(±)-(1 S ,2 R ,5 S)-5-Amino-2-fluorocyclohex-3-enecarboxylic Acid. A Potent GABA Aminotransferase Inactivator that Irreversibly Inhibits via an Elimination−Aromatization Pathway †

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(±)-(1*S*,2*R*,5*S*)-5-Amino-2-fluorocyclohex-3-ene Carboxylic Acid. A Potent GABA Aminotransferase Inactivator that Irreversibly Inhibits through an Elimination-Aromatization Pathway†

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Abstract

Inhibition of γ-aminobutyric acid aminotransferase (GABA-AT) raises the concentration of GABA, an inhibitory neurotransmitter in human brain, which could have therapeutic applications for a variety of neurological diseases including epilepsy. Based on studies of several previously synthesized conformationally-restricted GABA-AT inhibitors, (±)- (1*S*,2*R*,5*S*)-5-amino-2-fluorocyclohex-3-ene carboxylic acid (**12**) was designed as a mechanismbased inactivator. This compound was shown to irreversibly inhibit GABA-AT; substrate protects the enzyme from inactivation. Mechanistic experiments demonstrated the loss of one fluoride ion per active site during inactivation and the formation of *N*-*m*-carboxyphenylpyridoxamine 5′-phosphate (**26**), the same product generated by inactivation of GABA-AT by gabaculine (**8**). An elimination-aromatization mechanism is proposed to account for these results.

γ-Aminobutyric acid aminotransferase (GABA-AT, E.C. 2.6.1.19) is the enzyme responsible for the degradation of γ-Aminobutyric acid (GABA), one of the major inhibitory neurotransmitters in the mammalian central nervous system,[1] to succinic semialdehyde. Inhibition of this enzyme results in an increased concentration of GABA in the brain and could have therapeutic applications in neurological disorders including epilepsy,[2] Parkinson's disease,[3] Huntington's chorea,[4] and Alzheimer' disease.[5] In fact, vigabatrin (**1**, Figure 1), an irreversible inactivator of GABA-AT, is a drug for the treatment of epilepsy.[6] It has also been found that an increase in the availability of GABA blocks the effects of drug addiction.[7]

We recently reported several fluorine-containing conformationally-restricted analogues of GABA (Figure 1, **2**-**4**) as potential meschanism-based inactivators[8] of GABA-AT, but they turned out to have only minimal reversible inhibitory activity; the non-fluorinated parent compound also was devoid of substrate or inhibitory activity.[9] However, the corresponding

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SUPPORTING INFORMATION AVAILABLE

1H, 13C, and 19F spectra of all synthesized compounds in Scheme 1. This material is available free of charge via Internet at http://pubs.acs.org.

cyclopentane analogue of **2**, namely **5**, was shown to be a time-dependent inactivator of GABA-AT; [10] it inactivates the enzyme by an enamine mechanism.[11] Likewise, (1*R*,4*S*)-(+)-4-amino-2-cyclopentene-1-carboxylic acid (**6**) is a substrate and inhibitor of GABA-AT but not an inactivator,[12] whereas, the corresponding cyclohexene analogue (**7**) is a time-dependent inactivator of GABA-AT, which also was demonstrated to inactivate the enzyme by an enamine mechanism, in this case leading to a ternary complex between the enzyme, the PLP cofactor, and **7**.[13] It was reasoned that the flexible chair conformation of the cyclohexane ring is responsible for the inability of these compounds to inactivate the enzyme, and that the more rigid structures of the corresponding cyclopentane or cyclohexene analogues are more effective inactivators. This also is consistent with the observation that the natural product gabaculine (**8**) is a potent irreversible inhibitor of GABA-AT, which was the first compound to be shown to inactivate the enzyme via an aromatization mechanism.[14] There have been only three other compounds that were proposed to cause inactivation of any PLP-dependent enzyme via an aromatization pathway. One is isogabaculine (**9**), a tautomer of gabaculine, but no experimental support was provided to confirm the mechanism.[15] Another compound shown to inactivate GABA-AT by an aromatization mechanism is the natural product cycloserine (**10**).[16] Two hetero-dihydroaromatic analogues of gabaculine, (*S*)-4-amino-4,5-dihydro-2-thiophenecarboxylic acid (**11**, X = S)[17] and (*S*)-4-amino-4,5-dihydro-2-furancarboxylic acid (**11**, X = O)[18] were designed as potential irreversible inactivators of GABA-AT by the corresponding aromatization mechanism. Both gabaculine analogues are highly potent, irreversible inactivators of GABA-AT, and, therefore, it appeared reasonable, given their structural similarity to gabaculine, that **11** (X = S or O) also could inactivate the enzyme by an aromatization mechanism, although no evidence for that hypothesis was provided in the original work. We demonstrated that **11** (X = S) does, in fact, inactivate GABA-AT by an aromatization mechanism;[19] however, studies with 11 (X = O) showed that it does *not* inactivate GABA-AT by an aromatization mechanism (presumably because of the low aromaticity of a furan ring relative to thiophene and benzene).[20]

Given the potency of gabaculine (**8**), the inactivation properties of **1**, **5** and **7**, and the lack of binding activity for **2** and **3**, we made a composite structure having the form of **7** (which is a conformationally-constrained analogue of **1**), but with an added fluorine atom as in **5**, namely, **12**. This compound is comprised of moieties of other inactivators known to inactivate GABA-AT by a Michael addition mechanism (**1**) and an enamine mechanism (**5** and **7**). However, **12** could undergo enzyme-catalyzed elimination to give the tautomer of the intermediate formed during inactivation of GABA-AT with gabaculine, which inactivates GABA-AT by an aromatization mechanism (these three mechanisms are shown for **12** in Results and Discussion). Here we describe the synthesis and mechanistic studies of **12** as an inactivator of GABA-AT.

MATERIALS AND METHODS

General Materials and Methods

^{1}H and ^{13}C NMR spectra were recorded on Varian Mercury 400 MHz and Inova500 MHz NMR spectrometers. Chemical shifts are reported as δ values in parts per million (ppm) as referenced to chloroform (7.27 ppm for ^{1}H and 77.23 ppm for ^{13}C) or to methanol (4.87 ppm for CD_3OH and 49.15 ppm for ^{13}C). For compounds that are soluble only in deuterium oxide, the ^{1}H chemical shifts are referenced to DOH (4.80 ppm), and the ^{13}C chemical shifts are referenced to an external standard of 3-(trimethylsilyl)-1- propanesulfonic acid-d_6 sodium salt in deuterium oxide. All ^{19}F chemical shifts are referenced to an external standard of fluorotrichloromethane in deuterated chloroform. Mass spectra were obtained on Finnigan MAT900XL (EI) and VG70-250SE (ESI) mass spectrometers in the Analytical Service Laboratory at Northwestern University and on a 70-SE-4F mass spectrometer (FAB) in the

Mass Spectrometry Laboratory at University of Illinois. Elemental analyses were preformed by Atlantic Microlab, Inc. Flash column chromatography was carried out with standard silica gel (230-400 mesh) from Sorbent Technologies, Inc. TLC was run with EM Science silica gel 60 F254 precoated glass plates. Cation exchange chromatography was performed on Dowex 50WX8-200 ion-exchange resin (100-200 mesh). Melting points were measured on a Buchi B-540 melting point apparatus and are uncorrected. All reactions involving moisture sensitive reagents were conducted in oven-dried glassware under a nitrogen atmosphere. Enzyme assays were recorded on a Perkin-Elmer Lambda 10 UV/vis spectrophotometer. HPLC analysis was done with Beckman 125P pumps and a Beckman 166 detector. All the runs were monitored at 256 nm unless otherwise specified. An Alltech C18 column (4.6 × 250 mm, 5 μm) was used. Fluoride ion concentration measurements were obtained using an Orion Research model 702A pH meter with an Orion Research model 96-09 combination fluoride electrode.

All common reagents and solvents were purchased from either Aldrich Chemical Co. or Fisher Scientific without further purification except anhydrous ether and tetrahydrofuran, which were distilled over sodium metal under nitrogen, and anhydrous dichloromethane, which was distilled over calcium hydride. *N*-*m*-Carboxyphenylpyridoxamine 5′-phosphate (**26**) was synthesized by reductive amination of PLP and 3-aminobenzoic acid as described by Iskander et al.[21]

(cis,cis)-Methyl 2,5-diacetoxycyclohex-3-enecarboxylate (13)

The procedures by Smissman et al.[22] were followed. A solution of *trans*,*trans*-1,4-diacetoxy-1,3-butadiene (Fluka, 2.0 g, 11.8 mmol) and methyl acrylate (1.35 mL, 15.0 mmol) in anhydrous *m*-xylene (15 mL) was heated at reflux for 42 h. Most of the solvent was evaporated under reduced pressure, and the residue was flash chromatographed (ethyl acetate/hexanes, 1:10) to give a yellow oil, which was distilled at reduced pressure to give a pale yellow viscous oil (2.63 g, 87%) as a 5.9:1 mixture of **13** with its C-1 epimer; bp 142-144 °C/ 0.1 mmHg (ref.21 152-155 °C/ 3 mmHg). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.95-2.02 (q, 1 H, *J* = 12.3 Hz, H-6, *trans* to H-1), 2.02 (s, 3 H, $OCOCH_3$ at C-5), 2.10 (s, 3 H, $OCOCH_3$ at C-2), 2.39-2.42 (m, 1 H, H-6, *cis* to H-1), 2.79-2.83 (dt, 1 H, *J* = 13.5 Hz, 3.0 Hz, H-1), 3.71 (s, 3 H, $COOCH_3$), 5.34-5.37 (m, 1 H, H-5), 5.53-5.54 (m, 1 H, H-2), 5.91-5.93 (m, 1 H, H-4), 6.01-6.04 (m, 1 H, H-3). ^{13}C NMR ($CDCl^3$, 125 MHz) δ 20.95 ($OCO\mathit{CH}_3$ at C-5), 21.23 ($OCO\mathit{CH}_3$ at C-2), 25.10 (C-6), 41.92 (C-1), 52.17 ($COO\mathit{CH}_3$), 65.40 (C-5), 69.04 (C-2), 126.46 (C-4), 133.25 (C-3), 170.18 ($O\mathit{CO}CH_3$ at C-5), 170.54 ($O\mathit{CO}CH_3$ at C-2), 171.19 (COOMe). The C-1 epimer of **13** has a chemical shift of 2.93-2.98 ppm (m, 0.17 H) for H-1.

For the reaction in water,[23] a mixture of *trans*,*trans*-1,4-diacetoxy-1,3-butadiene (2.07 g, 12.2 mmol), methyl acrylate (15 mL, 0.17 mol), and water (15 mL) was heated at reflux for 17 h. Another portion of water (10 mL) and methyl acrylate (15 mL, 0.17 mol) was added, and the mixture was refluxed further for 12 h. The mixture was extracted once with ethyl acetate (70 mL), dried with sodium sulfate, and evaporated to give a yellow oil (3.9 g). NMR analysis showed a 10:2:1 ratio of **13** to its C-1 epimer to *trans*,*trans*-1,4-diacetoxy-1,3-butadiene. Some polymerized product from methyl acrylate was generated, but it did not interfere with the following hydrolysis reaction, at which stage it was removed by filtration.

Methyl 5-hydroxycyclohexa-1,3-dienecarboxylate (14) and (cis,cis)-methyl 2,5-dihydroxycyclohex-3-enecarboxylate (15)

To a solution of the above mixture of **13** epimers (2.06 g, ~8.0 mmol) in methanol (100 mL) cooled in an ice bath was added dropwise over 20 min a solution of sodium carbonate (1.0 *M*, 17.0 mL, 17.0 mmol). The mixture was stirred at 0 °C for 4 h, then it was adjusted to neutral pH by dropwise addition of 3 *N* hydrochloric acid while being kept cold. Most of the methanol was evaporated under reduced pressure, and the remaining solution was extracted with

dichloromethane (50 mL × 2). The combined organic layers were washed once with brine (20 mL), dried with sodium sulfate, evaporated and flash chromatographed (ethyl acetate/hexanes, 1:1) to give a colorless oil (0.30 g, 24%) of by-product **14**. The extracted aqueous layer was evaporated under reduced pressure to dryness. Ethyl acetate (100 mL) was added, and the mixture was heated to boiling. Upon cooling, the clear solution was transferred by decantation, and it was evaporated and chromatographed (ethyl acetate/hexanes, 1:1) to give a colorless oil (0.89 g, 64%) as a 9:2 mixture of **15** with its C-1 epimer. Crystallization from ethyl acetate/hexanes afforded diastereomerically pure **15** as a white solid (0.68 g, 49% for the pure isolated product).

For **14**: R_f = 0.39 (ethyl acetate/hexanes, 1:1). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.73 (bs, 1 H, OH), 2.61-2.67 (ddd, 1 H, *J* = 18.7 Hz, 7.7 Hz, 2.2 Hz, H-6), 2.90-2.95 (dd, 1 H, *J* = 19.0 Hz, 5.0 Hz, H-6), 3.78 (s, 3 H, $COOCH_3$), 4.37-4.41 (m, 1 H, H-5), 6.21-6.29 (m, 2 H, H-3 and H-4), 7.08-7.10 (m, 1 H, H-2). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 31.35 (C-6), 52.06 ($COO\mathit{CH}_3$), 63.30 (C-5), 125.01 (C-3), 127.07 (C-1), 131.63 (C-2), 133.43 (C-4), 167.63 (COOMe).

For **15**: R_f = 0.05 (ethyl acetate/hexanes, 1:1); mp 102.0-104.0 °C. ^{1}H NMR ($CDCl_3$, 500 MHz) γ 1.81-1.88 (m, 1 H, H-6, *trans* to H-1), 2.22-2.25 (m, 1 H, H-6, cis to H-1), 2.61-2.64 (dt, 1 H, *J* = 13.0 Hz, 2.7 Hz, H-1), 3.35 (bs, 2 H, OH), 3.74 (s, 3 H, $COOCH_3$), 4.18-4.21 (m, 1 H, H-5), 4.42 (m, 1 H, H-2), 5.84-5.91 (m, 2 H, H-3 and H-4). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 28.80 (C-6), 43.79 (C-1), 52.30 ($COO\mathit{CH}_3$), 63.81 (C-5), 66.96 (C-2), 128.40 (C-4), 135.24 (C-3), 174.08 (COOMe). HRMS (ESI) calcd for $C_8H_{13}O_4$ $(M + H)^+$ 173.0814, found 173.0819.

(±)-(1R,2S,5R)-Methyl 2-hydroxy-5-(4'-nitrobenzoyloxy)cyclohex-3-enecarboxylate (16)

To an ice bath-cooled solution of **15** (0.105 g, 0.61 mmol), *p*-nitrobenzoic acid (0.112 g, 0.66 mmol) and triphenylphosphine (0.185 g, 0.70 mmol) in anhydrous THF (20 mL) was added dropwise diisopropyl azodicarboxylate (0.14 mL, 0.70 mmol). The cooling bath was removed after 1 h, and the solution was stirred at room temperature for 15 h. The solvent was evaporated under reduced pressure, and the residue was flash chromatographed (ethyl acetate/hexanes, 1:2) to give a white solid (0.117 g, 60%); mp 104.1-106.0 °C; R_f = 0.31 (ethyl acetate/hexanes, 1:1). ^{1}H NMR ($CDCl^3$, 500 MHz) δ 2.26-2.29 (d, 1 H, *J* = 15.0 Hz, H-6, *cis* to H-1), 2.38-2.44 (dt, 1 H, *J* = 13.5 Hz, 4.3 Hz,H-6, *trans* to H-1), 2.86-2.87 (d, 1 H, *J* = 5.5 Hz, OH), 3.03-3.07 (dt, 1 H, *J* = 12.5 Hz, 3.2 Hz, H-1), 3.79 (s, 3 H, $COOCH_3$), 4.58-4.61 (q, 1 H, *J* = 4.5 Hz, H-2), 5.61-5.62 (m, 1 H, H-5), 6.09-6.12 (dd, 1 H, *J* = 9.7 Hz, 4.7 Hz, H-4), 6.20-6.23 (dd, 1 H, *J* = 9.7 Hz, 4.7 Hz, H-3), 8.18-8.20 (d, 2 H, *J* = 8.5 Hz, ArH), 8.28-8.30 (d, 2 H, *J* = 8.5 Hz, ArH). ^{13}C NMR ($CDCl^3$, 125 MHz) δ 25.65 (C-6), 41.41 (C-1), 52.42 ($COO\mathit{CH}_3$), 63.27 (C-2), 67.94 (C-5), 123.76 (Ar), 127.20 (C-4), 130.98 (Ar), 133.46 (C-3), 135.71 (Ar), 150.79 (Ar), 164.20 (ArCO), 174.16 (COOMe). HRMS (CI) calcd for $C_{17}H_{20}NO_7$ $(M + C2H5)^+$ 350.1234, found 350.1240; calcd for $C_{15}H_{14}NO_6$ $(M - OH)^+$ 304.0816, found 304.0814. Irradiation of H-1 resulted in a NOE of 2.7% for H-2, and no NOE for H-5.

(±)-(1R,4S,5R)-Methyl 4-hydroxy-5-(4'-nitrobenzoyloxy)cyclohex-2-enecarboxylate (17) and (±)-(1S,2R,5R)-methyl 2-fluoro-5-(4'-nitrobenzoyloxy)cyclohex-3-enecarboxylate (18)

To an ice bath-cooled solution of **16** (0.38 g, 1.2 mmol) in anhydrous dichloromethane (40 mL) over 3 Å molecular sieves was added (diethylamino)sulfur trifluoride (0.47 mL, 3.6 mmol) dropwise over a period of 10 min. The mixture was allowed to rise in temperature slowly to room temperature over 3 h, and the reaction was quenched with saturated sodium bicarbonate (10 mL). The separated organic layer was washed once with brine (10 mL), dried with sodium sulfate, evaporated, and flash chromatographed (ethyl acetate/hexanes, 1:10 to elute **18** followed by 1:1 ethyl acetate/hexanes to elute **17**) to give a white solid (0.25 g, 65%) after

crystallization from diethyl ether/hexanes as a 5:1 mixture of **18** with its C-2 epimer, and **17** as a yellow oil (89 mg, 23%).

For **17**: R_f = 0.40 (ethyl acetate/hexanes, 1:1). ^{1}H NMR ($CDCl^3$, 500 MHz) δ 2.18-2.22 (m, 1 H, H-6, *trans* to H-1), 2.44-2.50 (m, 1 H, H-6, *cis* to H-1), 3.41-3.42 (m, 1 H, H-1), 3.75 (s, 3 H, $COOCH_3$), 4.52 (s, 1 H, H-4), 5.58-5.60 (m, 1 H, H-5), 5.86-5.88 (d, 1 H, *J* = 10.0 Hz, H-3), 6.04-6.06 (d, 1 H, *J* = 10.0 Hz, H-2), 8.19-8.21 (d, 2 H, *J* = 8.5 Hz, ArH), 8.30-8.31 (d, 2 H, *J* = 8.5 Hz, ArH). ^{13}C NMR ($CDCl^3$, 125 MHz) δ 28.86 (C-6), 39.33 (C-1), 52.59 ($COOCH_3$), 65.94 (C-4), 71.94 (C-5), 123.87 (Ar), 127.43 (C-3), 129.35 (C-2), 131.03 (Ar), 133.43 (C-4), 135.56 (Ar), 150.92 (Ar), 164.61 (ArCO), 173.24 (COOMe). HRMS (CI) calcd for $C_{15}H_{16}NO_7$ $(M + H)^+$ 322.0921, found 322.0919; calcd for $C_{15}H_{14}NO_6$ $(M - OH)^+$ 304.0816, found 304.0815. The assignment of regio- and stereochemistry was based on 1D NOESY and COSY NMR studies.

For **18**: mp 85.5-87.0 °C; R_f = 0.89 (ethyl acetate/hexanes, 1:1). ^{1}H NMR ($CDCl^3$, 500 MHz) δ 2.13-2.19 (m, 1 H, H-6, *trans* to H-1), 2.31-2.34 (d, 1 H, *J* = 15.0 Hz, H-6, *cis* to H-1), 3.09-3.16 (m, 1 H, H-1), 3.79 (s, 3 H, $COOCH_3$), 5.34-5.45 (dd, 1 H, *J* = 47.5 Hz, 8.0 Hz, H-2), 5.56 (m, 1 H, H-5), 6.07-6.08 (m, 1 H, H-4), 6.14-6.19 (t, 1 H, *J* = 10.5 Hz, H-4), 8.22-8.24 (d, 2 H, *J* = 8.5 Hz, ArH), 8.31-8.32 (d, 2 H, *J* = 8.5 Hz, ArH). ^{13}C NMR ($CDCl^3$, 125 MHz) δ 29.23- 29.29 (d, *J* = 7.5 Hz, C-6), 42.20-42.36 (d, *J* = 19.9 Hz, C-1), 52.67 ($COOCH_3$), 66.42 (C-5), 86.86-88.22 (d, *J* = 170.2 Hz, C-2), 123.84 (Ar), 127.30-127.36 (d, *J* = 8.3 Hz, C-4), 131.09 (Ar), 131.86-132.04 (d, *J* = 23.3 Hz, C-3), 135.39 (Ar), 150.91 (Ar), 164.17 (ArCO), 172.95 (COOMe). ^{19}F NMR ($CDCl^3$, 376 MHz) δ -178.71--178.52 (dt, 1 F, *J* = 47.4 Hz, 12.2 Hz), - 177.46--177.33 (dm, 0.2 F, *J* = 46.8 Hz, from C-2 epimer of **18**). HRMS (CI) calcd for $C_{17}H_{19}NO_6F$ $(M + C_2H_5)^+$ 352.1191, found 352.1188. Irradiation of H-1 resulted in the following NOE: 0.5% for H-6 (*trans* to H-1), 1.4% for H-6 (*cis* to H-1), and 0.8% for H-2, indicating that the C-1 methyl ester group is *trans* to the C-2 fluorine. The C-2 epimer of **18** is much more labile to basic conditions than **18**, and it underwent almost complete elimination of fluorine in the following hydrolysis reaction.

(±)-(1S,2R,5R)-Methyl 2-fluoro-5-hydroxycyclohex-3-enecarboxylate (19)

To a solution of the mixture of epimers of **18** (0.25 g, 0.77 mmol) obtained above in methanol (20 mL) cooled in an ice bath was added dropwise over 40 min an ice-cooled solution of sodium carbonate (0.1 *M*, 10.0 mL, 1.0 mmol). TLC analysis showed the reaction was complete after 4 h, and the mixture was adjusted to neutral pH in an ice bath by dropwise addition of 3 *N* hydrochloric acid. Most of the methanol was evaporated under reduced pressure, and the remaining solution was extracted with ethyl acetate (30 mL × 2). The combined organic layer was washed once with brine (20 mL), dried with sodium sulfate, evaporated and flash chromatographed (ethyl acetate/hexanes, 1:2) to give a colorless oil (0.10 g) as a 3:1 mixture of **19** (57%) and **14** (19%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.74 (bs, 1 H, OH), 1.96-2.02 (dt, 1 H, *J* = 13.1 Hz, 3.5 Hz, H-6, *trans* to H-1), 2.08-2.11 (dd, 1 H, *J* = 7.5 Hz, 3.5 Hz, H-6, *cis* to H-1), 3.04-3.11 (m, 1 H, H-1), 3.76 (s, 3 H, $COOCH_3$), 4.28 (s, 1 H, H-5), 5.25-5.36 (dd, 1 H, *J* = 46.7 Hz, 8.2 Hz, H-2), 5.93- 5.99 (m, 2 H, H-3 and H-4). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 32.14-32.19 (d, *J* = 6.9 Hz, C-6), 41.60-41.75 (d, *J* = 19.9 Hz, C-1), 52.46 ($COOCH_3$), 62.78-62.80 (d, *J* = 3.0 Hz, C-5), 87.07- 88.40 (d, *J* = 167.9 Hz, C-2), 128.92-129.09 (d, *J* = 22.1 Hz, C-3), 131.73-131.81 (d, *J* = 9.2 Hz, C-4), 173.63 (COOMe). ^{19}F NMR ($CDCl_3$, 376 MHz) δ .177.87..177.69 (dt, 1 F, *J* = 47.1 Hz, 10.2 Hz). *m/z* (CI) 175, 157, 155, 137, 123, 95, 93. There was only 2% of the C-2 epimer of **19** as determined from ^{19}F NMR integration.

(±)-(1S,2R,5S)-Methyl 5-azido-2-fluorocyclohex-3-enecarboxylate (20)

To an ice bathcooled solution of the above mixture of **14** and **19** (91.5 mg, 0.41 mmol), diphenylphosphoryl azide (0.12 mL, 0.58 mmol), and triphenylphosphine (0.160 g, 0.60 mmol)

in anhydrous THF (10 mL) was added diisopropyl azodicarboxylate (0.13 mL, 0.58 mmol) dropwise. The cooling bath was removed after 1 h, and the solution was stirred at room temperature for 37 h. The solvent was evaporated under reduced pressure, and the residue was flash chromatographed (5% ether in hexanes) to give a colorless oil (42 mg, 52%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.76-1.84 (q, 1 H, *J* = 12.2 Hz, H-6, *trans* to H-1), 2.38-2.41 (m, 1 H, H-6, *cis* to H-1), 2.81-2.88 (m, 1 H, H-1), 3.78 (s, 3 H, $COOCH_3$), 4.10 (m, 1 H, H-5), 5.38-5.49 (dd, 1 H, *J* = 48.4 Hz, 8.5 Hz, H-2), 5.86-5.88 (d, 1 H, *J* = 10.5 Hz, H-4), 5.98-6.02 (t, 1 H, *J* = 10.7 Hz, H-3). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 30.39-30.44 (d, *J* = 5.4 Hz, C-6), 45.48-45.65 (d, *J* = 19.9 Hz, C-1), 52.66 ($COOCH_3$), 56.42-56.45 (d, *J* = 3.0 Hz, C-5), 86.64-87.98 (d, *J* = 168.7 Hz, C-2), 129.56-129.74 (d, *J* = 22.1 Hz, C-3), 130.02-130.09 (d, *J* = 9.0 Hz, C-4), 172.36 (COOMe). ^{19}F NMR ($CDCl_3$, 376 MHz) δ .175.73..175.58 (dm, 1 F, *J* = 48.9 Hz). *m/z* (CI) 200, 177, 143, 123, 95.

(±)-(1S,2R,5S)-Methyl 5-amino-2-fluorocyclohex-3-enecarboxylate (21)

A mixture of **20** (42 mg, 0.21 mmol) and triphenylphosphine (66 mg, 0.25 mmol) in THF (1.0 mL) and water (0.05 mL, 2.8 mmol) was stirred at room temperature for 41 h. The mixture was diluted in water (30 mL) and adjusted to pH 5 by addition of several drops of 3 *N* hydrochloric acid. The mixture was extracted with dichloromethane (10 mL × 3) to remove triphenylphosphine oxide, and the separated aqueous layer was evaporated under reduced pressure to give a white solid (42 mg, 95%) as the hydrochloric acid salt of the title compound; mp 164.0-166.0 °C (dec.). ^{1}H NMR (CD3OD, 500 MHz) δ 1.72-1.80 (q, 1 H, *J* = 12.2 Hz, H-6, *trans* to H-1), 2.40-2.42 (m, 1 H, H-6, *cis* to H-1), 2.86-2.94 (m, 1 H, H-1), 3.72 (s, 3 H, $COOCH_3$), 4.07 (m, 1 H, H-5), 5.29-5.40 (ddd, 1 H, *J* = 48.1 Hz, 8.9 Hz, 2.1 Hz, H-2), 5.86-5.88 (d, 1 H, *J* = 10.5 Hz, H-4), 6.04-6.08 (t, 1 H, *J* = 11.0 Hz, H-3). ^{13}C NMR (CD_3OD, 125 MHz) δ 30.14-30.20 (d, *J* = 6.9 Hz, C-6), 46.27-46.42 (d, *J* = 19.9 Hz, C-1), 48.35-48.37 (d, *J* = 2.3 Hz, C-5), 53.11 ($COOCH_3$), 87.88-89.23 (d, *J* = 169.4 Hz, C-2), 128.50-128.58 (d, *J* = 9.2 Hz, C-4), 132.40-132.57 (d, *J* = 22.1 Hz, C-3), 173.77 (COOMe). ^{19}F NMR (CD_3OD, 376 MHz) δ .177.66..177.49 (dm, 1 F, *J* = 47.1 Hz). HRMS (CI) calcd for $C_8H_{13}NO_2F$ $(M + H)^+$ 174.0925, found 174.0927.

(±)-(1S,2R,5S)-5-Amino-2-fluorocyclohex-3-enecarboxylic acid (12)

To an ice bathcooled solution of **21** (22 mg, 0.1 mmol) in water (5 mL) was added an ice-cooled lithium hydroxide solution (0.2 *N*, 5 mL, 1.0 mmol) dropwise. The cooling bath was removed after 30 min, and the solution was stirred at room temperature for 18 h. The mixture was then adjusted to pH 3, and the solvent was evaporated under reduced pressure to give a white solid. The crude material was loaded onto 2 g of Dowex 50WX8-200 ion-exchange resin, which had been preeluted with 50 mL of 0.5 *N* pyridine in water and 150 mL of water. The column was washed with water (100 mL), then the product was eluted with 50 mL of 0.5 *N* pyridine to give a white solid (11.4 mg, 68%) after evaporation of solvent and further recrystallization from methanol/ethyl acetate; mp 178.4-180.3 °C (dec.). ^{1}H NMR (CD_3OD, 400 MHz) δ 1.70-1.79 (q, 1 H, *J* = 12.1 Hz, H-6, *trans* to H-1), 2.40-2.43 (m, 1 H, H-6, *cis* to H-1), 2.79-2.88 (m, 1 H, H- 1), 4.05 (m, 1 H, H-5), 5.27-5.41 (dd, 1 H, *J* = 48.0 Hz, 8.4 Hz, H-2), 5.83-5.86 (d, 1 H, *J* = 10.4 Hz, H-4), 6.08-6.10 (t, 1 H, *J* = 11.0 Hz, H-3). ^{13}C NMR (CD_3OD, 125 MHz) δ 30.23-30.29 (d, *J* = 6.9 Hz, C-6), 46.31-46.67 (d, *J* = 20.5 Hz, C-1), 48.38 (C-5), 87.87-89.22 (d, *J* = 169.4 Hz, C-2), 128.40-128.47 (d, *J* = 9.2 Hz, C-4), 132.60-132.78 (d, *J* = 22.9 Hz, C-3), 175.04 (COOH). ^{19}F NMR (CD_3OD, 376 MHz) δ -177.63--177.50 0 (d, 1 F, *J* = 47.1 Hz). Anal. calcd for $C_7H_{10}NO_2F\eta 0.5H_2O$ C, 50.00, H, 6.59, N, 8.33; found C, 50.26, H, 6.31, N, 8.28.

Enzyme and Assays

GABA aminotransferase was isolated from pig brain by the published procedure.[24] Succinic semialdehyde dehydrogenase (SSDH) was isolated from GABAse, a commercially available mixture of SSDH and GABA-AT, using the method of Jeffery et al.[25] GABA-AT activity was assayed using a modification of the coupled assay of Scott and Jakoby.[26] The assay solution has final concentrations of 11 mM GABA, 1.1 mM $NADP^+$, 5.3 mM α-ketoglutarate, 2 mM β-mercaptoethanol, and excess SSDH in 50 mM potassium pyrophosphate buffer at pH 8.5. With this assay, the change in absorbance at 340 nm, corresponding to the formation of NADPH from $NADP^+$ at 25 °C, is proportional to the GABA-AT activity.

Time-Dependent Inactivation of GABA-AT by 12

GABA-AT (17.1 μm, 32 μL) was added at 25 °C to various concentrations of **12** (160 μL final volume) in 50 mM potassium pyrophosphate buffer, pH 8.5, containing 8 mM α-ketoglutarate and 2 mM β-mercaptoethanol at 25 °C. At timed intervals, aliquots (30 μL) were withdrawn and added to the assay solution (565 μL) followed by the addition of SSDH (5 μL, excess amount), and reaction rates were measured spectrophotometrically at 340 nm. K_I and k_{inact} values were determined by the method of Kitz and Wilson.[27]

Dialysis of Inactivated GABA-AT by 12

GABA-AT (17.1 μm, 20 μL) was added to a mixture of potassium pyrophosphate buffer (50 mM, 50 μL) and **12** (50 mM, 30 μL). A control substituted the solution of **12** with the same volume of potassium pyrophosphate buffer. Both solutions were incubated in the dark at room temperature for 1.5 h, and 30 μL of the mixture was tested for activity. The remaining solution was then transferred to a Slide-A-Lyzer 10K dialysis cassette and dialyzed against a 50 mM potassium pyrophosphate buffer containing 0.1 mM PLP, 0.1 mM α-ketoglutarate, and 2 mM β-mercaptoethanol (3 × 1 L, changed every 6 h) at 4 °C. After dialysis, equal amounts of solutions were assayed.

GABA Protection of the Inactivation of GABA-AT by 12

GABA-AT (17.1 μm, 20 μL) was added to **12** (final concentration 0.4 mM) in 50 mM potassium pyrophosphate buffer (80 μL) containing α-ketoglutarate (8 mM) and various concentrations of GABA (0, 3.4, 6.8 mM). At timed intervals, aliquots (30 μL) were withdrawn and added to the assay solution (565 μL). Excess succinic semialdehyde dehydrogenase (5 μL) was then added, and rates were measured spectrophotometrically at 340 nm at 25 °C.

Reverse Phase HPLC and Mass Spectral Analysis of the Inactivation Product of GABA-AT by 12

A mixture of GABA-AT (17.1 μm, 200 μL) and 12 (10 mM, 60 μL) were incubated at room temperature for 1 h, and an aliquot (5 μL) was assayed. Less than 1% of the enzyme activity remained. Aqueous trifluoroacetic acid (TFA, 10%, 40 μL) was then added, and the mixture was allowed to stand for 0.5 h. The denatured enzyme was then centrifuged for 15 min at 13,400 rpm with an Eppendorf Minispin centrifuge. The protein pellet was rinsed with 1% TFA (45 μL), vortexed, and centrifuged for 5 min. The supernatant solutions were combined and lyophilized to give a white solid. Water (115 μL), 10% TFA (**12** μL), and PLP (as an internal standard, 5 mM, 3 μL) were added, and the mixture was centrifuged again for 5 min at 13,400 rpm. The supernatant solution was then analyzed by reverse phase HPLC (see below for elution system). GABA-AT (17.1 μm, 40 μL) and 12 (10 mM, 40 μL) were subjected separately to the same procedures as above to give two controls. A standard solution was prepared by mixing synthetic **26** in DMSO (5 mM, 2 μL), PLP (5 mM, 4 μL), and 10% TFA (20 μL) in water (74 μL). These four samples (20 μL) were individually injected onto an Alltech Alltima C18 column (4.6 × 250 mm, 5 μm). Mobile phase A was 0.1% aqueous TFA and mobile phase B

was pure acetonitrile. UV absorption was monitored at 256 nm. The column was eluted with 2% B for 5 min, then a 10 min gradient from 2 to 80% B was applied, followed by a further 15 min elution with 80% B. Under these conditions, PLP elutes at 4.87 min, **12** at 10.77 min, and **26** at 12.42 min. A mixture of the inactivated enzyme (10 μL) and standard solution of **26** (10 μL) was also injected, and they coeluted to give a single peak at 12.42 min. The fractions from 12.3 to 14.0 min of ten injections were combined and concentrated in vacuo. LC-MS analysis was carried out using the same gradient as above except that 0.1% formic acid was used instead of TFA and methanol instead of acetonitrile. Mass spectra were acquired on a Micromass (Manchester, UK) hybrid quadrupole/time-of-flight mass spectrometer operated in positive ion mode. Capillary voltage was set at 3300V and cone voltage at 25V. Data were acquired at 6000 resolutions at *m/z* 500. Product ion spectra were acquired at a 22eV collision energy using argon as collision gas at 2.0×10-3mbar.

Fluoride Ion Release from (±)-(1R,2R,5S)-5-Amino-2-fluorocyclohex-3-ene-1-carboxylic Acid (12) During Inactivation of GABA-AT

GABA-AT (0.98 mg/mL, 2.3 μm) was incubated with **12** (20 μm) in a 50 mM potassium pyrophosphate buffer, pH 8.5, containing 2.0 mM β-mercaptoethanol and 4.7 mM α-ketoglutarate in a total volume of 280 μL. After 1 h incubation at 23 °C, no enzyme activity was observed. A sample (200 μL) of the inactivated enzyme solution was added to a mixture of 790 μL of 50 mM potassium pyrophosphate buffer, 10 μL of 1.19 μm sodium fluoride standard solution, and 1 mL of a low level total ionic strength buffer (57 mL of glacial acetic acid, 58 g of NaCl, and 0.30 g of sodium citrate diluted to 500 mL with H2O, pH 5.25) for the determination of the fluoride ion concentration with an Orion fluoride ion electrode. Human serum albumin (0.98 mg/mL) incubated with **12** (20 μm) was tested under the same conditions. The control contained identical components except that the protein was omitted. Diluted sodium fluoride solutions were used as calibration standards.

Time-dependent inactivation of GABA-AT by 8

GABA-AT (17.1 μm, 32 μL) was incubated at 25 °C with various concentrations of **8** (0.001~0.1 mM, 114 μL final volume) in 50 mM potassium pyrophosphate buffer, pH 8.5, containing 8 mM α-ketoglutarate and 2 mM β-mercaptoethanol. Aliquots (20 μL) of the incubation solution were added to the assay solution (575 μL, containing 11 mM GABA, 1.1 mM $NADP^+$, 5.3 mM α-ketoglutarate, 2 mM β-mercaptoethanol in 50 mM potassium pyrophosphate buffer, pH 8.5) followed by the addition of SSDH (5 μL, excess amount) at timed intervals. The change in absorbance at 340 nm was measured to determine the reaction rate and calculate the kinetic constants for the inactivation by the method of Kitz and Wilson.[26]

Determination of the formation of 8 from 12

The UV absorbance of various concentrations of **8** (0.004~0.020 mM) at 276 nm was measured to make a working curve. Solid **12** (0.26 mg) was dissolved in 409 μL of 50 mM potassium pyrophosphate buffer (pH 8.5), and an aliquot of 60 μL was withdrawn and diluted to 600 μL immediately to make a 0.40 mM solution. At timed intervals, the UV absorbance of the final solution at 276 nm was measured to monitor the reaction. A 50 mM potassium pyrophosphate buffer solution without **12** was used as the control solution.

RESULTS AND DISCUSSION

Synthesis of (±)-(1R,2R,5S)-5-Amino-2-fluorocyclohex-3-ene-1-carboxylic Acid (12)

The synthesis of **12** starts with a Diels-Alder reaction between trans,trans-1,4-diacetoxy-1,3-butadiene and methyl acrylate to give known compound **13** (Scheme 1).[22] In the

literature[22] it was assumed that only the all cis-**13** was formed, but in our hands, 17% of the corresponding C-1 epimer was obtained as well, and the diastereomers could not be separated by distillation. Sharpless et al. recently reported on the rate-accelerating effect of water on a series of reactions,[23] and we performed this Diels-Alder reaction "on water" to see whether there would be any difference in stereoselectivity. Although it was hard to drive the reaction to completion after 29 h of heating, the product was sufficiently pure that it needed no distillation. A similar yield and stereoselectivity were obtained. The hydrolysis of **13** was prone to elimination of either one acetoxyl group to give **14** or both acetoxyl groups to give methyl benzoate.[28] Under the best conditions we found (0.5 *M* Na_2CO_3, MeOH/H_2O 2:1), **14** was still formed. Compound **15** was separated from its C-1 epimer by recrystallization, then it was subjected to a Mitsunobu reaction with *p*-nitrobenzoic acid[29] to invert the configuration of the C-5 hydroxyl group. Fluorination of **16** with (diethylamino)sulfur trifluoride (DAST)[30] gave a rearranged byproduct **17** in addition to desired **18**. It is known that fluorination of an allylic alcohol will give mixed products,[29] and the hydroxyl group in **17** seemed to be shielded from further fluorine attack by the adjacent *p*-nitrobenzoate ester. Conversion of **18** to **19** was unsuccessful with either sodium azide in refluxing methanol[31] (no reaction) or trimethylsilyl iodide (TMSI) generated *in situ* (Me_3SiCl, NaI, acetonitrile)[32] at room temperature (no reaction) or 80 °C (decomposition). The *p*-nitrobenzoate ester, however, was very labile under weakly basic conditions and was cleaved by 0.1 *M* sodium carbonate in a 2:1 mixture of methanol and water, although **14** was again obtained as the by-product. A mixture of **19** and **14** could not be separated by flash chromatography, so another Mitsunobu reaction with diphenylphosphoryl azide (DPPA)[33] was performed on the mixture, and the desired compound **20** could be separated by flash chromatography in pure form. Direct hydrolysis of the methyl ester in **20** failed with pig liver esterase[34] (no reaction), TMSI,[31] or weak bases like sodium carbonate or lithium hydroxide (elimination). The azido group is likely activating the allylic fluorine, but it could be reduced to the amine with triphenylphosphine in THF/water,[35] and **21** was successfully hydrolyzed under weakly basic conditions to afford **12**.

Inactivation of GABA-AT by 12

Incubation of GABA-AT with **12** showed time- and concentration-dependent inhibition with the following kinetic constants: k_{inact} = 0.52 min^{-1}, K_I = 0.93 mM, k_{inact}/K_I = 0.56 $mM^{-1}min^{-1}$. Exhaustive dialysis of the correspondingly inactivated GABA-AT against potassium pyrophosphate buffer (50 mM, pH 8.5, 1 L × 3, changed every 6 h) containing PLP (0.1 mM) and α-ketoglutarate (0.1 mM)[6a] resulted in only a 4% recovery of enzyme activity as compared to the control. Thus the inhibition is irreversible. The presence of GABA slowed down the rate of inactivation of GABA-AT by **12**, suggesting that **12** binds to the active site of the enzyme. Both the double bond and the fluorine are important to the inhibition properties of **12**. Compounds **2**, **3**, and 3-aminocyclohexanecarboxylic acid, i.e., **2** or **3** without the fluorines, are not inhibitors of GABA-AT, but **7**-**9**, containing unsaturation, are irreversible inhibitors. This is in contrast to the corresponding cyclopentane compounds, which bind well to the enzyme either with[12] or without[10] unsaturation. As compared to **7**, incorporation of a single allylic fluorine results in a dramatic change in the inactivation mechanism and a 129-fold increase in inhibitory activity toward GABA-AT.

Release of Fluoride Ion During Inactivation of GABA-AT by 12

By measurement of the fluoride ion concentration it was found that 2.5 fluoride ions were released per enzyme dimer inactivated, but no fluoride ions were released in the absence of enzyme. To exclude the possibility that the fluoride ions were released by a non-inhibitory interaction between **12** and the protein, **12** was incubated with human serum albumin at the same protein concentration. No fluoride ions were released, which suggests that the fluoride ions were released by GABA-AT during inactivation.

Mechanism of Inactivation of GABA-AT by 12

As indicated above, **12** is comprised of moieties derived from compounds **1**, **5**, **7**, and **8**. Compound **1** inactivates GABA-AT by a Michael addition mechanism;[36] **5**[10] and **7**[13] inactivate GABA-AT by enamine mechanisms, and **8**[14] inactivates by an aromatization mechanism. Therefore, three different inactivation mechanisms can be envisioned for the irreversible inhibition of GABA-AT by **12**: a Michael addition mechanism (Scheme 2), an enamine mechanism (Scheme 3), and an aromatization mechanism (Scheme 4). The enamine pathway produces inactivation without loss of a fluoride ion, whereas the other two mechanisms require fluoride ion release. As indicated above, about one fluoride ion was released per active site after inactivation, but no fluoride ions were released in the absence of GABA-AT or if human serum albumin was substituted for GABA-AT. These results exclude the enamine mechanism. The Michael addition and enamine pathways would lead to a covalently modified active site residue, whereas the aromatization pathway produces a modified coenzyme (**26**). Compound **7**, which inactivates GABA-AT by an enamine mechanism,[13] is a poor mechanism-based inactivator of GABA-AT (k_{inact} = 0.01 min^{-1}, K_I = 2.3 mM, k_{inact}/K_I = 0.004 $mM^{-1}min^{-1}$), being 129 times less active than **12**. Because of the similar structures and conformations of **7** and **12**, it is difficult to rationalize their large difference in activity if they inactivate GABA-AT through the same inactivation pathway. Elimination of the fluoride ion from **12** leads to an intermediate (**22**), which is the protonated form of an intermediate proposed in the aromatization mechanism by gabaculine[14] (**25**, Schemes 4 and 5) and produces the identical product as that from gabaculine inactivation (**26**).

Because of the potency of gabaculine (**8**) as an inactivator of GABA-AT (k_{inact} = 6.4 $min^{-}1$, K_I = 29 μm, k_{inact}/K_I = 221 $mM^{-1}min^{-1}$),[14] it was a concern that **12** might eliminate one equivalent of HF to give **8** under normal assay conditions (50 mM potassium pyrophosphate buffer, pH 8.5), and **8** might be the actual inactivating species of the enzyme rather than **12**. One would expect, however, that this elimination reaction might be much slower than the enzymecatalyzed process. The fact that no lag time is observed for the inactivation of GABA-AT by **12** suggests that conversion to **8** is not involved in the inactivation process. Other evidence comes from ^{19}F NMR studies and inactivation studies with **8**. A 30 mM solution of **12** in deuterium oxide in 50 mM of potassium pyrophosphate buffer (pH 8.5) was monitored for ^{19}F signals, and no fluoride ion was detected after 3 hours of standing at room temperature. A 3 mM solution of potassium fluoride in the same buffer was prepared as the external standard of fluoride ion. As this is a high concentration of fluoride ion, further confirmation was made that **12**, not **8**, is responsible for inactivation.

Enzymatic testing showed that a 0.011 mM concentration of **8** (t½ = 4.58 min) inactivates the enzyme to the same extent as a 0.40 mM concentration of **12** (t½ = 4.52 min). The UV absorbance of **8** was used to determine whether **8** is responsible for inactivation by **12**. The UV absorption spectra of **8** (dashed line) and **12** (solid line) are shown in Figure 2A. Although the UV absorption spectrum of **12** overlaps with that of **8**, the extinction coefficient of **12** at 276 nm (0.31 mM^{-1}cm-1) is much smaller than that of **8** (6.7 mM^{-1}cm-1). The formation of **8** from **12**, therefore, results in increased absorbance. The absorbance of different concentrations of **8** at 276 nm was measured to make a standard curve (Figure 2B). Based on Figure 2B, the absorbance of a 0.011 mM concentration of **8** (the concentration to give the observed half-life with a 0.40 mM concentration of **12**) is 0.074 unit. The UV absorbance of a 0.40 mM solution of **12** in potassium pyrophosphate buffer (pH 8.5), however, showed no significant change over 32 minutes, indicating that an insufficient amount of **8** is generated during the total time of the experiment to account for the inactivation rate observed. Taking into consideration that **12** inactivates the enzyme in several minutes, it can be concluded that the inactivation of the enzyme by **12** is not caused by conversion to **8** prior to inactivation.

An aromatization mechanism was supported by HPLC and mass spectral analysis of the inactivated mixture of GABA-AT by **12** compared to a synthetic standard of the expected aromatization product[14b] (**26**, Figure 3). There appears to be only one major product formed from the inactivation of GABA-AT by **12** (T_R = 12.42 min), and it coelutes with synthetic **26**. Mass spectral analysis of this peak indicated that is has the identical elemental composition and product ion spectrum as synthetic **26** (Figure 4). This is consistent with the behavior previously observed for inactivation of GABA-AT by gabaculine; formation of **26** produces a tight-binding complex that is stable to dialysis and gel filtration.[14]

In conclusion, it was demonstrated that a certain degree of unsaturation is necessary for the cyclohexane-containing conformationally restricted analogues of GABA to bind well to GABA-AT and to cause irreversible inhibition. Compound **12**, a composite structure of **1**, **5**, **7**, and **8**, which inactivate GABA-AT by three different mechanisms, appears to inactivate GABA-AT (Scheme 4) by a composite of elimination (as demonstrated by loss of fluoride ion) and aromatization (as evidenced by the production of *N*-*m*-carboxyphenylpyridoxamine 5'phosphate (**26**), the same product generated by inactivation of GABA-AT with **8**).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

DIAD, diisopropylazodicarboxylate; DPPA, diphenylphosphoryl azide; GABA, γ-aminobutyric acid; GABA-AT, γ-aminobutyric acid aminotransferase; TMSI, trimethylsilyl iodide; TFA, trifluoroacetic acid.

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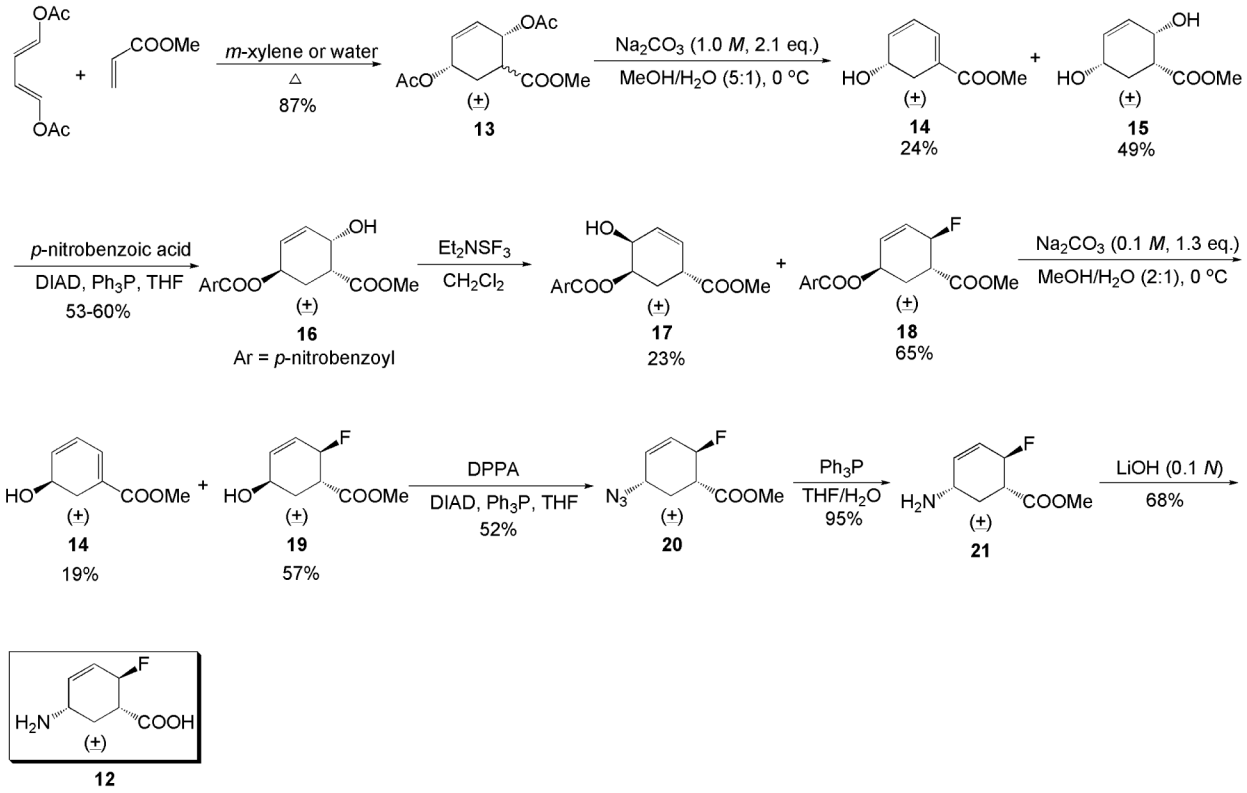
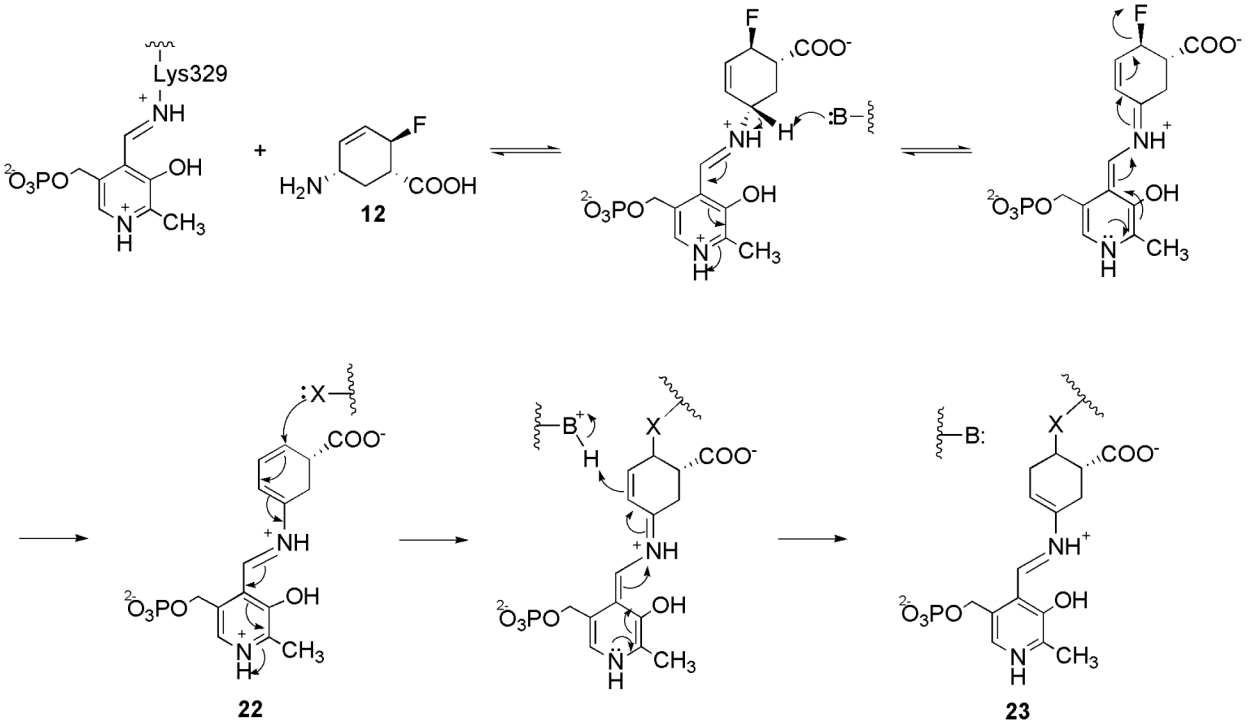
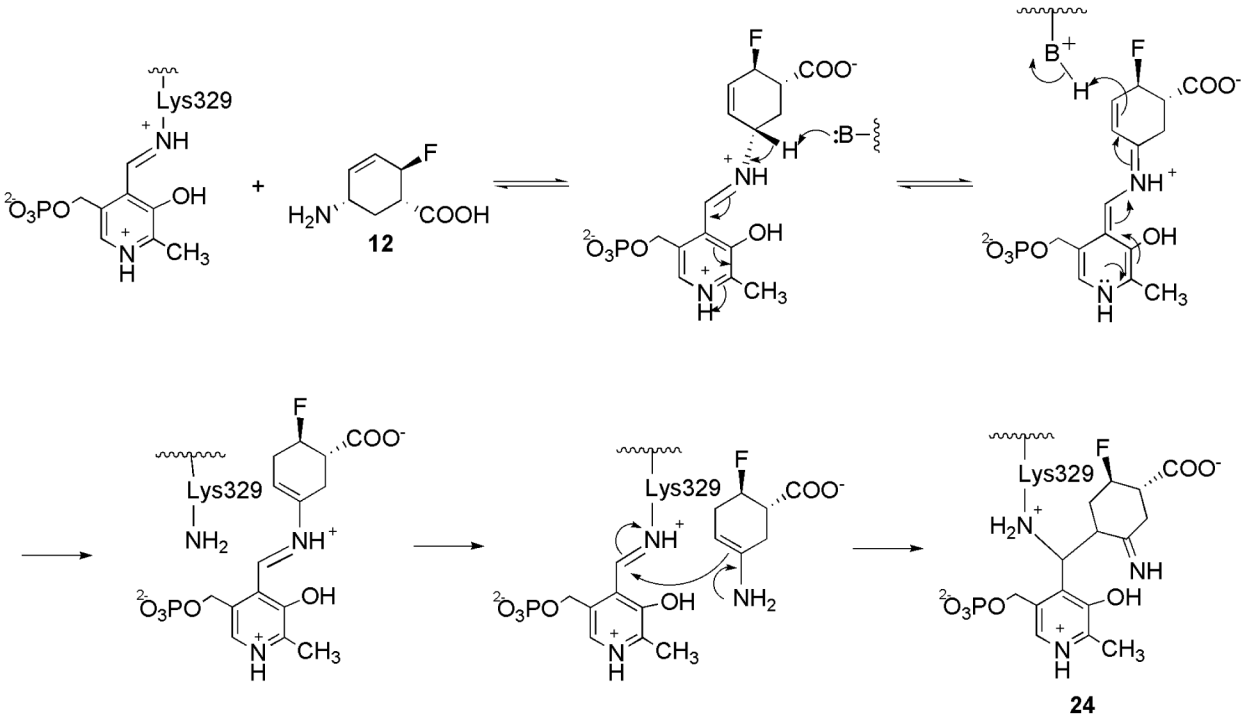


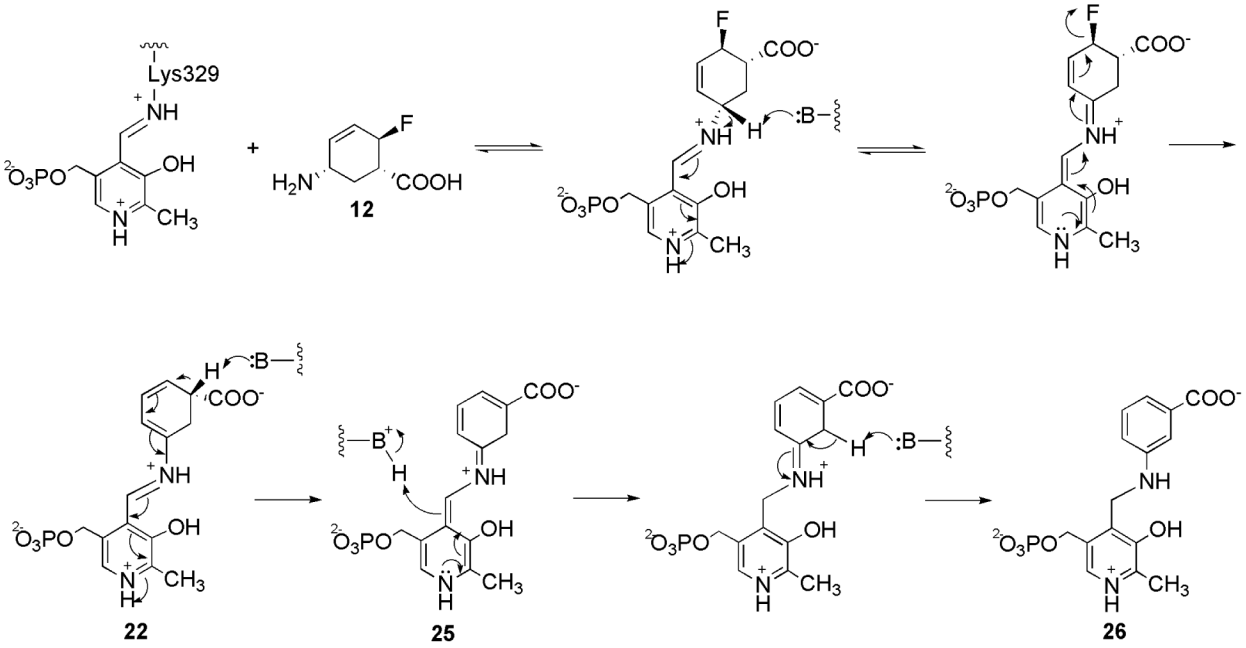
Scheme 1.
Synthesis of **12**.



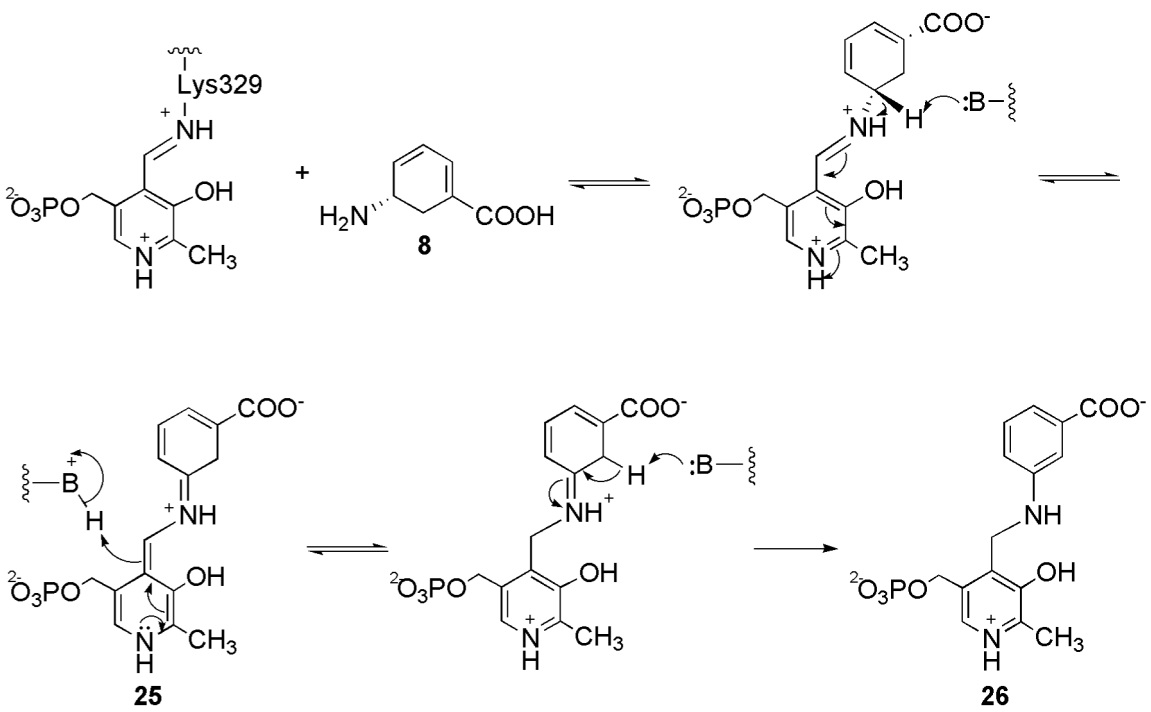
Scheme 2.
Potential Michael addition inactivation mechanism of GABA-AT by **12**



Scheme 3.
Potential enamine inactivation mechanism of GABA-AT by **12**



Scheme 4.
Potential aromatization inactivation mechanism of GABA-AT by **12**



Scheme 5.
Aromatization mechanism of inactivation of GABA-AT by gabaculine (**8**)

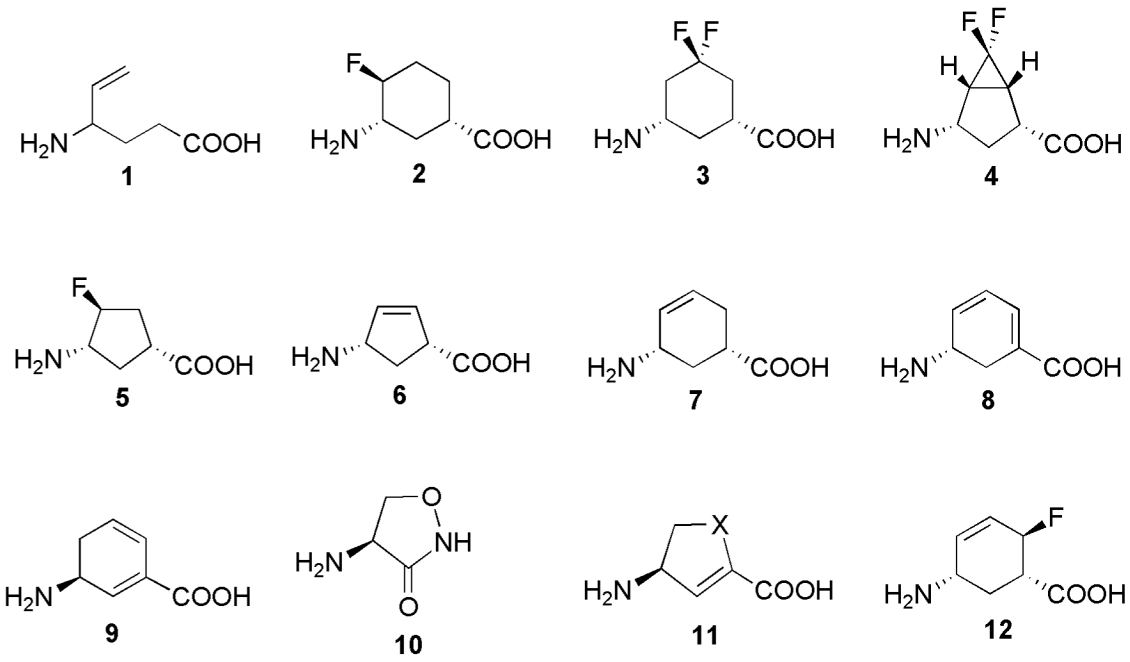


Figure 1.
GABA analogues

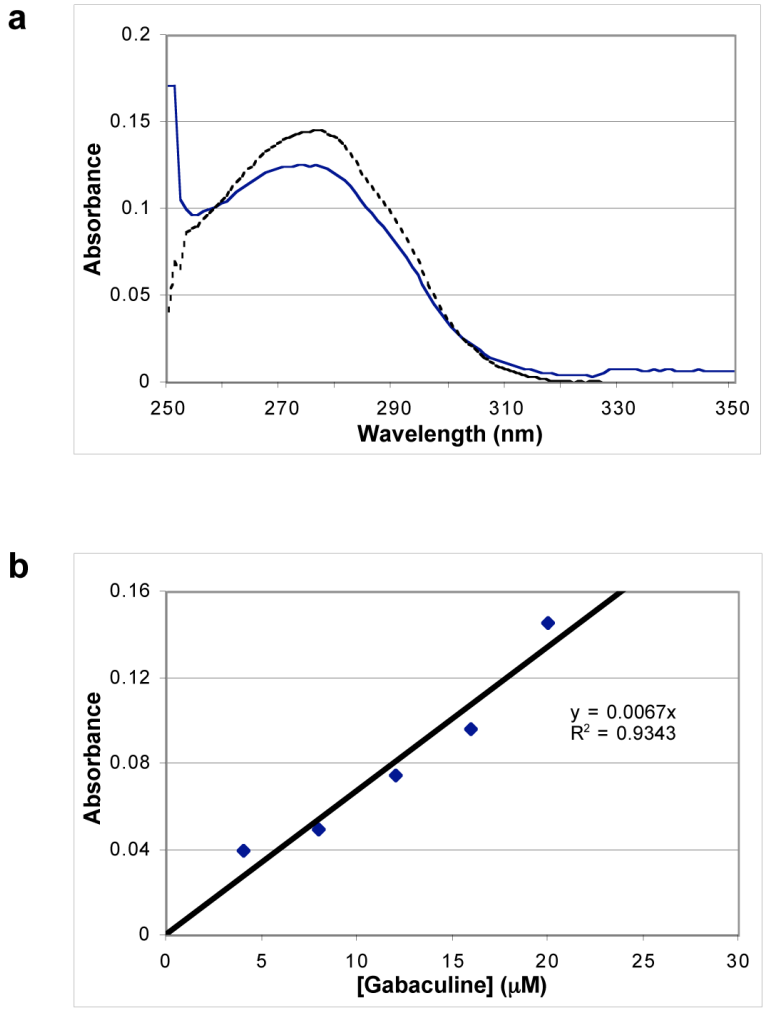


Figure 2.
(A) UV absorption spectra of **8** (dashed line) and **12** (solid line). (B) Plot relating UV absorbance at 276 nm with gabaculine concentration

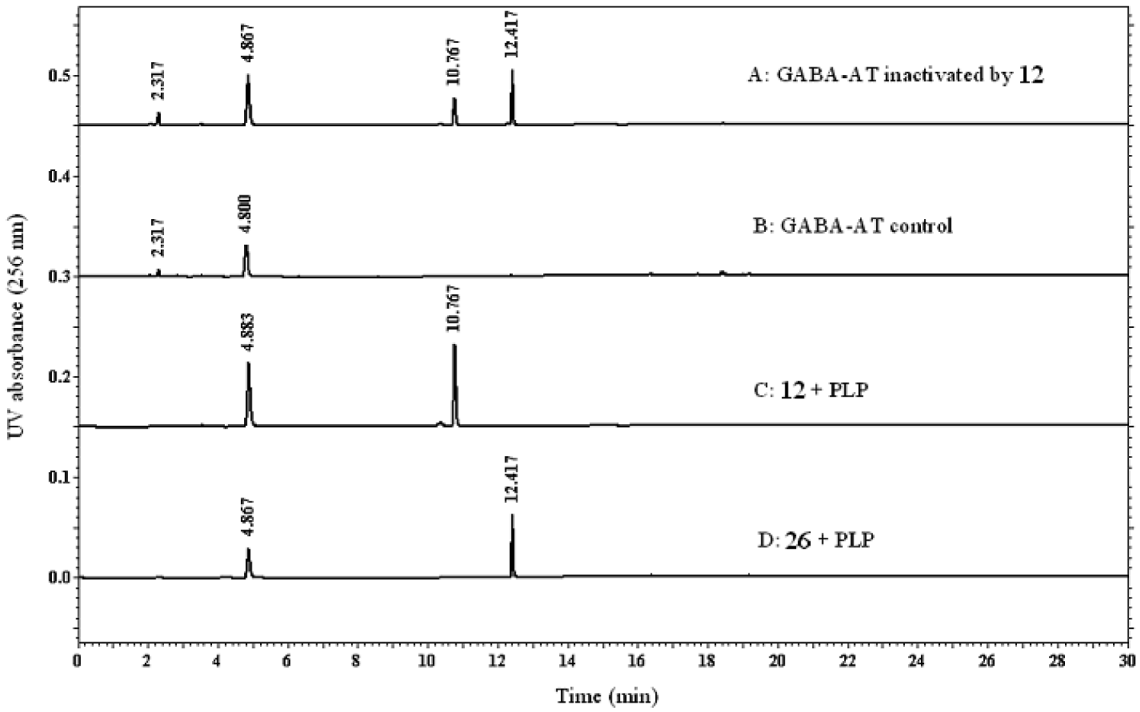


Figure 3.

HPLC analysis of the inactivation product of GABA-AT by **12**. The absorption peaks correspond to the standards: PLP (4.87 min), **12** (10.77 min), and **26** (12.42 min). PLP was added as the internal standard. See Experimental for details.

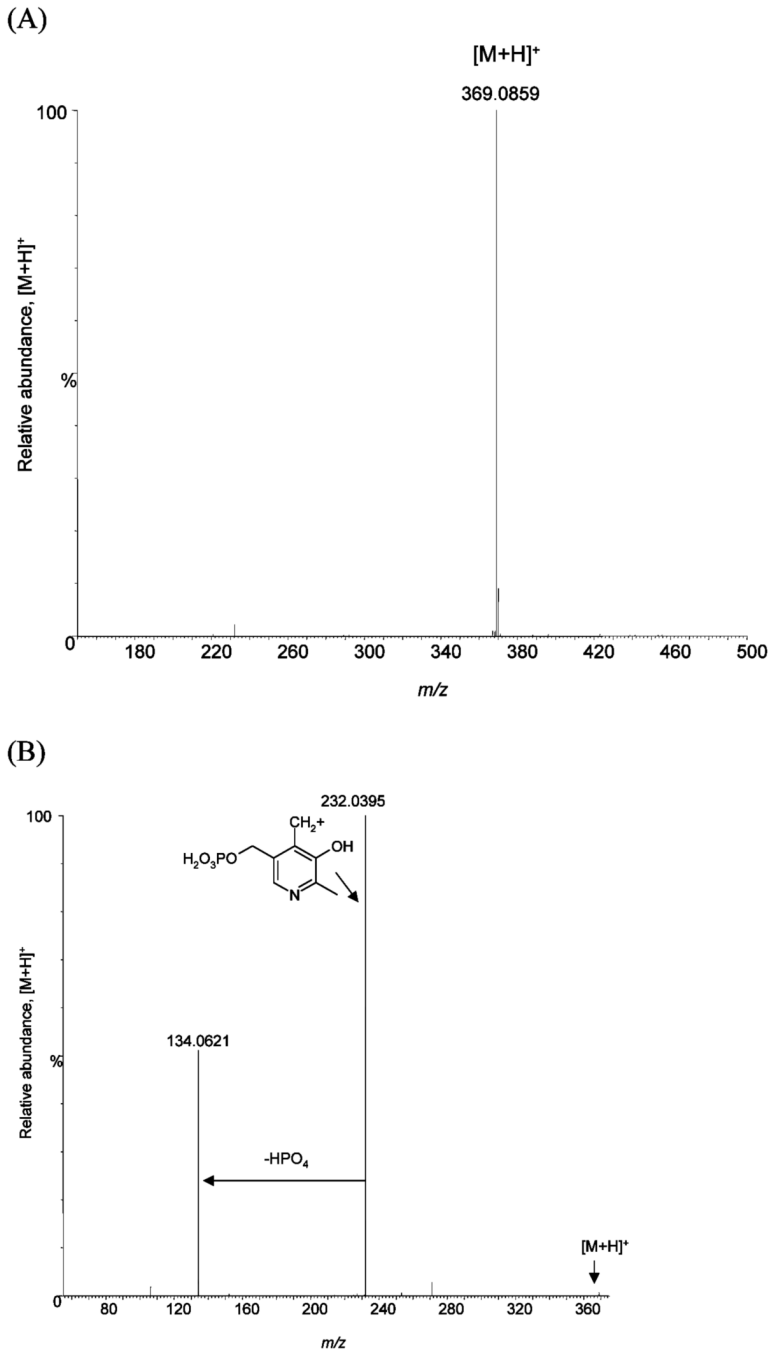


Figure 4.

(A) Positive ion electrospray mass spectrum of the PLP adduct from inactivated GABA-AT

(B) Product ion tandem mass spectrum of the PLP adduct from inactivated GABA-AT