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Actions of cyclic esters, *S*-esters, and amides of phenyl- and phenylthiophosphonic acids on mammalian and insect GABA-gated chloride channels

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Abstract

Cyclic esters, *S*-esters, and amides of phenyl(thio)phosphonic acid were synthesized to probe the interaction between noncompetitive antagonists of ionotropic γ-aminobutyric acid (GABA) receptors and their binding site. Some of these compounds competitively inhibited the specific binding of [^{3}H]EBOB, a noncompetitive GABA antagonist, to rat-brain and housefly-head membranes. The *trans* isomer of the ester bearing a *tert*-butyl group at the 5-position and a bromine atom at the *p*-position (**5t**) was most potent in rat receptors with an IC_{50} value of 40 nM, while the *trans* isomer of the *S*-ester bearing the same substituents (**10t**) was most potent in housefly receptors with an IC_{50} value of 55 nM. In both cases, the corresponding amide analogue (**12t**) was less potent. The potencies of **5t** and **12t** tended to decrease in the presence of GABA, particularly in housefly receptors, while that of **10t** remained unchanged. The rank order of activity in inhibiting [^{3}H]EBOB binding to housefly-head membranes in the presence of GABA (**10t** > **5t** > **12t**) was in accord with that of insecticidal activity. *S*-Ester **10t** depressed 10 μM and 300 μM GABA-induced $^{36}Cl^-$ influx into mouse cerebral synaptoneurosomes, whereas ester **5t** depressed 10 μM GABA-induced $^{36}Cl^-$ influx but not 300 μM GABA-induced flux. Amide **12t** was inactive at both GABA concentrations. These findings indicate that six-membered cyclic phenylthiophosphonic acid derivatives act as noncompetitive antagonists of GABA receptors and suggest that **10t** is able to bind to the receptor in the open, desensitized, and closed states, whereas the affinity of **5t** and **12t** is lower in the open and desensitized states than in the closed state. The derivatives have similar structures except for the heteroatoms at the 1- and 3-positions, so that the heteroatoms may play a unique role when antagonists bring the open state of the GABA-gated channel to the desensitized or closed state.

1. Introduction

The widely used organophosphorus (OP) insecticides exhibit their insecticidal effects by inhibiting acetylcholinesterase and thereby increasing the synaptic level of the excitatory neurotransmitter acetylcholine [1]. Although OP insecticides have also been shown to act on γ-aminobutyric acid (GABA) receptors, these activities have been observed only at higher concentrations [2,3]. However, bicyclic OP compounds such as PS-4 (Fig. 1) exert high insecticidal activity by antagonizing the action of the inhibitory neurotransmitter GABA [4], although TBPS (Fig. 1) has limited applicability as an insecticide due to its high toxicity to mammals [5]. Compounds of this group inhibit GABA-induced chloride flux into nerve or muscle cells by binding to an allosteric site probably within the GABA-gated chloride channel [6–11]. Furthermore, we have recently discovered a different class of OP compounds, DPTD and the dioxaphosphorinanes (**5t** and **5c**)(Fig. 1), which act in a similar manner [12,13].

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In the mammalian brain, $GABA_A$ receptors belong to a family of ligand-gated ion channels composed of multimeric subunits, and carry binding sites for more than 100 medicinal or toxic compounds [14,15]. Deficiencies in GABAergic neurotransmission have been implicated in a number of neurological abnormalities. Insects have similar receptors with different pharmacological properties [16,17], so that their GABA receptors are promising targets for insecticides. To date, structurally diverse compounds, several of which are shown in Fig. 1, have been reported to act as noncompetitive antagonists of GABA receptors. However, although molecular cloning studies have provided insights into the molecular structure of the receptors, the structure of the antagonist binding site and the molecular action mechanism of the antagonists remain uncertain. From the structure-activity relationships of antagonists, we previously proposed a model of their binding site, suggesting that a subsite located in the center of the binding site played an important role in an electrostatic interaction with the heteroatom(s) carried by the antagonists [13,18].

Further studies of the OP antagonists may lead to the development of novel insecticides [19] or agents useful for tomography [20]. Alternatively, analogues of dioxaphosphorinanes might serve as useful neuropharmacological probes to investigate the properties of the electrostatic interaction, since the oxygen atoms at the 1- and 3-positions can be replaced with sulfur or nitrogen atoms without significant structural change of other moieties. We report here the synthesis of the analogues and their characteristics as antagonists in mammalian and insect ionotropic GABA receptors.

2. Results

2.1 Chemistry

Reactions of 4-bromophenyl- and phenyl(thio)phosphonic dichloride with 2-alkyl-1,3-propanediol, -dithiol, and -diamine afforded the *trans* and *cis* isomers of cyclic esters, *S*-esters, and amides of phenyl(thio)phosphonic acid, respectively. Compounds **5**, **10**, and **12** were separated into *trans* (**5t**, **10t**, and **12t**, respectively) and *cis* isomers (**5c**, **10c**, and **12c**, respectively) by silica-gel column chromatography. Other compounds were used for assays without separating the isomers although the *trans*/*cis* ratios were determined by comparing their ^1H NMR spectra (Table 1).

The 400 MHz ^1H NMR spectra of **10t** and **10c** exhibited patterns of AA′BB′MX that approximated to the pattern A_2B_2MX ($X = {}^{31}P$). The large $J_{H4,6ax-H5ax}$ (10.5, 9.9 Hz) and small $J_{H4,6eq-H5ax}$ (2.7, 3.4 Hz) values for both isomers indicate the preference of a chair conformer with the *tert*-butyl group equatorial (Fig. 1). This conclusion was further supported by the analyses of the coupling constants between the 4,6-protons and the phosphorus nucleus [large $J_{P2-H4,6eq}$ (25.4, 25.1 Hz) and small $J_{P2-H4,6ax}$ (10.5, 9.9 Hz) values]. The down-

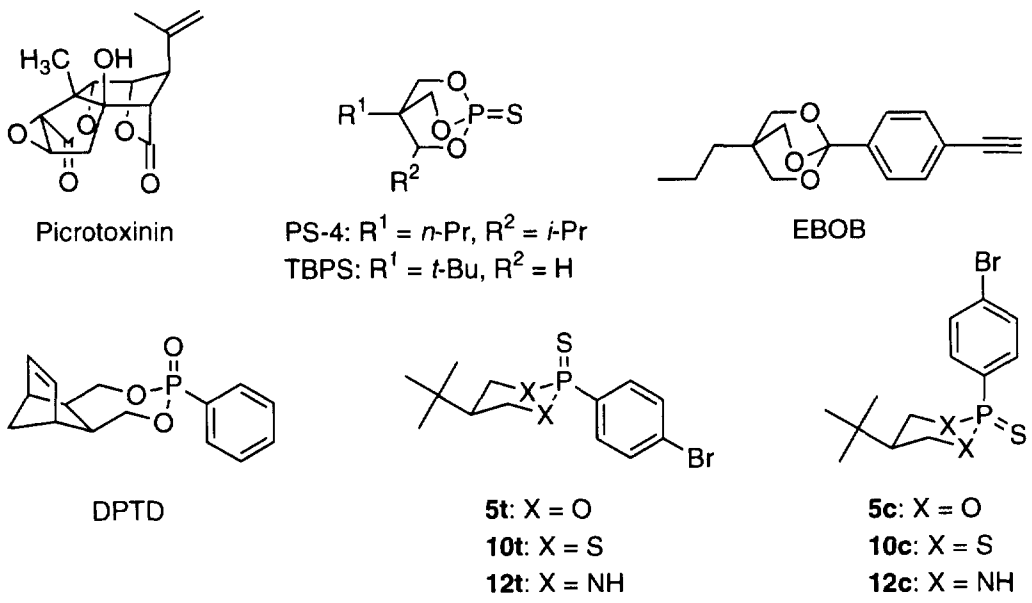


Fig. 1. Structures of noncompetitive antagonists of ionotropic GABA receptors. PS-4, 3-isopropyl-4-*n*-propyl-2,6,7-trioxa-1-phosphabicyclo[2.2.2]octane 1-sulfide; TBPS, 4-*tert*-butyl-2,6,7-trioxa-1-phosphabicyclo[2.2.2]octane 1-sulfide; EBOB, 1-(4-ethynylphenyl)-4-*n*-propyl-2,6,7-trioxabicyclo[2.2.2]octane; DPTD, 5-phenyl-2,3:8,7-*endo*-4,6-dioxa-5-phosphatricyclo[7.2.1.$0^{2,8}$]dodec-10-ene 5-oxide.

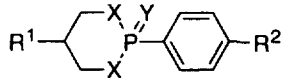
field shift of the axial 4,6-protons of **10t** can be explained by the deshielding effect of the axial P=S group. This interpretation is consistent with that reported for the *trans* and *cis* isomers of **9** [21]. A similar observation has been made for **5t** and **5c** [13]. ^{1}H NMR spectra of **12t** and **12c** showed complex multiplets for the 4,6-protons, so that the conformations of these amides in solution were not readily characterized. However, X-ray crystallographic analyses revealed that **12t** adopts a chair conformation with equatorial *tert*-butyl and equatorial *p*-bromophenyl groups in the solid state (Fig. 2).

2.2 *Effects on [^{3}H]EBOB binding to rat-brain and housefly-head membranes*

[^{3}H]EBOB (Fig. 1) is an available radioligand to label the binding site for noncompetitive antagonists of vertebrate and invertebrate GABA receptors. Therefore, phenylphosphonic acid derivatives were examined for their inhibitory effects on specific [^{3}H]EBOB binding to rat-brain and housefly-head membranes (Fig. 3 and Table 1).

Esters with a *tert*-butyl group at the 5-position (**3–5**) showed higher activities than those with *n*-propyl and *n*-butyl groups (**1** and **2**). In comparing **3** and **4**, the thiono analogue (**4**) proved to be more active than the oxon analogue (**3**), although **4** contained predominantly the *cis* isomer, which was presumed to be less active than the *trans* isomer based on the analogy to **5t** and **5c**. The introduction of a bromine atom into the *p*-position of the phenyl group of **4** to produce **5** resulted in increased inhibitory activity in rat receptors. The *trans* isomer (**5t**) was the most potent inhibitor of [^{3}H]EBOB binding, with an IC_{50} value of 0.040 μM. The *cis* isomer (**5c**) was about 17-fold less potent than **5t**. Although the three-dimensional structures of **5t** and **5c** are considerably different, Scatchard analyses of the binding data on rat receptors revealed that the inhibition was competitive

Table 1
Potencies of phenyl- and phenylthiophosphonic acid derivatives in inhibiting [^{3}H]EBOB binding to rat-brain and housefly-head membranes



Compound	R^1	R^2	X	Y	*trans*/*cis* (%)	IC_{50}(μM)	
						Rat	Housefly
Esters							
1	*n*-Pr	H	O	O	54/46	> 10(8.8[a])	> 10 (25.5[a])
2	*n*-Bu	H	O	O	55/45	> 10(7.8[a])	> 10 (18.6[a])
3	*t*-Bu	H	O	O	42/58	> 10(43.8[a])	> 10 (41.3[a])
4	*t*-Bu	H	O	S	22/78	2.12 (1.62–2.77[b])	3.53 (2.48–5.03[b])
5t	*t*-Bu	Br	O	S	100/0	0.040 (0.030–0.052[b])	0.88 (0.59–1.31[b])
5c	*t*-Bu	Br	O	S	0/100	0.66 (0.44–0.99[b])	> 10 (47.3[a])
S-Esters							
6	H	H	S	S	—	> 10 (18.8[a])	> 10 (35.4[a])
7	H	Br	S	S	—	> 10 (46.2[a])	≤10 (54.5[a])
8	*n*-Pr	H	S	S	81/19	≤10 (57.0[a])	0.97 (0.68–1.39[b])
9	*t*-Bu	H	S	S	90/10	1.19 (0.84–1.70[b])	0.21 (0.093–0.46[b])
10t	*t*-Bu	Br	S	S	100/0	0.12 (0.092–0.15[b])	0.055 (0.028–0.11[b])
10c	*t*-Bu	Br	S	S	0/100	1.06 (0.83–1.37[b])	0.32 (0.22–0.47[b])
Amides							
11	H	H	NH	S	—	> 10 (1.6[a])	> 10 (10.4[a])
12t	*t*-Bu	Br	NH	S	100/0	2.10 (1.34–3.29[b])	0.48 (0.24–0.93[b])
12c	*t*-Bu	Br	NH	S	0/100	> 10 (27.3[a])	> 10 (45.2[a])

[a]Inhibition (%) at 10 μM.
[b]95% confidence limit.

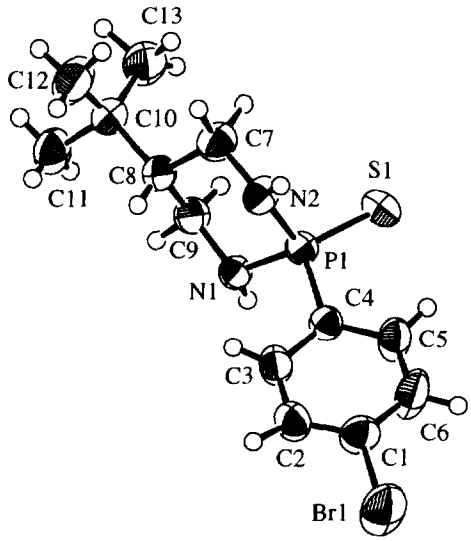


Fig. 2. Ortep drawing of **12t** from X-ray analyses.

(Fig. 4), indicating that both isomers act as noncompetitive antagonists of $GABA_A$ receptors. An increase in **5t** activity was also observed in housefly receptors by the introduction of the *p*-bromine atom, though this addition caused no corresponding increase in **5c** activity. Compound **5t** was 22-fold less potent in housefly than in rat receptors.

The introduction of a bromine atom into the *p*-position of *S*-ester **6** to produce **7** resulted in a slight increase in activity, and the introduction of an *n*-propyl group into the 5-position of **6** to give **8** enhanced the activity still further. At the 5-position, the *tert*-butyl group was more effective than the *n*-propyl group, as shown in **9**. The *trans* isomer bearing the *p*-bromine atom and the 5-*tert*-butyl group (**10t**) was most potent ($IC_{50}=0.12\,\mu M$ and $0.055\mu m$ in rat and housefly receptors, respectively), not only in a series of *S*-esters but also among all compounds in housefly receptors. In contrast to **5t**, **10t** was twofold more potent in housefly than in rat receptors. The *cis* isomer (**10c**) was about ninefold and about sixfold less potent than **10t** in rat and housefly receptors, respectively. Scatchard analyses of the effects of **10t** and **10c** revealed that the inhibition was competitive (plots not shown), indicating that both isomers act as noncompetitive antagonists of housefly GABA receptors.

The *trans* isomer (**12t**) of an amide having a structure corresponding to **5t** and **10t** exhibited a moderate activity, whereas the *cis* isomer (**12c**) was only very weakly active.

2.3 Effects on GABA-induced $^{36}Cl^-$ influx into mouse cerebral synaptoneurosomes

Electron microscopic analyses of the mouse cerebral synaptoneurosome fraction revealed the presence of sacs (synaptosomes) with attached resealed postsynaptic entities (neurosomes). Before assays, receptor desensitization was examined to see whether the synaptoneurosomes retained GABA receptor function. As shown in Fig. 5, the exposure of synaptoneurosomes to $20\,\mu M$ GABA for 15 s produced virtually complete desensitization. This finding indicates that the channel fluctuation between the open, desensitized, and closed states occurs properly.

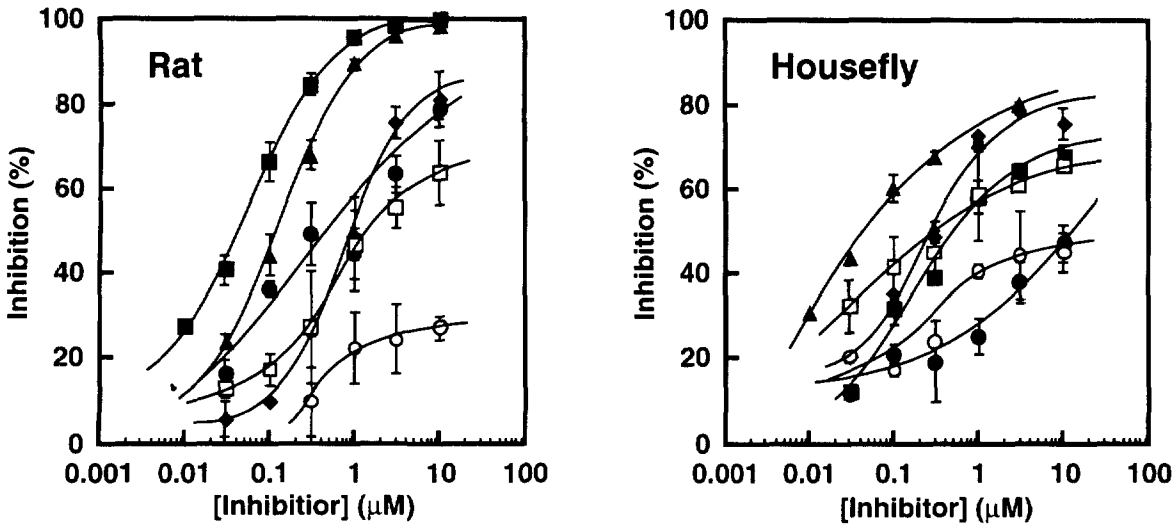


Fig. 3. Inhibition of [^{3}H]EBOB binding to rat-brain membranes (left) and housefly-head membranes (right) by phenyl(thio)phosphonic acid derivatives. ■, **5t**; ●, **5c**; ▲, **10t**; ◆, **10c**; □, **12t**; ○, **12c**.

Figure 6 shows the activities of **5t** and **10t** in inhibiting 10 μM and 300 μM GABA-induced $^{36}Cl^-$ influx into synaptoneurosomes when the synaptoneurosomes were preincubated with the compounds (20 μM) for 0 s, 5 s, and 20 min. $^{36}Cl^-$ influx induced by 10 μM GABA decreased with increasing time of preincubation with **5t** and **10t**. On the other hand, $^{36}Cl^-$ influx induced by 300 μM GABA was not decreased by prolonged preincubation; it was rather increased in the case of preincubation with **5t** for 20 min.

Figure 7 shows the activities of **5t** and **10t** in inhibiting 10 μM GABA-induced $^{36}Cl^-$ influx when synaptoneurosomes were preincubated with the compounds at various concentrations for 20 min. Both compounds inhibited $^{36}Cl^-$ influx in a concentration-dependent manner, but the inhibition did not reach 100% even at the highest concentration. The potencies of **5t** and **10t** were comparable.

Since the inhibition percentage of GABA-induced $^{36}Cl^-$ influx of **5t** and **10t** varied with GABA concentration (Fig. 6), **5t**, **5c**, **10t**, **10c**, **12t**, and **12c** were

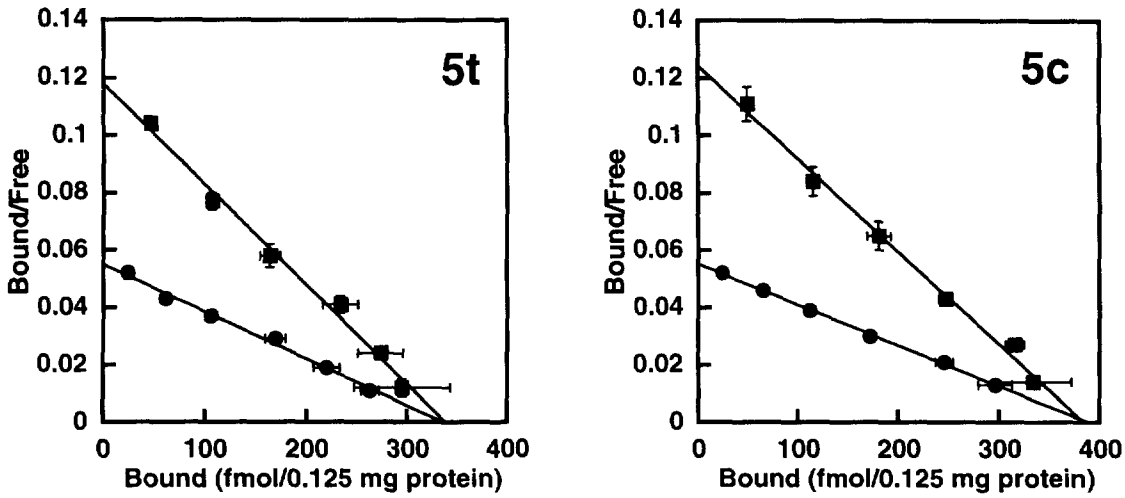


Fig. 4. Scatchard plots of [^{3}H]EBOB binding to rat-brain membranes in the absence (■) and presence (●) of 40 nM **5t** (left) and 660 nM **5c** (right).

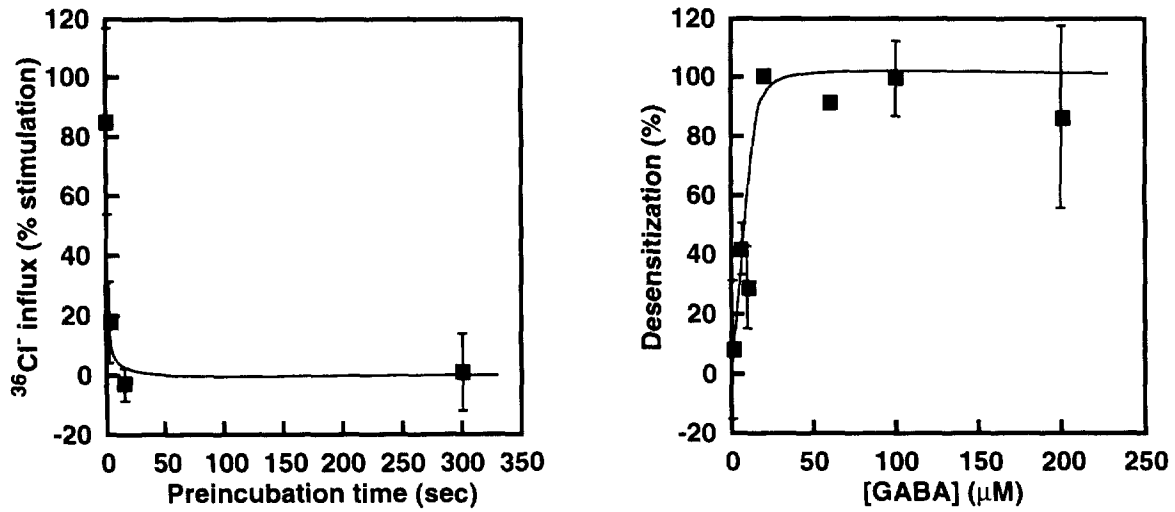


Fig. 5. Effects of GABA preincubation time (left) and GABA concentration (right) on GABA-induced $^{36}Cl^-$ influx. For desensitization experiments, synaptoneurosomes were exposed to 600 μM GABA for 0, 3, 15, and 300 s during a 20 min preincubation period before a 5 s influx was initiated by the addition of the equal volume of $Na^{36}Cl$ (left). In another experiment, synaptoneurosomes were exposed to 0–200 μM GABA for 15 s during a 20 min preincubation before a 5 s influx was initiated by the addition of the equal volume of a mixture of $Na^{36}Cl$ and 600 μM GABA (right).

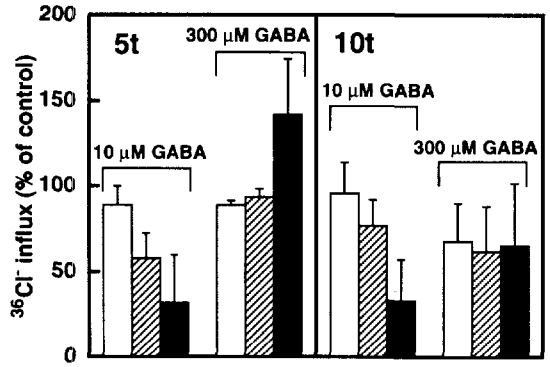


Fig. 6. Effects of preincubation time of compounds on GABA-induced $^{36}Cl^-$ influx. To evaluate the effects of preincubation time of compounds on the inhibition of GABA-induced $^{36}Cl^-$ influx, synaptoneurosomes were preincubated with 20 μM compounds for 0 s, 5 s, and 20 min before a 5 s influx was initiated by the addition of the equal volume of a mixture of $Na^{36}Cl$ and 20 or 600 μM GABA. Open bars, 0 s; hatched bars, 5 s; solid bars, 20 min.

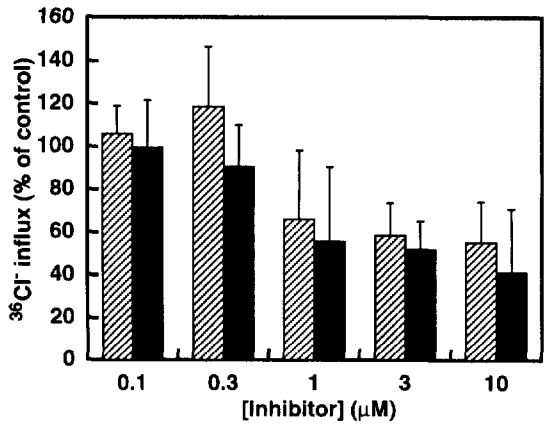


Fig. 7. Effects of **5t** and **10t** on 10 μM GABA-induced $^{36}Cl^-$ influx. For dose-response experiments, synaptoneurosomes were preincubated with 0.2–20 μM compounds for 20 min before a 5 s influx was initiated by the addition of the equal volume of a mixture of $Na^{36}Cl$ and 20 μM GABA. Hatched bars, **5t**; solid bars, **10t**.

examined at 10 μM for their inhibitory effects on $^{36}Cl^-$ influx induced by different concentrations of GABA (Table 2). When $^{36}Cl^-$ influx was induced by 10 μM GABA, the rank order of inhibitory activity was **10t** = **5t** > **5c** = **10c**. However, only **10t** exhibited marked inhibitory effects when $^{36}Cl^-$ influx was induced by 300 μM GABA. Amides **12t** and **12c** were almost inactive. As shown in Fig. 8, **10t** depressed maximal $^{36}Cl^-$ influx induced by 300 μM GABA whereas **5t** depressed the $^{36}Cl^-$ influx induced by low concentrations of GABA but not that induced by the high concentrations.

*2.4 Effects of GABA on the potencies of **5t**, **10t**, and **12t** in inhibiting [^{3}H]EBOB binding*

Since the concentration of GABA affected the activities of phenylthiophosphonic acid derivatives in inhibiting GABA-induced $^{36}Cl^-$ influx, the potencies of **5t**, **10t**, and **12t** in inhibiting [^{3}H]EBOB binding were examined in the presence of GABA (Table 3).

GABA was an inhibitor of [^{3}H]EBOB binding with an IC_{50} value of 1.05 μM and 162 μM in rat-brain and housefly-head membranes, respectively. In rat-brain membranes, the potency of **10t** increased in the presence of 0.30 μM GABA (a concentration causing 25% inhibition of [^{3}H]EBOB binding) but did not change in the presence of 4.0 μM GABA (a concentration causing 75% inhibition of [^{3}H]EBOB binding). The potency of **5t** did not change at 0.30 μM GABA but decreased at 4.0 μM GABA. The potency of **12t** decreased at 0.30 μM GABA but did not change at 4.0 μM GABA. In housefly-head membranes, the potencies of **5t** and **12t** decreased in the presence of 30 μM GABA (a concentration causing 25% inhibition of [^{3}H]EBOB binding), whereas the potency of **10t** did not change. The

Table 2
Effects of 10 μM **5**, **10**, and **12** on GABA-induced $^{36}Cl^-$influx into mouse cerebral synaptoneurosomes

Compound	$^{36}Cl^-$ influx (% of control)[a]			
	GABA (μM)			
	10	30	100	300
5t	22.2 ± 6.4	62.4 ± 17.6	101.0 ± 13.2	117.6 ± 15.3
5c	60.4 ± 14.8	101.9 ± 30.1	114.0 ± 30.1	102.5 ± 29.7
10t	16.9 ± 13.8	37.7 ± 10.1	52.7 ± 11.7	48.1 ± 3.2
10c	66.2 ± 6.9	84.4 ± 21.2	117.7 ± 23.4	78.9 ± 14.7
12t	88.7 ± 13.6	101.8 ± 21.4	90.2 ± 9.9	94.1 ± 18.0
12c	112.3 ± 12.0	88.9 ± 28.8	89.2 ± 14.7	79.9 ± 20.4

[a]Mean ± SD. Experimental conditions were identical to those described in Fig. 8

decrease in the potency of **12t** was profound; the IC_{50} value increased from 0.48 μM to > 10 μM.

2.5 *Insecticidal activity*

S-Ester **10t** and ester **5t** exhibited insecticidal activities to houseflies, with LD_{50} values of 0.10 μg/fly and 3.28 μg/fly, respectively, when synergized with piperonyl butoxide, a mixed-function oxidase inhibitor (Table 4). The corresponding *cis* isomers (**10c** and **5c**) and amides (**12t** and **12c**) were almost inactive.

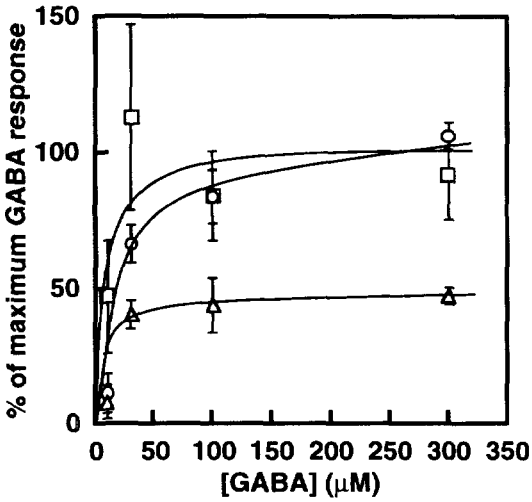


Fig. 8. Effects of **5t** and **10t** on GABA-induced $^{36}Cl^-$ influx. To examine the effects of GABA concentrations on potencies of compounds in inhibiting GABA-induced $^{36}Cl^-$ influx, synaptoneurosomes were preincubated with 20 μM compounds for 20 min before a 5 s influx was initiated by the addition of the equal volume of a mixture of $Na^{36}Cl$ and 20–600 μM GABA. □, None; ○, 10 μM **5t**; △, 10 μM **10t**.

Table 3
Potencies of **5t**, **10t**, and **12t** in inhibiting [^{3}H]EBOB binding to rat-brain and housefly-head membranes in the absence and presence of GABA

GABA (μM)	IC_{50} (μM)		
	5t	**10t**	**12t**
		Rat	
0	0.040 (0.030–0.052[a])	0.12 (0.092–0.15[a])	2.10 (1.34–3.29[a])
0.3	0.040 (0.032–0.051[a])	0.07 (0.056–0.088[a])	9.43 (4.97–17.90[a])
4.0	0.10 (0.075–0.14[a])	0.10 (0.078–0.14[a])	3.29 (1.94–5.58[a])
		Housefly	
0	0.88 (0.59–1.31[a])	0.055 (0.028–0.11[a])	0.48 (0.24–0.93[a])
30	2.23 (1.44–3.46[a])	0.058 (0.039–0.089[a])	> 10 (32.3[b])

[a]95% confidence limit.
[b]Inhibition (%) at 10 μM.

3. Discussion

Phenyl(thio)phosphonic acid derivatives competitively inhibited the specific binding of [^{3}H]EBOB to rat-brain and housefly-head membranes, indicating that these compounds act as noncompetitive antagonists of ionotropic GABA receptors. The *trans* isomer of the ester bearing the 5-*tert*-butyl and the *p*-bromo substituents (**5t**) was most active in rat receptors, while the *trans* isomer of the *S*-ester having the same substituents (**10t**) was most active in housefly receptors (Table 1 and Fig. 3). Thus, compounds **5t** and **10t** exhibited selectivity to some extent against rat and housefly receptors, respectively, in terms of affinity (IC_{50} values). Replacement and removal of the *tert*-butyl and bromine substituents resulted in decreased activity in the present study, but replacement of the bromine atom with an ethynyl group or other appropriate substituent could be expected to lead to more potent analogues as has been demonstrated for other GABA antagonists [22,23]. The amide **12t** was also an inhibitor of [^{3}H]EBOB binding in rat and housefly receptors, although less active than **5t** and **10t** (Table 1 and Fig. 3). The *cis* isomers of these compounds also exhibited moderate or weak inhibitory effects, although they had not been expected to show activities since their axial *p*-bromophenyl group seems to render these compounds less compatible with the binding-site model previously proposed [18].

It is assumed that [^{3}H]EBOB binding assays without GABA merely detect the potencies of compounds in the channel mainly in the closed state. To see whether phenyl(thio)phosphonic acid derivatives inhibit the function of $GABA_A$ receptors, $^{36}Cl^-$ influx assays were performed using mouse-brain synaptoneurosomes. The esters (**5t** and **5c**) and *S*-esters (**10t** and **10c**) exhibited inhibitory effects on 10 μM GABA-induced $^{36}Cl^-$

Table 4
Insecticidal activities of **5**, **10**, and **12** to houseflies

Compound	LD_{50} (μg/fly)[a]
5t	3.28 (3.05–3.54[b])
5c	> 10 (13.4[c])
10t	0.10 (0.093–0.11[b])
10c	> 10 (5.6[c])
12t	> 10 (0[c])
12c	> 10 (1.1[c])

[a]Synergized with piperonyl butoxide.
[b]95% confidence limit.
[c]Mortality (%) at 10 μg/fly.

influx, but the amides (**12t** and **12c**) were inactive (Table 2). The potencies of **5t** and **10t** increased with increasing time of preincubation of synaptoneurosomes with these compounds (Fig. 6). This finding implies that these compounds may slowly enter the channel in the closed state before receptor activation, since there are several lines of evidence that the antagonist binding site exists within the channel pore [24–26]. A similar phenomenon has been observed in the suppression of GABA-induced currents by picrotoxinin and TBPS [27,28].

As shown in Fig. 8, **10t** displayed a noncompetitive inhibition in the GABA concentration-response curve, i.e. the depression of the maximal GABA-induced $^{36}Cl^-$ influx without significant change in a GABA concentration to give a half-maximal response. However, compounds other than **10t** did not attenuate 300 μM GABA-induced $^{36}Cl^-$ influx (Table 2). Although the GABA concentration-response curve of **5t** resembled that of competitive GABA antagonism (Fig. 8), the noncompetitive GABA antagonism of this compound was confirmed by Scatchard analysis (Fig. 4). Taken together with the binding data, the $^{36}Cl^-$ influx data suggests that compounds such as **5t** and **12t** may bind to the antagonist binding site in the closed state, but that they easily dissociate from the binding site when the channel is brought to the open state by the binding of GABA.

The potencies of compounds in inhibiting [^{3}H]EBOB binding were complicated by the presence of GABA (Table 3). In rat receptors, the potencies of **5t** and **12t** were reduced in the presence of 4.0 μM GABA (a concentration causing 75% inhibition of [^{3}H]EBOB binding) and 0.30 μM GABA (a concentration causing 25% inhibition of [^{3}H]EBOB binding), respectively, whereas the potency of **10t** was enhanced in the presence of 0.30 μM GABA. In housefly receptors, the potencies of **5t** and **12t** were reduced in the presence of 30 μM GABA (a concentration causing 25% inhibition of [^{3}H]EBOB binding), while the potency of **10t** remained unchanged. In particular, the reduction in the potency of **12t** in the presence of GABA was remarkable. The rank order of insecticidal (in vivo) activities of **5t**, **10t**, and **12t** was in accord with that of the inhibitory effects on [^{3}H]EBOB binding in the presence of GABA (Tables 3 and 4). These findings suggest that the binding of GABA to the receptor induces GABA concentration-dependent conformational changes, or relaxation, in the antagonist binding site and that each compound has different affinities for the binding sites with different conformations. *S*-Ester **10t** can probably bind to the receptor in the open, desensitized and closed states, whereas the affinity of ester **5t** and amide **12t** is lower in the open and desensitized states than in the closed state. At present, it is believed that EBOB/picrotoxinin-type antagonists stabilize the desensitized or closed conformation of GABA-gated chloride channels to exert their antagonist effects [11,27–30]. In such an occasion, noncovalent bond interaction works between the atoms of ligands and those of the amino acid residues of the binding site [18]. Heteroatoms on the 1- and 3-positions of the phenylphosphonic acid derivatives may play a unique role in changing the state of the receptors from open to desensitized or closed.

In conclusion, the present results have revealed that six-membered cyclic phenylthiophosphonic acid derivatives bind to the site for noncompetitive antagonists in ionotropic GABA receptors. The derivatives have similar structures except for the heteroatoms at the 1- and 3-positions but exhibit different effects on the GABA-gated chloride channel. Appropriate electrostatic interaction between the heteroatoms and the binding site is likely of importance for high channel-blocking action. More detailed experiments on the effects might provide insights into the molecular mechanism by which noncompetitive antagonists block the channel.

4. Experimental

4.1 Instrumental analyses

Mass spectra were obtained with a Hitachi M-80B/M-0101 mass spectrometer. Nuclear magnetic resonance spectra were measured in $CDCl_3$ at 270 MHz with a JEOL JNM-GX 270 spectrometer or at 400 MHz with a JEOL JNM-A400 spectrometer. Melting point data were determined with a Yanako MP-500D apparatus and are uncorrected.

4.2 Chemicals

Dioxaphosphorinanes **1–5** were the same as those used in a previous study [13]. EBOB was synthesized by the method of Palmer *et al.* [31] [Propyl-2,3-^{3}H]EBOB (3126.5 and 1650.2 GBq/mmol) was purchased from Du Pont/NEN Research Products. $Na^{36}Cl$ (4366 kBq/ml, 2 mg Cl/ml and 3448.4 kBq/ml, 7.1 mg Cl/ml) was purchased from Amersham International plc.

4.3 Syntheses of dithiaphosphorinanes and hexahydrodiazaphosphorines

4.3.1 2-Phenyl-1,3,2-dithiaphosphorinane 2-sulfide (6)

Phenylthiophosphonic dichloride (0.50 g, 2.4 mmol) in dry ether (20 ml) was added dropwise to a stirred, ice-cold solution of 1,3-propanedithiol (0.26 g, 2.4 mmol) and triethylamine (0.49 g, 4.8 mmol) in dry ether (20 ml). The mixture was stirred at room temperature for 19 h. After the addition of water (20 ml) and chloroform (20 ml), the organic layer was separated, washed with water (20 ml), dried (Na_2SO_4) and concentrated. The

crude product was purified by silica-gel column chromatography (benzene) and recrystallized from carbon tetrachloride to give 0.19 g (22% yield) of **6**, mp 74.0°C. ^{1}H NMR δ: 2.06–2.30 (2H, m, H-5), 2.99–3.15 (2H, m, H-4, H-6), 3.44–3.59 (2H, m, H-4, H-6), 7.52–7.62 (3H, m, Ar), 8.14–8.23 (2H, m, Ar). EIMS *m/z* (rel. intensity): 246 (M^+)(21).

4.3.2 2-(4-Bromophenyl)-1,3,2-dithiaphosphorinane 2-sulfide (7).

Prepared from 4-bromophenylthiophosphonic dichloride [32] and 1,3-propanedithiol as described above. Recrystallization of the crude product from benzene gave **7** in 8% yield, mp 108.0°C. ^{1}H NMR δ: 2.07–2.30 (2H, m, H-5), 2.99–3.14 (2H, m, H-4, H-6), 3.43–3.58 (2H, m, H-4, H-6), 7.70 (2H, dd, J = 3.4 and 8.3 Hz, Ar), 8.04 (2H, dd, J = 8.3 and 14.9 Hz, Ar). EIMS *m/z* (rel. intensity): 326 ($M^+ + 2$)(80), 324 (M^+)(79).

*4.3.3 2-Phenyl-5-*n*-propyl-1,3,2-dithiaphosphorinane 2-sulfide (8)*

Prepared from phenylthiophosphonic dichloride and 2-*n*-propyl-1,3-propanedithiol [33] as described above. Silica-gel column chromatography (hexane:acetone = 9:1) of the crude product, followed by recrystallization from carbon tetrachloride, gave **8** (*trans* : *cis* = 81:19) in 5% yield, mp 59.0°C. ^{1}H NMR δ: 0.93–0.99 (*trans* and *cis* isomers, t, CH_3), 1.31–1.46 (*trans* and *cis* isomers, m, CH_2), 1.52–2.04 (*trans* and *cis* isomers, m, CH_2), 2.00–2.15 (*trans* and *cis* isomers, m, H-5), 2.82–2.91 (*cis* isomer, m, H-4, H-6), 3.06–3.15 (*trans* isomer, m, H-4, H-6), 3.27–3.37 (*trans* isomer, m, H-4, H-6), 3.47–3.55 (*cis* isomer, m, H-4, H-6), 7.53–7.60 (*trans* and *cis* isomers, m, Ar), 8.14–8.22 (*trans* and *cis* isomers, m, Ar), 8.21–8.27 (*trans* isomer, m, Ar). EIMS *m/z* (rel. intensity): 288 (M^+)(41).

*4.3.4 5-*tert*-Butyl-2-phenyl-1,3,2-dithiaphosphorinane 2-sulfide (9)*

Prepared from phenylthiophosphonic dichloride and 2-*tert*-butyl-1,3-propanedithiol [33] as described above. Recrystallization of the crude product from carbon tetrachloride gave **9** (*trans* : *cis* = 9:1) in 3% yield, mp 155.6°C. ^{1}H NMR δ: 0.90 (*cis* isomer, s, $C(CH_3)_3$), 1.05 (*trans* isomer, s, $C(CH_3)_3$), 1.92–2.04 (*trans* and *cis* isomers, m, H-5), 2.70–2.80 (*cis* isomer, m, H-4, H-6), 3.04–3.16 (*cis* isomer, m, H-4, H-6), 3.27–3.37 (*trans* isomer, m, H-4, H-6), 3.56–3.65 (*trans* isomer, m, H-4, H-6), 7.52 (*trans* and *cis* isomers, 2H, m, Ar), 8.08–8.18 (*cis* isomer, m, Ar), 8.21–8.27 (*trans* isomer, m, Ar). EIMS *m/z* (rel. intensity): 302 (M^+)(11).

*4.3.5 2-(4-Bromophenyl)-5-*tert*-butyl-1,3,2-dithiaphosphorinane 2-sulfide (10)*

2-*tert*-Butyl-1,3-propanedithiol was allowed to react with 4-bromophenylthiophosphonic dichloride in a manner described for **6**. Silica-gel column chromatography (hexane:acetone = 9:1) of the crude product, followed by recrystallization from ethyl acetate, gave **10t** (10% yield) and **10c** (6% yield). **10t**: mp 158.6°C. ^{1}H NMR δ: 1.04 (9H, s, $C(CH_3)_3$), 1.94 (1H, tt, J = 2.7 and 10.5 Hz, Hax-5), 3.32 (2H, ddd, J = 2.7, 14.2 and 25.4 Hz, H_{eq}-4 and H_{eq}-6), 3.57 (2H, ddd, J = 10.5, 10.5 and 14.2 Hz, H_{ax}-4 and H_{ax}-6), 7.69 (2H, dd, J = 3.4 and 8.5, Ar), 8.10 (2H, dd, J = 8.5 and 14.9 Hz, Ar). EIMS *m/z* (rel. intensity): 382 ($M^+ + 2$)(30), 380 (M^+)(27). **10c**: mp 178.0°C. ^{1}H NMR δ: 0.91 (9H, s, $C(CH_3)_3$), 2.00 (1H, tt, J = 3.4 and 9.9 Hz, H_{ax}-5), 2.27 (2H, ddd, J = 9.9, 9.9 and 14.3 Hz, H_{ax}-4 and H_{ax}-6), 3.11 (2H, ddd, J = 3.4, 14.5 and 25.1 Hz, H_{eq}-4 and H_{eq}-6), 7.67 (2H, dd, J = 3.4 and 8.8 Hz, Ar), 7.96 (2H, dd, J = 8.8 and 14.9 Hz, Ar). EIMS *m/z* (rel. intensity): 382 ($M^+ + 2$)(24), 380 (M^+)(22).

4.3.6 Hexahydro-2-phenyl-1,3,2-diazaphosphorine 2-sulfide (11)

1,3-Propanediamine was allowed to react with phenylthiophosphonic dichloride in a manner described for **6**. The crude product was purified by silica-gel column chromatography (chloroform) and recrystallized from cyclohexane/dichloromethane to give **11** in 17% yield, mp 115.0°C. ^{1}H NMR δ: 1.41–1.55 (1H, m, H-5), 1.73–1.82 (1H, m, H-5), 2.88 (2H, br.s, NH), 3.02–3.07 (2H, m, H-4, H-6), 3.27–3.40 (2H, m, H-4, H-6), 7.43–7.48 (3H, m, Ar), 7.81–7.90 (2H, m, Ar). EIMS *m/z* (rel. intensity): 212 (M^+)(100).

*4.3.7 2-(4-Bromophenyl)-5-*tert*-butylhexahydro-1,3,2-diazaphosphorine 2-sulfide (12)*

2-*tert*-Butyl-1,3-propanediamine [34] was allowed to react with 4-bromophenylthiophosphonic dichloride in a manner described for **6**. Silica-gel column chromatography (hexane:ethyl acetate = 9:1) of the crude product, followed by recrystallization from acetone and ethyl acetate, gave **12t** (7% yield) and **12c** (4% yield), respectively. **12t**: mp 177.7°C. ^{1}H NMR δ: 0.94 (9H, s, $C(CH_3)_3$), 1.75–1.83 (1H, m, H-5), 2.40 (2H, br.s, NH), 3.17–3.35 (4H, m, H-4, H-6,), 7.58 (2H, dd, J = 2.4 and 8.5 Hz, Ar), 7.94 (2H, dd, J = 8.5 and 13.7 Hz, Ar). EIMS *m/z* (rel. intensity): 248 ($M^+ + 2$)(19), 246 (M^+)(18). **12c**: mp 160.5°C. ^{1}H NMR δ: 0.82 (9H, s, $C(CH_3)_3$), 1.52–1.66 (1H, m, H-5), 2.77–2.86 (4H, m, H-4, H-6, NH), 3.31–3.42 (2H, m, H-4, H-6), 7.60 (2H, dd, J = 2.9 and 8.5 Hz, Ar), 7.71 (2H, dd, J = 8.5 and 12.9 Hz, Ar). EIMS *m/z* (rel. intensity): 248 ($M^+ + 2$)(33), 246 (M^+)(31).

4.4 X-Ray crystal structure analyses of 12t

Crystals of **12t** were grown from acetone. Crystal data: $C_{13}H_{20}BrN_2PS$, FW = 347.25, a = 21.835(2) Å, b = 14.200(2) Å, c = 12.233(2) Å, β = 122.916(7)°, V =

3184.1(7) Å^3, monoclinic, space group C2/c, Z = 8, D_{calc} = 1.45 g/cm^3, $\mu(CuK_\alpha)$ = 55.74 cm^{-1}. The data were collected on a Rigaku AFC7R diffractometer equipped with graphite-monochromated CuK_α radiation up to a maximum 2θ of 130°. The intensity data of 2839 independent reflections were collected, and 2324 with $I > 3\sigma(I)$ were used in the present X-ray analysis. The structure was solved by direct methods and refined by full-matrix least squares with anisotropic thermal parameters for all non-hydrogen atoms. The final R index was 0.051 (Rw = 0.088, S = 2.15). Crystal data and details of the structure determination, positional parameters, anisotropic thermal parameters, and bond distances and angles are reported in the supplementary material.

4.5 Preparation of rat-brain and housefly-head membranes

Rat-brain P_2 membranes were prepared from 5-week-old male Wistar rats by the method of Squires *et al.* [10] and stored at −80°C until use. The frozen pellets were thawed and suspended in buffer A (300 mM NaCl/10 mM sodium phosphate buffer, pH 7.5) just before the binding assays. Housefly-head P_2 membranes were prepared from 5- to 10-day-old adult houseflies (*Musca domestica* L.) of WHO strain by a modification of the method of Deng *et al.* [35] Briefly, the heads were homogenized in 0.25 M sucrose/10 mM Tris-HCl buffer (pH 7.5) with a glass-Teflon homogenizer, filtered through four layers of 64 μm mesh nylon screen and centrifuged at 500×*g* for 5 min. The supernatant was filtered through four layers of nylon screen and centrifuged at 25,000×*g* for 30 min. The pellets were suspended in buffer A and allowed to stand in an ice bath for 30 min. The suspension was centrifuged at 25,000×*g* for 30 min. The pellets were suspended in buffer A and used immediately for the binding assays without freezing.

4.6 Preparation of mouse-brain synaptoneurosomes

Synaptoneurosomes were prepared from male ddY mice (about 40 g each) according to Schwartz *et al.* [36] Briefly, cerebra were homogenized in buffer B (20 mM HEPES-Tris, 118 mM NaCl, 4.7 mM KCl, 1.18 mM $MgSO_4$, 2.5 mM $CaCl_2$, pH 7.4)(7 ml/1 g tissue) using a glass-glass homogenizer (five strokes). The homogenate was diluted with 30 ml of ice-cold buffer B and filtered by gravity through three layers of 160 μm mesh nylon screen placed in a Millipore Swinex filter holder. The filtrate was gently pushed through a 10 μm Millipore Mitex filter using a 5 ml syringe. The filtrate was centrifuged at 1000×*g* for 15 min. The pellets were gently suspended in 30 ml of buffer B and washed two more times by centrifugation (1000×*g*, 15 min). The final pellets were gently suspended in buffer B and used immediately for the $^{36}Cl^-$ influx assays.

4.7 [^{3}H]EBOB binding assays

[^{3}H]EBOB binding assays were carried out according to Cole and Casida [37] and Deng *et al.* [35] Briefly, test compounds were incubated with rat-brain membranes (125 μg protein) or housefly-head membranes (200 μg protein) and 0.5 nM [^{3}H]EBOB in 1.0 ml of buffer A at 37°C for 90 min (rats) or at 22°C for 70 min (houseflies). Specific binding was linear with tissue concentration until the above protein concentrations determined according to Bradford [38]. After incubation, the mixtures were filtered through GF/B filters and rapidly rinsed twice with 5 ml of ice-cold buffer A using a Brandel M-24 cell harvester. The radioactivity of [^{3}H]EBOB that specifically bound to membranes on the filters was measured with a Beckman LS 6000SE liquid scintillation spectrometer. Nonspecific binding was determined in the presence of 5 μM unlabeled EBOB. Test compounds and unlabeled EBOB were initially dissolved in DMSO and diluted in buffer A so that the final DMSO concentration was 0.4% (v/v). Each experiment was performed in triplicate and repeated 2 to 4 times.

4.8 $^{36}Cl^-$ influx assays

$^{36}Cl^-$ influx into synaptoneurosomes was measured according to Schwartz *et al.* [36] $Na^{36}Cl$ (18.5 kBq) maintained in buffer B at 30°C was added to synaptoneurosomes (200 μg protein) preincubated in buffer B at 30°C for 20 min (0.25 ml final volume). $^{36}Cl^-$ influx was terminated 5 s later by the addition of ice-cold buffer B (5 ml), followed by vacuum filtration through Whatman GF/C filters that had been presoaked in 0.05% polyethyleneimine. The filters were rapidly washed twice with 5 ml of ice-cold buffer B. The radioactivity of $^{36}Cl^-$ taken up into synaptoneurosomes on the filters was measured with a Beckman LS 6000SE liquid scintillation spectrometer. GABA-induced $^{36}Cl^-$ influx was defined as the difference between the amounts of $^{36}Cl^-$ taken up in the presence and absence of GABA. GABA-induced $^{36}Cl^-$ influx was linear with tissue concentration until the above protein concentration determined by the Bradford method [38]. Test compounds were initially dissolved in DMSO and diluted in buffer B so that the final DMSO concentration was 0.4 % (v/v). Each experiment was repeated 2 to 4 times.

4.9 Insecticidal assays

Acetone solutions (1 μl) of piperonyl butoxide (10 μg) were topically applied to the mesonotum of female adult houseflies (*Musca domestica* L., WHO susceptible strain, 3 to 5 days old, about 17 mg each). The flies were similarly dosed with test compounds after 1 h, placed in glass vials (5 cm in diameter and 10 cm in height) with a

sugar-soaked cotton pad, and held at 25°C. Three groups of 15 flies were used for each dosage. Mortality was recorded 24 h after the application of test compounds. The assay was repeated twice.

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