Orally Active and Potent Inhibitors of γ-Aminobutyric Acid Uptake[1]

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3-Pyrrolidineacetic acid (**1a**), certain piperidinecarboxylic acids—i.e., 3-piperidinecarboxylic acid (**2a**), 1,2,5,6-tetrahydro-3-pyridinecarboxylic acid (**3a**), and *cis*-4-hydroxy-3-piperidinecarboxylic acid (**4a**)—*cis*-3-aminocyclohexanecarboxylic acid (**5a**, *cis*-3-ACHC), and γ-aminobutyric acid (**6a**, GABA) itself are among the most potent inhibitors of [^{3}H]GABA uptake by neurons and glia in vitro. These hydrophilic amino acids, however, do not readily enter the central nervous system in pharmacologically significant amounts following peripheral administration. We now report that *N*-(4,4-diphenyl-3-butenyl)-3-piperidinecarboxylic acid (**2b**) is a specific GABA-uptake inhibitor that is more potent, more lipophilic and, in limited testing, as selective as **2a**. Similar results were obtained with the *N*-(4,4-diphenyl-3-butenyl) derivatives of **1a**, **3a**, and **4a**. By contrast, *N*-(4,4-diphenyl-3-butenyl) derivatives of **5a** and **6a** were not more potent than the parent amino acids and appear to inhibit GABA uptake, at least in part, by a nonselective mechanism of action. The *N*-(4,4-diphenyl-3-butenyl)amino acids **1b–4b** exhibit anticonvulsant activity in rodents following oral or intraperitoneal administration [Yunger, L.M.; et al. *J. Pharmacol. Exp. Ther.* **1984**, *228*, 109].

γ-Aminobutyric acid (GABA) is a major inhibitory neurotransmitter in the mammalian central nervous system (CNS);[2-4] it may also act as a neurotransmitter in peripheral organs.[5,6] Because decreased GABAergic activity has been implicated in the pathophysiology of several CNS diseases,[7,8] research has been directed toward the discovery of GABAmimetic substances, e.g., GABA-receptor agonists, GABA-uptake inhibitors, and inhibitors of GABA metabolism,[9-12] which may be clinically useful. 3-Pyrrolidineacetic acid (**1a**, homo-β-proline), certain piperidinecarboxylic acids—i.e., 3-piperidinecarboxylic acid (**2a**, nipecotic acid), 1,2,5,6-tetrahydro-3-pyridinecarboxylic acid (**3a**, guvacine), and *cis*-4-hydroxy-3-piperidinecarboxylic acid (**4a**)—*cis*-3-aminocyclohexanecarboxylic acid (**5a**, *cis*-3-ACHC), and GABA (**6a**) are among the most potent inhibitors of [^{3}H]GABA uptake by neurons and glia in vitro.[13-17] GABA and **1a** also have high affinity for GABA receptors.[14] In vivo, iontophoretically administered **3a** enhances the duration of recurrent inhibition mediated by synaptically released GABA.[18] However, **1a–6a** do not readily enter the CNS of animals in pharmacologically significant amounts following peripheral administration, presumably due to their hydrophilic character.[19-23]

One strategy for introducing pharmacologically significant concentrations of GABA-uptake inhibitors into the CNS is to administer their esters. The esters, being more lipophilic, can enter the CNS where they may be hydrolyzed to the amino acids. For example, **2a** was detected in the CNS of mice following peripheral administration of ethyl (*R*)-**2a** but not of (*R*)-**2a**.[20] Peripheral administration of ethyl (*R*)-**2a** elevates GABA levels in synaptosomes[24] and esters of **2a–4a** are reported to display anticonvulsant activity in animals following peripheral administration.[14,19,20,23,25-27] A second strategy has been to increase CNS penetration by replacing the carboxylate group with other, less acidic, moieties. For example, modification of **3a** gave 4,5,6,7-tetrahydroisoxazolo[4,5-c]pyridin-3-ol (THPO), a GABA-uptake inhibitor that elevates synaptosomal GABA levels and protects mice from seizures following systemic administration.[14,23,24]

Our strategy to discover orally active GABA-uptake inhibitors was to identify and alkylate positions of bulk tolerance on **1a–6a** with lipophilic groups. We now report that appropriate N-alkylation of **1a–4a** yields specific GABA-uptake inhibitors that are more potent, more li-

Table I. *N*-(4,4-Diphenyl-3-butenyl)amino Acids and Related Compounds

no.	mp, °C	yield, purified %	purifn solvent	formula[a]
1b	95–101	59	H_2O	$C_{22}H_{25}NO_2\cdot 1^1/_4H_2O$
2b	184–186	65	$MeOH–Et_2O$	$C_{22}H_{25}NO_2\cdot HCl$
(*R*)-**2b**	209–211	41	Me_2CO	$C_{22}H_{25}NO_2\cdot HCl$
(*S*)-**2b**	209–211	42	Me_2CO	$C_{22}H_{25}NO_2\cdot HCl$
Et **2b**	169–170	62	Me_2CO	$C_{24}H_{29}NO_2\cdot HCl$
2c	190–191	65	Me_2CO	$C_{22}H_{27}NO_2\cdot HCl$
2d	228 dec	80	6 N HCl	$C_{21}H_{23}NO_2\cdot HCl$
2e	182–184	24	$Me_2CO–Et_2O$	$C_{23}H_{27}NO_2\cdot HCl\cdot {}^1/_4H_2O$
3b	178–180	18	$Me_2CO–EtOAc$	$C_{22}H_{25}NO_2\cdot HCl$
Me **3b**	oil	69		$C_{23}H_{25}NO_2$
4b	174–177	70	Et_2O	$C_{22}H_{25}NO_3\cdot HCl\cdot {}^1/_4H_2O$
5b	234–235.5	90	H_2O	$C_{23}H_{27}NO_2\cdot HCl$
6b	144–145 dec	26	$MeCN–Me_2CO–Et_2O$	$C_{20}H_{23}NO_2\cdot HCl$

[a] All compounds analyzed satisfactorily for C,H,N.

(1) This paper has been presented in part. See: Lafferty, J. J.; Bondinell, W. E.; Dandrige, P. A.; Kaiser, C.; McDevitt, J. T.; Moonsammy, G. I.; Rush, J. A.; Setler, P. E.; Yunger, L. M.; Zarevics, P.; Zirkle, C. "Abstracts of Papers", 185th National Meeting of the American Chemical Society, Seattle, March 20–25, 1983; American Chemical Society: Washington, DC, 1983; MEDI 65.
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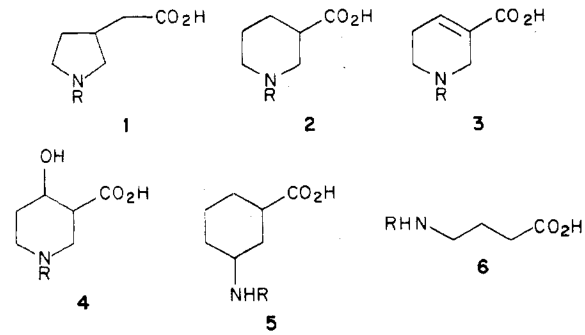
pophilic, and, in limited testing, at least as selective as the parent amino acids.

Chemistry. The methyl or ethyl esters of **1a–5a** were N-alkylated with 4,4-diphenyl-3-butenyl bromide to give the corresponding esters of **1b–5b**. The ethyl esters of **2d** and **2e** were prepared from ethyl **2a** and 3,3-diphenyl-2-propenyl bromide or 5,5-diphenyl-4-pentenyl bromide, respectively. Ethyl **2c** was prepared by catalytic reduction of ethyl **2b**. The esters of **1b–5b** and **2c–e** were hydrolyzed with 5–6 N HCl or saponified with 40% aqueous NaOH–MeOH to yield **1b–5b** and **2c–e** (Table I). (*R*)- and (*S*)-**2b** were prepared by diphenylbutenylation of ethyl (*R*)- and (*S*)-**2a** followed by hydrolysis (Table I).



a, R = H; **b**, R = $CH_2CH_2CH{=}CPh_2$; **c**, R = $CH_2CH_2CH_2CHPh_2$; **d**, R = $CH_2CH{=}CPh_2$; **e**, R = $CH_2CH_2CH_2CH{=}CPh_2$

Methyl 3-pyrrolidineacetate (methyl **1a**) was prepared from 1-benzyl-3-pyrrolidineacetonitrile by treatment with methanolic HCl followed by catalytic debenzylation. Methyl *cis*-4-hydroxy-3-piperidinecarboxylate (methyl **4a**) was prepared by $NaBH_4$ reduction of methyl 1-benzyl-4-oxo-3-piperidinecarboxylate, which gave a 1:1 mixture of diastereomeric alcohols as determined by GC–MS analysis. The diastereomers were separated by preparative HPLC to give the racemic cis and trans alcohols. The relative stereochemistry of the isomers was established by analysis of the vicinal coupling constants in the respective NMR spectra.[28] Methyl **4a** was obtained by catalytic debenzylation. A 6:1 ratio of cis and trans alcohols was obtained by $NaBH_4$ reduction of ethyl 1-(ethoxycarbonyl)-4-oxo-3-piperidinecarboxylate.[28] 5,5-Diphenyl-4-pentenyl bromide was obtained by treating 1,1-diphenyl-5-methoxy-1-pentanol, prepared from benzophenone and 4-methoxybutylmagnesium bromide, with HBr–HOAc.

N-(4,4-Diphenyl-3-butenyl)-γ-aminobutyric acid (**6b**) was prepared from *N*-benzyl-4,4-diphenyl-3-butenylamine and ethyl 4-bromobutyrate followed by debenzylation with BrCN and hydrolysis.

Table II. Inhibition of GABA and NE Accumulation

no.	GABA, IC_{50},[a] μM	no.	GABA, IC_{50},[a] μM	NE, IC_{50},[a] μM
1a	1.54 (1.23–1.94)	**1b**[c]	0.12 (0.10–0.15)	108.4 (98.3–118.5)
2a[b]	3.94 (2.55–6.08)	**2b**[d]	0.20 (0.16–0.26)	116.6 (102.8–130.4)
(*R*)-**2a**	1.69 (1.29–2.22)	(*R*)-**2b**	0.11 (0.08–0.13)	123.1 (117.9–128.2)
(*S*)-**2a**	9.88 (6.02–18.96)	(*S*)-**2b**	1.91 (1.67–2.17)	180.0 (172.5–187.5)
Et **2a**	52% (100)[e]	Et **2b**	+17% (100)[f]	
		2c	27% (10)[e]	
		2d	5% (10)[e]	
		2e	31% (1)[e]	
3a	4.92 (3.61–6.47)	**3b**[g]	0.20 (0.16–0.27)	58.2 (39.9–76.5)
Me **3a**	+4% (100)[f]	Me **3b**	43% (10)[e]	
4a[h]	4.56 (3.85–6.14)	**4b**[i]	0.26 (0.18–0.38)	33.6 (32.5–34.7)
5a	8.13 (5.07–13.01)	**5b**	54% (10)[e]	29.9 (29.6–30.2)
6a	2.60 (2.12–3.18)	**6b**	16.5 (13.2–20.6)	35.1 (28.3–41.9)
		imipramine	68% (100)[e]	0.027 (0.024–0.031)

[a] Numbers in parentheses represent Fieller's 95% confidence limits. [b] Inhibition of NE accumulation 16% at 1000 μM. [c] SK&F 100561. [d] SK&F 89976-A. [e] Percent inhibition (μM). [f] Percent increase (μM). [g] SK&F 100330-A. [h] Inhibition of NE accumulation 7% at 100 μM. [i] SK&F 100591-A.

Results

The ability of the amino acids **1a–6a** and of the *N*-(4,4-diphenyl-3-butenyl)amino acids **1b–6b** to inhibit sodium-dependent, high-affinity [^{3}H]GABA uptake was measured after preincubation for 15 min with rat brain synaptosomes and is expressed as IC_{50} values (Table II). The amino acids **1a–6a** had IC_{50}s between 1 and 9 μM in our assay; individual IC_{50}s are equal to (**1a**, **2a**, **4a**, **6a**) or 2–4-fold lower than (**3a**, **5a**) literature values measured in rat brain synaptosomes.[14,29,30] The key observation to be drawn from Table II is that *N*-(4,4-diphenyl-3-butenyl)-3-pyrrolidineacetic acid (**1b**) and the *N*-(4,4-diphenyl-3-butenyl)-3-piperidinecarboxylic acids **2b–4b** were, respectively, 12-, 19-, 24-, and 17-fold more potent that **1a–4a** and had IC_{50}s ranging from 120 to 260 nM. In contrast, *cis*-*N*-(4,4-diphenyl-3-butenyl)-3-aminocyclohexanecarboxylic acid (**5b**) was comparable in potency to **5a** while *N*-(4,4-diphenyl-3-butenyl)-γ-aminobutyric acid (**6b**) was less potent than GABA as an inhibitor of GABA uptake. Among the amino acid esters, ethyl **2a** was a weak inhibitor, as reported,[31] while methyl **3a** had no effect on GABA uptake at 100 μM. Likewise, esterification of **2b** and **3b**

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Table III. Interaction of Selected *N*-(4,4-Diphenyl-3-butenyl)amino Acids with GABA Receptors, GAD, GABA-T, and Benzodiazepine Receptors

compd	inhibn of binding, % (μM) [³H]muscimol	[³H]diazepam	enzyme inhibn, % (mM) GAD	GABA-T
1a	0.33 (0.24–0.45)[a]			
1b	20 (100)			
2a	164 (151–178)[a]		0 (1)	3 (1)
2b[b]	8 (100)	21 (100)	7 (1)	12 (1)
3a	27 (100)		0 (1)	7 (1)
3b	8 (100)	5 (100)	7 (1)	9 (1)
4a	10 (100)			
4b	48 (100)			
6a	0.029 (0.022–0.039)[a]			
6b	35 (100)			
muscimol	0.004 (0.003–0.006)[a]			
chlordiazepoxide		74 (1)		
isoniazide			42 (1)	
(aminooxy)acetic acid				83 (0.1)

[a] IC_{50}, μM, numbers in parentheses represent Feiller's 95% confidence limits. [b] [³H]WB4101 binding (α_1) displaced 12% at 10 μM; [³H]spiroperidol binding (D_2) displaced 22% at 50 μM; [³H]QNB binding (muscarinic cholinergic) displaced 12% at 100 μM; [³H]serotonin binding (5-HT_1) displaced 16% at 33 μM.

gave ethyl **2b**, which did not inhibit GABA uptake at 100 μM, and methyl **3b**, which was markedly less potent than **3b**.

The next step in evaluating **1b**–**6b** was to determine whether or not they were selective and specific inhibitors of GABA uptake.[32] The mechanism, i.e., selective or nonselective, by which **1b**–**6b** inhibit GABA uptake was assessed by comparing their IC_{50}s for GABA uptake with their IC_{50}s for *l*-norepinephrine (NE) uptake by rat brain synaptosomes. The results (Table II) show that **2a**, **4a**, and **1b**–**4b** were much more potent as inhibitors of GABA uptake than NE uptake while the reverse was true for imipramine. In contrast, **5b** and **6b** were only slightly more potent as inhibitors of GABA vs. NE uptake.

One criterion of specificity, stereoselectivity, was applied to **2b**. (*R*)- and (*S*)-**2b** were synthesized and their GABA-uptake inhibitory potencies were measured and compared with those of **2b** and of **2a**, (*R*)-**2a**, and (*S*)-**2a**.[31] The results, shown in Table II, indicate that the inhibitory activity of **2a** and of **2b** resides chiefly in their *R* enantiomers. Thus, (*R*)-**2a** was 5-fold more potent than (*S*)-**2a**, as reported,[31] while (*R*)-**2b** was 17-fold more potent than (*S*)-**2b**.

Having established the selectivity and specificity of action of **1b**–**4b** (see the Discussion section), the structure–activity relationships (SAR) of N-substituents on **2** were explored to determine what features of the diphenylbutenyl group contribute to the high inhibitory potency of **2b** and to develop more potent GABA-uptake inhibitors. These studies show, in part, that reduction of the olefinic double bond (**2c**) or shortening of the ethylene bridge (**2d**) in **2b** resulted in dramatic decreases in potency. Increasing the length of the ethylene bridge (**2e**) resulted in a smaller, but still significant, decrease in potency (Table II).

We next began to assess the selectivity of **1b**–**4b** as GABA-uptake inhibitors vs. their effect on other GABA receptors and binding sites by measuring their ability to displace [³H]muscimol binding from GABA receptors[33–37]

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Table IV. Dissociation Constants and Distribution Coefficients of **2a** and **2b**

no.	pK_a (CO_2H)[a]	pK_a (N)[a]	log *D*
2a	3.45[b]	10.32[b]	−2.66[c]
2b	3.32	9.36	1.14[d,e]

[a] Determined by the method of Albert and Sergeant.[38] [b] Similar values reported by Krogsgaard-Larsen et al.[14] [c] Reported by Yunger and Cramer.[39] [d] Determined by the method of Purcell.[40] [e] Log *P* = 3.10.

and by studying their effect on glutamic acid decarboxylase (GAD) and GABA transaminase (GABA-T). The results, tabulated in Table III, show that the *N*-(diphenylbutenyl)-3-piperidinecarboxylic acids **2b**–**4b** had low affinity for GABA receptors labeled by [³H]muscimol on rat synaptosomal membranes. Low affinity was also observed for **2a**–**4a** and reported for **2a**–**3a**.[37] *N*-(Diphenylbutenyl)-3-pyrrolidineacetic acid (**1b**) and *N*-(diphenylbutenyl)-γ-aminobutyric acid (**6b**) had low affinity for these GABA receptors while **1a** and GABA[37] had high affinity (Table III). Similar results have been reported for **1a**–**4a** and GABA utilizing displacement of [³H]GABA binding.[14] Studies with GAD and GABA-T (Table III) showed that the 3-piperidinecarboxylic acids **2a**, **2b**, **3a**, and **3b** had little or no effect on these enzymes at concentrations up to 1 mM.

Affinity for benzodiazepine receptors on rat synaptosomal membranes labeled with [³H]diazepam was measured because GABA and benzodiazepine receptors in the CNS can be cooperatively linked.[35,36] The results, shown in Table III, indicate that **2b** and **3b** had low affinity for these receptors. Finally the affinity of **2b** for several neurotransmitter receptors was determined; **2b** had low affinity for α_1-adrenergic, D_2-dopaminergic, muscarinic cholinergic, and 5-HT_1-serotoninergic receptors on rat synaptosomal membranes (Table III, footnote *b*).

Finally, selected physical properties of **2a** and **2b** were measured and are presented in Table IV. The pK_as of the carboxylic acid groups in **2a** and **2b** were similar while the pK_a of the amino nitrogen in **2b** was a log unit lower than that in **2a**. The distribution coefficient of **2b**, log *D* = 1.14 (1-octanol/phosphate buffer, pH 7.4), was 3 orders of magnitude higher than that of **2a**, log *D* = −2.66.

Discussion

The *N*-(4,4-diphenyl-3-butenyl)amino acids **1b**–**6b** are divided into two groups by the GABA-uptake inhibitory potency data, i.e., the potent, tertiary heterocyclic amines **1b**–**4b** and the less active secondary amines **5b** and **6b**. The next step in evaluating **1b**–**6b** was to determine whether or not they are selective and specific inhibitors of GABA uptake. The first test of selectivity requires that **1b**–**6b** inhibit GABA uptake by a selective vs. a nonselective mechanism of action, e.g., a nonselective inhibitor might disrupt all active uptake processes by an effect on the synaptosomal membrane. Therefore, the IC_{50} (GABA uptake) values of **1b**–**6b** were compared with their IC_{50} values for inhibition of NE uptake. The reasoning was that compounds whose IC_{50} (GABA uptake) and IC_{50} (NE uptake) are similar and in the micromolar range are probably inhibiting the uptake of these structurally dissimilar neurotransmitters by a nonselective mechanism. The wide

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differences in uptake inhibitory potencies displayed by **2a**, **4a**, and **1b**–**4b** show these compounds to be selective inhibitors of GABA uptake vs. NE uptake. By contrast, **5b** and **6b** are only slightly more potent as GABA-uptake inhibitors, suggesting that these compounds inhibit GABA uptake, at least in part, by a nonselective mechanism of action.

Specific inhibition of GABA uptake requires a structure-dependent interaction of the inhibitor with a macromolecular recognition site while nonspecific inhibition is induced solely by the physical properties of the inhibitor.[32] For example, two lines of evidence show that **2a** is a specific inhibitor of GABA uptake. First, **2a** is more potent than predicted by its physical properties alone.[32] Second, **2a** displays some stereoselectivity in its inhibition of GABA uptake, i.e., (*R*)-**2a** is 5-fold more potent than (*S*)-**2a**.[31] The macromolecular recognition site at which **2a** exerts its inhibition of GABA uptake may be the GABA binding site on the GABA uptake carrier. This is suggested by reports that the affinity of **2a** for the GABA binding site on the GABA-uptake carrier, as measured by sodium-dependent [^{3}H]-**2a** binding and by the ability of **2a** to displace sodium-dependent [^{3}H]GABA binding, is comparable to its GABA uptake inhibitory potency.[41,42] By contrast, chlorpromazine may be a nonspecific inhibitor of GABA uptake since its inhibitory potency, IC_{50} (GABA uptake) = 20 μM, is comparable to that predicted by its physical properties alone.[32] Furthermore, chlorpromazine has low affinity for the GABA binding site on the GABA-uptake carrier, IC_{50} (sodium-dependent GABA binding) = 600 μM, suggesting that it does not block GABA uptake at this site.[42] One of the criteria for specificity, i.e., stereoselectivity, was applied to **2b**. The *R* and *S* enantiomers of **2b** display a higher degree of stereoselectivity than those of **2a**, consistent with a specific mechanism of GABA-uptake inhibition. By contrast, **2b**, (*R*)-**2b**, and (*S*)-**2b** are less potent and nearly equiactive as inhibitors of NE uptake. The specificity of **1b**, **3b**, and **4b** as inhibitors of GABA uptake is suggested by their high inhibitory potency.

Since **1b**–**4b** inhibit GABA uptake by a selective and specific mechanism(s), the results in Table II for these compounds can be rationalized in structural terms by attributing their recognition and part of their affinity for the site(s) mediating GABA uptake to the amino acid moieties **1a**–**4a**. Any specific inhibition of GABA uptake by **5b** and **6b** would also be attributed to their amino acid moieties **5a** and **6a**. Several pieces of data support this intuitive assignment for **2b**. First, pK_a data (Table IV) show that **2b** retains the acidic carboxyl group and basic amino nitrogen believed to be important for the GABA-uptake inhibitory potency of **2a**.[14] Second, the inhibitory potencies of **2a** and **2b** and of **3a** and **3b** are greatly reduced or abolished by esterification (Table II). Even the weak inhibitory activity of the esters of **2a** and **3b** may reflect partial hydrolysis to the acid. Finally, the observation that the inhibitory potencies of **2a** and **2b** reside chiefly in their *R* enantiomers is consistent with a similar mode of binding to a common site of action.

The enhanced GABA-uptake inhibitory potency of **1b**–**4b** compared to that of **1a**–**4a** is attributed to a net increase in binding energy arising from the interaction of the diphenylbutenyl moieties with the site(s) of action. The interaction appears to have definite structural requirements, at least in the case of **2b**, since changes in the structure of the 4,4-diphenyl-3-butenyl group, as in **2c**–**e**, decrease inhibitory potency. In a different vein, the GABA-uptake inhibitory potency of **2b** and **3b** was not increased by a limited set of aryl substituents or by replacing one of the phenyl groups with a 2-thienyl group.[43]

Relative to interaction with other GABA receptors and binding sites, the low affinity of **1b** and **6b** for GABA receptors labeled by [^{3}H]muscimol (Table III) is attributed to an effect of the diphenylbutenyl groups since **1a** and GABA have high affinities for these sites. Perhaps the diphenylbutenyl moiety cannot be accommodated by the receptor or it may alter the conformation of the amino acid moieties in **1b** and **6b** so that they can no longer contribute to binding. The low affinities of **2b**–**4b** may be attributed to the diphenylbutenyl group or to the amino acid moieties since **2a**–**4a** have low affinities for these receptors. The inability of **2b** and **3b** to inhibit GAD or GABA-T (Table III) and their low affinity for benzodiazepine receptors are attributed to the amino acid moieties, since **2a** and **3a** have little or no effect on these enzymes at 1 mM (Table III) or on [^{3}H]diazepam binding.[44]

The increase in the distribution coefficient of **2b** as compared to **2a** (Table IV) is attributed to direct and indirect effects of the diphenylbutenyl group. The indirect effect operates through the decrease in the pK_a of the amino nitrogen in **2b** vs. **2a**, which decreases the percentage of N-protonated, and hence more hydrophilic, species in solution at physiological pH.[14] The distribution coefficient of **2b** is consistent with facile entry into the CNS by passive diffusion across the blood–brain barrier.[45]

The in vitro properties of **1b**–**4b** are reflected in their in vivo profiles. The increased lipophilicity of **2b**, and, presumably, **1b**, **3b**, and **4b**, explains, at least in part, their ability to enter the CNS, as evidenced by their ability to protect rodents from seizures following oral administration.[43] A combination of increased lipophilicity and high GABA-uptake inhibitory potency can also explain the ability of **1b**–**3b** to enhance central GABAergic activity. The latter is demonstrated by the ability of orally administered **1b**–**3b** to potentiate contralateral turning induced in rats by unilateral injection of GABA[46,47] and by the ability of **2b** and **3b** to block the tonic phase of seizures induced by the GABA-receptor antagonist bicuculline[34–36] but not by the glycine-receptor antagonist strychnine.[43] The data in Table III suggest that the central effects of **2b** and **3b** are not due to interaction with GAD, GABA-T, or receptors labeled by diazepam or, for **1b**–**4b**, muscimol. It is anticipated that orally active and potent GABA-uptake inhibitors such as **1b**–**4b** may be useful in elucidating the role of GABA in physiology and pathophysiology.

Experimental Section

Chemistry. Melting points were determined in open capillary tubes with a Thomas-Hoover Uni-Melt apparatus and are un-

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corrected. Elemental analyses were performed by the Analytical, Physical and Structural Chemistry Department of Smith Kline & French Laboratories. Where analyses are reported by symbols of the elements, results were within ±0.4% of the theoretical values. NMR spectra were obtained on a Varian EM-90 or a Perkin-Elmer R-24 spectrometer (Me_4Si). Mass spectra were recorded with a Hitachi Perkin-Elmer RMU-65 spectrometer. IR spectra were recorded on a Perkin-Elmer infrared spectrophotometer. IR and NMR spectral data were recorded for all numbered or named compounds and were judged to be consistent with the assigned structures. Optical rotations were measured with a Perkin-Elmer 241MC polarimeter. GC/mass spectral analysis was carried out on a Finnegan Model 3300; GC column 10% OV-17; 100/120 Chrom W (HP), 2.44 m × 2 mm, 190 °C; trimethylsilyl derivatives prepared with BSTFA (1% TMCS) in MeCN. Solutions were dried over Na_2SO_4 or $MgSO_4$ and concentrated with a Büchi rotary evaporator under water aspirator pressure. Silica gel (230–400 mesh) obtained from E. Merck was used for column chromatography. Jobin Yvon Chromatospac Prep column was packed with silica gel (15–60 μm) purchased from E. Merck. Preparative HPLC separations were carried out on a Waters Associates Prep LC/System 500 packed with Prep PAK 500 silica.

Methyl 1-Benzyl-3-pyrrolidineacetate Cyclohexylsulfamate. 1-Benzyl-3-pyrrolidineacetonitrile (12 g, 60 mmol)[48] was dissolved in 250 mL of MeOH saturated with HCl. After 16 h, the solvent was evaporated and the residue partitioned between $CHCl_3$ and 10% aqueous NaOH. The $CHCl_3$ phase was washed, dried, and concentrated. The residue was treated with cyclohexylsulfamic acid (10.7 g, 60 mmol) and the resulting solid was recrystallized three times from *i*-PrOH–Et_2O to yield 16 g (64%) of product: mp 102.5–103 °C. Anal. ($C_{20}H_{32}N_2O_5S$) C, H, N.

Methyl 3-Pyrrolidineacetate Cyclohexylsulfamate (Methyl 1a Cyclohexylsulfamate). Methyl 1-benzyl-3-pyrrolidineacetate cyclohexylsulfamate (10 g, 24 mmol) and 10% Pd/C (1 g) in 100 mL of EtOH were shaken under H_2 (60 psi) for 1 h at 50 °C. The mixture was cooled, filtered, and concentrated. The residue was crystallized from EtOH–Et_2O and then recrystallized twice from MeCN–Et_2O to yield 2.8 g (36%) of product: mp 68.5–69.5 °C. Anal. ($C_{13}H_{26}N_2O_5S$) C, H, N.

4,4-Diphenyl-3-butenyl Bromide.[49] Cyclopropyldiphenylcarbinol (101 g, 0.45 mol) was added to 400 mL of 48% aqueous HBr stirred at 5 °C. After 4 h, the mixture was extracted with CH_2Cl_2. The combined CH_2Cl_2 phases were washed, dried, and concentrated to give 128 g (99%) of product, which was used without further purification.

***N*-(4,4-Diphenyl-3-butenyl)-3-pyrrolidineacetic Acid (1b).** A mixture of methyl 3-pyrrolidineacetate cyclohexylsulfamate (44.5 g, 0.138 mol), 4,4-diphenyl-3-butenyl bromide (40.3 g, 0.14 mol), and K_2CO_3 (38.2 g, 0.276 mol) in 400 mL of Me_2CO was stirred and heated to reflux for 43 h. The mixture was cooled, filtered, and concentrated. The residue was partitioned between Et_2O and 1 N HCl. The aqueous phase was made alkaline with Na_2CO_3 and extracted with Et_2O, which was washed, dried, and concentrated. The residue was chromatographed with a Jobin Yvon Chromatospac on SiO_2 ($CHCl_3$–MeOH, 98:2) to yield 22.4 g (46%) of methyl *N*-(4.4-diphenyl-3-butenyl)-3-pyrrolidineacetate (methyl **1b**). A sample was treated with maleic acid and crystallized from EtOAc and then from toluene to give methyl *N*-(4,4-diphenyl-3-butenyl)-3-pyrrolidineacetate maleate (methyl **1b** maleate): mp 100.5–101.5 °C. Anal. ($C_{27}H_{31}NO_6$) C, H, N.

A suspension of methyl **1b** (17.9 g, 51 mmol) in 240 mL of 5 N HCl was stirred and heated under reflux for 16 h. The reaction was cooled, the aqueous phase was decanted, and the residue was treated with 15 mL of concentrated aqueous NH_4OH. The resulting solid was recrystallized twice from H_2O to give 10.7 g of **1b**.

***N*-(4,4-Diphenyl-3-butenyl)-3-piperidinecarboxylic Acid Hydrochloride (2b·HCl).** A mixture of ethyl 3-piperidinecarboxylate (ethyl **2a**; 3.1 g, 20 mmol), 4,4-diphenyl-3-butenyl bromide (5.7 g, 20 mmol), and K_2CO_3 (2.7 g, 20 mmol) in 100 mL of Me_2SO was stirred for 96 h. The mixture was diluted with H_2O and extracted with Et_2O. The Et_2O phase was washed, dried, and treated with HCl gas, and the resulting solid was recrystallized twice from Me_2CO to give 5 g of ethyl *N*-(4,4-diphenyl-3-butenyl)-3-piperidinecarboxylate hydrochloride (ethyl **2b**·HCl). Later preparations were carried out in refluxing Me_2CO as described for ethyl (*R*)-**2b**·HCl.

A suspension of ethyl **2b**·HCl (106 g, 0.265 mol) in 1.3 L of 5 N HCl was stirred and heated under reflux for 17 h and cooled and the aqueous phase decanted. The viscous residue was crystallized from Me_2CO and then from MeOH–Et_2O to give 64 g of **2b**·HCl.

(*R*)-*N*-(4,4-Diphenyl-3-butenyl)-3-piperidinecarboxylic Acid Hydrochloride [(*R*)-2b·HCl]. Ethyl (*R*)-**2a**[31] (7.8 g, 50 mmol), 4,4-diphenyl-3-butenyl bromide (14.4 g, 50 mmol), K_2CO_3 (13.8 g, 0.1 mol), and KI (0.2 g) in 150 mL of Me_2CO were stirred and heated under reflux for 20 h. The mixture was cooled and filtered and the filtrate was treated with HCl gas, concentrated, and treated with Et_2O. The resulting solid was recrystallized from Me_2CO to give 14.2 g (71%) of ethyl (*R*)-**2b**·HCl: mp 119–120 °C; $[\alpha]^{24}_{589}$ +8.3°, $[\alpha]^{24}_{578}$ +7.9°, $[\alpha]^{24}_{546}$ +8.6° (*c* 2, MeOH).

Ethyl (*R*)-**2b**·HCl (12.6 g, 31.6 mmol) was converted to (*R*)-**2b**·HCl as described for **2b**·HCl and the product crystallized from Me_2CO to give 4.8 g of (*R*)-**1b**·HCl: $[\alpha]^{25}_{589}$ +1.0°, $[\alpha]^{25}_{578}$ +0.9°, $[\alpha]^{25}_{546}$ +0.85° (*c* 5, MeOH).

(*S*)-*N*-(4,4-Diphenyl-3-butenyl)-3-piperidinecarboxylic Acid Hydrochloride [(*S*)-2b·HCl]. Ethyl (*S*)-**2a**[31] (7.8 g, 50 mmol) was converted to ethyl (*S*)-**2b**·HCl, 15.2 g (76%), as described for the *R* isomer: mp 119–120 °C; $[\alpha]^{24}_{589}$ −7.9°, $[\alpha]^{24}_{578}$ −8.1°, $[\alpha]^{24}_{546}$ −8.7° (*c* 2, MeOH). Ethyl (*S*)-**2b**·HCl (13 g, 32.5 mmol) was hydrolyzed as described for the *R* isomer and the product crystallized from Me_2CO to give 5.1 g of (*S*)-**1b**·HCl: $[\alpha]^{25}_{589}$ −1.16°, $[\alpha]^{25}_{578}$ −1.12°, $[\alpha]^{25}_{546}$ −1.0° (*c*, 5, MeOH).

***N*-(4,4-Diphenylbutyl)-3-piperidinecarboxylic Acid Hydrochloride (2c·HCl).** Ethyl **2b**·HCl (6 g, 15 mmol) in 200 mL of EtOH containing 10% Pd/C (0.4 g) was shaken under H_2 (50 psi) and filtered, and the filtrate was concentrated. The residue was crystallized from Me_2CO to give 4.6 g (76%) of ethyl *N*-(4,4-diphenylbutyl)-3-piperidinecarboxylate hydrochloride (ethyl **2c**·HCl): mp 140–142 °C. Ethyl **2c**·HCl was hydrolyzed in refluxing 6 N HCl and concentrated and the residue crystallized twice from Me_2CO to give 2.8 g of **2c**·HCl.

***N*-(3,3-Diphenyl-2-propenyl)-3-piperidinecarboxylic Acid Hydrochloride (2d·HCl).** Ethyl **2a** (0.55 g, 3.56 mmol), 3,3-diphenyl-2-propenyl bromide[49] (1.08 g, 4 mmol), and K_2CO_3 (0.42 g) in 25 mL of Me_2CO were stirred and heated under reflux for 16 h. The mixture was cooled and filtered, and the filtrate was concentrated. The residue was partitioned between Et_2O and 10% aqueous HCl. The aqueous phase was basified with 10% aqueous NaOH and extracted with CH_2Cl_2; the CH_2Cl_2 phase was concentrated and the residue chromatographed on SiO_2 (EtOAc–hexane, 1:9) to give 0.6 g (48%) of ethyl 1-(3,3-diphenyl-2-propenyl)-3-piperidinecarboxylate (ethyl **2d**).

Ethyl **2d** dissolved in 10 mL of 6 N HCl was stirred and heated under reflux for 1 h, cooled, and filtered to give 0.49 g of **2d**·HCl.

5,5-Diphenyl-4-pentenyl Bromide. 1-Bromo-4-methoxybutane (26.7 g, 160 mmol) in 300 mL of dry Et_2O was added dropwise to Mg turnings (3.9 g, 160 mmol) in 80 mL of Et_2O and the mixture was heated under reflux for 4 h. The mixture was cooled and a solution of benzophenone (14.6 g, 80 mmol) in 100 mL of dry Et_2O was added with stirring. The mixture was stirred at 25 °C for 18 h and cautiously quenched with H_2O and the Et_2O phase was dried and concentrated. The residue was crystallized from hexane–EtOAc to give 12.7 g (59%) of 1,1-diphenyl-5-methoxy-1-pentanol: mp 112–115 °C. A mixture of the pentanol (8.88 g, 32.8 mmol), 132 mL of HOAc, and 66 mL of distilled 48% aqueous HBr was stirred at 25 °C for 2 h and heated under reflux for 90 min. The mixture was cooled, diluted with ice and H_2O, and extracted with Et_2O. The combined Et_2O extracts were washed with 5% aqueous $NaHCO_3$ and H_2O, dried, and concentrated to give a brown oil. The oil was dissolved in hexane, stirred with SiO_2, filtered, and concentrated in vacuo to yield 3.2 g of product.

***N*-(5,5-Diphenyl-4-pentenyl)-3-piperidinecarboxylic Acid Hydrochloride (2e·HCl).** Ethyl **2a** (1.56 g, 10 mmol), 5,5-di-

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phenyl-4-pentenyl bromide (3 g, 10 mmol), and K_2CO_3 (2.76 g, 20 mmol) in 40 mL of Me_2CO were stirred and heated under reflux for 18 h. The mixture was cooled, filtered, and concentrated and 1 g of the resulting oil (3.5 g) was chromatographed on SiO_2 (EtOAc–hexane, 1:4) to give 0.6 g (16%) of ethyl *N*-(5,5-diphenyl-4-pentenyl)-3-piperidinecarboxylate (ethyl **2e**). A mixture of ethyl **2e** (0.6 g, 1.6 mmol), 20 mL of 6 N HCl, and 5 mL of *n*-BuOH was heated under reflux for 23 h, and concentrated. The resulting oil was stirred with Et_2O and crystallized twice from Me_2CO–Et_2O to give 0.15 g of **2e**·HCl.

***N*-(4,4-Diphenyl-3-butenyl)-1,2,5,6-tetrahydro-3-pyridinecarboxylic Acid Hydrochloride (3b·HCl).** Methyl 1,2,5,6-tetrahydro-3-pyridinecarboxylate[50] (methyl **3a**; 5 g, 28 mmol), 4,4-diphenyl-3-butenyl bromide (8.1 g, 28 mmol), and K_2CO_3 (10 g, 72 mmol) in 200 mL of Me_2CO were stirred and heated under reflux for 15 h. The mixture was cooled and filtered, and the filtrate was concentrated and partitioned between H_2O and EtOAc. The EtOAc phase was washed, dried, and concentrated, and the residue was chromatographed on SiO_2 (hexane; hexane–EtOAc, 9:1, 4:1) to give 6.75 g of methyl *N*-(4,4-diphenyl-3-butenyl)-1,2,5,6-tetrahydro-3-pyridinecarboxylate (methyl **3b**). Methyl **3b** (159 g, 0.46 mol) in 2.3 L of MeOH and 500 mL of 40% aqueous NaOH was stirred and heated under reflux for 45 min, cooled, and concentrated. The residue was diluted with H_2O and extracted with Et_2O. The aqueous phase was acidified with 3 N HCl and extracted with EtOAc and the EtOAc phase was washed, dried, and concentrated. The residue was triturated with Me_2CO, recrystallized from MeOH–Me_2CO–Et_2O, and triturated with Me_2CO–EtOAc (2:1) to give 31.8 g of **3b**·HCl.

Methyl *cis*-1-Benzyl-4-hydroxy-3-piperidinecarboxylate. $NaBH_4$ (7.4 g, 194 mmol) in 150 mL of EtOH was added dropwise to a solution of methyl 1-benzyl-4-oxo-3-piperidinecarboxylate (24 g, 97 mmol) in 160 mL of EtOH stirred at 0 °C. After 10 min, the mixture was diluted with H_2O, concentrated, and partitioned between $CHCl_3$ and H_2O, and the $CHCl_3$ phase was concentrated. A sample was trimethylsilylated and analyzed by GC/MS: GC t_R (peak 1) 30 min (50%), (peak 2) 33 min (50%); MS (peaks 1 and 2), *m/e* 322 (M + H). The residue was chromatographed on SiO_2 (EtOAc–cyclohexane, 1:1) to give a 1:1 mixture of cis and trans isomers of methyl 1-benzyl-4-hydroxy-3-piperidinecarboxylate, which were separated by HPLC on SiO_2 ($CHCl_3$–MeOH–NH_4OH, 98:2:0.2) to give 3.7 g (15%) of methyl *cis*-1-benzyl-4-hydroxy-3-piperidinecarboxylate [^{1}H NMR (90 MHz, $CDCl_3$) δ 4.18 (ddd, 1, $J_{4e,5a} \simeq 3.5$ Hz, $J_{4e,5e} \simeq 4.0$ Hz, $J_{4e,3a} \simeq 2.5$ Hz, H-4)] and 3.6 g (15%) of methyl *trans*-1-benzyl-4-hydroxy-3-piperidinecarboxylate [^{1}H NMR (360 MHz, $CDCl_3$) δ 3.77 (ddd, 1, $J_{4a,5a} = 10.8$ Hz, $J_{4a,5e} = 4.7$ Hz, $J_{4a,3a} = 9.8$ Hz, H-4)].

Methyl *cis*-4-Hydroxy-3-piperidinecarboxylate Hydrochloride (Methyl 4a·HCl). Methyl *cis*-1-benzyl-4-hydroxy-3-piperidinecarboxylate (3.7 g, 14.8 mmol) was converted to the hydrochloride, dissolved in 150 mL of MeOH containing 10% Pd/C (0.7 g), and shaken with H_2 (50 psi) for 1.5 h. The mixture was filtered and concentrated to yield 2.6 g (87%) of product: mp 149–151 °C.

***cis*-*N*-(4,4-Diphenyl-3-butenyl)-4-hydroxy-3-piperidinecarboxylic Acid Hydrochloride (4b·HCl).** Methyl *cis*-4-hydroxy-3-piperidinecarboxylate (1.6 g, 8.3 mmol), 4,4-diphenyl-3-butenyl bromide (3 g, 10.4 mmol), K_2CO_3 (1.5 g, 19 mmol), and KI (0.2 g) in 50 mL of DMF were stirred and heated under reflux for 18 h. The mixture was cooled, poured onto a mixture of ice and 5% aqueous $NaHCO_3$, and extracted with hexane. The combined hexane extract was washed, dried, and concentrated, and the residue was triturated with Et_2O to give 1.4 g (46%) of methyl *N*-(4,4-diphenyl-3-butenyl)-*cis*-4-hydroxy-3-piperidinecarboxylate (methyl **4b**). Methyl **4b** (1.4 g, 3.8 mmol) in 50 mL of 6 N HCl was stirred and heated under reflux for 2 h. The mixture was decolorized with charcoal, concentrated, dried by azeotroping with toluene, and concentrated. The residue was triturated with Et_2O to afford 1 g of **4b**·HCl.

***cis*-*N*-(4,4-Diphenyl-3-butenyl)-3-aminocyclohexanecarboxylic Acid Hydrochloride (5b·HCl).** A mixture of ethyl *cis*-3-aminocyclohexanecarboxylate[51] (7.1 g, 41.5 mmol), 4,4-diphenyl-3-butenyl bromide (11.9 g, 41.5 mmol), and K_2CO_3 (11.5 g, 83 mmol) in 225 mL of DMF was stirred at 25 °C for 72 h. The mixture was poured onto 600 mL of ice and extracted with toluene, and the combined toluene extracts were washed, dried, and concentrated. The residue was chromatographed by HPLC on SiO_2 (CH_2Cl_2–MeOH–NH_4OH, 97:3:0.2) and the product was converted to the hydrochloride and recrystallized from *i*-PrOH to afford 4.1 g (24%) of ethyl *cis*-*N*-(4,4-diphenyl-3-butenyl)-3-aminocyclohexanecarboxylate hydrochloride (ethyl **5b**·HCl): mp 156.5–158 °C. Anal. ($C_{25}H_{32}ClNO_2$) C, H, N. A suspension of ethyl **5b**·HCl (3.8 g, 9.2 mmol) in 44 mL of 5 N HCl was stirred and heated at reflux for 17 h and cooled. The aqueous phase was decanted and the viscous residue was crystallized from H_2O to give 3.2 g of **5b**·HCl.

***N*-(4,4-Diphenyl-3-butenyl)-γ-aminobutyric Acid Hydrochloride (6b·HCl).** 4,4-Diphenyl-3-butenyl bromide (10.4 g, 36 mmol) and NaN_3 (11.3 g, 174 mmol) in 50 mL of Me_2SO was heated to 90 °C for 30 min, poured into H_2O, and extracted with Et_2O. The Et_2O extracts were combined, washed, dried, and concentrated to give 4,4-diphenyl-3-butenyl azide. The azide was dissolved in 100 mL of EtOAc containing 10% Pd/C (1 g) and shaken under H_2 (50 psi) for 30 min. The mixture was filtered and concentrated. The residue was treated with ethereal HCl and recrystallized from EtOAc–Et_2O to give 4,4-diphenyl-3-butenylamine hydrochloride. The amine hydrochloride (9.0 g, 34 mmol) was converted to the free base. The base was refluxed azeotropically with benzaldehyde (4.4 g, 42 mmol) in 250 mL of toluene for 16 h. The mixture was concentrated, dissolved in 100 mL of MeOH, stirred at 0 °C, and treated with $NaBH_4$ (1.4 g) for 0.5 h. The reaction was quenched with HOAc, concentrated, and partitioned between dilute aqueous NH_4OH and EtOAc. The EtOAc phase was washed, dried, and concentrated. The residue was treated with ethereal HCl and recrystallized from EtOAc–Et_2O to give *N*-benzyl-4,4-diphenyl-3-butenylamine hydrochloride: mp 180.5–181.5 °C. Anal. ($C_{23}H_{24}ClN$) C, H, N.

A mixture of the *N*-benzylamine hydrochloride (4 g, 11.4 mmol), ethyl 4-bromobutyrate (2.2 g, 11.4 mmol), and K_2CO_3 (10 g) in 100 mL of DMF was refluxed for 2 h, poured into H_2O, and extracted with Et_2O. The Et_2O phase was washed, dried, and concentrated. The residue was chromatographed on SiO_2 (hexane–EtOAc, 9:1) to yield 4 g of ethyl *N*-benzyl-*N*-(4,4-diphenyl-3-butenyl)-γ-aminobutyrate. This amino acid ester (3 g, 7 mmol) was treated with BrCN (0.9 g, 8.9 mmol) in 100 mL of toluene at 50 °C under Ar. The mixture was stirred for 1 h, concentrated, and chromatographed on SiO_2 (EtOAc–hexane, 1:9) to yield 2.28 g of ethyl *N*-cyano-*N*-(4,4-diphenyl-3-butenyl)-γ-aminobutyrate. The *N*-cyano ester (2.28 g, 6.3 mmol) in 110 mL of 6 N H_2SO_4 was heated under reflux for 4 h. The mixture was diluted with H_2O, basified with aqueous NH_4OH, and extracted with Et_2O. The aqueous phase was acidified with aqueous HCl and the resulting solid was filtered and heated under reflux with 100 mL of 20% aqueous NaOH–MeOH (2:1). The mixture was cooled, acidified with 10% aqueous HCl, and extracted five times with EtOAc. The combined EtOAc extracts were dried and concentrated and the residue was crystallized from MeCN–Me_2CO–Et_2O to give 0.57 g of **6b**·HCl.

Biological Test Methods. All biological determinations were done with use of male Sprague–Dawley rats weighing 200–275 g obtained from Charles River Breeding Laboratories, Inc. Rats were decapitated and the brains removed; the appropriate brain areas were rapidly dissected on a chilled glass plate. Fluorescence was read on the Farrand Ratio Fluorometer (excitation filter, Corning No. 5840 and emission filter combination, Corning No. 4303 and 3387 with the latter facing the photo tube). Samples of **1a** and **4a** were kindly provided by Professor P. Krogsgaard-Larsen. [2,3-^{3}H]GABA, 28.2 Ci/mmol, *l*-[7-^{3}H]NE, 5.8 Ci/mmol, and [CH_3-^{3}H]diazepam, 60 Ci/mmol, were purchased from New England Nuclear Corp.

[^{3}H]GABA Uptake.[17,43] A crude synaptosomal fraction (P_2) was prepared from rat diencephalon–midbrain as described[52] with the following modifications. Tissue was homogenized in 10

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volumes of ice-cold 0.32 M sucrose with a Potter-Elvehjem homogenizer with Teflon pestle. Nuclei and cell debris were removed by centrifugation at 1000*g* for 10 min at 4 °C. The supernatant was centrifuged at 16500*g* for 20 min at 4 °C, and the resulting pellet was washed once with 0.32 M sucrose and then resuspended in one-half the original volume of 0.32 M glucose. The resulting P_2 fraction contained primarily mitochondria, synaptosomes, and, perhaps, gliosomes.[53,54] The assay buffer contained 128 mM NaCl, 1.2 mM $MgSO_4$, 5 mM KCl, 5 mM Na_2HPO_4, and 10 mM Tris base.[55] The pH was adjusted to 7.4 with HCl. The buffer was oxygenated for 25 min prior to use, during which time $CaCl_2$ (2.7 mM, final concentration) and (aminooxy)acetic acid (10 μM, final concentration) were added. Aliquots of the P_2 fraction, 25 μL (containing approximately 150 μg of protein) and a solution of the inhibitor or H_2O (10 μL) were diluted with 1 mL buffer and preincubated in a 37 °C water bath for 15 min. Accumulation was initiated by the addition of [^{3}H]GABA. The incubation time was 3 min. The final concentration of GABA was 1 μM; at this concentration, only the high-affinity component of GABA accumulation was observed. The K_m for GABA was 4.9 μM.[17] Accumulation was terminated and the tissue was collected by vacuum filtration over a 0.45-μm pore size Millipore filter. Each tube and filter was washed twice with 5 mL of cold buffer. The filters were blotted and dissolved in 10 mL Aquasol, and radioactivity was determined by liquid scintillation spectrometry. To control for binding and diffusion of [^{3}H]GABA, accumulation at 0 °C was subtracted from accumulation at 37 °C. The methods for determining accumulation at 0 °C was identical with that given above except that the tissue and media were not transferred to the 37 °C bath. Protein content of the P_2 fractions was determined as described.[56] Compounds were initially screened for GABA-uptake inhibition at 10, 1, and 0.1 μM by quadruplicate determinations with use of a pooled P_2 fraction of three rat diencephalon–midbrain sections. On the basis of these results, four to six concentrations of inhibitor that would produce a range of inhibition between 25% and 75% were chosen for use in subsequent studies to generate data for calculation of the IC_{50}. In most cases IC_{50} values were based on duplicate determinations in each of 3–12 different diencephalon–midbrain fractions.

[^{3}H]Norepinephrine uptake by rat brain hypothalamic synaptosomes[57] was carried out essentially as described.[58] The K_m for *l*-NE was 0.45 μM. Inhibition of *l*-NE uptake studies were carried out with 0.03 μM *l*-[^{3}H]NE. Specific uptake was determined by incubating control and test compounds at 0 °C and subtracting the values from those obtained at 37 °C.

Glutamic Acid Decarboxylase (GAD) Assay. Rat diencephalon–midbrain was homogenized by hand in 500 μL of cold 50 mM phosphate buffer, pH 6.5, containing 2 mg/mL of Triton X-100, with use of a microglass homogenizer. The GAD assay was carried out as described[59] with the following modifications. An aliquot (100 μL) of the tissue homogenates was added to 500 μL of GAD reagent on ice. The GAD reagent consisted of 100 mM phosphate buffer, pH 6.5, with 5 mM *l*-glutamate, 10 μM pyridoxal phosphate, and 10 mM 2-mercaptoethanol. Test compounds dissolved in 10 μL of H_2O were added to give a final concentration of 1 mM. The reaction was initiated by transferring the tubes to a 37 °C water bath where incubation proceeded for 30 min. The reaction was terminated by immersing the tubes in a 100 °C water bath for 4 min. Boiled enzyme blank samples underwent similar treatment. The samples were cooled on ice and a 25-μL aliquot was added to 1 mL of GABA reagent containing 100 mM Tris buffer, pH 9.0, with 1 mM α-ketoglutarate, 250 μM NADPH, and 5 mM 2-mercaptoethanol. The background fluorescence was read and the reaction was then initiated with the addition of 14 μg of GABAase. The reaction reached completion after 15 min, when the final fluorescence was measured.

GABA transaminase (GABA-T) assay was adapted from a published procedure.[60] Rat diencephalon–midbrain was homogenized in 3 mL of cold 50 mM Tris-HCl buffer, pH 8.0, containing 10 mM 2-mercaptoethanol and 2 mg/mL of Triton X-100, with a Potter-Elvehjem glass homogenizer with Teflon pestle. A 25-μL aliquot of homogenate was added to 500 μL of cold GABA-T reagent containing 0.2 M Tris-HCl buffer, pH 8, 2 mM α-ketoglutarate, 0.2 mM GABA, 0.1 mM 2-(aminoethyl)isothiouronium bromide, and 20 μM pyridoxal phosphate. Test compound dissolved in 10 μL of H_2O was added to give a final concentration of 1 mM. The reaction was initiated by placing the tubes in a shaking water bath at 37 °C for 30 min. The enzyme blanks were handled identically except that they were incubated at 4 °C. The reaction was terminated by immersing all tubes in a 100 °C water bath for 4 min. The samples were then cooled on ice and a 50-μL aliquot of the cooled mixture was added to 1 mL of glutamate reagent containing 0.1 M Tris-HCl, pH 8.6, 100 μM ADP, and 300 μM NAD. The background fluorescence was measured and the reaction was initiated by addition of 200 μg of glutamate dehydrogenase (20 mg/mL in glycerol) and allowed to proceed to completion (10 min). The final fluorescence was read, and the background fluorescence was subtracted from the total fluorescence to determine the amount of NADH formed. Blanks and glutamate standards were assayed simultaneously with the tissue samples.

[^{3}H]Muscimol binding to rat cerebellum membranes was carried out as described.[43] The K_D value was 1.56 nM, and displacement studies were run with 4 nM [^{3}H]muscimol. Nonspecific binding was determined with 100 μM GABA.

[^{3}H]Diazepam binding to rat frontal cortex membranes was determined as described.[52,61] The K_D value was 9.7 nM. Displacement studies were carried out with 1.5 nM [^{3}H]diazepam. Nonspecific binding was determined with 3 μM diazepam.

[^{3}H]WB4101 binding to rat cortical membranes was run as described.[62] The K_D value was 0.306 nM. Displacement studies were run with 0.22 nM [^{3}H]WB4101. Nonspecific binding was determined with 100 μM norepinephrine.

[^{3}H]QNB binding to rat striatal membranes was run as described.[63] The K_D value was 0.20 nM. Displacement studies were run with 0.23 nM [^{3}H]QNB. Nonspecific binding was determined with 1 μM atropine.

[^{3}H]Spiroperidol binding to rat striatal membranes was run as described.[64] The K_D value was 0.287 nM. Displacement studies were run with 0.22 nM [^{3}H]spiroperidol. Nonspecific binding was determined with 1 μM *d*-butaclamol.

[^{3}H]Serotonin binding to rat cortex membranes was run as described.[65] The K_D value was 0.349 nM. Displacement studies were run with 0.3 nM [^{3}H]-5-HT. Nonspecific binding was determined with 10 μM 5-HT.

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Registry No. **1b**, 89203-55-4; Me **1b**, 95273-96-4; Me **1b** maleate, 95273-97-5; (*R*)-**1b**·HCl, 95273-98-6; (*S*)-**1b**·HCl, 95273-99-7; Et **2a**, 5006-62-2; Et (*R*)-**2a**, 25137-01-3; Et (*S*)-**2a**, 37675-18-6; **2b**, 85375-85-5; **2b**-HCl, 85375-15-1; (*R*)-**26**·HCl, 85375-61-7; (*R*)-**26**, 95403-36-4; (*S*)-**26**·HCl, 85375-62-8; (*S*)-**26**,

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85375-85-5; Et **26**·HCl, 85375-14-0; Et **26**, 89203-62-3; Et (*R*)-**26**·HCl, 95274-16-1; Et (*S*)-**26**·HCl, 95274-17-2; **2c**, 95274-01-4; **2c**·HCl, 95274-00-3; Et **2c**·HCl, 95312-96-2; **2d**, 95274-03-6; **2d**·HCl, 95274-02-5; Et **2d**, 95274-04-7; **2e**, 95274-05-8; **2e**·HCl, 85375-40-2; Et **2e**, 85375-39-9; Me **3a**, 495-19-2; **3b**, 85375-88-8; **3b**·HCl, 85375-17-3; Me **3b**, 95274-06-9; Me **4a**·HCl, 85375-51-5; **4b**, 85375-91-3; **4b**·HCl, 85375-53-7; Me **4b**, 85375-52-6; **5b**, 95274-08-1; **5b**·HCl, 95274-07-0; Et **5b**·HCl, 95274-09-2; **6b**, 95274-11-6; **6b**·HCl, 95274-10-5; GABA, 56-12-2; methyl 1-benzyl-3-pyrrolidineacetate cyclohexylsulfamate, 95274-13-8; 1-benzyl-3-pyrrolidineacetonitrile, 55278-09-6; methyl 3-pyrrolidineacetate cyclohexylsulfamate, 95274-15-0; 4,4-diphenyl-3-butenyl bromide, 6078-95-1; 3,3-diphenyl-2-propenyl bromide, 4801-15-4; 5,5-diphenyl-4-pentenyl bromide, 85375-38-8; 1-bromo-4-methoxybutane, 4457-67-4; benzophenone, 119-61-9; 1,1-diphenyl-5-methoxy-1-pentanol, 85375-37-7; methyl *cis*-1-benzyl-4-hydroxy-3-piperidinecarboxylate, 85375-46-8; methyl 1-benzyl-4-oxo-3-piperidinecarboxylate, 57611-47-9; methyl *trans*-1-benzyl-4-hydroxy-3-piperidinecarboxylate, 85375-47-9; ethyl *cis*-3-aminocyclohexanecarboxylate, 62456-14-8; 4,4-diphenyl-3-butenyl azide, 95274-18-3; 4,4-diphenyl-3-butenylamine hydrochloride, 93007-57-9; 4,4-diphenyl-3-butenylamine, 93007-58-0; *N*-benzyl-4,4-diphenyl-3-butenylamine hydrochloride, 95274-19-4; ethyl 4-bromobutyrate, 2969-81-5; ethyl *N*-benzyl-*N*-(4,4-diphenyl-3-butenyl]-γ-aminobutyrate, 95274-20-7; ethyl *N*-cyano-*N*-(4,4-diphenyl-3-butenyl)-γ-aminobutyrate, 95274-21-8.

Methotrexate Analogues. 25. Chemical and Biological Studies on the γ-*tert*-Butyl Esters of Methotrexate and Aminopterin

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γ-*tert*-Butylaminopterin (γ-tBAMT), the first example of an aminopterin (AMT) γ-monoester, was synthesized, and new routes to the known N^{10}-methyl analogue γ-*tert*-butyl methotrexate (γ-tBMTX) were developed. The inhibitory effects of γ-tBAMT on the activity of purified dihydrofolate reductase (DHFR) from L1210 murine leukemia cells, the growth of L1210 cells and CEM human leukemic lymphoblasts in suspension culture, and the growth of several lines of human squamous cell carcinoma of the head and neck in monolayer culture were compared with the effects of γ-tBMTX and the parent acids AMT and methotrexate (MTX). Patterns of cross-resistance to γ-tBAMT, γ-tBMTX, and AMT among several MTX-resistant cell lines were examined. In vivo antitumor activities of γ-tBAMT and γ-tBMTX were compared in mice with L1210 leukemia. While the activity of γ-tBAMT was very close to that of γ-tBMTX in the DHFR inhibition assay, the AMT ester was more potent than the MTX ester against cells in culture and against L1210 leukemia in vivo. Only partial cross-resistance was shown against γ-tBMTX and γ-tBAMT in cultured cells that were resistant to MTX by virtue of a transport defect or a combination of defective transport and elevated DHFR activity.

γ-*tert*-Butyl *N*-(4-amino-4-deoxy-N^{10}-methylpteroyl)-L-glutamate (γ-tBMTX, **1**), a sterically hindered lipophilic ester of the anticancer drug methotrexate (MTX), was synthesized in our laboratory[1] with the aim of evaluating its activity against MTX-resistant tumors with a defect in their transport mechanism for MTX. Decreased MTX uptake has been observed in a variety of mammalian tumor cell lines with induced MTX resistance[2–9] and is thought to also contribute to resistance in the clinic.[10] Since γ-esterification removes one of the negative charges from the glutamate side chain, we speculated that this would promote uptake into cells by passive transport. Moreover, we considered that intracellular drug retention, which in the case of MTX involves enzymatic conversion to noneffluxing γ-polyglutamates,[11–15] might be achieved in **1** via hydrophobic interaction of the ester alkyl group with lipid-rich sites within the cell. With regard to binding to dihydrofolate reductase (DHFR), the target enzyme for MTX and other antifolates,[10] it is known that this enzyme has considerable tolerance for structural changes in the γ-terminal region of the glutamate moiety,[16,17] and we therefore assumed that once the ester crossed the cell membrane it would probably bind nearly as well as MTX

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