Articles

Novel Site-Directed Affinity Ligands for GABA-Gated Chloride Channels: Synthesis, Characterization, and Molecular Modeling of 1-(Isothiocyanatophenyl)-4-*tert*-butyl-2,6,7-trioxabicyclo[2.2.2]octanes

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p-, *m*-, and *o*-isothiocyanate derivatives (1-3, respectively) of *tert*-butylbicycloorthobenzoate (TBOB) were synthesized from 3-*tert*-butyloxetane-3-methanol (4) as the starting material. While 2 was readily obtained in four steps via catalytic hydrogenation of the *m*-nitro-*tert*-butylbicycloorthobenzoate (9) intermediate, 1 and 3 could not be obtained this way. 1 and 3 were instead synthesized by an alternative four-step approach which made use of the stability of the isothiocyanate moiety to strong Lewis acids such as boron trifluoride etherate, conditions that would isomerize isothiocyanato oxetane ester intermediates to their corresponding orthoesters. The *p*-isothiocyanate derivative of TBOB, compound 1, inhibited [^{35}S]-*tert*-butylbicyclophosphorothionate (TBPS) binding to rat cortical membranes with a potency (IC_{50} 62 nM) comparable to the parent compound while 2 and 3 were approximately 10-fold less potent (IC_{50} values 570 and 609 nM, respectively). Preincubating tissue with radioligand further reduced the potencies of 2 and 3 by approximately 1 order of magnitude (IC_{50} values 5400 and 7500 nM, respectively) while the potency of 1 (IC_{50} 90 nM) was only marginally affected by this procedure. Pretreatment of membranes with 1 and 2 followed by extensive washing resulted in a concentration-dependent inhibition of [^{35}S]TBPS binding. In contrast, preincubating tissues with up to 2.4 μM of 3 did not elicit an apparent acylation of [^{35}S]TBPS binding sites. Molecular modeling of the effective diameters of 1-3 in their thermodynamically most stable conformations indicates a relationship between these diameters and their relative efficacies as site-directed acylators; the smaller the diameter, the more potent the acylator. This hypothesis explains both the relative potencies of these compounds and their differential abilities to acylate the TBPS binding site.

Many pharmacologically important compounds exert their effects on neuronal excitability through γ-aminobutyric acid (GABA) gated benzodiazepine receptor coupled chloride channels. This "supramolecular complex" is thought to mediate the pharmacological actions of structurally diverse classes of compounds, including benzodiazepines, barbiturates, anesthetic steroids, and polychlorocycloalkane insecticides.[1] The class of compounds generally referred to as "cage" convulsants has been employed in electrophysiological[2-6] and pharmacological[2,7,8] studies of the benzodiazepine/GABA receptor chloride ionophore complex. Electrophysiological[4,6] and neurochemical[7,9] findings indicate that "cage" convulsants act at sites on or near the GABA-gated chloride channel.[3,6]

Site-directed acylating agents containing an isothiocyanate moiety have proven to be valuable tools in examining the molecular aspects of several receptor systems.[10-15] A series of isothiocyanate analogues of *tert*-butylbicycloorthobenzoate (TBOB)[2,8] were synthesized in order to further characterize binding sites for cage convulsants and their relationship to other components of the supramolecular complex.

Recently, we have shown that the *p*-isothiocyanate analogue (1) of TBOB is a potent, specific ligand which irreversibly inhibits the binding of [^{35}S]-*tert*-butylbicyclophosphorothionate ([^{35}S]TBPS) to sites on the GABA-gated chloride channel.[11] The meta derivative 2, while specific and irreversible, was approximately 1 order of magnitude less potent.[11] In the present study, we describe the synthesis of the *p*-, *m*-, and *o*-isothiocyanate derivatives of TBOB (1, 2, and 3, respectively) and com-

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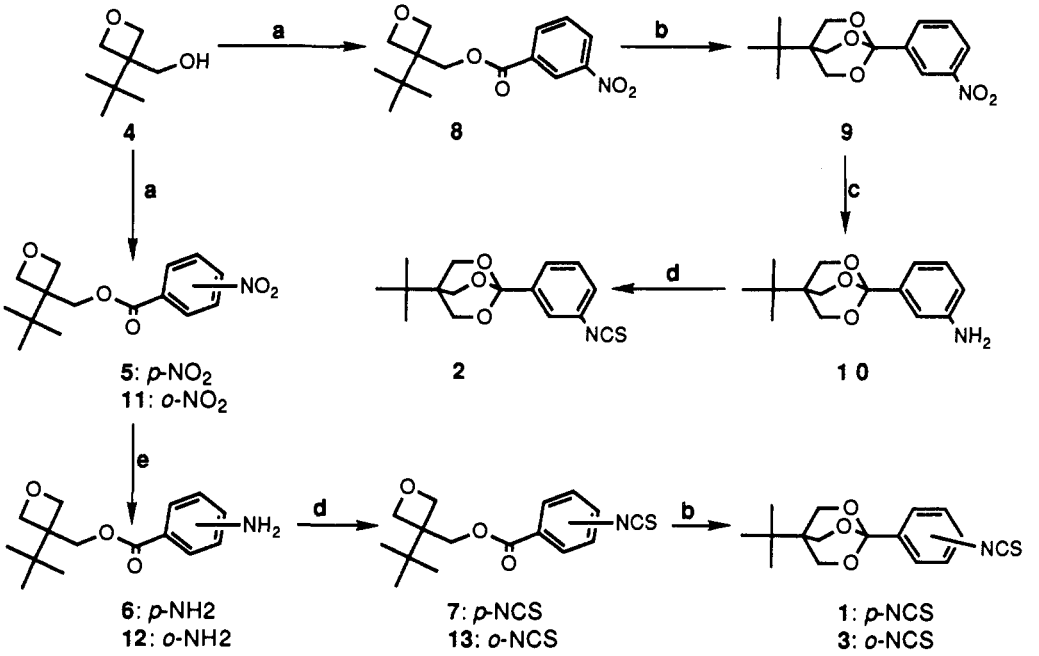
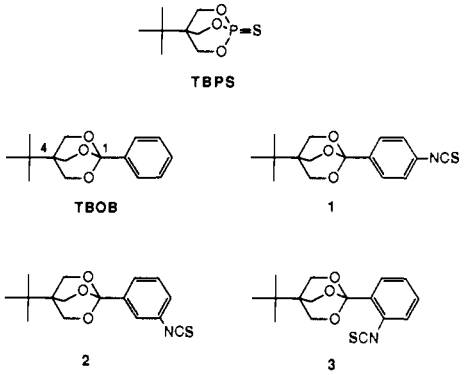
Scheme I. Synthesis of Isothiocyanate Analogues of 1-Phenyl-4-*tert*-butyl-2,6,7-trioxabicyclo[2.2.2]octane (TBOB)[a]



[a] (a) *o*-, *m*-, or *p*-nitrobenzoyl chloride, pyridine, CH_2Cl_2; (b) $BF_3{\cdot}OEt_2$, CH_2Cl_2, −70 °C → room temperature; (c) H_2, 5% Pd/$BaSO_4$, EtOH; (d) $CSCl_2$, aq $NaHCO_3$, $CHCl_3$; (e) H_2, 10% Pd/C, EtOAc.

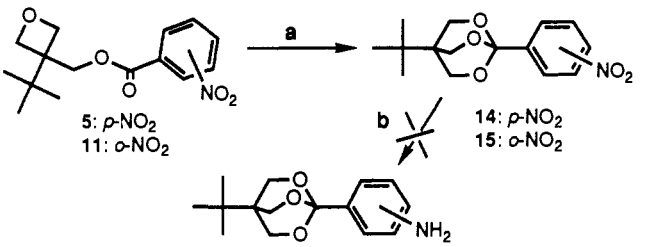
pare their neurochemical properties with respect to relative potencies and abilities to irreversibly inhibit radioligand binding to sites on the chloride ionophore. In addition, the molecular geometry of these compounds and the possible implications of the results with respect to the dimensions of the GABA-gated chloride channel are considered.



Chemistry

A successful synthetic strategy (Scheme I) leading to the desired isothiocyanate analogues **1–3** employed the readily available (from 3,3-dimethyl-1-butanol) 3-*tert*-butyloxetane-3-methanol (**4**).[2] The meta-substituted isothiocyanate derivative **2** was synthesized via coupling of **4** with *m*-nitrobenzoyl chloride (Scheme I) to give oxetane ester **8**. $BF_3{\cdot}OEt_2$-catalyzed isomerization[2,16] of **8** proceeded smoothly to give orthoester **9** in 45% recrystallized yield. Catalytic hydrogenation of **9** over 5% Pd/$BaSO_4$ yielded aniline **10** in 91% recrystallized yield. More vigorous hydrogenation conditions (10% Pd/C, EtOH) resulted in considerably lowered yields. Treatment of **10** with freshly redistilled thiophosgene[17] in a two-phase $CHCl_3$/$NaHCO_3$ mixture resulted in a 58% yield of the desired **2**. Application of the approach used for **2** for synthesis of isothiocyanate derivatives **1** and **3** was unsuccessful (Scheme II); the para-substituted nitro derivative **14**[2] (Scheme II) was synthesized as for **9**. Similarly, the *o*-nitro compound **15** was obtained in low yield by $BF_3{\cdot}OEt_2$-catalyzed isomerization of **11**. The step requiring catalytic hydrogenation of **14** and **15** to the corresponding anilines **16** and **17** however gave a complex mixture of products under a variety of conditions which included the use of the relatively mild 5% Pd/$BaSO_4$ as catalyst.

Scheme II. Attempted Synthesis of *o*- and *p*-Amino Derivatives of 1-Phenyl-4-*tert*-butyl-2,6,7-trioxabicyclo[2.2.2]octane (TBOB) as Potential Precursors to the *o*- and *p*-Isothiocyanate Derivatives[a]



[a] (a) $BF_3{\cdot}OEt_2$, CH_2Cl_2, −70 → 0 °C; (b) H_2, 5% Pd/$BaSO_4$, EtOH or 10% Pd/C, EtOAc or PtO_2, EtOAc.

Thus, an alternative approach (Scheme I) was investigated, based on the premise that the isothiocyanate moiety would be stable to Lewis acids such as $BF_3{\cdot}OEt_2$. Careful hydrogenation of nitrooxetane esters **5** and **11** over 10% Pd/C yielded anilines **6** and **12** in 86 and 74% yields, respectively; longer hydrogenation times for **5** and **11** resulted in considerably lowered yields, presumably due to hydrogenolysis of the oxetane ring. Treatment of **6** and **12** with thiophosgene[17] gave the corresponding isothiocyanates **7** and **13** in about 70% yield. $BF_3{\cdot}OEt_2$ catalyzed rearrangement of **7** and **13** proceeded smoothly to give the target compounds **1** and **3**. As observed for the *o*-nitro

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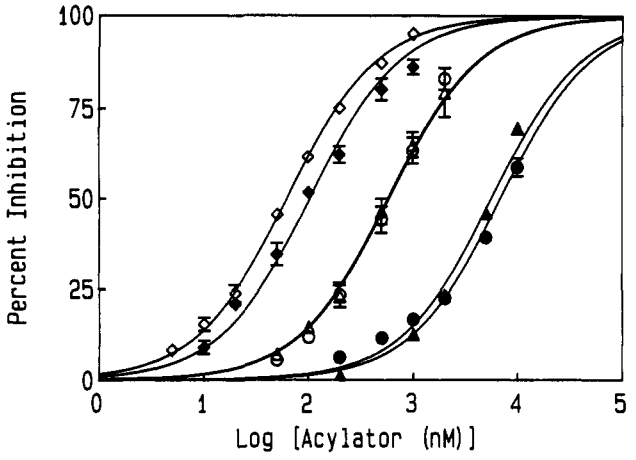

Figure 1. The effect of coincubation or preincubation with *p*-, *m*-, or *o*-NCS-TBOB (1, 2, and 3, respectively) on [^{35}S]TBPS binding in rat cortical homogenates. Tissue homogenates were prepared and radioligand binding performed as described in Methods. In experiments using the coincubation protocol (1, ◇; 2, △; 3, ○; $N \geq 3$), the [^{35}S]TBPS (5 nM) and acylators, in the concentrations indicated on the graph, were added to the tissue simultaneously. In experiments using the preincubation protocol (1, ◆; 2, ▲; 3, ●; $N = 1$), cortical homogenates were incubated with [^{35}S]TBPS for 60 min prior to the addition of the acylators. Percent inhibition was defined as fmol bound in the presence of acylators/fmol bound in the absence of acylator (control) × 100.

compound **15** (Scheme II), the yield of the *o*-NCS isomer **3** was low, possibly as a result of steric hindrance to bicycloorthoester formation by the bulky ortho substituent.

Results

[^{35}S]TBPS binding was inhibited by coincubation with **1–3**. The IC_{50} values were 62 ± 3, 750 ± 89, and 609 ± 78 nM for **1**, **2**, and **3**, respectively (Figure 1). The approximately 10-fold difference in binding potency between **1** and **2** reported here is similar to that reported by Lewin et al.[11] Preincubation of the cortical homogenates with [^{35}S]TBPS for 60 min prior to addition of **1** resulted in a reduction in potency to 90 nM. In contrast, the potencies of **2** and **3** were reduced by approximately 1 order of magnitude, to 5400 and 7500 nM, respectively by this procedure (Figure 1).

Pretreatment with an IC_{50} concentration (60 nM) of **1** followed by extensive washing resulted in a significant reduction in both the B_{max} and the apparent affinity (increased K_d) of [^{35}S]TBPS (Table I). These effects were similar in magnitude to those previously reported.[11] Analogous experiments with **2** (600–2400 nM) resulted in a concentration-dependent increase in the K_d of [^{35}S]TBPS with no significant difference in the B_{max} (see Table I). Preincubation with 600–2400 nM **3** resulted in no statistically significant changes in either the K_d or B_{max} of [^{35}S]TBPS.

The isothiocyanate moiety of **1–3** is capable of covalently reacting with a variety of bionucleophiles within the active site of a suitable receptor. Examples of such bionucleophiles include hydroxy (OH), thiol (SH), and amino (NH_2). For the purposes of our molecular modeling study, we chose the amino group of lysine as our hypothetical bionucleophile. In its global energy minimum conformation the thiourea **1a** derived from reaction of *p*-NCS-TBOB (**1**) with lysine is in an extended conformation (Figure 2), in which the distance from the α-carbon in lysine (C-α) to the C-4 carbon (see TBOB structure for numbering) is 15.6 Å. A conformation with C-α to C-4 = 13.7 Å (Figure 2) is 0.27 kcal/mol higher in energy (Table II). Conformations with shorter C-α to C-4 distances are more than 5 kcal/mol above the global energy minimum. According to the Boltzman distribution, the conformational composition of **1a** at 0 °C is, therefore, 62% in the "long" conformation and 38% in the "shorter" conformation. For the analogous *m*-thiourea (**2a**) the minimum-energy conformation has a C-α to C-4 distance of 11.4 Å (Figure 2); conformations with C-α to C-4 distance in the range of 12.5 Å are 0.6 kcal/mol above the global minimum, and conformations with C-α to C-4 distances of 13.7 and 15.6 Å are 0.16 and 0.56 kcal/mol above the minimum,

Table I[a]

compd	K_d ± SEM, nM	B_{max} ± SEM, fmol/assay
1 (nM)*		
0	47.0 ± 6.7	955.9 ± 36.3
60	72.2 ± 6.5	644.2 ± 110.9
2 (nM)**		
0	35.3 ± 1.5	1076.3 ± 53.6
600	44.5 ± 1.0	924.7 ± 100.0
1200	51.8 ± 2.8	889.0 ± 54.4
1500	55.8 ± 3.0	927.5 ± 60.6
2400	73.8 ± 2.9	1007.2 ± 97.6
3 (nM)		
0	46.7 ± 4.5	963.3 ± 101.4
600	57.3 ± 2.9	915.3 ± 78.4
1200	50.7 ± 2.4	817.3 ± 113.2
1500	56.3 ± 5.5	869.3 ± 72.9
2400	58.0 ± 4.6	1031.3 ± 50.8

[a] The effect of **1**, **2**, and **3** on [^{35}S]TBPS binding: Cortical homogenates were treated with the indicated concentrations of **1**, **2**, or **3**, followed by repeated washes and used in the TBPS binding assay as described in the Experimental Section. Values are derived from Scatchard plots of the receptor binding data and are expressed as the mean of three to five experiments ± standard error of the mean. (*) A one-way analysis of variance (ANOVA) demonstrated a significant effect of **1** on B_{max} (ANOVA df = 1, 8; $F = 7.137$; $p < 0.03$) and K_d (ANOVA df = 1, 8; $F = 7.231$; $p < 0.03$) of [^{35}S]TBPS. (**) Treatment with **2** resulted in a concentration-dependent increase in the K_d of [^{35}S]TBPS (ANOVA df = 4, 15; $F = 36.344$; $p < 0.0001$).

Table II[a]

Conformational Properties of Thioureas **1a**, **2a**, and **3a**

compd no.	desig in Figure 2	Cα–C4, Å	ΔE, kcal/mol	% composn at 0 °C
1a	A	15.56	0	62
	B	13.76	0.27	38
2a		11.44	0	57
		12.45	0.60	10
	C	13.70	0.16	22
	D	15.57	0.56	11
3a	E	10.45	0	100

Conformational Parameters of Isothiocyanates **1**, **2**, and **3**

	desig in Figure 3	minimum effective diameter, Å	heat of formation, kcal/mol
1	A	6.59	76
2			
conformation I	B	8.09	76
conformation II	C	9.96	76
3			
conformation I	D	8.09	84
conformation II	E	9.96	78

[a] Conformational properties of the thioureas **1a**, **2a**, and **3a** and isothiocyanates **1**, **2**, and **3**. The conformations were obtained as described in the Molecular Modeling section. The composition was calculated by using the Boltzmann equation. For isothiocyanates, only conformations in which the isothiocyanato group is coplanar with the phenyl ring (Figure 3) are shown; out-of-plane conformations are of considerably higher energy. The data were obtained as described in the Molecular Modeling section.

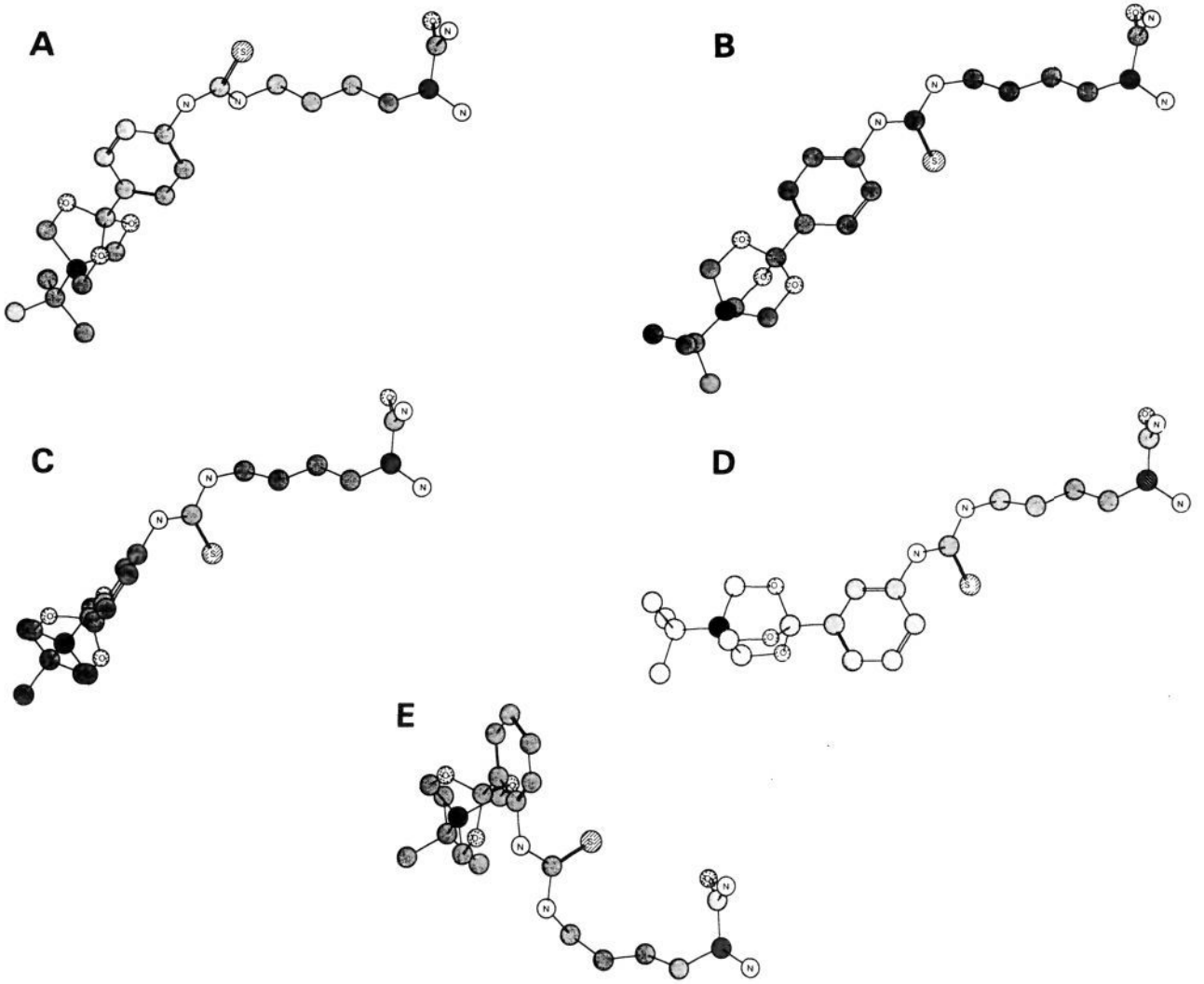

Figure 2. Minimum-energy conformations for the thioureas **1a**, **2a**, and **3a** formed by acylation of a lysine residue with *p*-, *m*-, or *o*-NCS-TBOB. These conformations were obtained as described in the Molecular Modeling section. They were generated in Chem 3D using the SYBYL coordinates and annotated as black and white figures using MacDraw. The structures of the thioureas are organized as follows: (A) global minimum conformation of **1a** (Cα-C4) = 15.56 Å; (B) conformation of **1a** with Cα-C4 = 13.76 Å; (C) conformation of **2a** with Cα-C4 = 15.57 Å; (D) conformation of **2a** with Cα-C4 = 13.70 Å; (E) global minimum conformation of **3a** (Cα-C4) = 10.45 Å; more detailed information concerning these conformations can be found in the Discussion.

respectively. The conformational composition of **2a** at 0 °C is 11% in the "long" conformation, 22% in the shorter conformation, and 57% in "too short" conformations. The global energy minimum conformation of the *o*-thiourea (**3a**) has a C-α to C-4 distance of 10.5 Å with no conformations with 15.6 or 13.7 Å distances within 5 kcal/mol of the global minimum.

The "minimum" molecular diameters of TBOB, **1**, **2**, and **3** are presented in Table II. Two conformations of **2** and **3** were optimized: in conformation I, the isothiocyanate group was maintained at a 20° wide angle to the center-line axis while in conformation II a 100° angle was maintained. Isothiocyanate **1** had a slightly larger effective diameter than TBOB (6.59 Å vs 6.42 Å). Conformers I and II had the same molecular dimensions in isomers **2** and **3** due to the symmetry of the systems (Figure 3). The smaller conformer (I) was significantly larger than either TBOB or **1**, with an effective diameter of 8.09 Å. The diameter of conformer II was 9.96 Å (see Table II). The heats of formation for conformations I and II of **2** were essentially identical, indicating that **2** can exist in either conformation. For **3**, the heat of formation of conformer I was 6 kcal/mol higher than that of conformer II. Therefore, at 25 °C, **3** will exist entirely in conformation II.

Discussion

Both the potencies and the characteristics of **1**–**3** as ligands for the chloride ionophore indicate that each positional isomer affects the [^{35}S]TBPS binding site in a unique manner. Thus, placement of the isothiocyanate moiety at the meta (**2**) or ortho (**3**) positions resulted in a 10-fold reduction in potency relative to the para (**1**) position when assayed by simultaneous addition of inhibitor and radioligand. However, when tissues were preincubated with [^{35}S]TBPS prior to addition of the affinity ligands, the potency of **1** was only slightly diminished, whereas the potencies of **2** and **3** were reduced by about 10-fold. An explanation consistent with the apparent change in potencies relates to the significantly slower association rate of TBPS-type ligands relative to TBOB and TBOB-related compounds.[8] Thus, coincubation of TBPS and a TBOB-derived ligand leads to rapid occupation of the receptor by the TBOB-derived ligand, requiring a long incubation period for equilibrium to be achieved. Preincubation with the more slowly associating TBPS may result in a more valid comparison of ligand

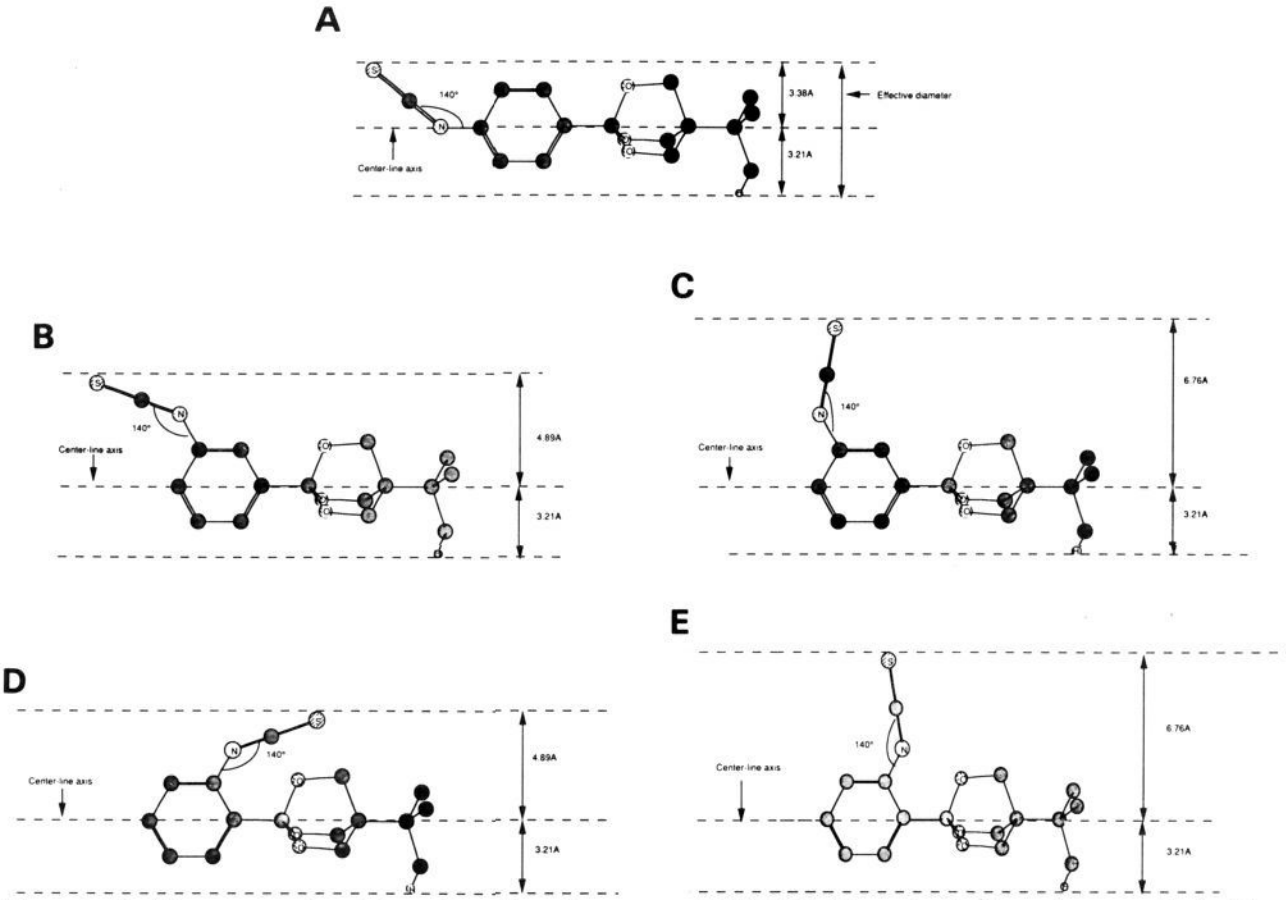

Figure 3. Graphic representation of the conformations of *p*-, *m*-, or *o*-NCS-TBOB. The molecular dimensions were obtained from the MNDO minimized conformations. The structures of the isothiocyanates are organized as follows: (A) *p*-NCS-TBOB (**1**); (B) small conformation of *m*-NCS-TBOB (**2**); (C) large conformation of *m*-NCS-TBOB (**2**); (D) small conformation of *o*-NCS-TBOB (**3**); (E) large conformation of *o*-NCS-TBOB (**3**).

potencies. This situation has been reported for TBPS-derived vs TBOB-derived ligands[8] with a 2.5-fold difference in apparent potencies when preincubation versus coincubation experiments were compared. In the case of the isothiocyanato TBOB derivatives, the 10-fold difference observed for **2** and **3** suggests a higher association rate for these ligands than for TBOB, probably due to the electron-withdrawing inductive effect of the isothiocyanate substituent.[11] On the basis of this effect, the substantially smaller change (1.5-fold) observed for the potency of **1** in preincubation with TBPS vs coincubation experiments was initially somewhat surprising, since inductive effects for meta and para substituents are virtually identical. However, this result is completely consistent with the irreversible nature of **1**.[11] Thus, since the rate of association of **1** with the receptor significantly exceeds that of TBPS, **1** will displace TBPS. In addition, if a large proportion of the receptor-bound ligand becomes irreversibly coupled such that it continues to occupy the receptor, the apparent potency would remain high. On the other hand, the large effects on the potencies of **2** and **3** in preincubation vs coincubation experiments suggests little or no irreversible effects by these ligands on [^{35}S]TBPS binding. The large difference between the potencies of **1** and **2** despite their similar inductive effects further supports this idea. Specifically, if only a small percentage of the covalently bonded meta isomer (**2**) actually occupied the receptor site, it would appear to have an appreciable dissociation rate and, therefore, a lower apparent potency than the para isomer (**1**) with its relatively low dissociation rate. The ortho isomer (**3**), with the largest electron-withdrawing inductive effect, should have exhibited the greatest potency. Since this is not the case, **3** may be covalently bonded to the protein in a fashion or at a locus which does not allow it to occupy or interfere with the [^{35}S]TBPS binding site.

Additional support for this hypothesis was obtained by examining the effects of preincubation of rat cortical membranes with **1**, **2**, or **3** on [^{35}S]TBPS binding. Thus, Scatchard analysis of [^{35}S]TBPS binding reveals that under the described preincubation and washing conditions, treatment with **1** elicits a reduction in both the affinity (K_d) and number (B_{max}) of [^{35}S]TBPS binding sites, while **2** diminishes the apparent affinity and **3** has no effect on either the affinity or the number of binding sites. Both **1** and **2** have thus been characterized as irreversible ligands of the [^{35}S]TBPS site. The observed effects have been attributed to acylation of several nucleophiles in the vicinity of the [^{35}S]TBPS binding site, some hindering access (decreasing the apparent affinity) and others blocking access (reducing the apparent B_{max}) of the radioligand to the binding site.[11] Consistent with this explanation, the results of the current investigation may be interpreted as suggesting that **2** acylates only sites which hinder (but do not completely prevent) access to the binding site while **3** does not acylate bionucleophiles in the vicinity of the [^{35}S]TBPS site. The large difference in potency between **1** and **2**, which has been attributed to the significantly larger effective diameter of **2**,[11] supports the notion that the differences in the effects of **1**–**3** on [^{35}S]TBPS binding are related to the details of their molecular structures. The effect of **2** on [^{35}S]TBPS binding, observed in the preincubation and washing experiments, indicates that **2** bonds

covalently in the vicinity of the receptor. Since 3 does not affect [^{35}S]TBPS binding under analogous conditions, it is tempting to propose that it does not bond covalently. However, this explanation appears unlikely since the two ligands have similar potencies and identical substrates (isothiocyanate groups) for attack by bionucleophiles. An alternative explanation is that 3 does acylate the same bionucleophiles as 2, but that the distance from the site of acylation to the TBPS binding site is not optimal for either preventing or hindering TBPS binding. This proposal leads to an image of a lipophilic receptor site into which the "cage" portion of the molecule, i.e. the *tert*-butyltrioxabicyclo[2.2.2]octane moiety, fits. The presence of a bioamine near the receptor site will lead to the formation of a thiourea by the reaction of the amine with the isothiocyanate portion of 1–3. If the distance from the site of attachment of 1–3 to the receptor is optimized, acylation will lead to occupation of the receptor, preventing TBPS binding. If the distance deviates slightly from the optimum, some hindrance to binding may be observed. If the deviation from the optimal distance is too great in either direction, no effect would be observed. This model also predicts that when the distance from the receptor to the bionucleophile is optimal, site-specific acylation would occur, leading to high apparent potency. Conversely, when the distance of the bionucleophile from the receptor is too large or too small, site-specific acylation would occur either less frequently or not at all, resulting in a lower apparent potency. The range of distances from the α-carbon atom (C-α) of lysine (a model bioamine which is likely to react with the isothiocyanate to give the thiourea) to C-4 in the "cage" for all the conformers within 5 kcal/mol of the global minimum energy conformation of the thioureas **1a**, **2a**, and **3a** resulting from the reaction of lysine with **1**, **2**, and **3**, respectively, is shown in Table II. Inspection of the table shows that, in the lowest energy conformation of **1a**, this distance is around 15.6 Å while in the minimum-energy conformations of **2a** and **3a**, this distance is considerably shorter (11.4 and 10.5 Å, respectively). The meta isomer, **2a**, has low-energy conformations (0.2 kcal/mol above the global minimum) in which the C-α to C-4 distance is 13.7 Å; it can also achieve the longer distance of 15.6 Å in conformations 0.6 kcal/mol above the global energy minimum. No conformers of **3a** within 5 kcal/mol of the global energy minimum possess either of these distances. These observations suggest that if acylation of a bioamine either 15.6 or 13.7 Å distant from the receptor site were to occur, preincubation with **1** or **2** would affect [^{35}S]TBPS binding (reducing B_{max}) while in the shorter (13.7 Å) conformations, they interfere with access to the TBPS binding site, causing an increase in the K_d. The reverse is equally possible; i.e. the shorter conformation may be required for a B_{max} effect and the longer conformation may affect K_d. At present, the available data do not resolve these alternatives.

It should be noted that although the actual distances from the membrane surface to the cage moiety will depend on the specific bionucleophile interacting with the ligand, the relative distances do not if the same bionucleophile is involved with the three isothiocyanato TBOBs. Thus, this interpretation (based on modeling of a lysine derived urea) is independent of the specific bionucleophile.

The aspects of the molecular geometry of **1–3** discussed above present a consistent hypothesis to account for the receptor binding data. It is, however, possible that the minimum effective diameters of the three compounds also contribute to the potency and efficacy of these ligands as site-directed acylators. Comparing the low-energy conformations of **1–3** demonstrates that the most potent compound (**1**) has the smallest effective diameter (6.59 Å). This effective diameter is considerably smaller than the diameter of **2** in either conformation I (8.09 Å) or II (9.96 Å), both of which are equally populated, and of **3**, which exists only in conformation II (9.96 Å). These data suggest that access to TBPS binding sites may be constrained by the diameter of the molecule. This would lead to the hypothesis that the effective diameter of the channel can accommodate a molecule approximately 6.4–6.6 Å in diameter (the effective diameter of the parent compound TBOB is 6.42 Å and the diameter of **1** is 6.59 Å) but is less able to accommodate a molecule as large as 10 Å across. If the TBPS binding site is located within the chloride channel, the larger effective diameter of **2** and **3** would severely limit their access to a binding site within the channel, resulting in lower affinities, and in the case of **3**, an inability to acylate the site. If **1**, **2**, or **3** enter the channel oriented along the vertical axis, the data presented here suggest a diameter that is at least 6.42 Å but less than 10 Å. This estimate of channel diameter is slightly larger than the 5.6 Å proposed by Bormann et al.[18] based on the relative permeability of monoatomic and polyatomic anions through the GABA-gated chloride channel.

It is appropriate at this point to note that the data for **2** are similar to but not in total agreement with previous observations.[11] Whereas in the previous study preincubation of cortical homogenates with **2** resulted in a decrease in both K_d and B_{max} for [^{35}S]TBPS binding, only the K_d was decreased in the current investigation. It should be noted, however, that in the earlier study the isothiocyanates were added to a 1:30 (original wet weight:volume) cortical homogenate, while in this work a 1:15 cortical homogenate was used. When studies with **1** and **2** were repeated using the original conditions, the initial observations were replicated (data not shown). These results suggest that a change in tissue concentration may alter the nature of the receptor–ligand interaction.

The present study indicates that the distance from the α-carbon in the lysine moiety to C-4 in the cage portion of the molecule appears to correlate with acylator potency and efficacy. As the distance in the lower energy, more frequently occurring conformations of the thioureas resulting from acylation of a bionucleophile is decreased from **1a**, to **2a**, to **3a**, the acylators **2** and **3** are both less potent and less efficaceous as site-directed acylators of the TBPS binding site. In addition, the effective diameter of the molecule in its thermodynamically most stable conformation appears to correlate with its relative effectiveness as a site-directed acylator; the smaller the diameter, the more potent the acylator. The specificity and potency of **1** makes it a potentially valuable tool in the identification of the TBPS binding site and the elucidation of its relation to other components of the supramolecular complex.

Experimental Section

Materials and Methods. Chemistry. Melting points were determined on a Thomas-Hoover capillary apparatus and are uncorrected. GC (gas chromatography) was determined on a Hewlett-Packard 5880A instrument fitted with a 30-m SE-30 capillary column and a flame-ionization detector. Elemental analyses were performed at Atlantic Microlabs, Atlanta, GA; all crystalline compounds furnished analyses within ±0.4% of the theoretical value for carbon, hydrogen, and nitrogen, and are denoted by the symbols C, H, N. Chemical-ionization mass spectra (CIMS) were obtained with a Finnigan 1015 mass spectrometer.

(18) Bormann, J.; Hamill, O. P.; Sakmann, B. *J. Physiol.* **1987**, *382*, 243–286.

Electron-ionization mass spectra (EIMS) and high-resolution mass measurements (HRMS) were obtained with a V.G. Micro Mass 7070F mass spectrometer. ^{1}H NMR spectra were obtained from $CDCl_3$ solutions with a Varian XL-300 spectrometer. Infrared (IR) spectra were determined with a Beckman 4230 IR spectrophotometer using KBr pellets for crystalline compounds and liquid films for compounds that were liquids or oils. Thin-layer chromatography (TLC) was performed on 250 μM Analtech GHLF silica gel plates. Column chromatography was performed on basic alumina (Brockmann I, 150 mesh).

3-[[(*m*-Nitrobenzoyl)oxy]methyl]-3-*tert*-butyloxetane (8). To a stirred and cooled (ice bath) solution of oxetane (4)[2] (2.0 g, 13.9 mmol) and *p*-nitrobenzoyl chloride (2.84 g, 15.3 mmol) in dry THF (30 mL) was added dropwise triethylamine (TEA) (3.86 mL, 27.8 mmol). After 1 h at room temperature, the precipitate of TEA·HCl was filtered and washed with THF (10 mL), and the solvent was evaporated in vacuo at room temperature to give pure 8 (3.30 g, 81%) after recrystallization from hot isooctane: mp 77–78 °C; ^{1}H NMR δ 1.08 (s, 9 H, *t*-Bu), 4.54 (s, 2 H, $COOCH_2$), 4.59, 4.65 (AB q, 4 H, J_{gem} = 6.6 Hz), 7.69 (t, 1 H, *J* = 8.1 Hz), 8.45 (dd, 2 H, *J* = 1.6 Hz, *J* = 8.1 Hz), 8.92 (t, 1 H, *J* = 1.7 Hz); IR 3140, 3100, 2970, 1740, 1535, 1360, 1300, 1150, 990, 720 cm^{-1}; CIMS M + H = 294. Anal. ($C_{15}H_{19}NO_5$) C, H, N.

1-(*m*-Nitrophenyl)-4-*tert*-butyl-2,6,7-trioxabicyclo[2.2.2]octane (9). To a stirred solution of 8 (2.82 g, 9.62 mmol) in dry CH_2Cl_2 (80 mL) at −78 °C was added (via syringe) freshly distilled BF_3·Et_2O (3.0 mL), and the solution was allowed to warm to room temperature and stir for a further 12 h. Triethylamine (30 mL, excess) was added to the reaction mixture to destroy the excess reagent followed by ether (200 mL). Addition of the ether resulted in an insoluble crystalline precipitate which was filtered and discarded. The solution was washed with water (3 × 100 mL) and the solvent was evaporated to give crude product. Purification on Brockmann I basic alumina, eluting with CH_2Cl_2–petroleum ether (1:2), and recrystallization from 95% EtOH afforded 1.28 g (45%) of 9: mp 166–168 °C; ^{1}H NMR δ 0.93 (s, 9 H, *t*-Bu), 4.21 (s, 6 H, CH_2), 7.52 (t, 1 H, *J* = 8.1 Hz), 7.92–7.95 (m, 1 H), 8.19–8.23 (m, 1 H), 8.49 (t, 1 H, *J* = 1.9 Hz); IR 3100, 2960, 1615, 1530, 1475, 1350, 1125, 1020 cm^{-1}; CIMS M + H = 294. Anal. ($C_{15}H_{19}NO_5$) C, H, N.

1-(*m*-Aminophenyl)-4-*tert*-butyl-2,6,7-trioxabicyclo[2.2.2]octane (10). A solution of 9 (0.50 g, 1.71 mmol) in EtOH (50 mL) containing 5% Pd/$BaSO_4$ was stirred at room temperature and atmospheric pressure under an atmosphere of hydrogen gas. TLC (EtOAc–hexane, 1:4) indicated that the reaction was complete after 20 min. The solution was filtered through a pad of Celite, the solvent evaporated, and the product recrystallized from 2-propanol to give 10 (0.41 g, 91%): mp 176–177 °C; ^{1}H NMR δ 0.91 (s, 9 H, *t*-Bu), 3.66 (br s, 2 H, NH_2), 4.17 (s, 6 H, CH_2), 6.63–6.68 (m, 1 H), 6.94 (t, 1 H, *J* = 1.7 Hz), 7.00 (dd, 1 H, *J* = 1.2 Hz, *J* = 7.8 Hz), 7.13 (t, 1 H, *J* = 7.6 Hz); IR 3440, 3360, 2960, 1610, 1460, 1340, 1130, 990 cm^{-1}; CIMS M + H = 264. Anal. ($C_{15}H_{21}NO_3$) C, H, N.

1-(*m*-Isothiocyanatophenyl)-4-*tert*-butyl-2,6,7-trioxabicyclo[2.2.2]octane (2). To a rapidly stirred solution of 10 (100 mg, 0.38 mmol) and potassium carbonate (0.30 g, 2.17 mmol) in a mixture of pentene-stabilized $CHCl_3$ (20 mL) and water (20 mL) was added via syringe freshly redistilled thiophosgene (31.9 μL, 0.42 mmol), and the solution was stirred for 20 min. The organic layer was separated, diluted to 50 mL with $CHCl_3$, and washed with water (2 × 50 mL). Evaporation of the solvent in vacuo and recrystallization of the residue from 2 mL of 2-propanol afforded 67 mg (58%) of crystalline 2 as colorless laminae: mp 146–147 °C; ^{1}H NMR δ 0.92 (s, 9 H, *t*-Bu), 4.18 (s, 6 H, CH_2), 7.17–7.20 (m, 1 H), 7.32 (t, 1 H, *J* = 8.3 Hz), 7.50–7.53 (m, 2 H); IR 2970, 2120 (NCS), 1590, 1340, 1225, 1130, 1010 cm^{-1}; CIMS M + H = 306. Anal. ($C_{16}H_{19}NO_3S$) C, H, N.

3-[[(*p*-Nitrobenzoyl)oxy]methyl]-3-*tert*-butyloxetane (5). To a stirred mixture of 4 (1.56 g, 10.83 mmol), dry pyridine (6 mL), and CH_2Cl_2 (20 mL) was added *p*-nitrobenzoyl chloride (2.01 g, 10.83 mmol) and the solution stirred for 12 h at room temperature. The reaction mixture was diluted with ether (200 mL), washed with water (100 mL), 1.0 M aqueous HCl (4 × 50 mL), and saturated $NaHCO_3$ (2 × 20 mL), and dried over anhydrous Na_2SO_4. Recrystallization of the product from 30 mL of ethanol afforded 2.15 g (68%) of pure 5: mp 121.5–122.5 °C; ^{1}H NMR δ 1.07 (s, 9 H, *t*-Bu), 4.51 (s, 2 H, $COOCH_2$), 4.60, 4.66 (AB q, 4 H, J_{gem} = 6.6 Hz), 8.30 (d, 4 H, *J* = 2.1 Hz); IR 3130, 2960, 1710, 1600, 1520, 1280, 1100, 980, 710 cm^{-1}; CIMS M + H = 294. Anal. ($C_{15}H_{19}NO_5$) C, H, N.

3-[[(*p*-Aminobenzoyl)oxy]methyl]-3-*tert*-butyloxetane (6). A solution of 5 (1.55 g, 5.29 mmol) in EtOAc (30 mL) containing 0.15 g of 10% Pd/C was hydrogenated for 40 min at 50 psi. The reaction mixture was filtered through a pad of Celite, and the Celite was washed with a further 40 mL of EtOAc. Evaporation of the solvent from the filtrate afforded a colorless oil which crystallized on standing. Recrystallization of the product from 2,2,4-trimethylpentane–2-propanol afforded 1.20 g (86%) of 6: mp 124–125 °C; ^{1}H NMR δ 1.06 (s, 9 H, *t*-Bu), 4.08 (br s, 2 H, NH_2), 4.39 (s, 2 H, $COOCH_2$), 4.59, 4.64 (AB q, 4 H, J_{gem} = 6.3 Hz, CH_2), 6.66, 7.93 (AB q, 4 H, *J* = 8.6 Hz); IR 3340, 3220, 2950, 1680, 1600, 1510, 1280, 1170, 1110, 970 cm^{-1}; CIMS M + H = 264. Anal. ($C_{15}H_{21}NO_3$) C, H, N.

3-[[(*p*-Isothiocyanatobenzoyl)oxy]methyl]-3-*tert*-butyloxetane (7). Freshly redistilled thiophosgene (0.29 mL, 3.76 mmol) was added via syringe to a stirred solution of 6 (0.90 g, 3.42 mmol) in a mixture of saturated $NaHCO_3$ (25 mL) and $CHCl_3$ (25 mL). Reaction was complete after 20 min at room temperature as determined by TLC (EtOAc–hexane, 1:4). The organic layer was separated and the aqueous layer was washed with a further 25 mL of $CHCl_3$, and the combined organic layer was washed with water (20 mL) and evaporated in vacuo to give the crude product. Recrystallization of the product from 2-propanol (5 mL) afforded 0.72 g, 69% of 7: mp 98–99 °C; ^{1}H NMR: δ 1.07 (s, 9 H, *t*-Bu), 4.45 (s, 2 H, $COOCH_2$), 4.60, 4.63 (AB q, 4 H, J_{gem} = 6.4 Hz, CH_2), 7.30, 8.11 (AB q, 4 H, *J* = 8.6 Hz); IR 2950, 2110 (NCS), 1705, 1595, 1270, 1110, 980 cm^{-1}; CIMS M + H = 306. Anal. ($C_{16}H_{19}NO_3S$) C, H, N.

1-(*p*-Isothiocyanatophenyl)-4-*tert*-butyl-2,6,7-trioxabicyclo[2.2.2]octane (1). To a stirred solution of 7 (0.60 g, 1.97 mmol) in alcohol-free CH_2Cl_2 (10 mL) at −70 °C was added, via syringe, freshly distilled boron trifluoride etherate (1.5 mL, 12.2 mmol) and the solution allowed to warm to room temperature. Reaction was complete after 24 h at room temperature. Triethylamine (3.4 mL, 24.4 mmol) was added to destroy the excess BF_3·OEt_2, and the solution was diluted to 200 mL with ether. The ether layer was washed with water (5 × 50 mL), dried over Na_2SO_4, and evaporated to give 1 (0.60 g, quantitative) as a crystalline solid. Recrystallization from 10 mL of EtOH at 0 °C afforded 0.30 g (50%) of 1 as colorless plates: mp 191–192 °C; ^{1}H NMR δ 0.92 (s, 9 H, *t*-Bu), 4.18 (s, 6 H, CH_2), 7.19, 7.58 (AB q, 4 H, *J* = 8.5 Hz); IR 2960, 2120 (NCS), 1335, 1120, 1070, 990, 820 cm^{-1}; CIMS M + H = 306. Anal. ($C_{16}H_{19}NO_3S$) C, H, N.

3-[[(*o*-Nitrobenzoyl)oxy]methyl]-3-*tert*-butyloxetane (11). Reaction of 4 (1.56 g, 8.66 mmol) with *o*-nitrobenzoyl chloride as described for 5 and recrystallization of the product from isooctane afforded 11 (2.54 g, quantitative): mp 62–63.5 °C; ^{1}H NMR δ 1.03 (s, 9 H, *t*-Bu), 4.47 (s, 2 H, CH_2COO), 4.50, 4.56 (AB q, 4 H, J_{gem} = 6.6 Hz), 7.66–7.74 (m, 2 H), 7.80 (dd, 1 H, *J* = 1.7 Hz, *J* = 7.3 Hz), 7.95 (dd, 1 H, *J* = 1.3 Hz, *J* = 7.7 Hz); IR 2970, 1725, 1535, 1295, 1140, 990 cm^{-1}; CIMS M + H = 294. Anal. ($C_{15}H_{19}NO_5$) C, H, N.

3-[[(*o*-Aminobenzoyl)oxy]methyl]-3-*tert*-butyloxetane (12). Hydrogenation of 11 (2 g, 6.83 mmol) in EtOAc as described for 6, and recrystallization of the product from isooctane afforded 12 (1.43 g, 80%): mp 92–94 °C; ^{1}H NMR δ 1.06 (s, 9 H, *t*-Bu), 4.41 (s, 2 H, $COOCH_2$), 4.63 (s, 4 H, CH_2), 5.75 (br s, 2 H, NH_2), 6.67 (m, 2 H), 7.31 (m, 1 H), 7.97 (dd, 1 H, *J* = 1.4 Hz, *J* = 8.2 Hz); IR 3450, 3330, 2960, 1695, 1620, 1485, 1240, 1095, 975 cm^{-1}; CIMS M + H = 264. Anal. ($C_{15}H_{21}NO_3$) C, H, N.

3-[[(*o*-Isothiocyanatobenzoyl)oxy]methyl]-3-*tert*-butyloxetane (13). Reaction of 12 (1 g, 3.80 mmol) with thiophosgene as described for 7 and recrystallization of the product from 2-propanol–isooctane (10 mL) afforded 13 (0.86 g, 74%): mp 66–68 °C; ^{1}H NMR δ 1.07 (s, 9 H, *t*-Bu), 4.51 (s, 2 H, CH_2COO), 4.63 (s, 4 H), 7.34–7.39 (m, 2 H), 7.56 (dd, 1 H, *J* = 1.8 Hz, *J* = 8.0 Hz), 8.10 (dd, 1 H, *J* = 1.5 Hz, *J* = 8.3 Hz); IR 2960, 2100 (NCS), 1730, 1595, 1480, 1260, 1080, 990 cm^{-1}; CIMS M + H = 306. Anal. ($C_{16}H_{19}NO_3S$) C, H, N.

1-(*o*-Isothiocyanatophenyl)-4-*tert*-butyl-2,6,7-trioxabicyclo[2.2.2]octane (3). Treatment of 13 (0.70 g, 2.29 mmol) with BF_3·OEt_2 (3.0 mL, 24.4 mmol) at room temperature overnight

followed by quenching with triethylamine (6.8 mL, 48.8 mmol) and purification of the product by column chromatography as described for 1 afforded **3** as an oil; recrystallization of the product from 10 mL of *n*-hexane furnished **3** (0.27 g, 39%) as small colorless needles: mp 115–116.5 °C; ^{1}H NMR δ 0.93 (s, 9 H, *t*-Bu), 4.27 (s, 6 H, CH_2), 7.16 (dd, 1 H, J = 1.4 Hz, J = 7.7 Hz), 7.23–7.31 (m, 2 H), 7.71 (dd, 1 H, J = 1.7 Hz, J = 7.6 Hz); IR 2960, 2100 (NCS), 1600, 1470, 1330, 1240, 1125, 1000, 750 cm^{-1}; CIMS M + H = 306. Anal. ($C_{16}H_{19}NO_3S$) C, H, N.

1-(*o*-Nitrophenyl)-4-*tert*-butyl-2,6,7-trioxabicyclo[2.2.2]-octane (15). Compound 11 (2.62 g, 8.94 mmol) in 80 mL of dry CH_2Cl_2 was treated with BF_3·OEt_2 (6.0 mL, 48.8 mmol) and stirred for 48 h at room temperture. Treatment of the reaction mixture with triethylamine (excess) and isolation of the product as described above for **9** afforded **15** (0.37 g, 14%) as colorless needles (2-propanol, 5 mL): mp 198–199 °C; ^{1}H NMR δ 1.04 (s, 9 H, *t*-Bu), 4.19 (s, 6 H, CH_2), 7.44 (m, 2 H), 7.55–7.93 (complex m, 2 H); IR 2960, 2900, 1530, 1370, 1330, 1125, 1000, 770, 740 cm^{-1}; CIMS M + H = 294. Anal. ($C_{15}H_{19}NO_5$) C, H, N. Attempts to improve the yield failed.

Biochemical and Biological Methods. Tissue Preparation. Adult male Sprague-Dawley rats (approximately 200 g) supplied by Taconic Farms (Germantown, NY) were decapitated, and the cerebral cortices removed on ice. The tissue was weighed and disrupted with a Brinkman Polytron (setting 5–6 for 15 s) in 50 volumes of 50 mM potassium phosphate buffer, pH 7.4, containing 100 mM sodium chloride. Cortical homogenates were centrifuged at 20000*g* (4 °C) for 20 min. The resulting pellets were resuspended in an equal volume of buffer containing sodium chloride and recentrifuged. This "washing" procedure was repeated a total of five times. The final pellet was resuspended in 10–15 volumes chloride free potassium phosphate buffer. Membranes were prepared and used on the same day as the assay.

For experiments in which cortical homogenates were pretreated with **1**, **2**, or **3** prior to incubation with the radioligand, aliquots of a twice-washed 1:15 cortical homogenate were incubated on ice for 1 h with **1** (60 nM), **2** (600–2400 nM), or **3** (600–2400 nM) in the presence of 200 mM sodium chloride. The reaction was terminated by dilution with approximately 10 volumes of potassium phosphate buffer, pH 7.4, containing 100 mM sodium chloride and centrifuged at 20000*g*, at 4 °C for 20 min. Resuspension of the pellet in the same volume of chloride containing buffer and centrifugation was carried out for a total of five washes. The final pellet was resuspended in 15 volumes of chloride-free buffer.

Receptor Binding. [^{35}S]TBPS. One hundred and fifty microliters of cortical homogenate (approximately 10 mg original wet weight) was added to tubes containing potassium phosphate buffer, pH 7.4, 200 mM NaCl, 2–5 nM [^{35}S]TBPS and unlabeled TBPS (0–160 nM) in a total volume of 500 μL. Nonspecific binding was defined with use of 20 μM picrotoxinin. Incubations were carried out at room temperature (25 °C) for 2 h and terminated by rapid filtration over Whatman GF/B glass fiber filters with two 5-mL washes with ice-cold 50 mM potassium phosphate buffer. Radioactivity retained on the filters was determined by liquid scintillation spectrometry.

When inhibition of [^{35}S]TBPS binding was measured by the simultaneous addition of radioligand and acylators, 5 nM radioligand was employed with 5–1000 nM **1** and 50–10000 nM **2** and **3**. For sequential addition of [^{35}S]TBPS and acylators, cortical homogenates were first incubated with 5 nM radioligand for 60 min. The acylators were then added and the incubations continued for an additional 60 min. All other conditions were maintained as discussed above.

Molecular Modeling. The optimized conformations of the thioureas **1a**, **2a**, and **3a** (Figure 2) produced by acylation of a lysine residue by **1**, **2**, and **3**, respectively, were obtained by MNDO calculations using SYBYL software (version 5.1, TRIPOS Assoc., a division of Evans and Sutherland, St. Louis, MO). Each optimized conformation was then subjected to conformational search with either (a) no constraint on the distance from the α-carbon in the lysine moiety (C-α) to C-4 (for numbering, see TBOB structure) in the cage portion of the molecule, or (b) a 13.6–13.8 Å C-α to C-4 distance constraint within 5 kcal/mol of the global energy minimum, or (c) a 15.6–15.7 Å C-α to C-4 distance constraint within 5 kcal/mol of the global energy minimum. Each conformation obtained from the conformational search routine was then optimized with use of Maximin2. The energies and distances reported in Table II are for the Maximin2 optimized conformations.

The global energy minimum conformations of TBOB, **1**, **2**, and **3** were also calculated by MNDO. The molecular dimensions (Figure 3) were obtained 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 a methyl hydrogen on the *tert*-butyl group to the center-line axis was found to be 3.21 Å. For the isothiocyanate derivatives, the distance from the sulfur atom was used; for the para isomer, this distance was determined to be 3.38 Å, while for the ortho and meta isomers, a distance range of 4.89–6.76 Å was found. The "effective diameters" were defined as the sum of the radii; for the isothiocyanate derivatives, the effective diameter was taken as the sum of the distance from the sulfur atom and the *tert*-butyl hydrogen to the center-line axis (Table II); for TBOB, the effective diameter was taken as twice the distance from one of the methyl hydrogens of the *tert*-butyl group to the center-line axis.

Registry No. **1**, 119963-45-0; **2**, 119963-44-9; **3**, 132981-30-7; **4**, 99250-47-2; **5**, 132981-25-0; **6**, 132981-26-1; **7**, 132981-27-2; **8**, 132981-24-9; **9**, 119963-47-2; **10**, 119963-48-3; **11**, 132981-28-3; **12**, 133008-41-0; **13**, 132981-29-4; **15**, 132981-31-8; [^{35}S]-TBPS, 98774-25-5; *p*-$O_2NC_6H_4COCl$, 122-04-3; $CSCl_2$, 463-71-8; *o*-$O_2NC_6H_4COCl$, 610-14-0.

Benzyloxazolidine-2,4-diones as Potent Hypoglycemic Agents

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A series of benzyloxazolidine-2,4-diones, containing oxazole-based side chains, were found to lower blood glucose levels in the genetically obese ob/ob mouse. Incorporation of a benzofuran structural element in these compounds provides greatly enhanced in vivo potency. The syntheses and structure–activity relationships for this series are detailed.

Diabetes mellitus is a complex, chronic, progressive disease which eventually can adversely affect a number of organs, as well as the nervous and vascular systems. Of the estimated six million individuals diagnosed with dia-

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