4A–I** by using the corresponding *N*′,*N*′-(alkane-α,ω-diyl) bis(2-chloroacetamide).

The structures, appearance, and yields of target compounds **4A–I** are listed in Table 4, and their ^{1}H NMR and elemental analysis data are given in the Table 3 and Table 4.

4.3. In vitro AChE inhibitory bioassay

The inhibitory activity against AChE was evaluated by using a modified Ellman's method reported by Zhao et al.[17,18] The adult *Drosophila melanogaster* and *Musca domestic* heads were used as the source of AChE. All the compounds were carefully measured and the results are shown in Table 1. The results were collected from at least three independent measurements.

4.4. In vivo insecticide activity assay

4.4.1. Insecticidal test for *Lipaphis erysimi*

The activities of insecticidal compounds **4A–I** against *Lipaphis erysimi* were tested by using leaf-dip method reported previously.[19–21] Chinese cabbage leaves with 45 apterous adults were dipped in the diluted Triton X-100 (0.2 g/L) solution for 10 s, the leaves were then placed in a plastic dish until they were dry. The dried leaves were transported to a conditioned room (25 ± 1 °C, 50% RH). The aqueous solution of Triton X-100 (0.1 mg/L) was used as the control. Omethoate and chlprpyrifos were also evaluated against *L. erysimi* and utilized as the positive controls. Each test had three repetitions. The mortality was assessed after 24 h, and data were corrected and subjected to prohibit analysis as before.

4.4.2. Insecticidal test for *Tetranychus cinnbarinus*

The insecticide activities of the title compounds against *Tetranychus cinnbarinus* were evaluated using the method similar to that against *Lipaphis erysimi* as described. Cotton leaves with approximately 80 *Tetranychus cinnbarinus* adults were selected, and dipped into the solutions containing the prepared compounds for 5 s. The omethoate and chlprpyrifos were taken as the positive controls. The aqueous solution of Triton X-100 (0.1 mg/L) was used as the control. Percentage mortalities were evaluated 24 h after treatment, and three replicates were carried out.

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