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meta- and *para*-Isothiocyanato-*t*-butylbicycloorthobenzoate: Irreversible ligands of the γ-Aminobutyric Acid-Regulated Chloride Ionophore

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SUMMARY

The *meta*- and *para*-isothiocyanato derivatives of *t*-butylbicycloorthobenzoate (TBOB) were synthesized by catalytic reduction of the corresponding nitro compounds, followed by treatment with thiophosgene. *p*-NCS-TBOB (2) inhibited the binding of both [^{3}H]TBOB and [^{35}S]*t*-butylbicyclophosphorothionate (TBPS) with potencies (IC_{50} of 61 and 23 nM, respectively) similar to the parent compound. In contrast, the *meta* derivative (*m*-NCS-TBOB, 1) was more than 1 order of magnitude less potent (IC_{50} of 1588 and 149 nM, respectively). The IC_{50} values for both 1 and 2 were strongly dependent on the tissue concentration, in a manner characteristic of irreversible inhibitors. Moreover, preincubation of tissue with these compounds, followed by extensive washing, resulted in a concentration-dependent reduction in the number of [^{35}S]TBPS binding sites and in the apparent affinity of this radioligand. Similar effects were not observed in tissues treated in identical fashion with either TBOB or picrotoxin. Preincubation with *p*-NCS-TBOB at concentrations that significantly inhibit [^{35}S]TBPS or [^{3}H]TBOB binding did not affect radioligand binding to either benzodiazepine or γ-aminobutyric acid receptors. These findings suggest that *m*- and *p*-NCS-TBOB bind irreversibly to sites labeled by cage convulsants such as TBOB and TBPS, which are on or near GABA-gated chloride channels. *p*-NCS-TBOB should prove useful in determining the molecular characteristics of the benzodiazepine receptor-coupled GABA-gated chloride ionophore.

During the past decade, photoaffinity labeling and acylating agents have been important tools for the characterization of both benzodiazepine (1–5) and $GABA_A$ receptors (6, 7), constituents of the benzodiazepine/GABA receptor chloride channel complex ("supramolecular complex"). However, a specific, high affinity irreversible ligand of the benzodiazepine receptor-coupled, GABA-gated chloride ionophore has not been developed. Electrophysiological and biochemical experiments suggest that sites on or near this chloride ionophore may be the locus of action of substances such as "cage" convulsants like picrotoxin and TBPS (8–12), barbiturates (13–16), and polychlorocycloalkane insecticides (12, 17). the apparent lability of this site in solution (18, 19), coupled with the lack of a suitable irreversible ligand, has hampered both a detailed biochemical characterization of the chloride ionophore and resolution of its relationship with the other constituents of the supramolecular complex.

We now report the synthesis and characterization of the *metal* and *para* isothiocyanato derivatives of the cage convulsant TBOB. These compounds are specific, site-directed, acylating agents for sites on or near GABA-gated chloride channels labeled by cage convulsants such as [^{3}H]TBOB and [^{35}S]TBPS and, as such, are potentially important tools for characterization of the GABA-gated chloride ionophore.

Materials and Methods

Tissue preparation. Adult male Sprague-Dawley rats (150–200 g; Taconic Farms, Germantown, NY) were killed by decapitation. The cerebral cortices were carefully dissected, weighed, and disrupted with a Brinkman Polytron (setting 5–6) for 15 sec in 50 volumes of ice-cold 50 mM phosphate buffer (pH 7.4) containing 100 mM NaCl. This homogenate was centrifuged at 20,000 × *g* (4°) for 20 min. The resulting pellet was resuspended in 50 volumes of buffer using the Polytron (5 sec) and recentrifuged. This procedure was repeated for a total of six washes. For use in GABA and benzodiazepine binding assays, the last wash was with chloride-free buffer. Pellets were resuspended in 20–35 volumes of phosphate buffer (no NaCl, 4°) for [^{35}S]TBPS, [^{3}H]TBOB, [^{3}H]FNZ, and [^{3}H]DMCM binding assays. Pellets were resuspended in 10 volumes of buffer for [^{3}H]muscimol and [^{3}H]SR95531 assays.

[^{35}S]TBPS BINDING. Binding assays were carried out in a total volume of 0.5 ml, containing 50 mM phosphate buffer, 200 mM NaCl, 0.2–0.6 mg of protein, and 2–4 nM radioligand. In tissue dilution

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ABBREVIATIONS: GABA, γ-aminobutyric acid; TBPS, *t*-butylbicyclophosphorothionate; TBOB, *t*-butylbicycloorthobenzoate; FNZ, flunitrazepam; DHDMBB, 2,2-bis(hydroxymethyl)-3,3-dimethylbutyl-*p*-isothiocyanatobenzoate; DMCM, 6,7-dimethoxy-4-ethyl-3-carbomethoxy-β-carboline

experiments requiring a 1.0-ml incubation volume, 0.1-0.2 mg of protein/assay was used. The suspensions were incubated at room temperature for at least 90 min. Incubations were terminated with two 5-ml washes of buffer through Whatman GF/B filters, using a Brandel M-24R filtering manifold (Brandel Instruments, Gaithersburg, MD). Radioactivity retained on the filters was counted in a Beckman LS 5801 liquid scintillation counter. Nonspecific binding was defined in the presence of 20 μM picrotoxinin.

[^{3}H]TBOB binding. Binding assays were carried out as described above, except that the incubation period was 30 min and the radioligand concentration was 4–6 nM. The radioligand (supplied as a toluene solution) was diluted with 10 volumes of ethanol followed by the appropriate volume of buffer to insure a homogeneous solution. Nonspecific binding was defined as above.

[^{3}H]Muscimol and [^{3}H]SR95531 binding. [^{3}H]Muscimol and [^{3}H]SR95531 binding was assayed at radioligand concentrations of 2 and 40 nM and 7–15 and 25–37 nM, respectively. GABA (1 mM) and SR95531 (100 μM) were used to define the nonspecific binding. Assays were carried out in 0.5-ml volumes containing 50 mM phosphate buffer and 0.4-0.6 mg of protein. Incubations at 4° were terminated after 30 min by two 5-ml washes with cold (4°) buffer.

[^{3}H]FNZ and [^{3}H]DMCM binding. [^{3}H]FNZ and [^{3}H]DMCM binding was assayed in 1.0 ml containing 50 mM phosphate buffer, 0.2–0.4 nM radioligand, and 0.1–0.15 mg of protein; nonspecific binding was defined by 10 μM flurazepam. Incubations (4°) were terminated after 60 min with two 5-ml washes with 4° buffer.

Tissue preincubation. Tissue was prepared as described above, but only two washes were performed. Preincubation with the drug (acylator, reversible ligand, or vehicle) was performed under the assay conditions, i.e., using 50 mM phosphate buffer containing 200 mM sodium chloride at a tissue concentration of 1.3–2.0 mg of protein/ml. The drug (in a 10 μM solution) was added to the tissue suspension and maintained at 4° for the required preincubation period. The reaction was terminated by dilution with 20–25 volumes of 50 mM phosphate buffer and centrifugation at 20,000 × *g* for 20 min. The pellet was resuspended in an equal or greater volume of ice-cold buffer and maintained at 4° for 5 min before centrifugation. This procedure was repeated for a total of five washes. The resulting pellet was resuspended in 20 volumes of ice-cold 50 mM phosphate buffer for a tissue concentration of 0.15–0.75 mg of protein/assay.

Protein determination. Protein content of the tissue samples was determined by the Miller (20) modification of the method of Lowry *et al.* (21), using bovine serum albumin as the standard.

Molecular modeling. The molecular dimensions of TBOB and its derivatives were estimated by measuring the distance from the center-line axis of each compound to the outside edge of the Van der Waals radius of the most distant atom. For TBOB, the distance of one of the methyl hydrogens of the *t*-butyl group to the center-line axis was found to be 3.21Å. For the isothiocyanate derivatives, the distance from the sulfur atoms was used; for **2** this distance was determined to be 3.38Å, whereas for **1** a distance range of 4.89–6.76 Å was found. The effective diameters were taken as the sum of the radii; for the isothiocyanate derivatives the "minimum diameter" was taken as the sum of the distance from the sulfur atom and of the *t*-butyl hydrogen atom to the center-line axis. Computer-assisted molecular modeling was performed using SYBYL software (version 5.1; TRIPOS Associates, a subdivision of Evans and Sutherland, St. Louis, MO).

Materials. The *meta* (**1**) and *para* (**2**) isothiocyanato derivatives of TBOB were prepared by reduction of the appropriate nitro derivatives to their amino precursors, followed by treatment of each with thiophosgene. DHDMBB (**3**) (an analog of **2** lacking the cage structure) was a hydrolytic by-product in the synthesis and purification of **2**. The chemical structures were confirmed by spectroscopy (nuclear magnetic resonance, infrared, mass) and the chemical purity was ascertained by thin layer and gas chromatography. The compounds were stable when stores as solids at −20°. Ethanol solutions (1 μM) were stable for several months when stored at −20°. Experimental details of the syntheses will be described in a subsequent publication.

[^{35}S]TBPS (specific activity, 60–100 Ci/mmol), [^{3}H]FNZ (specific activity, 80–90 Ci/mmol), [^{3}H]DMCM (specific activity, 80–90 Ci/mmol), [^{3}H]SR95531 (specific activity, 45.7 Ci/mmol), and [^{3}H]muscimol (specific activity, 20 Ci/mmol) were purchased from Dupont-New England Nuclear (Boston, MA). [^{3}H]TBOB (specific activity, 45.8 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). Picrotoxinin and GABA were purchased from Sigma Chemical Co. (St. Louis, MO), and flurazepam was a gift from Hoffmann LaRoche (Nutley, NJ).

Results

Both *m*- and *p*-NCS-TBOB (**1** and **2**; Fig. 1) inhibited the binding of [^{3}H]TBOB and [^{35}S]TBPS to well washed membranes from rat cerebral cortex. At protein concentrations of 0.4–0.6 mg/assay, the IC_{50} values of *p*-NCS-TBOB (**2**) to inhibit [^{3}H]TBOB and [^{35}S]TBPS binding (61±7 and 23±1 nM, respectively) were similar to those of TBOB (37±3 and 26±2 nM, respectively), whereas the *m*- derivative (**1**) was more than 1 order of magnitude less potent (IC_{50} of 1588±50 and 149±18 nM, respectively) (Table 1). An analog of **2** lacking the cage structure, *p*-NCS-DHDMBB (**3**), inhibited [^{35}S]TBPS binding with an IC_{50} of >20 μM (Table 1).

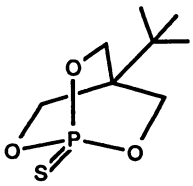
The effective molecular diameter for TBOB was found to 6.42Å, whereas the diameters of **1** and **2** were 8.09–9.96 Å and 6.73 Å, respectively.

Preincubation of membrane suspensions with either *m*- or *p*-NCS-TBOB followed by extensive washing resulted in a persistent inhibition of both [^{35}S]TBPS (Table 2; Fig. 2) and [^{3}H]TBOB (data not shown) binding. In contrast, comparable treatment of tissue with either 350 nM TBOB or picrotoxinin (Table 2) did not affect either [^{35}S]TBPS or [^{3}H]TBOB binding. Similarly, preincubation of membranes with **2** followed by extensive washing did not affect radioligand binding to either benzodiazepine or GABA receptors (fig. 3 legend). The extent of apparent acylation (i.e., the inhibition of radioligand binding by preincubation with the inhibitors followed by extensive washing) was dependent on both the preincubation time and the inhibitor concentration (Figs. 2–4). Scatchard analysis of radioligand binding in extensively washed membranes preincubated with *m*- or *p*-NCS-TBOB revealed a concentration-dependent reduction in both the apparent number of [^{35}S]TBPS binding sites and the apparent affinity of this radioligand (Fig. 4; Table 2). The IC_{50} values for inhibition of [^{3}H]TBOB binding by *p*-NCS-TBOB were found to vary with tissue concentration; the data fitted well with an exponential curve (Fig. 5). In contrast, inhibition of [^{35}S]TBPS binding by TBPS varied only slightly (in a linear fashion) with tissue concentration (Fig. 5).

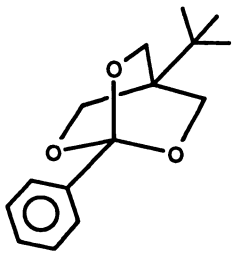
Discussion

The availability of compounds that irreversibly label benzodiazepine and GABA receptors has resulted in significant insights into the structure and organization of the supramolecular complex. However, the lack of specific irreversible ligands for the GABA-gated chloride ionophore, together with its apparent lability in solution (18, 19), has impeded studies of its molecular architecture and relationship to benzodiazepine and GABA receptors.

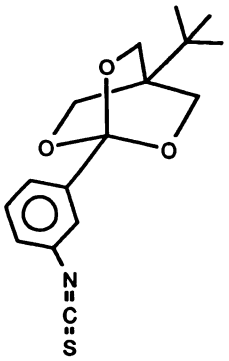
Because cage convulsants like TBPS, TBOB, and picrotoxin



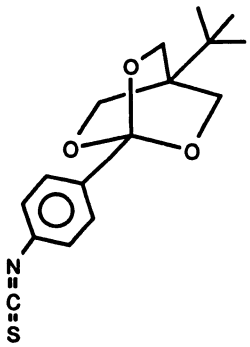
TBPS



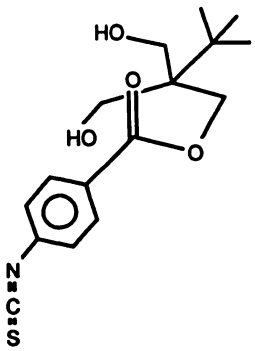
TBOB



m-NCS-TBOB
1



p-NCS-TBOB
2



p-NCS-DHMDMBB
3

Fig. 1. Structures of cage convulsants, isothiocyanato derivatives, and analogs.

TABLE 1

Inhibition of [^{35}S]TBPS and [^{3}H]TBOB binding to rat cortical membranes

Membranes were prepared and radioligand assays were performed as described in Materials and Methods. Values represent the mean ± standard error of three experiments or more (indicated in parentheses) using at least six inhibitor concentrations. The radioligand concentration was 5 nM for [^{3}H]TBOB and 3 nM for [^{35}S]TBPS. The tissue concentration was 0.4–0.6 mg of protein/assay.

Inhibitor	IC_{50} for Inhibition of Binding of	
	[^{3}H]TBOB	[^{35}S]TBPS
TBPS	ND[a]	38 ± 3 (6)
TBOB	37 ± 3	26 ± 2
m-NCS-TBOB (1)	1,588 ± 50	149 ± 18
p-NCS-TBOB (2)	61 ± 7 (4)	23 ± 1
p-NCS-DHDMBB (3)	ND	21,000 ± 876

[a] ND, not determined.

TABLE 2

Effect of preincubation on [^{35}S]TBPS binding

Membranes were prepared and radioligand binding was performed as described in Materials and Methods. Values represent the mean of three experiments or more (indicated in parentheses) with at least six inhibitor concentrations; the desired concentrations of [^{35}S]TBPS were achieved by adding TBPS (up to 1280 nM) to 2–4 nM [^{35}S]TBPS. The tissue concentration was 0.3–1.2 mg of protein/ml. K_d and B_{max} were calculated from saturation isotherms using GraphPad (ISI Software) or Lundon-1 (Lundon Software). K_{d^*} and B_{max^*}, are values obtained in preincubated tissue; K^*_d and B_{max^d} are values obtained in identically treated membranes preincubated with vehicle. Typical values for [^{35}S]TBPS binding are K_d = 70 ± 10 nM and B_{max} = 2.0 ± 0.2 pmol/mg of protein. Preincubation with 1 was for 30 min at 0°; preincubation with 2 was for 15 min at 0°.

Preincubation with (Ligand)	Concentration	K_{d^*}/K_{d^d}	B_{max^*}/B_{max^d}
	nM		
Picrotoxinin	350	0.97	0.93
TBOB	350	0.97	0.93
m-NCS-TBOB (1)	350	1.94	0.80
	700	2.27	0.59
	1400	2.91	0.38
p-NCS-TBOB (2)	25	1.03	0.95
	50	1.19	0.79
	100	1.60	0.75
	250	2.84	0.58 (6)
	500	3.63	0.30 (6)

are thought to bind to sites located in or near GABA-gated chloride channels (8–11), we embarked on a program to investigate the applicability of irreversible cage convulsants to the identification and characterization of binding sites located on or near GABA-gated chloride channels. Derivatives of the cage convulsant TBOB containing the acylating isothiocyanate moiety were selected as prototypes. The electrophilic isothiocyanate moiety on these compounds was expected to react irreversibly with bionucleophiles, particularly free amino groups on lysine, arginine, and histidine. A similar approach has been used to irreversibly acylate opiate (22), benzodiazepine (1, 2), and "peripheral" benzodiazepine receptors (23).

The effects of **1** and **2** on [^{35}S]TBPS and [^{3}H]TBOB binding were first examined under "equilibrium" binding conditions. The *p*-isothiocyanato derivative (**2**) was found to inhibit the binding of these radioligands with a potency comparable to that of the parent compound TBOB, whereas the *m*-isothiocyanato derivative (**1**) was more than 1 order of magnitude less potent (Table 1). At low receptor (tissue) concentrations, the potency of **2** to inhibit [^{3}H]TBOB binding increased, whereas at high receptor concentrations, it decreased dramatically. A curvilinear relationship between the IC_{50} of **2** and the receptor concentration was observed (Fig. 5), comparable to that reported by Lueddens *et al.* (23) for AHN 086, an irreversible

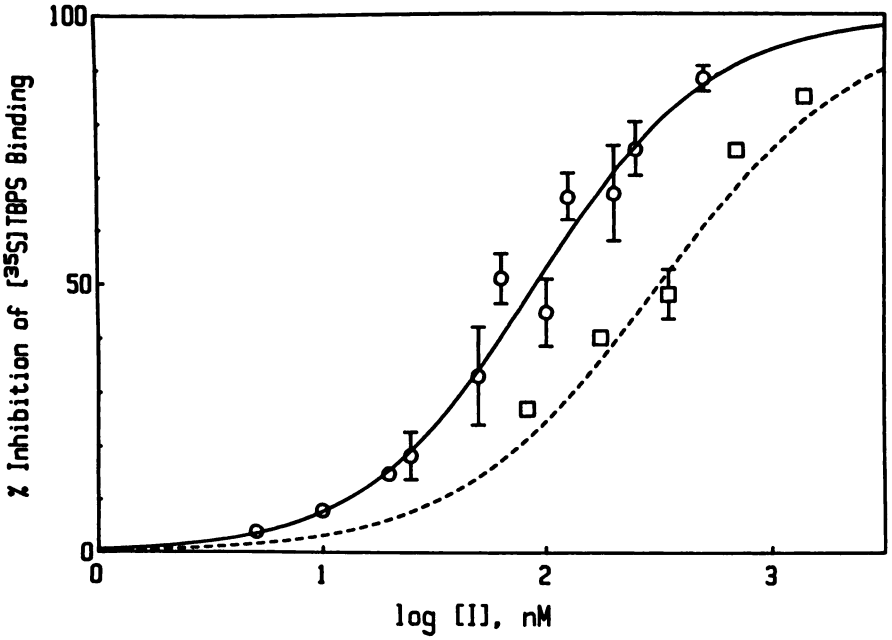


Fig. 2. Inhibition of [^{35}S]TBPS binding in tissues preincubated with acylating agents. Membranes were prepared as described in Materials and Methods. Tissues were preincubated with the indicated concentrations of **1** (for 30 min) or **2** (for 15 min) and extensively washed as described in Materials and Methods. Percent inhibition of [^{35}S]TBPS binding is defined as fmol bound in treated tissue/fmol bound in untreated (control) tissue × 100. Values represent the mean ± standard error of at least three experiments; values without standard error bars represent a single experiment. Concentrations were 2–4 nM for the radioligand and ○, 0.15–0.75 mg of protein/assay for the tissue. **1**; □, **2**. Essentially identical results were obtained when tissues were preincubated with **1** or **2** and [^{3}H]TBOB was used as the radioligand (data not shown).

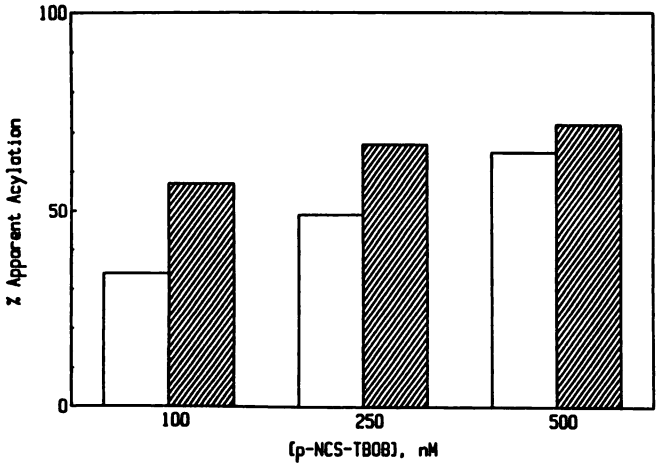


Fig. 3. Effect of time an acylator concentration on the extent of apparent acylation by **2**. Similar results were obtained for **1** (data not shown). For assay conditions see legend to Fig. 2. Percent apparent acylation is the percent inhibition of [^{35}S]TBPS binding (see legend to Fig. 2). The preincubation period was either 15 (□) or 30 (▨) min. Tissue preincubated with 250 nM **2** for 15 min exhibited [^{3}H]FNZ (0.2–0.4 nM), [^{3}H]DMCM (0.2–0.4), [^{3}H]muscimol (2 and 40 nm), and [^{3}H]SR95531 (7–37 nM) [^{3}H]FNZ, [^{3}H]DMCM, [^{3}H]muscimol, and [^{3}H]SR95531 binding that was not different from tissue treated with vehicle under the same conditions (data not shown).

inhibitor of peripheral benzodiazepine receptors. A similar relationship between the tissue concentration and the potency of **1** to inhibit [^{35}S]TBPS binding was observed (data not shown) whereas the expected linear relationship was obtained for TBPS (Fig. 5). These observations suggest that, at low tissue concentrations, **1** and **2** acylate the receptor, rendering it inaccessible to the radioligand. Thus, a more detailed biochemical characterization was performed, predominantly with **2**, to determine whether this compound was a specific, site-directed, acylator.

The observation that preincubation of rat cortical membranes with either **1**, or **2** produces an inhibition of both [^{3}H]TBOB and [^{35}S]TBPS binding that persists after extensive washing (Figs. 2–4; Table 2) strongly suggests that both compounds act as irreversible acylators. In contrast, no inhibition of radioligand binding was observed when tissues were preincubated and washed under identical conditions with reversible ligands like TBOB and picrotoxinin (Table 2). Furthermore, the extent of apparent acylation in tissues preincubated with either **1** or **2** was dependent both on the length of the preincubation period (Fig. 3) and concentration of the acylator (Figs. 2–4). The potency difference between **1** and **2** (Table 1) and the finding that **3** (a TBOB derivative containing an acylating moiety but lacking the cage structure) did not significantly inhibit radioligand binding (Table 1) suggest the observed effects on radioligand binding are due to a site-directed acylation of GABA-gated chloride channels rather than a nonspecific acylation due to the presence of the isothiocyanate moiety. Furthermore, the observation that radioligand binding to benzodiazepine or GABA receptors was not significantly affected (Fig. 3 legend) by preincubation with **2** further suggests a specific acylation of sites associated with the chloride ionophore.

Although the findings of Lawrence *et al.* (24) strongly suggest that [^{3}H]TBOB and [^{35}S]TBPS bind to the same site, TBOB, **1**, and **2** were significantly more potent as inhibitors of [^{35}S]TBPS than [^{3}H]TBOB binding (Table 1). Because [^{35}S]TBPS reaches equilibrium more slowly than [^{3}H]TBOB (10, 24, 25), the apparent higher potencies of these derivatives to inhibit [^{35}S]TBPS binding could be due to determination of the IC_{50} under nonequilibrium conditions. Increasing the incubation period to 180 min resulted in a higher B_{max} and lower K_d of [^{35}S]TBPS, suggesting that, under the conditions used in the present study, equilibrium was not achieved during the 90-min incubation period (Tables 1 and 3). This hypothesis was further supported by the observation that no inhibition of [^{35}S]TBPS binding was found when a protocol (25) was used in which tissues were preincubated with radioligand for 60 min, followed by a 90-min coincubation with 350 nM **1** (Table 3). This finding is consistent with the very low affinity of *m*-NCS-TBOB determined with [^{3}H]TBOB. Nonetheless, both 90- and 180-min coincubations with *m*-NCS-TBOB (**1**) reduced the B_{max} of [^{35}S]

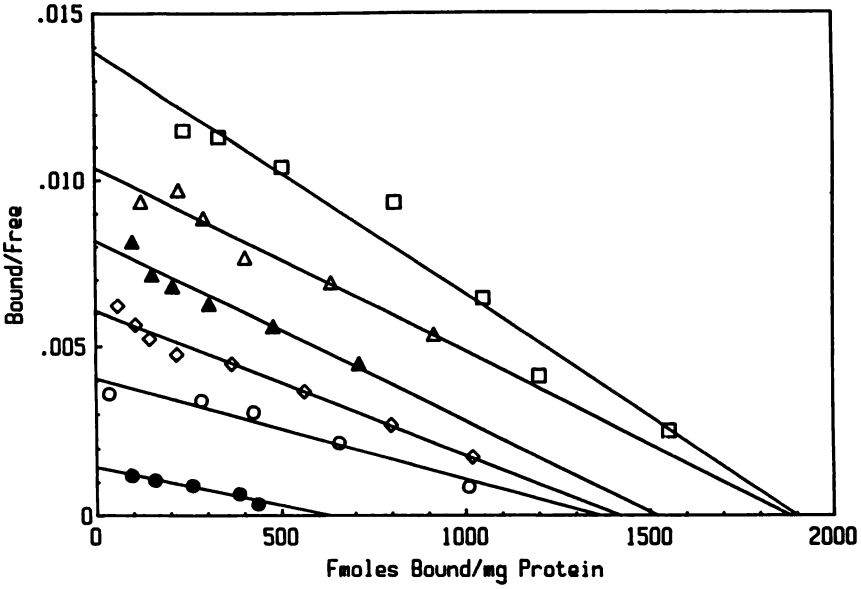


Fig. 4. Effect of preincubation with acylating agents on [^{35}S]TBPS binding. Membranes were prepared and radioligand binding was performed as described in Materials and Methods. Tissue suspensions were preincubated with the indicated concentrations of **2** for 15 min at 4°, then extensively washed as described in Materials and Methods. The desired concentration of [^{35}S]TBPS were achieved by adding TBPS 9–1280 nM) to 2–4 nM [^{35}S]TBPS. The tissue concentration was 0.7–1.5 mg of protein/ml. K_d and B_{max} were calculated from saturation isotherms using GraphPad (ISI Software) and Lundon-1 (Lundon Software). *Symbols* refer to values obtained for tissues preincubated with various concentrations of **2**: □, 0 nM (control); △, 25 nM; ▲, 50 nM; ◇, 100 nM; ○, 250 nM; ●, 500 nM.

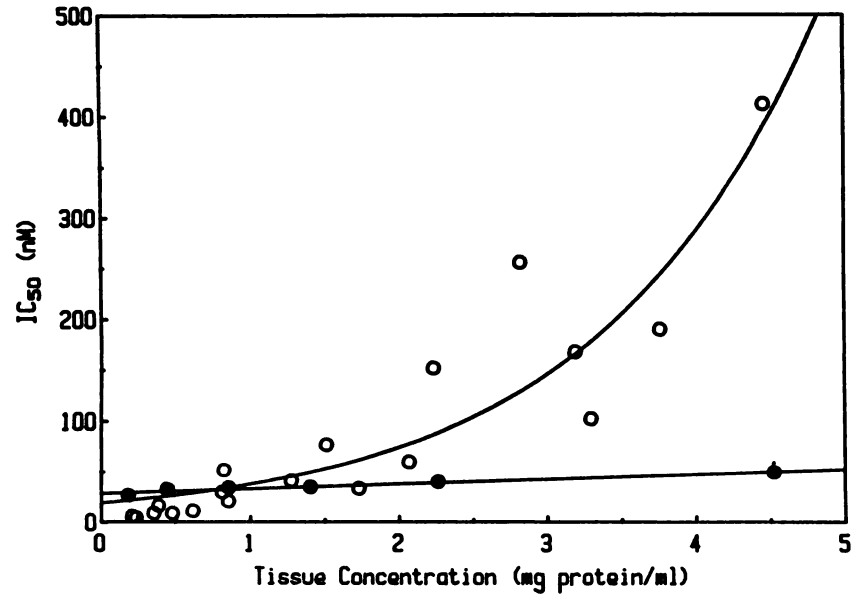


Fig. 5. Effect of tissue concentration on IC_{50}. Membranes were prepared and radioligand binding was performed as described in Materials and Methods. The IC_{50} for TBPS was determined (●) by inhibition of [^{35}S] TBPS binding; the desired concentrations were achieved by adding TBPS (up to 320 nM) to 3–4 nM [^{35}S]TBPS. The IC_{50} of **2** (○) was determined by inhibition of [^{3}H]TBOB (7–8 nM) binding by the addition of **2** (up to 2560 nM). For tissue concentrations <0.5 mg of protein/ml, 1-ml assay volumes were used. Each experiment had at least six inhibitor concentrations and each point was in duplicate. The IC_{50} values were calculated using GraphPad (ISI Software) and Lundon-1 (Lundon Software).

TABLE 3

Effect of incubation conditions on [^{35}S]TBPS binding

Membranes were prepared and radioligand binding was performed as described in Materials and Methods. Values represent single determinations with at least six concentrations of inhibitor in duplicate. All *r* values are >0.99. The radioligand concentration was 3.5 nM and the tissue concentration was 0.24 mg of protein/assay.

Conditions	K_d	B_{max}
	nM	*fmol/mg of protein*
TBPS		
90 min	38	660
180 min	25	800
TBPS + 350 nM *m*-NCS-TBOB (**1**)		
90 min	72	480
180 min	38	480
TBPS, 60 min, followed by 350 nM *m*-NCS-TBOB, 90 min	37	620

TBPS by 27% and 40%, respectively, suggesting that **1** binds irreversibly to the [^{35}S]TBPS binding site.

Structure-activity studies of a series of TBOB derivatives and analogs (26) suggest that an increase in the polarization of the benzylic carbon-carbon bond (i.e., the bond between C-1 and the phenyl group) to increase the δ^+ (partial positive charge) at the benzylic carbon C-1 and the partial negative charge δ^- of the C-1 phenly results in increased potency. Because an isothiocyanato substituent is inductively electron withdrawing, substitution of TBOB with an isothiocyanato group (particularly in the *meta* position) should lead to a compound with potency greater than or equal to that of the parent compound. The similar potencies of **2** and TBOB are consistent with this prediction (Table 3). However, the observation that the potency of **1** is more than 1 order of magnitude lower than that of either **2** or TBOB suggests that a serious steric constraint may be present. Because electrophysiological and biochemical findings (8, 9, 11) have suggested that cage convulsants like TBOB and TBPS bind inside the lumen of the chloride channel, their affinities may depend on the molecular diameter of the ligand relative to the channel pore size. Computer-aided molecular modeling shows that, whereas placement of an isothiocyanate group in the *para* position (**2**) alters

the minimum diameter of the cylinder-like TBOB molecule (Fig. 1) only slightly (6.42 Å for TBOB versus 7.04 Å for **2**), a *m*-isothiocyanato group (**1**) leads to an effective molecular diameter of 8.09–9.96 Å. Thus, it could be argued that, whereas TBOB and **2** can "fit" into the chloride channel, **1** might be marginally too large and would have to adopt a high energy "small" conformation for binding within it. If this explanation is valid, it may be concluded that the diameter of the GABA-gated chloride ion channel must be on the order of 8 Å. Based on the permeability sequence of large polyatomic anions, a pore diameter of 5.6 Å has been proposed (27). However, this diameter of the chloride ionophore would not permit the binding of TBPS or TBOB and its derivatives within the chloride channel.

Scatchard analysis of [^{35}S]TBPS binding in membranes preincubated with **1** and **2** revealed concentration–dependent reductions in both the apparent affinity of this ligand and numbers of binding sites (Fig. 4; Table 2). These observations suggest that **2** could acylate several nucleophiles in the vicinity of the [^{35}S]TBPS binding site, some partially hindering access (resulting in an increased K_d) and others completely blocking access of the radioligand (resulting in a reduction in B_{max}). Alternatively, these data could be explained by a heterogeneity of binding sites for these cage convulsants. This latter proposal is consistent with demonstration of multiple conductance states of GABA-gated chloride channels (27, 28) as well as differences in the regional distribution of [^{35}S]TBPS binding sites and benzodiazepine receptors in cerebellum (29).

The availability of a specific acylating ligand of GABA-gated chloride channels may prove to be valuable in examining the molecular arrangement of benzodiazepine receptors, GABA receptors, and their associated chloride ionophore. Schofield *et al.* (30) have reported that a chloride channel modulated by benzodiazepines, GABA, and barbiturates can be expressed in *Xenopus* oocytes by the introduction of RNAs coding for the benzodiazepine and GABA receptors. Although these findings suggest that the chloride channel may be composed of α and β subunits, biochemical and immunological evidence suggests that the stoichiometry and arrangement of these units may vary (reviewed in Ref. 31). Moreover, the presence of endogenous chloride-selective channels in *Xenopus* oocytes (32) suggests the possibility that other proteins may be required for a fully functional chloride ionophore. The availability of acylating ligands of GABA-gated chloride channels, particularly in a radioactive form, would be useful in addressing these issues as well as defining the locus of action of barbiturates, pyrazolopyridines, and other drugs thought to exert their actions at GABA-gated chloride channels.

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