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Design of a Conformationally Restricted Analogue of the Antiepilepsy Drug Vigabatrin that Directs Its Mechanism of Inactivation of γ-Aminobutyric Acid Aminotransferase

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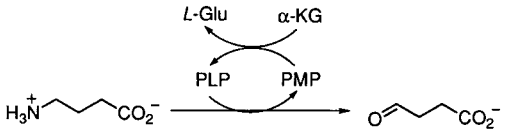
Abstract: The antiepilepsy drug vigabatrin (**1**, 4-aminohex-5-enoic acid, γ-vinylGABA) is known to be a mechanism-based inactivator of the pyridoxal phosphate (PLP)-dependent enzyme γ-aminobutyric acid aminotransferase (GABA-AT). Inactivation has been shown to proceed by two divergent mechanisms (Nanavati, S. M.; Silverman, R. B. *J. Am. Chem. Soc.* **1991**, *113*, 9341−9349). The major pathway involves γ-proton removal, tautomerization into the PLP ring, followed by Michael addition of an active site lysine residue at the conjugated vinyl group to give a stable covalent adduct with the protein (Scheme 2, pathway a). The minor inactivation mechanism also involves γ-proton removal, but tautomerization occurs through the vinyl group, followed by an enamine rearrangement that leads to attachment of the inactivator to the PLP, which is bound to the protein (Scheme 2, pathway b). The cause for the two different inactivation pathways was hypothesized to be potential overlap of the incipient carbanion with the π-orbitals of both the PLP and the vinyl group. With use of the crystal structure data for GABA-AT recently reported (Storici, P.; Capitani, C.; De Biase, D.; Moser, M.; John, R. A.; Jansonius, J. N.; Schirmer, T. *Biochemistry* **1999**, *38*, 8628−8634) a computer model of vigabatrin bound to the PLP was constructed and energy minimized. This model indicated that the major Michael addition pathway could only occur if the vinyl group were allowed to rotate by 180°. A conformationally rigid analogue of vigabatrin, *cis*-3-aminocyclohex-4-ene-1-carboxylic acid (**9**), was designed to prevent bond rotation and block the Michael addition pathway. A detailed study of the mechanism of inactivation of GABA-AT by **9** revealed that it inactivates by a single mechanism, the enamine pathway.

Introduction

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian central nervous system.[1] The major pathway for its degradation is via transamination with α-ketoglutarate (α-KG), catalyzed by the pyridoxal 5′-phosphate (PLP)-dependent enzyme GABA aminotransferase (GABA-AT, E.C. 2.6.1.19; Scheme 1).[2] In this reaction GABA is degraded to succinic semialdehyde and the PLP is converted to pyridoxamine 5′-phosphate (PMP), which is restored to PLP by transamination with α-KG, generating the excitatory neurotransmitter L-glutamate. Inhibition of this enzyme results in an increase in availability of GABA and could have therapeutic applications in neurological disorders including epilepsy,[3] Parkinson's disease,[4] Huntington's chorea,[5] and Alzheimer's disease.[6] Recently, it has been found that an increase in GABA also blocks the effects of drug addiction.[7]

Scheme 1



The selective inactivator of GABA-AT, vigabatrin (**1**; 4-aminohex-5-enoic acid; γ-vinylGABA),[8,9] a mechanism-based inactivator[10] of the enzyme, is already successfully utilized in the treatment of epilepsy[11] and is in clinical trials for the

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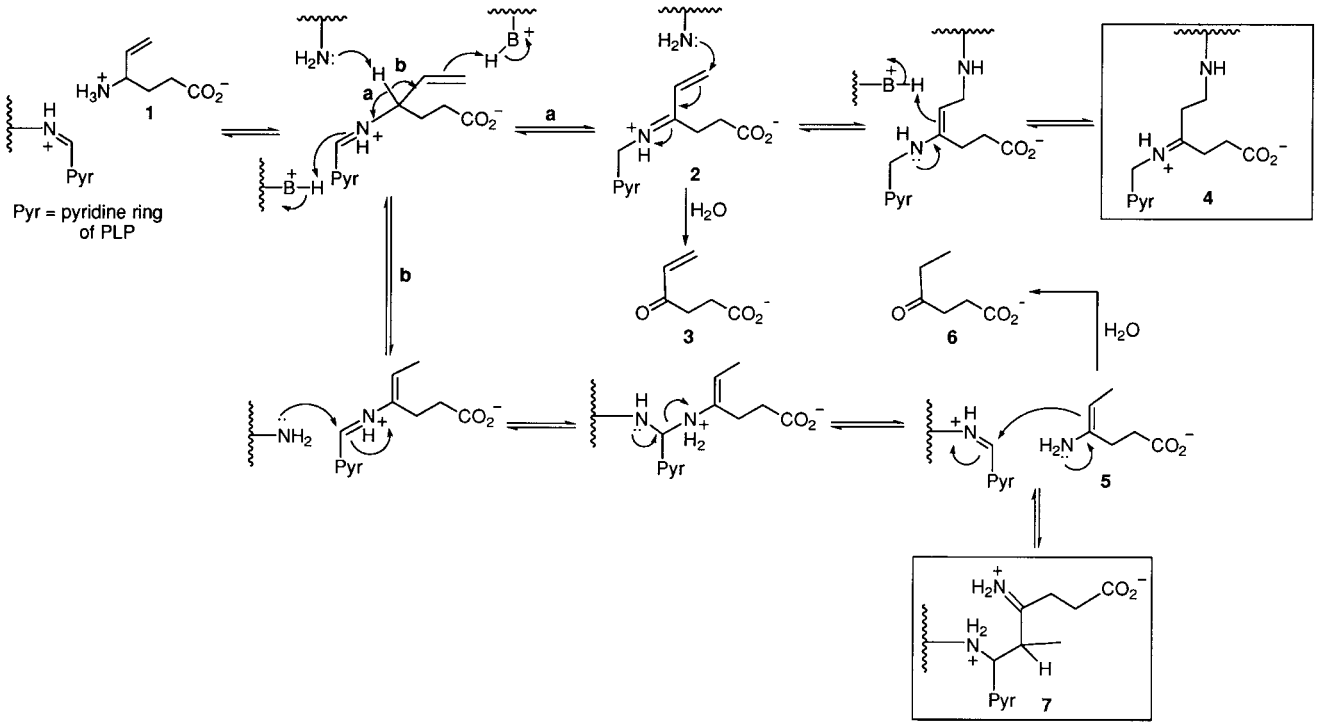
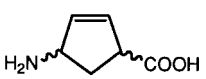
Scheme 2



treatment of drug addiction.[12] A detailed study of the mechanism of inactivation of GABA-AT by vigabatrin revealed that it acts by two principal inactivation pathways: a Michael addition mechanism (Scheme 2, pathway a) and an enamine mechanism (pathway b).[13] These two pathways occur via deprotonation of the γ-carbon followed by tautomerization through either the PLP ring (pathway a, leading to Michael addition) or the vinyl double bond (pathway b, leading to enamine formation). Presumably, the reason that these two pathways occur is because the orientations of the pyridoxal ring π-system and the vinyl π-orbitals relative to the electrons in the γ-C−H bond of γ-vinyl-GABA allow delocalization of the electrons in either direction, although the Michael addition pathway is favored 70:30 relative to the enamine pathway. The protein nucleophile that participates in the Michael addition reaction is known to be Lys-329 (for the pig liver enzyme), the lysine residue that holds the PLP at the active site.[14]

Vigabatrin, like all other CNS drugs, has a variety of side effects of unknown causes. In addition to it acting as an inactivator of GABA-AT, vigabatrin also acts as a substrate, producing **3**, a potent electrophile, from hydrolysis of **2**. Hydrolysis of the enamine product (**5**) from pathway b gives ketone **6**, which does not possess the electrophilicity of **3**. Therefore, we thought that it would be intriguing to determine if vigabatrin could be redesigned so that it inactivated GABA-AT only by the presumably less noxious minor pathway b. This might be possible if the overlap of the electrons in the γ-C−H bond with the π-electrons of PLP and the vinyl group could be controlled. To that end, a series of conformationally rigid vigabatrin analogues was synthesized in which the vinyl group was attached directly to the GABA backbone to give a cyclopentene ring (**8**; both cis-isomers and the racemic trans-isomer were made).[15] Unfortunately, none of these compounds was a time-dependent inhibitor; the (+)-cis isomer was an excellent substrate, and the (−)-cis and the trans compounds were competitive inhibitors. Apparently, the conformational constraint prevented both Michael addition and enamine inactivation pathways.



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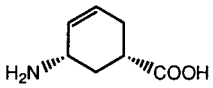

(±)-9

Toward the end of 1999 the crystal structure of GABA-AT from pig liver was reported.[16] We report here computer modeling of vigabatrin bound to GABA-AT using the coordinates of this crystal structure and how that model led to the design of a conformationally rigid vigabatrin analogue that inactivates GABA-AT only via the minor enamine inactivation mechanism.

Results and Discussion

An energy-minimized molecular model of vigabatrin bound to PLP within the active site of GABA-AT is shown in Figure 1 (see also Figure 3c in ref 16). The carboxylate of vigabatrin coordinates to Arg-192, which presumably also occurs with the carboxylate of GABA. Lys-329 is the lysine residue that holds the PLP at the active site. It appears that Lys-329 also is responsible for γ-proton abstraction from vigabatrin (and, presumably, also from GABA). However, the lowest energy

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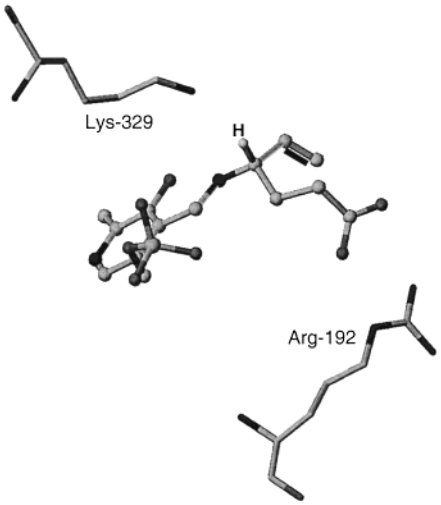

Figure 1. Molecular model of vigabatrin bound to show Lys-329 is not in correct orientation for Michael addition.

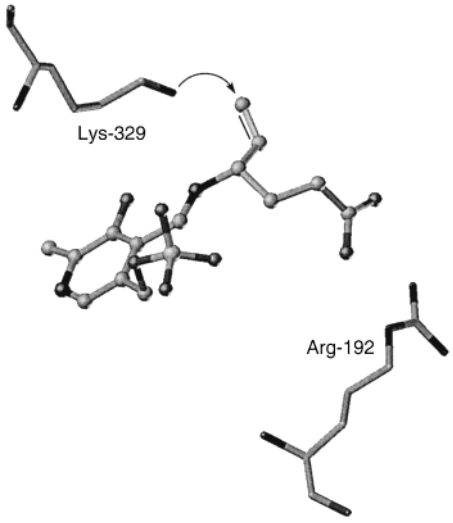

Figure 2. Michael addition requires the vinyl group to rotate toward the active site nucleophile Lys-329.

structure for bound vigabatrin has the vinyl group pointing away from Lys-329, which precludes Michael addition of this residue (known as the nucleophile for this reaction)[14] after tautomerization. For Michael addition to occur, the vinyl group has to rotate approximately 180° (Figure 2). Constraining this vinyl group so that it cannot rotate should preclude the Michael addition (major) pathway, which was the case with the cyclopentene analogues described above. However, the rigidity of the cyclopentene ring, apparently, also precluded the enamine mechanism. More flexibility in the ring system should give it a better chance to undergo the requisite enamine reaction to inactivate the enzyme.

Consequently, (*d,l*)-*cis*-3-aminocyclohex-4-ene-1-carboxylic acid (**9**) was synthesized as a conformationally restricted analogue of vigabatrin with more flexibility than the corresponding cyclopentene analogue *cis*-**8**. A molecular model of **9** bound to GABA-AT (Figure 3) shows that Lys-329 can abstract the C-3 proton, but the alkene cannot rotate, prohibiting the Michael addition pathway (Scheme 3, pathway a). Molecular modeling predicts that, as in the case of vigabatrin in which the 4*S* isomer was shown to be the active isomer,[17] the active

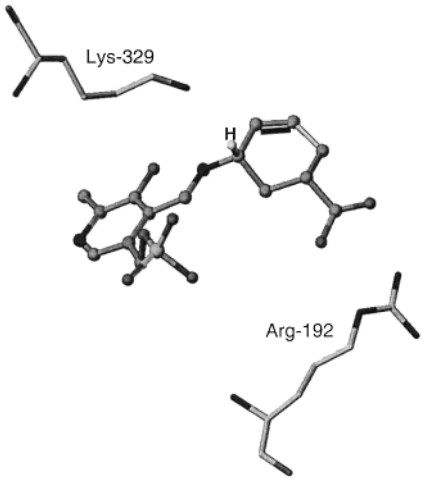

Figure 3. Molecular model of **9** bound to show Lys-329 is not in correct orientation for Michael addition.

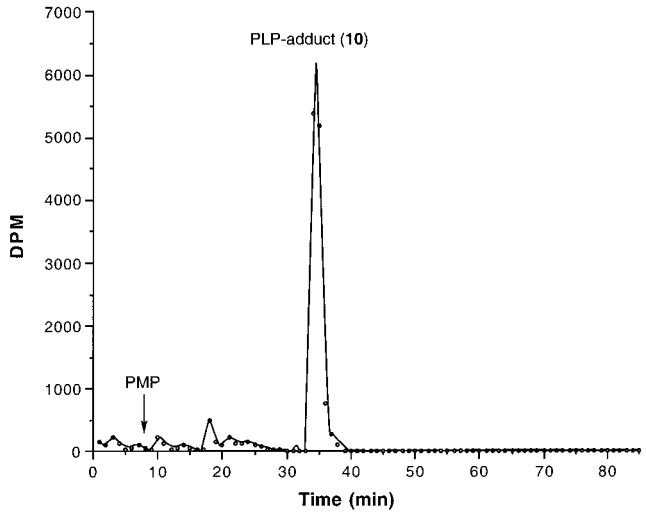

Figure 4. Reverse-phase HPLC of the cofactor products formed by inactivation of [^{3}H]PLP-GABA-AT by **9**.

enantiomer of **9** should be the (1*S*,3*S*) isomer (the one shown in Figure 3). The (1*R*,3*R*)-enantiomer bound so that the carboxylate coordinates with Arg-192 puts the C-3 proton pointing away from Lys-329, prohibiting deprotonation (data not shown).

Compound **9** was found to be a time-dependent inactivator of GABA-AT; the kinetic constants are $K_I = 2.3$ mM and $k_{inact} = 0.01$ min^{-1} at pH 8.5. Gel filtration did not restore enzyme activity. Two inactivation mechanisms can be considered (Scheme 3); pathway a, the Michael addition mechanism, would produce pyridoxamine 5′-phosphate (PMP) upon denaturation of the inactivated species, and pathway b, the enamine mechanism, would give a modified cofactor (**10**), which was synthesized as a chromatography standard. Inactivation of [^{3}H]-PLP-reconstituted GABA-AT with **9** followed by denaturation and HPLC separation gave a largely predominant radioactive peak (at $T_R = 34$ min), corresponding to **10**; little or no PMP was detected (Figure 4). The peak at $T_R = 34$ min was further identified as **10** by LC-MS. All of the radioactivity bound to the enzyme was accounted for in this experiment. Therefore,

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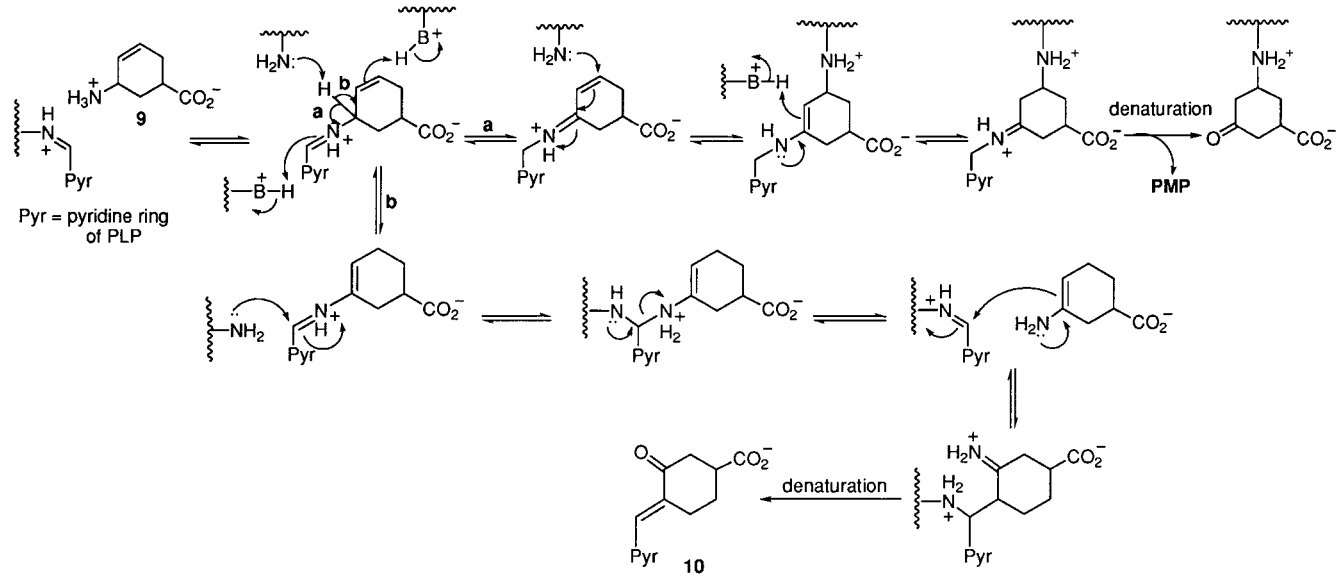
Scheme 3



unlike vigabatrin, **9** inactivates GABA-AT exclusively by an enamine mechanism (the minor pathway for vigabatrin).

Rotation about the vinyl group in vigabatrin could account for the observation of two inactivation mechanisms (Scheme 2). Upon rotation of the vinyl group, the ammonium ion of Lys-329 becomes equidistant to the iminium carbon of the vigabatrin-PLP Schiff base and to the terminal carbon of the vinyl group (Figure 5; both 3.5 Å). If protonated Lys-329 (protonation occurs upon deprotonation of the γ-carbon of vigabatrin) is the acid group that donates a proton to both the iminium carbon, leading to pathway a (Scheme 2), and to the terminal vinyl carbon, leading to pathway b (Scheme 2), then that would account for the occurrence of both inactivation pathways. The reason for the preference in pathway a may be the time required for the vinyl bond to rotate, which results in a lower probability for protonation of the vinyl group (pathway b).

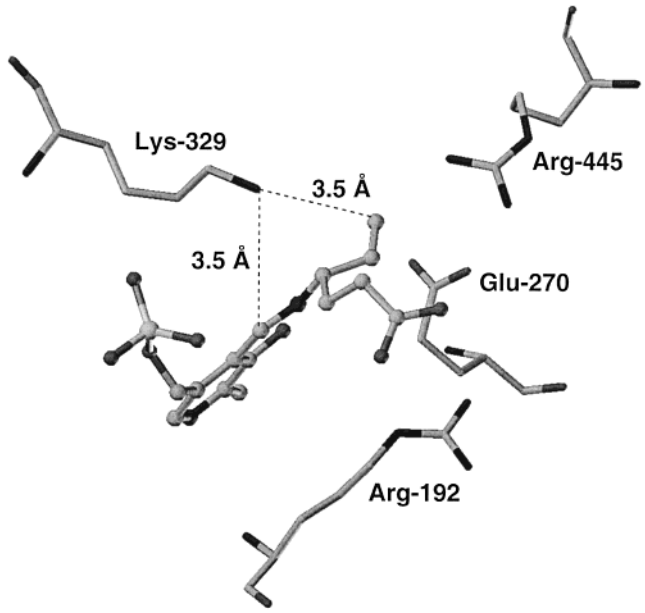


Figure 5. Molecular model of vigabatrin bound to PLP with the vinyl group rotated toward Lys-329 to show the distance of the ammonium group from two protonation sites.

In conclusion, based on computer modeling of vigabatrin bound in the active site of GABA-AT, it was possible to design a conformationally restricted analogue (**9**) that inactivated the enzyme by only one of two possible inactivation mechanisms observed for vigabatrin (the minor pathway). These results support the concept of stereoelectronic control in enzymatic reactions and the importance of reaction energetics in determination of the mechanism. This approach should be useful to the design of mechanism-based inactivators of other enzymes that proceed by multiple inactivation mechanisms.

Experimental Section

General Methods. ^{1}H and ^{13}C NMR spectra were recorded on Varian Gemini 300 MHz and Inova 500 MHz NMR spectrometers. Chemical shifts are reported as δ values in parts per million (ppm) 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. Mass spectral analyses were performed by the Analytical Services Laboratory in the Department of Chemistry at Northwestern University. Combustion analyses were performed by Oneida Research Laboratories, NY. Thin-layer chromatography (TLC) was run with silica gel 60 F_{254} precoated glass plates (EM Separations Technology) using standard visualization techniques. Flash column chromatography was carried out with silica gel 60 (230−400 mesh) from Merck. Cation exchange chromatography was performed on Dowex 50 resin (BioRad AG50W-X8, 100−200 mesh, biotech grade). Unless otherwise noted, all reactions were carried out under a nitrogen atmosphere. An Orion Research Model 701 pH meter with a general combination electrode was used for pH measurements. Enzyme assays were recorded on a Perkin-Elmer Lambda 10 UV/vis spectrophotometer. Radioactivity was measured by liquid scintillation counting using a Packard Tri-Carb 2100TR counter and Packard Ultima Gold scintillation cocktail. HPLC analyses were performed using a Beckman System Gold system with a 125 solvent delivery module and a 166 UV detector.

Reagents. All chemicals used in synthetic procedures were purchased from Aldrich or Fischer Scientific and used as received unless otherwise noted. α-Ketoglutarate, β-mercaptoethanol, $NADP^+$, Sephadex G-50, buffer salts, and other reagents used in the enzymological studies were purchased from Sigma.

Synthesis of *cis*-3-Aminocyclohex-4-ene-1-carboxylic acid (9). (*d,l*)-*cis*-3-Phthalimidocyclohex-4-ene-1-carboxylic acid was prepared from cyclohex-3-ene-1-carboxylic acid by the procedure of Allan et

al.[18] The phthalimide (490 mg, 1.80 mmol) was refluxed in methanol (10 mL) and hydrazine (570 μL, 18.0 mmol) for 30 min, and the solvent was removed under vacuum. To this was added 6 M HCl (10 mL) in an ice bath, and the precipitate was removed by filtration. The neutral amino acid was isolated from a Dowex 50W-X8 cation exchange column by washing the column with water and then eluting with 1 M pyridine. Subsequent recrystallization from ethanol/water gave the cis amino acid **9** (121 mg, 48%). ^{1}H NMR (D_2O) δ 1.65−2.20 (4H, m, CH_2 and CH_2), 2.53 (1H, m, $CHCO_2$), 3.93 (1H, m, CHN), 5.57 (1H, br d, J 10 Hz, =C(4)H), 6.05 (1H, m, =C(5)H); ^{13}C NMR (D_2O) δ 32.6 (C6), 34.6 (C2), 45.2 (C1), 52.1 (C3), 126.9 (C4), 137.4 (C5), 188.0 (CO_2); HRMS (EI) (*m*/*z*) $[M - H]^+$ calcd for $C_7H_{10}NO_2$ 140.0712, found 140.0716. Anal. Calcd for $C_7H_{11}NO_2$: C, 59.56; H, 7.85; N, 9.92. Found: C, 59.45; H, 7.80; N, 9.76.

Synthesis of Standard for the Enamine Reaction (10). Aldol condensation of PLP with 3-oxo-1-cyclohexanecarboxylic acid at pH 7 gave **10** after HPLC purification.

Molecular Modeling. All procedures were performed using SYBYL molecular modeling software version 6.5 or 6.6 (Tripos, Inc., St. Louis, MO) operating under IRIX 6.5. The coordinates from the X-ray crystal structure of GABA-AT[16] were used for the modeling studies. The docking analysis was performed using the FlexX default parameters, and the complex exhibiting the highest interaction energy was selected. Water molecules were removed as they were found not to be located within the active site region, and hydrogen atoms on the backbone and side chains were added based on the standard average bond angles and lengths. Dictionary charges from the AMBER force field were added. A sphere of 10 Å around the cofactor PLP was defined as the binding region for the docking experiment. The investigated ligands were created as external aldimines starting from geometrically optimized fragments. The potential energies were minimized to obtain a comparable starting point for each compound. The Powell conjugate gradient minimizer within the MAXIMIN procedure was used with the parameters of the MMFF94 force field.

Enzymes and Assays. GABA aminotransferase was isolated from pig brains as described previously.[19] The purified enzyme was approximately 95% homogeneous by SDS-PAGE electrophoresis (Coomassie blue). Succinic semialdehyde dehydrogenase (SSDH) was isolated from GABAse (Sigma), and GABA-AT activity was monitored spectrophotometrically via a coupled assay as previously described.[20]

Inactivation of [^{3}H]PLP-Reconstituted GABA-AT by 9 and HPLC Analysis. GABA-AT, which had been reconstituted with [^{3}H]-PLP as previously described,[21] was incubated at 25 °C and protected from light in 100 mM potassium phosphate buffer containing 112 mM **9**, 5 mM α-ketoglutarate, and 5 mM β-mercaptoethanol at pH 7.4. A control was run with the same concentrations of each reagent, excluding inactivator, and a second control was run with 40 mM GABA containing no inactivator or α-ketoglutarate. Following incubation and denaturation, the first control should release the cofactor as PLP, while the second should release PMP. When the enzyme in the inactivator solution was less than 7% active, excess small molecules were removed by running the solutions over Sephadex G-50 using the Penefsky spin method.[22] The pH of each solution was adjusted to 11−12 using 1 M KOH, then they were incubated at room temperature for 1 h. Enough TFA to quench the base and make a 10% v/v TFA solution was added. After being allowed to stand at room temperature for 10 min, the denatured enzyme solutions were placed into pre-rinsed Centricon 10 microconcentrators and were centrifuged for 20 min at 5000 rpm in a Du Pont Sorvall RC5B Plus Centrifuge, using an SA 600 rotor to achieve complete separation of the protein and the effluents. Each Centricon was rinsed with 1 mL of the buffer (containing 100 mM potassium phosphate and 5 mM β-mercaptoethanol at pH 7.4), vortexed, and then centrifuged for a further 20 min. This was repeated three times, and the rinses were added to the supernatants, and then freeze-dried. Analysis of the incubation mixture was carried out by dissolving the resulting solid in 100 μL of water, and then injecting the samples onto an Alltech Econosil C18 reverse-phase HPLC column (4.6 × 250 mm, 10 μm). HPLC analysis was done using the mobile phase H_2O containing 0.1% TFA flowing at 0.5 mL/min for 15 min. The flow rate was increased to 1.0 mL/min over the next 5 min, and then a solvent gradient to 100% methanol with 0.1% TFA was run over the next 20 min. The flow rate was further increased to 2.0 mL/min over 5 min and maintained for 15 min, and then a 10 min solvent gradient to 100% water with 0.1% TFA was run, followed by changing the flow rate to 0.5 mL/min over 5 min. Under these conditions, PLP elutes at 17 min and PMP at 8 min. Fractions were collected every minute, and the elution of radioactivity was followed by liquid scintillation counting. The incubation mixture with fractions, which during previous experiments with [^{3}H]PLP-reconstituted GABA-AT with **9** contained substantial amounts of radioactivity, was analyzed by LC/MS using electrospray (ES^+) ionization, which confirmed the occurrence of the modified cofactor **10**: $[M + H]^+$ calcd for $C_{15}H_{18}NO_8P$ 371.286, found 372.1.

Time-Dependent Inhibition of GABA-AT. GABA-AT (15 μL, 2.12 mg/mL) was incubated at 25 °C in 150 μL total volume of a solution containing various concentrations of **9**, 1 mM α-ketoglutarate, 5 mM β-mercaptoethanol, and 50 mM potassium diphosphate at pH 8.5. An identical sample, which contained no inhibitor, served as the control. At time intervals over 60 min, 10 μL aliquots were assayed for enzyme activity.

Substrate Protection from Inactivation of GABA-AT. GABA-AT (15 μL, 2.12 mg/mL) was incubated at 25 °C in 150 μL total volume of a solution containing 5 mM **9**, 5 mM α-ketoglutarate, 5 mM β-mercaptoethanol, 0−10 mM GABA, and 0.1 M potassium diphosphate at pH 8.5. A sample in which the inhibitor was omitted served as the control. At time intervals over 60 min, 10 μL aliquots were removed and assayed for enzyme activity.

Irreversible Inactivation of GABA-AT. GABA-AT (15 μL, 2.12 mg/mL) was incubated at 25 °C in 150 μL total volume of a solution containing 5 mM **9**, 5 mM α-ketoglutarate, 5 mM β-mercaptoethanol, and 0.1 M potassium diphosphate at pH 8.5. Aliquots (10 μL) were removed at 0.5 and 17.5 h and assayed for activity. A portion of the incubation mixture (100 μL) was applied to a Penefsky spin column prepared with Sephadex G50 in a Bio-Spin disposable chromatography column (BioRad) according to the published procedure. Small molecules were removed by spinning for 2 min on an IEC Clinical centrifuge. A 10 μL aliquot was removed and assayed for enzyme activity. An identical sample, which contained no inactivator, served as the control. Experiments were run in duplicate.

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