Synthesis and Structure–Activity Relationships of a Series of Insecticidal Dioxatricyclododecenes Acting as the Noncompetitive Antagonist of $GABA_A$ Receptors

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Chlorinated analogues of 5-substituted 2,3:8,7-*endo*-4,6-dioxatricyclo[7.2.1.$0^{2,8}$]dodec-10-ene (DTD) and 2,3:8,7-*endo*-4,6-dioxa-5-thiatricyclo[7.1.1.$0^{2,8}$]dodec-10-ene 5-oxide were prepared. With $-CH_2-$, -CH(Me)-, and -S(O)- at the 5 position, the presence of chlorine atoms in the norbornene moiety was important in exerting toxic effects to houseflies, while with -C*H*(*n*-Pr)- and -CH(Ph-CN-4)- the introduction of chlorine atoms resulted in a loss of toxic activity. The effect of the presence of chlorine atoms on the potency in inhibiting [^{35}S]*tert*-butylbicyclophosphorothionate (TBPS) binding to rat brain membranes was found to be correlated to their insecticidal activity. A good correlation ($r = 0.807$) was obtained between insecticidal activity and the potency in inhibiting [^{35}S]TBPS binding for 29 compounds, including known noncompetitive antagonists of $GABA_A$ receptors. The Scatchard analysis indicated that both types of analogues, 1,9,10,11,12,12-hexachloro-DTD and 5-(4-cyanophenyl)-DTD, act competitively at the TBPS binding site in the GABA-gated chloride channel. These two types of analogues, however, appear to interact with slightly different subsites within the TBPS binding site.

INTRODUCTION

Cyclodiene insecticides and lindane (γ-BHC) have been shown to exert their toxic effects through their interaction with γ-aminobutyric acid (GABA)-gated chloride channels in the nervous system (Matsumura and Ghiasuddin, 1983; Tanaka et al., 1984; Lawrence and Casida, 1984; Abalis et al., 1985b). These chlorinated insecticides inhibit GABA-induced chloride flux by binding to the specific picrotoxinin binding site within the $GABA_A$ receptor (Ghiasuddin and Matsumura, 1982; Bloomquist and Soderlund, 1985; Gant et al., 1987; Ogata et al., 1988; Tokutomi et al., 1993). While there are structural similarities between cyclodiene insecticides, e.g., heptachlor epoxide, and picrotoxinin, which gave a clue to the elucidation of their action mechanism in an initial study (Matsumura and Ghiasuddin, 1983), structurally different types of compounds such as bicyclophosphorus esters, bicycloorthocarboxylates (Ozoe and Eto, 1986; Casida and Palmer, 1988), and dithianes (Elliott et al., 1992; Palmer and Casida, 1992; Wacher et al., 1992) are also thought to act similarly at the same or overlapping site. Such structural diversity of ligands makes it difficult to understand the molecular topography of this toxicologically important binding site.

To study structural requirements for the interaction of bridged bicyclic compounds with the picrotoxinin binding site, a number of analogues of bicyclo[3.2.1]oct-6-en-3-one and bicyclo[2.2.1]hept-2-ene were previously synthesized as model compounds which have partial structural similarities to both cyclodiene insecticides and picrotoxinin (Ozoe and Matsumura, 1986). In a subsequent study (Ozoe et al., 1990), 5-substituted 2,3:8,7-*endo*-4,6-dioxatricyclo-[7.2.1.$0^{2,8}$]dodec-10-enes (DTDs) were synthesized based on structural comparison of endosulfan, a cyclodiene insecticide, and bicycloorthocarboxylates. Structure–activity studies of these compounds (Ozoe and Matsumura, 1986; Ozoe et al., 1990; Akamatsu et al., 1992) revealed that active ligands had electronegative and sterically bulky moieties (hydrophobic) capable of interacting with at least two of four important subsites in the binding site. In the present study, we additionally synthesized DTDs with chlorine atom(s) in the 1, 9, 10, 11, and 12 positions and examined their insecticidal activity as well as potencies as $GABA_A$ receptor antagonists at insect and mammalian nervous systems.

MATERIALS AND METHODS

Chemicals. Compounds **4**, **19**, **21**, **23**, **30**, **32–44**, and **52–62**, *tert*-butylbicyclophosphorothionate (TBPS), *tert*-butylbicycloorthobenzoate (TBOB), and 3-isopropyl-4-*n*-propylbicyclophosphorothionate (PS-4) were available from our previous studies (Ozoe and Matsumura, 1986; Ozoe et al., 1990, 1992a,b). Sources for other chemicals were as follows: Sigma Chemical Co., St. Louis, MO (picrotoxinin); Wako Pure Chemical Industries, Ltd., Japan (α-endosulfan, β-endosulfan, and δ-BHC); BHC Kogyo-Kai, Japan (γ-BHC); and Tokyo Kasei Kogyo Co., Ltd., Japan (α-BHC and β-BHC). [^{35}S]TBPS (>60 Ci/mmol) was purchased from DuPont/NEN Research Products, Boston, MA.

Synthesis. Analogues of 2,3:8,7-*endo*-4,6-dioxatricyclo-[7.2.1.$0^{2,8}$]dodec-10-ene (DTD) with chlorine atom(s) in the norbornene moiety were synthesized in the current study. Hexachloro analogues were prepared by the cyclization of the corresponding *endo* diol with an aldehyde or with an acetal. Pentachloro analogues were synthesized by the UV (254 nm) irradiation, $NaBH_4$–vitamin B_{12}, or Bu_3SnH–AIBN reduction of the hexachloro analogues. Tetrachloro analogues were synthesized by the UV (254 nm) irradiation of the hexachloro analogues followed by $NaBH_4$–vitamin B_{12} reduction, except for a 1,9,10,11-tetrachloro analogue (**9**), which was obtained by the reaction of 5,6-bis(hydroxymethyl)-1,2,3,4-tetrachlorobicyclo-

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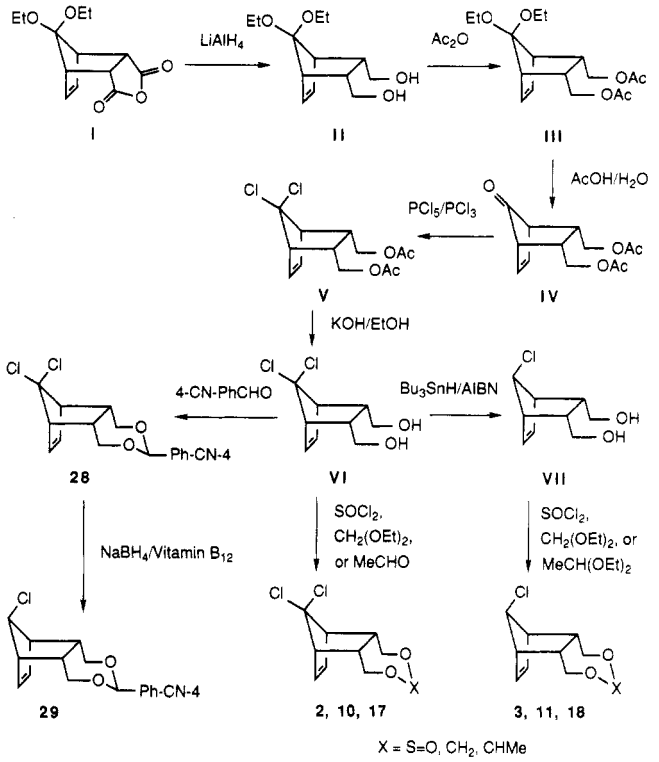


Figure 1. Synthesis of the 12,12-dichloro and *anti*-12-chloro analogues of DTD and α-endosulfan.

[2.2.1]hept-2-ene with diethoxymethane. 12,12-Dichloro analogues were synthesized via 10 steps from cyclopentanone. The synthetic route from the fifth step is shown in Figure 1. 12-Chloro analogues were obtained by the $NaBH_4$–vitamin B_{12} reduction of a 12,12-dichloro analogue or by the reaction of a corresponding monochloro diol with an acetal or with thionyl chloride. The configurations at the 2 and 8 positions were determined to be *endo* based on the pattern of ^1H NMR spectra. Dechlorination at the 12 position of **8**, **14**, **16**, **25**, and **27** was judged to have occurred at the position *anti* to the double bond, from the finding that the NMR signals of their 2- and 8-protons were markedly shifted upfield. The *anti*-12-proton signals of **16** and **27** splited into fine doublets arising from long-range coupling with the olefinic proton. On the other hand, in **3**, **6**, **11**, **18** and **29**, which have a 12-chlorine atom, the NMR signals of 2- and 8-protons were little shifted relative to the corresponding signals in **2**, **5**, **10**, **17**, and **28**, which have two 12-chlorine atoms. Also, no long-range coupling between a 12-proton and the olefinic proton was observed in **3**, **11**, **18**, and **29**. Therefore the 12-chlorine atoms of these compounds were judged to be in the *anti* configuration.

Photochemical dechlorination process was carried out with an Eikohsha EL-J-60 low-pressure mercury arc lamp (30 W, 3 kV, 0.02 A). Structures and purity of the compounds synthesized were confirmed by mass spectroscopy, nuclear magnetic resonance (NMR) spectroscopy, and thin-layer chromatography. Chemical ionization (isobutane) and electron impact (70 eV) mass spectra were obtained with a Hitachi M-80B/M-0101 mass spectrometer. ^1H NMR spectra were measured in $CDCl_3$ at 270 MHz with a JEOL JNM-GX 270 spectrometer. Melting points were determined on a Yanako MP-500D apparatus and are shown as uncorrected. These data are given in the supplementary material.

1,9,10,11,12,12-Hexachloro-2,3:8,7-*endo*-4,6-dioxatricyclo-[7.2.1.0^{2,8}]dodec-10-enes (5, 13, 20, 22, and 24). Synthesis of **24** has been described in a previous paper (Ozoe et al., 1990). Compound **5** was similarly prepared from 1,2,3,4,7,7-hexachloro-5,6-bis(hydroxymethyl)bicyclo[2.2.1]hept-2-ene (Riemschneider et al., 1961) and freshly distilled diethoxymethane in 19% yield. Compounds **13**, **20**, and **22** were prepared in 51%, 14%, and 31% yields by similar methods using 1,1-diethoxyethane, propionaldehyde, and butyraldehyde instead of diethoxymethane, respectively.

1,9,10,11,12-Pentachloro-2,3:8,7-*endo*-4,6-dioxatricyclo-[7.2.1.0^{2,8}]dodec-10-enes (6, 14, and 25). A mixture of **24** (47 mg, 0.10 mmol), $NaBH_4$ (150 mg, 3.9 mmol), and vitamin B_{12} (20 mg) in dimethoxyethane (7.5 mL) and absolute MeOH (5 mL) was heated under reflux. After 2 h, $NaBH_4$ (150 mg, 3.9 mmol) was added, and heating was continued for an additional 1 h. The mixture was partitioned between water and CH_2Cl_2. The CH_2Cl_2 layer was washed, dried (Na_2SO_4), and concentrated. The residue was purified by column chromatography on silica gel (benzene) and recrystallization from CCl_4 to give **25** (23 mg, 52%). Compound **5** (450 mg, 1.2 mmol) was added to a mixture of 2,2′-azobis(isobutyronitrile) (AIBN) (2.5 mg) and tributyltin hydride (Bu_3SnH) (350 mg, 1.2 mmol) in benzene (5 mL), and the mixture was heated under reflux for 5 h and then concentrated. The residue was purified by column chromatography on silica gel (hexane) to give **6** (62 mg, 15%). Compound **14** was obtained by the $NaBH_4$–vitamin B_{12} reduction of the corresponding hexachloro analogue (**13**).

1,9,10,12,12-Pentachloro-2,3:8,7-*endo*-4,6-dioxatricyclo-[7.2.1.0^{2,8}]dodec-10-enes (7, 15, and 26). Compound **24** (150 mg, 0.32 mmol) in MeOH (300 mL) was irradiated with UV (254 nm) for 20 min. The mixture was concentrated, and the residue was purified by column chromatography on silica gel (benzene) to give **26** (30 mg, 21%). Compounds **7** and **15** were similarly obtained from **5** and **13**, respectively.

1,9,10,*syn*-12-Tetrachloro-2,3:8,7-*endo*-4,6-dioxatricyclo-[7.2.1.0^{2,8}]dodec-10-enes (8, 16, and 27). Compounds **8**, **16**, and **27** were obtained from **7**, **15**, and **26**, respectively, by the method described for **14** and **25**.

1,9,10,11-Tetrachloro-2,3:8,7-*endo*-4,6-dioxatricyclo[7.2.1.0^{2,8}]-dodec-10-ene (9). A solution of 1,2,3,4-tetrachlorocyclopentadiene (Wilcox and Zajacek, 1964) (1.4 g, 6.9 mmol) and *cis*-2-butene-1,4-diol (1.8 g, 20.4 mmol) in 1,4-dioxane (10 mL) was heated in a sealed tube at 120 °C for 15 h. The solution was concentrated and purified by column chromatography on silica gel (acetone/hexane = 5:1) to give 5,6-bis(hydroxymethyl)-1,2,3,4-tetrachlorobicyclo[2.2.1]hept-2-ene as an oil (0.23 g, 11%). Compound **9** was obtained in 6% yield from this diol and diethoxymethane by a method similar to that for **5**.

12,12-Dichloro-2,3:8,7-*endo*-4,6-dioxatricyclo[7.2.1.0^{2,8}]dodec-10-enes (10, 17, and 28) (Figure 1). Cyclopentanone was converted to the diethyl acetal (bp 37–38 °C/7 mmHg) in 95% yield (Böeseken and Tellegen, 1938). The acetal of cyclopentadienone was obtained by the bromination of cyclopentanone diethyl acetal followed by dehydrobromination (Allred and Anderson, 1967). The crude cyclopentadienone acetal was immediately used in the next step. The Diels–Alder adduct (**I**, mp 81–82 °C) between cyclopentadienone diethyl acetal and maleic anhydride was reduced to the corresponding diol (**II**, oil) with $LiAlH_4$ in 95% yield. The diol (8.5 g, 35 mmol) was mixed with Ac_2O (85 mL) and pyridine (85 mL) and stirred at room temperature for 24 h. The reaction mixture was diluted with CH_2Cl_2, washed with 5% HCl and water, and dried on Na_2SO_4. The acetate (**III**, mp 72–73 °C, 8.2 g, 25 mmol) thus obtained (yield 75%) as white crystals upon evaporation was further stirred in a mixture of AcOH (50 mL) and water (10 mL) at 60 °C for 6 h, at room temperature for 2 days. After evaporation, the residue was recrystallized from EtOH to give its ketone (**IV**, mp 96–97 °C, 3.8 g, 60%). To a solution of **IV** (1.0 g, 4.0 mmol) in PCl_3 (15 mL) in a NaCl/ice bath was added PCl_5 (1.3 g, 6.2 mmol) in portions, and the mixture was poured into an ice/water mixture after stirring at room temperature for 24 h. The benzene extract of the aqueous solution was washed with 5% $NaHCO_3$ and water, dried over Na_2SO_4, concentrated, and recrystallized from *i*-PrOH to give **V** (mp 75–77 °C, 1.0 g, 83%). Potassium hydroxide (85%) (320 mg, 4.9 mmol) was added to **V** (470 mg, 1.5 mmol) in 95% EtOH (100 mL), and the solution was stirred at room temperature for 4 h. The mixture was concentrated to one-third of the original volume and partitioned between water and $CHCl_3$. The $CHCl_3$ layer was washed with water, dried (Na_2SO_4), and concentrated. Recrystallization of the residue from benzene yielded **VI** (mp 112–113 °C, 270 mg, 80%). Compound **VI** (100 mg, 0.45 mmol) was heated under reflux with *p*-cyanobenzaldehyde (60 mg, 0.46 mmol) and *p*-toluenesulfonic acid monohydrate (20 mg) in benzene (20 mL) in a flask equipped with a Dean-Stark water separator for 20 min. The mixture was washed with 5% Na_2CO_3

and water, dried (Na_2SO_4), and concentrated. The residue was purified by recrystallization from EtOH to give **28** (80 mg, 53%). Compounds **10** and **17** were similarly obtained by the reaction of **VI** with diethoxymethane or with acetaldehyde.

12,12-Dichloro-2,3:8,7-*endo*-4,6-dioxa-5-thiatricyclo[7.2.1.0^{2,8}]-dodec-10-ene 5-Oxide (2). To a solution of diol **VI** (110 mg, 0.49 mmol) in $CHCl_3$ (10 mL) was added $SOCl_2$ (60 mg, 0.50 mmol). After stirring at room temperature for 2 h, the mixture was washed with water, dried (Na_2SO_4), and concentrated. The residue was recrystallized from Et_2O and hexane to give **2** (81 mg, 61%).

***anti*-12-Chloro-2,3:8,7-*endo*-4,6-dioxatricyclo[7.2.1.0^{2,8}]dodec-10-enes (11, 18, and 29).** Compound **29** was obtained from **28** by the method described for **14** and **25**. Compounds **11** and **18** were prepared by the method described for the 12,12-dichloro analogues from corresponding acetals and 7-chloro-5,6-bis-(hydroxymethyl)bicyclo[2.2.1]hept-2-ene (**VII**), which was obtained (yield 52%, mp 64–67 °C) by heating a benzene (5 mL) solution of Bu_3SnH (140 mg, 0.48 mmol), AIBN (0.63 mg), and **VI** (107 mg, 0.48 mmol) under reflux for 12 h, followed by concentration, column chromatography on silica gel (acetone/hexane = 2:5), and recrystallization from benzene and hexane.

***anti*-12-Chloro-2,3:8,7-*endo*-4,6-dioxa-5-thiatricyclo[7.2.1.0^{2,8}]-dodec-10-ene 5-Oxide (3).** Compound **3** was obtained in 58% yield from **VII** and $SOCl_2$ by the method described for **2**.

2,3:8,7-*endo*-4,6-Dioxatricyclo[7.2.1.0^{2,8}]dodec-10-ene (12). Compound **12** was synthesized from diethoxymethane and 5,6-bis(hydroxymethyl)bicyclo[2.2.1]hept-2-ene according to the general method described in a previous paper (Ozoe et al., 1990).

5-(4-Ethynylphenyl)-2,3:8,7-*endo*-4,6-dioxatricyclo[7.2.0.1^{2,8}]-dodec-10-ene (31). A solution containing 5-(4-bromophenyl)-DTD (Ozoe et al., 1990) (0.80 g, 2.5 mmol), (trimethylsilyl)-acetylene (0.50 g, 4.6 mmol), triphenylphosphine (30 mg), and palladium(II) acetate (15 mg) in dry Et_3N (2.5 mL) was heated under reflux for overnight and concentrated. To the residue cooled to 0 °C were added THF (10 mL) and a THF solution of 1 M tetrabutylammonium fluoride (3 mL, 3 mmol). After stirring for 2 h, the mixture was concentrated and partitioned between water and CH_2Cl_2. The CH_2Cl_2 layer was dried (Na_2SO_4) and concentrated to an oil. The oil was purified by column chromatography on silica gel (acetone/hexane = 1:10) and recrystallization from hexane to give **31** (35 mg, 5%).

Bioassays. Acetone solutions (1 μL) of piperonyl butoxide (PB, 10 μg) were topically applied to the mesonotum of female adult houseflies (*Musca domestica* L., WHO susceptible strain, 3–5 days old). The flies were 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 and LD_{50} values were obtained by the Probit method.

Preparation of Rat Brain and Housefly Head Membranes. Rat brain membranes were prepared according to the method of Squires et al. (1983). Five-week old, male Wistar rats were killed by cervical dislocation, and their forebrains were removed and stored frozen at −20 °C. The tissue was thawed and homogenized in 10 volumes of ice-cold 1 mM EDTA using a Teflon-glass homogenizer. The homogenate was centrifuged at 1000*g* for 10 min. The supernatant was then centrifuged at 25000*g* for 30 min. The resulting pellet was suspended in 1 mM EDTA and dialyzed three times (2 h each) in cellophane tubing against 2 L of distilled/deionized water at 4 °C. After the dialysis, the inner solution was centrifuged at 25000*g* for 30 min, and the pellet was stored frozen at −20 °C. The frozen pellet was thawed and suspended in 5 mM Tris-HCl buffer (pH 7.5) containing 0.2 M KBr and 1 mM EDTA (buffer A).

Heads of adult houseflies (WHO susceptible strain) were homogenized in 20 volumes of ice-cold 0.25 M sucrose containing 1 mM EDTA with a glass homogenizer. After filtering through gauze, the homogenate was centrifuged at 1000*g* for 10 min. The supernatant was centrifuged at 25000*g* for 30 min. The resulting pellet was superficially rinsed with 5 mM sodium phosphate buffer (pH 7.0) containing 0.2 M NaCl and 10 μM PB (buffer B) and suspended in buffer B.

Binding Assays. A test compound at various concentrations was incubated with 1.0 pmol [^{35}S]TBPS and rat brain membranes (0.25 mg protein) in 1.0 mL of buffer A at 25 °C for 90 min. After the incubation, the membranes were collected by rapid filtration on Whatman GF/C filter and washed twice by 5 mL of ice-cold buffer A. The filter was placed in a scintillation vial, and membrane-bound radioactivity was counted in toluene–Methyl Cellosolve-based scintillation fluid. Nonspecific binding was determined in the presence of 10 μM unlabeled TBPS. Protein content was determined by Bradford's method (1976) with bovine serum albumin as standard. IC_{50} values were obtained based on the inhibition percentage of specific binding by the Probit method. In the Scatchard analysis, rat membranes were incubated with increasing concentrations (2.0–300.0 nM) of [^{35}S]TBPS. Various concentrations of unlabeled TBPS were added to a fixed concentration (2.0 nM) of [^{35}S]TBPS to achieve the desired final concentrations. The amount of specifically bound [^{35}S]TBPS and the concentration of free [^{35}S]TBPS were plotted according to the method of Scatchard (1949).

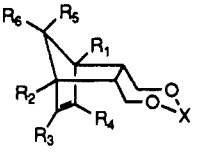
Assays on housefly head membranes were similarly performed under the following conditions: [^{35}S]TBPS, 5.0 pmol; protein, 0.75 mg per tube; incubation, 20 °C, 50 min; assay and rinse buffers, buffer B. One to three concentrations of each test compound were used to estimate an approximate IC_{50} value.

RESULTS

Insecticidal Activity. Table I shows the insecticidal activity of DTDs topically applied on PB-pretreated houseflies. In endosulfan analogues, the *anti*-12-chloro analogue (**3**) and deschloroendosulfan (**4**) had moderate insecticidal activity against houseflies, and the 12,12-dichloro analogue (**2**) was less active with the mortality of 38% at 10 μg/fly. In DTDs without 5-substituents, the hexachloro analogue (**5**) was the most active, followed by the 1,9,10,11,*anti*-12-pentachloro (**6**), 1,9,10,12,12-pentachloro (**7**), 1,9,10,11-tetrachloro (**9**), and 1,9,10,*syn*-12-tetrachloro (**8**) analogues. The 12,12-dichloro (**10**), *anti*-12-chloro (**11**), and non-chlorinated (**12**) analogues were inactive at 10 μg/fly. The insecticidal activity decreased with dechlorination. In DTDs with a methyl group in the 5 position, only the hexachloro analogue (**13**) was moderately active. Replacement of the methyl group of **13** by an ethyl or *n*-propyl group resulted in a complete loss in insecticidal activity. Complete dechlorination of the 5-*n*-propyl analogue (**22**) gave an analogue (**23**) with a modest activity, whereas the dechlorinated analogue (**21**) of the 5-ethyl analogue (**20**) was inactive at 10 μg/fly. In DTDs with a 4-cyanophenyl group in the 5 position, all chlorinated analogues (**24–29**) were inactive at 10 μg/fly, but non-chlorinated analogue **30** showed a high activity. Replacement of the 4-cyanophenyl group of **30** with a 4-ethynylphenyl group gave the most active analogue (**31**) with an LD_{50} value of 6.5 ng/fly among a series of DTDs.

Potency in Inhibiting [^{35}S]TBPS Binding. All endosulfan analogues including the insecticidally almost inactive dichloro analogue (**2**) showed the potency to inhibit binding of [^{35}S]TBPS, a specific, high-affinity ligand for the picrotoxinin binding site, to rat brain membranes (Table I). In the case of 5-unsubstituted DTDs, the *anti*-12-chloro (**11**) and non-chlorinated (**12**) analogues were inactive, but all other compounds, including an insecticidally inactive analogue (**10**), had IC_{50} values of <5 μM. In 5-methyl-DTDs, the hexachloro (**13**), pentachloro (**14** and **15**), and tetrachloro (**16**) analogues showed low activities, although three of these compounds (**14**, **15**, and **16**) were insecticidally inactive. The 5-ethyl and 5-*n*-propyl analogues, including an insecticidally active analogue (**23**), were inactive. In the case of 5-(4-cyanophenyl)-DTDs, the 12,12-dichloro (**28**), *anti*-12-chloro (**29**), and non-chlorinated (**30**) analogues were active compounds as judged by [^{35}S]TBPS binding test results, but two of them were insecticidally inactive. 5-(4-Ethynylphenyl)-DTD was

Table I. Insecticidal Activity and Potency in Inhibiting [^{35}S]TBPS Binding



								[^{35}S]TBPS binding	
								insecticidal activity[a]	housefly head	rat brain
no.	R_1	R_2	R_3	R_4	R_5	R_6	X	LD_{50} (μg/fly)	inhibition (%)	IC_{50} (μM)
1	Cl	Cl	Cl	Cl	Cl	Cl	S=O	0.042[b]	78 ± 10 (10^{-6} M) 27 ± 17 (10^{-7} M)	0.014
2	H	H	H	H	Cl	Cl	S=O	>10		0.20
3	H	H	H	H	Cl	H	S=O	5.3	84 ± 16 (10^{-5} M) 16 ± 9 (10^{-6} M)	6.7
4	H	H	H	H	H	H	S=O	0.86[b]	74 ± 9 (10^{-6} M) 15 ± 6 (10^{-7} M)	1.6
5	Cl	Cl	Cl	Cl	Cl	Cl	CH_2	0.11	74 ± 13 (10^{-6} M) 38 ± 16 (10^{-7} M) 10 ± 17 (10^{-8} M)	0.14
6	Cl	Cl	Cl	Cl	Cl	H	CH_2	0.40	88 ± 14 (10^{-6} M) 53 ± 17 (10^{-7} M) 16 ± 8 (10^{-8} M)	1.2
7	Cl	Cl	Cl	H	Cl	Cl	CH_2	0.82	91 ± 12 (10^{-6} M) 50 ± 10 (10^{-7} M) 15 ± 3 (10^{-8} M)	0.088
8	Cl	Cl	Cl	H	H	Cl	CH_2	3.9	96 ± 15 (10^{-6} M) 37 ± 2 (10^{-7} M) 14 ± 6 (10^{-8} M)	0.59
9	Cl	Cl	Cl	Cl	H	H	CH_2	2.5	92 ± 18 (10^{-6} M) 44 ± 19 (10^{-7} M) 5 ± 17 (10^{-8} M)	2.5
10	H	H	H	H	Cl	Cl	CH_2	>10	51 ± 11 (10^{-5} M)	4.3
11	H	H	H	H	Cl	H	CH_2	>10	36 ± 15 (10^{-5} M)	>10
12	H	H	H	H	H	H	CH_2	>10	86 ± 8 (10^{-5} M) 25 ± 8 (10^{-6} M)	>10
13	Cl	Cl	Cl	Cl	Cl	Cl	CH-CH_3	1.5	102 ± 11 (10^{-5} M) 76 ± 10 (10^{-6} M) 25 ± 16 (10^{-7} M)	3.7
14	Cl	Cl	Cl	Cl	H	Cl	CH-CH_3	>10		1.6
15	Cl	Cl	Cl	H	Cl	Cl	CH-CH_3	>10		3.6
16	Cl	Cl	Cl	H	H	Cl	CH-CH_3	>10		6.9
17	H	H	H	H	Cl	Cl	CH-CH_3	>10		>10
18	H	H	H	H	Cl	H	CH-CH_3	>10		>10
19	H	H	H	H	H	H	CH-CH_3	>10[b]		>10
20	Cl	Cl	Cl	Cl	Cl	Cl	CH-C_2H_5	>10	98 ± 11 (10^{-5} M) 52 ± 3 (10^{-6} M) 21 ± 5 (10^{-7} M)	>10
21	H	H	H	H	H	H	CH-C_2H_5	>10[b]		
22	Cl	Cl	Cl	Cl	Cl	Cl	CH-n-C_3H_7	>10	70 ± 6 (10^{-5} M) 37 ± 3 (10^{-6} M)	>10
23	H	H	H	H	H	H	CH-n-C_3H_7	7.6[b]		>10
24	Cl	Cl	Cl	Cl	Cl	Cl	CH-C_6H_4-CN-4	>10	36 ± 6 (10^{-5} M)	>10
25	Cl	Cl	Cl	Cl	H	Cl	CH-C_6H_4-CN-4	>10		>10
26	Cl	Cl	Cl	H	Cl	Cl	CH-C_6H_4-CN-4	>10		>10
27	Cl	Cl	Cl	H	H	Cl	CH-C_6H_4-CN-4	>10		>10
28	H	H	H	H	Cl	Cl	CH-C_6H_4-CN-4	>10		2.4
29	H	H	H	H	Cl	H	CH-C_6H_4-CN-4	>10		0.74
30	H	H	H	H	H	H	CH-C_6H_4-CN-4	0.11[b]	34 ± 5 (10^{-5} M)	0.092
31	H	H	H	H	H	H	CH-C_6H_4-C≡CH-4	0.0065		0.0032

[a] Synergized with PB. [b] Taken from Ozoe et al. (1990).

the most potent inhibitor with an IC_{50} value of 3.2 nM among compounds assayed. The results of Scatchard plots analysis in the presence of **5** (0.1 μM) and **30** (0.1 μM) are shown together with a control in Figure 2. The data indicate that both compounds are competitive inhibitors of [^{35}S]TBPS binding to rat brain membranes.

In [^{35}S]TBPS binding assays with housefly head membranes, most analogues with LD_{50} of <5 μg/fly were found to show inhibitory activity with approximate IC_{50} values of 0.1–1 μM (Table I). Compound **30** with the 5-(4-cyanophenyl) group was the only exception. No significant correlation was observed to exist between LD_{50} values and approximate IC_{50} values among this series of compounds (plots not shown): e.g., an approximate IC_{50} value of α-endosulfan (**1**) appeared lower, and those of 5-unsubstituted tetrachloro analogues (**8** and **9**) appeared higher than those expected from their LD_{50} values, respectively.

Relationship between Insecticidal Activity and Potency of Inhibiting [^{35}S]TBPS Binding to Rat Brain Membranes. The LD_{50} values (mol/fly) of many test compounds are plotted against their IC_{50} values (M) in terms of [^{35}S]TBPS binding inhibition to rat brain membranes in Figure 3, along with previously tested compounds (Ozoe et al., 1990) and standard insecticidal $GABA_A$ receptor antagonists known to act at the picrotoxinin binding site. A correlation coefficient of 0.674 was

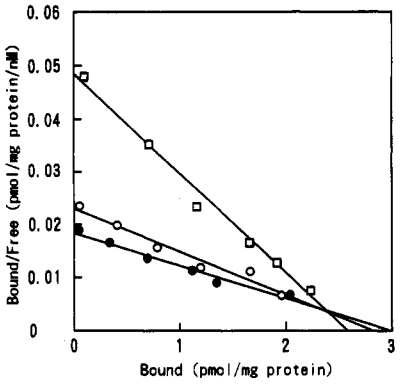


Figure 2. Scatchard analysis of DTD inhibition of [^{35}S]TBPS binding to rat brain membranes. Data are means of two to three experiments, each done in duplicate. □, control (without inhibitor), K_d = 53 nM, B_{max} = 2.6 pmol/mg protein; ○, in the presence of **5** (0.1 μM), K_d = 122 nM, B_{max} = 2.8 pmol/mg protein; ●, in the presence of **30** (0.1 μM), K_d = 161 nM, B_{max} = 3.0 pmol/mg protein.

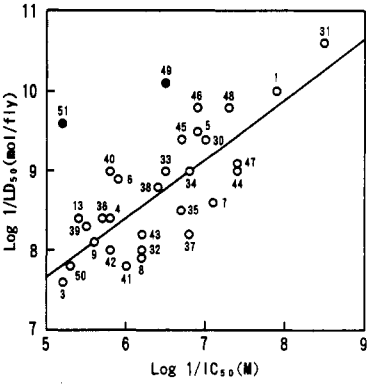


Figure 3. Relationship between potency in inhibiting [^{35}S]TBPS binding to rat brain membranes and synergized insecticidal activity against houseflies. Compounds **49** (γ-BHC) and **51** (PS-4) were omitted in drawing the linear regression line (log 1/LD_{50} = 3.956 + 0.743 log 1/IC_{50}; n = 29, s = 0.450, r = 0.807, $F_{1,27}$ = 50.3). Numbers 1–31 refer to compounds in Table I. Compounds **32–42** are DTDs with a following 5-substituent: **32**, Ph; **33**, Ph-Br-4; **34**, Ph-Cl-4; **35**, Ph-F-4; **36**, Ph-NO_2-4; **37**, Ph-Cl-3; **38**, Ph-Cl_2-3,4; **39**, 3-*c*-hexenyl; **40**, *c*-hexyl; **41**, *n*-Bu; **42**, *n*-pentyl. Other compounds included are as follows: **43**, 5-phenyl-2,3:8,7-*endo*-4,6-dioxa-5-phosphabicyclo[7.2.1.$0^{2,8}$]dodec-10-ene 5-oxide; **44**, 5-(4-cyanophenyl)-2,3:8,7-*endo*-4,6-dioxatricyclo[7.2.1.$0^{2,8}$]dodecane; **45**, picrotoxinin; **46**, β-endosulfan; **47**, TBOB; **48**, TBPS; **49**, γ-BHC; **50**, δ-BHC; **51**, PS-4. Data on the insecticidal activity of **1**, **4**, **30**, **32–44**, **46**, and **47** were taken from Ozoe et al. (1990), that (injection/+PB) of **45** from Ozoe et al. (1983), that (injection) of **48** from Palmer and Casida (1985), and that of **51** from Ozoe et al. (1992a).

obtained for all compounds but was improved (r = 0.807) by omitting two outliers, γ-BHC (**49**) and PS-4 (**51**). The following DTDs were not included in Figure 3 (compound no., 5-substituent, LD_{50} (μg/fly), IC_{50} (μM)): **52**, Ph-Cl-2, >10, >10; **53**, Ph-Cl_2-2,4, >10, 3.1; **54**, Ph-CF_3-4, >10, 1.0; **55**, Ph-Me-4, >10, 1.0; **56**, Ph-OCH_3-4, >10, 1.1; **57**, Ph-$N(CH_3)_2$-4, >10, >10; **58**, *c*-octyl, 0.75, >10; **59**, CH_2Ph, 6.1, >10; **60**, *n*-hexyl, >10, 3.7; **61**, *n*-heptyl, >10, 8.4. The following compounds were also not included (LD_{50} (μg/fly), IC_{50} (μM)): 5-(4-cyanophenyl)-2,3:8,7-*exo*-4,6,12-trioxatricyclo[7.2.1.$0^{2,8}$]dodec-10-ene (**62**), 0.93, >10; α-BHC (**63**), >10, 5.6; β-BHC (**64**), >10, >10.

DISCUSSION

The present study results demonstrate that, in the case of DTDs with a small 5-substituent (H and Me), the presence of chlorine atoms in the norbornene moiety is the most important factor in conferring toxic effects to houseflies. However, such an effect of chlorine substitution could not be observed in the compounds with a large 5-substituent (*n*-Pr and Ph-CN-4). Dechlorination of the *syn*-12-chlorine atom of **5** did not induce an increase in insecticidal activity, unlike the case with α-endosulfan (Brooks and Mace, 1987). Dechlorination of the *syn*-12 chlorine atom resulted in an increase in insecticidal activity in the 12,12-dichloro endosulfan-type analogue (**2**) but not in other 12,12-dichloro analogues (**10**, **17**, and **28**). Thus, the effect of 12-chlorine atoms on insecticidal activity was not necessarily consistent with the finding that, in picrotoxinin analogues, their *cis*-isopropyl groups probably equivalent to the *syn*-12 chlorine atom are less important for toxicity (Kuwano et al., 1980; Ozoe and Matsumura, 1986). A reduction in insecticidal activity by dechlorination also was found to occur among the endosulfan analogues: the insecticidal activity of deschloroendosulfan (**4**) was comparable to that of 5-unsubstituted pentachloro DTD **7**, but that was much lower than that of α-endosulfan (**1**). The high activity of α-endosulfan as compared to 5-unsubstituted hexachloro DTD **5** indicates the importance of electronegativity of the –O–S(O)–O– moiety.

The ability of selected analogues to inhibit [^{35}S]TBPS binding to housefly head membranes was determined to assess their intrinsic activity. Approximate IC_{50} values for all compounds tested did not satisfactorily correlate with LD_{50} values (plots not shown), probably because in the latter *in vivo* cases transporting, partitioning, metabolizing, and other processes are expected to play significant roles in modifying their toxicities. This finding indicates that the interaction of metabolically susceptible and resistant members of DTD and endosulfan analogues with the target site in houseflies cannot be explored by the [^{35}S]TBPS binding assay alone, unlike the case with the structure–activity studies of bicyclophosphorothionates which appear to be more homogeneous (Cohen and Casida, 1986; Ozoe et al., 1992a,b). However, another possibility for the lack of correlation in the fly head preparation could be the presence of interfering substances, heterogeneity of TBPS binding sites, the low ratio of specific to nonspecific binding, etc., all resulting from the crude nature of the fly head preparations (i.e., mixture of neural and nonneural tissues). Similar studies with [^{3}H]-ethynylorthobenzoate, which has been reported to be an improved radioligand (Deng et al., 1991), will be required to further examine the correlation between *in vivo* and *in vitro* data, although this ligand is not commercially available.

Meanwhile, a high correlation (r = 0.807) was obtained for the relationship between LD_{50} values for houseflies and IC_{50} values on rat brain membranes when γ-BHC and PS-4 were omitted (Figure 3). This finding suggests that the picrotoxinin binding sites of houseflies and rats are basically similar in structure and physical nature. Probably this is because the picrotoxinin binding site is located within the chloride channel (Havoundjian et al., 1986; Martini et al., 1991; ffrench-Constant et al., 1993; Tokutomi et al., 1993), whose amino acid sequence is highly conserved between vertebrates and invertebrates (Darlison, 1992). Information on differences in housefly and rat GABA receptors might be due to a higher neural content of the latter preparation. However, much more work would be needed to prove such a possibility. Despite the high insecticidal activity, γ-BHC had a relatively large IC_{50} value. This may be due to the fact that the insect GABA receptor is more sensitive to γ-BHC than the mammalian counterpart as revealed by [^{3}H]ethynylorthobenzoate binding assays (Cole et al., 1992). Alternatively, this may

be related to the finding that γ-BHC has other site(s) of action than the GABA receptor, e.g., voltage-dependent chloride channels (Abalis et al., 1985a; Matsumoto et al., 1988; Thompson et al., 1990). Bicyclophosphorothionate PS-4 was another compound which showed a large deviation from the line drawn in Figure 3. This compound has been shown to have higher potency on a housefly GABA receptor than the counterpart in rats (Ozoe et al., 1992b). As shown in Table I and described in the Results section, there are several compounds that have insecticidal activity but no activity on the rat TBPS binding site or vice versa. It is interesting to note that the non-chlorinated DTDs with a C_3–C_5 *n*-alkyl group in the 5 position (**23**, **41**, and **42**) had insecticidal activity, whereas inhibition of [^{35}S]-TBPS binding ($IC_{50} < 10$ μM) was observed in analogues with a C_4–C_7 *n*-alkyl group (**41**, **42**, **60**, and **61**). Additional experiments are necessary to determine whether this is due to differences between houseflies and rats in the structure and nature of TBPS binding sites.

The effect of chlorine atoms in the norbornene moiety with respect to their potency in inhibiting [^{35}S]TBPS binding was analogous to that on their insecticidal activity. The presence of four to six chlorine atoms definitely enhanced the potency in analogues with a small 5-substituent. In contrast their presence caused a loss in potency in analogues with a large 5-substituent. Nevertheless, it is evident from the Scatchard analysis that active compounds of both types act at the TBPS binding site competitively. Previously we have proposed a hypothesis that the TBPS binding site consists of four subsites where critical interactions with active compounds occur (Ozoe et al., 1990), i.e., subsite A which might interact with electronegative parts of compounds such as –O–CH_2–O– and –O–S(O)–O– moieties; subsite B which might accept electronegative moieties such as the olefinic chlorines; subsite C which could interact with the chlorine atom(s) in the 12 position or the non-chlorinated norbornene moiety; and subsite D which might accommodate the 5-substituent such as a 4-cyanophenyl group. We have further speculated that subsites A, C, and D are aligned linearly and that subsite A plays the most important role. A minimum structural requirement for an active ligand at the TBPS binding site is probably to possess moieties capable of interacting with at least two, including subsite A, of the four subsites. The lack of activity of **24**, which seems to have moieties to interact with all four subsites, is probably due to the inadequate spacing of interacting moieties. Efforts are currently underway to verify this hypothesis by three-dimensional quantitative structure–activity relationship analyses [cf. Akamatsu et al. (1992)]. Recently, Squires et al. (1989) have reported that cyclodiene insecticides and non-chlorinated GABA antagonists might occupy the TBPS binding site in different ways. The current study results show that these two different groups of ligands bind to the critical site in the same orientation but that the bulkiness and the angles of the chlorine substitution on the norbornene ring are very critical in determining their binding capabilities to their binding site.

ABBREVIATIONS USED

AIBN, 2,2′-azobis(isobutyronitrile); B_{max}, apparent maximum number of binding sites; Bu_3SnH, tributyltin hydride; DTD, 2,3:8,7-*endo*-4,6-dioxtricyclo[7.2.1.$0^{2,8}$]-dodec-10-ene; GABA, γ-aminobutyric acid; K_d, apparent equilibrium dissociation constant; PB, piperonyl butoxide; PS-4, 3-isopropyl-4-*n*-propylbicyclophosphorothionate or 3-isopropyl-4-*n*-propyl-2,6,7-trioxa-1-phosphabicyclo[2.2.2]-octane 1-sulfide; TBPS, *tert*-butylbicyclophosphorothionate or 4-*tert*-butyl-2,6,7-trioxa-1-phosphabicyclo[2.2.2]-octane 1-sulfide; TBOB, *tert*-butylbicycloorthobenzoate or 4-*tert*-butyl-1-phenyl-2,6,7-trioxabicyclo[2.2.2]octane; UV, ultraviolet.

ACKNOWLEDGMENT

This study was supported in part by Grant-in-Aid for Scientific Research 02660096 from the Ministry of Education, Science, and Culture of Japan and Grants ES05707 and ES01963 from the National Institute of Environmental Health Sciences, Research Triangle Park, NC.

Supplementary Material Available: Melting points and spectral (MS and ^{1}H NMR) data of DTDs **2**, **3**, **5**–**18**, **20**, **22**, **25**, **26**–**29**, and **31** (3 pages). Ordering information is given on any current masthead page.

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Received for review March 22, 1993. Revised manuscript received August 10, 1993. Accepted August 26, 1993.[⊗]

[⊗] Abstract published in *Advance ACS Abstracts*, October 15, 1993.