Inhibition and Substrate Activity of Conformationally Rigid Vigabatrin Analogues with γ-Aminobutyric Acid Aminotransferase

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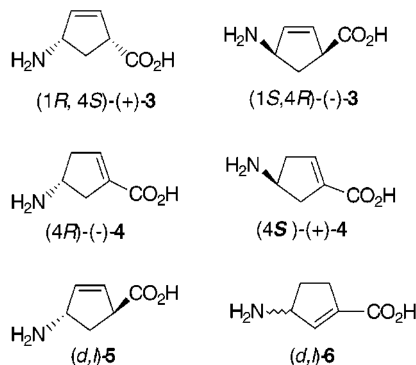
Several cyclopentene GABA analogues were synthesized as conformationally rigid analogues of the epilepsy drug vigabatrin and tested as inhibitors and substrates of γ-aminobutyric acid aminotransferase (GABA-AT). None of these compounds produced time-dependent inhibition. (1*R*,4*S*)-(+)-4-Amino-2-cyclopentene-1-carboxylic acid ((+)-**3**), (4*R*)-(−)-4-amino-1-cyclopentene-1-carboxylic acid ((−)-**4**), and *d,l*-3-amino-1-cyclopentene-1-carboxylic acid (**6**) are good substrates. The K_m and k_{cat} values for the latter two compounds are very similar to those of GABA, suggesting that they bind in an orientation similar to that of GABA. The K_m value for (+)-**3** is 24 times lower than that for GABA, although its k_{cat} value is only one-fourth that for GABA; nonetheless, it is a better substrate for GABA-AT than is GABA. All of these compounds, as well as the enantiomers of **3** and **4** and *d,l-trans*-4-amino-2-cyclopentene-1-carboxylic acid (**5**), are competitive inhibitors of GABA-AT. These results demonstrate the effects of the carboxylate group orientation and the stereochemistry of the amino and carboxylate groups on the substrate activity and inhibitor activity, and this should be important to the future design of inhibitors of GABA-AT.

Introduction

Selective blockage of the catabolytic pathway of the inhibitory neurotransmitter, γ-aminobutyric acid (GABA), has proven to be an effective means to terminate epileptic seizures resulting from a decrease in the brain concentration of GABA.[1] The enzyme that catalyzes the degradation of GABA, GABA aminotransferase (GABA-AT, EC 2.6.1.19), is a pyridoxal 5′-phosphate (PLP)-dependent enzyme.[2] Compounds that inactivate this enzyme exhibit anticonvulsant activity.[1,3] One of the most effective inactivators of GABA-AT is the antiepileptic drug vigabatrin ((4*S*)-4-amino-5-hexenoic acid (**1**); γ-vinyl-GABA),[4] which was designed as a mechanism-based inactivator of this enzyme (Scheme 1).[5] Vigabatrin was shown to inactivate GABA-AT by at least two principal mechanisms: a Michael addition mechanism (pathway a) and an enamine mechanism (pathway b).[6] These two pathways occur via deprotonation of the γ-carbon followed by either tautomerization through the PLP ring (**2**, pathway a, leading to Michael addition) or tautomerization through the vinyl double bond (pathway b, leading to enamine formation). In the absence of the crystal structure of GABA-AT, the position of the double bond of enzyme-bound **1** is unknown. (The crystal structure of GABA-AT was recently reported; see: Storici, P.; Capitani, G.; De Biase, D.; Moser, M.; John, R. A.; Jansonius, J. N.; Schirmer, T. Crystal Structure of GABA-Aminotransferase, a Target for Antiepileptic Drug Therapy. *Biochemistry* **1999**, *38*, 8628–8634.) Presumably, the reason that two different inactivation mechanisms arise is because the orientations of the pyridoxal ring system relative to the electrons in the γ-C–H bond of vigabatrin allow for delocalization of those electrons in either direction. As a possible approach to prohibiting one of the possible tautomerizations and enhancing the other, a series of conformationally rigid vigabatrin and related analogues (**3**–**6**), which were previously shown to be excellent mimetics of GABA during studies of the $GABA_A$ receptor and GABA uptake,[7] were synthesized.[8,9] Furthermore, the binding affinities of the different geometric and enantiomeric compounds may reveal information regarding the binding of the various groups at the active site and provide a basis for further inhibitor design. Surprisingly, unlike vigabatrin, none of these analogues exhibit time-dependent inhibition of GABA-AT; however, all of the analogues are competitive inhibitors, and analogues (+)-**3**, (−)-**4**, and **6** are very good substrates for GABA-AT.



Chemistry

The enantiomers of **3**[8] were synthesized by acid hydrolysis of the corresponding commercially available enantiopure lactams **7** (Scheme 2). The enantiomers of

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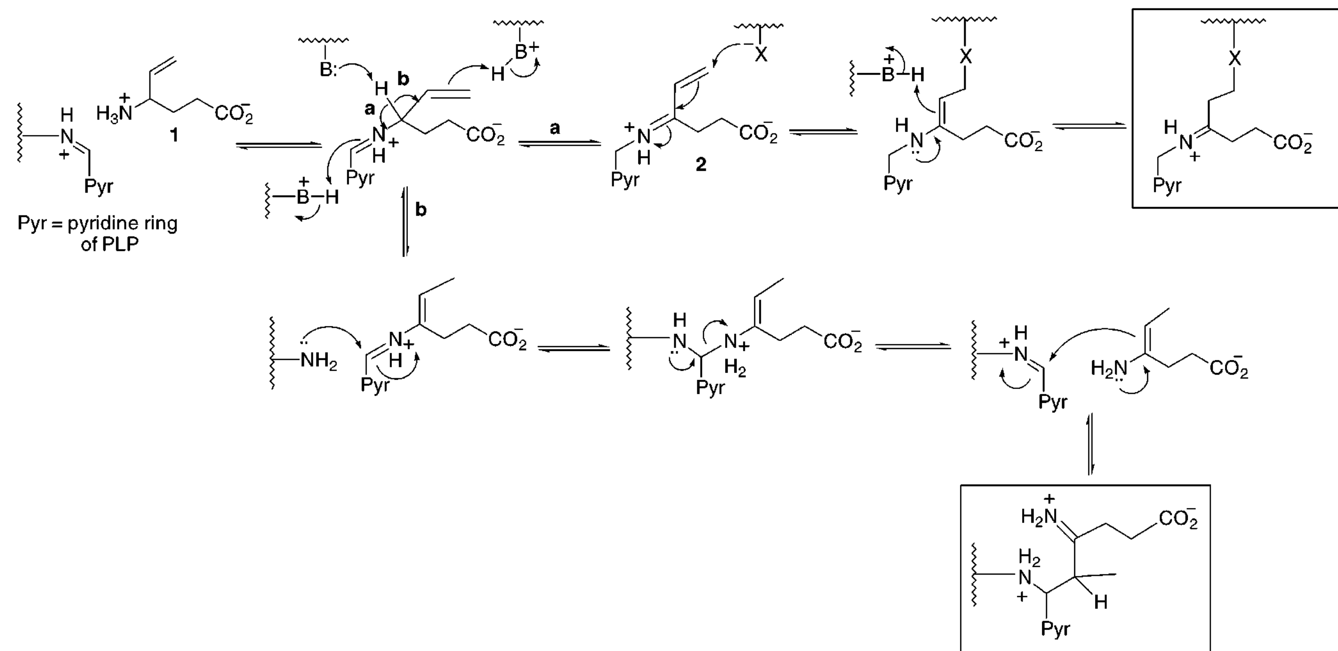
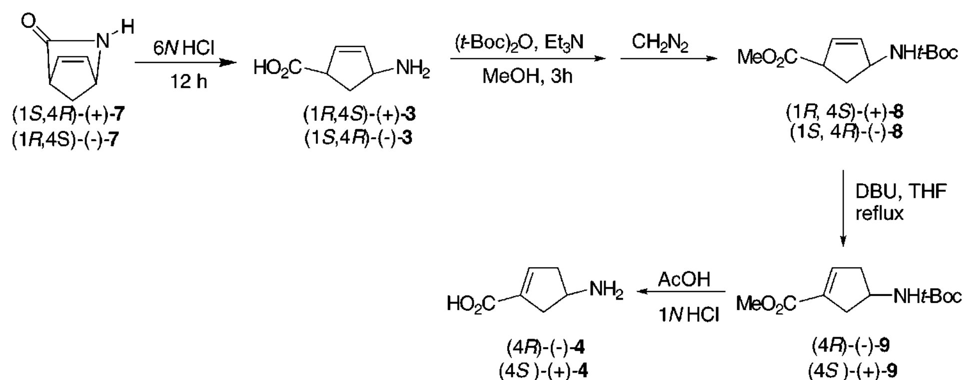
Scheme 1



Scheme 2



4[8] were synthesized by Boc-protection of each enantiomer of **3**, esterification, base treatment, and deprotection (Scheme 2). Following published procedures, analogues **5**[8] and **6**[9] were synthesized in racemic forms.

Results and Discussion

Surprisingly, incubation of GABA-AT with compounds **3**–**6** resulted in no time-dependent loss of enzyme activity. Given the potency of vigabatrin as a time-dependent inactivator[6] and the similarity of the structures, particularly of (+)-**3** and **5**, to vigabatrin, these compounds are expected to be inactivators. Incubation of (+)-**3** (2 mM) with GABA-AT resulted in an immediate loss of enzyme activity (30%), presumably the result of competitive reversible inhibition. However, the enzyme activity gradually recovered over 40 min, presumably the result of transamination of the compound. Although (+)-**3** is not an inactivator, it is an excellent substrate (Table 1), having a K_m value 24 times lower than that of GABA; the specificity constant (k_{cat}/K_m) for this compound as a substrate (117 min^{-1} mM^{-1}) is almost 6 times that of GABA (20.4 min^{-1} mM^{-1}). Therefore, it is effectively being converted into an intermediate that should resemble that derived from vigabatrin (see **2**, Scheme 1), but it is not active as an inactivator. Apparently, constraining the double bond of vigabatrin into the five-membered ring of these cyclopentene analogues misorients it so it is not properly juxtaposed for nucleophilic attack by the enzyme, nor is it able to act as an effective enamine for addition to the enzyme-bound PLP (such as in pathway b, Scheme 1).

Table 1. Kinetic Constants for the Cyclopentene Analogues

compd	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})	K_i (mM)
GABA	2.4 ± 0.07	49	20.4	
(1*R*,4*S*)-(+)-**3**	0.1 ± 0.01	11.7	117	0.052 ± 0.004
(1*S*,4*R*-)-(−)-**3**	no turnover			2.7 ± 0.6
(4*R*)-(−)-**4**	1.6 ± 0.1	37.5	23.4	1.2 ± 0. 2
(4*S*)-(+)-**4**	no turnover			72 ± 3
d,l-**5**	no turnover			38 ± 5
d,l-**6**	2.3 ± 0.4	40	17.4	0.6 ± 0.1

In addition to (+)-**3**, (−)-**4** and *d,l*-**6** also are good substrates for GABA-AT, having k_{cat}/K_m values comparable to that of GABA. Note that (1*R*,4*S*)-(+)-**3** and (4*R*)-(−)-**4** have the same stereochemistry at C-4; the rules for nomenclature change the notation. Therefore, both of these compounds have the γ-proton in the same stereochemical position as the proton that is removed

from GABA during turnover (the 4-*pro-S* proton).[10] The corresponding enantiomers of **3** and **4** are not substrates. The K_m values for (−)-**4** and *d,l*-**6** are 16 and 23 times, respectively, higher than that for (+)-**3**, although they are comparable to that of GABA. This suggests that binding of GABA to the enzyme is similar to that for (−)-**4** and *d,l*-**6** (presumably, it is (4*R*)-**6** that is the substrate), in which the carboxylate is planar to the cyclopentene ring. The carboxylate group in (+)-**3**, which is not planar to the ring, must be in a more favorable position than that in bound GABA, (−)-**4**, or *d,l*-**6**. Although the binding energies are lower, the rate constants for (−)-**4** and *d,l*-**6** are about 4 times higher than that for (+)-**3**. Since the rate-determining step is γ-proton removal,[11] removal of this proton in (+)-**3** must be a lower energy process than in the case of GABA, (−)-**4**, or *d,l*-**6**.

It is intriguing that *d,l*-**5** is neither an inactivator nor a substrate, even though one of the enantiomers (1*S*,4*S*) has the correct stereochemistry at C-4 for deprotonation. Apparently, the carboxylate group, which is on the opposite face of the ring as in (+)-**3**, interacts with the enzyme in such a way as to misorient the C-4 proton, preventing its removal by the active site base. The other enantiomer has the wrong stereochemistry at C-4. Nonetheless, **5** is a weak competitive inhibitor of the enzyme, and in fact, all of the compounds tested are competitive reversible inhibitors (Table 1). Compound (+)-**3** is the most potent with a K_i value of 52 μM. Compounds (+)-**4** and *d,l*-**5** are very poor inhibitors.

In conclusion, these results demonstrate the importance of the orientation of the carboxylate group, as well as the stereochemistry of the amino group, on the binding of the compound to GABA-AT and suggest future directions for the design of inhibitors.

Experimental Section

General Methods. Optical spectra and GABA-AT assays were recorded on a Perkin-Elmer Lambda 10 UV/vis spectrophotometer. ^{1}H NMR spectra were recorded on a Varian Gemini 300-MHz NMR spectrometer. Chemical shifts are reported as δ values in parts per million downfield from Me_4Si (δ 0.0) as the internal standard in $CDCl_3$. For samples run in D_2O, the HOD resonance was arbitrarily set at 4.80 ppm. IR spectra were taken with a Bio-Rad FTS60 spectrophotometer. An Orion Research model 701 pH meter with a general combination electrode was used for pH measurements. Mass spectra were obtained on a VG Instrument VG70-250SE high-resolution spectrometer with a Maspec Data System. Flash column chromatography was carried out with Merck silica gel 60 (230−400 mesh ASTM). TLC was run with EM Science silica gel 60 F254 precoated glass plates.

Reagents. All reagents were purchased from Aldrich Chemical Co. without further purification except anhydrous ether and tetrahydrofuran, which were distilled over sodium metal under nitrogen, and anhydrous dichloromethane, which was distilled over calcium hydride.

Methyl (1*S*,4*R*)-4-[(*tert*-Butyloxycarbonyl)amino]cyclopent-2-ene-1-carboxylate, (−)-8. Crude (1*S*,4*R*)-4-amino-2-cyclopentene-1-carboxylic acid, (−)-**3**, $[\alpha]^{23.5}$ = −236° (*c* 1.7, H_2O; 98% ee)[8b] (0.50 g, 3.0 mmol), obtained from acid hydrolysis of the corresponding lactam (1*S*,4*R*)−(+)-**7** (Aldrich Chemical Co.), was added to a solution of methanol (10 mL) containing triethylamine (1.6 mL, 12 mmol) and di-*tert*-butyl dicarbonate (1.46 g, 6.6 mmol) at room temperature. After being stirred for 3 h the reaction mixture was concentrated under vacuum. The resultant mixture was dissolved in dichloromethane (10 mL), and excess ethereal diazomethane solution (10 mL) was added. After being stirred for 1 h, the solution was diluted with ethyl acetate (100 mL). The combined organic solution was washed with 5% HCl solution (15 mL), saturated $NaHCO_3$ solution (15 mL), and brine (15 mL) and then dried over anhydrous $MgSO_4$. After removal of solvents under reduced pressure, the residue was purified by silica gel chromatography to afford a colorless oil (0.86 g, 98%): R_f = 0.26 (EtOAc:hexane, 1:4); $[\alpha]^{23.5}$ = −51.4° (*c* 5.95, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 5.89 (2H, m, H_2, H_3), 4.79 (1H, m, H_4), 3.71 (3H, s, OCH_3), 3.48 (1H, m, H_1), 2.51 (1H, dt, *J* 14, 8.5 Hz, H_{5a}), 1.84 (1H, dt, *J* 14, 4.1 Hz, H_{5b}), 1.45 (9H, s, −O-*t*-Bu); EI-MS 175, 159, 145, 129, 101, 81; HRMS calcd for $C_6H_3F_2NO_3$ 175.0081, found 175.0081.

Methyl (4*S*)-4-[(*tert*-Butyloxycarbonyl)amino]cyclopent-1-ene-1-carboxylate, (+)-9. Compound (1*S*,4*R*)−(−)-**8** (0.88 g, 3.6 mol) was dissolved in a solution of anhydrous THF (8 mL) and DBU (0.65 g, 4.2 mmol). The resultant solution was heated at reflux for 10 h. After cooling, the resultant solution was concentrated under reduced pressure. The residue was purified by silica gel chromatography to afford a white solid (0.8 g, 95%): R_f = 0.26 (EtOAc:hexane, 1:4); $[\alpha]^{23.5}$ = +35.8° (*c* 4.63, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 6.70 (1H, t, *J* 2.2 Hz, H_2), 4.34 (1H, s, broad, H_4), 3.71 (3H, s, $-OCH_3$), 2.90 (2H, m, H_{5a} and H_{5b}), 2.40 (2H, m, H_{3a} and H_{3b}), 1.45 (9H, s, −O-*t*-Bu); EI-MS 241, 185, 168, 153, 141, 124, 57; HRMS calcd for $C_{12}H_{19}NO_4$ 241.1314, found 241.1315.

(4*S*)-4-Amino-1-cyclopentene-1-carboxylic Acid, (+)-4. Compound (4*S*)−(+)-**9** (0.7 g, 2.9 mmol) was added to a solution of acetic acid (6 mL) and 1 M HCl (6 mL). The resultant solution was stirred and heated to 100 °C for 1 h. After removal of solvents under vacuum, the residue was purified with an ion-exchange column (AG 50w-8x, H^+ form), eluting with water and then 1 M pyridine solution. After evaporation of solvents, the resultant solid was further purified by recrystallization from ethanol and ether to afford a white solid (0.3 g, 77%): $[\alpha]^{23.5}$ = +32° (*c* 2.14, H_2O);[8a] ^{1}H NMR ($CDCl_3$) δ 6.32 (1H, m, H_2), 3.98 (1H, m, H_4), 2.95 (2H, m, H_{5a} and H_{5b}), 2.55 (2H, m, H_{3a} and H_{3b}); EI-MS 127, 109, 82, 80, 56; HRMS calcd for $C_6H_9NO_2$ 127.0633, found 127.0635.

Methyl (1*R*,4*S*)-4-[(*tert*-Butyloxycarbonyl)amino]cyclopent-2-ene-1-carboxylate, (+)-8. This compound was prepared as described above from (1*R*,4*S*)-4-amino-2-cyclopentene-1-carboxylic acid, (+)-**3**, [α] = +241° (*c* 5.14, H_2O; >99% ee).[8b] All NMR data and MS data are identical to those of the enantiomer; the optical rotation was $[\alpha]^{23.5}$ = +51.9° (*c* 4.65, $CHCl_3$).

Methyl (4*R*)-4-[(*tert*-Butyloxycarbonyl)amino]cyclopent-1-ene-1-carboxylate, (−)-9. This compound was prepared as described above from (1*R*,4*S*)−(+)-**8**. All NMR data and MS data are identical to those of the enantiomer; the optical rotation was $[\alpha]^{23.5}$ = −36.1° (*c* 4.41, $CHCl_3$).

(4*R*)-4-Amino-1-cyclopentene-1-carboxylic Acid, (−)-4. This compound was prepared as described above from (4*R*)−(−)-**9**. All NMR data and MS data are identical to those of the enantiomer; the optical rotation was $[\alpha]^{23.5}$ = −31.9° (*c* 2.41, H_2O).[8a]

Enzymes and Assays. Pig brain GABA-AT[12] (specific activity 3.9 units/mg), GABAase, and succinic semialdehyde dehydrogenase were obtained and assayed as previously described.[13]

Determination of Kinetic Constants for Substrates. GABA analogues of varying concentrations (e.g. 0.11, 0.21, 0.32, 0.54. 1.07, and 1.60 mM for (+)-**3**) were incubated at 25 °C with GABA-AT (0.004 unit) in 50 mM potassium pyrophosphate buffer, pH 8.5, containing 5 mM β-mercaptoethanol and 5 mM [5-^{14}C]2-ketoglutarate (0.1 mCi/mmol) for 60 min. The resulting [^{14}C]glutamate was isolated and quantified as described previously.[12] The ranges of concentrations for (−)-**4** and *d,l*-**6** are 0.65−6.5 and 0.8−8.5 mM, respectively. Controls consisted of the entire incubation mixture with either enzyme or substrate omitted. Nonlinear regression analysis of the Michaelis−Menten data was carried out for the calculation of the kinetic constants according to Cleland.[14]

Inhibition of GABA-AT by GABA Analogues. The activity of GABA-AT (0.004 unit) upon the introduction of varying

concentrations of GABA analogues (e.g. 0.022, 0.045, 0.062, 0.090, and 0.122 mM for (+)-**3**) was determined spectrophotometrically, by measuring the formation of NADH upon the conversion of succinic semialdehyde dehydrogenase at 25 °C and at several GABA concentrations (e.g. 1.77, 2.47, 3.51, 5.20, and 8.45 mM) in 100 mM potassium pyrophosphate buffer, pH 8.5, containing 5 mM β-mercaptoethanol.[15] Kinetic parameters were derived from Dixon[16] and Cornish−Bowden[17] plots with at least five different concentrations of GABA analogues for every concentration of GABA. The ranges of concentrations for those analgues are as follows: 0−10 mM for (−)-**3**, 0−4.9 mM for (−)-**4**, 0−18 mM for (+)-**4**, 0−24 mM for *d,l*-**5**, and 0.0−6.5 mM for *d,l*-**6**.

Time-Dependent Inactivation of GABA-AT by Analogues (+)-3, (−)-3, and *d,l*-5. Incubation mixtures were set up consisting of GABA-AT (0.02 unit), 5 mM α-ketoglutarate, 5 mM β-mercaptoethanol, and a range of GABA analogue concentrations from 0−10 mM in 100 mM potassium pyrophosphate buffer, pH 8.5, at 25 °C. Periodically, 20-μL aliquots were withdrawn and assayed spectrophotometrically for remaining enzyme activity in a total volume of 600 μL.

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