Pergamon

Bioorganic & Medicinal Chemistry 7 (1999) 1581–1590

BIOORGANIC & MEDICINAL CHEMISTRY

Isolation and Characterization of the Product of Inactivation of γ-Aminobutyric Acid Aminotransferase by Gabaculine

Mengmeng Fu and Richard B. Silverman*

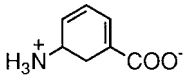
Department of Chemistry and Department of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Evanston, IL 60208-3113, USA

Received 3 December 1998; accepted 2 February 1999

Abstract—Gabaculine (5-amino-1,3-cyclohexadienylcarboxylic acid, **1**), a naturally occurring neurotoxin isolated from *Streptomyces toyocaenis*, has been shown to be a mechanism-based inactivator of γ-aminobutyric acid aminotransferase (GABA-AT) (Rando, R. R. *Biochemistry* **1977**, 16, 4604). Inactivation results from reaction of gabaculine with the pyridoxal 5′-phosphate (PLP) cofactor. Two HPLC systems for isolating this inactivator-PLP adduct are described as well as a detailed characterization of the adduct, including the ultraviolet–visible spectrum, electrospray mass spectra, and NMR spectrum. The same spectral characterization of the chemically synthesized gabaculine-PLP adduct is also reported.

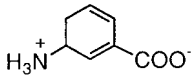
Introduction

Gabaculine (5-amino-1,3-cyclohexadienylcarboxylic acid, **1**) is a naturally occurring neurotoxin isolated from *Streptomyces toyocaenis*.[1] Soon after its isolation, it was found that gabaculine is an exceedingly potent irreversible inactivator of pyridoxal 5′-phosphate (PLP)-dependent mouse brain γ-aminobutyric acid aminotransferase (GABA-AT; EC 2.6.1.19) both in vitro and in vivo.[2] When administered intraperitoneally, brain GABA-AT is rapidly inactivated, and the brain levels of the inhibitory neurotransmitter GABA rise by 15–20-fold, suggesting that gabaculine can cross the blood-brain barrier.[3] Unfortunately, it is too toxic to be useful as a pharmaceutical agent, probably because it inactivates several other enzymes as well.[4] But because of its remarkable properties, gabaculine has since been used as a powerful pharmacological tool in a variety of GABAergic neurotransmission studies.[5–13]



1

Mechanistic studies of the inactivation of bacterial GABA-AT by gabaculine were carried out in the mid-1970s 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 same mechanism and product was proposed in the inactivation of GABA-AT by isogabaculine (**4**).[16]



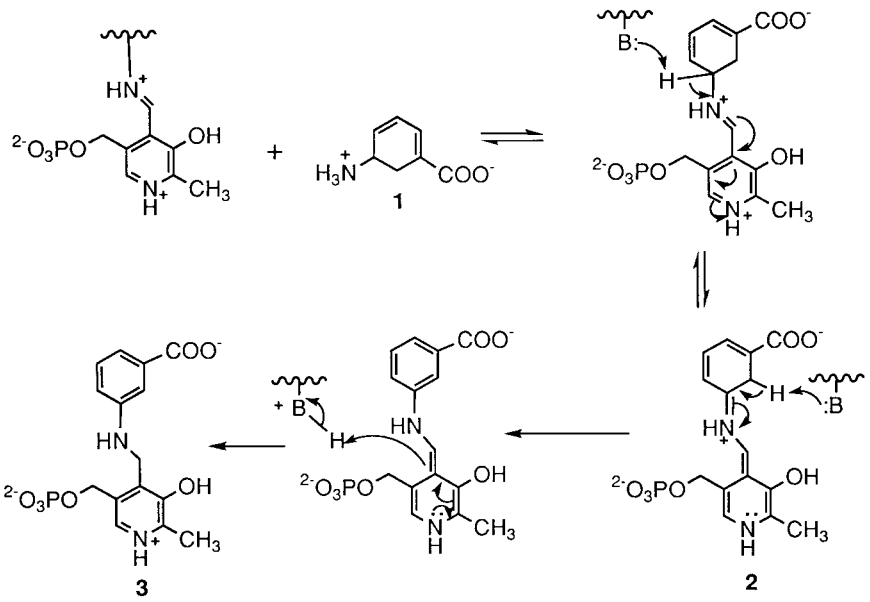
4

It was suggested that β-proton removal is an enzyme-catalyzed event for those PLP enzymes that have a basic group at the active site to catalyze this process.[17] Two related syntheses of the hypothesized modified cofactor (**3**) were carried out; one was the reaction of PLP and gabaculine at elevated temperature, and the other was the reaction of PLP with *m*-anthranilic acid followed by sodium borohydride reduction.[14] On chemical grounds, both of these reactions are expected to give **3**, but the characterizations of these synthetic products consisted of thin-layer chromatography, its absorption and fluorescence spectra, and an elemental analysis. The product of the enzymatic reaction was then compared to the synthetic product by thin-layer chromatography and electrophoresis. We thought that a more complete characterization of the synthetic and enzymatic products was warranted and report here the electrospray mass spectral and NMR spectral analyses of these products, which support the original hypothesis of Rando.[15]

Key words: γ-Aminobutyric acid aminotransferase; gabaculine; pyridoxal 5′-phosphate; electrospray mass spectrometry.

* Corresponding author. Tel.: (847) 491-5653; fax: (847) 491-7713; e-mail: agman@chem.nwu.edu

0968-0896/99/$ - see front matter
PII: S0968-0896(99)00081-4



Scheme 1.

Results

Time-dependent inactivation of GABA-AT by gabaculine

Inactivation of GABA-AT by gabaculine was concentration and time-dependent; K_I and k_{inact} values were determined by the method of Kitz and Wilson[18] to be 29 μM and 6.4 min^{-1}, respectively, at room temperature. The K_I and k_{inact} values at 0°C were determined to be 259 μM and 1.4 min^{-1}, respectively.

Inactivation of [^{3}H]PLP-GABA-AT by gabaculine, product isolation, and analysis by reverse phase HPLC

There is apparently only one major product formed from the inactivation of [^{3}H]PLP-GABA-AT by gabaculine as shown with two different HPLC elution systems ($T_R = 36$ min, Fig. 1A; $T_R = 10.8$ min, Fig. 1B). This product coelutes with synthetic **3**. Less than 1% of the radioactivity was detected in the protein pellet.

UV–visible spectroscopic studies of the purified gabaculine inactivation product

The UV–visible spectrum of the purified gabaculine inactivation product had a maximum at 312 nm (in 2 M NH_4OH) and a shoulder at 245 nm (Fig. 2; —). This is consistent with the UV–visible spectrum of synthetic **3** (80 nmol, Fig. 2; - - - -). From the absorption at 312 nm, it was estimated that, after purification, approximately 62% (50 nmol) of the product was recovered.

Electrospray ionization mass spectral studies of synthetic 3

Negative mode electrospray ionization mass spectrometry (ESIMS$^-$) of **3** showed the expected molecular ions ([M−H]$^-$, $m/z = 367$, Fig. 3). The other prominent peaks in the spectrum could be assigned as in Scheme 2. Positive mode electrospray ionization mass spectrometry

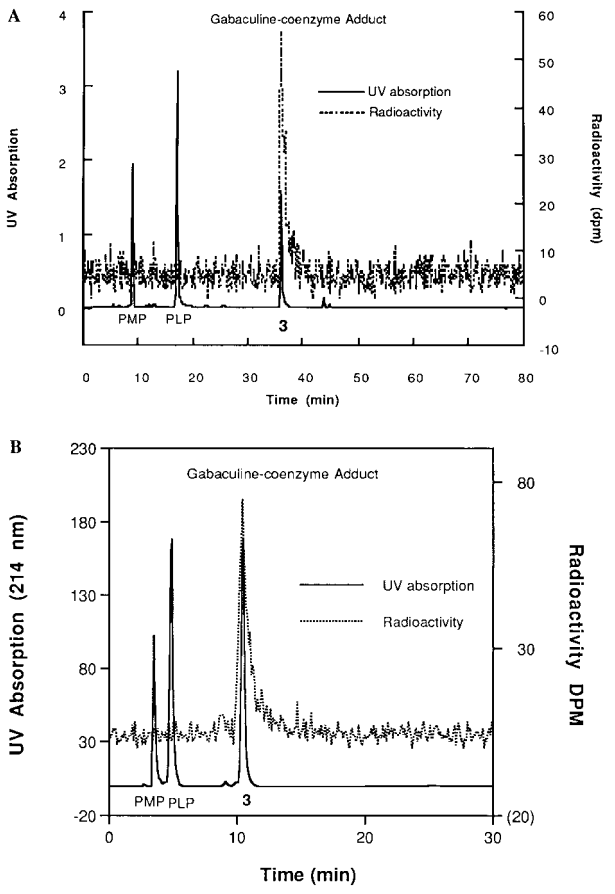


Figure 1. Reverse phase HPLC analysis of the product isolated from [^{3}H]PLP-GABA-AT inactivated by gabaculine. Radioactivity (- - - -) and absorption (—) are plotted versus retention time of the standards and [^{3}H] labeled product. The absorption peaks correspond to the standards: PMP (8 min), PLP (16 min), **3** (36 min) in Figure 1A and PMP (3.5 min), PLP (4.9 min), **3** (10.8 min) in Figure 1B. See solvent system A in the Experimental for details for Figure 1A and solvent system B for Figure 1B.

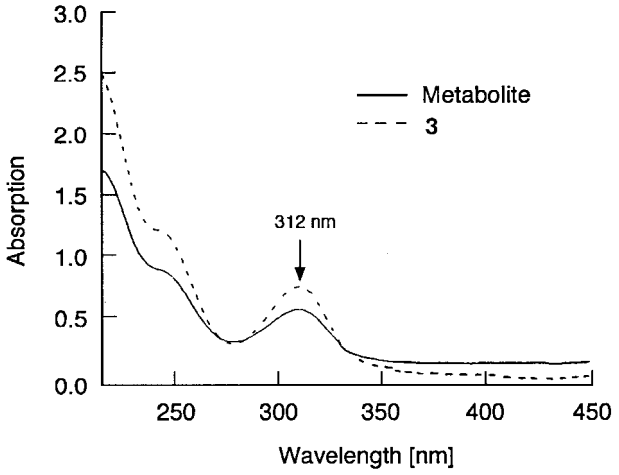


Figure 2. UV–visible spectra of the purified gabaculine inactivation product and synthetic **3**.

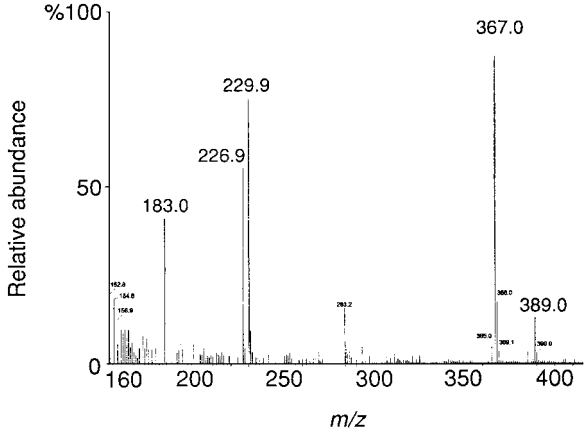


Figure 3. Electrospray ionization mass spectrum (negative mode) of **3**.

(ESIMS$^+$) of **3** also showed the expected molecular ions ($[M+H]^+$, $m/z = 369$, Fig. 4). Attempts to get a MS/MS spectrum from the ESIMS$^-$ peak failed because of the overwhelming HO_3PO^- signal. MS/MS was therefore carried out in the ESIMS$^+$ mode on the peak m/z 369. Two daughter ion peaks were detected (Fig. 5), which could be assigned as in Scheme 3.

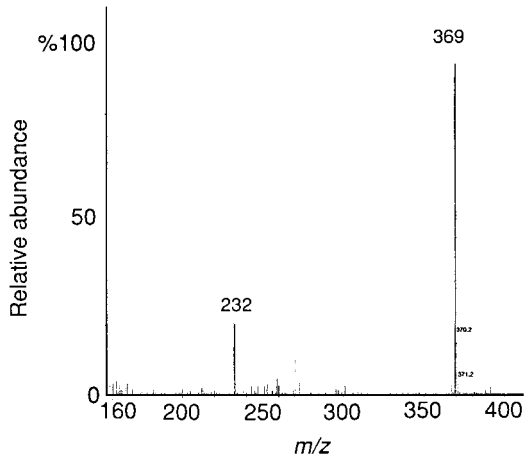


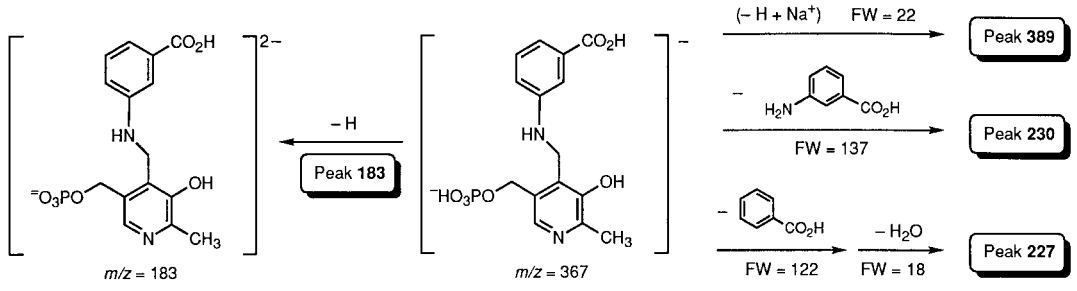
Figure 4. Electrospray ionization mass spectrum (positive mode) of **3**.

Electrospray ionization mass spectral studies of the highly purified gabaculine inactivation product

The ESIMS$^-$, ESIMS$^+$, and MS/MS experiments were performed on the purified gabaculine inactivation product under the same sets of conditions as synthetic **3**. Similar fragmentation patterns from the respective molecular ions were observed (Figs 6–8, respectively).

NMR Studies of 3 and the purified gabaculine inactivation product

The ^{1}H NMR spectra obtained after 4096 scans of both synthetic **3** (carried through the same conditions as the isolation of the gabaculine inactivation product) and the purified gabaculine inactivation product are compared in Figure 9. Despite the small amount of sample used, all of the peaks expected from the ^{1}H NMR spectrum of **3** were observed both in the spectrum of pretreated synthetic **3** and the purified gabaculine inactivation product: (D_2O, 400 MHz) δ 2.51 (3 H, s), 4.58 (2 H, s), 5.08 (2 H, d, $J = 6.8$ Hz), 7.04 (1 H, m), 7.35 (3 H, m), 7.85 (1 H, s). Many of the peaks that are not assigned from both samples have similar coupling constants and chemical shifts, suggesting that these impurities may have been similarly introduced during the purification process. The concentration of the purified gabaculine inactivation product is less than 0.1 mM, and the concentration of the pretreated **3** is about 0.25 mM.



Scheme 2.

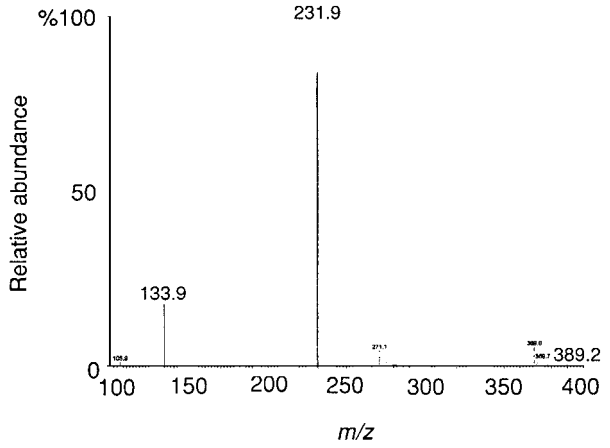


Figure 5. MS/MS of peak m/z 369 from the EIMS$^+$ spectrum of **3**.

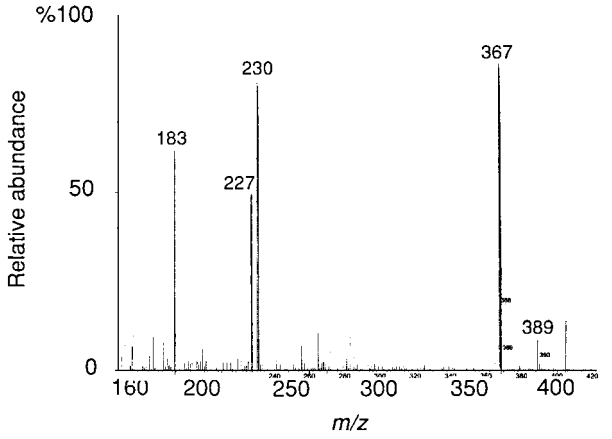


Figure 6. Electrospray ionization mass spectrum (negative mode) of the purified gabaculine inactivation product.

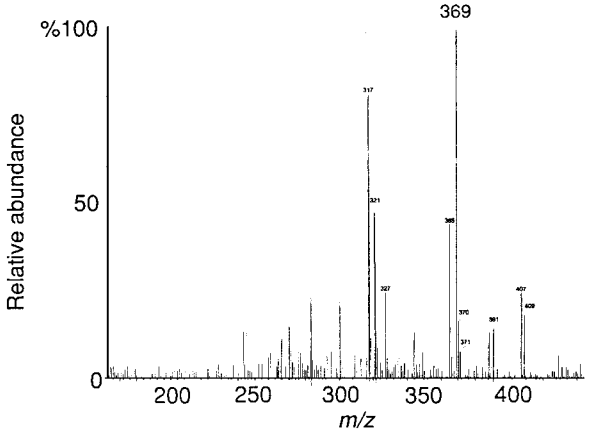


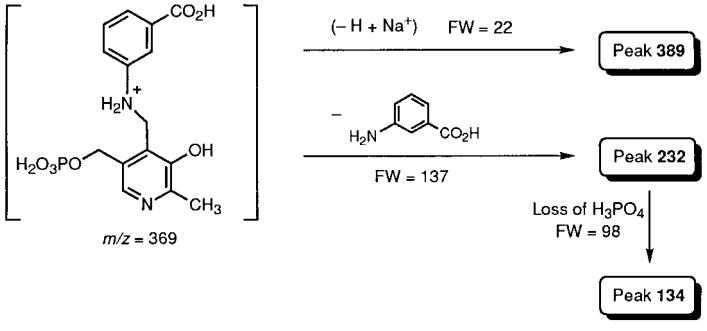
Figure 7. Electrospray ionization mass spectrum (positive mode) of the purified gabaculine inactivation product.

Discussion

Soon after the isolation of gabaculine from *Streptomyces toyocaenis*,[1] an aromatization mechanism (Scheme 1) was proposed by Rando[14,15] to account for the bacterial GABA aminotransferase inactivation by gabaculine. The results of these studies successfully demonstrated the mechanism-based nature of the inactivation of GABA-AT by gabaculine. The conclusion that catalytic conversion of this inhibitor necessarily precedes the observed inactivation was made because (1) gabaculine is only an irreversible inhibitor of the enzyme when the cofactor is in the PLP form; (2) there is a deuterium isotope effect on the rate of the inactivation when a deuterium is incorporated into the 5 position of gabaculine; (3) the pH versus rate of inactivation profile is similar to that of substrate turnover; and (4) external nucleophilic trapping agents, such as β-mercaptoethanol, have no effect on the rate of inactivation.

Inactivation is not the result of modification of an active site residue.[2] When bacterial GABA-AT was inactivated by [2−^{3}H]gabaculine, radioactivity incorporation occurred simultaneously with enzyme inactivation. Denaturation of the radiolabelled enzyme resulted in complete loss of radioactivity from the protein. Intermediate **2** (Scheme 1) is a Michael acceptor and is, in principle, capable of alkylating an active-site residue (Scheme 4), although this does not occur.

The enzymatic formation of *m*-carboxyphenylpyridoxamine phosphate (**3**, Scheme 1) was supported[2] by thin-layer chromatographic and paper electrophoretic behavior. The enzymatic product isolated from denatured [2−^{3}H] gabaculine-inactivated GABA-AT comigrated with the carrier, namely synthetic **3**. Dephosphorylation of the inactivated radiolabelled inactivation product by alkaline phosphatase produced a compound that behaved identically by thin-layer chromatography and paper electrophoresis as synthetic *m*-carboxyphenylpyridoxamine



Scheme 3.

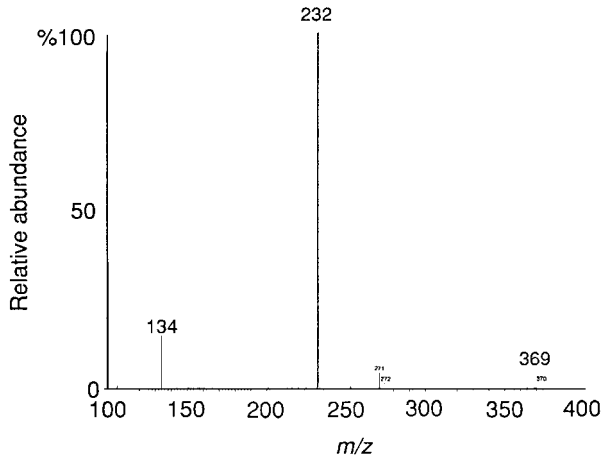


Figure 8. MS/MS of peak m/z 369 from the EIMS$^+$ spectrum of the purified gabaculine inactivation product.

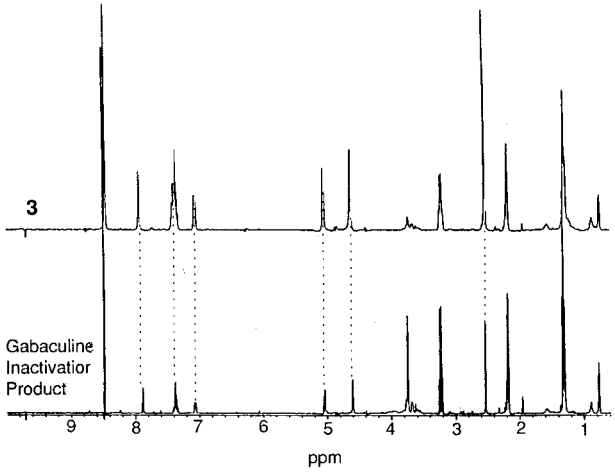


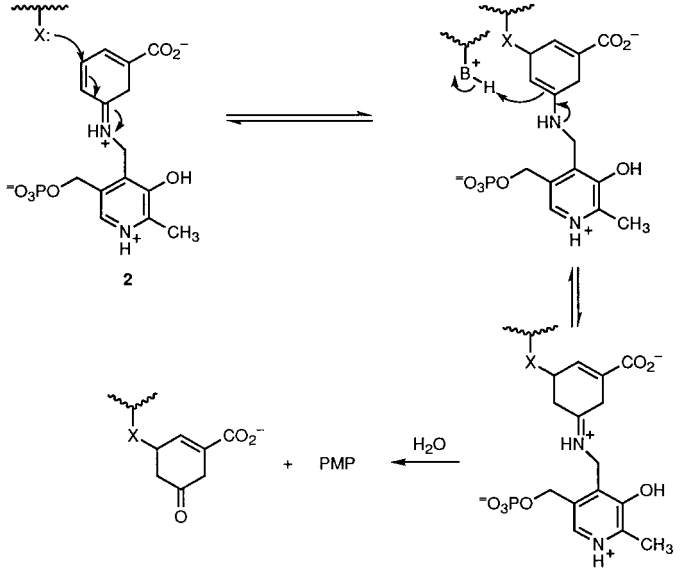
Figure 9. ^{1}H NMR of **3** and the purified gabaculine inactivation product.

(**3** without the phosphate group). To show the formation of only one enzymatic product and to characterize the product of the enzyme-catalyzed reaction more fully, HPLC was used to isolate the product, and the mass spectrum and NMR spectrum are now reported.

As shown by reverse phase HPLC analysis in Figure 1, there is only one radiolabelled product from the inactivation of [^{3}H]PLP-GABA-AT by gabaculine. This radiolabelled product comigrates with synthetic *m*-carboxyphenylpyridoxamine phosphate (**3**) and behaves differently from that of PLP and PMP when analyzed under the same HPLC conditions. This result is consistent with the thin-layer chromatographic and paper electrophoretic studies done by Rando.[2] Further characterizations of both the synthetic and enzymatic products were carried out. After isolation and purification, the single product obtained had a UV–vis spectrum virtually identical to that of synthetic **3** (Fig. 2). A maximum absorption in this range could be an indication of the formation of a pyridoxamine adduct.[19]

Electrospray ionization mass spectrometry (ESIMS) of the gabaculine inactivation product was also carried out (Figs. 6 and 7). Both negative and positive ionization mode spectra were the same as those for the synthetic **3** (Figs. 3 and 4). The most prominent peak in the ESIMS$^+$ of the modified coenzyme (Fig. 7) is the molecular ion with m/z 369, corresponding to **3**. This peak was analyzed by electrospray ionization tandem mass spectrometry (MS/MS) (Fig. 8), and the molecular ion (m/z 369) and the major daughter ions at m/z 232 and 134 also were found in the MS/MS of synthetic **3** (Fig. 5). The possible fragmentation sites are shown in Scheme 3; all are consistent with the proposed product structure **3**.

Further support for structure **3** for the inactivation product was gathered by proton NMR spectroscopy



Scheme 4.

studies. The NMR spectra for the gabaculine inactivation adduct and for synthetic **3** are identical (Fig. 9).

Additional support for the aromatization mechanism comes from structural studies of ornithine aminotransferase inactivated by gabaculine.[20] These studies show that aromatic–aromatic interactions occur between the enzyme-bound gabaculine molecule and active site residues Tyr-85 and Phe-177. This ornithine aminotransferase-gabaculine complex provides the first structural evidence that the potency of the inhibitor results from energetically favorable aromatic interactions with residues in the active site.

Conclusion

All of the evidence for the structure of the product of inactivation of GABA-AT by gabaculine supports **3**. This strongly supports the aromatization-type mechanism (Scheme 1).

Experimental

Analytical methods

GABA-AT assays and UV–visible 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 done with Pierce Slide-A-Lyzer dialysis cassettes (molecular weight cut-off is 10,000) unless otherwise specified. Radioactivity was determined either with a Radiomatic FLO-ONE\Beta Series A-200 liquid flow scintillation counter or a Packard TRI-CARB 2100TR liquid scintillation analyzer. The scintillation fluids used were Packard ULTIMA-FLO^{TM}M and ULTIMA GOLD. Electrospray ionization mass spectra were acquired on a Micromass Quattro II mass spectrometer (Fisons Instruments, Manchester, UK). 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 down field from tetramethylsilane (TMS) in $CDCl_3$ or DMSO and from sodium 3-(trimethylsilyl)propionate in D_2O. Coupling constants are reported in Hertz. Melting points were determined on a Mel-Temp capillary tube melting point apparatus and are uncorrected. A Beckman Microfuge B was used for microcentrifugations and an IEC clinical centrifuge was used for the Penefsky spin method. Cavitator ultrasonic cleaner from Mettler Electronics Corp. was used for ultrasonications. Column chromatography was done with Whatman DE52 (a pre-swollen microgranular anion exchanger: diethylamino ethyl cellulose). 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 μm, or semi-prep Econosil 10 μm 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). 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.

Reagents

All reagents were purchased from Sigma Chemical Co. except the following: trifluoroacetic acid (TFA), 3-aminobenzoic acid, sodium borohydride, amyl alcohol, diethylamine, and all of the NMR solvents were purchased from Aldrich; Centricon 10 microconcentrators were purchased from Amicon; HPLC mobile phases and organic solvents were purchased from Fisher; unpacked chromatography columns for Penefsky spin method,[21,22] Dowex 50 and Dowex 1 resins, sodium dodecyl sulfate, were purchased from Bio-Rad; ultrapure urea was a product of ICN Biomedicals; [^{3}H]sodium borohydride was obtained from Amersham. All of the reagents used for gel electrophoresis were purchased from Bio-Rad. Pig brains were purchased from American Meat Protein Corporation (Ames, IA). All of the buffers and solvents used for HPLC or FPLC analyses were filtered through Gelman 0.45 μ membranes. Buffer A (100 mM potassium phosphate buffer, pH 7.4, containing 0.25 mM β-mercaptoethanol) was used in most of the enzyme incubations. Water used in enzyme related experiments is deionized and doubly distilled.

Synthesis of *N*-*m*-carboxyphenylpyridoxamine phosphate (3)

N-*m*-Carboxyphenylpyridoxamine phosphate (**3**) was prepared according to a modification of the method of Iskander et al.[18] Thus PLP (4.94 g, 20 mmol) was dissolved in dry methanol (80 mL, distilled over magnesium with iodine under nitrogen directly before use) containing KOH (2.24 g, 40 mmol). To the resulting yellow solution, a mixture of 3-aminobenzoic acid (2.74 g, 20 mmol) in dry methanol (80 mL) containing KOH (1.12 g, 20 mmol) was added dropwise. The mixture turned into a bright reddish-orange solution. After the addition, the reaction mixture was stirred for an additional 30 min at room temperature, then sodium borohydride (0.76 g, 20 mmol) was added portionwise at 0–5°C. The reaction mixture was stirred for a further 20 min, filtered, the filtrate acidified with glacial acetic acid to pH 6 and concentrated in vacuum to dryness. The solid was dissolved in water (5 mL) and loaded onto a 2×50 cm Whatman DE 52 resin packed column (prewashed and equilibrated with the eluting buffer). The compound was eluted with an aqueous solution containing 0.3 M acetic acid and 0.3 M pyridine. Fractions of 10 mL were collected and checked by TLC (acetone/*t*-amyl alcohol/water/diethylamine, 3/4/2/1, silica gel).

The fractions with Rf of 0.51 were combined and cooled to 0°C. The white needle-shaped crystals formed were filtered and washed with the recrystallization buffer. After being vacuum dried, the product (**3**) was obtained in 30% yield (2.4 g). The UV–visible spectrum had a maximum at 312 nm (in 2 M NH_4OH); ^1H NMR (DMSO, 300 MHz) δ 2.41 (3 H, s), 4.32 (2 H, s), 4.96 (2 H, d, *J* 6.8 Hz), 6.87–6.90 (1 H, m), 7.18–7.20 (2 H, m), 7.26 (1 H, d, *J* 1.16 Hz), 8.00 (1 H, s).

Synthesis of tritiated PLP ([4−^{3}H]PLP)

Tritiated PLP ([4-^{3}H] PLP) was synthesized using a variation of the method of Stock et al.[23] PLP (140 mg, 0.569 mmol) was dissolved in 0.5 M sodium hydroxide (2.5 mL). The solution was protected from light and cooled to 0°C. [^{3}H]Sodium borohydride from Amersham (100 μCi, 15 Ci/mmol) was dissolved in 0.3 M solution of sodium borohydride in 0.1 M NaOH (450 μL). This solution was then added to the PLP solution prepared above and stirred for 40 min at 0°C. The reaction was monitored by following the 390 nm absorption change of PLP (UV–visible scan of an aliquot of the reaction mixture in 500 μL of 2 M NH_4OH). Further sodium borohydride (total about 2.3 mg) was added portionwise until the 390 nm absorption change reached its minimum. The solution was very light yellow at this point, and the reaction was quenched with concentrated HCl (120 μL). Activated manganese dioxide (75 mg), which was prepared as described by Silverman and Invergo,[24] was added to the solution stirred at room temperature for about 2 h. Additional manganese dioxide (total about 160 mg) was added in three portions until the return of the 390 nm peak reached its maximum. The solution was then divided into two centrifuge tubes and brought to pH 8 with 1 M NaOH. After being centrifuged in an IEC clinical centrifuge at maximum speed, the pellet was dark brown, and the supernatant was dark yellow. Both of the supernatants were combined, and the solvent was removed by bulb-to-bulb distillation in a closed system. The brownish yellow [^{3}H] PLP was purified on a Dowex 1 column (1.5×7.5 cm) which had been cleaned by washing with 5 M acetic acid, 1 M NaOH, and 5 M acetic acid once more. Between each of these washes, the column was washed with water until the eluent was neutral. Finally, the column was equilibrated in 4 M sodium acetate. The [^{3}H] PLP solution was applied to the column and was eluted with water (10 mL), followed by a gradient of water (250 mL) and 5 M acetic acid (250 mL). A Pharmacia Biotech peristaltic pump was attached to the column and was run at 1.5 mL/min while the fraction collector changed fractions every 10 min. The fraction with the highest absorption at 390 nm and all of the other fractions with higher than 10% of that highest absorption were combined, and the solvent was removed by lyophilization. The [^{3}H]PLP was further purified using three HPLC systems sequentially. All of the systems utilized an Econosil 10 micron C18 semi-prep HPLC column with solvent flowing at 1 mL/min. Detection was at 254 nm. The first purification was done using H_2O/0.1% TFA as the mobile phase. Five-milliliter fractions were collected; those containing radioactivity and coeluting with authentic PLP were combined, and the solvent was removed by lyophilization. The second column was run with 100 mM potassium phosphate, pH 7 as the mobile phase. Fractions of 1 mL were collected, and those coeluting with unlabeled PLP and containing radioactivity were combined and lyophilized. The third purification was the same as the first one except that 1 mL fractions were collected; those containing radioactivity and coeluting with authentic PLP were combined, and the solvent was removed by lyophilization. The tritiated PLP obtained had a specific radioactivity of 8.38 Ci/mmol, a concentration of 0.28 mM, and a radiopurity of 99%.

Enzymes and assays

Succinic semialdehyde dehydrogenase (SSDH) was obtained from GABAase, a commercially available mixture of GABA-AT and SSDH, by the reported procedure.[25] 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.[26] 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 diphosphate, 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.

Further purification of GABA-AT isolated from pig brains

GABA-AT (55 mg, 0.52 μmol) was isolated from pig brains (2.5 kg) using a modification of the procedure of Churchich and Moses.[27] The purity of the enzyme prior to the modification is 77%, and major impurities came from proteins having molecular weights larger and smaller than that of GABA-AT according to gel electrophoresis analysis. A published size exclusion chromatography procedure[28] was, therefore, modified to further purify the isolated GABA-AT (stored in 25 mL of 350 mM KH_2PO_4 buffer, pH 7.0, containing 1 mM β-mercaptoethanol). This solution was concentrated (to 2.5 mL) in an Amicon ultrafiltration cell (#8050) containing a PM 10 membrane at a N_2 pressure of 70 psi. The concentrated enzyme was filtered through a 0.45 μ GHP Acrodisc purchased from Gelman. The combined filtered enzyme and rinses were injected onto a 16/60 Pharmacia Hiprep Sephacryl S-200 high resolution column, prewashed and equilibrated with two column volumes of buffer H (50 mM Na_2HPO_4 buffer, pH 7, containing 0.15 M NaCl, 1 mM β-mercaptoethanol). The enzyme was then eluted with buffer H at 0.2 mL/min for 400 min. The elution was monitored at 280 nm. Fractions (1 mL) were collected and assayed. All of the enzyme-containing fractions were analyzed by gel electrophoresis. Active enzyme fractions of more than 90% purity (fractions from 210 to 250 min) were

collected and combined. The enzyme was then dialyzed against buffer A (3×1L, buffer changed every 2 h). The enzyme obtained (40 mg) had a specific activity of 8.5 units/mL, a protein concentration of 2.1 mg/mL, and a purity of 95%. When this purified enzyme (95% pure, 10 mg) was subjected to the same purification process as discussed above, a 99% pure enzyme (8 mg) was obtained.

Preparation of [^{3}H]PLP-reconstituted GABA-AT

GABA-AT (3 units, 6.8 nmol) was incubated with 194 mM GABA in buffer A at room temperature in the dark for 30 min. After being cooled to 4°C, monobasic potassium phosphate was added slowly to the incubation mixture over a period of 10 min to make a 0.5 M, pH 5.5 KH_2PO_4 solution. This mixture was dialyzed first against 1L of 0.5 M KH_2PO_4 buffer, containing 0.25 mM β-mercaptoethanol for 1 h, then against 4 L of 100 mM potassium phosphate buffer, pH 7.0, containing 0.25 mM β-mercaptoethanol for 2 h at 4°C. An assay showed less than 1% activity remaining. This *apo*GABA-AT was incubated with a 250-fold excess of [^{3}H]PLP (30 μCi, 1.7 μmol, freshly purified by HPLC) at room temperature until maximum activity returned. Assays were performed every hour to monitor the return of activity, which generally took about 4 h. About 60% of the activity returned. The excess [^{3}H]PLP was then removed from the reconstituted enzyme by running portions (200 μL) over Sephadex G-50 packed columns prewashed with buffer A, using the Penefsky spin method.[23] All of the enzyme samples were combined and dialyzed against buffer A (2×4 L, changed every 2 h) at 4°C. The [^{3}H]PLP-GABA-AT obtained had a specific activity of 1.06 units/mL, a protein concentration of 0.59 mg/mL, and a specific radioactivity of 0.011 μCi/mg. The yield of the preparation was 39% (1.06 units, 0.59 mg; yield calculated based on the enzyme activity).

Time-dependent inactivation of GABA-AT by gabaculine

GABA-AT (10 μL; final concentration 0.275 μM) was added to solutions of gabaculine (90 μL; final concentrations of 0, 5, 6, 8, 10, 14, 16, 20 μM, seven pre-incubation mixtures for each concentration) in buffer A, containing 100 μM α-ketoglutarate at 25°C. At timed intervals, aliquots (85 μL) were withdrawn and added to the assay solution (515 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][19] was constructed to determine K_I and k_{inact} values for gabaculine. The inactivation also was carried out at 0°C under the same conditions except for different gabaculine concentrations (final concentrations of 200, 250, 300, 400, 500, 600, 700, 800, 900 μM).

Inactivation of [^{3}H]PLP-GABA-AT by gabaculine, denaturation, product isolation, and analysis by reverse phase HPLC.

A solution of [^{3}H]PLP-GABA-AT (0.59 mg/mL, 50 μL; 7400 dpm), α-ketoglutarate (5 mM), gabaculine (2 mM) in a total of 100 mL of Buffer A (100 mM potassium phosphate buffer, pH 7.4, containing 0.25 mM β-mercaptoethanol) was incubated, while protected from light, at 25°C for 4h, and the reaction mixture was assayed. By this time, less than 1% of the enzyme activity remained. Two controls also were run simultaneously, one with no gabaculine and one with no α-ketoglutarate or gabaculine, but containing GABA (40 mM). Excess inactivator was removed by running the solutions over Sephadex G-50 packed columns prewashed with water, using the Penefsky spin method.[23] The solutions obtained were again assayed; no return of enzyme activity was detected. All three solutions obtained were subjected to the following denaturation process.

The pH of the samples were adjusted to 12 using 1 M KOH and were incubated at room temperature for 1 h. Then enough trifluoroacetic acid (TFA) was added to quench the base and make 10% v/v TFA solutions. White denatured protein appeared in the solution after the acid addition. After being allowed to stand at room temperature for 10 min, the denatured enzyme solutions were placed into Centricon 10 micro-concentrators and were centrifuged for 15 min at 5,000 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 pellets were 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 pellets obtained were redissolved in water (1 mL) and were counted for radioactivity. The effluent and rinses were combined and lyophilized to dryness. The solid obtained from lyophilization was dissolved in water (500 μL) to make a stock solution. The final sample was prepared by taking the stock sample (100 μL) and adding a standard (20 μL, containing 4 mM each of PLP, PMP, and **3**) which had been subjected to the same basification and acidification steps. The samples were subjected to two different reverse phase HPLC systems. The final sample (pH adjusted to 1 by 10% aqueous TFA) was injected onto an Alltech Alltima C18 column (4.6×250 mm, 5μm). 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 CPPp 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 detected radioactivity of the isolated gabaculine inactivation product were plotted against the retention time. For the other system, the column was isocratically eluted with 2% aqueous CH_3CN for 5 min then a 10 min gradient from 2 to 80% aqueous CH_3CN was applied. The flow rate was 1 mL/min. Under these conditions, PLP elutes at 4.9 min, PMP at 3.5 min, and CPPp at 10.8 min. The HPLC eluent was monitored at 214 nm and analyzed for radioactivity with the FLO-ONE\Beta Series A-200 liquid flow scintillation counter. After being calibrated

with the data from the two controls, the UV absorption at 214 nm and detected radioactivity of the isolated gabaculine inactivation product were plotted against the retention time.

UV–visible spectroscopic studies of the purified gabaculine inactivation product

The gabaculine inactivation product was prepared, denatured, isolated, and purified as described above from an incubation mixture containing 1.2 mL (80 nmol) of GABA-AT, 10 mM α-ketoglutarate, and 2 mM gabaculine. After denaturation the gabaculine inactivation product was purified using the 0.1% TFA to 50% aqueous acetonitrile elution system. The 35 to 37 min HPLC fractions were collected and freeze-dried. The dried sample was then dissolved in 2 M NH_4OH (500 μL), placed in a Fisher 1 cm cuvette, and scanned from 450 nm to 214 nm. Synthetic **3** (80 nmol) was also dissolved in 2 M NH_4OH (500 μL) and scanned under the same conditions.

Electrospray ionization mass spectroscopic (ESIMS) studies of the highly purified gabaculine inactivation product

Care was taken in all of the experimental procedures to eliminate the introduction of glycerol, polyethylene glycol, or other detergent related polymers into samples intended for mass spectroscopic studies. 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 gabaculine (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 for the UV–visible spectroscopic study was followed except that the 2–80% aqueous acetonitrile elution system was used. The 10–12.5 min HPLC fractions were collected and freeze-dried. The same purification procedure was applied to synthetic **3** (80 nmol) to serve as a control for analysis. The dried samples prepared above were dissolved in 50% v/v aqueous CH_3CN (50 μL) giving solutions with a product concentration of about 1.6 mM and a control 3 concentration of 1.6 mM. The pH of the solution was brought to about 10 with the addition of 1 M NH_4OH for negative mode electrospray ionization mass spectrometry ($ESIMS^-$) and to 4 by 1% formic acid addition for positive mode electrospray ionization mass spectrometry ($ESIMS^+$) analysis. The control synthetic **3** sample was run first to find the optimum conditions. Aliquots of 10-μL each (16 nmol) were loop injected into the mass spectrometer. All data were acquired in the multichannel analysis (MCA) mode, and a CsI/NaI mixture was used to calibrate the instrument. The resolution of the instrument was 15.0 at both low and high mass. The mass range was scanned from m/z 160 to 600 in 1.99 s. For MS/MS analysis, argon was used as the collision gas at a pressure of ca. 2.2×10^{-3} mbar. The collision energy was 16 eV. The mass range was scanned from m/z 100 to 400.

NMR studies of the purified gabaculine inactivation product

GABA-AT (2 mL, 160 nmol) was inactivated with gabaculine (2 mM) in buffer A containing 10 mM α-KG at room temperature in the dark for 4 h. The same inactivation, denaturation, and product isolation procedures described above for the UV–visible spectral study were followed. The 35–37 min HPLC fractions were collected and freeze dried. The same purification was applied to synthetic **3** (160 nmol) to serve as a control for analysis. The control and purified product samples were dissolved in 1 mL of D_2O and freeze dried. The D_2O exchanged samples were each dissolved in 350 μL of D_2O, placed in Schigemi BMS-005T NMR tubes, and ^1H NMR spectra were taken on a Varian Unity Plus 400 NMR spectrometer.

Acknowledgements

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

References

1. Mishima, H.; Kurihara, H.; Kobayashi, K.; Miyazawa, S.; Terahara, A. *Tetrahedron Lett*. **1976**, 537.
2. Rando, R. R.; Bangerter, F. W. *J. Am. Chem. Soc*. **1976**, *98*, 6762.
3. Rando, R. R.; Bangerter, F. W. *Biochem. Biophys. Res. Commun*. **1977**, *76*, 1276.
4. Wood, J. D.; Kurylo, E.; Tsui, D. S.-K. *Neurosci. Lett*. **1979**, *14*, 327.
5. Behar, K. L.; Boehm, D. *Magn. Reson. Med*. **1994**, *31*, 660.
6. Watts, S. D. M.; Atkins, A. M. *UCLA Symp. Mol. Cell. Biol., New Ser*. **1987**, *60*, 525.
7. Gammon, D. W.; Gingrich, H. L.; Sander, G.; Stewart, R. R.; Van Der Werf, P. A. *ACS Symp. Ser*. **1987**, *356*, 122.
8. Prince, D. J.; Djamgoz, M. B. A.; Karten, H. J. *Neurochem. Int*. **1987**, *11*, 23.
9. Palfreyman, M. G.; Schechter, P. J.; Buckett, W. R.; Tell, G. P.; Koch-Weser, J. *Biochem. Pharmacol*. **1981**, *30*, 817.
10. Aurisano, N.; Bertani, A.; Reggiani, R. *Phytochemistry* **1995**, *38*, 1147.
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.
13. Bernasconi, R.; Maitre, L.; Martin, P.; Raschdorf, F. *J. Neurochem*. **1982**, *38*, 57.
14. Rando, R. R.; Bangerter, F. W. *J. Am. Chem. Soc*. **1977**, *99*, 5141.
15. Rando, R. R. *Biochemistry* **1977**, *16*, 4604.
16. Metcalf, B. W.; Jung, M. J. *Mol. Pharmacol*. **1979**, *16*, 539.
17. Soper, T. S.; Manning, J. M. *J. Biol. Chem*. **1982**, *257*, 13930.
18. Kitz, R.; Wilson, I. B. *J. Biol. Chem*. **1962**, *237*, 3245.

19. Khomutov, R. M.; Karpeiskii, M. Y.; Severin, E. S. *Chemical and Biological Aspect of Pyridoxal Catalysis. I. U. B. Symposium Series*; Pergamon Press: New York, 1963; pp 313–321.
20. Shah, S. A.; Shen, B. W.; Brunger, A. T. *Structure* **1997**, *5*, 1067.
21. Penefsky, H. *J. Biol. Chem.* **1977**, *252*, 2891.
22. Penefsky, H. *Methods Enzymol.* **1979**, *56*, 527.
23. Stock, A.; Ortanderl, F.; Pfleiderer, G. *Biochem. Z.* **1966**, *344*, 353.
24. Silverman, R. B.; Invergo, B. J. *Biochemistry* **1986**, *25*, 6817.
25. Hopkins, M. H.; Bichler, K. A.; Su, T.; Chamberlain, C. L.; Silverman, R. B. *J. Enzyme Inhib.* **1992**, *6*, 195.
26. Scott, E. M.; Jakoby, W. B. *J. Biol. Chem.* **1958**, *234*, 932.
27. Churchich, J. E.; Moses, U. *J. Biol. Chem.* **1981**, *256*, 1101.
28. De Biase, D.; Barra, D.; Bossa, F.; Pucci, P.; John, R. A. *J. Biol. Chem.* **1991**, *266*, 20056.