Unusual Mechanistic Difference in the Inactivation of γ-Aminobutyric Acid Aminotransferase by (*E*)- and (*Z*)-4-Amino-6-fluoro-5-hexenoic Acid

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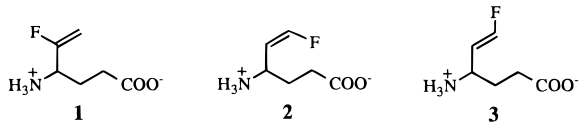
Received August 14, 1995[⊗]

Abstract: The mechanisms of inactivation of γ-aminobutyric acid (GABA) aminotransferase by (*Z*)- (**2**)- and (*E*)-4-amino-6-fluoro-5-hexenoic acid (**3**) were studied. The kinetic constants of inactivation for **2** and **3** were approximately the same. Inactivation of [7-^{3}H]PLP-reconstituted GABA aminotransferase by **2** and **3** also gave similar results for the two isomers: 63% (**2**) and 66% (**3**) of the radioactivity remained covalently attached to the enzyme; 31% (**2**) and 29% (**3**) were released as PLP; 5% (**2**) and 4% (**3**) of the radioactivity emerged as PMP. Treatment of GABA aminotransferase with either [^{3}H]-**2** or [^{3}H]-**3** led to the incorporation of 1.0 equiv of tritium into the enzyme after gel filtration. Urea denaturation at pH 7.0, however, released about 0.3 equiv of the tritium from the enzyme, and urea denaturation at pH 2.4 released 0.35 equiv of the tritium. About 85% of the released radioactivity was identified as 4-amino-6-oxohexanoic acid (**31**) and the remainder as a mixture of 4-oxo-5-hexenoic acid (**10**) and the product of Michael addition of β-mercaptoethanol to **10**. Neither inactivator produced any amine metabolites during inactivation. The first divergence from similarity between the two isomers was in the isolation of nonamine metabolites. Inactivator **2** generated two nonamine metabolites, whereas **3** produced only one. The additional metabolite with **2** was identified as **10**. The metabolite in common may be the normal transamination product or something derived from it. To confirm this possibility, it was shown that both isomers undergo transamination; **2** is transaminated 1.4 ± 0.3 times, and **3** is transaminated 0.7 ± 0.3 time. Fluoride ion release also was monitored, and it was found that **2** released 1.4 ± 0.2 F^- and **3** released 0.9 ± 0.05 F^-. The additional F^- release for **2** is expected, given that it produces an additional metabolite that requires release of F^- for its formation. Absorption spectra of GABA aminotransferase inactivated with **2**, **3**, and 4-amino-5-fluoropentanoic acid (**35**) showed an absorbance at 430 nm that was missing in the spectrum of native enzyme. Taken together, these results indicate that **2** and **3** inactivate GABA aminotransferase by multiple mechanisms, but at least the major inactivation mechanism is different for the two isomers. The results for **2** can be rationalized by three different mechanisms. All are initiated by Schiff base formation of the inactivator with the active site PLP followed by γ-proton removal. The major pathway (about 65%) proceeds by isomerization of the fluorovinyl double bond (Scheme 2) and produces a ternary complex between the enzyme, the coenzyme, and the inactivator (**13**); a small amount of inactivation (about 3%) may result from formation of a weakly stable covalent adduct with an active site nucleophile (**14**). The other two inactivation pathways proceed by isomerization of the aldimine double bond of the Schiff base with PLP. Scheme 7 (about 30%) results in the formation of a weakly stable adduct that decomposes upon denaturation at neutral pH to 4-amino-6-oxohexanoic acid (**31**). Scheme 6 (about 5%) could account for the formation of PMP, although this amount may be outside experimental error. The 430 nm absorbance observed in the absorption spectrum may correspond to the conjugated ternary complex **13**, which has a structure similar to that of the known product of inactivation of GABA aminotransferase by **35** and 4-amino-5-hexenoic acid (**37**). The same mechanisms of inactivation by **3** are relevant except that, to avoid the formation of **10**, an S_N2 mechanism (Scheme 3) is invoked in place of the elimination mechanism (Scheme 2). This difference in mechanism may be the result of hydrogen bonding of the fluorine atoms in different orientations at the active site (Scheme 10). The inactivation mechanisms for **2** and **3**, therefore, are different from each other as well as from 4-amino-5-fluoro-5-hexenoic acid (Silverman, R. B.; Bichler, K. A.; Leon, A. J. *J. Am. Chem. Soc.* **1996**, *118,* 1241−1252).

γ-Aminobutyric acid (GABA) aminotransferase (EC 2.6.1.19) is a pyridoxal 5′-phosphate (PLP)-dependent enzyme that catalyzes the transamination of GABA and α-ketoglutarate to give succinic semialdehyde and L-glutamate. In the preceding paper[1] the mechanisms of inactivation of GABA aminotransferase by 4-amino-5-fluoro-5-hexenoic acid (**1**), a compound



1 **2** **3**

reported by Kolb et al.[2] to be a mechanism-based inactivator of this enzyme, were shown to be initiated by Schiff base formation of the inactivator with the active site PLP followed by γ-proton removal and elimination of the fluoride ion to give

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⊗ Abstract published in *Advance ACS Abstracts,* January 15, 1996.

(1) Silverman, R. B.; Bichler, K. A.; Leon, A. J. *J. Am. Chem. Soc.* **1996**, *118,* 1241−1252.

(2) Kolb, M.; Barth, J.; Heydt, J. G.; Jung, M. J. *J. Med. Chem.* **1987**, *30,* 267−272.

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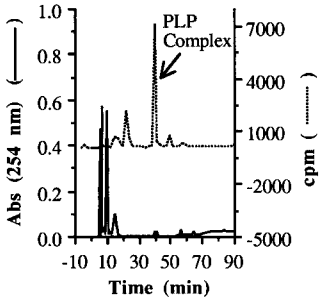


Figure 1. HPLC of the Schiff base complex between PLP and **2**.

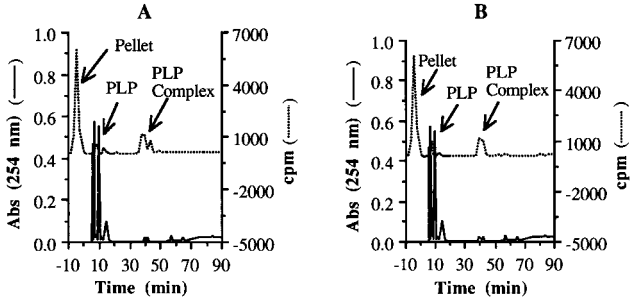


Figure 2. HPLC trace of the inactivation of [^{3}H]PLP-reconstituted GABA aminotransferase by **2** (A) or **3** (B) following denaturation without removal of excess inactivator (see Experimental Section for details).

a conjugated allene. Three different reactions on this allene led to three different inactivation pathways.[1] The 6-fluoro isomer, (*Z*)-4-amino-6-fluoro-5-hexenoic acid (**2**), also was reported by Kolb et al.[2] and was found to be a much more potent inactivator of GABA aminotransferase than was **1**. We now have synthesized the diastereomer of **2**, (*E*)-4-amino-6-fluoro-5-hexenoic acid (**3**), and have found that, although the inactivation potencies for **2** and **3** are the same, the inactivation mechanisms are different. Furthermore, despite the fact that **1**−**3** are all monofluorovinyl GABA analogues, the inactivation mechanisms for **2** and **3** are completely different from those of **1**. The results of these studies are described in this paper.

Results

Syntheses of 2 and 3. Our synthetic route to compound **2** is a shortened modification of that described by Kolb et al.[2] and is shown in the preceding paper (see Scheme 1 in that paper).[1] In the step that makes the pyrrolidinone (KO*t*-Bu/THF), Kolb et al.[2] do not get the pyrrolidinone precursor to **3** (i.e., compound **10** in Scheme 1 of the preceding paper); instead, they observe double elimination to give 5-ethynyl-2-pyrrolidinone, a compound that we did not obtain. In our hands, 5-[(*E*)-fluorovinyl]-2-pyrrolidinone (compound **10** in the preceding paper) was obtained, which was acid hydrolyzed to **3**.

Kinetics of Inactivation of GABA Aminotransferase with 2 and 3. The kinetic constants for the inactivation of GABA aminotransferase by both **2** and **3** were determined using the method of Kitz and Wilson.[3] The K_I and k_{inact} values are 0.17 mM and 0.06 min^{-1}, respectively, for **2** and 0.14 mM and 0.05 min^{-1}, respectively, for **3**.

Inactivation of [^{3}H]PLP-Reconstituted GABA Aminotransferase by 2 and 3 without Removal of Excess Inactivator. It was found that without removal of the excess inactivator, a Schiff base complex between the inactivators and released PLP formed; under the given elution conditions, the retention time for the Schiff base of either isomer was about 40 min (Figure 1). The total for the radioactivity of PLP and the Schiff base of PLP with inactivator was 31% for **2** (Figure 2A) and 29% of the radioactivity for **3** (Figure 2B). With both inactivators, however, the majority of the radioactivity remained attached to the protein: 63% for **2** (Figure 2A) and 66% for **3** (Figure 2B). PMP accounted for 5% of the radioactivity with **2** and 4% with **3** (this may exceed experimental error).

Equivalents of [^{3}H]-2 and [^{3}H]-3 Bound to GABA Aminotransferase after Inactivation. Inactivation of GABA aminotransferase by [^{3}H]-**2** and [^{3}H]-**3** followed by gel filtration resulted in 1.05 ± 0.05 equiv of inactivator remaining bound to the enzyme.

Equivalents of [^{3}H]-2 and [^{3}H]-3 Bound to GABA Aminotransferase after Inactivation and Denaturation with Urea at pH 7.0. Following inactivation, gel filtration, and denaturation in 6 M urea, pH 7.0, [^{3}H]-**2** resulted in covalent attachment of 0.65 ± 0.05 equiv of radioactivity and [^{3}H]-**3** led to the attachment of 0.70 ± 0.05 equiv.

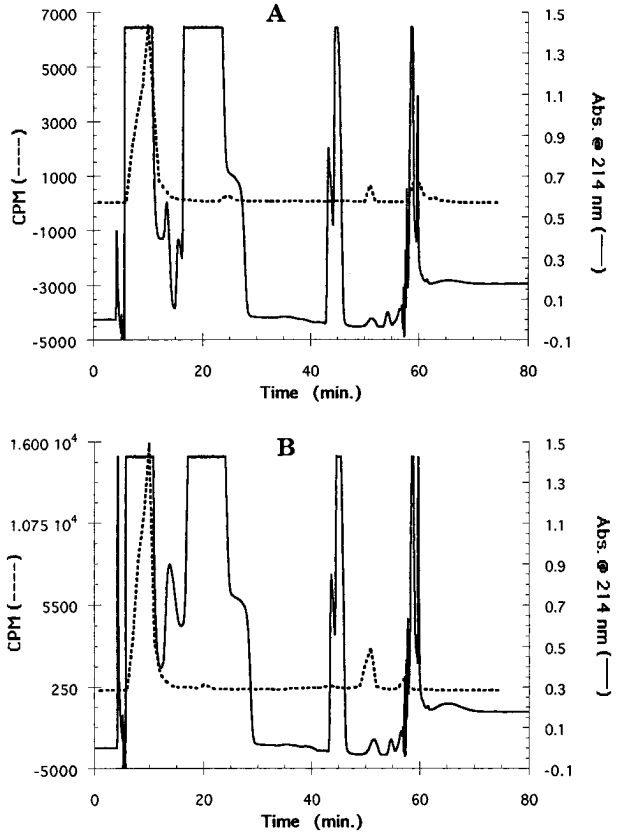


Figure 3. HPLC trace of the products released from [^{3}H]-**2**-inactivated (A) and [^{3}H]-**3**-inactivated and 6 M urea-denatured GABA aminotransferase at pH 7.

Identification of Products Released by Urea Denaturation of [^{3}H]-2- and [^{3}H]-3-Inactivated GABA Aminotransferase. Following inactivation of GABA aminotransferase by [^{3}H]-**2** or [^{3}H]-**3**, gel filtration, and microdialysis against 6 M urea in potassium phosphate, pH 7.0, buffer containing β-mercaptoethanol, the released products in the denatured enzyme solution were monitored by HPLC. With [^{3}H]-**2**, one major and three minor peaks were observed (Figure 3A). The major peak (9 min) contained 86.5% of the radioactivity and peaks at 25, 50, and 60 min contained 2%, 3%, and 8.5% of the radioactivity, respectively. With [^{3}H]-**3**, there also were one major (9 min, 84%) and three minor (43 min, 4%; 50 min, 10%; and 60 min, 2%) peaks (Figure 3B). The peak at 9 min was identified by independent synthesis as coming from 4-amino-6-oxohexanoic acid (**31**). The peak at 50 min coelutes with **5**, and the peak at 60 min coelutes with **4**.

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

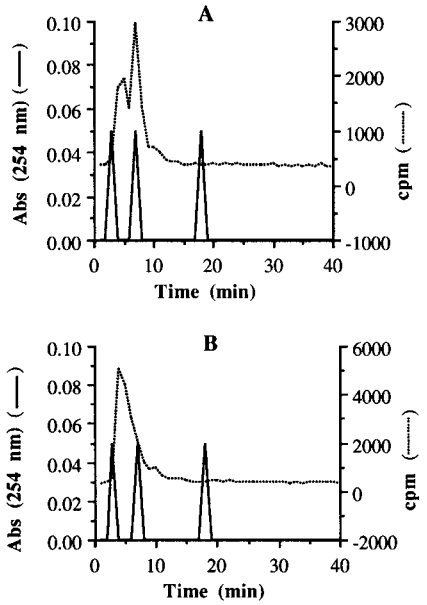
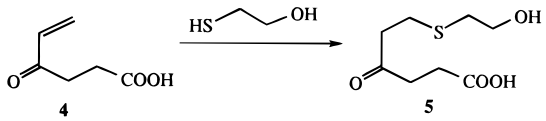


Figure 4. HPLC analysis of nonamine metabolites formed during inactivation of GABA aminotransferase with [^{3}H]-**2** (A) and [^{3}H]-**3** (B).

Scheme 1. Michael Addition of β-Mercaptoethanol to 4-Oxo-5-hexenoic Acid



Equivalents of [^{3}H]-2 and [^{3}H]-3 Bound to GABA Aminotransferase after Inactivation and Denaturation with 8 M Urea, pH 2.4. Following inactivation, gel filtration, and denaturation in 8 M urea, pH 2.4, both [^{3}H]-**2** and [^{3}H]-**3** led to the covalent attachment of 0.65 equiv of radioactivity.

Equivalents of [^{3}H]-2 and [^{3}H]-3 Bound to GABA Aminotransferase after Inactivation and Denaturation with Trifluoroacetic Acid. Following inactivation, gel filtration, and trifluoroacetic acid denaturation, 1.09 equiv of radioactivity from [^{3}H]-**2** and 1.06 equiv of radioactivity from [^{3}H]-**3** remained bound to the enzyme.

Analysis of Amine Products Released from GABA Aminotransferase after Inactivation with [^{3}H]-2 and [^{3}H]-3. By dansylation, no amine metabolites could be detected with either [^{3}H]-**2** or [^{3}H]-**3**.

Analysis of Nonamine Metabolites Formed during Inactivation of GABA Aminotransferase with [^{3}H]-2 and [^{3}H]-3. The HPLC trace of the nonamines generated after inactivation of GABA aminotransferase with [^{3}H]-**2** is shown in Figure 4A. Two peaks of radioactivity were detected. The peak at 7 min corresponded by HPLC comparison to the β-mercaptoethanol Michael adduct (**5**, Scheme 1) with 4-oxo-5-hexenoic acid (**4**). The peak at 5 min is unknown, but may be the normal transamination product.

The HPLC trace for the same experiment with [^{3}H]-**3** (Figure 4B) shows a broad peak at 5 min.

Transamination Events per Inactivation of GABA Aminotransferase with 2 and 3. GABA aminotransferase, which had been inactivated by **2** in the presence of [5-^{14}C]-α-ketoglutarate, produced 1.4 $\pm$ 0.3 equiv (five experiments) of [5-^{14}C]glutamate per inactivation event, which represents the number of transaminations per inactivation event. When **3** was used, only 0.7 $\pm$ 0.3 transamination (five experiments) was detected.

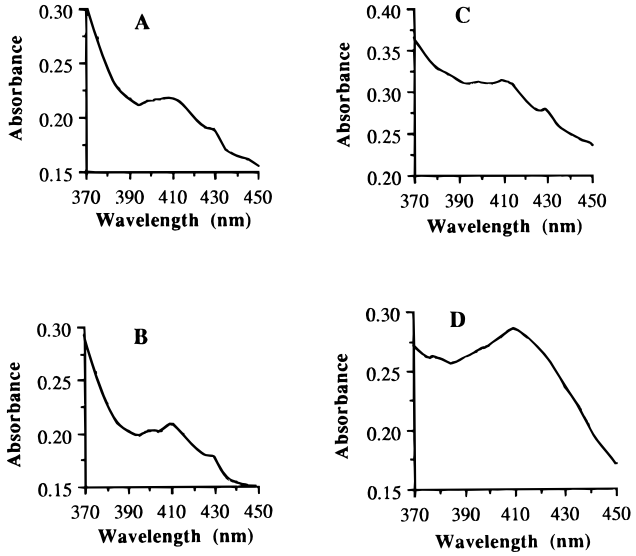


Figure 5. Visible absorption spectrum of the product of inactivation of GABA aminotransferase by **2** (A), **3** (B), or **32** (C). (D) Visible absorption spectrum of GABA aminotransferase (see Experimental Section for details).

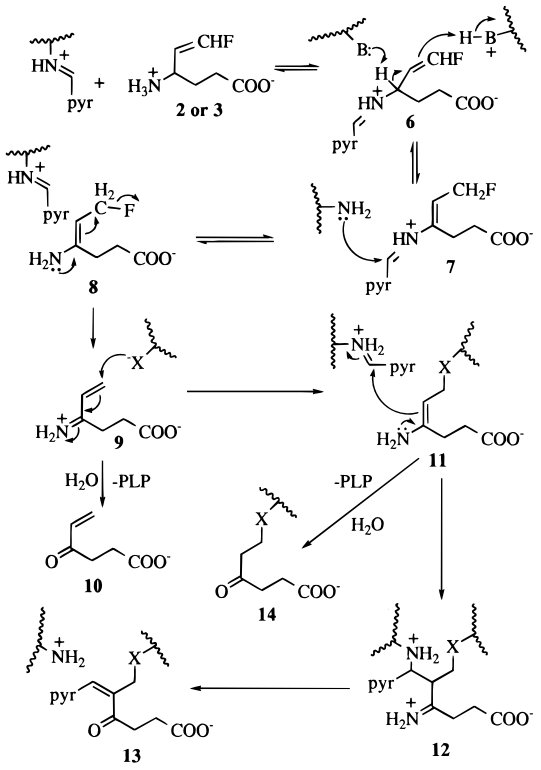
Fluoride Ion Release during Inactivation of GABA Aminotransferase with 2 and 3. Inactivation of GABA aminotransferase by **2** was monitored for fluoride ion release. Because of the sensitivity of the system to pH fluctuations, the pH values of the inactivated samples and controls were carefully monitored. After correction for the controls, it was found that 1.4 $\pm$ 0.2 fluoride ions (four experiments) were released per inactivation event. With **3**, 0.9 $\pm$ 0.05 equiv of F^- (four experiments) was released.

UV−vis Spectrometry of the Covalent Adduct Formed between GABA Aminotransferase and 2 or 3. Scans of GABA aminotransferase inactivated with **2** (Figure 5A), **3** (Figure 5B), and 4-amino-5-fluoropentanoic acid (Figure 5C) from 370 to 450 nm showed no significant differences. However, there was an absorbance at 430 nm in all three spectra that was absent in that of the native enzyme (Figure 5D).

Discussion

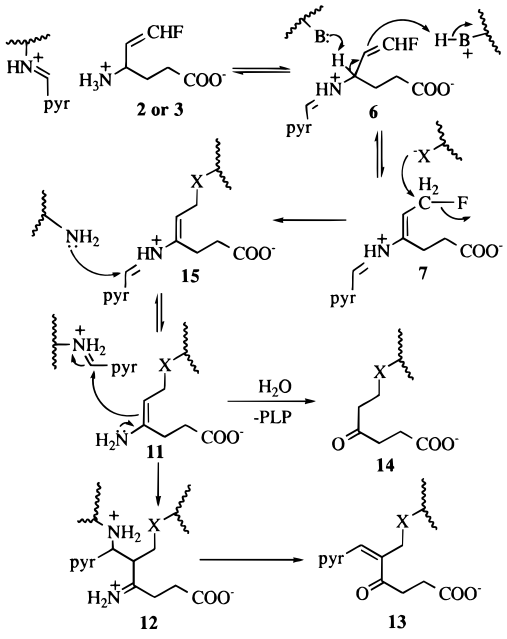
The catalytic mechanism of PLP-dependent GABA aminotransferase was discussed in the preceding paper (see Scheme 3 in that paper); it uses a standard transamination mechanism.[1] On this basis, numerous inactivation mechanisms can be envisioned for **2** and **3**. Several possibilities are shown in Schemes 2−7. These mechanisms, however, can be differentiated by investigation of a combination of fluoride ion release, coenzyme structure, and formation of adducts and metabolites, as summarized in Table 1. All of the mechanisms are initiated by the normal Schiff base formation (**6**) of **2** or **3** with the active site PLP and γ-proton removal. Mechanism 1 (Scheme 2) continues with γ-proton removal and protonation on the carbon containing the fluorine (**7**) followed by transfer of the PLP back onto the active site lysine (**8**) and elimination of fluoride ion to give the reactive intermediate **9**, which can either hydrolyze to 4-oxo-5-hexenoic acid (**10**) or undergo Michael addition to give enamine **11**. Intermediate **11** can either hydrolyze to enzyme adduct **14** or undergo enamine addition to the enzyme-bound PLP (**12**). Elimination of the lysine from **12** would give a stable ternary complex of the inactivator, the coenzyme, and the protein (**13**). The second mechanism (Scheme 3) starts out the same as Scheme 2 up to **7**. This intermediate has an activated fluoride leaving group, which is susceptible to S_N2 addition by an active

Scheme 2. First Potential Mechanism of Inactivation of GABA Aminotransferase by **2** or **3**[a]



[a] The symbol pyr stands for the pyridine ring of the PLP.

Scheme 3. Second Potential Mechanism of Inactivation of GABA Aminotransferase by **2** or **3**[a]

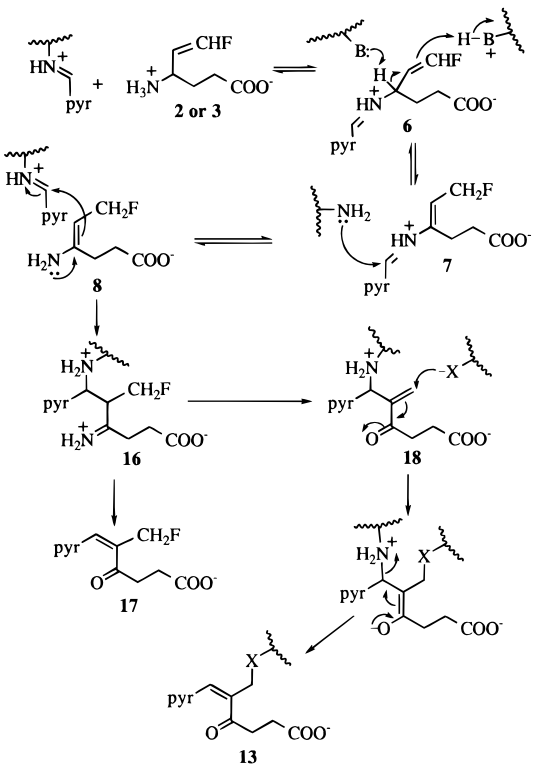


[a] The symbol pyr stands for the pyridine ring of the PLP.

site nucleophile to give **15**. Although fluoride ion, generally, is not a very good leaving group in S_N2 reactions, Poulter et al.[4] have shown that 3-(fluoromethyl)-3-butenyl diphosphate inactivates isopentenyl diphosphate isomerase by an S_N2 displacement of the fluoride ion. Transfer of the PLP back onto the protein gives enamine **11**, which can produce **13** and/or **14** as described above for the first mechanism. The third mechanism (Scheme 4) is the same as the first mechanism (Scheme

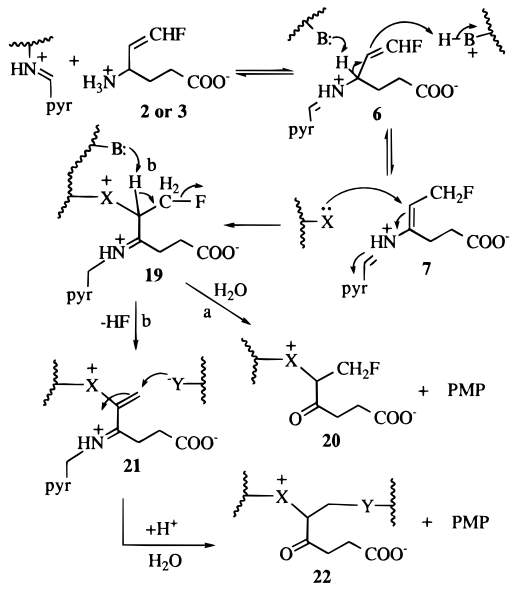
(4) Poulter, C. D.; Muehlbacher, M.; Davis, D. R. *J. Am. Chem. Soc.* **1989**, *111*, 3740−3742.

Scheme 4. Third Potential Mechanism of Inactivation of GABA Aminotransferase by **2** or **3**[a]



[a] The symbol pyr stands for the pyridine ring of the PLP.

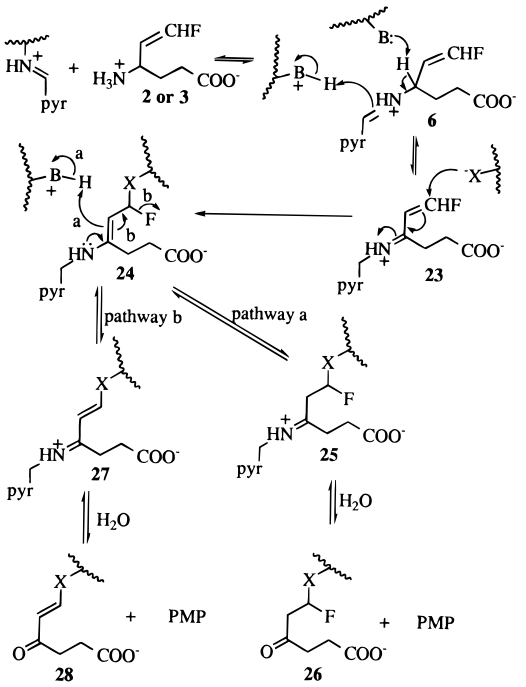
Scheme 5. Fourth Potential Mechanism of Inactivation of GABA Aminotransferase by **2** or **3**[a]



[a] The symbol pyr stands for the pyridine ring of the PLP.

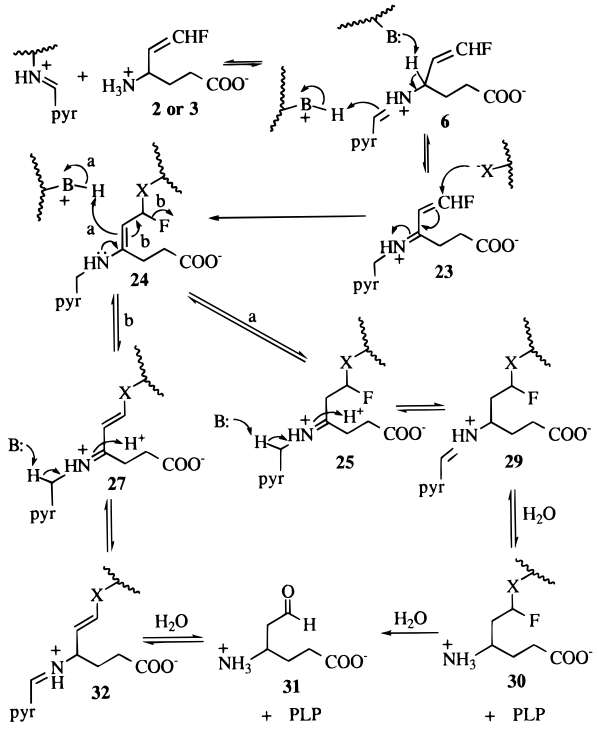
2) up to **8**. Enamine addition to the enzyme-bound PLP gives the ternary complex **16**, which, upon elimination of the lysine or the fluoride, would give, respectively, either modified coenzyme **17** or adduct **13** via **18**. Scheme 5 depicts a fourth mechanism, again via intermediate **7**. Michael addition to **7** would generate ternary complex **19**. Hydrolysis of **19** would give adduct **20** and PMP (pathway a), whereas elimination of HF (pathway b) would give **21**, which is susceptible to another Michael addition and hydrolysis to **22** and PMP. A fifth mechanism (Scheme 6) starts as the normal transamination mechanism to **23**. Michael addition would give the ternary complex **24**, which could be protonated (pathway a) to **25** and

Scheme 6. Fifth Potential Mechanism of Inactivation of GABA Aminotransferase by **2** or **3**[a]



[a] The symbol pyr stands for the pyridine ring of the PLP.

Scheme 7. Sixth Potential Mechanism of Inactivation of GABA Aminotransferase by **2** or **3**[a]



[a] The symbol pyr stands for the pyridine ring of the PLP.

hydrolyzed to **26** and PMP or undergo elimination of fluoride ion to **27** and hydrolysis to adduct **28** and PMP (pathway b). The last mechanism (Scheme 7) starts out the same as Scheme 6 to **25** and **27**. Isomerization of the PMP ketimine **25** to the corresponding PLP aldimine (**29**) followed by hydrolysis would release PLP and 4-amino-6-oxohexanoic acid (**31**). Isomerization of PMP ketimine **27** to the corresponding PLP aldimine (**32**) followed by hydrolysis also would give PLP and **31**.

With these potential inactivation mechanisms as working hypotheses, studies directed at gaining evidence that would favor one or more of these mechanisms were carried out. Compounds **2** and **3** were synthesized by a modification of the method of Kolb et al.,[2] as described in Scheme 1 of the preceding paper.[1] However, **3** was not obtained by Kolb et al.;[2] instead, in the potassium *tert*-butoxide elimination step, they obtained 5-(1-fluorovinyl)-2-pyrrolidinone, (*Z*)-5-(2-fluorovinyl)-2-pyrrolidinone, and the double-elimination product, 5-ethynyl-2-pyrrolidinone. They did not report the formation of the (*E*)-5-(2-fluorovinyl)-2-pyrrolidinone. In our hands, the addition of stronger base (but not KO*t*-Bu) resulted in the formation of the double-elimination product as well. It appears, then, that Kolb et al.[2] converted the *E*-isomer into the acetylene.

Table 1. Expected Differences for the Inactivation Mechanisms for 2 and 3

mechanism	fluoride release	cofactor release	unstable adduct release
Scheme 2	yes	PLP	maybe 4-oxo-5-hexenoic acid
Scheme 2	yes	enzyme bound	maybe 4-oxo-5-hexenoic acid
Scheme 3	yes	PLP	no
Scheme 3	yes	enzyme bound	no
Scheme 4	no	modified cofactor	release of modified cofactor
Scheme 4	yes	enzyme bound	no
Scheme 5	no	PMP	no
Scheme 5	yes	PMP	no
Scheme 6	no	PMP	no
Scheme 6	yes	PMP	no
Scheme 7	yes	PLP	4-amino-6-oxohexanoic acid

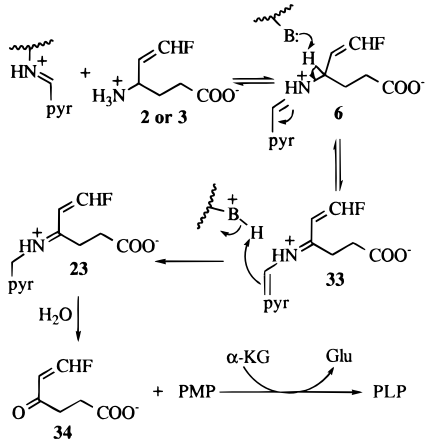
The inactivation kinetic constants for **2** and **3** were found to be almost identical. The fate of the coenzyme after inactivation of GABA aminotransferase by **2** and **3** was examined with the use of [7-^{3}H]PLP-reconstituted GABA aminotransferase (Figure 2). Again, the results for **2** and **3** were virtually identical. Most of the radioactivity (63% for **2** and 66% for **3**) after inactivation and denaturation remained bound to the protein, suggesting that a covalent adduct was formed between the coenzyme and the protein. This is consistent with adduct **13** in Schemes 2−4. With **2** as the inactivator, 31% of the radioactivity remained as PLP (actually a mixture of PLP and the imine of PLP with **2**), as would be expected for the route to adduct **14** in Schemes 2 and 3 or to metabolite **31** in Scheme 7, and 5% of the radioactivity was found as PMP, suggesting that a small fraction of the turnovers (if this small amount has not exceeded experimental error) may proceed by mechanisms like those shown in Schemes 5 and 6. Similar results were obtained with **3**.

Inactivation of GABA aminotransferase with [2-^{3}H]-**2** and [2-^{3}H]-**3** also gave very similar results. Following gel filtration, 1.0 equiv of tritium was attached to the enzyme for both inactivators. Upon urea denaturation at pH 7.0 or 2.4, 0.30−0.35 equiv of tritium was released from the enzyme inactivated by either compound. Gel filtration followed by trifluoroacetic acid denaturation, however, did not release radioactivity from the enzyme. The lack of release of radioactivity by trifluoroacetic acid denaturation also was observed during the inactivation of GABA aminotransferase by 3-amino-4-fluorobutanoic acid.[5] It appears that rapid precipitation of the protein by trifluoroacetic acid traps the radioactivity within the denatured protein. Neither inactivator produced amine metabolites during inactivation.

To this point, **2** and **3** have given identical results. The first difference, however, was observed when nonamine metabolites

(5) Silverman, R. B.; Roscher, C. L. C. *Bioorg. Med. Chem.* **1996**, in press.

Scheme 8. Transamination of **2** or **3**[a]



[a] The symbol pyr stands for the pyridine ring of the PLP.

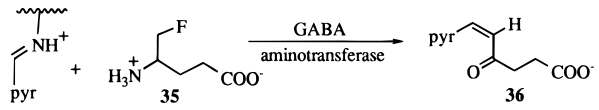
were monitored (Figure 4). Compound **2** was turned over to give two nonamine metabolites, whereas **3** produced only one nonamine metabolite. The metabolite that was found in the reaction mixture with **2** that was not found with **3** was identified as the product of addition of β-mercaptoethanol to 4-oxo-5-hexenoic acid (**5**, Scheme 1). β-Mercaptoethanol is routinely put in the enzyme incubation mixtures to keep the enzyme reduced. The 4-oxo-5-hexenoic acid (**10**) appears as a potential metabolite in Scheme 2, the hydrolysis product of **9** in competition with Michael addition to **9** by the enzyme (**11**). Isolation of the Michael addition product (**5**) is strong evidence for a pathway that produces **10**, such as the one shown in Scheme 2. The common metabolite for **2** and **3** is believed to be the normal transamination product (**34**, Scheme 8) or its hydrolysis product (**34** except with OH in place of F), but we were unable to synthesize this compound to use as a standard.

The hypothesized formation of **34** suggests that transamination is a viable pathway. In fact, as evidenced by the normal transamination assay, the conversion of [^{14}C]-α-ketoglutarate to [^{14}C]glutamate, it was found that both **2** and **3** undergo transamination, but **2** is transaminated about twice as much as is **3** (1.4 vs 0.7 equiv). Transamination requires the enzyme to protonate the isomerized substrate−coenzyme imine (**33**, Scheme 8) on the coenzyme rather than on the fluorovinyl double bond. Since **2** and **3** are diastereomers, it is reasonable that this equilibrium would be different for the two inactivators, leading to different amounts of transamination. This also suggests that the two inactivator−coenzyme complexes have different binding interactions, as is expected for diastereomers.

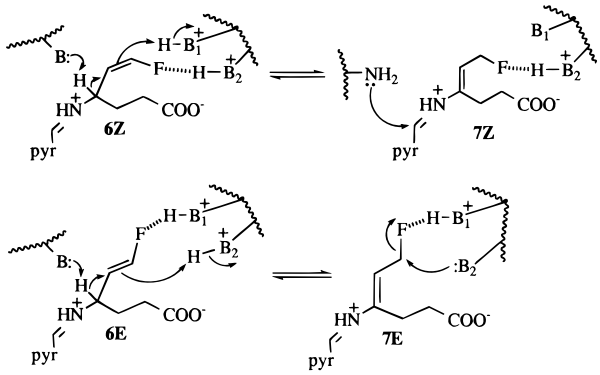
If **2** and **3** inactivate GABA aminotransferase by most of the proposed mechanisms, then fluoride ion release must occur. In fact, we observed fluoride ion release from both inactivators, but different amounts. Compound **2** released about 1.5 equiv of F^-, whereas **3** released only about 1 equiv of F^-. This is consistent with the above results. Inactivation by any of the routes shown in Schemes 2 and 3 and half of the routes in Schemes 4−7 requires the loss of F^-. Compound **2** also was found to produce a nonamine (**5**, Scheme 1), which requires loss of F^-. This could account for why **2** inactivation always generated more F^- than did inactivation by **3**.

The observed results are consistent with at least three different inactivation mechanisms in which compounds **2** and **3** appear to be proceeding by different pathways. In the inactivation mechanism(s) by **2** it is necessary to account for an additional nonamine metabolite not found with **3**, twice as much transamination as **3**, and 50% more F^- release as **3**. Given the relatively minor difference in structure between **2** and **3**, it is

Scheme 9. Reaction of **32** with GABA Aminotransferase



Scheme 10. Hydrogen Bonding and Isomerization of the Schiff Bases of 2 and 3 with Enzyme-Bound PLP[a]



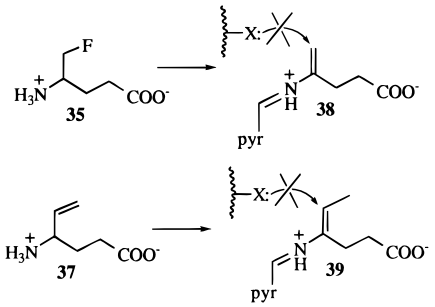
[a] The symbol pyr stands for the pyridine ring of the PLP.

reasonable to suggest that they both proceed by a common mechanism to a point. One way to rationalize these results is to propose that **2** proceeds by the mechanism in Scheme 2. This mechanism accounts for the additional nonamine (identified as **10**) and generates two different covalent adducts. Ternary complex **13** could account for the 63% of the radioactivity from [^{3}H]PLP that remains covalently attached to denatured enzyme. Adduct **14**, especially if X is a good leaving group, such as a cysteine, glutamate, aspartate, or tyrosine, may be susceptible to β-elimination to 4-oxo-5-hexenoic acid (**4**, Scheme 1) upon denaturation at neutral pH via the enol. Evidence for the formation of **13** comes from the radioactivity results and a spectral study. The same amount of [^{3}H]PLP and [^{3}H]-**2** or -**3** (about 0.65−0.7 equiv) becomes covalently attached to the enzyme under denaturing conditions, consistent with a ternary complex. Furthermore, 4-amino-5-fluoropentanoic acid (**35**, Scheme 9) inactivates GABA aminotransferase by an enamine mechanism that converts the PLP into adduct **36**.[6] The visible spectrum of this adduct (Figure 5C) has a long wavelength absorbance at 430 nm, consistent with the extended conjugated system. The visible spectra of the enzyme inactivated with **2** and **3** (Figure 5A,B) showed the same absorbance, which is absent in the control enzyme that was not inactivated (Figure 5D). These spectra support the proposed structure for **13**, which is very similar to that of **36**.

The inactivation mechanism with **3** has to proceed without formation of **10**, but with the formation of **13** and **14**, if that is what **2** produces, since the results are the same for the two inactivators except for the absence of **10** when **3** is the inactivator. The mechanism depicted in Scheme 3 would accomplish this. A condition, however, must be set on the mechanisms in Schemes 2 and 3 to differentiate them, since it appears that the partitioning between these mechanisms occurs at intermediate **7**, which is common to both mechanisms. A rationalization for why intermediate **7** in Scheme 2 could transfer the PLP to the enzyme, but intermediate **7** in Scheme 3 would undergo an S_N2 reaction, is that the geometries of the fluorine atom may be different with **2** and **3** (Scheme 10). Fluorine is a good hydrogen bond acceptor.[7] When **2** and **3** bind to the

(6) Silverman, R. B.; Invergo, B. J. *Biochemistry*, **1986**, *25*, 6817−6820.
(7) (a) Shebakami, M.; Sekiya, A. *Bull. Chem. Soc. Jpn.* **1993**, *66*, 315−316. (b) Fann, Y.-c.; Ong, J.-l.; Nocek, J. M.; Hoffman, B. M. *J. Am. Chem. Soc.* **1995**, *117*, 6109−6116.

Scheme 11. Proposed Intermediate in the Inactivation of GABA Aminotransferase by **32** and **34**[a]



[a] The symbol pyr stands for the pyridine ring of the PLP.

enzyme, the fluorine may bind in different orientations, possibly to different acid groups. Isomerization would give **7**, but with the fluorine remaining in different orientations; presumably, the fluorine orientation with **2** is incompatible with its S_N2 displacement, but with **3** it may be juxtaposed correctly for displacement (Scheme 10).

If **14** in Schemes 2 and 3 represents the covalent adduct that is unstable to denaturation in urea at neutral pH, then the breakdown product of **14** should be **10** by β-elimination of the X group. However, when the covalent adduct from inactivation by [^{3}H]-**2** was subjected to conditions that released 0.3 equiv of radioactivity in the presence of β-mercaptoethanol as the trapping agent, only 3% of the released radioactivity comigrated with the β-mercaptoethanol adduct of **10** (i.e., **5**); another 8.5% of the radioactivity comigrated with the α,β-unsaturated ketone (**4**), which may not have been trapped. When [^{3}H]-**3** was the inactivator, only 10% of the released radioactivity comigrated with **5** and 2% with **4**. This suggests that only about 12% of the unstable adduct can be represented as structure **14**. The large majority of the released radioactivity (86.5% from [^{3}H]-**2** and 84% from [^{3}H]-**3**), however, was found to comigrate with **31** (Scheme 7). Therefore, it appears that there are really two different unstable adducts formed during inactivation, **14** (from either Scheme 2 or 3) and **31** (from Scheme 7). The formation of **31** is not unexpected, since this should be a byproduct of the normal transamination pathway via **23**. Because of the reactivity of **23**, a β-fluoro-α,β-unsaturated iminium salt, an active site nucleophile (X^-) could react in competition with hydrolysis of the PMP-ketimine that gives PMP and the transamination product (**34**, Scheme 8). If X^- is a lysine residue, **32** or **30** (Scheme 7) would rapidly hydrolyze at neutral pH.

To account for the 4−5% of PMP that was observed as a product of the inactivation with **2** and **3**, the mechanisms in Schemes 5 and 6 can be considered. The mechanisms in Scheme 5 are unlikely because it was shown that intermediates very similar in structure to **7**, which were proposed for the inactivation of GABA aminotransferase by **35**[5] and by γ-vinyl-GABA (**37**),[8] i.e., **38** and **39**, respectively (Scheme 11), did *not* undergo the Michael addition that is shown in Scheme 5. Instead, they only underwent transfer of the PLP to the active site lysine (as in Schemes 2 and 4). More likely, since transamination is observed with **2** and **3**, it is reasonable that in addition to hydrolysis of **23** to give **34**, Michael addition could occur a small percentage of the time, leading to **26** or **28** and PMP (Scheme 6), particularly because the alkene is activated with a fluorine atom.[9] Furthermore, the mechanism up to **25** and **27** in Scheme 6 is the same as the one in Scheme 7 that was proposed to give the unstable adduct (**30** or **32**). Therefore, starting from the normal transamination mechanism to **23**, one of three pathways could evolve: hydrolysis to the transamination product (Scheme 8), Michael addition of an active site nucleophile to **23** (Scheme 6 or 7) followed by hydrolysis to **26** or **28** and PMP (Scheme 6), and/or isomerization and then hydrolysis to **29** or **32**, which may further hydrolyze to **31** and PLP (Scheme 7).

One other mechanism that may be possible is the one that leads to **13** shown in Scheme 4; no evidence, however, was found in the experiments with [^{3}H]PLP-reconstituted GABA aminotransferase for the formation of **17**. One of the metabolites isolated in the preceding paper[1] when 4-amino-5-fluoro-5-hexenoic acid reacts with GABA aminotransferase was the same as **17** except with a hydroxyl group in place of the fluorine. No metabolite from either **2** or **3** was observed by HPLC analysis in the region where the hydroxy analogue of **17** from 4-amino-5-fluoro-5-hexenoic acid was detected. Given that a leaving group is β to the enamine in **8** (Scheme 4), it may seem unlikely that attack of the enzyme-bound PLP would occur in preference to elimination of the fluoride ion. However, if the fluorine is hydrogen bonded to the protein, as we suggested could be the case, then the C−F bond may be misoriented for β-elimination, thereby allowing addition to the PLP instead, as is shown in Scheme 4. Although this mechanism can account for the 63−66% of radioactivity bound to the [^{3}H]PLP-reconstituted enzyme after inactivation, it does not account for the formation of **10** or the unstable adduct and, therefore, is not likely.

Conclusion

As in the case of 4-amino-5-fluoro-5-hexenoic acid,[1] there are multiple pathways for the inactivation of GABA aminotransferase by **2** and **3**. The mechanisms in Schemes 2 and 7 are most consistent with the results for **2**, and those in Schemes 3 and 7 are likely for **3**. Transamination (Scheme 8) and possibly a small amount of inactivation by the mechanism in Scheme 6 also may be important.

It is apparent from the results described here and in the preceding paper that minor structural changes in molecules can lead to vastly different inactivation mechanisms, and when multiple inactivation pathways with comparable energies exist, it is likely that multiple inactivation pathways will result.

Experimental Section

Analytical Methods and Reagents. The analytical methods and reagents used, as well as the syntheses of 5-vinyl-2-pyrrolidinone, the mixture of 5-(1-bromo-2-fluoroethyl)-2 pyrrolidinone and 5-(2-bromo-1-fluoroethyl)-2 pyrrolidinone, [7-^{3}H]pyridoxal 5′-phosphate ([^{3}H]PLP), and 4-oxo-5-hexenoic acid, were described in the preceding paper.[1] 4-Amino-6-oxohexanoic acid (**31**) was synthesized as described earlier.[10]

(*Z*)-5-(2-Fluorovinyl)-2-pyrrolidinone. After 3.30 g (1.57 mmol) of the mixture of 5-(2-bromo-1-fluoroethyl)-2 pyrrolidinone and 5-(1-bromo-2-fluoroethyl)-2 pyrrolidinone[1] in 20 mL of THF was cooled to −78 °C in a dry ice/acetone bath, 63 mL of a 1.0 M solution of potassium *tert*-butoxide in THF was added dropwise. While stirring, this reaction was warmed to −30 °C and maintained between −20 and −30 °C for 2 h. It was cooled to −78 °C and quenched with 2.7 mL of glacial acetic acid. After 15 mL of ether was added, the precipitate that formed was removed by filtration and washed with ether. Solvents were evaporated, resulting in 1.93 g (14.9 mmol, 95% yield) of a 2:5:1 mixture of 5-(1-fluorovinyl)-2-pyrrolidinone, (*Z*)-5-(2-fluorovinyl)-2-pyrrolidinone, and (*E*)-5-(2-fluorovinyl)-2-pyrrolidinone.[1] The mixture was column chromatographed, 100 mg at a time, using 15 g of TLC grade silica and a 5:1 ethyl acetate/hexane solvent system. The three compounds have only slight differences in their elution rates, with R_f

(8) Nanavati, S. M.; Silverman, R. B. *J. Am. Chem. Soc.* **1991**, *113*, 9341−9349.

(9) Silverman, R. B.; Abeles, R. H. *Biochemistry* **1976**, *15*, 4718−4723.

(10) Burke, J. R.; Silverman, R. B. *J. Am. Chem. Soc.* **1991**, *113*, 9329−9340.

values approximately 0.01 unit apart and centered at 0.30. The middle spot of the three is (*Z*)-5-(2-fluorovinyl)-2-pyrrolidinone. After several columns, first on the mixture of the three compounds and then on the fractions eluting from those columns that also contained mixtures, 258 mg (2.0 mmol, 22% yield) of pure (*Z*)-5-(2-fluorovinyl)-2-pyrrolidinone was isolated as a light yellow oil: ^{1}H NMR ($CDCl_3$) δ 6.5 (dd, 1 H, CHF, $J_{HH} = 5$, $J_{HF} = 83$), 4.86 (ddd, 1 H, CHCF, $J_{HHcis} = 5$, $J_{FH} = 40$, $J_{HH} = 9$), 4.7 (m, 1 H, CHN), 2.4 and 1.8 (m, 3 H, 1 H, CH_2CH_2); ^{19}F NMR ($CDCl_3$) δ −127 (dd, $J_{FH} = 83$, $J_{FHtrans} = 40$).

After prolonged storage in a desiccator at −20 °C, the (*Z*)-5-(2-fluorovinyl)-2-pyrrolidinone was dissolved in ethyl acetate. A brown solid precipitated, which was removed by filtration. Most of the solvent was evaporated, and the remaining solution was purified over a 15 g TLC grade silica gel column with a 5:1 ethyl acetate/hexane solvent system, which yielded 116 mg of (*Z*)-5-(2-fluorovinyl)-2-pyrrolidinone as a yellow oil.

(*E*)-5-(2-Fluorovinyl)-2-pyrrolidinone. The compound with the lowest R_f of the three described for the synthesis of (*Z*)-5-(2-fluorovinyl)-2-pyrrolidinone is (*E*)-5-(2-fluorovinyl)-2-pyrrolidinone. After several 15 g TLC grade silica gel columns were run, again first on 100 mg of the mixture of the three compounds and then on those fractions containing mixtures of the *Z*- and *E*-isomers, 118 mg (0.91 mmol, 49% yield) of pure (*E*)-5-(2-fluorovinyl)-2-pyrrolidinone was isolated as a light yellow solid: ^{1}H NMR ($CDCl_3$) δ 6.7 (dd, 1 H, CHF, $J_{HH} = 11$, $J_{FH} = 82$), 5.4 (ddd, 1 H, CHCF, $J_{HHtrans} = 11$, $J_{HF} = 17$, $J_{HH} = 9$), 4.1 (m, 1 H, CHN), 2.4 and 1.9 (m, 3 H, 1 H, CH_2CH_2); ^{19}F NMR ($CDCl_3$) δ −128 (dd, $J_{FH} = 82$, $J_{FHcis} = 17$); high-resolution electron impact mass spectrometry calcd for $C_6H_8FNO_2$ 129.0590, found 129.0587.

After prolonged storage in a desiccator at −20 °C, the (*E*)-5-(2-fluorovinyl)-2-pyrrolidinone was dissolved in ethyl acetate. A resulting precipitate was removed by filtration and, after evaporation of most of the solvent, the remaining lactam was purified over a 15 g TLC grade silica gel column utilizing a 5:1 mixture of ethyl acetate/hexane as solvent, which resulted in 62 mg of (*E*)-5-(2-fluorovinyl)-2-pyrrolidinone as a white solid.

(*Z*)-4-Amino-6-fluoro-5-hexenoic acid (2). To 46 mg (0.36 mmol) of (*Z*)-5-(2-fluorovinyl)-2-pyrrolidinone was added 3.5 mL of 1 N HCl. The solution was stirred at 95 °C for 20 h. The solvent was evaporated, and the compound was washed twice with 3 mL of deionized water. The product, in 1 mL of water, was applied to a 5 × 70 mm Dowex 50 column. The column was washed with 5 mL of deionized water, and then the product was eluted with 1 N NH_4OH. Fractions of 1 mL were collected. Ninhydrin-positive fractions were combined, and the solvent was removed by lyophilization, yielding 41.9 mg (0.28 mmol, 79% yield) of **2** as a white solid: ^{1}H NMR (D_2O) δ 6.82 (dd, 1 H, CHF, $J_{HF} = 83$, $J_{HH} = 5$), 4.99 (ddd, 1 H, CHCF, $J_{HHcis} = 5$, $J_{HH} = 10$, $J_{FH} = 40$), 4.31 (m, 1 H, CHN), 2.3 (m, 2 H, CH_2CO), 2.1 (m, 1 H, C*H*CHN), 1.8 (m, 1 H, C*H*CHN); ^{19}F NMR (D_2O) δ −118 (dd, $J_{FH} = 83$, $J_{FHtrans} = 40$); high-resolution electron impact mass spectrometry calcd for $C_6H_{10}FNO_2$ 147.0696, found 147.0681.

(*E*)-4-Amino-6-fluoro-5-hexenoic acid (3). To 42 mg (0.33 mmol) of (*E*)-5-(2-fluorovinyl)-2-pyrrolidinone was added 3 mL of 1 N HCl. The solution was stirred at 90 °C for 20 h. The solvent was evaporated and the product purified on a 5 × 70 mm Dowex 50 column as described in the synthesis of **2**. Ninhydrin-positive fractions were combined, and the solvent was evaporated, leaving 41.3 mg (0.28 mmol, 85% yield) of **3** as a white solid: ^{1}H NMR (D_2O) δ 6.92 (dd, 1 H, CHF, $J_{FH} = 81$, $J_{HH} = 11$), 5.39 (ddd, 1 H, CHCF, $J_{FH} = 17$, $J_{HH} = 11$ $J_{HH} = 11$), 3.82 (m, 1 H, CHN), 2.4 (m, 2 H, CH_2CO), 2.1 (m, 1 H, C*H*CHN), 1.9 (m, 1 H, C*H*CHN); ^{19}F NMR (D_2O) δ −119 (dd, $J_{FH} = 81$, $J_{FHcis} = 17$); high-resolution fast atom bombardment mass spectrometry calcd for $C_6H_{11}FNO_2$ 148.0774, found 148.0789. Anal. Calcd for $C_6H_{10}NO_2F$: C, 48.97; H, 6.85; N, 9.52. Found: C, 48.40; H, 6.78; N, 9.44.

[^{3}H]-(*Z*)-5-(2-Fluorovinyl)-2-pyrrolidinone. To 23.2 mg (0.18 mmol) of (*Z*)-5-(2-fluorovinyl)-2-pyrrolidinone under argon was added 3 mL of THF followed by 7 μL of ^3H_2O (5 Ci/mL, corrected for decay, 0.36 mmol, 2 equiv) and then 0.55 mL of 1.0Mpotassium *tert*-butoxide in THF (0.55 mmol, 3 equiv). The mixture was stirred under argon for 10 days. Another 7 μL of ^3H_2O was added to quench the reaction, and, after 2 h of stirring, 2 drops of glacial acetic acid was added. The solvents were removed by bulb-to-bulb distillation in a closed evacuated system; about 2 mL of water was added, and bulb-to-bulb distillation was repeated three times, yielding 10.0 mg (0.08 mmol, 43% yield) of a yellow solid that comigrated with unlabeled (*Z*)-5-(2-fluorovinyl)-2-pyrrolidinone by TLC with ethyl acetate as the mobile phase.

[^{3}H]-(*E*)-5-(2-Fluorovinyl)-2-pyrrolidinone. To 20.9 mg (0.16 mmol) of [^{3}H]-(*E*)-5-(2-fluorovinyl)-2-pyrrolidinone under argon was added 3 mL of THF followed by 6 μL of ^3H_2O (5 Ci/mL, corrected for decay, 0.33 mmol, 2 equiv) and 0.5 mL of 1.0 M potassium *tert*-butoxide in THF. The reaction was stirred under argon for 11 days, at which time 6 μL of ^3H_2O was added and stirring continued for 1 h. To completely quench the reaction, 2 drops of glacial acetic acid was added. A precipitate was removed by filtration and was rinsed well with THF. The THF was removed in an evacuated bulb-to-bulb distillation system. About 2 mL of water was added, and bulb-to-bulb distillation was repeated three times. The reaction yielded 12.3 mg (0.095 mmol, 59% yield) of a yellow powder which comigrated with unlabeled (*E*)-5-(2-fluorovinyl)-2-pyrrolidinone on a TLC plate using ethyl acetate as the mobile phase.

[^{3}H]-(*Z*)-4-Amino-6-fluoro-5-hexenoic acid ([^{3}H]-2). To 10.0 mg (0.08 mmol) of [^{3}H]-(*Z*)-5-(2-fluorovinyl)-2-pyrrolidinone was added approximately 4 mL of 1 N HCl. The solution was stirred at 92 °C for 20 h. The solvent was evaporated, and the product, in 1 mL of water, was purified over a 5 × 70 mm Dowex 50 column. The column was washed with 5 mL of water, the amino acid was eluted with 1 N NH_4OH, and fractions of 1 mL were collected. Those fractions containing radioactivity were combined. The solvent was removed by rotary evaporation, leaving 1.9 mg (0.013 mmol, 16% yield) of a light brown solid, which comigrated with unlabeled **2** on TLC using a 3:1:1 *n*-butanol/HOAc/H_2O solvent system. Approximately 4 mg of unlabeled **2** was added to this compound. The diluted [^{3}H]-**2** had a specific activity of 3.1 mCi/mmol and was 97% radiopure.

[^{3}H]-(*E*)-4-Amino-6-fluoro-5-hexenoic acid ([^{3}H]-3). Approximately 4 mL of 1 N HCl was added to 12.3 mg (0.084 mmol) of [^{3}H]-(*E*)-5-(2-fluorovinyl)-2-pyrrolidinone, and the solution was stirred at 93 °C for 19 h. The solvents were evaporated, and the product, in 1 mL of water, was purified by Dowex 50 (5 × 70 mm) ion exchange chromatography (5 × 70 mm). The column was washed with 5 mL of water, and then the product was eluted with 1 N NH_4OH. Fractions of 1 mL were collected. Those fractions containing radioactivity were freeze-dried, yielding 7.1 mg (0.048 mmol, 51% yield) of brown solid, which comigrated with unlabeled **3** using a 3:1:1 *n*-butanol/HOAc/H_2O solvent system. About 8 mg of unlabeled **3** was added, resulting in [^{3}H]-**3** which had a specific activity of 11.3 mCi/mmol and was 99% radiopure.

Enzymes and Assays. All of the enzymes and assays used were described in the preceding paper.[1]

Kinetics of Inactivation of GABA Aminotransferase by 2 and 3. The procedure described in the preceding paper[1] for 4-amino-5-fluoro-5-hexenoic acid was followed using 0.01, 0.02, 0.05, 0.1, 0.2, and 0.5 mM of inactivators.

Inactivation of [^{3}H]PLP Reconstituted GABA-Aminotransferase by 2 and 3 without Removal of Excess Inactivator. This was done as described for 4-amino-5-fluoro-5-hexenoic acid in the preceding paper.[1]

Schiff Base Adduct Formed between PLP and 2 or 3. This was done as described for 4-amino-5-fluoro-5-hexenoic acid in the preceding paper.[1]

Inactivation of GABA Aminotransferase by [^{3}H]-2 and [^{3}H]-3. In a typical experiment, GABA aminotransferase (5.7 nmol) was incubated in a total volume of 390 μL with 2.0 mM [^{3}H]-**2** or [^{3}H]-**3**, 2.0 mM α-ketoglutarate, and 5 mM β-mercaptoethanol in 100 mM potassium phosphate, pH 7.4, for 6 h. After the enzyme solutions were less than 3% active, they were applied to Sephadex G-50 columns and eluted using the Penefsky spin method[11] with 100 μL of 100 mM potassium phosphate, pH 7.4, used to rinse the columns. This enzyme solution was then used in the various experiments described below.

Equivalents of [^{3}H]-2 and [^{3}H]-3 Bound to GABA Aminotransferase after Inactivation. This was done as described for 4-amino-5-fluoro-5-hexenoic acid in the preceding paper.[1]

(11) Penefsky, H. S. *Methods Enzymol.* **1979**, *56*, 527−530.

Equivalents of [^{3}H]-2 and [^{3}H]-3 Bound to GABA Aminotransferase after Inactivation and Denaturation with Urea, pH 7.0. This was done as described for 4-amino-5-fluoro-5-hexenoic acid in the preceding paper.[1]

Equivalents of [^{3}H]-2 and [^{3}H]-3 Bound to GABA Aminotransferase after Inactivation and Denaturation with 8 M Urea, pH 2.4. GABA aminotransferase was inactivated and purified over Sephadex G-50 as described above. Approximately 450 μL of the enzyme solutions was dialyzed against 500 mL of 8 M urea, pH 2.4, for 4 h and then another 500 mL of 8 M urea, pH 2.4 for 12 h. A protein assay using BSA standards in 8 M urea, pH 2.4, was run on the dialyzed enzyme solutions, and a 300 μL aliquot was counted by liquid scintillation counting.

Equivalents of [^{3}H]-2 and [^{3}H]-3 Bound to GABA Aminotransferase after Inactivation and Denaturation with Trifluoroacetic Acid. GABA aminotransferase was inactivated and purified over Sephadex G-50 as described above. A 135 μL portion of the inactivated enzyme sample was added to 15 μL of trifluoroacetic acid (TFA). These solutions were mixed well and then were incubated at room temperature for 5 min. The enzyme that had precipitated was removed by microcentrifugation. The supernatants were separated, and the pellets were rinsed three times with deionized water. Microcentrifugation was repeated after each rinse. A 200 μL aliquot of 2 M potassium hydroxide was added to each pellet. These were vortexed several times and allowed to dissolve for 2 h. An equivalents bound determination was done by first performing a protein assay using BSA in 2 M KOH as the protein standard and then counting 100 μL of the enzyme solution by liquid scintillation counting. Equivalents of inactivator remaining bound to the enzyme were determined by the ratio of moles of inactivator present, based on the specific activity of the inactivator, to the moles of active enzyme used.

Identification of Products Released by Urea Denaturation of [^{3}H]-2- and [^{3}H]-3-Inactivated GABA Aminotransferase. GABA aminotransferase was inactivated with [^{3}H]-**2** or [^{3}H]-**3** and purified over Sephadex G-50[11] as described above. Each inactivated enzyme solution (250 μL) was dialyzed against 4 mL of 6 M urea containing 21 mM β-mercaptoethanol and 50 mM potassium phosphate, pH 7.0, buffer for 18 h. Released products were analyzed by HPLC (Alltech Econosil C_{18} column; 4.6 $\times$ 250 mm, 10 μm). The sample was eluted with a 0.1% trifluoroacetic acid in a water/methanol gradient (0% MeOH for 20 min followed by an increase to 15% MeOH over the next 15 min, elution at 15% methanol was continued for 10 min, and then 100% MeOH over the next 2 min) at a flow rate of 0.5 mL/min for the first 20 min and then 1.0 mL/min for the next 55 min with detection at 214 nm.

Analysis of Amine Metabolites Formed during Inactivation of GABA Aminotransferase by [^{3}H]-2 and [^{3}H]-3. GABA aminotransferase was inactivated as described under Inactivation of GABA Aminotransferase by [^{3}H]-**2** and [^{3}H]-**3** except for the purification over Sephadex G-50. One-fourth of this solution was applied to a Centricon 30 and centrifuged using a JA-20 rotor at 5000 rpm for 5 min. The Centricon was rinsed three times with 200 μL of deionized water, followed each time by centrifugation. The final spin was for 10 min to get all of the liquid through the membrane. The filtrate was applied to a prewashed 5 $\times$ 70 mm Dowex 50 column. The column was washed with 5.5 mL of water and then eluted with 10 mL of 1.5 N HCl. The acidic elution solution was freeze-dried. Amine metabolites were analyzed by dissolving the solid in 125 μL of 40 mM Li_2CO_3 and adding 100 μL of that solution to a micro-test tube. The same amount of 20 mM dansyl chloride in acetonitrile was added, and the mixture was stirred at room temperature for 1 h, protected from light. The entire reaction mixture was then injected into the HPLC equipped with an Alltech Econosil C_{18}, 10 μm 4.6 $\times$ 250 mm column. The flow rate was 1.0 mL/min, and a solvent system consisting of 80% H_2O/20% methanol, containing 0.6% acetic acid and 0.03% triethylamine, was used. After 2 min, a 60 min gradient into 100% methanol was begun. Fractions were collected every 2 min for 70 min and counted by liquid scintillation counting.

Analysis of Nonamine Metabolites Formed during Inactivation of GABA Aminotransferase by [^{3}H]-2 and [^{3}H]-3. This was done as described for 4-amino-5-fluoro-5-hexenoic acid in the preceding paper.[1]

Transamination Events per Inactivation of GABA Aminotransferase by 2 and 3. This was done as described for 4-amino-5-fluoro-5-hexenoic acid in the preceding paper except using 2.0 mM **2** and **3**.[1]

Analysis of Fluoride Ion Release during Inactivation of GABA Aminotransferase by 2 and 3. This was done as described for 4-amino-5-fluoro-5-hexenoic acid in the preceding paper except using 2.0 mM **2** and **3**.[1]

UV−vis Spectrometry of Covalent Adduct Formed between GABA Aminotransferase and 2 and 3. GABA aminotransferase (6.0 nmol) in a solution containing 2 mM **2** or **3**, 2 mM α-ketoglutarate, 5 mM β-mercaptoethanol, and 100 mM potassium phosphate, pH 7.4, in a total volume of 450 μL was incubated at room temperature. A third inactivation was done under the same conditions using 2 mM 4-amino-5-fluoropentanoic acid (**35**) in place of **2** or **3**. A control sample of enzyme containing no inactivator also was incubated. After 5 h, the inactivating enzyme samples were >95% inactivated. Each was transferred to a cuvette, and UV−vis scans were run in the visible region from 350 to 700 nm.

Acknowledgment. We are grateful to the National Institutes of Health (NS15703) for financial support of this research.

JA952789X