Mechanism of Inactivation of γ-Aminobutyric Acid Aminotransferase by (*S*)-4-Amino-4,5-dihydro-2-thiophenecarboxylic Acid

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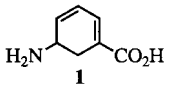
Abstract: (*S*)-4-Amino-4,5-dihydro-2-thiophenecarboxylic acid ((*S*)-**6**) was previously synthesized (Adams, J. L.; Chen, T. M.; Metcalf, B. W. *J. Org. Chem.* **1985**, *50*, 2730−2736.) as a heterocyclic mimic of the natural product gabaculine (5-amino-1,3-cyclohexadienylcarboxylic acid), a mechanism-based inactivator of γ-aminobutyric acid aminotransferase (GABA-AT) (Rando, R. R. *Biochemistry* **1977**, *16*, 4604). Inactivation of GABA-AT by (*S*)-**6** is time-dependent and protected by substrate. Two methods were utilized to demonstrate that, in addition to inactivation, about 0.7 equiv per inactivation event undergoes transamination. Inactivation results from the reaction of (*S*)-**6** with the pyridoxal 5′-phosphate (PLP) cofactor. The adduct was isolated and characterized by ultraviolet−visible spectroscopy, electrospray mass spectrometry, and tandem mass spectrometry. All of the results support a structure (**11**) that derives from the predicted aromatization inactivation mechanism (Scheme 2) originally proposed by Metcalf and co-workers for this compound. This is only the third example, besides gabaculine and L-cycloserine, of an inactivator of a PLP-dependent enzyme that acts via an aromatization mechanism.

Introduction

Gabaculine (5-amino-1,3-cyclohexadienylcarboxylic acid, **1**),



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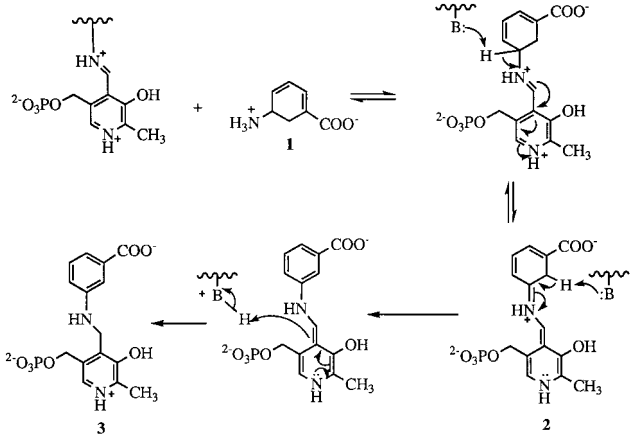
a natural product found in *Streptomyces toyocaenis*,[1] was shown to be an exceedingly potent irreversible inactivator of the pyridoxal 5′-phosphate (PLP)-dependent enzyme γ-aminobutyric acid aminotransferase (GABA-AT, EC 2.6.1.19) both in vitro and in vivo.[2] Gabaculine crosses the blood−brain barrier, and, as a result of inactivation of GABA-AT, causes a rise in the concentration of GABA in the brain.[3] Unfortunately, gabaculine is too toxic to be useful as a pharmaceutical agent, probably because it inactivates several other enzymes as well.[4] However, gabaculine has been used as a powerful pharmacological tool in a variety of GABAergic neurotransmission studies.[5−13]

Scheme 1



Mechanistic studies of the inactivation of bacterial GABA-AT by gabaculine were carried out by Rando and co-workers, and an aromatization mechanism was proposed, leading to the formation of a modified PLP cofactor (**3**, Scheme 1).[14,15] The structure of the PLP-gabaculine adduct was recently characterized fully.[16] A tautomer of gabaculine, isogabaculine (**4**), also

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(1) Mishima, H.; Kurihara, H.; Kobayashi, K.; Miyazawa, S.; Terahara, A. *Tetrahedron Lett.* **1976**, *7*, 537.

(2) Rando, R. R.; Bangerter, F. W. *J. Am. Chem. Soc.* **1976**, *98*, 6762.

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(7) Gammon, D. W.; Gingrich, H. L.; Sander, G.; Stewart, R. R.; VanDerWerf, P. A. *ACS Symp. Ser.* **1987**, *356*, 122.

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(11) Faingold, C. L.; Marcinczyk, M. J.; Casebeer, D. J.; Randall, M. E.; Arneric, S. P.; Browning, R. A. *Brain Res.* **1994**, *640*, 40.

(12) Michalik, M.; Nelson, J.; Erecinska, M. *Diabetes* **1993**, *42*, 1506.

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(14) Rando, R. R.; Bangerter, F. W. *J. Am. Chem. Soc.* **1977**, *99*, 5141.

(15) Rando, R. R. *Biochemistry* **1977**, *16*, 4604−4610.

(16) Fu, M.; Silverman, R. B. *Bioorg. Med. Chem.*, **1999**, *7*, in press.

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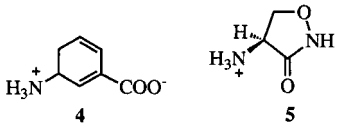
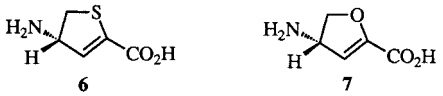
inactivates GABA-AT, and the same mechanism and product was proposed, although experimental support is lacking.[17]

Until recently, gabaculine was the only known inactivator of GABA-AT for which an aromatization mechanism was supported by experiment. Last year, however, we showed that the antibiotic L-cycloserine (**5**) also inactivates GABA-AT via an



4 **5**

aromatization mechanism.[18] With gabaculine as the lead compound, two other dihydroaromatic GABA analogues, (*S*)-4-amino-4,5-dihydro-2-thiophenecarboxylic acid (SADTA, (*S*)-**6**) and (*S*)-4-amino-4,5-dihydro-2-furancarboxylic acid (**7**), were



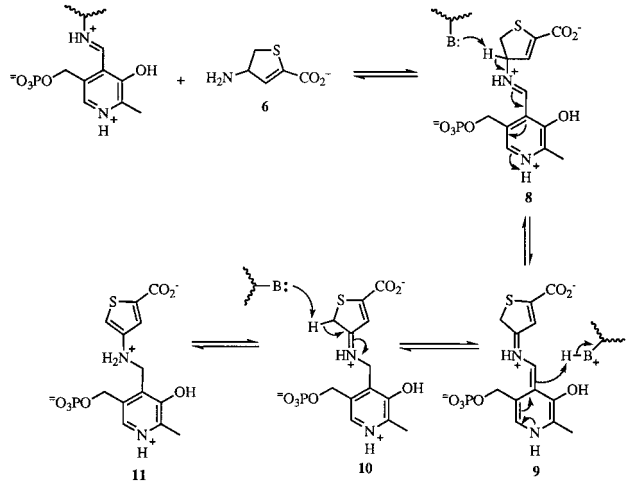
6 **7**

synthesized by Metcalf and co-workers and shown to be irreversible inactivators of GABA-AT,[19] but no mechanistic studies were carried out. The mechanism of inactivation for both of these compounds was proposed to be identical to that of gabaculine on the basis of structure analogy (Scheme 2 shows the mechanism for **6**), although no experimental support was provided.

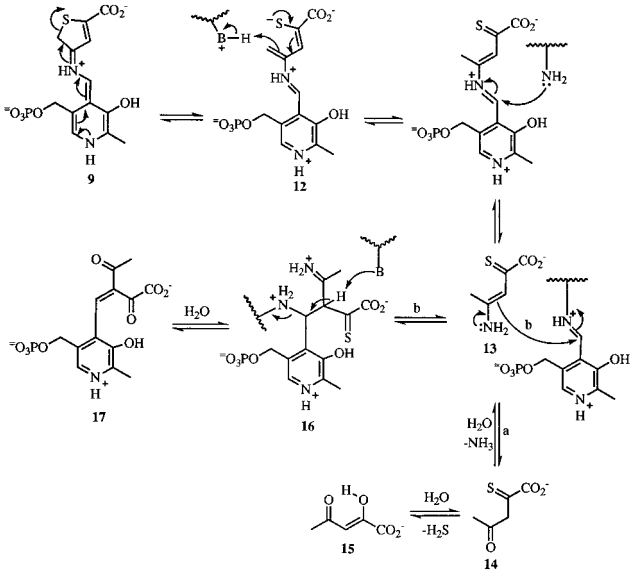
However, there are at least three general mechanisms of inactivation of GABA-AT, referred to as the aromatization mechanism, the enamine mechanism, and the Michael addition mechanism.[20] Rando had considered the Michael addition mechanism as a possibility for inactivation of GABA-AT by gabaculine but dismissed it when a modified PLP was isolated.[15] Unlike gabaculine, **6** and **7** are theoretically capable of undergoing all three inactivation mechanisms (Schemes 2−4). In the enamine mechanism (Scheme 3), intermediate **9** from the aromatization mechanism (Scheme 2) can partition by cleavage of the C−S bond into **12**. Protonation followed by transfer of the PLP back onto the active site lysine residue gives enamine **13**, which can either undergo hydrolysis to **14** and/or **15** or undergo enamine addition to the enzyme-bound PLP to give **16**. Elimination of the covalent adduct from the lysine residue upon denaturation would give the modified cofactor structure **17**.

The Michael addition pathway, again starting from intermediate **9** (Scheme 4), involves the nucleophilic addition of an active site residue to the α,β-unsaturated iminium ion of **9** to give covalent adduct **18**. Carbon−sulfur bond cleavage with concomitant protonation produces **19**, which could undergo hydrolysis to a covalent adduct (**20**) and pyridoxamine 5′-phosphate (PMP, **21**). All three of these mechanisms are energetically reasonable, but because gabaculine undergoes aromatization, it is reasonable that the corresponding dihydrothiophene analogue also should favor that route. However, thiophene has a lower aromatic stabilization energy (29 kcal/mol) than benzene (36 kcal/mol).[21] Therefore, it is not clear that the dihydrothiophene analogue, (*S*)-**6**, is as susceptible to aromatization as is the dihydrobenzene analogue, gabaculine. The results described here establish that the aromatization mechanism also is the highly preferred pathway for the dihydrothiophene analogue **6**, which, therefore, acts like gabaculine.

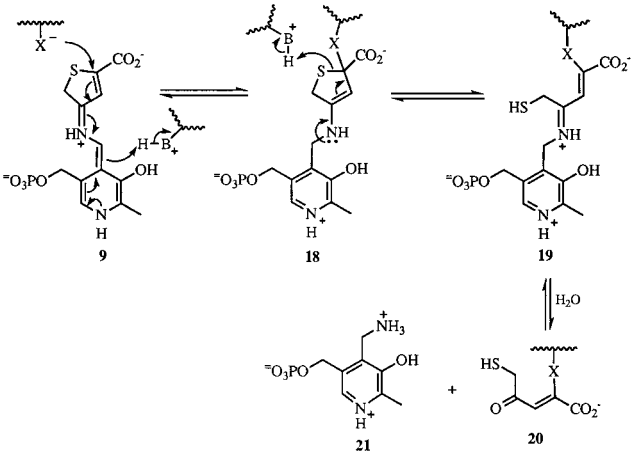
Scheme 2



Scheme 3



Scheme 4



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(21) (a) Bird, C. W. *Tetrahedron* **1985**, *41*, 1409−1414. (b) Bird, C. W. *Tetrahedron* **1986**, *42*, 89−92.

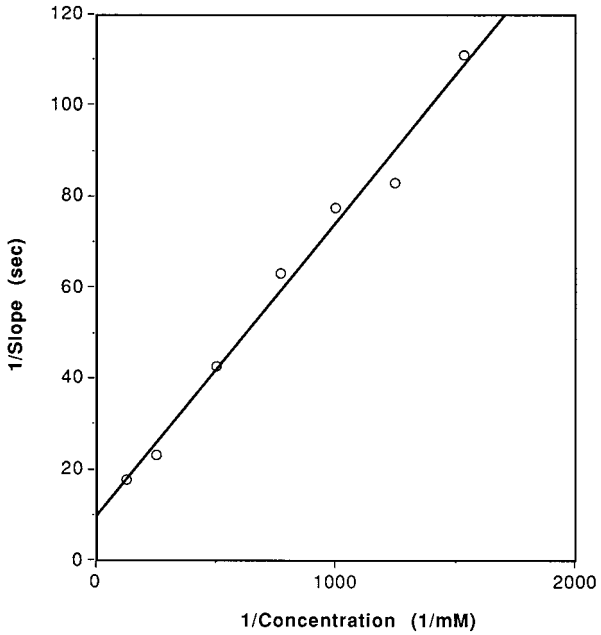


Figure 1. Time-dependent inactivation of GABA-AT by (*S*)-**6** at 0 °C. See the Experimental Section for details.

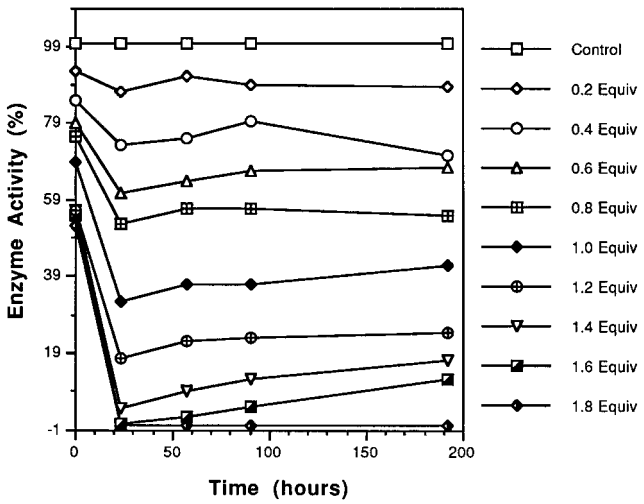


Figure 2. Time-dependent inactivation of GABA-AT by various equivalents of (*S*)-**6** at pH 6.5. See the Experimental Section for details.

Results

Inactivation of GABA-AT by 6. Inactivation of GABA-AT by (*S*)-**6** was time- and concentration-dependent. The rate of inactivation was so fast that accurate measurements could not be obtained at 25 °C. At 0 °C the K_I and k_{inact} values were determined by the method of Kitz and Wilson[22] to be 6.6 μM and 6.1 min^{-1}, respectively (Figure 1). Less than 2% of the enzyme activity was detected from fast gel filtration and exhaustive dialysis of the **6**-inactivated enzyme, while the enzyme in the controls remained active after such treatments. Compound (*S*)-**6**, therefore, irreversibly inactivates purified pig brain GABA-AT. No inactivation was observed by 2 mM (*R*)-**6**, even after 2 h, that is, after the trace amounts of the (*S*)-isomer were removed.

Time-Dependent Inactivation of GABA-AT by Various Equivalents of 6 at pH 6.5. By incubation of the enzyme with various equivalents of inactivator, the turnover number of the inactivation was determined. Inactivation of GABA-AT with various equivalents of **6** at pH 6.5 and 7.4 gave similar results (Figure 2 shows the results for pH 6.5). A plot of the number of equivalents of inactivator versus enzyme activity gives the turnover number (about 1.7), which is plotted in Figure 3 for the inactivation of GABA-AT by (*S*)-**6** at both pH 6.5 and 7.4. Therefore, the pH does not affect the partition ratio.

(22) Kitz, R.; Wilson, I. B. *J. Biol. Chem.* **1962**, *237*, 3245−3249.

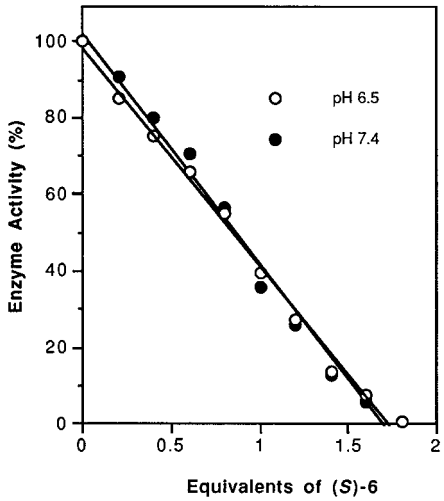


Figure 3. Determination of the turnover number for the inactivation of GABA-AT by (*S*)-**6**. The data from Figure 2 are replotted as enzyme activity vs equivalents of (*S*)-**6**.

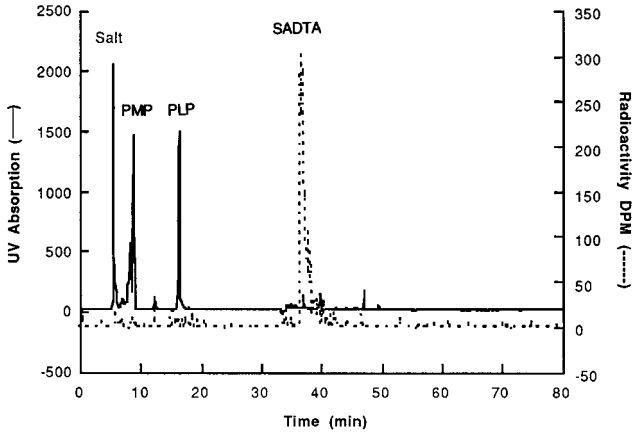


Figure 4. Reversed-phase HPLC analysis of the product isolated from [^{3}H]PLP-GABA-AT inactivated by (*S*)-**6**. Radioactivity and absorption are plotted versus the retention time of the standards and [^{3}H]-labeled product. The absorption peaks correspond to the standards: PMP (8 min), PLP (16 min). See the Experimental Section for details.

Transamination Events Per Inactivation of GABA-AT by 6. The average number of transaminations per inactivation, as determined from the conversion of [^{14}C]α-ketoglutarate to [^{14}C]-glutamate, is 0.6 ± 0.1.

Inactivation of [^{3}H]PLP-Reconstituted GABA-AT by 6, Product Isolation, and Analysis by Reverse-Phase HPLC. Only one major product was detected by HPLC (T_R = 37 min) from the inactivation of [^{3}H]PLP-reconstituted GABA-AT by **6** (Figure 4). More than 99% of the radioactivity was released from the enzyme in this experiment.

UV−Vis Spectroscopic Studies of the Purified Inactivation Product. A comparison of the UV−vis spectra of the purified products of inactivation of GABA-AT by (*S*)-**6** and gabaculine[16] are shown in Figure 5.

Synthesis and Electrospray Mass Spectrum of 23. 4-Amino-2-thiophenecarboxylic acid (**22**),[23] which was prepared from thiophene-2-carboxylic acid, was condensed with PLP then

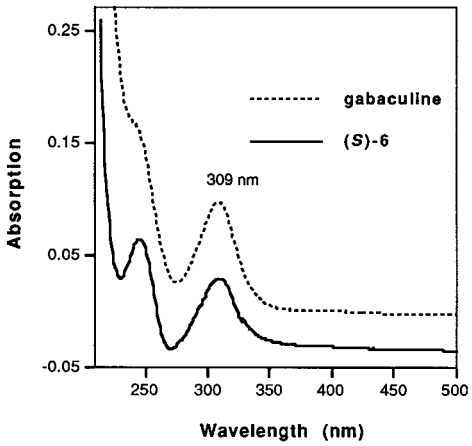
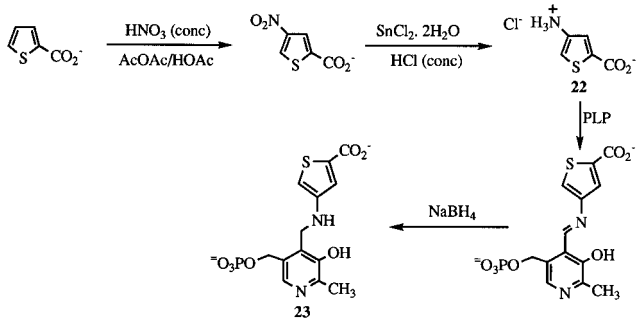
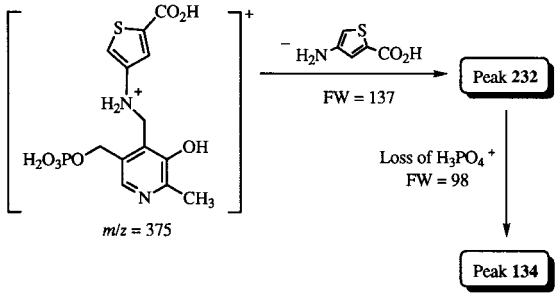


Figure 5. UV−Vis spectra of the products produced from inactivation of GABA-AT by gabaculine (---) and by (*S*)-**6** (—). See the Experimental Section for details.

Scheme 5



Scheme 6



reduced with sodium borohydride to give **23** (Scheme 5). The positive mode electrospray ionization mass spectrum (Figure 6) is consistent with the structure of **23** (Scheme 6).

Electrospray Ionization Mass Spectrum of the (*S*)-6 Inactivation Product. LC/MS analysis of the isolated (*S*)-**6** inactivation product was carried out. Positive mode electrospray ionization mass spectrometry (ESIMS$^+$) showed a molecular ion ([M + H]$^+$, m/z = 375) and its potassium adduct ([M + K]$^+$, m/z = 413, Figure 7). The MS/MS spectrum of the molecular ion peak at m/z 375 (Figure 8) showed a fragmentation pattern similar to that obtained from **23** and from gabaculine.[16]

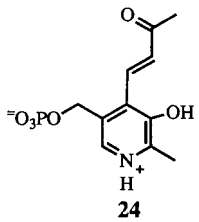
Discussion

Initially, treatment of GABA-AT with both (*S*)- and (*R*)-**6** led to time-dependent inactivation. However, chiral HPLC of each synthetic compound demonstrated that the (*R*)-isomer contained about 0.5% of the (*S*)-isomer. Following HPLC purification of the (*R*)-isomer, no inactivation of purified pig brain GABA-AT was observed (data not shown). The (*R*)-isomer also is not a substrate for the enzyme; no [5-^{14}C]glutamate production is observed when GABA-AT is incubated with (*R*)-**6** in the presence of [5-^{14}C]α-ketoglutarate.

Time-dependent inactivation of purified pig brain GABA-AT by (*S*)-**6** demonstrates that it is an extremely potent inactivator (Figure 1). In fact, it is so potent that the rate of inactivation was too fast to measure with a standard spectrophotometer at 25 °C, so the kinetics were measured at 0 °C. Furthermore, the potency (k_{inact}/K_I) of (*S*)-**6** is about 170 times greater than that of gabaculine; the k_{inact} is over four times greater (6.1 vs 1.4 min^{-1}), and the K_I is about 40 times lower (6.6 vs 260 μM) at 0 °C. It is not clear what type of additional binding interactions occur with the heterocycle relative to benzene, but it is known that isogabaculine (**4**) has an affinity for GABA-AT 10−20 times greater than that reported for gabaculine, and the half-life of enzyme activity at high concentrations of isogabaculine is much shorter,[17] making **4** and (*S*)-**6** almost equipotent. Possibly, the rigidity in the dihydroaromatic rings of **4** and (*S*)-**6**, as a result of the 1,2-double bond, is important.

Less than 2% of the enzyme activity returns upon dialysis or fast gel filtration of the (*S*)-**6**-inactivated enzyme, suggesting that an irreversible inhibition occurs. This irreversible inhibition competes with the normal catalytic turnover; the presence of the substrate GABA significantly reduces the inactivation process.

The three mechanisms of inactivation of GABA-AT by (*S*)-**6**, the aromatization mechanism (Scheme 2), the enamine mechanism (Scheme 3), and the Michael addition mechanism (Scheme 4), can be differentiated by where the inactivator (*S*)-**6** becomes attached and what products are expected to be released upon denaturation of the inactivated enzyme (Table 1). If the aromatization mechanism is followed, upon denaturation, the (*S*)-**6**-PMP adduct (**11**, which is equivalent to synthetic **23**) would be released. The product of an aromatization mechanism should have the same reverse-phase HPLC retention time, UV−vis λ_{max} and mass spectrum as those of **23**. A different modified coenzyme (**17**) would be released if the enamine mechanism is followed. This modified (*S*)-**6**-cofactor adduct (**17**) is structurally similar to **24**, a PLP-acetone adduct made earlier.[24] Because of



24

the extra carboxycarbonyl group in **17** as compared to **24**, a longer retention time in the HPLC and a bathochromic shift of the λ_{max} in the UV−vis study is predicted for this adduct (Table 1). Commercially available PMP (**21**) served as the control for the enzymatic PMP release following a Michael addition inactivation; PMP has a HPLC T_R = 8 min and a λ_{max} = 309 nm.

Attempts to prepare **11** following synthetic procedures similar to that for the synthesis of **3** as described by Rando et al.[14] resulted in complete decomposition of the starting materials and/or gave no detectable amount of **11**. Compound (*S*)-**6** is heat sensitive (decomposition at about 50 °C), so the elevated

(23) Jung, F. H. WO 93/19070, PCT/GB93/00603, Zeneca Ltd., France, 1993.

(24) Silverman, R. B.; Roscher, C. L. C. *Bioorg. Med. Chem.* **1996**, *4*, 1521−1535.

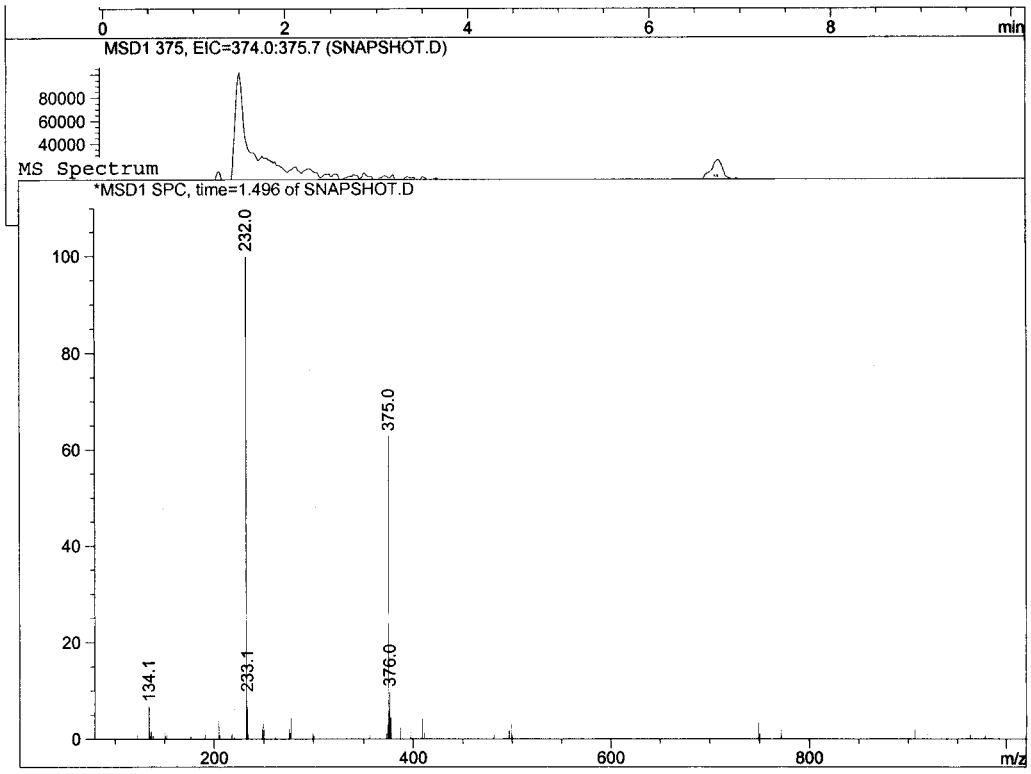


Figure 6. Electrospray ionization mass spectrum of **23**.

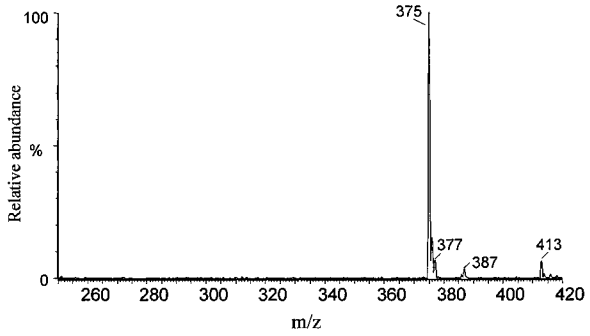


Figure 7. Electrospray ionization mass spectrum of **11**. See the Experimental Section for details.

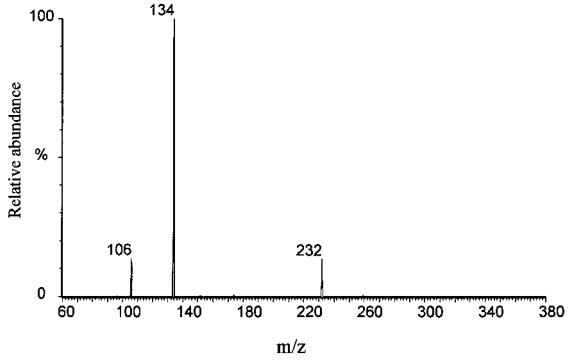
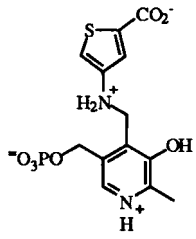
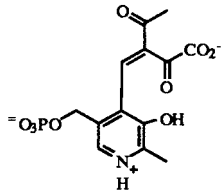
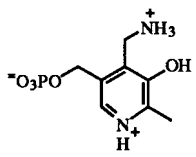


Figure 8. MS/MS spectrum of **11**. See the Experimental Section for details.

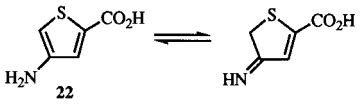
Table 1. Comparison of the Proposed Products Released by Inactivation of GABA-AT with (*S*)-**6**

adduct	predicted HPLC T_R	predicted UV−vis λ_{max}	expected *m*/*z*
 **11**	∼37 min T_R (**23**) = 37 min	∼309 nm λ_{max} (**23**) = 309 nm	*m*/*z* = 374 $C_{13}H_{15}N_2O_7PS$
 **17**	>31 min T_R (**24**) = 31 min	>405 nm λ_{max} (**24**) = 405 nm	*m*/*z* = 359 $C_{13}H_{14}NO_9P$
 **21**	8 min	309 nm	*m*/*z* = 248 $C_8H_{13}N_2O_5P$

temperatures used may have been responsible. 4-Amino-2-thiophenecarboxylic acid (**22**) was synthesized by a modification of a known procedure.[25] Incubation of **22** with PLP followed by treatment with sodium borohydride (Scheme 5) gave a product that was characterized by electrospray ionization mass spectrometry (Figure 6), although some decomposition (about 35%) occurred during handling. Most aminothiophenes are unstable, and decomposition occurs via polymerization of the thiophene ring,[26] possibly because of the ease of tautomerization of aminothiophenes (Scheme 7). In fact, the C-5 proton of **22** readily exchanges in D_2O, as observed in the NMR spectrum

(25) Jung, F. H. *Carbapenem Derivatives as Antibiotics and Intermediates Thereof*; WO 93/19070, PCT/GB93/00603, Zeneca Limited: France, 1993.

Scheme 7



(data not shown), consistent with a rapid tautomerization. This also accounts for the considerable decomposition observed during purification of the inactivation adduct (**11**).

When (*S*)-**6** was incubated with [^{3}H]PLP-reconstituted GABA-AT, all of the radioactivity was released from the protein upon denaturation. Only one radioactive product was detected by reversed-phase HPLC analysis, and it has a retention time of 37 min (Figure 4). This product comigrates with synthetic **11** (**23**). The UV−vis spectrum of this product (Figure 5) shows a λ_{max} at 309 nm, which is the same as the λ_{max} for the gabaculine inactivation adduct under the same conditions, consistent with the modified pyridoxamine structure.[27]

Positive ion electrospray LC/MS of the isolated inactivation product was carried out (Figure 7). The peak eluting in the LC at 9.1 min was detected to contain mostly a molecular ion ([M + H]$^+$) with *m*/*z* 375 and its potassium adduct (*m*/*z* = 413). The *m*/*z* 375 molecular ion shows an A + 2 isotope with 7.1% abundance, which suggests the presence of a sulfur atom (the theoretical ratio for the proposed structure is 7.4%). The MS/MS spectrum of this molecule (Figure 8) is dominated by the ions at *m*/*z* = 232, 134, and 106. The ion at *m*/*z* 232 represents the loss of the group attached to the reduced coenzyme pyridoxamine group with the formation of a stable benzylium-type structure. The high tendency for the formation of this type of ion precluded the direct observation of the ion corresponding to the attached group. The 232 and 134 fragment ions were further used in precursor ion scan experiments to check for the presence of other possible pyridoxal ring-containing adducts, that is, the parent ions of these fragments. Only ion 375 was found, confirming that this ion represents the only product of inactivation (data not shown). The mass difference between 232 and 134 is 98, which suggests that this molecule contains a phosphate group. These results are the same as those obtained with the corresponding synthetic compound (**23**, Figure 6). To get further support for the identities of the fragments, tandem mass spectra of **3**[16] were acquired. A comparison of the spectra for the (*S*)-**6** inactivation adduct and for **3** shows that they both contain daughter peaks at *m*/*z* 232, 134, and 106. This confirms the presence of a common moiety, the substituted pyridinium ring.

To determine if (*S*)-**6** also is a substrate in addition to being an inactivator, the transamination events per inactivation were determined in two different ways. GABA-AT was incubated with fractional amounts of (*S*)-**6** at either pH 6.5 or 7.4 until the loss of enzyme activity ceased (Figures 2 and 3). The extrapolated number of equivalents of inactivator required to get complete inactivation at either pH value was 1.7; one equivalent of (*S*)-**6** goes toward inactivation, leaving 0.7 equiv for enzymatic conversion to a product. Virtually the same result was obtained when the number of enzymatic turnovers was determined using [5-^{14}C]α-ketoglutarate and measuring the amount of [5-^{14}C]glutamate product that is produced, a measure of the amount of enzyme converted to the PMP form. In this experiment 0.6 ± 0.1 transamination events per inactivation were determined. This suggests that **10** (Scheme 2) partitions only about 40% by hydrolysis to give PMP and 60% to **11** and inactivation.

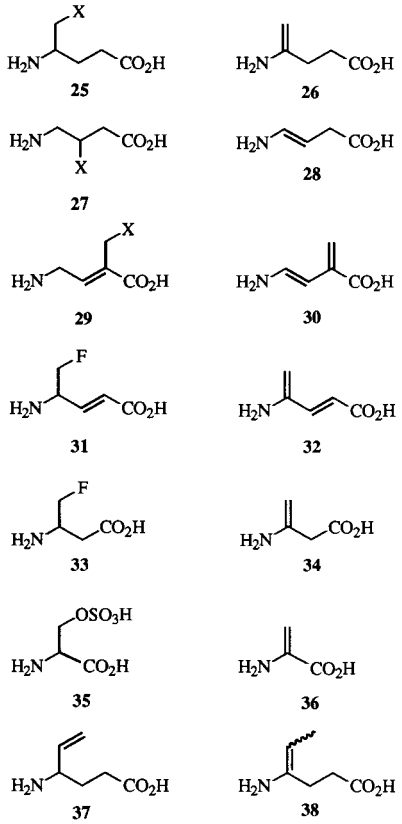


Figure 9. Comparison of the structures of the proposed enamine intermediates from a variety of known inactivators of GABA-AT

So why is the aromatization mechanism preferred over the enamine and Michael addition pathways? A variety of compounds previously have been designed to inactivate GABA-AT[20] and other PLP-dependent enzymes by enamine mechanisms.[28] The structures for many of the earlier compounds can be generalized by **25** (X = F, Cl, Br, OH), **27** (X = F, Cl, OH), and **29** (X = F, Cl, OH), and the corresponding proposed enamine intermediates that would be released are depicted as **26**, **28**, and **30** (Figure 9). However, only compounds **25** actually inactivate GABA-AT; **27** and **29** undergo elimination of the leaving group and, presumably, release of the corresponding enamines (**28** and **30**), but these enamines are hydrolyzed without inactivation. A comparison of the proposed intermediates of these compounds suggests that the enamines embedded in the GABA backbone (**28**, **30**) are inactive. Possibly, when the carboxylates of these intermediates are held by the active site Arg-192 and Lys-203 residue, as is believed to be the case with the substrate GABA,[29] enamines **28** and **30** are positioned away from the active-site electrophile (presumably, the PLP bound Lys-329 aldimine) and, consequently, fail to react prior to their release from the active site. The unsubstituted *exo*-enamine **26**, however, is apparently positioned close enough to the enzyme-bound PLP to allow nucleophilic attack to occur. This observation is consistent with the fact that the same lysine residue that binds to the PLP (Lys-329) also removes the γ-*S* proton from GABA, so it is close to the C-4 position of bound

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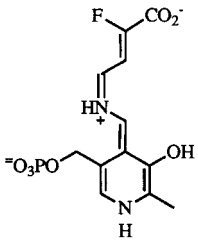
GABA. Later studies with other enamine inactivators (**31**,[30] **33**,[22] and **35**[31]) show the involvement of structurally similar enamines (**32**, **34**, and **36**), all of which have enamine double bonds that are unsubstituted and positioned α- to the amino group, away from the amino acid backbone. Interestingly, γ-vinyl GABA (**37**), whose enamine structure (**38**) is like that of the active enamine structures, has the double bond of the enamine directed away from the GABA backbone but has an additional methyl group, which could impart steric hindrance to an enamine addition. This may account for why this inactivator proceeds by two different inactivation mechanisms (enamine and Michael addition), and the enamine pathway accounts for only 30% of the total inactivation.[32] With regard to (*S*)-**6**, the potential enamine intermediate that would form (**13**, Scheme 3) has its double bond in the GABA backbone (and is further stabilized by the carbon−sulfur double bond) and, therefore, does not correspond to the "active" enamine form.

The proposed Michael addition acceptor, the α,β-unsaturated imine intermediate **9**, is similar to another proposed Michael addition mechanism intermediate (**39**) from inactivator **40**.[33] In



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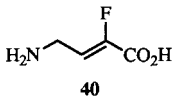

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both cases, the α,β-unsaturated imine intermediates are activated by an electron-withdrawing group, a sulfur in **9** and a fluorine in **39**. However, deprotonation of **9** to give the aromatized adduct **11** must be a lower energy process than Michael addition to **39**. These rationalizations explain why the Michael and enamine mechanisms are not as favored as the aromatization mechanism for **6**.

The energy differences between the three mechanisms discussed here are relatively small, so minor perturbations in structure could have drastic effects in the inactivation mechanism preference. However, the lower aromatization stabilization energy of thiophene (29 kcal/mol) relative to that for benzene (36 kcal/mol)[22] is not sufficiently low to cause a change in the preferred inactivation mechanism. It is not clear how much smaller the aromatic stabilization energy would have to be to change the mechanism, but studies are underway on the corresponding dihydrofuran analogue (**7**), which would aromatize to a furan having an aromatic stabilization energy of only 16 kcal/mol.[22]

Conclusion

Evidence based on the UV-visible spectrum, electrospray ionization mass spectrometry, and tandem mass spectrometry supports structure **11** as the product of inactivation of GABA aminotransferase by (*S*)-**6**. These results indicate that the predicted aromatization inactivation mechanism (Scheme 2) proposed by Metcalf and co-workers[19] for this compound occurs. This is only the third example, besides gabaculine[14−16] and L-cycloserine,[18] of an inactivator of a PLP-dependent enzyme that acts via an aromatization mechanism.

Experimental Section

Analytical Methods. GABA-AT assays and UV−vis spectra were recorded on a Perkin-Elmer Lambda 10 spectrophotometer. Measurements of pH were performed on an Orion 701-A pH meter with a Ross 8301 combination electrode. All of the dialyses were performed with Pierce Slide-A-Lyzer dialysis cassettes (molecular weight cutoff is 10 000) unless otherwise specified. Radioactivity was determined either with a Radiomatic FLO-ONE\Beta Series A-200 liquid flow scintillation counter or with a Packard TRI-CARB 2100TR liquid scintillation analyzer. The scintillation fluids used were Packard ULTIMA-FLOM and ULTIMA GOLD. Electrospray ionization mass spectra were acquired on a Micromass Quattro II mass spectrometer (Fisons Instruments, Manchester, UK); the LC system coupled to the spectrometer consisted of an ABS 140A syringe pump, a Rheodyne injector model 7725 equipped with a 50 μL loop, and a 2 × 250 mm Hypersil BDS C18 column. HRMS was done on a VG70−250SE spectrometer on EI mode. NMR spectra were recorded on either a Varian Unity Plus 400 MHz or a Varian Gemini 300 MHz spectrometer. Chemical shifts are reported as δ values in parts per million downfield from tetramethylsilane (TMS) in $CDCl_3$ and d6-DMSO or from sodium 3-(trimethylsilyl)propionate in D_2O. Coupling constants are reported in Hertz. Data are presented as follows: chemical shift (integrated intensity, multiplicity, coupling constant). For centrifugations, a Du Pont Sorvall RC5B Plus centrifuge was used with either an SLA-3000 or an SA600 rotor. Beckman Microfuge B was used for microcentrifugations. IEC clinical centrifuge was used for the Penefsky spin method. Cavitator ultrasonic cleaner from Mettler Electronics Corp. was used for ultrasonications. Chromatography refers to purification by flash chromatography on F. Merck Silica gel 60 (230−400 mesh). The eluting solvents are listed in order of the sequence used to elute the product. Unless otherwise noted, all reactions were run under N_2. Thin-layer chromatography (TLC) was performed using Whatman PE SIL/UV silica gel plates with UV indicator. Amines were visualized on TLC plates by dipping the plate into a solution of ninhydrin in *n*-butanol and then heating. Other compounds were visualized with I_2 or phosphomolybdic acid in ethanol followed by heating. HPLC analysis was done with Beckman 125P pumps and a Beckman 166 detector. All of the runs were monitored at 254 nm, unless otherwise specified. The HPLC columns used were Alltech C18 analytical Alltima, Hypersil Elite 5 micron, or semi-prep Econosil 10 micron columns. Enzyme purification was carried out on a Pharmacia Biotech FPLC system (consisting of a conductivity monitor, a UV-MII detector, and two P-500 pumps) with 16/60 Pharmacia Hiprep Sephacryl S-200 high-resolution column. Electrophoresis was carried out on a Bio-Rad Mini-Protean II electrophoresis cell with Bio-Rad 12% Tris-HCl 10 well, 30 μL comb ready gel, using a Bio-Rad Model 1000/500 power supply. Melting points were determined on a Mel-Temp capillary tube melting point apparatus and are uncorrected.

Reagents. All reagents were purchased from either Aldrich Chemical Co. or Sigma Chemical Co. except the following: Centricon 10 microconcentrators were purchased from Amicon; HPLC mobile phases and organic solvents, fuming nitric acid, glacial acetic acid, and concentrated hydrochloric acid were purchased from Fisher; unpacked chromatography columns for Penefsky spin method,[34] Dowex 50 and Dowex 1 resins, sodium dodecyl sulfate, and all of the reagents used for gel electrophoresis were purchased from Bio-Rad; ultrapure urea was a product of ICN Biomedicals; and [^{3}H]sodium borohydride and [5-^{14}C]-α-ketoglutarate were obtained from Amersham. Pig brains were a generous gift of the American Meat Protein Corp. (Ames, IA); Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide) was obtained from Pierce. All of the buffers and solvents used for HPLC or FPLC analyses were filtered through Gelman 0.45 μm membranes. Buffer A (100 mM potassium phosphate buffer, pH 7.4, containing

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0.25 mM β-mercaptoethanol) was used in most of the enzyme incubations. Water used in enzyme-related experiments was doubly distilled. Compound (*S*)-**6** was received as a generous gift from Marion Merrell Dow Pharmaceuticals; **24** was synthesized according to the procedure of Silverman and Roscher.[24]

(*R*)-4-Amino-4,5-dihydro-2-thiophenecarboxylic Acid (*R*)-6. This compound was synthesized according to the published procedure by Adams et al.[19a] except for the purification of the final product. Prior to the recrystallization of the final product from 2 M NH_4OH, it was purified by cation-exchange chromatography, and the fractions containing (*R*)-**6** were lyophilized to dryness. The optical and chemical purity of the crystals obtained were checked with Marfey's reagent and analyzed by reverse-phase HPLC. These crystals were further fractionally recrystallized from 2 M NH_4OH, and the purity of the crystals obtained was checked with the same analytical procedure. The same optical and chemical purity analysis was carried out with the (*S*)-**6** obtained from Marion Merrell Dow Pharmaceuticals.

4-Amino-2-thiophene Carboxylic Acid (22). 4-Amino-2-thiophene carboxylic acid was synthesized using a modification of a reported patent procedure.[23] 2-Thiophenecarboxylic acid (6.4 g, 50 mM) was suspended in acetic anhydride (15 mL). Fuming nitric acid (16 mL) in glacial acetic acid (25 mL) was then added slowly over 1 h with stirring, while keeping the temperature of the reaction mixture below 30 °C. The reaction mixture was stirred at ambient temperature for 2 h. TLC showed 20% conversion of the starting material at this point. Additional glacial acetic acid (75 mL) was added slowly over 30 min with stirring, while keeping the temperature of the reaction mixture below 30 °C. After being stirred for another 2 h, TLC showed complete conversion of the starting material. While being cooled in an ice bath with stirring, the reaction was neutralized with NaOH pellets to pH 2−3. The reaction mixture was then transferred to a separatory funnel and diluted with water (200 mL) and ether (200 mL). The organic layer was washed with water and brine, dried over $MgSO_4$, filtered, and concentrated at reduced pressure. Upon workup, a yellow solid (4.5 g) was obtained. Chromatography (a gradient of 2%, 5%, 10%, 20% HOAc/hexane) of several small portions of the product afforded 4-nitro-2-thiophenecarboxylic acid (3 g, 34%). ^{1}H NMR ($CDCl_3$): δ 8.35 (d, $J = 1.5$ Hz, 1 H), 8.54 (d, $J = 1.5$ Hz, 1 H), and 5-nitro-2-thiophenecarboxylic acid (1.5 g, 17%). ^{1}H NMR ($CDCl_3$): δ 7.79 (d, $J = 4.3$ Hz, 1 H), 7.91 (d, $J = 4.3$ Hz, 1 H).

The 4-nitro-2-thiophene carboxylic acid obtained above (3 g, 17.1 mmol) was added with stirring to a solution of $SnCl_2 \cdot 2H_2O$ (9.75 g, 43.2 mmol) in concentrated HCl (30 mL). The mixture was stirred for 6 h at ambient temperature. The resulting reaction mixture was filtered. The solid obtained was washed with toluene and vacuum-dried to give 4-amino-2-thiophene carboxylic acid (**22**, 2 g, 67%) as an off-white solid. ^{1}H NMR (D_2O): δ 6.49 (d, $J = 1.8$ Hz, 1 H), 7.11 (d, $J = 1.8$ Hz, 1 H). HRMS: calculated for $C_5H_5NO_2S$ 143.1600, found 143.1681.

Synthesis of Adduct 23. Pyridoxal 5′-phosphate (248 mg, 1 mmol) and **22** (179 mg, 1 mmol) were dissolved in water (5 mL), and the pH of the reaction mixture was adjusted to 10 with 1 N KOH. After being stirred at 40 °C overnight in the dark, the reaction mixture was adjusted to pH 8 with 1 N HCl. The reaction mixture was cooled to −10 °C, and sodium borohydride (75.6 mg, 2 mmol) was added portionwise; then the reaction mixture was stirred at room temperature for 8 h. The pH of the reaction mixture was adjusted to 1, and the precipitate that formed was removed by filtration. The filtrate was purified by reversed-phase HPLC as follows: 0−10 min 5% mobile phase B (0.1% TFA in acetonitrile) and 95% mobile phase A (0.1% TFA in water) followed by 10−50 min gradient of 5−80% mobile phase B/95−20% mobile phase A at a flow rate of 21 mL/min, detection at 254 nm. The product-containing fractions were collected, lyophilized to dryness, redissolved in D_2O, and lyophilized to a light yellow solid (27 mg, 7%).

Enzymes and Assays. GABA-AT was purified as previously described.[35] Succinic semialdehyde dehydrogenase (SSDH) was obtained from GABAase, a commercially available mixture of GABA-AT and SSDH, by the reported procedure.[36] Protein assays were carried out using bovine serum albumin (BSA) and Pierce Coomassie protein assay reagent for standard curves. GABA-AT activity assays were carried out using a modification of the coupled assay developed by Scott and Jakoby.[37] The assay solution contained 110 mM GABA, 5.3 mM α-KG, 1.1 mM $NADP^+$, and 5 mM β-mercaptoethanol in 200 mM potassium pyrophosphate, pH 8.5. For each assay, excess SSDH was used. The amount of activity remaining in an enzyme solution was determined by adding an aliquot of enzyme solution to the assay solution with SSDH and monitoring the change in absorption at 340 nm at 25 °C, as a result of the conversion of $NADP^+$ to NADPH by SSDH.

Time-Dependent Inactivation of GABA-AT by (*S*)-6 (Figure 1). GABA-AT (10 μL; final concentration 0.05 μM) was added to solutions of (*S*)-**6** (90 μL; final concentrations of 0, 0.65, 0.8, 1, 1.3, 2, 4, and 8 μM, seven preincubation solutions for each concentration were made), in buffer A, containing 80 μM α-ketoglutarate at 0 °C. At timed intervals, aliquots (85 μL) were withdrawn and added to the assay solution (510 μL) containing excess succinic semialdehyde dehydrogenase (5 μL). Rates were measured spectrophotometrically at 340 nm at 25 °C, and the logarithm of the remaining activity was plotted against time for each concentration of inhibitor. A secondary plot of 1/slope of these lines versus 1/[inactivator][22] was constructed to determine K_I and k_{inact} values for (*S*)-**6**.

Inactivation of GABA-AT by (*S*)-6 and Dialysis of the Inactivated Enzyme. GABA-AT (15 μL; final concentration 1 μM) was added to (*S*)-**6** (135 μL, 2 mM) in buffer A, containing 10 mM α-ketoglutarate at 25 °C. Another portion of GABA-AT (15 μL) was added to buffer A (135 μL) at 25 °C to serve as a control. Both solutions were incubated in the dark at room temperature for 4 h and were monitored for activity. When the (*S*)-**6**-inactivated enzyme exhibited no activity, both solutions were allowed to pass through Sephadex G-50 columns prewashed with buffer A, using the Penefsky spin method.[34] The solutions obtained were again assayed; no enzyme activity was detected from the (*S*)-**6**-inactivated enzyme, while the activity of the control remained unchanged. The enzyme solutions were then transferred to Slide-A-Lyzer dialysis cassettes and dialyzed against buffer A (3 × 1 L, changed every 3 h) at 4 °C. After the dialyses, the solutions were assayed, and no return of enzyme activity was detected from the (*S*)-**6**-inactivated enzyme, while the control enzyme solution remained active.

GABA Protection of the Inactivation of GABA-AT by (*S*)-6. GABA-AT (15 μL; final concentration 1 μM) was added to (*S*)-**6** (135 μL, 20 μM) in buffer A, containing 11 mM GABA and 1 mM α-ketoglutarate at 25 °C. At time intervals, aliquots (10 μL) were withdrawn and added to the assay solution (585 μL) containing excess succinic semialdehyde dehydrogenase (5 μL). Rates were measured spectrophotometrically at 340 nm at 25 °C, and the logarithm of the remaining activity was plotted against time. The inactivation was also carried out under the same conditions in the presence of different GABA concentrations: 0, 22, 33, 88, and 176 mM.

Time-Dependent Inactivation of GABA-AT by Various Equivalents of (*S*)-6 at pH 6.5 (Figure 2) and 7.4. GABA-AT (15 μL; final concentration 1 μM) was added to solutions of (*S*)-**6** (135 μL; final concentrations of 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, and 1.8 μM; these concentrations are equal to 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, and 1.8 equiv, respectively, of the inactivator compared to the enzyme concentration) in 100 mM potassium phosphate buffer, pH 6.5, containing 50 μM α-ketoglutarate and 10 μM β-mercaptoethanol at 25 °C. At timed intervals, aliquots (10 μL) were withdrawn and added to the assay solution (585 μL) containing excess succinic semialdehyde dehydrogenase (5 μL). Rates were measured spectrophotometrically at 340 nm at 25 °C, and the logarithm of remaining activity was plotted against time for each concentration of inhibitor. After being incubated at room temperature for 200 h, each of these solutions were exhaustively dialyzed against buffer A at 4 °C. The enzyme activity left in each of the solutions was assayed, and the protein concentration was determined. The values of the remaining enzyme activity were corrected by the protein concentration of these solutions and plotted against the equivalents of (*S*)-**6** used. The same experiment was carried out at pH 7.4.

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Transamination Events Per Inactivation of GABA-AT by (*S*)-6. The number of turnovers, or transamination events, of GABA-AT during the inactivation by (*S*)-**6** was determined by incubating GABA-AT (0.39 nmol) with (*S*)-**6** (1 mM), 10 mM [^{14}C]-α-ketoglutarate (280 mCi/mmol from Amersham, diluted to 0.88 mCi/mmol with cold α-ketoglutarate), and 5 mM β-mercaptoethanol. Controls were run containing everything except the enzyme, two containing everything except inactivator, and two containing only the labeled α-ketoglutarate in buffer. After being incubated at room temperature and protected from light for 4 h, the inactivation sample was less than 1% active. Each sample was denatured by adding enough 20% trichloroacetic acid to make an 8% (w/v) solution. These were mixed well and then applied to prewashed 5 × 70 mm Dowex 50 columns. The columns were washed with water (6 mL) and then eluted with 2 M NH_4OH (0.5 mL), followed by 2 M NH_4OH (5 mL), and then another 2.0 mL of 2 M NH_4OH to be certain that all of the radioactivity had eluted from the column. Each of the four solutions for each sample was collected separately in scintillation vials, and radioactivity was measured by liquid scintillation counting. The number of counts eluting with the ammonium hydroxide solution, after being corrected by controls, is indicative of the amount of [^{14}C]glutamate present in the sample. By conversion of the number of counts to millicurie units then to millimoles on the basis of the specific activity of the original [^{14}C]-α-ketoglutarate, the millimoles of glutamate formed during the inactivation was calculated. The ratio of millimoles of glutamate produced to millimoles of active enzyme used in the sample is the number of transaminations occurring per inactivation event. This experiment was run in duplicate, and the data obtained were averaged.

Inactivation of [^{3}H]PLP-GABA-AT by (*S*)-6, Product Isolation, and Analysis by Reverse-Phase HPLC (Figure 4). [^{3}H]PLP-reconstituted GABA-AT[38,39] (50 μL, 0.59 mg/mL) was incubated with (*S*)-**6** (2 mM) and α-ketoglutarate (5 mM) in a total volume of 100 μL of buffer A, while protected from light, at 25 °C for 4 h, and the reaction mixture assayed. By this time, less than 1% of the enzyme activity remained. Excess inactivator was removed by running the solution over Sephadex G-50 packed columns prewashed with water, using the Penefsky spin method.[34] The solution obtained was again assayed; no return of enzyme activity was detected. Two controls were run simultaneously, one that was identical but contained no inactivator, and one that contained no inactivator or α-ketoglutarate, but contained 40 mM GABA (to determine the extent of conversion of the PLP to PMP). All three solutions obtained were subjected to a potassium hydroxide denaturation process as follows. The pH of the enzyme was adjusted to 12 using 1 M KOH and was incubated at room temperature for 1 h. Then enough trifluoroacetic acid (TFA) was added to quench the base and make a 10% v/v TFA solution. White denatured protein appeared in the solution after the acid addition. After standing at room temperature for 10 min, the denatured enzyme solution was placed into a Centricon 10 microconcentrator and was centrifuged for 15 min at 5000 rpm with a Du Pont Sorvall RC5B Plus centrifuge, using an SA600 rotor to achieve a complete separation of the protein and the effluents. The protein was rinsed with 0.1% aqueous TFA (50 μL), vortexed, and then centrifuged for a further 10 min. This process was repeated three times. The protein obtained was redissolved in buffer A (500 μL) and was counted for radioactivity. The effluent and rinses were combined and lyophilized to dryness. The solid obtained from the lyophilization was dissolved in water (500 μL) to make a stock solution. The final sample was prepared by adjusting the pH to 1 with 10% aqueous TFA. To a 100 μL aliquot of the final sample was added a 20 μL aliquot solution containing 4 mM each of PLP and PMP standards, which had been subjected to the same basification and acidification steps; this sample was injected into the HPLC Alltech Alltima C18 column (4.6 × 250 mm, 5 μ). Mobile phase A was 0.1% aqueous TFA flowing at 0.5 mL/min for 15 min. Then a 5 min gradient was run to 50% mobile phase B (80% aqueous acetonitrile). The column was eluted with 50% mobile phase B for 20 min. Under these conditions, PLP elutes at 16 min, PMP at 8 min, and **3** at 36 min. The HPLC eluents were analyzed for radioactivity with a Radiomatic FLO−ONE\Beta Series A-200 liquid flow scintillation counter. After being calibrated with the data from the two controls, the UV absorption at 254 nm and the detected radioactivity of the isolated (*S*)-**6** inactivation product were plotted against the retention time.

UV−Vis Spectrum of the Purified (*S*)-6 Inactivation Product (Figure 5). GABA-AT (1.2 mL, 80 nmol) was inactivated with (*S*)-**6** (2 mM) in buffer A, containing 10 mM α-ketoglutarate in the dark. After being incubated at room temperature for 4 h, the reaction mixture was assayed. Less than 1% of the enzyme activity remained. Excess inactivator was removed by running the portions of the inactivation solutions (200 μL) over Sephadex G-50 packed columns prewashed with water, using the Penefsky spin method.[34] The solutions obtained were again assayed; no enzyme activity was detected. The isolation and purification of the product was achieved as described above. The 35−37 min HPLC fractions were collected and lyophilized. The dried sample was dissolved in 2 M NH_4OH (500 μL), placed in a Fisher 1 cm cuvette, and scanned from 500 to 214 nm. The gabaculine inactivation product (**3**) (80 nmol) also was dissolved in 2 M NH_4OH (500 μL) and scanned under the same conditions.

Electrospray Ionization Mass Spectral Studies of the (*S*)-6 Inactivation Product (Figures 7 and 8). Care was taken in all of the experimental procedures to eliminate the introduction of glycerol, poly(ethylene glycol), or other detergent-related polymers into samples. All glassware used was treated sequentially with 30% v/v nitric acid in water, water, methanol, chloroform, methanol, and water. The enzyme isolated from pig brain was further purified by dialysis against buffer A (4 × 4 L, changed every 4 h). The dialysis tubing used, Spectra/por 2, molecular porous dialysis membrane from Baxter, was soaked in water (3 × 2 L, changed every 4 h) before use. The purified GABA-AT (1.2 mL, 80 nmol) was inactivated with (*S*)-**6** (2 mM) in buffer A containing 10 mM α-ketoglutarate at room temperature in the dark for 4 h. The same inactivation, denaturation, and product isolation procedure described above was followed except that the inactivation product was not subjected to HPLC purification. The lyophilized sample was dissolved in 50% aqueous CH_3CN (500 μL) to obtain a sample concentration of approximately 0.16 mM. Aliquots of 10 μL each (16 nmol) were loop injected onto a Hypersil BDS C18 column (2 × 250 mm), connected to the mass spectrometer. The column was isocratically eluted with 5% aqueous CH_3CN for 5 min, and then a 10 min gradient from 5 to 100% CH_3CN was applied. The flow rate was 0.2 mL/min. During the isocratic portion of the run the flow was directed to the waste to avoid contamination of the electrospray source with salts and other nonvolatile material from the sample. Positive ion electrospray mass spectra were acquired. Typical operational parameters were as follows: source temperature 135 °C; capillary 3.6 kV; counter electrode 0.5 kV; cone voltage 25 V. The mass axis was calibrated using a PEG solution according to the manufacturer's instructions, and unit mass resolution was used for all measurements. The instrument was scanned from 250 to 420 amu at a scan rate of 2 s/scan. Tandem mass spectra were acquired at 35 eV collision energy and 3×10^{-3} mbar gas (argon) pressure.

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