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Bicycloorthocarboxylate Convulsants

Potent $GABA_A$ Receptor Antagonists

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SUMMARY

4-*t*-Butyl-1-(4-bromophenyl)-bicycloorthocarboxylate antagonizes γ-aminobutyric acid (GABA)-mediated relaxation at a functional insect nerve-muscle synapse, mimicking the action of picrotoxinin, suggesting that it causes GABA antagonism through blockade of the chloride ionophore. It is also a potent $GABA_A$ receptor antagonist, inhibiting the binding of [^{35}S]*t*-butyl-bicyclophosphorothionate ([^{35}S]TBPS) to EDTA/water-dialyzed human brain P_2 membranes. Structure-activity relationships of 74 1,4-*bis*-substituted bicycloorthocarboxylates, mostly new compounds, reveal that for high potency as a $GABA_A$ receptor antagonist the optimal 4-substituent is a C_4 to C_6 branched chain alkyl or cycloalkyl group (e.g., *t*-butyl, *s*-butyl, or cyclohexyl) and the optimal 1-substituent is a phenyl moiety with one or more electron-withdrawing groups (e.g., 4-cyano, 4-bromo, 4-chloro, 3,4-dichloro, or pentafluoro). Bicycloorthocarboxylate inhibitors of [^{35}S]TBPS binding with IC_{50} values of 5–10 nM exceed by several-fold the potency of any $GABA_A$ receptor antagonist previously reported. The 4-*t*-butyl-1-(4-azidophenyl) analog, synthesized as a candidate photoaffinity label, gives an IC_{50} of 315 nM. The potency of bicycloorthocarboxylates for decreasing [^{35}S]TBPS binding generally correlates with their toxicity, i.e., compounds without inhibitory activity in this brain receptor assay are of low toxicity on intraperitoneal administration to mice, and the analogs most potent as inhibitors are generally those most toxic to mice (e.g., IC_{50} of 5 nM and LD_{50} of 0.06 mg/kg for 4-*t*-butyl-1-(4-cyanophenyl)-bicycloorthocarboxylate). The effects of phenyl substituents on the potency of the orthobenzoates as $GABA_A$ receptor antagonists are similar to those on toxicity. In contrast to the 1-substituted phenyl compounds, 4-*t*-butyl-1-ethynyl-bicycloorthocarboxylate and its 4-*i*-propyl analog are very toxic (LD_{50} 0.4–2 mg/kg) but have only moderate inhibitory potency (IC_{50} 480–2900 nM), a pattern noted for many 1-alkyl-bicycloorthocarboxylates, suggesting that even within this series there may be different types of receptor-inhibitor interactions. 1-(4-Chlorophenyl)-4-cyclohexyl-bicycloorthocarboxylate is particularly sensitive to oxidative detoxification based on its 10-fold synergism of toxicity by piperonyl butoxide and marked potency loss in a coupled [^{35}S]TBPS receptor/microsomal oxidase assay. Some benzodiazepines and phenobarbital protect against poisoning by 1-(4-bromophenyl)- and 1-ethynyl-4-*t*-butyl-bicycloorthocarboxylates and their 1-(4-bromophenyl)-4-cyclohexyl analog. The exceptional potency and high structural specificity of the bicycloorthocarboxylates indicate they are useful probes in further studies of the GABA receptor/ionophore complex.

INTRODUCTION

A variety of cyclic and bicyclic compounds are convulsants (1–4) that block GABA[1]-mediated inhibitory transmission by acting as noncompetitive $GABA_A$ receptor antagonists (5–12). Potent toxicants acting in this way include the PTX analogs (3, 6, 7, 9), polychlorocycloalkanes (4, 10), and two types of 2,6,7-trioxabicyclo[2.2.2]

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[1] The abbreviations used are: GABA, γ-aminobutyric acid; $GABA_A$ receptor, bicuculline-sensitive sites (11); Me, methyl; Et, ethyl; Pr, propyl; Bu, butyl; Pen, pentyl; Hex, hexyl; Hept, heptyl; Ph, phenyl; *n*, normal; *i*, iso; *s*, secondary; *t*, tertiary; *c*, cyclo; IC_{50}, concentration producing 50% inhibition; i.p., intraperitoneal; K_D, dissociation binding constant; LD_{50}, dose producing 50% mortality; NMR, proton nuclear magnetic resonance; PB, piperonyl butoxide; PTX, picrotoxinin; TBPS, *t*-butyl-bicyclophosphorothionate; [^{35}S]TBPS, radioligand for the TBPS receptor; TBOB, *t*-butyl-bicycloorthobenzoate or 4-*t*-butyl-1-phenyl-bicycloorthocarboxylate; TBPO, *t*-butyl-bicyclophosphate.

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octanes, i.e., the bicyclophosphorus esters (1, 2, 4, 5, 8, 11) and bicycloorthocarboxylates (1, 2, 8). The receptor(s) for these convulsants is partially characterized by binding studies using brain membranes and the PTX analog [^{3}H]dihydropicrotoxinin (6), the bicyclophosphorus ester [^{35}S]TBPS (8), and the bicycloorthocarboxylate [^{3}H]TBOB (13) as radioligands.

1,4-*bis*-Substituted bicycloorthocarboxylates are of continued interest for four reasons. First, there appears to be a possible correlation between potency in decreasing [^{35}S]TBPS binding to a brain receptor and mouse toxicity, but this is based on data for only four bicycloorthocarboxylates (1, 2, 8); a more extensive and critical evaluation is required. Second, some 4-*t*-butyl-bicycloorthocarboxylates are very potent inhibitors of [^{35}S]TBPS binding, i.e., TBOB and its 1-*n*-butyl analog are one-half to one-third the potency of TBPS at the rat brain receptor (8) and TBOB is only 1.6-fold less potent than TBPS at the human brain receptor (12). Considering the possible variants at the 1- and 4-positions, it appears likely that compounds can be designed with increased convulsant activity and potency at the receptor. Third, synthesis in the bicycloorthocarboxylate series, such as the azidophenyl analog, may also provide the needed photoaffinity label for the receptor(s). Finally, preliminary structure-mouse toxicity studies indicate that bicycloorthocarboxylates with 1-substituents that are small (H, Me) or large (Ph, *n*-Bu) are more potent than those of intermediate size (Et, *i*-Pr) suggesting that more than one type of receptor interaction may be involved (1, 2, 8). The present study uses a variety of bicycloorthocarboxylates as probes for examining the conformation of the binding site(s) for GABA$_A$ receptor antagonists.

MATERIALS AND METHODS

GABA-mediated synaptic transmission assay. This assay used a cockroach (*Periplaneta americana* L.) nerve-muscle preparation. The metathoracic coxal depressor muscle (no. 177d; nomenclature according to Carbonell (14)) and its innervating nerve (nerve root 5) were isolated and perfused with physiological saline (15). Motoneurons were stimulated selectively with extracellular suction electrodes attached to nerve 5, and muscle tension was recorded with a force transducer (Cambridge Instruments, Cambridge, MA). Tension in the muscle was elevated by 1-Hz stimuli applied to an excitatory peptidergic motoneuron (D_s) (15). Transient relaxation of the muscle was elicited by stimulation of GABA-releasing motoneurons using 8-Hz pulse trains of 1-sec duration applied every 10 sec (see Ref. 16, Fig. 1 for explanation of GABAergic motoneuron stimulation). The bicycloorthocarboxylate was bath-applied to the muscle preparation in physiological saline to test for antagonism of GABA-mediated relaxation events. PTX was used as a positive control for GABA inhibition.

[^{35}S]TBPS receptor assay. EDTA/water-dialyzed P_2 membranes, prepared from human brain by a described procedure (8, 12), were held at −80° and resuspended as needed at 0.5 mg of protein (17)/ml in 10 mM Na phosphate, 200 mM NaCl, pH 7.4, buffer. P_2 suspension (0.5 ml) was added to 0.5 ml of buffer containing 2 pmol of [^{35}S]TBPS (~100 Ci/mmol, New England Nuclear) and the candidate inhibitor in 5 μl of dimethyl sulfoxide. [^{35}S]TBPS binding after 60 min incubation at 37° was determined by the filtration method (18) on a Brandel M-24 cell harvester (Brandel Instruments). Nonspecific binding was determined with 2 nmol of unlabeled TBPS. This binding assay is characterized by a K_D of 61 ± 12 nM and a maximum receptor density of 6.6 ± 2.2 pmol/mg protein (12). IC_{50} values were taken directly from Hill plots. In all cases the Hill coefficients were not significantly different from 1.0. The 95% confidence limits had an average range of ±58% of the IC_{50} values as calculated by computer-assisted linear regression analyses of the Hill plot data. This procedure was varied when specifically stated by using [^{3}H]TBOB (13) instead of [^{35}S]TBPS with all other assay conditions unchanged.

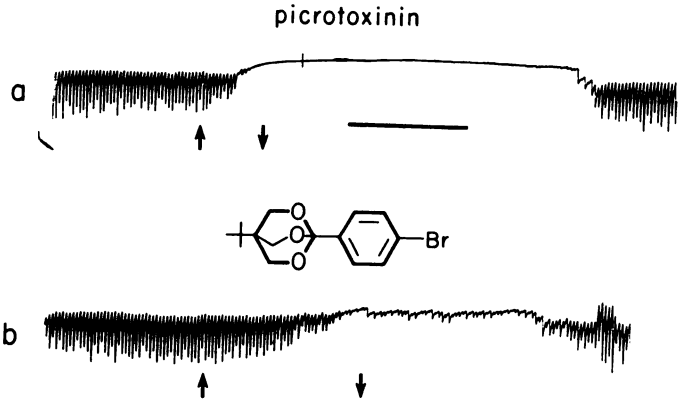

FIG. 1. *Actions of 4-t-butyl-1-(4-bromophenyl)-bicycloorthocarboxylate (**25**) and PTX on GABA-mediated relaxation of the cockroach coxal depressor muscle*

Transient relaxation events in the muscle (downward deflections) are caused by brief trains of 8-Hz stimuli applied every 10 sec to GABAergic inhibitory motoneurons. a. Application of 5 μM PTX (upward arrow) results in a rapid and complete block of GABA-mediated relaxation events. b. Application of 5 μM **25** causes a similar inhibition of the relaxation events, although the onset of the blockade is slower and less complete (90–95% amplitude, as compared with complete block by PTX). Removal of the toxin with a saline wash (downward arrow) permits recovery of the relaxations after about 10 min. Calibration mark: 5 min. These studies were carried out by Michael E. Adams of Zoecon Corporation (Palo Alto, CA).

The coupled metabolic activation/detoxification system involved an identical receptor assay as described above in 1-ml volume but fortified with mouse liver microsomes (25 μg of protein, noninduced) and NADPH (2 μmol for the oxidase system and 0 μmol for the control) (10). Specific [^{35}S]TBPS binding is identical in the presence and absence of NADPH. However, correction is required for controls both with and without NADPH because total binding is ~11% higher with this cofactor due to enhanced nonspecific binding attributable to phosphorothionate oxidation and binding of the liberated sulfur to membranes (10).

Mouse toxicity assays. LD_{50} values were determined from mortality data 24 hr after i.p. administration of the trioxabicyclooctanes to male albino Swiss-Webster mice (18–22 g) with methoxytriglycol (50 μl) as the carrier vehicle. In some cases the mice were pretreated i.p. with the oxidase inhibitor PB at 150 mg/kg (administered in 25 μl of methoxytriglycol) 1 hr before the toxicant or with sodium phenobarbital at 100 mg/kg or a benzodiazepine at 10 mg/kg (given in water and methoxytriglycol) 5 and 15 min, respectively, before the toxicant. Five to nine mice were used at each concentration in determining the reported LD_{50} values.

Spectroscopy. NMR spectra were obtained at 300 MHz with a Bruker WM-300 spectrometer for samples dissolved in deuterochloroform. Mass spectrometry involved a Hewlett-Packard 5985 system using chemical ionization at 230 eV with methane (0.8 torr).

Chemicals. The bicyclophosphorus esters were from our earlier syntheses (1, 2). The benzodiazepines were provided by Richard F. Squires (Rockland Research Institute, Orangeburg, NY). Sources or preparations for the bicycloorthocarboxylates are given by Palmer and Casida (19) or in our earlier reports (1, 2). Syntheses are detailed below for two bicycloorthocarboxylates (**30, 43**) highly potent as both inhib-

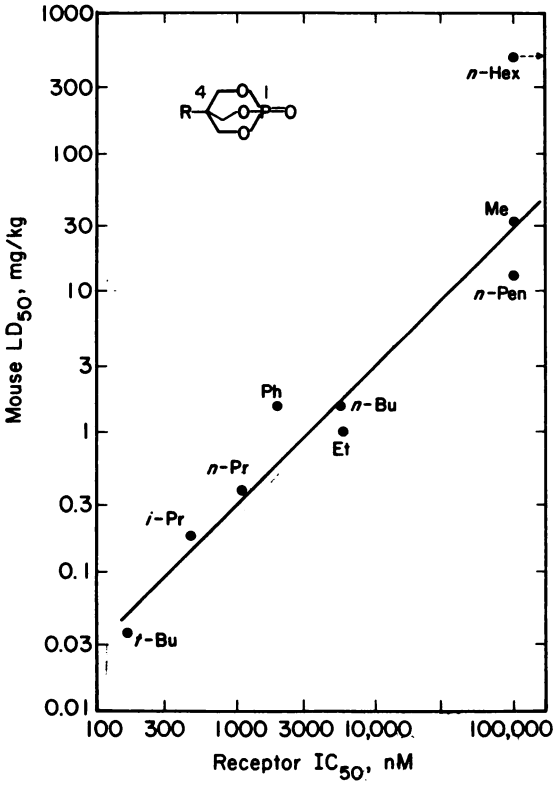

FIG. 2. *Relation of potency for decreasing [^{35}S]TBPS binding to the human brain receptor and toxicity to mice of 4-substituted bicyclophosphates*

Designations are indicated for the 4-substituents. Assay conditions are given in Table 1. LD_{50} data for the *n*-Hex and Ph compounds are from Ref. 1 and the others are from Ref. 2. An arrow indicates <50% inhibition or mortality at the highest dose tested, and the direction of the arrow refers to the receptor or mouse assay. Least squares correlation coefficient $r = 0.892$ ($n = 8$) based on compounds with actual values for both IC_{50} and LD_{50}.

itors and convulsants, one compound of high toxicity but relatively low inhibitory activity (**65**) and a candidate photoaffinity label (**32**).

Preparation of orthobenzoates **30**, **32**, and **43** involved an improvement of a described synthesis (20) of 2-*t*-butyl-2-hydroxymethyl-1,3-propanediol (referred to as the triol) which was then converted to 3-*t*-butyl-3-hydroxymethyloxetane (referred to as the oxetane) (similar procedure in Ref. 21) for coupling with the appropriate 4-substituted benzoyl chloride followed by rearrangement (22). 3,3-Dimethylbutanal (0.26 mol) (from pyridinium chlorochromate oxidation (23) of 3,3-dimethyl-1-butanol (Aldrich)) was mixed with formalin (215 ml, 2.65 mol of formaldehyde) and NaOH (0.33 mol in 270 ml of water) and stirred overnight at 50°. Following neutralization with dilute H_2SO_4 and evaporation to dryness, the portion of the residue soluble in *i*-propanol was subjected to gradual heating at 0.5 mm Hg (to complete the reduction step) and then stripped of products volatile at up to 100°, yielding a solid residue. Recrystallization from hexane-chloroform gave the triol as white crystals (yield 31%), m.p. 204–207°. A mixture of the triol (0.21 mol), diethyl carbonate (0.21 mol), and KOH (0.1 g in 5 ml of ethanol) was heated to reflux under nitrogen for 15 min. Ethanol was then distilled off at atmospheric pressure and the oxetane sublimed (90–110°, 25 mm Hg) as a white solid (62%), m.p. 163–165°. $[M+1]^+$ 145, δ0.95 [9H, s, Me_3C], 1.7–1.8 (1H, broad—OH), 3.75 (2H, s, CH_2OH), 4.55 (4H, s, CH_2OCH_2).

Synthesis of 4-N_3Ph compound **32** was initiated by addition of a solution of 4-azidobenzoyl chloride (3.5 mmol) (from treatment of 4-azidobenzoic acid (24) with oxalyl chloride) to the oxetane (3.5 mmol) and dry pyridine (2 ml) in dry dichloromethane (20 ml). The mixture

TABLE 1

Effect of 4-substituent of 1-(4-chlorophenyl)-bicycloorthocarboxylates and their 1-cyclohexyl analogs on their potency for inhibiting [^{35}S]TBPS binding to the human brain receptor and on their toxicity to mice

IC_{50} values for decreasing specifically bound [^{35}S]TBPS by the bicycloorthocarboxylates were determined with EDTA/water-dialyzed P_2 membranes of human brain (0.5 mg of protein) and 2 nM [^{35}S]TBPS in 1.0 ml of 10 mM Na phosphate, 200 mM NaCl, pH 7.4, buffer with incubation for 60 min at 37°. When IC_{50} values were not attained the highest concentration tested is followed by the percentage of inhibition in parenthesis. LD_{50} values are for i.p.-treated mice or, in parentheses, for mice treated i.p. with PB at 150 mg/kg for 1 hr before i.p. administration of the test compound. Data for the 1-*c*-Hex analogs are (compound no., 4-substituent, IC_{50}, LD_{50}): **12**, 4-Et > 10^5 (30%), 170; **13**, 4-*c*-Hex 217, 42; **14**, 4-Ph 3500, 150. PB alters the toxicity of compounds **1–3**, **5**, **6**, **9**, and **11–14** by less than 2-fold.

4-Substituent	Compound no.	Receptor IC_{50}	LD_{50}
		nM	*mg/kg*
Et	**1**	413	13
n-Pr	**2**	176	9.1
i-Pr	**3**	45	2.3
n-Bu	**4**	200	50 (10)
s-Bu	**5**	6	2.5
t-Bu	**6**	7	1.1
c-Pen	**7**	21	6.1 (2.5)
c-Hex	**8**	13	52 (5.0)
Ph	**9**	100	54
4-MePh	**10**	>10^5 (10%)	Insoluble
NO_2	**11**	>10^5 (20%)	150

was stirred overnight at room temperature, extracted with water, dried (Na_2SO_4), and evaporated to leave the 4-azidobenzoyl oxetane (95%) as a residue which was not purified further. δ1.05 [9H, s, Me_3C], 4.45 (2H, s, CH_2OCO), 4.60 (4H, s, CH_2OCH_2), 7.05 (2H, d aromatic), 8.1 (2H, d, aromatic). Boron trifluoride etherate (1 ml) was added to a solution of the azidobenzoyl oxetane (3.3 mmol) in dry dichloromethane (30 ml) under nitrogen at −70°. The solution was allowed to warm to room temperature, quenched with triethylamine, evaporated to dryness, and partitioned between dichloromethane and water. The organosoluble fraction was chromatographed on basic alumina with dichloromethane as the elutrient to give compound **32** as a pale yellow solid which was recrystallized from hexane-chloroform (68%), m.p. 122–124°. $[M+1]^+$ 290, δ0.90 [9H, s, Me_3C], 4.15 [6H, s, $(CH_2O)_3$], 7.0 (2H, d, aromatic), 7.6 (2H, d, aromatic). 4-CNPh derivative **30**, prepared by the same procedure from 4-cyanobenzoyl chloride, was obtained as white crystals (74%), m.p. 207–208°. $[M+1]^+$ 274, δ0.90 [9H, s, Me_3C], 4.15 [6H, s, $(CH_2O)_3$], 7.6–7.7 (4H, q, aromatic). F_5Ph analog **43**, similarly prepared from pentafluorobenzoyl chloride, was obtained as pale yellow crystals (27%), m.p. 163–165°. $[M+1]^+$ 339, δ0.90 [9H, s, Me_3C], 4.15 [6H, s, $(CH_2O)_3$].

A different type of procedure was used to prepare 1-ethynyl compound **65**. A mixture of the triol (20 mmol), triethyl 2-bromoorthopropionate (20 mmol) (25), and 4-toluenesulfonic acid (10 mg) was heated to give the 1-bromoethyl-bicycloorthoester intermediate as a liquid (86%) which was then stirred at reflux in dry toluene under nitrogen overnight with KO-*t*-Bu (3 eq). The resulting mixture was poured into ice water and extracted with ether. Evaporation of the ether gave the 1-vinyl-bicycloorthoester (79%) which was immediately treated with bromine (1 eq) in carbon tetrachloride at 0°. The solution was evaporated and the residue treated with KO-*t*-Bu (6 eq) with work-up as above and then recrystallization from hexane-chloroform to give **65** (55%) as white needles, m.p. 195–197°. $[M+1]^+$ 197, δ0.85 [9H, s, Me_3C], 2.50 [1H, s, C≡CH], 4.05 [6H, s, $(CH_2O)_3$].

TABLE 2

Effect of 1-(substituted-phenyl) group of 4-alkyl-bicycloorthocarboxylates and their 4-phenyl analogs on their potency for [^{35}S]TBPS displacement from the human brain receptor and on their toxicity to mice

For conditions and terms see the text and Table 1. Data for the 4-Ph series are (compound no., phenyl substituent, IC_{50}, LD_{50}): **44**, H 5500, >300; **45**, 4-F 216, 37. PB altered the toxicity of compounds **15–17**, **19–23**, and **25–45** by less than 2-fold.

Substituent on 1-phenyl group	Compound no.				Receptor IC_{50}				LD_{50}			
	4-*n*-Pr	4-*i*-Pr	4-*t*-Bu	4-*c*-Hex	4-*n*-Pr	4-*i*-Pr	4-*t*-Bu	4-*c*-Hex	4-*n*-Pr	4-*i*-Pr	4-*t*-Bu	4-*c*-Hex
						nM				*mg/kg*		
H	**15**	**16**	**17**	**18**	1,500	692	49	41	10	5.9	1.3	5.6 (2.3)
2-F			**19**				16				0.70	
2-Cl			**20**				95				12	
3-Cl			**21**				13				1.6	
3-PhO		**22**				1100				>200		
4-F			**23**	**24**			42	21			0.77	3.5 (1.2)
4-Cl	**2**	**3**	**6**	**8**	176	45	7	13	9.1	2.3	1.1	52 (5.0)
4-Br			**25**	**26**			10	19			1.2	4.8
4-CF_3			**27**				92				38	
4-NO_2		**28**	**29**			255	55			8.5	2.9	
4-CN			**30**	**31**			5	10			0.060	0.60
4-N_3			**32**				315				15	
4-$MeSO_2$	**33**				>10,000 (15%)				81			
4-MeS	**34**				>10,000 (30%)				200			
4-MeO		**35**				706				25		
4-Me		**36**				684				25		
4-*t*-Bu			**37**				2,200				84	
3,4-Cl_2			**38**	**39**			10	50			0.88	39
3-NO_2,4-Cl	**40**				45				9.0			
3,4-OCH_2O	**41**				2,660				>200			
F_5		**42**	**43**			26	8			3.5	1.1	

TABLE 3

Effect of 1-substituent of 4-i-propyl- and 4-t-butyl-bicycloorthocarboxylates and their 4-n-propyl analogs on their potency in inhibiting [^{35}S]TBPS binding to the human brain receptor and on their toxicity to mice

For conditions and terms see the text and Table 1. Toxicity data for compound **47** from Ref. 2. Data for the 4-*n*-Pr analogs are (compound no., 1-substituent, IC_{50}, LD_{50}): **69**, 1-*n*-Bu 2000, 36; **70**, 1-(1-bicyclo[2.2.1]heptanyl), >10,000 (15%), 210; **71**, 1-(2-bicyclo[2.2.1]heptanyl), 19,000, 155; **72**, 1-(cyclohex-3-enyl) 6300, 200; **73**, 1-(5-bromo-2-furyl) 151, 7.6. Comparable data in the 4-*c*-Hex series are: **13**, 1-*c*-Hex 217, 42; **74**, 1-*c*-Hept 1360, >100. PB altered the toxicity of compounds **46–74** by less than 2-fold.

1-Substituent	Compound no.		Receptor IC_{50}		LD_{50}	
	4-*i*-Pr	4-*t*-Bu	4-*i*-Pr	4-*t*-Bu	4-*i*-Pr	4-*t*-Bu
			nM		*mg/kg*	
H		**46**		7,200		9.5
Me		**47**		23,000		6.0
Et		**48**		10^5		145
n-Pr		**49**		1,150		13
i-Pr		**50**		> 10,000 (0%)		>500
n-Bu		**51**		118		1.4
s-Bu	**52**		>10^5 (10%)		>500	
n-Pen		**53**		27		6.6
neo-Pen	**54**		>10^5 (15%)		>200	
n-Hex	**55**		1,700		>200	
c-Pr	**56**		>10,000 (15%)		450	
c-Bu	**57**		>10^5 (30%)		375	
c-Pen	**58**		>10,000 (15%)		150	
c-Hex	**59**	**60**	4,900	970	180	25
c-Hept	**61**	**62**	8,800	1,285	>200	92
Vinyl	**63**		>10^5 (0%)		>150	
Ethynyl	**64**	**65**	2,900	480	2.0	0.43
1-BrEt	**66**		>10^5 (10%)		140	
1,2-Br_2Et	**67**		>10^5 (0%)		60	
Benzyl		**68**		7,300		>150

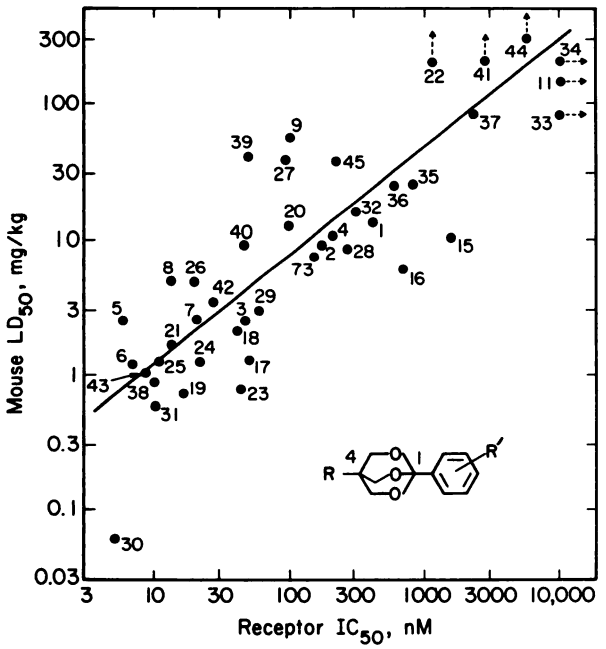

FIG. 3. *Relation of potency in inhibiting [^{35}S]TBPS binding to the human brain receptor and toxicity to mice of 4-substituted 1-(substituted-phenyl)-bicycloorthocarboxylates (**1–9, 11, 15–45**) and a 1-(5-bromo-2-furyl) analog (**73**)*

Assay conditions are given in Table 1. Compound numbers refer to structures given in Tables 1–3. The receptor potency of **11** is plotted as >10,000 nM. LD_{50} data are without PB synergism except for compounds **4, 7, 8, 18,** and **24**. Compound **10** was not sufficiently soluble for LD_{50} determination. Arrow designations and correlation coefficient are defined in Fig. 1. $r = 0.616$ ($n = 36$).

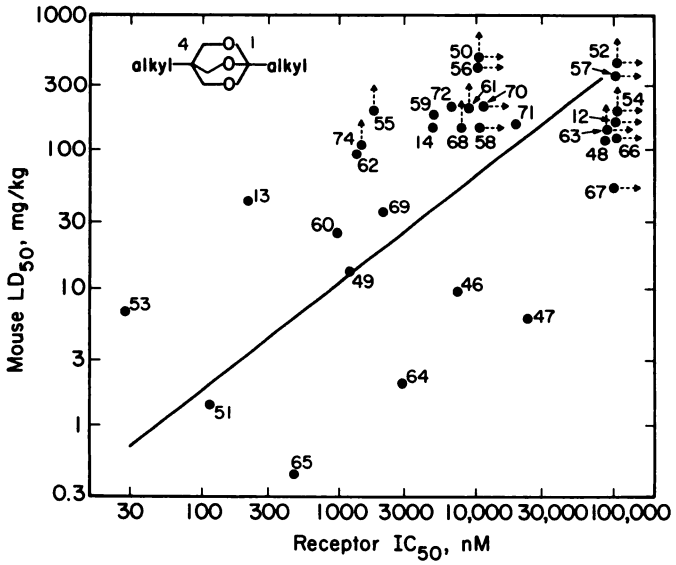

FIG. 4. *Relation of potency in inhibiting [^{35}S]TBPS binding to the human brain receptor and toxicity to mice of 1,4-bis-alkyl-bicycloortho-carboxylates (**12, 13, 47–72, 74**) and analogs with a 4-phenyl (**14**) or 1-hydrogen (**46**) substituent*

Assay conditions are given in Table 1. Compound numbers refer to structures given in Tables 1 and 3. LD_{50} data are without PB synergism. Arrow designations and correlation coefficient are defined in Fig. 1. $r = 0.331$ ($n = 16$).

TABLE 4

Effect of microsomal oxidases on potency of three bicycloorthocarboxylates in decreasing [^{35}S]TBPS binding

The receptor assay was identical to that in Table 1 in 1-ml volume but involving fortification with mouse liver microsomes (25 μg of protein, noninduced) and NADPH (2 μmol for the oxidase system and 0 μmol for the control).

Compound[a]	Compound no.	nM[a]	Inhibition, %[a]	
			Control	Oxidase
t-Bu-C$(CH_2O)_3$C-PhCl-4	**6**	7	29 ± 1	34 ± 1
c-Hex-C$(CH_2O)_3$C-PhCl-4	**8**	13	50 ± 9	11 ± 7
c-Hex-C$(CH_2O)_3$C-PhBr-4	**26**	19	41 ± 1	7 ± 1

[a] Chlorinated cyclodienes examined as internal standards showed the expected (10) activation of isodrin (300 nM, 38 ± 3% inhibition for the control and 55 ± 1% inhibition for the oxidase) with little or no effect on endrin (100 nM, 49 ± 4% control, 47 ± 1% oxidase) or endosulfan sulfate (30 nM, 32 ± 3% control, 29 ± 1% oxidase).

RESULTS

Action of bicycloorthocarboxylates as GABA antagonists and $GABA_A$-receptor antagonists. Bicycloorthocarboxylate **25** at 5 μM antagonizes GABA-mediated relaxation events in the cockroach coxal depressor muscle causing a 90–95% reduction in the amplitude of the relaxations within 5 min of application; the effect is reversible with an 8-min saline wash (Fig. 1b). PTX causes a stronger effect than **25** at 5 μM, completely blocking the GABA response and totally eliminating the relaxations within 2 min of treatment; a longer wash is also necessary (13–15 min) to achieve recovery of GABA transmission (Fig. 1a).

The bicycloorthocarboxylate radioligand [^{3}H]TBOB behaves identically to the known $GABA_A$ receptor antagonist radioligand [^{35}S]TBPS in its sensitivity to inhibition by GABA (IC_{50} 1.5–1.6 μM), taurine (IC_{50} 1.5–1.7 mM), and glycine (18–22% inhibition at 10 mM) when studied in human brain P_2 membranes.

Relation of bicyclophosphate structure to potency in decreasing [^{35}S]TBPS binding and in causing toxicity to mice. The bicyclophosphate series serves as a useful model in evaluating a possible relation between receptor potency and toxicity. IC_{50} values in this series are as follows (nM or % inhibition at highest concentration tested): 4-Me 10^5 (40%), 4-Et 5900, 4-*n*-Pr 1100, 4-*i*-Pr 480, 4-*n*-Bu 5700, 4-*t*-Bu (TBPO) 170, 4-*n*-Pen 10^5, 4-*n*-Hex $>10^5$ (25%), and 4-Ph 2000. The IC_{50} for TBPS is 62 nM under the same assay conditions. There is a remarkably good correlation ($n = 0.892$) between potency at the receptor and toxicity considering that the receptor assays are made with a human brain preparation and the toxicity determinations with mice (Fig. 2). The potency order conferred by the 4-substituent is the same in both tests, i.e., *t*-Bu > *i*-Pr > *n*-Pr > Ph, *n*-Bu or Et > *n*-Pen or Me > *n*-Hex.

Relation of bicycloorthocarboxylate structure to potency in decreasing [^{35}S]TBPS binding. Branched-chain alkyl and cycloalkyl groups are the most effective 4-substituents in the 1-(4-ClPh) series (Table 1). Thus, in comparing compounds **1–8**, *i*-Pr, *s*-Bu, *t*-Bu, *c*-Pen, and *c*-Hex are much more effective than Et, *n*-Pr, and *n*-Bu. Less effective 4-substituents are Ph, 4-MePh, and NO_2 (**9–**

TABLE 5

Effect of phenobarbital and two benzodiazepines on the toxicity of three bicycloorthocarboxylates to mice

Protective factors are given as the increase in LD_{50} on pretreatment with sodium phenobarbital at 100 mg/kg or a benzodiazepine at 10 mg/kg administered i.p. 5 and 15 min, respectively, before the convulsant. LD_{50} values for the bicycloorthocarboxylates alone are given in Tables 2 and 3.

Structure[a]	Compound designation	Protective factor[a]		
		Phenobarbital	Diazepam	Bromazepam
t-Bu-C($CH_2O)_3$C-PhBr-4	**25**	1.9	1.8	2.6
c-Hex-C($CH_2O)_3$C-PhBr-4	**26**	9.0	5.8	7.0
t-Bu-C($CH_2O)_3$C-C≡CH	**65**	3.4	3.1	3.4

[a] The comparison compound TBPO gives protective factors of 3.3, 1.7, and 2.6 with phenobarbital, diazepam, and bromazepam, respectively.

11). These relationships are supported by the 4-*c*-Hex > 4-Ph > 4-Et activity sequence in the 1-*c*-Hex series (**12–14**) (Table 1), the 4-*t*-Bu ≧ 4-*c*-Hex > 4-*i*-Pr > 4-*n*-Pr > 4-Ph sequence for the substituted orthobenzoates (Table 2), and the 4-*t*-Bu > 4-*i*-Pr > 4-*n*-Pr sequence when the 1-substituent is alkyl or ethynyl (Table 3).

Substituents on the 1-Ph group have a large influence on the potency in decreasing [^{35}S]TBPS binding (Table 2). Halogen and CN substituents generally increase the displacement potency relative to H whereas MeO and Me have little effect and N_3, $MeSO_2$, MeS, *t*-Bu, OCH_2O, and PhO confer weaker inhibitory activity. The most potent compounds (IC_{50} ≦ 10 nM) are generally in the 4-*t*-Bu series and have 4-ClPh (**5, 6**), 4-BrPh (**25**), 4-CNPh (**30, 31**), 3,4-Cl_2Ph (**38**), or F_5Ph (**43**) substituents in the 1-position (Tables 1 and 2).

The size of the 1-substituent in the *n*-alkyl series greatly influences effects on [^{35}S]TBPS binding, with a potency sequence of H > Me > Et < *n*-Pr < *n*-Bu < *n*-Pen, indicating that inhibitor potency is better with small and large than with intermediate-sized groups (Table 3). In contrast to the 4-position, branching at the α-carbon of the 1-position reduces the effectiveness for displacement, i.e., *i*-Pr < *n*-Pr and *c*-Hex < *n*-Hex. In the 1-cycloalkyl series, the optimal ring size is *c*-Hex > *c*-Hept > *c*-Pen, *c*-Bu, or *c*-Pr. 1-Ethynyl (**64, 65**) confers higher potency than vinyl, 1-BrEt, 1,2-Br_2Et, or benzyl (**63, 66–68**). A variety of other 1-substituents (**69–74**) confer displacement activity but not of unusually high magnitude (Table 3).

Relation of bicycloorthocarboxylate structure to mouse toxicity. Several of the new compounds are highly toxic to mice (Tables 1–3). The most toxic is 4-*t*-Bu-1-(4-CNPh)-bicycloorthocarboxylate (**30**) which is similar in potency to TBPO (**2**), i.e., i.p. LD_{50} ~0.05 mg/kg. Other highly toxic 4-*t*-Bu-bicycloorthobenzoates (LD_{50} < 1 mg/kg) are those with 2-F (**19**), 4-F (**23**) and 3,4-Cl_2 (**38**) substituents. The 4-*t*-Bu-1-ethynyl compound (**65**) is the most toxic bicycloorthocarboxylate containing only carbon, hydrogen, and oxygen.

Relation of the potency of bicycloorthocarboxylates for effects on [^{35}S]TBPS binding to their mouse toxicity. Two types of correlations are evident. First, bicycloorthocarboxylates inactive in decreasing [^{35}S]TBPS binding are generally of low toxicity to mice and potent inhibitors are usually of high toxicity (Figs. 3 and 4, Tables 1–3). Second, 4-alkyl-1-(substituted-phenyl) derivatives are usually more potent inhibitors relative to their toxicity (Fig. 3) than are the corresponding 1,4-*bis*-alkyl compounds (Fig. 4). This division into two classes is provisional because some compounds fall into overlapping regions, e.g., 1-Ph compounds **15, 16**, and **23** and 1-alkyl compounds **13** and **53**.

Metabolic detoxification and activation. All of the bicycloorthocarboxylates were tested for PB synergism to evaluate their ease of oxidative detoxification. Only five of the compounds show a synergism factor of greater than 2-fold, i.e., 1-(4-ClPh) compounds with 4-*n*-Bu (**4**), 4-*c*-Pen (**7**), and 4-*c*-Hex (**8**) substituents, the 1-Ph-4-*c*-Hex analog (**18**), and the 1-(4-FPh)-4-*c*-Hex compound (**24**), with the largest ratio being 10 for compound **8** (Tables 1–3). The coupled receptor-microsomal oxidase assay, used with only three of the bicycloorthocarboxylates, shows a small effect of the oxidase on the potency of 4-*t*-Bu compound **6** and distinct detoxification of 4-*c*-Hex derivatives **8** and **26** (Table 4).

Symptomology and cumulative effects. Compounds **25, 26**, and **65**, representing three types of bicycloorthocarboxylates, were examined in greater detail. Their poisoning signs on i.p. administration are similar to those of TBPO involving sequential sedation for 2–10 min, spasms characterized by nonperiodic alternate contraction and relaxation of muscles and raising of the tail for 5–20 min, and convulsions with severe flexion jerks of the legs for 5–30 min before death or progressive improvement via the sedation phase. The action of **65** is initiated and proceeds most rapidly and of **26** most slowly. Compound **26** also differs from **25** and **65** in the cumulative effects of hourly treatments each of which is equivalent to half the single dose LD_{50} value; mice survive an average of 3.7 such treatments with **26** *versus* 6–7 treatments with **25** and **65**.

Effect of phenobarbital and benzodiazepines on the toxicity of bicycloorthocarboxylates to mice. The toxicity of the three bicycloorthocarboxylates examined is decreased on pretreatment with phenobarbital and two benzodiazepines with a greater protective effect for **26** than for **25** or **65**; similar protective effects are noted against TBPO toxicity (Table 5). Protection against poisoning by **25** under the standard conditions (Table 5) is superior for bromazepam as compared to clonazepam, diazepam, fletazepam, flurazepam, medazepam, and nitrazepam.

DISCUSSION

Bicycloorthocarboxylate [^{3}H]TBOB and the known GABA$_A$ receptor antagonist [^{35}S]TBPS (8) have a com-

mon binding site, and the binding of these radioligands is similarly influenced by various GABA mimetics and antagonists in rat brain membranes (13) as confirmed here by GABA, taurine, and glycine in human brain membranes. On this basis, and contrary to expectations (2), trioxabicyclooctanes with small and large substituents at the 1-position probably act at the same receptor. Structural features of the human brain trioxabicyclooctane receptor can, therefore, be partially defined by structure-activity relationships developed in the present study for inhibition of [^{35}S]TBPS binding by various bicycloorthocarboxylates in comparison with the bicyclophosphates.

A representative bicycloorthocarboxylate (the 4-*t*-Bu-1-(4-BrPh) analog) antagonizes GABA-mediated relaxation at a functional insect nerve-muscle synapse. The antagonism is similar to that caused by PTX, which is known to specifically block conductance at the GABA-activated chloride ionophore (26), indicating that the bicycloorthocarboxylates also cause GABA antagonism through blockade of the chloride ionophore.

The potency of bicycloorthocarboxylates in decreasing [^{35}S]TBPS binding is strongly influenced by the nature of the substituents at both the 1- and 4-positions indicating that each terminal region of the molecule is critical in binding. The 4-substituent is optimally a C_4 to C_6 branched-chain alkyl or cycloalkyl group such as *t*-Bu, *s*-Bu, or *c*-Hex in both the bicycloorthocarboxylate (1, 2) and bicyclophosphate (1, 2, 20, 27) series. These size and steric features apparently confer appropriate hydrophobic interaction of the 4-substituent at one region of the binding site. The 1-substituent is optimally a Ph moiety with one or more electron-withdrawing groups, i.e., activity is generally increased by halogens and CN. Fluoro is a better substituent than Cl in the 2-position of the Ph group and Cl than F in the 4-position indicating that steric features in the ring may play an intramolecular role. Alternatives for the substituted Ph group in the 1-position are ethynyl, *n*-Pr, *n*-Bu, *n*-Pen, *c*-Hex, or *c*-Hept, but except for *n*-Bu and *n*-Pen, they are much less effective than substituted Ph at this second hydrophobic binding site.

Correlations between potency at the TBPS receptor and toxicity indicate that the 1-alkyl- and 1-ethynyl-bicycloorthocarboxylates and the bicyclophosphorus esters may act somewhat differently than the 1-(substituted phenyl)-bicycloorthocarboxylates such as TBOB. In addition, although TBPS and TBOB bind at the same hydrophobic receptor site of the chloride channel, there are two important differences in their interactions with this site, i.e., [^{35}S]TBPS binding is dependent on temperature and the modulation of its binding by GABA is dependent on salt concentration whereas [^{3}H]TBOB binding is independent of both temperature and a salt concentration effect on GABA modulation (13). These findings suggest that there are substituent-dependent differences in the degree to which receptor binding is related to a block in GABA-mediated neurotransmission.

The peak brain concentration is reached within 1 hr after i.p. treatment of mice with TBPO and its 4-methyl analog without significant localization in the cerebellum or other brain regions (28). The concentration in the brain relative to that in the blood is much higher for the *t*-butyl- than the methyl-bicyclophosphate (28). Accessibility to the brain tissue is, therefore, one factor determining the relative toxicity of the bicyclophosphates and by analogy probably of the bicycloorthocarboxylates as well.

The bicycloorthocarboxylates examined all appear to act directly rather than following metabolic activation. Their mouse toxicity is not limited by PB-sensitive oxidative detoxification, with a few exceptions involving 4-halophenyl, 4-phenyl, 1-*n*-Bu, 1-*c*-Pen, and 1-*c*-Hex substituents. Although not specifically examined, metabolic detoxification may involve opening of the bicyclic structure via OCH_2 hydroxylation (28) or oxidation of aryl or alkyl substituents.

This study led to the recognition of the most potent known $GABA_A$ receptor antagonists based on their inhibitory activity in the TBPS binding assay. Thus, the potency of TBOB, which is similar to that of TBPO and TBPS (5, 13), is increased 5–10-fold on introducing 4-cyano, 4-bromo, 4-chloro, 3,4-dichloro, and pentafluoro substituents in the phenyl group. The toxicity of the bicycloorthocarboxylates is alleviated by phenobarbital and some benzodiazepines in the same manner as with the bicyclophosphate $GABA_A$ receptor antagonists. Bicycloorthocarboxylates such as the highly potent 4-*t*-Bu-1-(4-CNPh) analog should be useful probes in further studies on the GABA receptor-ionophore complex.

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