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Inactivation of γ-aminobutyric acid aminotransferase by (*S*)-4-amino-4,5-dihydro-2-furancarboxylic acid does not proceed by the expected aromatization mechanism

Mengmeng Fu[†] and Richard B. Silverman*

Department of Chemistry and Department of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Evanston, IL 60208-3113, USA

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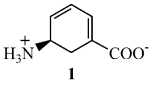
Abstract—Inactivation of pyridoxal 5′-phosphate (PLP)-dependent γ-aminobutryic acid aminotransferase by (*S*)-4-amino-4,5-dihydro-2-furancarboxylic acid (SADFA) gives pyridoxamine 5′-phosphate, not the expected SADFA-PLP aromatization product. Inactivation appears to proceed by a Michael addition/hydrolysis mechanism instead.

The natural product gabaculine (5-amino-1,3-cyclohexadienylcarboxylic acid, **1**) is a highly 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.[1] Inactivation of GABA-AT causes a rise in the concentration of GABA in the brain,[2] which results in an anticonvulsant effect. However, gabaculine is too toxic for medical use, probably because of its reactivity with a variety of other enzymes.[3] Mechanistic studies by Rando and co-workers support an aromatization mechanism for gabaculine, leading to the formation of a modified PLP cofactor (**2**, Scheme 1).[4,5]



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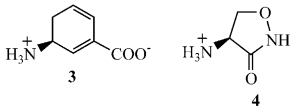
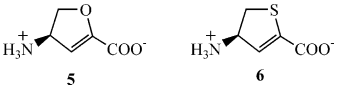
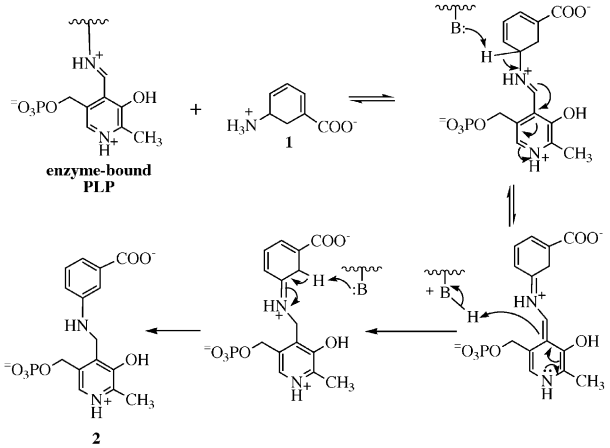


3 **4**



5 **6**

The structure of the PLP-gabaculine adduct was characterized fully.[6] Since the proposed aromatization mechanism for gabaculine was published, there have been only three other compounds that have been proposed to cause inactivation of any PLP-dependent enzyme via an aromatization pathway. One is isogabaculine (**3**), a tautomer of gabaculine, but no experimental support has been provided to confirm the



Scheme 1. Mechanism of inactivation of GABA-AT by gabaculine.

* Corresponding author. Tel.: +1-847-491-5653; fax: +1-847-491-7713; e-mail: agman@chem.northwestern.edu; URL: http://www.chem.northwestern.edu/~agman/index.htm
† Current address: Biotechnology Core Facility, Centers for Disease Control and Prevention, Atlanta, GA 30333, USA.

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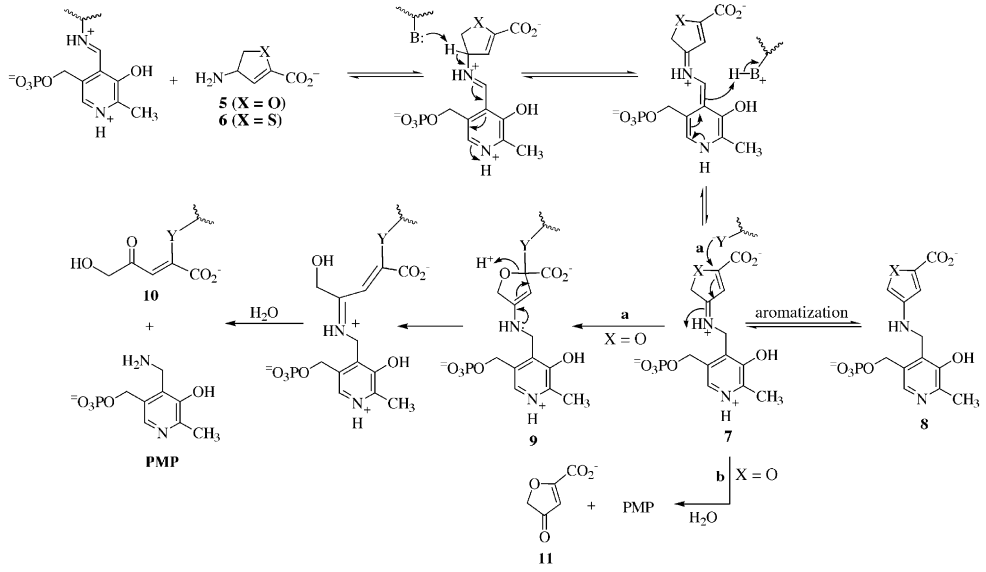

Scheme 2. Proposed mechanisms of inactivation of GABA-AT by **5** and **6**.

mechanism.[7] Another compound shown to inactivate GABA-AT by an aromatization mechanism is the natural product cycloserine (**4**).[8] In the mid-1980s, two dihydroheteroaromatic analogues of gabaculine, (*S*)-4-amino-4,5-dihydro-2-furancarboxylic acid (**5**, SADFA)[9] and (*S*)-4-amino-4,5-dihydro-2-thiophenecarboxylic acid (**6**, SADTA)[10] were designed and synthesized as potential irreversible inactivators of GABA-AT by the corresponding aromatization mechanism (Scheme 2). Both gabaculine analogues are highly potent, irreversible inactivators of GABA-AT, and, therefore, it appeared reasonable, given their structural similarity to gabaculine, that both **5** and **6** also could inactivate the enzyme by an aromatization mechanism, although no evidence for that assumption was provided in the original work. We were able to demonstrate that **6** does, in fact, inactivate GABA-AT by an aromatization mechanism;[11] the modified SADTA–PLP adduct (**8**, X = S; Scheme 2) was isolated and characterized. Here we report a study of the mechanism of inactivation of GABA aminotransferase by the dihydrofuran **5** and show that it does *not* follow the assumed aromatization mechanism.

Inactivation of GABA-AT by **5** was concentration and time-dependent; the K_I and k_{inact} values were determined by the method of Kitz and Wilson[12] to be 6.3 μM and 2.4 min^{-1}, respectively, at pH 7.4 and 0 °C (Fig. 1). The kinetic analysis had to be run at 0 °C because the rate was too fast to monitor at 25 °C. Less than 2% of the enzyme activity was detected after fast gel filtration or dialysis at 4 °C of the **5**-inactivated enzyme, while the control enzyme remained fully active. Compound **5**, therefore, irreversibly inactivates purified pig brain GABA-AT. The rate of inactivation by **5** is diminished in the presence of GABA, which suggests a competitive inhibition. When the enzyme was treated with an increasing number of equivalents of **5** at pH 7.4 and allowed to incubate until no further change in activity was observed (about 160 h), then the percentage of enzyme activity remaining plotted against the number of equiv of **5** used, the intercept at zero enzyme activity was 4.7 equiv. This value represents the turnover number (the number of molecules of **5** acted on per active site), which indicates that the partition ratio (the number of molecules of product generated per inactivation event) is 3.7. This is consistent with the number of transamination events per inactivation (3.2 ± 0.2), as determined by measuring the conversion of [5-^{14}C]-α-ketoglutarate to [5-^{14}C]glutamate during inactivation by **5**. However, when the determination of the number of equivalents of **5** was repeated at pH 6.5, a turnover number of 15.1 (partition ratio of 14.1) was measured. This suggests a change in partitioning between product formation and inactivation as a function of pH (vida infra).

Inactivation of [^{3}H]PLP-reconstituted GABA-AT[8] with **5**, denaturation and hydrolysis by potassium hydroxide, and reversed-phase HPLC analysis (Fig. 2) showed that all of the radioactivity comigrates with pyridoxamine 5′-phosphate (PMP); the protein contained <1% of tri-

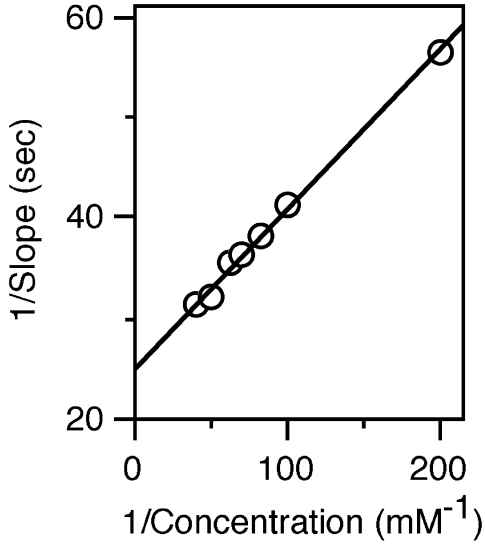

Figure 1. Kitz and Wilson plot for inactivation of GABA-AT by SADFA in 100 mM potassium phosphate buffer, pH 7.4 and 0 °C.

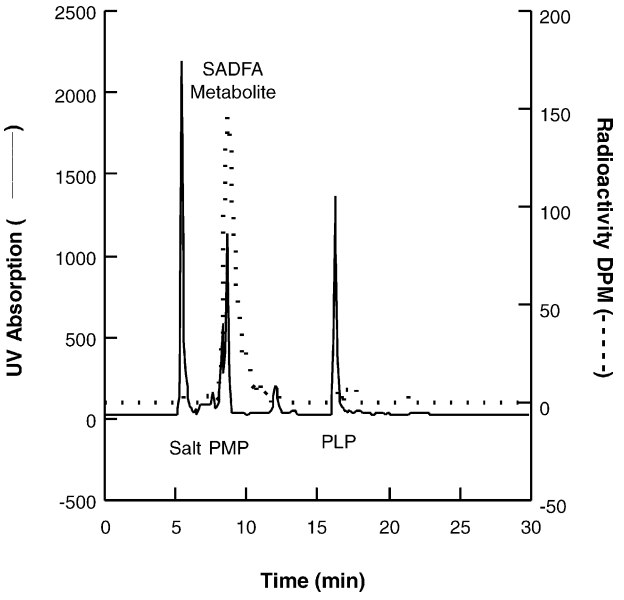

Figure 2. Revised-phase HPLC analysis of the product isolated from [^{3}H]PLP-GABA-AT inactivated by SADFA: radioactivity and absorption are plotted versus retention time of the standards and [^{3}H] labeled product. The absorption peaks correspond to the standards: PMP (8 min), PLP (16 min).

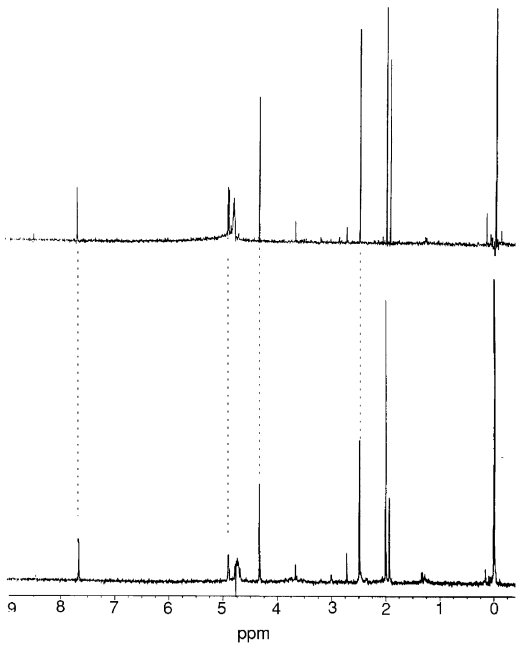

Figure 3. ^{1}H NMR spectra of PMP and the purified SADFA inactivation product.

tium. When trifluoroacetic acid was the denaturant and DEAE ion exchange HPLC was carried out, again, the only radioactive peak corresponded to that of the PMP standard (data not shown). The UV–visible spectrum of this product peak (using non-tritiated PLP) corresponded to that for standard PMP. Positive-ion mode electrospray ionization mass spectrometry gave an $[M+H]^+$ peak at m/z 249, as does the standard PMP: MS/MS of the m/z 249 peak produced three daughter ion peaks at m/z 151, 134, and 106 (as does standard PMP), corresponding to the loss of the phosphate group (151), then the ammonium group (134), and two methylene groups (106). The ^{1}H NMR spectrum (D_2O, 600 MHz) of the purified product corresponds to the NMR spectrum of standard PMP carried through the same procedure as the product from the inactivation (Fig. 3).

The identification of PMP as the product of denaturation of GABA-AT inactivated by **5** is inconsistent with the aromatization mechanism (Scheme 2), which, should have produced the condensed heteroaromatic-coenzyme adduct (**8**, X = O). PMP could arise by normal transamination to intermediate **7** (X = O), followed either by Michael addition to give **9**, then hydrolysis to PMP and **10** (pathway a) or by direct hydrolysis of **7** (pathway b) (Scheme 2). Because of the much lower aromatic stabilization energy of furans (16 kcal/mol) relative to benzenes (36 kcal/mol),[13] **8** (X = O) may not be favored.

An experiment to try to differentiate pathways a and b was carried out. Pathway b produces GABA-AT in the reduced (PMP) form. Incubation of that form of the enzyme with PLP should lead to exchange of PMP for PLP and rapid reactivation of the enzyme, whereas incubation of the modified enzyme from pathway a with PLP may not produce reactivation. Three control experiments were run simultaneously. In one control (**A**), **5** was omitted with no addition of GABA (this leaves the enzyme unchanged); in another control (**B**), the GABA-AT was reduced with GABA in the absence of α-ketoglutarate (this generates the PMP form of the enzyme); in the last control GABA-AT was inactivated with gabaculine (**C**), known to give a stable modified coenzyme form of the enzyme. Dialysis of all four of these enzyme solutions against 100 mM potassium phosphate buffer, pH 7.4, containing 0.25 mM β-mercaptoethanol and 1 mM PLP at 4 °C gave after 2 h the following results: **A** had full enzyme activity, **B** had 60% activity, **C** showed no enzyme activity, and the **5**-inactivated enzyme was 10% active; after 24 h of dialysis, **A** and **B** were fully active (**B** was fully active after 4 h), **5**-inactivated enzyme was 60% active, and **C** was about 10% active; at 48 h, **A**, **B**, and the **5**-inactivated enzyme were all fully active, but **C** was still $<20\%$ active. This indicates that the structure of **5**-inactivated GABA-AT is different from that of gabaculine-inactivated GABA-AT. The difference between the rate of reactivation with the PMP form of the enzyme (**B**) and with **5**-inactivated enzyme indicates that it is not just the PMP form of the enzyme. If it is assumed that the conversion of **7** (X = O) to PMP and **11** is rapid, which should be the case because hydrolysis of the product-PMP imine is the normal catalytic reaction for this enzyme, then pathway a is supported. The cause for reactivation of the enzyme inactivated via pathway a may be the result of a slow hydrolysis of **10**. Pathway a also is consistent with the observation that the partition ratio is much larger at pH 6.5 than at pH 7.4. Presumably, turnover to product is represented by pathway b; the partition ratio, then, should be the same at both pH values if pathway b is responsible for inactivation. At the higher pH, the active site nucleophile Y^- in pathway a would be more

deprotonated and, therefore, more reactive, leading to more nucleophilic attack and a lower partition ratio than at the lower pH where there should be less of an anionic form of the nucleophile. To differentiate these mechanisms more conclusively, radioactively-labeled **5** needs to be synthesized; however, we have been unsuccessful at this synthesis.

In summary, (*S*)-4-amino-4,5-dihydro-2-furancarboxylic acid (**5**) is a mechanism-based inactivator of GABA aminotransferase, but aromatization of intermediate **7** (X = O) does not appear to be involved in the inactivation, unlike the inactivation of GABA aminotransferase by gabaculine and by **6**. Because of the lower aromatic stabilization energy of furans relative to benzenes, intermediate **7** (X = O) may not undergo aromatization to **8** (X = O), but, rather, is susceptible to Michael addition by an active site residue and hydrolysis.

Acknowledgements

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