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Syntheses of (*Z*)- and (*E*)-4-Amino-2-(trifluoromethyl)-2-butenoic Acid and Their Inactivation of γ-Aminobutyric Acid Aminotransferase

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Abstract—(*Z*)- and (*E*)-4-amino-2-(trifluoromethyl)-2-butenoic acid (**4** and **5**, respectively) were synthesized and investigated as potential mechanism-based inactivators of γ-aminobutyric acid aminotransferase (GABA-AT) in a continuing effort to map the active site of this enzyme. The core α-trifluoromethyl-α,β-unsaturated ester moiety was prepared via a Reformatsky/reductive elimination coupling of the key intermediates *tert*-butyl 2,2-dichloro-3,3,3-trifluoropropionate and *N,N*-bis(*tert*-butoxycarbonyl)glycinal. Both **4** and **5** inhibited GABA-AT in a time-dependent manner, but displayed non-pseudo-first-order inactivation kinetics; initially, the inactivation rate increased with time. Further investigation demonstrated that the actual inactivator is generated enzymatically from **4** or **5**. This inactivating species is released from the active site prior to inactivation, and as a result, **4** and **5** cannot be defined as mechanism-based inactivators. Furthermore, **4** and **5** are alternate substrates for GABA-AT, transaminated by the enzyme with K_m values of 0.74 and 20.5 mM, respectively. Transamination occurs approximately 276 and 305 times per inactivation event for **4** and **5**, respectively. The enzyme also catalyzes the elimination of the fluoride ion from **4** and **5**. A mechanism to account for these observations is proposed.

Introduction

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian central nervous system and is catabolized by the pyridoxal 5′-phosphate (PLP)-dependent enzyme GABA aminotransferase (GABA-AT, E.C. 2.6.1.19).[1] Inhibition of this enzyme results in an increase in availability of GABA in the central nervous system, which can have a beneficial effect in neurological disorders in which disturbances in GABA metabolism have been implicated.[2] These diseases include epilepsy,[3] Parkinson's disease,[4] Huntington's chorea,[5] and Alzheimer's disease.[6]

Previously in this laboratory, (*Z*)-4-amino-2-(fluoromethyl)-2-butenoic acid (**1**) was prepared and evaluated as a potential inactivator of GABA-AT.[7] The first steps of the proposed mechanism of inactivation for this compound (Scheme 1) follow the normal catalytic mechanism for GABA-AT, that is, Schiff base formation to the PLP cofactor, followed by deprotonation at the γ-carbon. Subsequent elimination of HF leads to the formation of a PLP-bound Michael acceptor (**2**) which could undergo attack by an active-site nucleophile. However, **1** showed no time-dependent inhibition of the enzyme. Rather, **1** was a potent reversible inhibitor of and substrate for GABA-AT. The compound also showed enzyme catalyzed fluoride ion release, demonstrating that elimination does occur. Two possible phenomena could explain the lack of time-dependent inhibition. First, no active-site nucleophile is properly juxtaposed for attack at the bound reactive Michael acceptor. However, Silverman and George demonstrated later that (*Z*)-4-amino-2-fluoro-2-butenoic acid (**3**) inactivates GABA-AT via a Michael-addition mechanism,[8] suggesting that a nucleophilic residue is positioned in the active site near the α-position of the enzyme bound GABA backbone. A second explanation is that the PLP-bound reactive intermediate (**2**) may have been insufficiently electrophilic to undergo attack by an active site nucleophile.

Key words: Amino acids and derivatives; γ-aminobutyric acid aminotransferase; mechanism-based inactivation.

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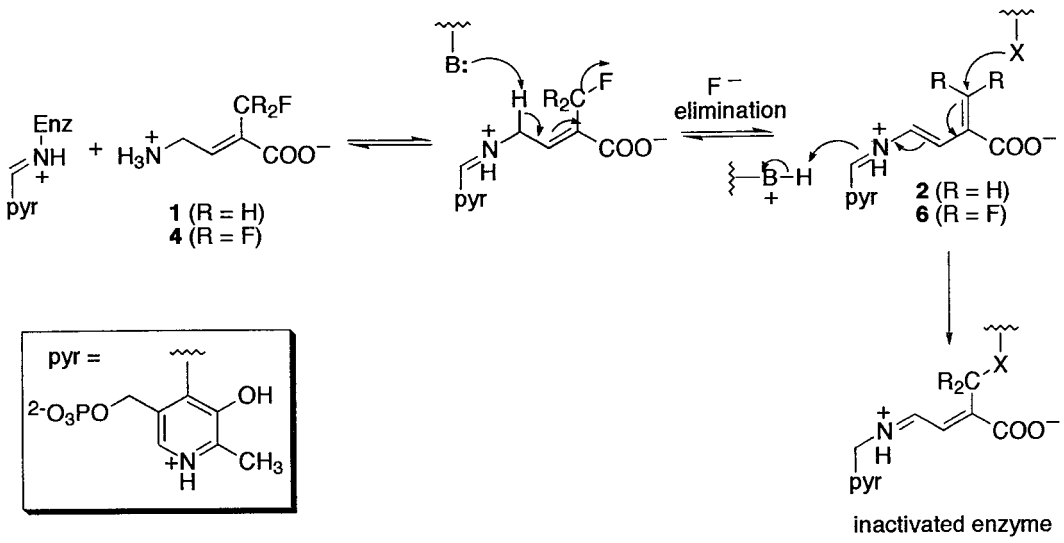
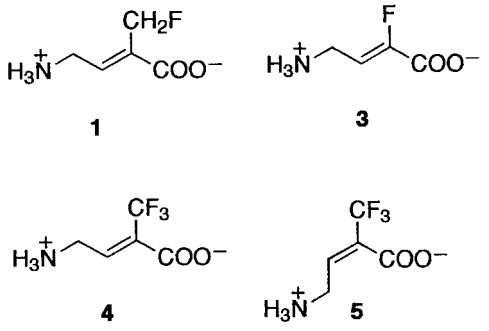


Scheme 1. Proposed mechanism of inactivation of GABA-AT by **1** or **4**.



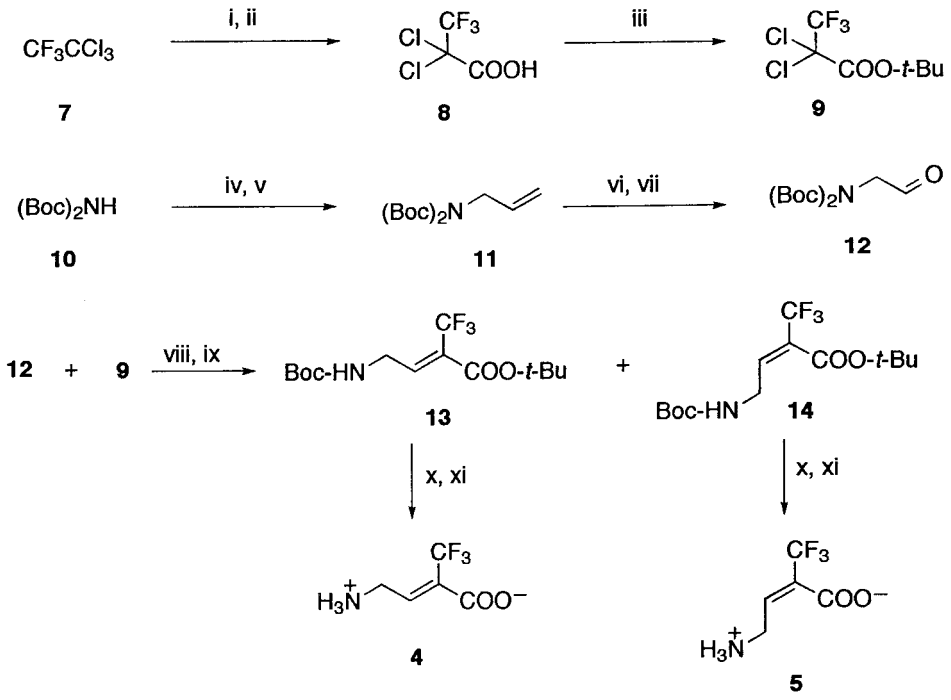
Several precedents in the literature have illustrated the increase of the electrophilicity of a Michael acceptor by substitution of hydrogen atoms with electronegative fluorine.[9–12] As part of a continuing effort to map the active site of GABA-AT, we now report the synthesis of (*Z*)- and (*E*)-4-amino-2-(trifluoromethyl)-2-butenoic acid (**4** and **5**, respectively) and their biological properties as potential mechanism-based inactivators of GABA-AT. In the proposed mechanism of inactivation for **4** (Scheme 1), the reactive intermediate (**6**) is activated for Michael addition by substitution of the terminal alkene with fluorine.

Results and Discussion

Synthesis

Initial efforts toward the synthesis of **4** focused on substitution of a GABA-like backbone, a strategy successfully used in the preparation of **1**.[7] Attempts were made to introduce the trifluoromethyl group via electrophilic,[13] nucleophilic,[14] or radical reactions.[15] After these efforts proved fruitless, a convergent synthesis was pursued which could incorporate all of the key functionalities in a single step. The literature cites only one general method for the synthesis of α-trifluoromethyl-α,β-unsaturated esters. Allmendinger and Lang described the synthesis of these molecules via a one-pot Reformatsky/reductive elimination coupling of methyl 2,2-dichloro-3,3,3-trifluoropropionate with a variety of aldehydes.[16] This methodology was successfully employed in the preparation of **4** and **5** (Scheme 2).

Preparation of 2,2-dichloro-3,3,3-trifluoropropionic acid (**8**) from 2,2,2-trichlorotrifluoroethane (**7**) followed the procedure of Lang and Klingert.[17] The core α-trifluoromethyl-α,β-unsaturated ester moiety proved to be sensitive to basic and nucleophilic conditions. Therefore, selection of appropriate protecting groups for the amino and carboxylate functionalities was essential. To facilitate deprotection of the carboxylate group under acidic conditions, the acid (**8**) was converted to the *tert*-butyl ester (**9**), which was used in place of the methyl ester employed in the literature methodology.[16] Initially, *N*-phthalimidoglycinal[18] was used in the coupling step; however, a more labile alternative was deemed necessary. Both *N*-(benzyloxycarbonyl)-[19] and *N*-(*t*-butoxycarbonyl)glycinal[20] were found to be unstable compounds that gave low yields during the coupling step. As a result, di-Boc protection was selected as an appropriate alternative. This protecting group had been employed previously in the preparation of other GABA analogues.[21] *N,N*-Bis(*t*-butoxycarbonyl)-glycinal (**12**) was prepared via a Gabriel-type synthesis. Conversion of di-*t*-butyl iminodicarboxylate (**10**) to its stable, isolable potassium salt[21] followed by alkylation with allyl bromide yielded *N,N*-bis(t-butoxycarbonyl)-allylamine (**11**). Ozonolysis yielded the stable aldehyde (**12**). Coupling of **12** and **9** via the method of Allmendinger and Lang[16] yielded a mixture of the mono-(*t*-butoxycarbonyl) (Boc) (**13** and **14**, major) and di-Boc (minor) products with Z/E ratios of approximately 1/1. The diastereomers (**13** and **14**) were not separable by flash silica gel chromatography. Instead, preparative-scale C_{18} reversed-phase HPLC was employed to separate the isomers cleanly. The protecting groups of **13** and **14** were removed with TFA followed by conversion to the non-hygroscopic free amino acids (**4** and **5**, respectively) using Dowex 50 ion exchange chromatography.



Scheme 2. Synthesis of **4** and **5**. (i) Zn, CO_2, DMF; (ii) aq. HCl; (iii) $(CH_3)_2C{=}CH_2$, concd H_2SO_4, CH_2Cl_2; (iv) KOH, EtOH; (v) allyl bromide, DMF, 100°C; (vi) O_3, −78°C, CH_2Cl_2; (vii) Me_2S; (viii) Zn–Cu, Ac_2O, 4 Å molecular sieves, THF, 66°C; (ix) C_{18} reversed-phase HPLC; (x) TFA, CH_2Cl_2; (xi) Dowex 50.

Enzymology

Compounds **4** and **5** inhibited GABA-AT in a time-dependent and concentration-dependent manner. However, both compounds displayed non-pseudo-first-order inactivation kinetics (Fig. 1). The rate of inactivation initially increased with time. The initial lag in inactivation suggests that the presumed inactivator is being converted into another species, which is the actual inactivator.[22] At high concentrations of **4** the rate of inactivation decreased with time later in the incubation period, and the enzyme was not fully inactivated (10–20% activity remaining). This decrease in the inactivation rate suggests that product inhibition may occur.

When **4** or **5** was incubated with the enzyme for 2.5 h followed by addition of a fresh aliquot of GABA-AT, the lag in inactivation was not observed (Fig. 2). The formation of the actual inactivator was shown to be a result of enzymatic, rather than nonenzymatic conversion. Compound **4** was preincubated in buffer for 21 h, and then incubated with enzyme. The initial lag in inactivation was still observed, indicating that enzyme was necessary for production of the inactivating species (Fig. 3). Furthermore, when GABA-AT is incubated with **4** or **5** in the presence of β-mercaptoethanol (5 mM), an electrophile trapping agent, inactivation is completely prevented (Fig. 4). These experiments indicate that the actual inactivator is a moderately reactive, electrophilic species, which escapes from the active site, then as it builds in concentration, returns to inactivate the enzyme. Because inactivation occurs subsequent to release of the activated species, **4** and **5** cannot be classified as mechanism-based inactivators.[22]

Substrate protection experiments demonstrated that inactivation occurs at the active site. Addition of GABA to the incubation of **4** or **5** with the enzyme results in a significant decrease in inactivation rate (Fig. 5). The substrate could potentially interfere with either the conversion of the presumed inactivator to the actual inactivator or with the inactivation step. Incubation of the enzyme with the inactivator for 2.5 h, followed by concomitant addition of fresh enzyme and substrate, shows a decrease in the inactivation rate when the substrate is present (Fig. 2). This experiment demonstrates that GABA protects the inactivation step, that is, inactivation occurs at the active site. However, this experiment does not rule out the possibility that the conversion step may also be shut down in the presence of the substrate.

Inactivation of GABA-AT with **4** or **5** followed by removal of excess inactivator by gel filtration results in no return of enzyme activity, indicating the inactivation is irreversible. This experiment suggests that a covalent bond between the actual inactivating species and the enzyme active site forms.[22]

In addition to converting **4** and **5** into a reactive, inactivating species, GABA-AT also transaminates these compounds. Approximately 276 and 305 transaminations occurred per inactivation event when GABA-AT was incubated with **4** and **5**, respectively, indicating that transamination is the preferred pathway. Since **4** and **5** are both substrates for GABA-AT and display non-pseudo-first-order inactivation kinetics, the K_m and k_{cat} values were estimated by quenching the transamination reaction early (after 10 min), during which time negligible inactivation had occurred. The values, determined

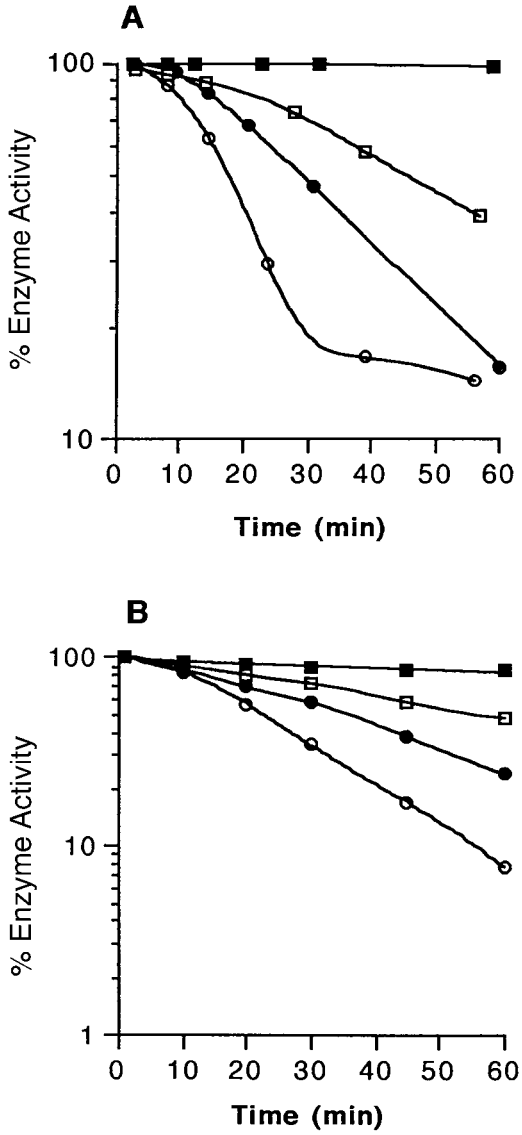


Figure 1. Time-dependent inhibition of GABA-AT by (A) 0.0 (■), 0.5 (□), 1.0 (●), and 5.0 (○) mM **4** or by (B) 0.0 (■), 1.0 (□), 2.0 (●), and 6.0 (○) mM **5**.

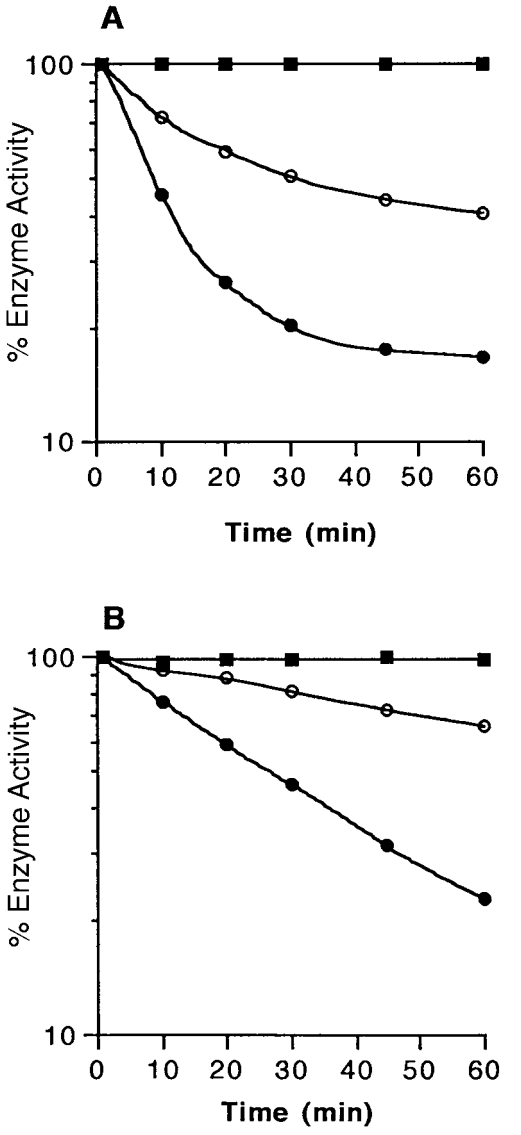
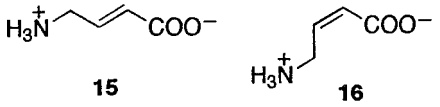


Figure 2. Time-dependent inhibition following addition of fresh enzyme to a solution of GABA-AT preincubated for 2.5 h with (A) 0 mM **4** (■), 1 mM **4** and 5 mM GABA (○), and 1 mM **4** (●) or with (B) 0 mM **5** (■), 5 mM **5** and 10 mM GABA (○), and 5 mM **5** (●).

using Hanes plots, are summarized in Table 1. The K_m value for GABA agrees with previously reported values.[23] Interestingly, **4** is a much better substrate than **5**. This result agrees with the previously reported observation that *trans*-4-aminocrotonic acid (**15**) is a good substrate for GABA-AT, while the *cis*-isomer (**16**) is not a substrate.[24] These observations support the hypothesis that GABA binds to the active site preferentially in its extended conformation.



The proposed mechanism of inactivation when compounds **4** and **5** were designed relied on an enzyme-catalyzed release of fluoride ion to generate the reactive intermediate (**6**) (Scheme 1). In fact, a time-dependent, enzyme-catalyzed release of fluoride is observed (Fig. 6). The rate of release increased with time and did not correlate to the loss of enzyme activity. Inactivation ceased at about 10–20% enzyme activity remaining. Addition of gabaculine, a potent inactivator of GABA-AT,[25] after 70 min, completely inactivated the enzyme. However, fluoride ion release was still observed even in the absence of enzyme activity, indicating that nonenzymatic fluoride release was occurring. A possible explanation for these observations could be that there is an initial, enzyme-catalyzed elimination of fluoride ion from the Schiff base formed between **4** and the PLP cofactor (Scheme 1). Subsequent release of the presumed activated species from the active site could lead to a further, nonenzymatic release of fluoride ion as a result of hydrolysis. This phenomena could account for the lack of correlation between inactivation and fluoride ion release.

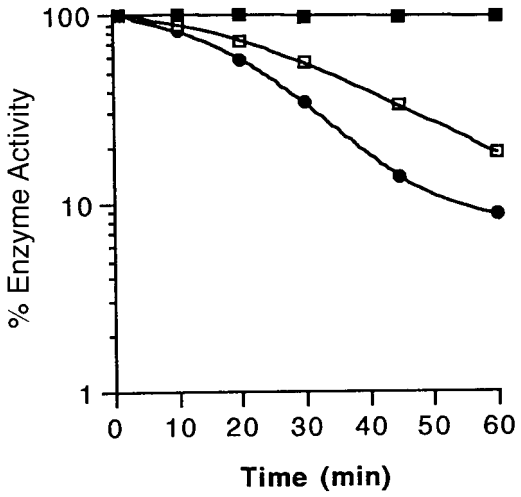
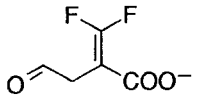


Figure 3. Time-dependent inhibition of GABA-AT by **4** preincubated in buffer for 21 h: 0 mM (■), 2 mM after 21 h in buffer (□), and 2 mM freshly prepared (●).

A revised proposal for the mechanism of inactivation of GABA-AT by **4** is presented in Scheme 3. Following Schiff base formation between **4** and the PLP cofactor, abstraction of the γ-proton could lead to either a transamination reaction (pathway a) or a fluoride elimination reaction (pathway b). All of the data presented in this paper indicate that inactivation occurs subsequent to release of the activating species. Therefore, the PLP-bound Michael acceptor (**6**) does not undergo nucleophilic attack by an enzyme active site residue as originally proposed (Scheme 1). Rather, we propose that this species is hydrolyzed from the cofactor and released from the active site as a reactive enamine (**17**), which could readily hydrolyze further to the corresponding aldehyde (**18**). Compound **18** is proposed as the actual inactivating species (pathway c). Various examples of activated esters and acids of the general structure **19** are known to act as Michael acceptors with concomitant loss of fluoride ion.[26–30]



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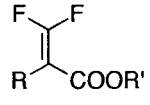


R' = H, ester

19

The carboxylate group of **18**, which would be in its ionized state under the incubation pH conditions, may temper the reactivity of the activated difluoromethylene moiety enough to allow this species to exist in aqueous solution without immediate hydrolysis. The observation that inactivation occurs subsequent to release of the activated species suggests that an active site nucleophile is not properly positioned for attack at the difluoromethylene position of bound species **6**, the same conclusion reached in the analysis of **1**.[7] Perhaps, the activated species (**18**) released into solution returns to the active site, binding in a different conformation which allows it to undergo nucleophilic attack. A similar

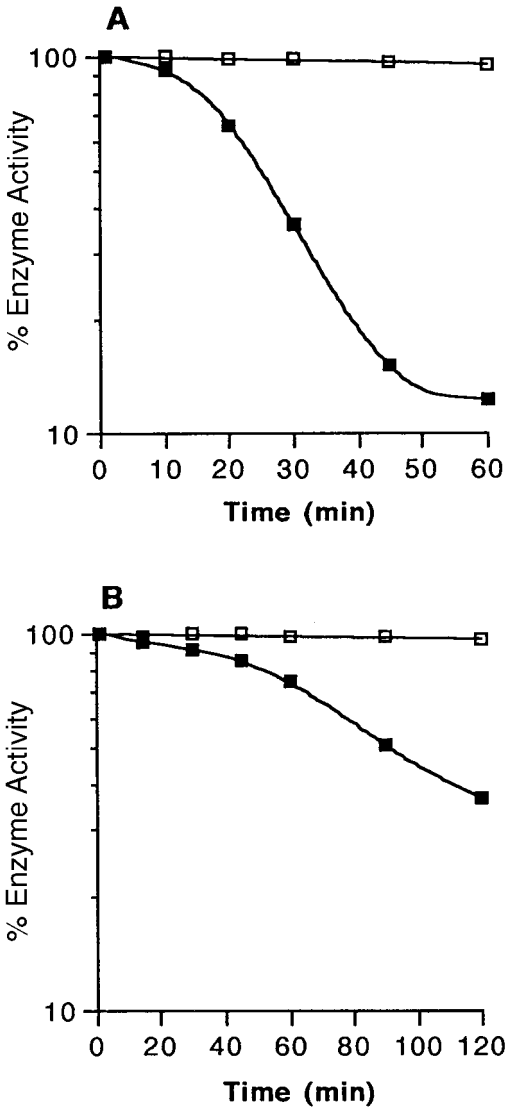


Figure 4. Effect of β-mercaptoethanol (β-ME) on the rate of inactivation of GABA-AT by (A) 5 mM **4** and 5 mM β-ME (□) and 5 mM **4** and 0 mM β-ME (■) or (B) 10 mM **5** and 5 mM β-ME (□) and 10 mM **5** and 0 mM β-ME (■). Control samples containing no inactivator showed no loss of enzyme activity in the prescence or absence of β-ME.

mechanism is envisioned for **5**, which would lead to the formation of **18** as well. Therefore, both isomers would produce the same inactivating species. Release of species **18** could produce succinic semialdehyde (SSA, **20**) by initial hydrolysis of the difluoromethylene functionality to produce the malonic acid analogue (**21**) followed by decarboxylation (Scheme 3). When **4** was incubated with GABA-AT and succinic semialdehyde dehydrogenase (SSDH), SSDH activity was observed (Fig. 7). However, whether this activity was due to the production of SSA or some other aldehyde could not be demonstrated. An HPLC assay was developed to detect SSA in the incubation samples. GABA-AT was incubated with **4**, and the carbonyl metabolites produced were derivatized with 2,4-dinitrophenylhydrazine (2,4-DNP) and extracted from the incubation mixture. The derivatized samples were analyzed by HPLC. No peaks were detected which coeluted with a synthetic standard

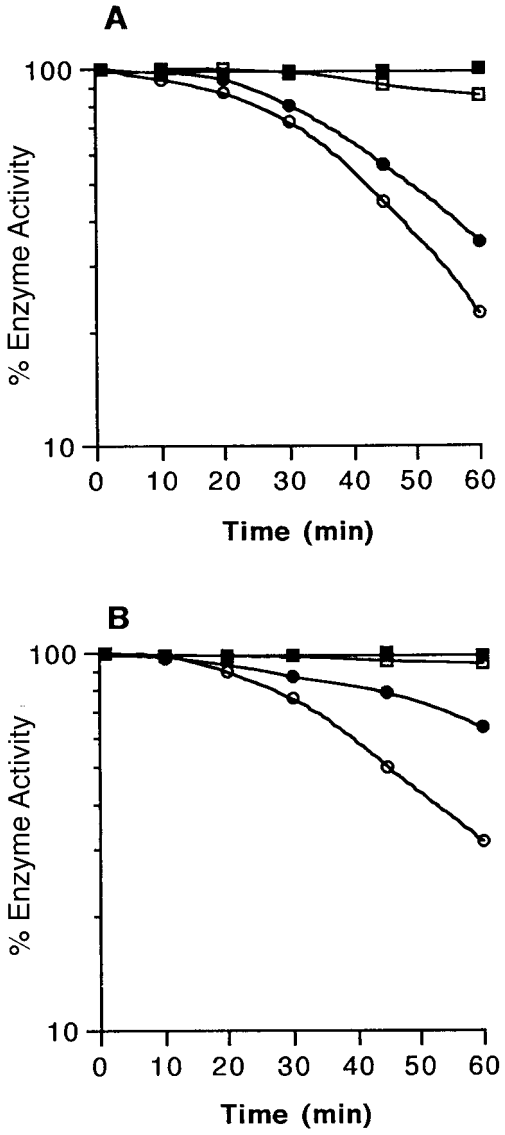
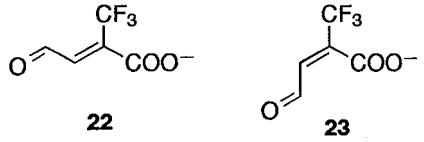


Figure 5. Effect of GABA on the rate of inactivation of GABA-AT by (A) 1 mM **4** and 0.0 (○), 0.5 (●), and 5.0 (□) mM GABA or (B) 5 mM **5** and 0 (○), 2 (●), and 10 (□) mM GABA. Inactivator and GABA were omitted in the control (■).

of the 2,4-DNP derivative of SSA[31] (Fig. 8A). However, a peak not present in the controls was observed, eluting at 16.6 min. Direct LC–MS analysis of the sample was performed. The peak eluting at 16.6 min had a mass of 347 (M-H) consistent with the 2,4-DNP derivative of the expected turnover product **22**. MS/MS analysis of the 347 molecular ion produced a daughter fragment with a mass of 283, which would be consistent with the loss of the carboxylate group and one fluoride.



Similar results were obtained when the experiment was repeated with **5** (Fig. 8B). A peak was observed eluting

Table 1. Kinetic parameters for the transamination of GABA, **4**, and **5** by GABA-AT

Substrate	[K_m (mM)]	[V_{max} (M/min)]	[k_{cat} (min^{-1})]	[k_{cat}/K_m (min^{-1} mM^{-1})]
GABA	1.15	2.90×10^{-5}	111	96.5
4	0.74	6.08×10^{-6}	23.2	31.4
5	20.5	7.07×10^{-6}	27.0	1.3

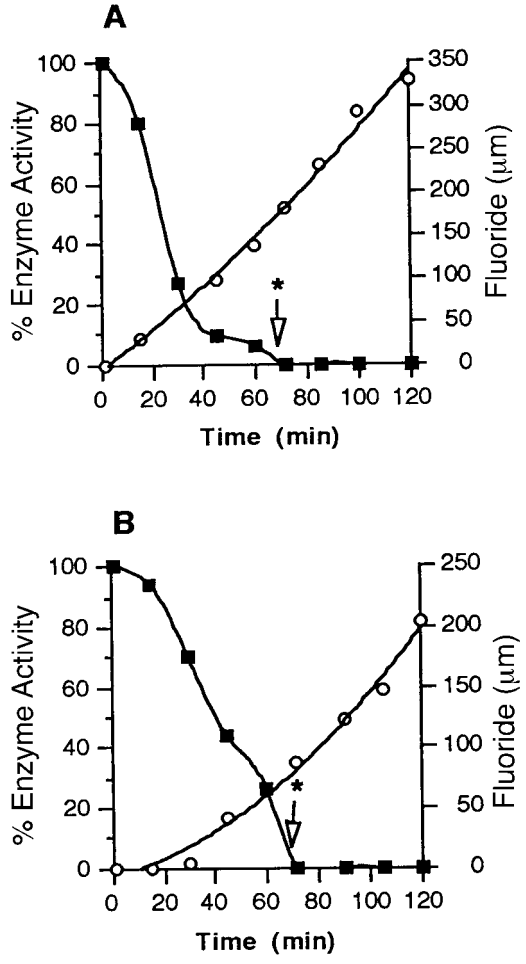
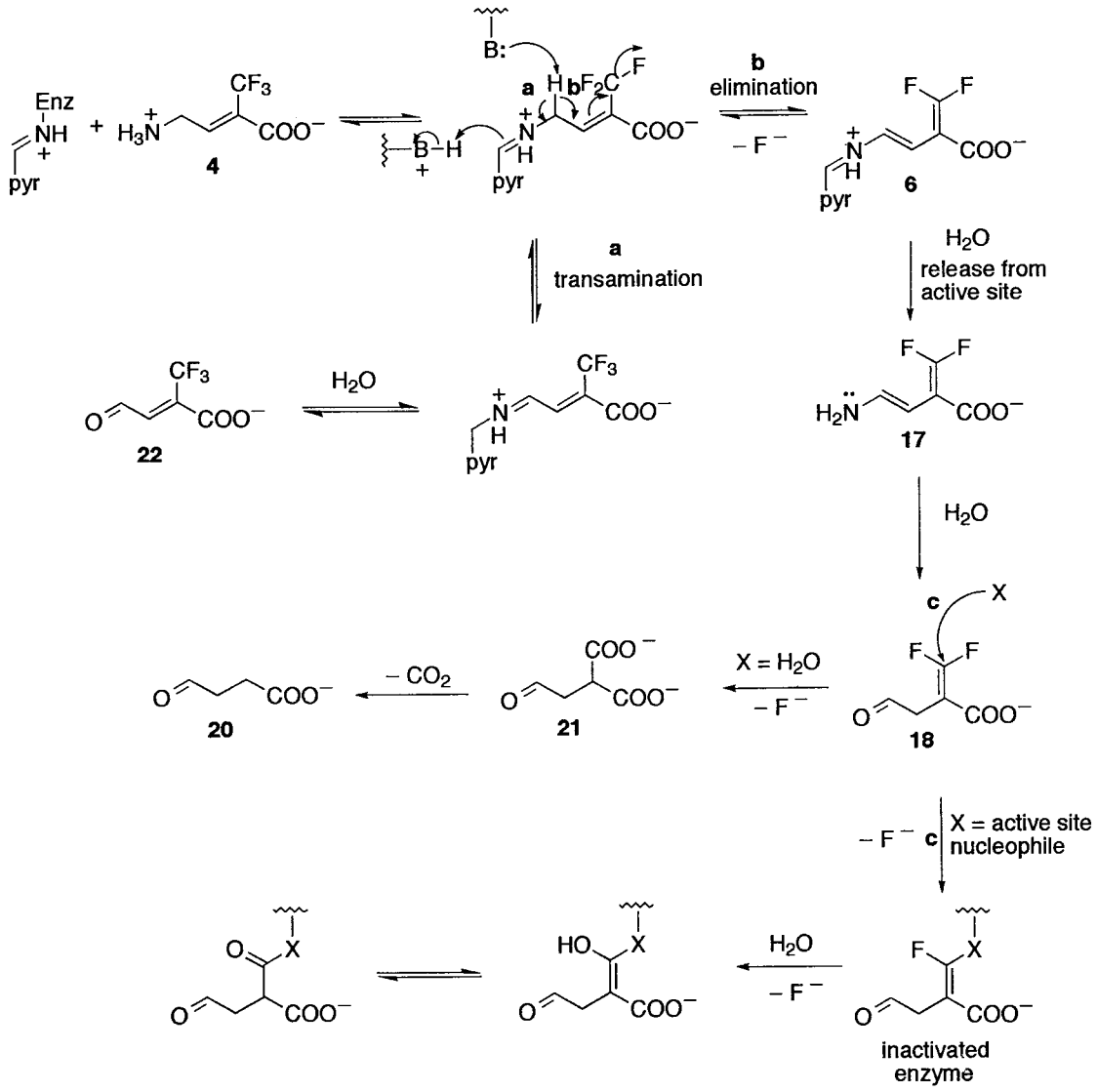


Figure 6. Loss of GABA-AT activity and release of fluoride ion from inactivator. Parallel experiments were performed where enzyme was incubated with (A) 2 mM **4** or (B) 5 mM **5**. Enzyme activity (■) and fluoride ion concentration (○) were determined. * After 70 min, gabaculine was added to a final concentration of 5 mM to completely inactivate the enzyme.

at 19.9 min which was not present in the controls. This peak had a mass of 347 (M-H), and the major daughter fragment was 283, consistent with the 2,4-DNP derivative of the expected turnover product **23**. These aldehyde turnover products may be alternate substrates for SSDH and may account for the activity observed (Fig. 7). Furthermore, **22** is likely to be responsible for the product inhibition observed in the inactivation kinetics. Over time, as the concentration of **22** builds up, it may compete with the inactivator at the enzyme active site,



Scheme 3. Proposed mechanism of inactivation of GABA-AT by **4** with release of activated species.

diminishing the effectiveness of the inactivator.[22] Interestingly, less product inhibition is observed with **5**, consistent with the notion that **23** does not bind as readily to the active site as does **22**. This observation provides further evidence that GABA binds to the active site in its extended conformation.[24] Even though production of SSA was not observed, this experiment does not rule out the possibility that it is formed. Since transamination is the predominant pathway, the HPLC assay may have been insufficiently sensitive to detect small quantities of SSA. Also, if the decarboxylation of **21** does not occur under these conditions, SSA would not be observed.

Conclusion

Compounds **4** and **5** irreversibly inhibit GABA-AT, and inactivation occurs at the active site; however, these compounds are not mechanism-based inactivators. Our observations suggest an active site nucleophile is not properly positioned to carry out an attack on the PLP-bound Michael acceptor.

Experimental

General methods

^{1}H, ^{19}F, and ^{13}C NMR spectra were recorded on a Varian Gemini 300 MHz spectrometer. Proton chemical shifts are reported as δ values in ppm downfield from Me_4Si in $CDCl_3$ or from TSP in D_2O. Fluorine-19 shifts are reported relative to $CFCl_3$ in $CDCl_3$ or D_2O. Carbon-13 shifts are reported relative to $CHCl_3$ in $CDCl_3$ or 1,4-dioxane (δ 65.4) in D_2O. Melting points were determined on a Fisher Johns melting point apparatus and are uncorrected. Combustion analyses were performed by Oneida Research Laboratories, NY. Thin-layer chromatography was performed using silica gel 60 F_{254} plates (EM Separations Technology) using standard visualization techniques. Column chromatography

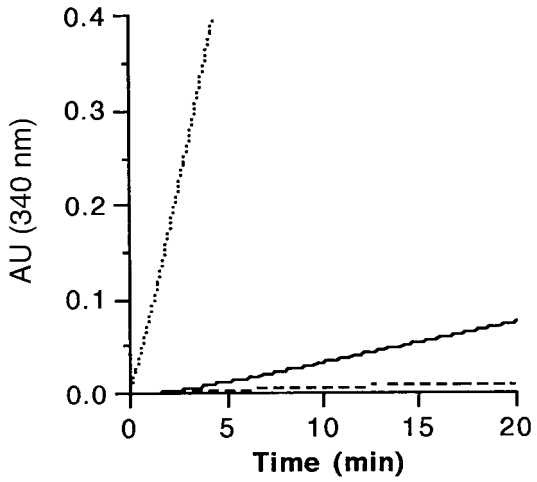


Figure 7. SSDH substrate activity of metabolites produced by incubation of 10 mM **4** with GABA-AT (solid line). Replacing **4** with 10 mM GABA served as a positive control (dotted line). Omission of substrate served as a negative control (dashed line).

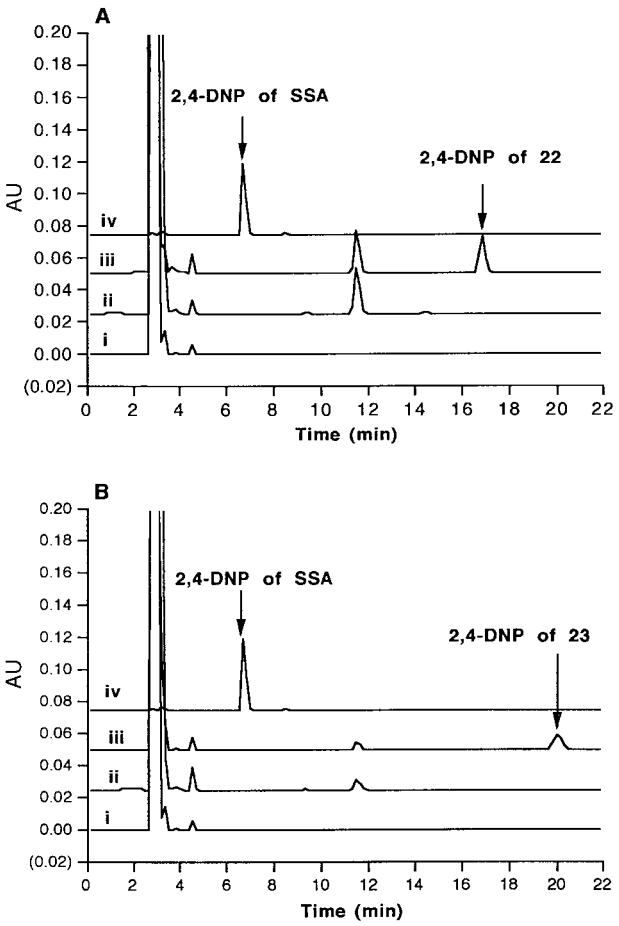


Figure 8. HPLC analysis of the metabolites produced during the incubation of GABA-AT with (A) 5 mM **4** or (B) 5 mM **5** followed by derivatizaton with 2,4-DNP: (i) enzyme only control, (ii) inactivator only control, (iii) enzyme + inactivator, and (iv) synthetic standard of the 2,4-DNP derivative of SSA.

was carried out with silica gel 60 (230–400 mesh, 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. 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. Mass spectral analyses were performed by the Analytical Services Laboratory at the Department of Chemistry, Northwestern University. Electron impact high resolution mass spectra (HRMS) were obtained with a VG70-50SE spectrometer. Electrospray mass spectra were obtained on a Micromass Quatro II spectrometer with the sample introduced using a Hewlett Packard Series 1100 liquid chromatography system. Fluoride ion concentration measurements were obtained using an Orion Research Model 720A pH meter equipped with an Orion Research Model 96-09 combination fluoride electrode.

Reagents

All chemicals used in synthetic procedures were purchased from Fischer Scientific or Aldrich Chemical Co. and used as received unless otherwise noted. Tetrahydrofuran (THF) was distilled under nitrogen from sodium metal with sodium benzophenone ketyl as an indicator. Methylene chloride (CH_2Cl_2) was distilled under nitrogen from powdered calcium hydride. Anhydrous dimethyl formamide (DMF) was used as purchased. 2,2,2-Trichlorotrifluoroethane was distilled under nitrogen from powdered 4 Å molecular sieves immediately before use.[17] Zinc dust was activated immediately before use.[32] Molecular sieves were dried at 275 °F and stored in a desiccator prior to use. GABA was recrystallized from ethanol-water. α-Ketoglutarate, β-mercaptoethanol, $NADP^+$, Sephadex G50, buffer salts, and other reagents used in the enzymological studies were purchased from Sigma Chemical Co.

2,2-Dichloro-3,3,3-trifluoropropionic acid (8). The method of Lang and Klingert was modified as follows.[17] To a suspension of activated zinc dust (65.4 g, 1 mol) in anhydrous DMF (500 mL) was added freshly distilled 2,2,2-trichlorotrifluoroethane (203.5 g, 1.09 mol) via cannula over several minutes. An ice bath was used to maintain the ensuing exothermic reaction just below reflux (46°C). After the initial exothermic reaction subsided, the mixture was stirred at room temperature for 1 h. The reaction mixture was quickly filtered through a pad of Celite to remove unreacted zinc, and then CO_2 (from dry ice, passed through a drying tube packed with Drierite) was introduced into the dark reddish brown filtrate through a gas dispersion tube for 18 h. The reaction solution was poured into a mixture of 20% HCl (500 mL) and ice (300 g) and extracted with ether (Et_2O) (5×300 mL). The pooled ether extracts were washed with 2% HCl (200 mL), dried over $MgSO_4$, and evaporated to a dark brown oil. The crude material was fractionally distilled twice, to give **8** as a pale-yellow oil (40.33 g, 94–96°C/29 mm Hg). Compound **8** could not

be purified to homogeneity;[33] it was used without further purification; ^{1}H NMR ($CDCl_3$): δ 10.88 (s, 1H); ^{19}F NMR ($CDCl_3$): δ −76.4 (s); ^{13}C NMR ($CDCl_3$): δ 120.7 (q, J = 283 Hz), 164.47 (CCl_2 hidden beneath $CDCl_3$). HRMS (EI) (m/z): $[M+H]^+$ calcd for $C_3H_2Cl_2F_3O_2$, 196.9384; found, 196.9391.

***tert*-Butyl 2,2-dichloro-3,3,3-trifluoropropionate (9).** The esterification reaction was divided equally in two Ace pressure tubes (38 mL). A solution of **8** (9.85 g, 50 mmol) in CH_2Cl_2 (5 mL) containing concd H_2SO_4 (0.14 mL, 5 mmol) was cooled to −78°C, and isobutylene (17–18 g, ca. 300 mmol) was condensed into the tube. The vessel was sealed, and the reaction mixture was stirred at room temperature for 70 h. The solution was cooled again to −78°C, and the vessel opened. After allowing the contents to slowly warm to room temperature, the contents of both reaction vessels were diluted with ether (250 mL), washed with 1 M $NaHCO_3$ (75 mL) and brine (50 mL), dried over $MgSO_4$, and evaporated. The crude product was chromatographed (pentanes) to give **9** as a clear colorless oil (17.94 g, 71%); ^{1}H NMR ($CDCl_3$): δ 1.56 (s, 9H); ^{19}F NMR ($CDCl_3$): δ −76.0 (s); ^{13}C NMR ($CDCl_3$): δ 27.3, 30.3, 87.3, 121.0 (q, J = 283 Hz), 159.2. HRMS (EI) (m/z): $[M\text{-}CH_3]^+$ calcd for $C_6H_6Cl_2F_3O_2$, 236.9697; found, 236.9699.

***N,N*-Bis(*tert*-butoxycarbonyl)allylamine (11).** A solution of allyl bromide (11.0 mL, 127.5 mmol) in anhydrous DMF (500 mL) was cooled to 0–5°C, and potassium bis(*tert*-butoxycarbonyl)aminide (21.7 g, 85.0 mmol), prepared from bis-(*tert*-butoxycarbonyl)aminide (**10**) according to the procedure of Allan et al.[21] was added portionwise over 5 min. The mixture was heated to 100°C for 2 h, allowed to cool, and poured into cold water (1000 mL). The mixture was extracted with ether (4×250 mL), and the pooled ether layers were washed with water (150 mL) and brine (150 mL), dried over Na_2SO_4, and evaporated. The crude product was chromatographed (hexanes, then 1:1 hexanes:ether) to give **11** as a colorless, crystalline solid, mp 42–43°C (lit. 47–48°C)[34] (21.79 g, 100%); ^{1}H NMR ($CDCl_3$): δ 1.50 (s, 18H), 4.17 (d, J = 5.5 Hz, 2H), 5.12 (dd, J = 10.4, 1.4 Hz, 1H), 5.17 (dd, J = 17.3, 1.5 Hz, 1H), 5.78-5.91 (m, 1H); ^{13}C NMR ($CDCl_3$): δ 27.9, 48.4, 82.2, 116.1, 133.6, 152.2, HRMS (EI) (m/z): $[M\text{-}C_4H_8]^+$ calcd for $C_9H_{15}NO_4$, 201.1001; found, 201.0996.

***N,N*-Bis(*tert*-butoxycarbonyl)glycinal (12).** A solution of **11** (20.59 g, 80 mmol) in CH_2Cl_2 (500 mL) was cooled to −78°C, and O_3 was introduced through a gas dispersion tube until a blue color persisted. Methyl sulfide (29.5 mL, 400 mmol) was added, and the clear colorless solution was slowly allowed to warm to room temperature over 17 h. The solvent was evaporated, and the crude product was chromatographed (4:1 hexanes: EtOAc) to give **12** as a clear, colorless oil which solidified to a colorless wax upon standing (11.51 g, 55%); ^{1}H NMR ($CDCl_3$): δ 1.51 (s, 18H), 4.39 (d, 2H), 9.55 (s, 1H); ^{13}C NMR ($CDCl_3$): δ 27.8, 55.1, 83.3, 151.6, 196.6 HRMS (EI) (m/z): $[M+H]^+$ calcd for $C_{12}H_{21}NO_5$, 260.1498; found, 260.1481.

***(Z)*- and *(E)*-*tert*-Butyl 4-[(*tert*-butoxycarbonyl)amino]-2-(trifluoromethyl)-2-butenoate (13 and 14).** The method of Allmendinger and Lang[16] was modified as follows. A suspension of activated zinc dust (4.90 g, 75 mmol), copper(I) chloride (0.74 g, 7.5 mmol), and powdered 4 Å molecular sieves (2.5 g) in THF (50 mL) was refluxed for 15 min. After cooling to room temperature, acetic anhydride (2.6 mL, 27.5 mmol) was added in one portion followed by the dropwise addition of a solution of **12** (6.48 g, 25 mmol) and **9** (6.96 g, 27.5 mmol) in THF (25 mL). Following a brief induction period (ca. 5 min), an exothermic reaction ensued which was controlled with a 0–5°C bath. Once the initial exothermic reaction subsided, the mixture was refluxed for 1 h. Upon cooling, the reddish brown mixture was diluted with ether (150 mL) and was filtered through Celite. The filtrate was washed with saturated NH_4Cl (50 mL), 5% $NaHCO_3$ (50 mL), and brine (50 mL), dried over Na_2SO_4, and evaporated to a dark red oil. The crude product was chromatographed (85:15 hexanes:EtOAc) to yield the mono-Boc product as a yellow wax (3.60 g, 44%) as a 1:1 mixture of the (*Z*)- and (*E*)-isomers (**13** and **14**, respectively). The minor di-Boc product was isolated as a viscous yellow oil (1.49 g, 14%) containing a 1:1 mixture of the (*Z*)- and (*E*)-isomers. Isomers **13** and **14** were separated on an Alltech Econosil C_{18} preparative scale HPLC column (250×22 mm, 10 μm) using a mobile phase of 35:35:30 MeCN:MeOH:H_2O at a flow rate of 10 mL/min, monitoring at 254 nm. Multiple injections (1.5 mL) were made of a solution of the isomeric mixture in mobile phase (ca. 70 mg/mL). Under these chromatographic conditions **13** and **14** eluted at 25 and 27 min, respectively. Fractions were collected manually. The organic portions of the fractions were evaporated, and the remaining aqueous layer was extracted with ether several times. The pooled ether extracts were washed with brine, dried over Na_2SO_4, and evaporated, yielding pure **13** as a colorless crystalline solid, mp 67–68°C (0.976 g) and **14** as a clear, colorless, viscous oil (0.723 g). **13**: ^{1}H NMR ($CDCl_3$): δ 1.46 (s, 9H), 1.51 (s, 9H), 4.15 (br s, 2H), 4.87 (br s, 1H), 7.03 (t, J = 5.8 Hz, 1H); ^{19}F NMR ($CDCl_3$): δ-59.5 (s); ^{13}C NMR ($CDCl_3$): δ 27.9, 28.3, 38.7, 80.11, 82.9, 122.3 (q, J = 274 Hz), 124.8 (q, J = 31 Hz), 150.2, 155.7, 161.1. HRMS (EI) (m/z): M^+ calcd for $C_{14}H_{22}F_3NO_4$, 325.1501; found, 325.1501. **14**: ^{1}H NMR ($CDCl_3$): δ 1.46 (s, 9H), 1.52 (s, 9H), 4.23 (br s, 2H), 4.94 (br s, 1H), 6.77 (*t*, J = 5.2 Hz, 1H); ^{19}F NMR ($CDCl_3$): δ −65.0 (s); ^{13}C NMR ($CDCl_3$): δ 27.9, 28.2, 39.3, 79.8, 83.2, 121.6, (*q*, J = 273 Hz), 125.2 (*q*, J = 30 Hz), 147.9, 155.8, 160.9. HRMS (EI) (m/z): M^+ calcd for $C_{14}H_{22}F_3NO_4$, 325.1501; found, 325.1502.

***(Z)*-4-Amino-2-(trifluoromethyl)-2-butenoic acid (4).** To a solution of ester **13** (0.651 g, 2 mmol) in CH_2Cl_2 (12 mL) was added TFA (3.1 mL, 40 mmol) dropwise. The solution was stirred at room temperature for 30 min, and then diluted with ether (25 mL). The mixture was extracted with water (2×15 mL), and the pooled aqueous extracts were washed with ether (20 mL) and applied to a plug (6 mL) of Dowex 50 resin. The plug was washed with water (100 mL), and the product was eluted with 0.1 M pyridine (250 mL). The

ninhydrin active fractions were pooled, evaporated, and dried under vacuum over P_2O_5 to give **4** as an off-white solid, mp 215–220°C dec. (0.164 g, 50%); ^{1}H NMR (D_2O): δ 4.06 (m, 2H), 6.74 (*t*, J = 6.5 Hz, 1H); ^{19}F NMR (D_2O): δ −58.5 (s); ^{13}C NMR (D_2O): δ 37.8, 123.4 (q, J = 273 Hz), 132.5 (q, J = 29 Hz), 136.5, 169.5. HRMS (EI) (m/z): $[M+H]^+$ calcd for $C_5H_7F_3NO_2$, 170.0429; found, 170.0410. Anal. calcd for $C_5H_6F_3NO_2$: C, 35.51; H, 3.58; N, 8.28. Found: C, 35.42; H, 3.50; N, 8.14.

(*E*)-4-Amino-2-(trifluoromethyl)-2-butenoic acid (5). The procedure described for **4** was employed in the conversion of **14** (0.488 g, 1.5 mmol) to **5** as a white solid, mp 212–215°C dec. (0.079 g, 31%); ^{1}H NMR (D_2O): δ 3.93 (3, J = 6.6 Hz, 2H), 6.47 (t, J = 6.6 Hz, 1H); ^{19}F NMR (D_2O): δ −64.0 (s); ^{13}C NMR (D_2O): δ 38.2, 122.7 (q, J = 273 Hz), 130.1, 135.2 (q, J = 29 Hz), 169.0. HRMS (EI) (m/z): $[M+H]^+$ calcd for $C_5H_7F_3NO_2$, 170.0429; found, 170.0422. Anal. calcd for $C_5H_6F_3NO_2$: C, 35.51; H, 3.58; N, 8.28. Found: C, 35.33; H, 3.55; N, 8.18.

Enzymes and assays

GABA-AT was isolated from pig brain according to the procedure of Churchich and Moses[35] with minor modifications. Subsequent to purification by hydroxylapatite chromatography, the enzyme was concentrated to approximately 2 mL. The concentrated enzyme was further purified on a Pharmacia Hiprep Sephacryl S-200 16/60 high resolution gel filtration column using 0.05 M potassium phosphate at pH 7 containing 0.15 M NaCl and 1 mM β-mercaptoethanol as the mobile phase. GABA-AT containing fractions were collected, concentrated, and stored until use at −80°C in 0.1 M potassium phosphate at pH 7 containing 1 mM β-mercaptoethanol. The purified enzyme was approximately 95% homogeneous by SDS-PAGE electrophoresis (Coomassie blue). Succinic semialdehyde dehydrogenase (SSDH) was isolated from GABAse (Sigma) as described previously.[36] GABA-AT activity was monitored spectrophotometrically via a coupled assay as described previously.[36,37]

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 **4** or **5**, 1 mM α-ketoglutarate, and 50 mM potassium pyrophosphate 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.

Incubation of 4 or 5 with GABA-AT prior to addition of fresh enzyme. GABA-AT (15 μL, 2.12 mg/mL) was incubated at 25°C in 150 μL total volume of a solution containing 2 mM **4** or 10 mM **5**, 5 mM α-ketoglutarate, and 0.1 M potassium pyrophosphate at pH 8.5. An identical sample, which contained no inactivator, served as the control. After a 2.5 h incubation period, fresh GABA-AT (15 μL) and 0–10 mM GABA were added. Over the next 60 min, at various time intervals, 10 μL aliquots were removed and assayed for enzyme activity.

Incubation of 4 with buffer prior to inactivation of GABA-AT. A 20 mM solution of **4** in 50 mM potassium pyrophosphate at pH 8.5 was incubated at 30°C for 21 h. This solution was diluted to a final concentration of 2 mM in 150 μL total volume of a solution containing GABA-AT (15 μL, 2.12 mg/mL), 1 mM α-ketoglutarate, and 50 mM potassium pyrophosphate at pH 8.5 and incubated at 25°C. Controls were run using either a freshly prepared solution of **4** or no inactivator. At time intervals over 60 min, 10 μL aliquots were removed and assayed for enzyme activity.

Effect of β-mercaptoethanol on inactivation of GABA-AT. GABA-AT (15 μL, 2.80 mg/mL) was incubated at 25°C in 150 μL total volume of a solution containing 5 mM **4**, 5 mM α-ketoglutarate, 0 or 5 mM β-mercaptoethanol (β-ME), and 0.1 M potassium phosphate at pH 7.4. An identical sample, which contained no inactivator, served as the control. At time intervals over 60 min, 10 μL aliquots were removed and assayed for enzyme activity. The experiment was repeated using 10 mM **5** over 120 min.

Protection from inactivation of GABA-AT by addition of substrate. GABA-AT (15 μL, 2.12 mg/mL) was incubated at 25°C in 150 μL total volume of a solution containing 5 mM **4** or 10 mM **5**, 5 mM α-ketoglutarate, 0–10 mM GABA, and 0.1 M potassium pyrophosphate 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 **4**, 5 mM α-ketoglutarate, and 0.1 M potassium pyrophosphate at pH 8.5. Aliquots (10 μL) were removed at 0.5 and 90 min and assayed for activity. A portion of the incubation mixture (100 μL) was applied to a Penefsky spin column[38] prepared with Sephadex G50 in a Bio-Spin disposable chromatography column (BioRad) according to a 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. The experiment was repeated using 10 mM **5** with an incubation time of 3 h.

Transaminations per inactivation event. GABA-AT (20 μL, 2.80 mg/mL) was incubated at 25°C in 200 μL total volume of a solution containing 5 mM **4** or **5**, 5 mM [1-^{14}C]-α-ketoglutarate (0.0495 μCi, 49.0 μCi/mmol, 2.48 μCi/mL), and 0.1 M potassium pyrophosphate at pH 8.5 for 90 min. Incubations were run in triplicate. Samples containing all but enzyme and all but inactivator served as controls. Samples containing unlabeled α-ketoglutarate were run concomitantly and used to determine the enzyme activity remaining after the 90 min incubation period. The transamination reactions were quenched by addition of 20% TCA (50 μL), mixed, and applied to prewashed 1 mL Dowex 50 columns. The

tubes were rinsed with 250 μL of water, and the rinse was applied to the column. The columns were washed with water (2×3 mL) and 2 N NH_4OH (0.75 mL). The [1-^{14}C]-glutamate produced was then eluted with 4 and 3 mL aliquots of 2 N NH_4OH. Each of the five washes was collected in separate 20 mL scintillation vials, mixed with 10 mL scintillation cocktail, and counted for radioactivity. The concentration of glutamate produced during the reaction was calculated by subtracting the counts in the controls from the inactivated samples, and then converting the number of counts to mmol based on the specific activity of the [1-^{14}C]-α-ketoglutarate used in the experiment. The ratio of the concentration of glutamate produced to the concentration of inactivated enzyme used in the sample is the number of transaminations per inactivation event.[37]

GABA-AT catalyzed transamination of 4 and 5. GABA-AT (2 μL, 2.80 mg/mL) was incubated at 25°C in 200 μL total volume of a solution containing various concentrations of substrate (**4**, **5** or GABA), 5 mM [1-^{14}C]-α-ketoglutarate (0.0495 μCi, 49.0 μ Ci/mmol, 2.48 μCi/mL), and 0.1 M potassium pyrophosphate at pH 8.5. Transamination reactions were quenched after 10 min, at which time only a negligible amount of inactivation would have occurred. Samples containing all but enzyme and all but substrate served as controls. The amount of [1-^{14}C]-glutamate produced was determined as described previously. Kinetic constants (K_m and V_{max}) were determined from linear regression analyses (correlation coefficients > 0.996) of Hanes plots.

Correlation of time-dependent fluoride ion release with time-dependent inhibition. GABA-AT (100 μL, 1.82 mg/mL) was incubated at 22°C in 1 mL total volume of a solution containing 2 mM **4** or 5 mM **5**, 5 mM α-ketoglutarate, and 0.1 M potassium pyrophosphate at pH 8.5. An identical sample which contained no enzyme served as the control. At various time intervals over 120 min, 100 μL aliquots of the reaction mixture were removed, diluted to 1 mL in 20 μL plastic scintillation vials with a fluoride standard solution giving a final background fluoride concentration of 100 μM, and quenched by addition of 1 mL total ionic strength adjusting buffer (TISAB II, Orion). The 100 μM background fluoride standard was added to keep the amount of fluoride in the sample in the linear portion of the standard curve.[39] The samples were analyzed for fluoride ion with a fluoride selective electrode (calibrated with a standard curve prepared with NaF standard solutions). After 70 min, gabaculine was added to give a final concentration of 5 mM. The net fluoride ion release was determined by subtracting the fluoride released in the control from the enzyme containing sample. Concomitantly, the enzyme activity of an identical sample (prepared to a final volume of 150 μL) was assayed at various time intervals as described previously. Again, at 70 min, gabaculine was added to a final concentration of 5 mM.

SSDH substrate activity of metabolites produced by incubation of 4 with GABA-AT. Compound **4** (10 mM) was incubated at 30°C in 600 μL total volume of a solution containing GABA-AT (1 μL, 2.80 mg/mL), excess SSDH, 10 mM α-ketoglutarate, 1 mM $NADP^+$, and 0.1 M potassium pyrophosphate at pH 8.5. SSDH activity was monitored continuously for 20 min by recording the increase in absorbance at 340 nm (corresponding to the production of NADPH). Positive and negative controls were run by replacing **4** with GABA (10 mM) or buffer, respectively.

Analysis of carbonyl metabolites produced during incubation of 4 or 5 with GABA-AT. Compound **4** or **5** (5 mM) was incubated at 25°C in 200 μL total volume of a solution containing GABA-AT (2 μL, 1.82 mg/mL), 5 mM α-ketoglutarate, and 0.1 M potassium pyrophosphate at pH 8.5. Identical samples which contained no enzyme or no inactivator served as controls. After 60 min, the samples were quenched by addition of 200 μL of 0.5% 2,4-dinitrophenylhydrazine (2,4-DNP) in 2 N HCl. The derivatization reaction was heated at 80°C for 15 min. Upon cooling, the sample was extracted with ethyl acetate (500 μL). A 350 μL aliquot of the extract was back extracted with 350 μL of 50 mM borate buffer pH 9. A 250 μL aliquot of the aqueous extract was acidified with 600 μL 0.1 M phosphate buffer pH 2 and extracted with ethyl acetate (500 μL). A 350 μL aliquot of the extract was concentrated to dryness at 40°C under a stream of N_2. The extract was reconstituted in 50 μL of mobile phase, and 10 μL was injected into the HPLC system. Products were separated on an Alltech Altima C_{18} analytical column (5 μm, 250×4.6 mm), using an isocratic mobile phase mixture of 70% 50 mM ammonium acetate pH 6 buffer and 30% acetonitrile at a flow rate of 1 mL/min monitored at 340 nm. For LC–MS analyses, the products were separated on a narrow bore Hewlett–Packard Hypersil BDS C_{18} column (5 μm, 250×2.0 mm), using an isocratic mobile-phase mixture of 70% 50 mM ammonium acetate pH 7 buffer and 30% acetonitrile at a flow rate of 0.2 mL/min. The column effluent was monitored at 340 nm and then directly introduced into the electrospray mass spectrometer.

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