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Stereospecific Action of Pyrethroid Insecticides on the γ-Aminobutyric Acid Receptor–Ionophore Complex

Abstract. *The potent α-cyano-3-phenoxybenzyl pyrethroids, including cypermethrin, deltamethrin, and fenvalerate, act stereospecifically to inhibit binding to rat brain synaptic membranes of sulfur-35–labeled* t*-butylbicyclophosphorothionate, a new radioligand for the picrotoxinin binding site. Scatchard analysis indicates that picrotoxinin inhibition of* t*-butylbicyclophosphorothionate binding is competitive whereas cypermethrin inhibition possibly involves a closely associated site in the γ-aminobutyric acid receptor–ionophore complex. Studies with 37 pyrethroids reveal an absolute correlation, that is, no false positives or negatives, between mouse intracerebral toxicity and in vitro inhibition: all toxic cyano compounds but none of their nontoxic stereoisomers are inhibitors;* cis *isomers are more potent than* trans *isomers as both toxicants and inhibitors; and noncyano pyrethroids are much less potent or are inactive.*

Pyrethroid esters of (S)-α-cyano-3-phenoxybenzyl alcohol with various 3-substituted-2,2-dimethylcyclopropanecarboxylic acids (such as cypermethrin and deltamethrin) or 2-substituted-3-methylbutyric acids (such as fenvalerate) are potent and widely used insecticides (Fig. 1) (*1*). The poisoning syndrome of these pyrethroids is referred to as type 2 to differentiate it from the apparently different symptomology and mode of action for the earlier pyrethroids, referred to as type 1 (*2–6*). Unique type 2 features for the neurotoxicity of the cyanophenoxybenzyl esters are (i) inactivity in inducing repetitive firing after stimulation of the cockroach cercal sensory nerve (*3*); (ii) primary action in the central nervous system of mammals (*4*); (iii) prominent symptoms of sinuous writhing and profuse salivation in mammals (*5*); and (iv) delay by diazepam of the symptoms of poisoning (*6*). The type 1 pyrethroid action is similar in many respects to that of DDT (*3, 7*) but the type 2 syndrome of intracerebrally administered pyrethroids more closely approximates that of the convulsant picrotoxinin (PTX) (*4, 6*). Deltamethrin and a variety of cage convulsants, including bicyclophosphorus esters, inhibit binding of [^{3}H]dihydropicrotoxinin (the dihydro derivative of PTX shown in Fig. 1) to rat brain synaptic membranes, whereas the essentially nontoxic αR epimer of deltamethrin is inactive (*8*). These observations suggest a possible relation between the type 2 pyrethroid action and the γ-aminobutyric acid (GABA) receptor complex, but a more definitive approach is needed. We find, by using a new radioligand for the PTX binding site, namely [^{35}S]*t*-butylbicyclophosphorothionate, or [^{35}S]TBPS, (Fig. 1) (*9*), that (S)-α-cyanophenoxybenzyl esters act as inhibitors in a stereospecific manner related to their toxicity by binding to a site closely associated with but possibly distinct from that of TBPS and PTX.

The binding of [^{35}S]TBPS (*10*) was recently characterized by using rat brain synaptic membranes treated with EDTA and dialyzed to remove endogenous GABA (*9*). Similar results are obtained with our standard procedure (Fig. 2) using undialyzed membranes with respect to *p*H and temperature dependence, drug specificity and potency, and percent specific binding. TBPS binding is apparently to a single population of high-affinity sites (linear Scatchard plot) and is inhibited competitively by PTX, that is, the dissociation constant (K_d) increased with no change in the maximum number of binding sites (B_{max}) (Fig. 2B), supporting the earlier indications that PTX and TBPS bind to the same site (*9*). Like PTX, the bicyclophosphates (*11*) are indirect or noncompetitive GABA-A antagonists (*9, 12*). Inhibition by (1R,αS)-*cis*-cypermethrin of TBPS binding, on the other hand, appears to be mixed or possibly noncompetitive (it increases K_d and decreases B_{max}, although in neither case to a significant extent) (Fig. 2B). This suggests that the pyrethroid binding domain may be distinct from that of TBPS and PTX or that they only partially overlap. Inhibition of binding is concentration-dependent with both PTX and (1R,αS)-*cis*-cypermethrin, and although complete for PTX, it is maxi-

Table 1. Stereospecific correlation between in vitro potency of α-cyano-3-phenoxybenzyl pyrethroids as inhibitors of [^{35}S]TBPS binding and their toxicity. Inhibitory potencies (standard error < 10 percent of the mean; $N = 6$) are relative to (1R,αS)-*cis*-cypermethrin at 5 μ*M*, which gave 37 percent inhibition (assays as in legend to Fig. 2 but at 2 to 3 n*M* [^{35}S]TBPS and 0.5 mg of protein per milliliter). Total and nonspecific binding in the controls were typically about 3000 and 350 count/min, respectively. Toxicities are also relative to (1R,αS)-*cis*-cypermethrin with a mouse intracerebral LD_{50} of 1.4 nmole per gram of brain tissue determined by injection in 3 μl of methoxytriglycol into the third ventricle [data from (*4*) or determined by an identical procedure]. Values tabulated as 0 for inhibition are < 5 and for toxicity are < 0.1. Deltamethrin was not assayed as the S-acid isomers. Comparable values (relative binding, toxicity) for pyrethroids with other types of acid moieties are: (1R,αS)-*cis*-cyphenothrin (54, 14) (1R,αS)-*trans*-cyphenothrin (31, 7) and (RS)-fenpropathrin (56, 11). The isomers indicated by asterisks are potent insecticides and the other isomers are essentially inactive (*1, 2*).

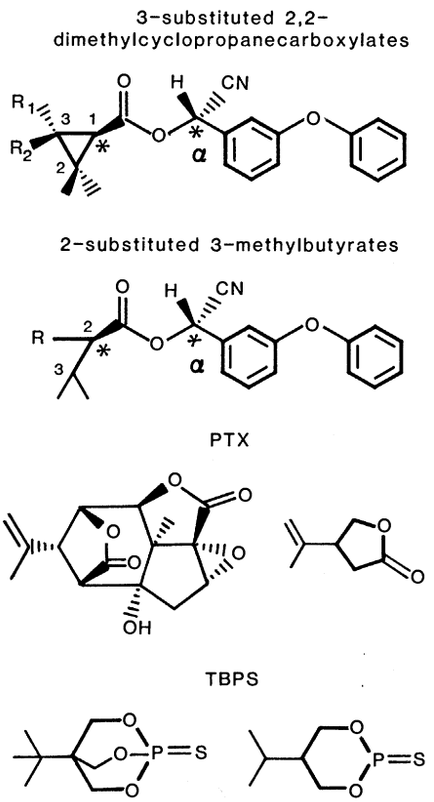
Pyrethroid	Assay	Relative potency		
		S-alcohol		R-alcohol
		R-acid	S-acid	R- and S-acid
3-(2,2-Dihalovinyl)-2,2-dimethylcyclopropanecarboxylates				
cis-Cypermethrin	Inhibition	100*	0	0
	Toxicity	100*	0	0
trans-Cypermethrin	Inhibition	54*	0	0
	Toxicity	43*	0	0
Deltamethrin	Inhibition	73*		0
	Toxicity	57*		0
2-Substituted-3-methylbutyrates				
Fenvalerate	Inhibition	0	43*	0
	Toxicity	0	57*	0
Fluvalinate	Inhibition	50*	0	
	Toxicity	34*	0	

mally 60 to 70 percent with the pyrethroid, possibly a consequence of its low aqueous solubility. Further evidence that pyrethroids and PTX differ in their interactions with the TBPS-PTX receptor comes from the observation that R 5135 (a steroid derivative with bicuculline-like activity) partially reverses TBPS binding inhibition by deltamethrin but not by PTX (*9*). Neurophysiological and toxicological differences are also evident. For example, cypermethrin, deltamethrin, and fenvalerate reduce the conductance increase caused by bath-applied GABA in the crayfish claw opener muscle, but they act more slowly than PTX (*2, 13*), and the poisoning signs, although similar, are not identical (*4, 6, 14*).

The toxicological relevance of the [^{35}S]TBPS receptor assay was evaluated by a pyrethroid structure-activity study comparing the inhibition of ligand binding by 37 stereoisomers and analogs with the intracerebral median lethal dose (LD_{50}) for mice, a criterion chosen because the mouse brain level of pyrethroids producing the type 2 syndrome is closely related to their toxicity (*4, 15*).

No false positives or false negatives appeared in correlating inhibition with toxicity in assays of 19 cyanophenoxybenzyl esters at 5 μM (Table 1). Of the eight cypermethrin isomers, only the two 1R,αS compounds inhibit [^{35}S]TBPS binding; they are also the only toxic isomers. The same correlation applies to two isomers of deltamethrin, also a dihalovinyldimethylcyclopropanecarboxylate, and to four isomers of fenvalerate and two isomers of fluvalinate, which are 2-substituted-3-methylbutyrates. The *cis* isomers of cypermethrin and cyphenothrin inhibit ligand binding 1.8 times more effectively than the *trans* isomers and they are also about twice as toxic (Table 1). The same *cis-trans* relations are evident with fenpyrithrin, a pyridyl analog of cypermethrin (*16*).

Pyrethroids without the α-cyano substituent do not inhibit [^{35}S]TBPS binding to rat brain synaptic membranes at 5 μM, and even at 10 μM little or no inhibition is evident. The compounds examined include four isomers each of phenothrin and permethrin (structures of cyphenothrin and cypermethrin, respectively, but with hydrogen replacing the cyano substituent), four isomers of resmethrin, (1R,*cis*)- and (1R,*trans*)-eth-



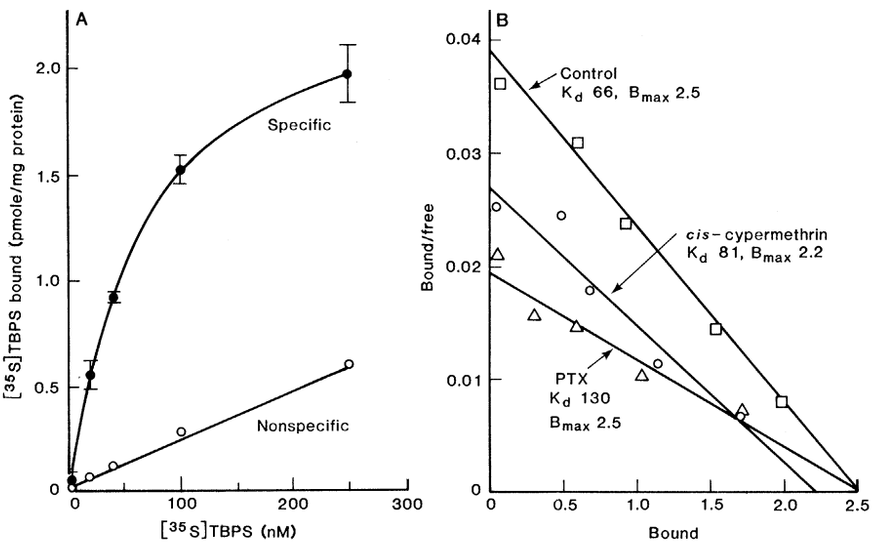


Fig. 1 (left). Structures of two types of (S)-α-cyano-3-phenoxybenzyl pyrethroids and of the noncompetitive GABA-A receptor antagonists PTX and TBPS. The 3-substituents of the *cis*-2,2-dimethylcyclopropanecarboxylates are: cyphenothrin $R_1 = (CH_3)_2C{=}CH{-}$, $R_2 = H{-}$; cypermethrin $R_1 = Cl_2C{=}CH{-}$, $R_2 = H{-}$; deltamethrin $R_1 = Br_2C{=}CH{-}$, $R_2 = H{-}$; and fenpropathrin $R_1 = R_2 = CH_3{-}$. R_1 and R_2 are reversed in the less toxic *trans* isomers. Fenpropathrin was used as the racemate. The 2-substituents of the 3-methylbutyrates are: fenvalerate R = 4-Cl-phenyl- and fluvalinate R = 2-Cl, 4-CF_3-phenyl-NH–. Asterisks indicate asymmetrical centers at which only the designated pyrethroid isomer is active. The partial structures show portions of the TBPS and PTX (*17*) molecules that are potentially equivalent, based on comparison of molecular models, in their interaction with the TBPS-PTX binding site of the GABA receptor–ionophore complex. Fig. 2 (right). (A) Specific binding of [^{35}S]TBPS to rat brain synaptic membranes as a function of [^{35}S]TBPS concentration. Synaptic membranes (P_2 fraction) were prepared by a described procedure (*18*) at 2 mg of protein (125 mg wet brain weight equivalent) per milliliter of ice-cold 5 mM sodium phosphate buffer (*p*H 7) containing 0.2M sodium chloride. Protein was determined by the method of Bradford (*19*). For assay 0.5 ml of cold membrane preparation was added to 0.5 ml of buffer and 5 μl of dimethyl sulfoxide (DMSO) at 37°C containing [^{35}S]TBPS (*10*) (2 to 250 nM; concentration varied by the addition of unlabeled TBPS). These mixtures were incubated for 30 minutes at 37°C with shaking, quickly diluted with 5 ml of ice-cold buffer, and filtered under vacuum through Whatman GF/C filters using an Amicon VFM1 filtration manifold. Immediately following filtration, the filters were rinsed rapidly with two 5-ml portions of ice-cold buffer, air-dried, and radioassayed by liquid scintillation counting. Specific binding is defined as the difference in labeling of samples incubated with [^{35}S]TBPS only (total binding) and those incubated in the presence of 2 μM unlabeled TBPS added initially in 5 μl of DMSO (nonspecific binding). Standard errors of the mean (eight determinations; four separate experiments) for specific binding are indicated by error bars and for nonspecific binding were between 2 and 5 percent of the mean except for the value at 2 nM (13 percent). (B) Scatchard analysis of [^{35}S]TBPS specific binding without inhibitor [control, the same data as in (A)] and in the presence of 0.5 μM PTX and 5 μM (1R,αS)-*cis*-cypermethrin. Inhibitor assays (four determinations; two separate experiments) used 5 μl of DMSO as the carrier. Values for B_{max} (picomoles per milligram of protein) and K_d (nanomolar) were determined and statistically evaluated by computer-assisted linear regression analysis. The 95 percent confidence intervals for K_d were 56 to 84, 86 to 255, and 53 to 168 for the control, PTX, and cypermethrin, respectively. Equivalent values for B_{max} were 2.2 to 2.9, 1.4 to 3.6, and 1.2 to 3.2, respectively. Lineweaver-Burk plots (not shown) of the same data gave similar values: K_d of 61, 165, and 58 and B_{max} of 2.4, 2.9, and 1.8 for control, PTX, and *cis*-cypermethrin, respectively.

anomethrin, kadethrin, and *S*-bioallethrin, ten of which are potent insecticides (*1*) or are highly toxic when injected into mammals (*2, 4*).

Specific binding of [^{35}S]TBPS is inhibited by the toxic isomers of each α-cyanophenoxybenzyl pyrethroid and to a greater degree by the *cis* than the *trans* isomers, in agreement with their relative toxicity. Binding is not inhibited by any nontoxic stereoisomer, presumably because the site is stereospecific relative to each chiral center in the pyrethroid. Pyrethroids producing the type 1 syndrome are essentially inactive in this assay with brain synaptic membranes, indicating that their primary action may be elsewhere. The mechanism of toxicity of the cyanophenoxybenzyl pyrethroids therefore appears to involve an interaction with the TBPS-PTX receptor by binding to a closely associated though possibly not identical site in the GABA receptor–ionophore complex.

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A Cellulolytic Nitrogen-Fixing Bacterium Cultured from the Gland of Deshayes in Shipworms (Bivalvia: Teredinidae)

Abstract. *A novel bacterium has been isolated in pure culture from the gland of Deshayes in six species of teredinid bivalves. It is the first bacterium known to both digest cellulose and fix nitrogen, and it is a participant in a unique symbiotic relation with shipworms that may explain how teredinids are able to use wood as their principal food source.*

It has been reported that adult shipworms, wood-boring bivalves of the family Teredinidae, can grow on a diet of wood alone unsupplemented by filter feeding (*1*). However, there is considerable disagreement over the origin of the enzymes necessary for the digestion of cellulose and the means by which these mollusks meet their nitrogen requirements when feeding on wood. We describe here the isolation and purification of a cellulolytic, nitrogen-fixing bacterium from the gland of Deshayes in six species of shipworms: *Lyrodus pedicellatus* (Quatrefages), *Bankia gouldi* (Bartsch), *Teredo navalis* Linnaeus, *Teredo furcifera* Von Martens, *Teredo bartschi* Clapp, and *Psiloteredo healdi* (Bartsch). Its presence in enormous numbers in the gland of Deshayes (Fig. 1a) may account for the ability of shipworms to digest cellulose and to balance their nitrogen budget.

The gland of Deshayes is unique to the Teredinidae. It was discovered more than a century ago by Deshayes (*2*) and subsequently named and described by Sigerfoos (*3*). To date, functions ascribed to this gland have been inferred largely from circumstantial evidence. They include the production of cellulolytic enzymes (*4*) and the synthesis of amino acids (*5*). The gland consists of a brown, irregular mass of tissue lining the

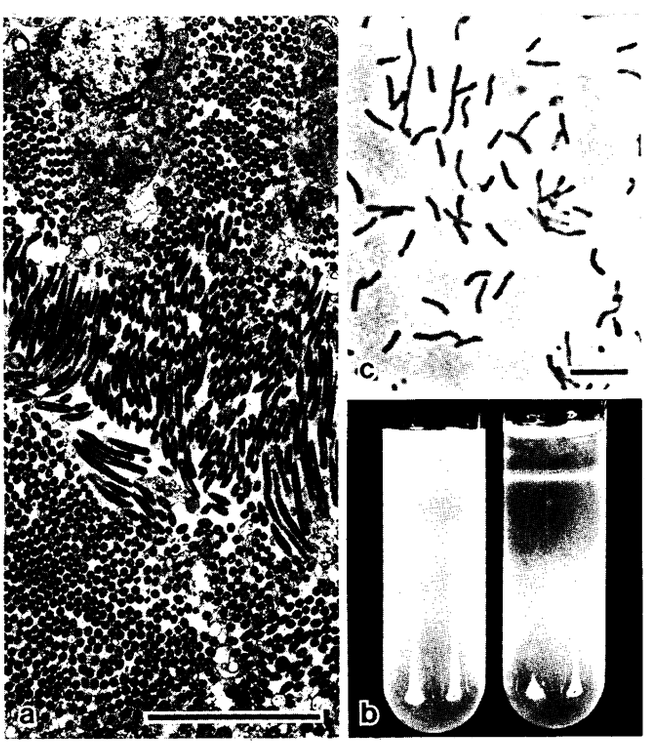

Fig. 1. (a) Transmission electron micrograph of a thin section of the gland of Deshayes from *L. pedicellatus*, showing numerous Gram-negative, rod-shaped bacteria. Scale bar, 5 μm. (b) Bacterial isolate from *L. pedicellatus*, shown growing on colloidal cellulose agar medium (tube on right). Note the distinct band of cells below the agar surface and the disappearance of the colloidal cellulose both above and below this band. The tube at left contains uninoculated medium; colloidal cellulose is uniformly distributed. (c) Phase-contrast photomicrograph of the bacterium isolated from the gland of Deshayes in *L. pedicellatus*. Scale bar, 5 μm.