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Mechanism of Inactivation of GABA Aminotransferase by (*E*)- and (*Z*)-(1*S*,3*S*)-3-Amino-4-fluoromethylenyl-1-cyclopentanoic Acid

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Abstract

When γ -aminobutyric acid (GABA), the major inhibitory neurotransmitter in the mammalian central nervous system, falls below a threshold level, seizures occur. One approach to raise GABA concentrations is to inhibit GABA aminotransferase (GABA-AT), a pyridoxal 5'-phosphate-dependent enzyme that degrades GABA. We have previously developed (1*S*,3*S*)-3-amino-4-difluoromethylene-1-cyclopentanoic acid (CPP-115), which is 186 times more efficient in inactivating GABA-AT than vigabatrin, the only FDA-approved inactivator of GABA-AT. We also developed (*E*)- and (*Z*)-(1*S*,3*S*)-3-amino-4-fluoromethylenyl-1-cyclopentanoic acid (**1** and **2**, respectively), monofluorinated analogs of CPP-115, which are comparable to vigabatrin in inactivating GABA-AT. Here we report the mechanism of inactivation of GABA-AT by **1** and **2**. Both produce a metabolite that induces disruption of the Glu270-Arg445 salt bridge to accommodate interaction between the metabolite formyl group and Arg445. This is the second time that Arg445 has interacted with a ligand and is involved in GABA-AT inactivation, thereby confirming the importance of Arg445 in future inactivator design.

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ASSOCIATED CONTENT

Supporting Information

Turnover of **1** and **2** by GABA-AT, fluoride ion release results, cofactor release results, middle-down proteomics data, high resolution mass spectrometric analysis, crystallographic data collection and processing statistics, molecular modeling. This material is available free of charge via the Internet at <http://pubs.acs.org>.

The authors declare no competing financial interest.

INTRODUCTION

There are two major neurotransmitters that regulate brain neuronal activity: *L*-glutamate, an excitatory neurotransmitter, and γ -aminobutyric acid (GABA), an inhibitory neurotransmitter.¹ When GABA concentrations in the brain fall below a threshold level, convulsions occur. Low levels of GABA are linked to not only epilepsy,² but also many other neurological disorders including Parkinson's disease,³ Alzheimer's disease,⁴ Huntington's disease,⁵ and cocaine addiction.⁶ One of the principal methods to raise the GABA level in human brain is to use small molecules that cross the blood-brain barrier and inhibit the activity of γ -aminobutyric acid aminotransferase (GABA-AT), a pyridoxal 5'-phosphate (PLP)-dependent enzyme that degrades GABA.⁷ Indeed, a FDA-approved inactivator of GABA-AT is the current antiepileptic drug vigabatrin (Figure 1), sold under the brand name Sabril.⁸ However, a large dose of vigabatrin (1 – 3 g) needs to be taken daily to be effective,^{9–11} and many serious side effects, including psychosis¹² and permanent vision loss,¹³ arise from its usage in 25–40% of patients. Therefore, there is an important need for an alternative to vigabatrin.

Our group has recently developed a compound, CPP-115 (Figure 1), that is 186 times more efficient in inactivating GABA-AT than vigabatrin. Unlike vigabatrin,¹⁴ CPP-115 did not inactivate or inhibit off-target enzymes, such as alanine aminotransferase and aspartate aminotransferase.¹⁵ CPP-115 was tested in a multiple-hit rat model of infantile spasms,¹⁶ and the results showed that it suppressed spasms at doses of 0.1–1 mg/kg/day, >100-fold lower than those for vigabatrin. CPP-115 produced longer spasm suppression than vigabatrin (3 days vs. 1 day) and had a much larger margin of safety. CPP-115 was granted Orphan Drug Designation by the FDA for the treatment of infantile spasms and has completed a Phase I clinical trial.

We have studied the inactivation of GABA-AT by CPP-115 and discovered that the resulting metabolite forms a tightly-bound complex with the enzyme via electrostatic interactions of the two carboxylate groups in the metabolite with Arg192 and Arg445 in the active site (Scheme 1).¹⁷ The inactivation was initiated by Schiff base formation of CPP-115 with the active site PLP, followed by γ -proton removal and catalytic hydrolysis of the difluoromethylenyl group to give the PLP-bound dicarboxylate metabolite. We also discovered that the Glu270-Arg445 salt bridge in the active site was disrupted, leading to the formation of a new binding pocket for the inactivator.

Previously, (*E*)- and (*Z*)-(1*S*,3*S*)-3-amino-4-fluoromethylenyl-1-cyclopentanoic acid (**1** and **2**, respectively, in Figure 1), monofluorinated analogs of CPP-115, were synthesized and evaluated as potential mechanism-based inactivators of GABA-AT.¹⁸ Compounds **1** and **2** showed concentration- and time-dependent inhibition of GABA-AT with K_I values of 250 μ M and 530 μ M, respectively. Although **1** and **2** bound better to GABA-AT than vigabatrin (K_I 1.3 mM), the inactivation rate constants for **1** (k_{inact} 0.25 min⁻¹) and **2** (k_{inact} 0.74 min⁻¹) were smaller than that for vigabatrin (k_{inact} 2.2 min⁻¹); consequently, the efficiency constants for **1** (k_{inact}/K_I 1.0 mM⁻¹min⁻¹) and **2** (k_{inact}/K_I 1.4 mM⁻¹min⁻¹) were comparable to that of vigabatrin (k_{inact}/K_I 1.7 mM⁻¹min⁻¹). However, despite their similarities in structure and potency, the inactivation mechanism of GABA-AT by **1** and **2** may be very

different. For example, diastereomers **3** and **4** (Figure 1) also differ only as (*E*)- and (*Z*)-fluoroalkenes, but they have vastly different mechanisms of inactivation of GABA-AT.¹⁹ Because different inactivation mechanisms can occur by minor structural changes, we were interested to determine how **1** and **2** might undergo inactivation of GABA-AT. Furthermore, if they inactivate by a mechanism that disrupts the Arg445-Glu270 salt bridge to provide a new binding pocket, this would confirm the importance of Arg445 in the design of new GABA-AT inactivators. Here we report our mechanistic studies on the inactivation of GABA-AT by **1** and **2**.

RESULTS

Turnover of **1** and **2** by GABA-AT

GABA-AT inactivated by **1** and **2** was assayed for transamination by monitoring the conversion of α -ketoglutarate to glutamate. In the co-incubation samples of GABA-AT with the analogs, continuous formation of glutamate was observed in both samples, even though the rates of formation gradually decreased (Supplementary Information Figure S1). Compound **2** produced glutamate at a greater rate than **1**, which accounts for its larger k_{inact} value than that of **1**. GABA-AT inactivated by **1** and **2** seemed to be releasing glutamate very slowly, which may account for their inability to completely inactivate the enzyme even at high concentrations. The average number of transaminations per inactivation was determined after 24 hours of inactivation to allow sufficient time for the enzyme to release glutamate. The partition ratios, the ratios of product released to enzyme inactivated, for **1** and **2** were 380 ± 11 and 273 ± 10 , respectively; vigabatrin was used as a positive control, which gave an average number of transaminations per inactivation of 2.7 ± 0.1 (Supplementary Information Figure S2). The partition ratio for CPP-115 with GABA-AT was reported to be about 2000, releasing cyclopentanone-2,4-dicarboxylate and two other precursors of this compound.¹⁷

Fluoride Ion Release During Inactivation

A fluoride ion electrode was used to determine if fluoride ions were released during the inactivation of GABA-AT by **1** or **2**. Interestingly, fluoride ions were continually released during inactivation, even after the activity of the enzyme had diminished to almost zero (Supplementary Information Figure S3). After normalization of the values with the controls, it was found that 202 ± 15 and 179 ± 11 equiv of fluoride ions were released slowly over a period of 30 h for **1** and **2**, respectively. Because α -ketoglutarate is essential to regenerate PLP from PMP, the amount of fluoride ion released in a single turnover can be calculated in the absence of α -ketoglutarate. As GABA-AT is only active as a homodimer, one turnover equates to two molecules of inactivator. A continual release of fluoride ions was not observed when α -ketoglutarate was omitted. Only 2.3 ± 0.3 equivalents of fluoride ions were detected, which accounts for the release of one fluoride ion by a single turnover per enzyme active site (Supplementary Information Figure S4). However, when α -ketoglutarate was added to this mixture, the fluoride ion concentration continued to increase. No fluoride ions were detected in the control experiment when no enzyme was present.

Cofactor Fate During Inactivation

To determine the fate of the PLP coenzyme, GABA-AT was reconstituted with [^3H]PLP and then inactivated with **1** or **2**. The released radioactive compounds from each incubation mixture were analyzed (Figures 2A and 2B). The experiments were performed with two controls: the inactivator was omitted in a negative control (all radioactivity should be labeled PLP), and the inactivator was replaced by GABA with α -ketoglutarate omitted in a positive control (all radioactivity should be PMP). The negative control released all of its radioactivity as PLP (Supplementary Information Figure S5A), while the positive control released radioactivity almost all as PMP but also as a small amount of PLP (Supplementary Information Figure S5B). Given that the positive control with GABA should produce only PMP, the PLP released from this sample represents the portion of the enzyme that was inactive, formed during reconstitution of *apo*-GABA-AT with [^3H]PLP. After the background radioactivity from the control experiments (Supplementary Information Figures S5A and S5B) was subtracted from the inactivation experiments (Figures 2A and 2B), both **1** and **2**-inactivated [^3H]PLP-reconstituted GABA-AT were found to release 100% of its cofactor as [^3H]PMP.

Proteomics After Inactivation

Top-down proteomics was run on samples of GABA-AT inactivated by **1** and **2**.²⁰ However, the resolution was low, and the mass shift for each peak, compared to that of native GABA-AT, was inconsistent, producing no robust information (data not shown). Middle-down proteomics was then run on samples of GABA-AT inactivated by **1** or **2**; in this experiment, the samples were treated with NaBH_4 , followed by Glu-C digestion, before being submitted to mass spectral analysis. A sample of GABA-AT with no inhibitor underwent similar treatment and was used as a control. The masses of peptides suspected to be covalently modified were interrogated, most likely bound to Lys329, to identify unmodified peptides in the control and any corresponding modified peptides in the inhibited samples. However, the results showed no additional mass on any peptide (Supplementary Information Figures S6 and S7). The active site peptide did show more PLP bound in the control enzyme sample, as would be expected because the PLP in the native enzyme is covalently bound to Lys329.

UV Absorption During Inactivation

An increase in the UV absorption at 300–320 nm was observed during inactivation of GABA-AT by **1** and **2** to confirm the formation of vinylogous amide. *trans*-Vinylogous amide compounds generally absorb in the range 285 – 305 nm with molar extinction constants of 25,000 to 35,000 $\text{L mol}^{-1} \text{cm}^{-1}$, and *cis*-vinylogous amide compounds generally absorb in the range 300 – 320 nm with molar extinction constants of 10,000 to 20,000 $\text{L mol}^{-1} \text{cm}^{-1}$, so they could be easily observed even at μM concentrations.²¹ Because the UV absorption peak of a vinylogous amide might overlap with that of PMP, the experiments were performed with two controls: the inactivator was replaced by GABA in the presence or absence of α -ketoglutarate. When α -ketoglutarate is present, PLP and PMP are in a dynamic equilibrium. When α -ketoglutarate is omitted, all of the PLP is converted to PMP. UV absorption spectra showed an absorption peak in the range 300 – 320 nm for both **1**- and **2**-inactivated GABA-AT (Figure 4), suggesting the formation of a *cis*-

vinyllogous amide. The control experiments show little change in the range 300 – 320 nm (Supporting Information S9). The formation of a *cis*-vinyllogous amide is faster in **2**-inactivated GABA-AT than in **1**-inactivated GABA-AT, consistent with the larger k_{inact} value for **2** than that for **1**.

Metabolites Formed During Inactivation

Mass spectrometric analysis (using ESI-mass spectrometry) was performed to search for the metabolites released during inactivation of GABA-AT by **1** and **2**. After GABA-AT inactivation by **1** and **2**, followed by denaturation and filtration, a metabolite was identified from both sample solutions that was not present in the control sample: $[m/z]$ 155.0335 (Figures 3A shows results for **1**; Supplementary Information Figure S8A shows results for **2**). This parent ion was selected for fragmentation using normalized collision energies. Fragmentation data for m/z 155.0335 (Figures 3B shows results for **1** and Supplementary Information Figure 8B shows results for **2**) confirmed the structure of 3-formyl-4-oxocyclopentane-1-carboxylic acid (see Figure 3A for structure).

X-Ray Crystallography of GABA-AT Inactivated by **1** and **2**

To understand how time-dependent inactivation of GABAAT by **1** or **2** could occur without covalent modification of the protein or cofactor, **1**- and **2**-inactivated and dialyzed GABAAT were crystallized (Supplementary Information Table S1 gives the crystallographic data and refinement statistics). The crystal structures of the native enzyme and the inactivated enzymes, obtained to 1.7 Å resolution, were compared to analyze the difference in overall structure (Supplementary Information Figure S10) and in the active site (Figure 5 shows the structure with **1** bound, and Supplementary Information Figure S11 shows the structure with **2** bound). The active site of the inactivated GABA-AT was investigated to understand the ligand-enzyme interactions; the omit maps support the ligand interpretation.

Inactivation of GABA-AT by **1** and **2** and Stability of the Complex

GABA-AT was incubated with excess **1** and **2** at room temp. After 25 h of incubation, the enzyme activity of **1**- and **2**-inactivated GABA-AT was 1.4% and 0.3%, respectively, consistent with previous experiments.¹⁸ The mixture was dialyzed against bulk buffer with α -ketoglutarate and PLP. Aliquots at different time intervals were collected and assayed for the return of enzyme activity (Figure 6). After 72 h of dialysis, the enzyme activity of **1**- and **2**-inactivated GABA-AT returned and stabilized at 23% and 21%, respectively. Identical experiments were repeated for 12 h of incubation, and the results were similar to those with 25 h of incubation.

DISCUSSION

To deduce the mechanism for the inactivation of GABA-AT by **1** or **2**, we considered a variety of likely inactivation mechanisms (Schemes 2–5), then designed experiments to differentiate them. All of the inactivation mechanisms are initiated by Schiff base formation of **1** or **2** with the active site PLP, followed by γ -proton removal, similar to those shown in Scheme 1. In Mechanism 1 (Scheme 2), following γ -proton abstraction of **5**, tautomerization

leads to an α,β -unsaturated intermediate (**6**), which is attacked by the active site lysine residue or another base to covalently modify the enzyme (**7**). Fluorine elimination leads to inactivation of the enzyme (**8**). Hydrolysis of **8** gives **9** with release of PMP as the cofactor. If the X in **7** is OH from attack by water, elimination of the fluoride ion will result in a stable formyl group (**10**, Scheme 3). Hydrolysis of **10** gives **11** with release of PMP as the cofactor. A third potential mechanism involves allylic tautomerization of aldimine **5** to form **12** (Scheme 4). Because **12** also is a reactive electrophile, it may undergo Michael addition to form adduct **13**, which could be hydrolyzed to give **14** and release PMP. In the final potential mechanism (Scheme 5), intermediate **12** generates an enamine (**15**), which then can proceed through four different pathways. In pathway a, **15** undergoes enamine attack on the Lys329-bound PLP to form covalent adduct **16**, which hydrolyzes to covalent adduct **17**. In pathway b,c **15** undergoes fluoride ion elimination to generate reactive Michael acceptor **18** and PLP; hydrolysis of **18** gives **19**. Mechanism b,d involves attack on **18** by an active site nucleophile, which gives covalent adduct **20**. Tautomerization and hydrolysis of **20** gives **21**. Mechanism b,e is the same as b,d except that water is the nucleophile to give **22**; tautomerization and hydrolysis gives **23**. All of these mechanisms can be differentiated by the determination of whether fluoride ion is released, by the fate of the cofactor, and by the final metabolites or adducts formed; these possibilities are summarized in Table 1.

A single turnover experiment (Supplementary Information Figure S4) demonstrated that one fluoride ion was released from **1** and **2**. Therefore, Mechanisms 3 and 4a, which release no fluoride ions during inactivation, can be excluded. During inactivation the PLP cofactor is converted to PMP (Figure 2 and Supplementary Information Figure S5). Therefore, Mechanisms 4b,c, 4b,d, and 4b,e, which release the cofactor as PLP during inactivation, can be excluded; only Mechanisms 1 and 2 remain. All attempts to detect covalently modified GABAAT by mass spectrometry failed. These experiments suggest that **1** and **2** do not covalently modify GABA-AT, which is Table 1. Expected Differences in the Various Inactivation Mechanisms Mechanism Fluoride release Cofactor release Final adduct/ Metabolite release inconsistent with Mechanism 1, although it is possible that **9** (Scheme 2) could be hydrolyzed to **11** (Scheme 3). Mass spectrometry was able to identify **11** (Supplementary Information Scheme 3) as the metabolite generated during inactivation, which is consistent with Mechanism 2. An increase in UV absorption at 300–320 nm, observed during the inactivation of GABA-AT by **1** and **2** (Figure 4), confirmed the formation of a *cis*-vinylogous amide.

To determine the structure of the inactivated enzyme, X-ray crystallography was carried out. Consistent with Mechanism 2, there is no covalent adduct; instead, **11** is bound in a Schiff base with the cofactor, but it is not tightly bound (Figure 5 shows the structure with **1** bound, and Supplementary Information Figure S11 shows the structure with **2** bound). There appears to be considerable flexibility in the formyl group side chain. When the structure of the metabolite was fitted into the electron cloud, one carbon in the cyclopentane ring did not have electron density around it (Supplemental Information Figures S12 and S13). This could be attributed to wobbling of the ring (weak binding) inside the active site. The crystal structures, however, clearly showed that the metabolite contained the PLP ring and that it did not covalently modify the enzyme. On the basis of the evidence from fluoride release,

cofactor release, metabolite formation, proteomics, UV absorption spectra, and X-ray crystallography, the most consistent mechanism is that shown in Scheme 6.

To test the stability of the metabolite in the active site and whether there was a reversible component to the inactivation, time-dependent reactivation of GABA-AT was studied. The results showed that after 25 h of incubation with excess **1** and **2**, followed by 72 h of dialysis, the enzyme activity of **1**- and **2**-inactivated GABA-AT returned and stabilized at 23% and 21%, respectively. This suggests that the inactivation of GABA-AT by **1** and **2** includes both an irreversible component and a reversible component.

In the inactivation of GABA-AT by CPP-115, we discovered that the resulting metabolite (same as **11** except with a carboxylate in place of the formyl group) forms a tightly-bound complex via electrostatic interactions between the two carboxylate groups of the CPP-115 metabolite and Arg192 and Arg445 in the active site; a conformational change disrupts the Glu270-Arg445 salt bridge in the active site, leading to the formation of a new binding pocket for the inactivator.¹⁷ Here, the salt bridge between Arg445 and Glu270 has also been broken, and Glu270 is rotated away from its original position to accommodate, depending on the resonance structure (**24**, Scheme 6), either a hydrogen bonding interaction between the formyl group and Arg445 or a weak electrostatic interaction between the enolate of the formyl group in **24** and Arg445 (Figure 5 shows the structure with **1** bound, and Supplementary Information Figure S11 shows the structure with **2** bound). The rotation of Glu270, however, is less than that in the case of CPP-115, in which Glu270 completely rotates away to accommodate a full second guanidinium–carboxylate electrostatic interaction with Arg445 (Figure 7 shows an overlay of **1**-inactivated GABA-AT and CPP-115-inactivated GABAAT; Supplementary Information Figure S14 shows an overlay of **2**-inactivated GABA-AT and CPP-115-inactivated GABAAT). The crystal structures of four different **1**-inactivated GABA-AT monomers show that Glu270 only partially rotates away and maintains a partial electrostatic interaction with Arg445 (Figure 5), suggesting that the potential electrostatic interaction of the metabolite of **1** and Arg445 is weaker than the interaction of the metabolite of CPP-115 and Arg445. This is reasonable considering the difference in electron density on a carboxylate group (CPP-115 inactivated) vs that on a formyl group (**1**- or **2**-inactivated). The crystal structures of two different **2**-inactivated GABA-AT monomers (Supplementary Information Figure S11A and S11D) show Glu270 maintains a full electrostatic interaction with Arg445, while the crystal structures of two other **2**-inactivated GABAAT monomers (Supplementary Information Figure S11B and S11C) show Glu270 maintains a partial electrostatic interaction with Arg445. The inability of **1** and **2** to completely inactivate the enzyme could be attributed to this weak interaction of **24** with GABA-AT. It has been proposed that the Glu270-Arg445 salt bridge only disassociates during the second half of catalysis, i.e., the regeneration of PLP from PMP, to aid in the binding of the second carboxylate group of α -ketoglutarate.²² Many research groups have attempted to cocrystallize GABAAT with ligands such as α -ketoglutarate or L-glutamate, but they have not yet been successful.^{22, 23} However, by computer modeling using GOLD docking,²⁴ when the PLP-aldimine of **1** ((*E*)-**5**, Scheme 2) was docked into the active site of GABAAT in which the carboxylate of (*E*)-**5** forms a salt bridge with Arg192 as all GABA-related ligands do, the fluoromethylenyl group clashes with the Arg445–

Glu270 salt bridge; in order for this ligand to fit, the Arg445-Glu270 salt bridge must dissociate (Supplementary Information Figure S15), suggesting that the induced-fit rotation of Glu270 occurs immediately after transimination (the first step) so as to accommodate the side chain. The stability of **24** (up to 80%) in the active site could even further suggest that in **1**- and **2**-inactivated GABAAT, the enzyme alternates between two conformations, in which the Glu270-Arg445 salt bridge is open or closed, and in the correct final conformation (80%), **24** does not wobble randomly but moves in sync with the two conformations of GABA-AT, thus remaining in the active site. When the complex does not move in sync (20%), the product is washed out during dialysis; the remaining complex (80%) represents non-covalent irreversibly inhibited enzyme. To find an explanation for why no covalent modification takes place in the case of both **1** and **2**, we initiated molecular docking calculations of intermediate **6** (both (*E*)- and (*Z*) forms) using GOLD. The computer model shows that Lys329 is $> 4.3 \text{ \AA}$ from the fluoromethylenyl electrophilic center, which is too great a distance for nucleophilic attack (Supplementary Information Figure S16). This is only the second example showing that Arg445 can interact directly with a ligand and be involved in the inactivation of GABA-AT.

CONCLUSIONS

Similar to CPP-115, **1** and **2** were rationally designed to inactivate GABA-AT via a covalent Michael addition mechanism. However, the results described here indicate that they both inactivate GABA-AT by mechanism-based formation of a metabolite that induces a conformational change and forms a complex with the enzyme via electrostatic interactions with Arg192 and Arg445 (**24**, Scheme 6). After their formation, some metabolites, having wrong conformations, are slowly released from the active site, which accounts for the inability to completely inactivate the enzyme by **1** or **2** and the extended period of time that fluoride ions are released relative to the rate of inhibition of the enzyme. Other metabolites with suitable conformations stay in the active site, thus inactivating the enzyme. The crystal structures of **1**- and **2**-inactivated GABAAT reveal that the Arg445-Glu270 salt bridge in the active site is disrupted during inactivation, and Glu270 rotates away from its original position to accommodate a weak electrostatic or hydrogen bonding interaction between the formyl group in **24** and Arg445. These results confirm the possibility of additional binding energy with Arg445 and that future inactivators may be designed to take advantage of the formation of this new binding pocket.

EXPERIMENTAL PROCEDURES

Analytical Methods

GABA-AT assays were recorded on a Synergy H1 hybrid multi-mode microplate reader (Biotek, USA) with transparent 96-well plates (Greiner bio-one, USA). Measurements of pH were performed on a Fisher Scientific AP71 pH/mV/°C meter with a pH/ATC electrode. Determinations of fluoride ion concentration were performed on the same meter with a Thermo Scientific 9609BN combination fluoride electrode. Small-scale dialyses were performed with EMD Chemicals D-Tube™ Mini dialyzer (molecular weight cutoff of 12–14 kDa). Radioactivity was determined with a Packard TRI-CARB 2100TR liquid scintillation

analyzer using Perkin-Elmer ULTIMA GOLD scintillation fluid. Eppendorf Minispin plus tubes were used for microcentrifugation. HPLC analysis was done with Beckman 125P pumps and a Beckman 166 detector. All of the runs were monitored at 254 nm. The HPLC column used was a Phenomenex Gemini-NX C18 analytical column (5 micron, 250 × 4.60 mm).

Reagents

All reagents and materials were purchased from Sigma-Aldrich Co., except the following: Centrifugal filters (molecular weight cut-off value of 10 kDa and 30 kDa) were purchased from EMD Millipore; Dowex 50 and sodium dodecyl sulfate were purchased from Bio-Rad; [³H]sodium borohydride was purchased from American Radiolabeled Chemicals, Inc.; all of the buffers and solvents used for FPLC analyses were filtered through GE Healthcare 0.45 μm nylon membranes.

Enzyme and Assays

GABA-AT (2.65 mg/mL, specific activity 2.1 unit/mg) was purified from pig brain by the procedure described previously.²⁵ Succinic semialdehyde dehydrogenase (SSDH) was purified from GABase, a commercially available mixture of SSDH and GABA-AT, using a known procedure.²⁶ GABA-AT activity was assayed using a published method.²⁷ GABase (*Pseudomonas fluorescens*) and succinic semialdehyde were purchased from Sigma-Aldrich. The final assay solution consisted of 11 mM GABA, 1.1 mM NADP⁺, 5.3 mM α-ketoglutarate, 2 mM β-mercaptoethanol, and excess SSDH in 50 mM potassium pyrophosphate buffer, pH 8.5. The change in UV absorbance at a wavelength of 340 nm at 25 °C caused by the conversion of NADP⁺ to NADPH is proportional to the GABA-AT activity. Enzyme assays were recorded with a Perkin-Elmer Lambda 10 UV/Vis spectrophotometer and a Biotek Synergy H1 using 96-well plate.

Syntheses of (E)- and (Z)-(1S,3S)-3-Amino-4-fluoromethylenyl-1-cyclopentanoic Acid (**1** and **2**, respectively)

These compounds were synthesized according to the published procedure by Pan *et al.*¹⁸

Inactivation of GABA-AT by **1** and **2**, and Dialysis of the Inactivated Enzyme

Potassium pyrophosphate buffer (500 μL, 50 mM, pH 8.5) containing GABA-AT (230 μg, 2.09 nmol), α-ketoglutarate (5 mM), β-mercaptoethanol (5 mM), and **1** or **2** (0.85 mg, 8.7 mM) was protected from light and incubated at room temp for 16 h. An aliquot of the inactivated GABA-AT (5 μL) was microcentrifuged (4×5 min, 13,400 rpm) through a 10 kDa MW cutoff centrifugal filter against 4×400 μL of potassium pyrophosphate buffer (50 mM, pH 8.5) containing α-ketoglutarate (5 mM) and β-mercaptoethanol (5 mM) to afford a 75 μL enzyme solution. PLP (3 μL, 500 μM) was added, and the resulting mixture was protected from light and incubated for 1 h at room temp.

Transamination Events per Inactivation of GABA-AT with **1** or **2** with and without pre-incubation

The method to detect glutamate followed from an established method with some modification.²⁸ GABA-AT (5 μ g) was added with 2 mM **1** or **2**, 5 mM α -ketoglutarate, and 50 mM potassium pyrophosphate (pH 8.5) in a total volume of 50 μ L. The mixtures were preincubated at room temperature for 24 h, protected from light. The mixtures with and without preincubation were each added to 50 μ L of an assay mixture that contained 50 mM potassium pyrophosphate (pH 8.5), 0.2 mM Amplex[®] Red, horseradish peroxidase (1.25 U), and glutamate oxidase (2 mU) to make a total volume of 100 μ L. The solution was incubated at 37 °C for 5 min with gentle shaking. Fluorescence was measured with excitation at 530 nm and emission at 590 nm using black 96-well plates. A standard curve was obtained by measuring varying concentrations of glutamate (0, 0.1, 0.5, 2.5, 5, 10, and 20 μ M).

Analysis of Fluoride Ion Release during Inactivation of GABA-AT by **1** or **2**

GABA-AT (450 μ L) was incubated in 100 mM potassium pyrophosphate buffer at pH 8.5 containing 2 mM **1** or **2** and 2.5 mM α -ketoglutarate in a total volume of 1510 μ L. A control containing everything but GABA-AT was also incubated. The incubation was protected from light and was carried out at room temp. At different incubation times, an aliquot (100 μ L) was removed from the incubation samples, mixed with 1.9 mL of total ionic strength adjustment buffer (TISAB), and their relative potentials were measured using a fluoride ion selective electrode. A standard curve was obtained prior to reading the fluoride ion release from the samples to obtain a conversion formula between potential (mV) and fluoride ion concentrations. The readings from the control sample were subtracted from the inactivated sample, and the concentration was divided by the concentration of GABA-AT to get the equivalents of fluoride ion released per inactivation event.

One-Turnover Experiment to Determine Fluoride Ion Release during Inactivation of GABA-AT by **1** or **2** without α -Ketoglutarate

The same experiment was run as above but without α -ketoglutarate to test the amount of fluoride ion released during one turnover. When there is no α -ketoglutarate in the mixture to regenerate PLP, the reaction stops at one turnover per active site.

Radioactive Labeling of [7-³H]-PLP with Tritiated NaBH₄

[7-³H]-PLP was synthesized according to a published procedure.¹⁷

Inactivation of [7-³H]PLP-Reconstituted GABA-AT by **1** or **2**

GABA-AT that had been reconstituted with [7-³H]PLP was incubated with **1** or **2** (2 mM) in 100 mM potassium phosphate buffer containing, α -ketoglutarate (3 mM) and β -mercaptoethanol (3 mM) in a total volume of 100 μ L at pH 7.4 at room temperature, protected from light. A negative control was run under identical conditions as above, excluding the inactivator. A positive second control was run with 3 mM GABA in the absence of inactivator and α -ketoglutarate. The first control should release cofactor as PLP, and the second control should release cofactor as PMP. After incubation for 24 h, the activity of GABA-AT was less than 1% of control, and the solutions were adjusted to pH 11

with 1 M KOH and incubated for 1 h. Trifluoroacetic acid (TFA) was added to quench the base and make the solution 10% v/v TFA. The resulting denatured enzyme solution was microcentrifuged for 5 min at 10,000 rpm after standing at room temp for 10 min. A small amount of white solids was seen at the bottom of the tube. The supernatants were collected individually. To rinse the pellets, 50 μ L of 10% TFA was added to each tube, vortexed, and microcentrifuged for another 5 min. This process was repeated three times. The supernatant and rinses were combined and lyophilized. Cofactor analysis was carried out by dissolving the solids obtained from lyophilization with 100 μ L of a solution containing 1 mM PLP and 1 mM PMP standards, and then injecting the samples into the HPLC through a Phenomenx Gemini C18 column (4.6 mm $\times$ 150 mm, 5 μ). The mobile phase used was 0.1% aqueous TFA flowing at 0.5 mL/min for 25 min. The flow rate was increased to 1 mL/min from 25 to 30 min, and then a solvent gradient to 95% methanol was run over 30 min. Under these conditions, PLP eluted at 12 min and PMP at 6 min. Fractions were collected every minute, and the radioactivity was measured by liquid scintillation counting.

Top-down Native Spray Proteomics

For native spray studies of the intact GABA-AT enzyme, the reactions in the mass spectrometric analysis section were buffer exchanged into 100 mM ammonium acetate buffer using Millipore 30 kD MWCO filters. All experiments were performed with a modified Q Exactive mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) using direct ESI infusion in the nanoflow regime. ESI spray voltage and pressure within the instrument was modulated in order to observe the intact GABA-AT dimer. However, we were unable to observe and define specific differences between the sets of samples.

Middle-down Proteomics of the Inactivated GABA-AT by 1 or 2

Both inhibited and control GABA-AT reactions were first reduced with sodium borohydride, as described previously,²⁹ and then digested with endopeptidase Glu-C (protease V8). The resulting peptides were analyzed via nanocapillary LC/MS using a 100 mm $\times$ 75 μ m ID Jupiter C18 (Phenomex) column in-line with an Orbitrap Elite mass spectrometer (ThermoFisher, Waltham, MA). All MS methods included the following events: (1) FT scan, m/z 400–2000, and (2) data-dependent MS/MS on the top 5 peaks in each spectrum from scan event 1 using higher-energy collisional dissociation (HCD) with normalized collision energy of 25. All data were analyzed using QualBrowser, part of the Xcalibur software packaged with the ThermoFisher Orbitrap Elite.

Mass Spectrometric Analysis (using ESI-mass spectrometry) of the Inactivated GABA-AT by 1 or 2

GABA-AT (30 μ g) was incubated in 50 mM ammonium bicarbonate buffer (pH 7.4) containing 2 mM **1** or **2** and 1 mM α -ketoglutarate in a total volume of 100 μ L at room temperature in the dark for 24 h. A control containing everything except inactivator was also incubated. After 24 h, GABA-AT in the inactivated sample was less than 1% active vs control. Formic acid (1 μ L) was added to each reaction mixture, and both were centrifuged in a 0.5 mL 10 kDa MWCO centrifuge tube at 14000 g for 10 min or until most of the solution had passed through. An additional 20 μ L of 50 mM ammonium bicarbonate was

added above the filter and centrifuged for 3 min. Flow through (20 μ L) was injected onto a Luna C18(2) column (100 A, 2 $\times$ 150 mm, 5 μ , Phenomenex). A 60 min gradient (Agilent 1100 HPLC, solvent A = 5% acetonitrile and 0.1% formic acid; solvent B = 0.1% formic acid in acetonitrile) was run from 2 – 80% B over 40 min. The LC was directly connected to a Thermo Fisher™ Q Exactive mass spectrometer. The top five most abundant ions in negative ion mode were selected for fragmentation using normalized collision energies.

UV Absorption During Inactivation of GABA-AT by **1** and **2**

Absorption of potassium pyrophosphate buffer (120 μ L, 50 mM, pH 8.5) containing GABA-AT (6 μ g, 0.054 nmol, 0.45 μ M), α -ketoglutarate (3.3 mM), β -mercaptoethanol (3.3 mM), and **1** and **2** (300 μ M) was observed in the UV range 290 – 400 nm at room temp over time. Control experiments were identical to those of **1** and **2** except GABA was used in place of **1** and **2**, and with the presence or absence of α -ketoglutarate (3.3 mM).

Inactivation of GABA-AT by **1** and **2** and Dialysis of the Inactivated Enzyme

GABA-AT (3 μ g, 0.027 nmol, 0.67 μ M) was incubated with **1** and **2** (1.5 mM) in potassium pyrophosphate buffer (40.6 μ L, 50 mM, pH 8.5) containing α -ketoglutarate (2.5 mM), β -mercaptoethanol (2.5 mM) at room temp for 25 h. An identical solution of GABA-AT without **1** and **2** was used as a control. Each of the enzyme solutions was transferred to a D-Tube Mini dialyzer and exhaustively dialyzed against potassium pyrophosphate buffer (3 $\times$ 200 mL, 50 mM, pH 8.5) containing α -ketoglutarate (5 mM), β -mercaptoethanol (5 mM), and PLP (0.1 mM) at 4 °C. The dialysis buffer was exchanged three times at 3 h intervals,. The enzyme activity remaining in each of the solutions was assayed at various time intervals. Identical experiments were repeated with 12 h of incubation.

Crystallization and Data Collection

The crystallization and data collection were performed according to a published procedure.¹⁷

Phasing, Model Building, and Refinement

Molecular replacement for the inactivated GABA-AT was carried out using the program Phaser³⁰ from the CCP4 software suite.³¹ An isomorphous structure model (PDB code: 1HOV)³² of native GABA-AT from pig brain including 4 monomers was used as the starting search model; PLP and other ligands including water were removed from the search model before use. The molecular search in Phaser produced a good structural solution. The rigid body refinement was followed by restrained refinement using Refmac5³³ and further manual model inspection and adjustments were conducted using the program COOT³⁴. The coordinates of the final PLP-inactivator adducts were built in the program JLigand³⁵; the adduct coordinates were regularized, and then the chemical restrains were generated in JLigand. The PLP-inactivator adducts were fitted into the residual electron density in COOT after the rest of the structure, including most of the solvent molecules, had been refined. The R_{cryst} and R_{free} for inactivated GABA-AT were satisfactory and are shown in Supporting Information Table S1. All structural figures were made in either UCSF Chimera³⁶ or PyMol (The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrodinger LLC).

Molecular Modeling

Molecular modeling studies were performed using the GOLD software package, version 5.3 (Cambridge Crystallographic Data Center, Cambridge, UK). The GABA-AT active site was defined as a sphere enclosing residues within 10 Å around the ligand. The 3D structures of pertinent ligands bound to PLP were built using ChemBio Ultra (version 14.0) and were energy minimized using an MMFF94 force field for 3000 iterations. The energy-minimized structures were docked into the binding site of GABA-AT and scored using ChemPLP fitness function. All poses generated by the program were visualized; however, the pose with the highest fitness score was used for elucidating the binding characteristics. Pymol (version 1.1) was used for generating images.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Some inactivators of GABA-AT

A

Reversed Phase HPLC and Radioactivity (2 mM 1)

B

Reversed Phase HPLC and Radioactivity (2 mM 2)

Figure 2.
HPLC trace of the inactivation of [³H]PLP-reconstituted GABA-AT by **1** (2 mM) (A) and **2** (2 mM) (B)

A **B** **Figure 3.**

(A) High resolution mass spectrum of metabolite released from a reaction incubation of **1** and GABA-AT. (B) Fragmentation and assigned structures of peak m/z 155 from a reaction incubation of **1** and GABA-AT.

Figure 4.
UV absorption spectra during the inhibition of GABA-AT by **1** (A) and **2** (B).

Figure 5.
Superimposition of the crystal structures of four **1**-inactivated GABA-AT (green) and native GABA-AT (cyan) monomers.

Figure 6.
Reactivation of the inactivated GABA-AT by **1** (A) and **2** (B)

Figure 7.
Superimposition of the crystal structures of four **1**-inactivated GABA-AT (green) and CPP-115-inactivated GABA-AT (pink) monomers.

Scheme 1.
Inactivation of GABA-AT by CPP-115

Scheme 2. First potential mechanism of inactivation of GABA-AT by **1** or **2**

Pyr = Pyridine ring

Scheme 3.

Second potential mechanism of inactivation of GABA-AT by **1** or **2**

Scheme 4.
Third potential mechanism of inactivation of GABA-AT by **1** or **2**

Scheme 5.

Fourth potential mechanism of inactivation of GABA-AT by 1 or 2

Scheme 6. Most consistent mechanism of inactivation of GABA-AT by **1** or **2**

Table 1

Expected Differences in the Various Inactivation Mechanisms

Mechanism	Fluoride release	Cofactor release	Final adduct/Metabolite release
1	Yes	PMP
2	Yes	PMP
3	No	PMP
4a	No	Enzyme bound
4b,c	Yes	PLP
4b,d	Yes	PLP
4b,e	Yes	PLP