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4-(OXOALKYL)-SUBSTITUTED GABA ANALOGUES AS INACTIVATORS AND SUBSTRATES OF GABA AMINOTRANSFERASE

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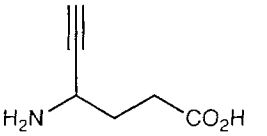
On the basis of the mechanism of inactivation of γ-aminobutyric acid (GABA) aminotransferase by γ-ethynyl GABA that was reported recently (Burke and Silverman, manuscript submitted), three 4-(oxoalkyl)-substituted GABA analogues, 4-amino-5-oxohexanoic acid (**7**), 4-amino-5-oxopentanoic acid (**8**), and 4-amino-6-oxohexanoic acid (**9**) were synthesized and tested as inactivators. Only **8** was an inactivator of the enzyme. A mechanism for the inactivation by **8** is proposed as well as rationalizations for the lack of inactivation by **7** and **9**.

KEY WORDS: γ-Aminobutyric acid, γ-aminobutyric acid aminotransferase, 4-amino-5-oxohexanoic acid, 4-amino-5-oxopentanoic acid, 4-amino-6-oxohexanoic acid, enzyme inactivation.

INTRODUCTION

When brain levels of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) fall below a critical amount, convulsions can occur.[1-5] One of the treatments of epileptic convulsions, therefore, has focused on the inhibition of the enzyme responsible for the degradation of GABA, namely, GABA aminotransferase (EC 2.6.1.19). Selective inhibition of this enzyme has been shown to raise brain GABA levels and provide protection against seizures[6,7] γ-Ethynyl GABA (**1**) is a mechanism-based inactivator of GABA aminotransferase,[8-11] and our detailed investigation of its mechanism[11]



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indicated that the inactivation of GABA aminotransferase by γ-ethynyl GABA proceeds through attack of the ε-amino group of an active-site lysine residue into reactive allene. **3** (Scheme 1). This leads to the enamine **4**, which is in equilibrium with imine **5** and conjugated enamine **6**; the latter is believed to be responsible for the covalently modified irreversibly-inactivated enzyme adduct.[11] On the basis of the proposed intermediate **5**, it was reasoned that the active-site lysine may be able to condense with the carbonyl moiety of a series of γ-(oxoalkyl)-substituted GABA analogues, namely, 4-amino-5-oxohexanoic acid (**7**), 4-amino-5-oxopentanoic acid

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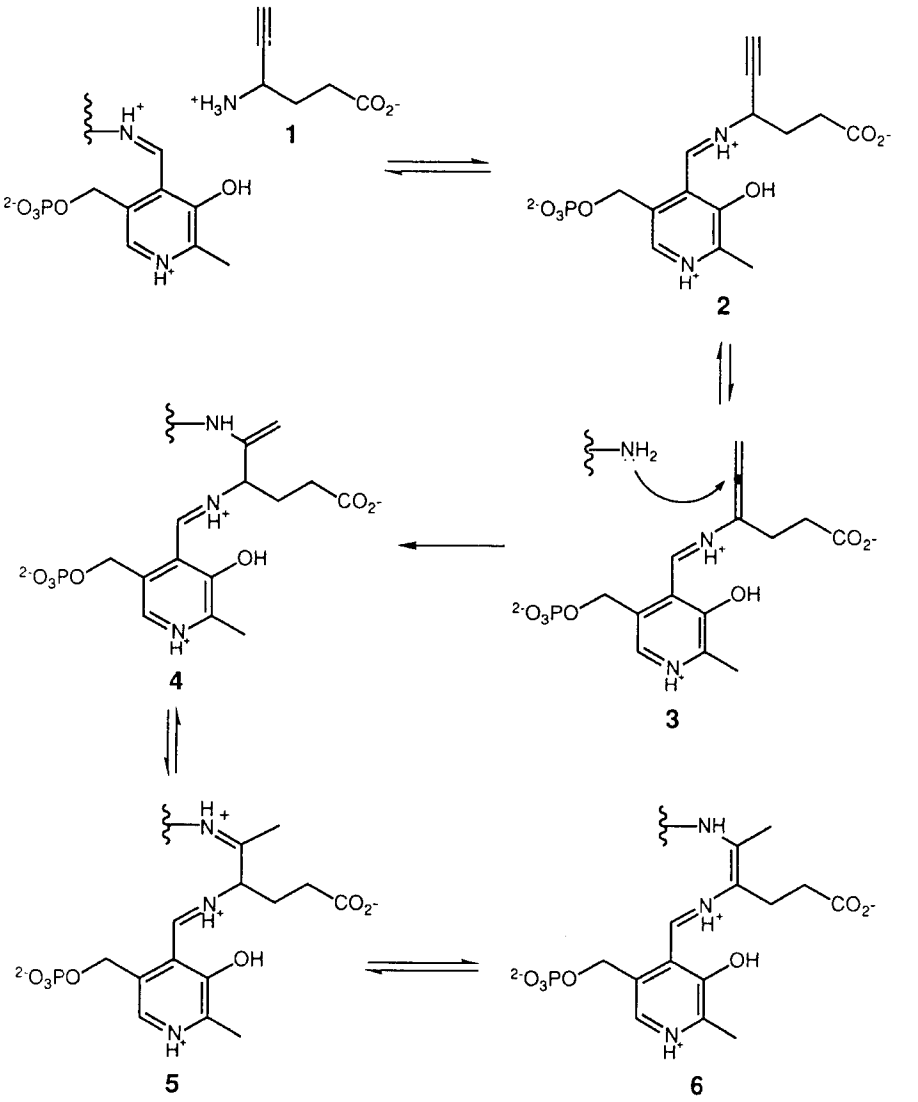
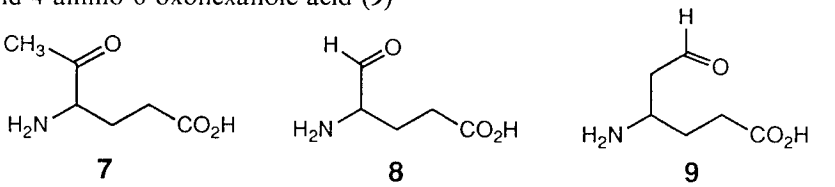


SCHEME 1 Proposed mechanism of inactivation of GABA aminotransferase by γ-ethynyl GABA (Note: the carbon–carbon double bonds shown are not meant to imply E or Z configuration).

(**8**), and 4-amino-6-oxohexanoic acid (**9**)



once these compounds form the Schiff base with the active site pyridoxal phosphate. Each of these molecules may form an imine with the active site lysine residue which

then may be able to tautomerize to a stable enamine, thereby resulting in inactivated enzyme. In addition, this may give an indication of the maximum distance from C-4 of a GABA backbone structure that, in general, an electrophilic species can be located for reaction with the active-site lysine. The results with these compounds as potential inactivators of GABA aminotransferase are described here.

MATERIALS AND METHODS

Analytical Methods

GABA aminotransferase assays were recorded on a Perkin-Elmer Lambda 1 spectrophotometer. An Orion Research Model 601 pH meter with a general combination electrode was used for pH measurements. Radioactivity was measured by liquid scintillation counting using a Beckman LS-3133T counter and Research Products International 3a70b scintillation cocktail. [^{14}C]-Toluene (4×10^5 dpm/ml) from New England Nuclear was used as an internal standard.

Reagents

The syntheses of 4-amino-5-oxohexanoic acid hydrochloride (**7**),[12] 4-amino-5-oxopentanoic acid hydrochloride (**8**),[13] and 4-amino-6-oxohexanoic acid hydrochloride (**9**),[11] were previously reported. γ-Aminobutyric acid, α-ketoglutarate, and β-mercaptoethanol were purchased from Sigma. [5-^{14}C]-α-Ketoglutarate (24.2 mCi/mmol) was obtained from Amersham.

Enzymes and Assays

Pig brain GABA aminotransferase was purified to homogeneity using the published procedure.[14] Succinic semialdehyde dehydrogenase was isolated from GABAse (Boehringer Mannheim Biochemicals) using the method of Jeffery *et al.*[15] All buffers and enzyme solutions were prepared with deionized distilled water. Unless noted, GABA aminotransferase activity was assayed using a modification of the coupled assay of Scott and Jakoby.[16] The assay solution had final concentrations of 10 mM GABA, 1 mM NADP, 5 mM α-ketoglutarate, 5 mM β-mercaptoethanol and excess succinic semialdehyde dehydrogenase 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 aminotransferase activity.

*Time-Dependent Inactivation of GABA Aminotransferase with 4-Amino-5-Oxopentanoic Acid (**8**)*

Solutions of GABA aminotransferase (0.14 nM) in 100 mM potassium phosphate buffer containing 4 mM α-ketoglutarate, 1 mM β-mercaptoethanol, and varying concentrations of **8** (0, 5, 10, 15, 25, 35, and 50 mM) were incubated at 25°C at pH 7.4. Aliquots were periodically assayed for enzyme activity. A plot of $[I]t_{1/2}$ versus [I] was used to determine the kinetic constants k_{inact} (the first-order rate constant for conversion of E-I to inactive enzyme) and K_I (the apparent dissociation constant) from the slope ($In2/k_{inact}$) and the x-intercept ($-K_I$). This plot is analogous to the Hanes plot

for normal enzyme-substrate kinetics and has the same advantages over the common Kitz and Wilson[17] plot that a Hanes plot has over a Lineweaver-Burk plot.[18,19]

GABA Protection of GABA Aminotransferase from Inactivation by (**8**)

To a solution of 600 μl of 100 mM potassium phosphate containing 50 mM GABA, 50 mM **8**, 4 mM α-ketoglutarate, and 1 mM β-mercaptoethanol at pH 7.4 was added GABA aminotransferase (86.4 pmol, 20 μl). A control was prepared that was identical except that no GABA was present. After incubation at 25°C for 14 h, the samples were assayed for enzyme activity.

γ-(Oxoalkyl)-GABA Analogues as Substrates for GABA Aminotransferase

Solutions of GABA aminotransferase (0.14 nM) in 100 mM potassium phosphate buffer containing 3.8 mM [5-^{14}C]-α-ketoglutaric acid (sp. act. 1.45 mCi/mmol), 1 mM β-mercaptoethanol, and varying concentrations of **7**, **8**, or **9** (0, 2.5, 4.0, 7.0, 11.0, 16.0, 22.0 mM) in a total volume of 200 μl were incubated for 2 h at 25°C. After being quenched with 25 μl of 20% trichloroacetic acid, the [5-^{14}C]-glutamate produced was isolated as previously reported[20] and the radioactivity content measured by liquid scintillation counting. GABA also was assayed under these conditions as described except the solutions (0, 0.18, 0.48, 0.75, and 1.08 mM GABA) were only incubated for 40 min before being quenched with trichloroacetic acid. Kinetic constants (K_M and k_{cat}) were obtained for all four substrates from Hanes plots.[11]

RESULTS AND DISCUSSION

Of the three γ-oxoalkyl analogues (**7**, **8**, and **9**), only **8** showed time- and concentration-dependent inactivation of GABA aminotransferase, although the inactivation was only pseudo-first order through the first 20% of inactivation after which, presumably,

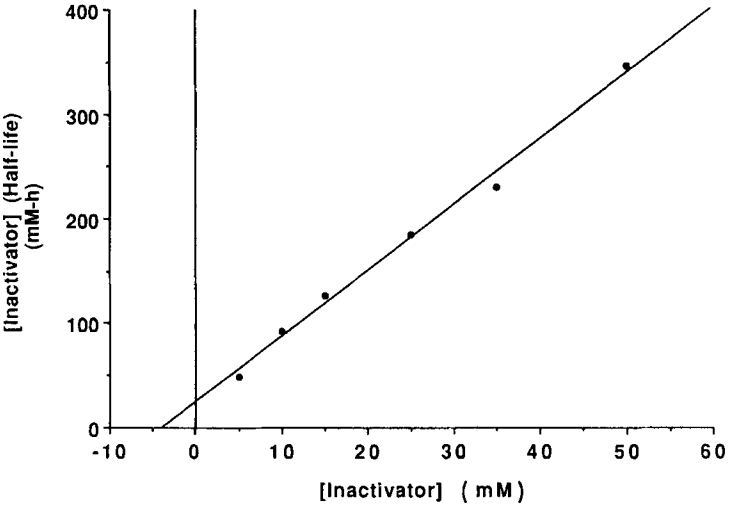
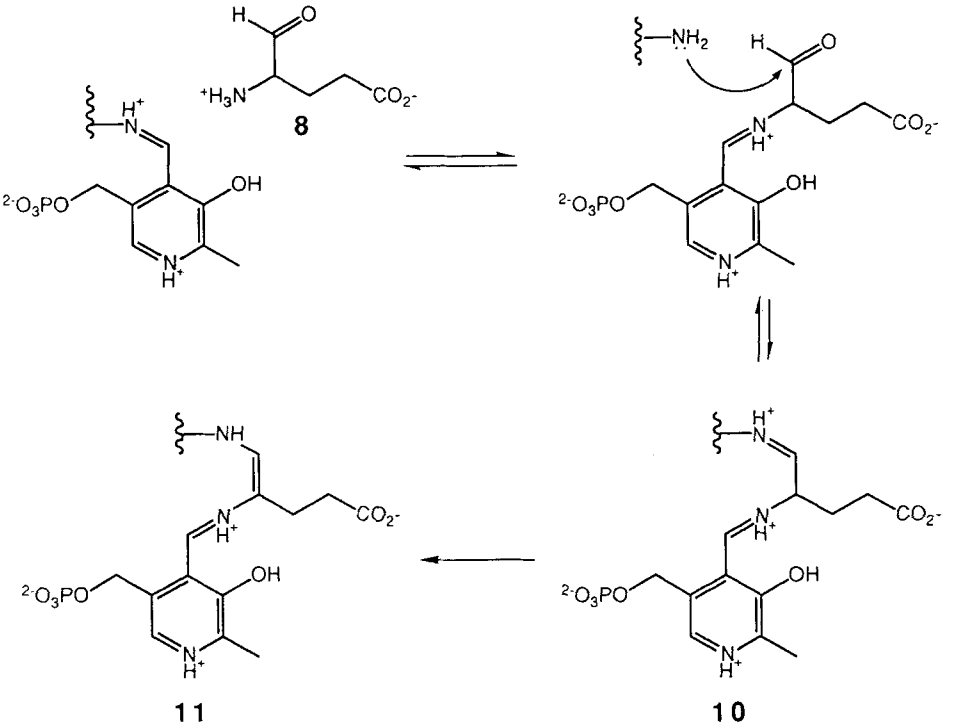


FIGURE 1 Plot of the [Inactivator] versus [Inactivator] (t1/2) for the inactivation of GABA aminotransferase by **8**.

TABLE I
Kinetic constants for GABA and γ-(oxoalkyl)-substituted GABA analogues as substrates for GABA aminotransferase. See Experimental section for details

Substrate	K_M (mM)	k_{cat} (min^{-1})
GABA	0.8	8.9
7	1.5	0.12
8	21.7	0.91
9	22.5	0.72

a metabolite of the inactivator is formed in high enough concentrations so that it competes with the inactivator for the active site.[21] A plot of the [**8**] versus [**8**]$t_{1/2}$ (Figure 1) was used to determine the kinetic constants for the inactivation of GABA aminotransferase by **8**; the initial apparent dissociation constant (K_I) and the first-order rate constant (k_{inact}) for conversion of the E-I complex to inactive enzyme were determined to be 4.0 mM and 0.0018 min^{-1}, respectively. When GABA aminotransferse was incubated with 50 mM **8** in the presence of 50 mM GABA, the enzyme retained all of its activity after 14 h, while a control without GABA present had only 29% of its original activity. This prevention of inactivation by **8** suggests that inactivation by **8** is active-site directed. The irreversible nature of the inactivation was

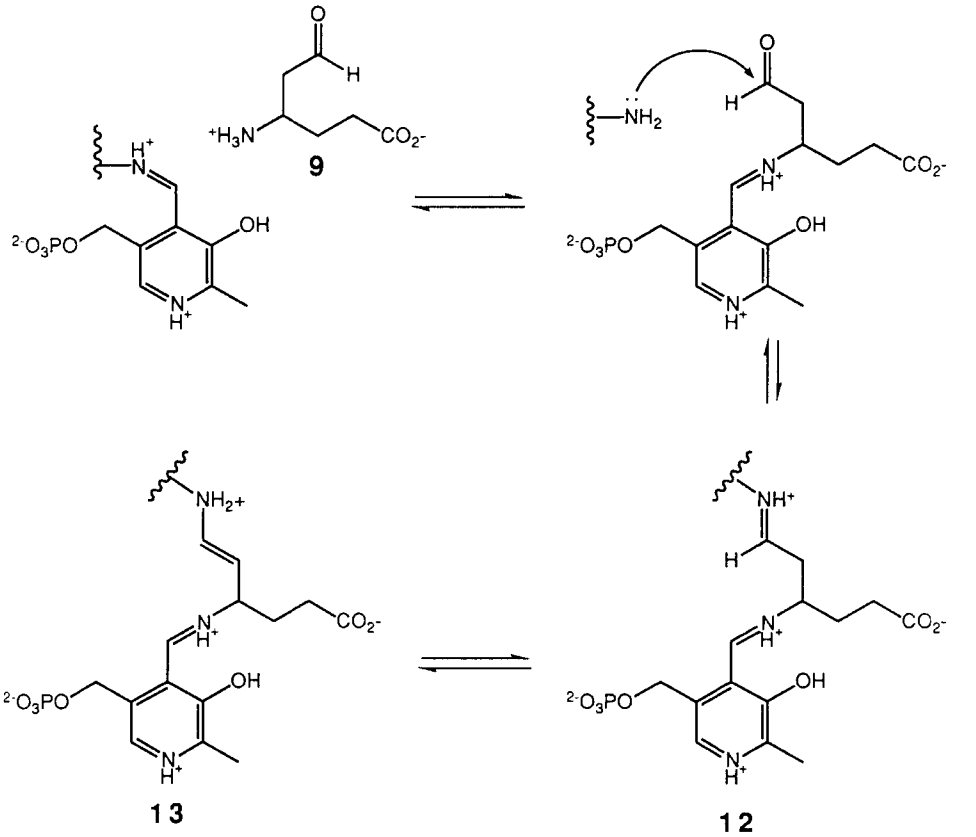


SCHEME 2 Proposed mechanism of inactivation of GABA aminotransferase by **8** (note: the carbon–carbon double bonds shown are not meant to imply E or Z configuration).

indicated by the finding that GABA aminotransferse, which had been inactivated with **8**, did not regain any enzyme activity upon extensive dialysis.

All three compounds were shown to be substrates for GABA aminotransferase with kinetic constants as shown in Table I. For comparison, the kinetic constants for GABA under these conditions also were determined. Since all three compounds are substrates, they are getting into the active site and combining with the cofactor. The fact that only **8** is an inactivator would seem to indicate that the active-site lysine residue is only able to condense with the carbonyl of **8** to become covalently attached in an irreversible manner. Since **7** also should be expected to condense with the active-site lysine in the same way, the fact that it does not inactivate the enzyme indicates that once **7** has condensed with the cofactor, either the ketone carbonyl is not in the proper orientation for condensation with the free lysine residue, that the ketone, being more stable than the corresponding aldehyde, is not reactive enough to condense with the lysine residue, or that the equilibrium between the ketone and the imine lies far in favor of the unbound ketone.

The fact that **9** is not an inactivator of GABA aminotransferase may suggest that its aldehyde carbonyl is too far removed to condense with the active-site lysine



SCHEME 3 Possible condensation between an active-site lysine residue and the carbonyl of **9** (note: the carbon–carbon double bonds shown are not meant to imply E or Z configuration).

residue. Another explanation for why **8** inactivates the enzyme whereas **9** does not, and one which is consistent with the proposed mechanism for inactivation of GABA aminotransferase by γ-ethynyl GABA,[11] can be seen by comparing the enamine tautomers that would result from condensation between the aldehyde carbonyl groups of these compounds and the active-site lysine residue (see Schemes 2 and 3). The imine **10**, formed from **8** (Scheme 2), could tautomerize readily into the enamine **11**, which strongly resembles the stabilized putative inactivated enzyme adduct **6** (Scheme 1). Such a highly stabilized enamine may be formed essentially irreversibly and, therefore, give an inactivated enzyme. If the imine **12** is formed from **9** (Scheme 3), tautomerization into the enamine **13** would not be stabilized by conjugation with the cofactor and, therefore, would not be expected to be irreversible. These results are consistent with the mechanism of inactivation of GABA aminotransferase by γ-ethynyl GABA previously proposed.[11]

Acknowledgment

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