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Inactivation of GABA transaminase by 4-acryloylphenol

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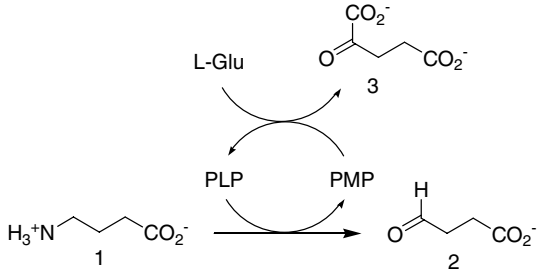
Abstract—Previous study showed that 4-hydroxybenzaldehyde is a competitive inhibitor of GABA transaminase. As a result, 4-acryloylphenol was synthesized as a 4-hydroxybenzaldehyde analogue, and shown to inactivate potently the enzyme in a time-dependent manner. The inactivation was protected by α-ketoglutarate, indicating that it occurs at the active site of the enzyme. β-Mercaptoethanol also prevented the enzyme from inactivation. The possible mechanism involving a Michael addition was proposed to rationalize the inactivation.

γ-Aminobutyric acid (GABA, **1**) is the major inhibitory neurotransmitter in the mammalian central nervous system.[1] The major pathway for its degradation is via transamination with α-ketoglutarate (**3**) catalyzed by the pyridoxal 5′-phosphate (PLP)-dependent enzyme GABA transaminase (GABA-T, E.C. 2.6.1.19) (Scheme 1).[2] GABA-T has been considered as a target for neuroactive drugs,[3,4] because its inhibition in brain tissues increases the concentration of GABA and could have therapeutic applications in neurological disorders including epilepsy, Parkinson's disease, Huntington chorea, and Alzheimer's disease.[5] Selective inactivation by vigabatrin, a mechanism-based inhibitor of the enzyme,[6] is already successfully applied in treatment of epilepsy.[7] Silverman and co-workers have reported a great lot of GABA-T inactivators and rationalized their inactivation mechanisms for decades. So far most of the GABA-T inhibitors are GABA analogues without benzene ring, mainly including vigabatrin (**4**) analogues,[5,8,9] 4-amino-5-halopentanoic acid (**5**) analogues,[10] and (*Z*)-4-amino-2-butenoic acid (**6**) analogues.[11] Vigabatrin analogues inactivate the enzyme by two pathways: a Michael addition mechanism (Scheme 2, pathway a) and an enamine mechanism (pathway b).[8] The mechanism of the inactivation of GABA-T by **5** was shown to proceed only via an enamine pathway.[10] Moreover, hydrazine analogues, a class of general inhibitors of PLP-dependent enzymes, were found to be potent GABA-T inhibitors.[12]



Scheme 1. The reaction catalyzed by GABA-T.

It has previously been showed that 4-hydroxybenzaldehyde (**7**) potently inhibits GABA-T.[13] Recently, based on the result, we found that the compound gives competitive inhibition of GABA-T with respect to α-ketoglutarate.[14] Our results indicated that 4-hydroxybenzaldehyde is similar to α-ketoglutarate and succinic semialdehyde (**2**) in structure, suggesting that replacement of the carboxylic acid by the phenol moiety is accepted by the enzyme. Consequently, we attempt to design novel 4-hydroxybenzaldehyde analogues as GABA-T inhibitors. As a result of the effort, 4-acryloylphenol (**8**, Scheme 3), a novel 4-hydroxybenzaldehyde analogue, was synthesized and preliminarily assayed, exhibiting more inhibitory potency than 4-hydroxybenz-

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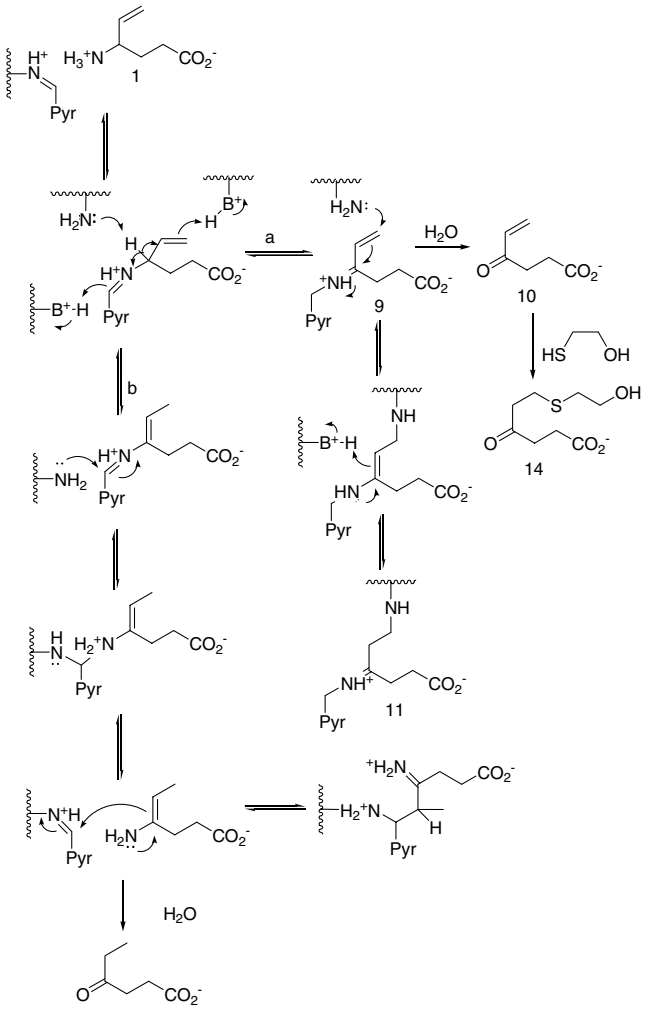
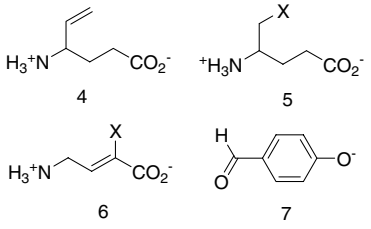
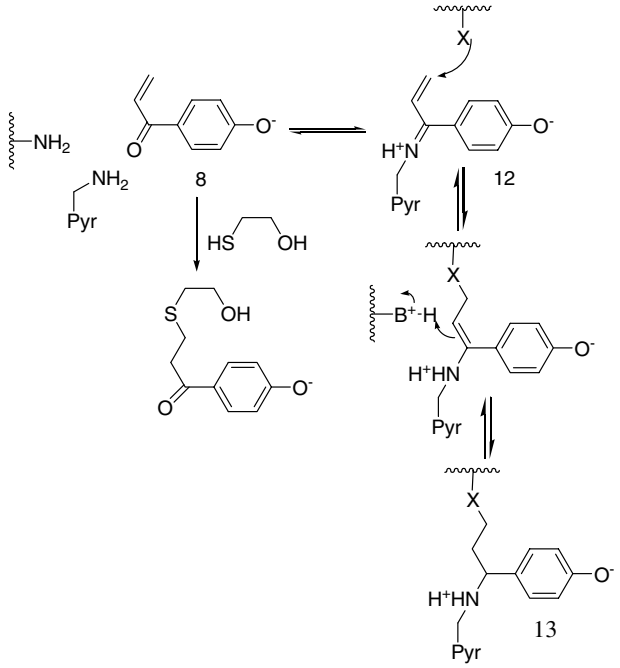


Scheme 2. Mechanism of inactivation of GABA-T by vigabatrin.



aldehyde.[14] In this paper, we further study inactivation of GABA-T by **8**.

GABA-T and succinic semialdehyde dehydrogenase (SSADH) were isolated from rat brains by a modified procedure,[11,15] respectively. Time-dependent inactivation of GABA-T by **8** was carried out using a modified assay of Qiu and Silverman.[10] GABA-T (0.008 unit) was added to solutions of **8** (120 μL in final, with varied concentrations of **8**), in 100 mM potassium pyrophosphate buffer, pH 8.5, containing 5 mM GABA at 25 °C. At timed intervals, aliquots (30 μL) were withdrawn and added to the assay solution (1 mL) in 100 mM potassium pyrophosphate, pH 8.5, containing 5 mM GABA, 5 mM α-ketoglutarate, 0.5 mM NAD^+, and excess SSADH. Amount of NADH generated was measured



Scheme 3. Possible mechanism of inactivation of GABA-T by **8**.

by a HITACHI FL-4500 Fluorescence Spectrophotometer (excitation 355 nm, emission 459 nm). The logarithm of the remaining activity was plotted against time for each concentration of inhibitor. A secondary plot of $t_{1/2}$ obtained from the first plot versus 1/[inactivator] was constructed to determine K_I and k_{inact} values[16] for the inactivator.

Compound **8** potently inhibited GABA-T in a time-dependent and concentration-dependent manner (Fig. 1, $K_I = 470$ μM, $k_{inact} = 0.061\ min^{-1}$, and $k_{inact}/K_I = 0.129\ mM^{-1}\ min^{-1}$). Surprisingly, K_I value is so low, indicating that the phenol moiety has even a stronger affinity for the active site of the enzyme than the carboxylic acid of its substrate. The results are supported by the fact that (3-hydroxybenzyl)-hydrazine inhibits more potently GABA-T than methylhydrazine.[12]

Inactivation by **8** was prevented by addition of α-ketoglutarate to the incubation, indicating that the inactivation occurs at the active site and **8** is bound to

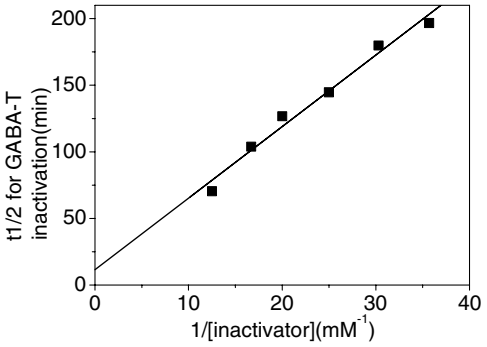


Figure 1. Time-dependent inactivation of GABA-T by **8**.

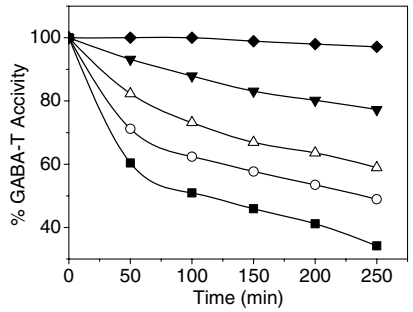


Figure 2. Effect of α-ketoglutarate and β-mercaptoethanol on the inactivation rate of GABA-T by 60 μM **8** (■), 60 μM **8** and 10.0 (○), 20.0 mM (△) α-ketoglutarate, 3.0 (▼), and 6.0 (♦) mM β-mercaptoethanol.

PMP form of the coenzyme to form Schiff base **12** (Fig. 2). So far, most of the published GABA-T inactivators are bound to PLP form of the enzyme as GABA analogues. Like **7**, however, **8** appears to be an analogue of α-ketoglutarate. Furthermore, as shown in Figure 2, addition of β-mercaptoethanol to the incubation of **8** with the enzyme led to a significant decrease in inactivation rate. **8** was possibly chemically trapped in the presence of β-mercaptoethanol, resulting in the protection from inactivation (Scheme 3). Inactivation of GABA-T with **8** followed by removal of excess inactivator using gel filtration results in no return of enzyme activity, indicating that the inactivation is irreversible. However, **7** is a reversible inhibitor, and major difference in inhibitory manner resulting from introduction of a vinyl group is very interesting and important.

The Michael addition mechanism of inactivation by vigabatrin may be used in rationalization of the inactivation by **8**. In the Michael addition mechanism, vigabatrin undergoes azaallylic isomerization via the normal catalytic process of GABA-T, leading to a reactive intermediate (**9**, Scheme 2). This intermediate is hydrolyzed partly to give the transamination product **10**. The remainder undergoes nucleophilic attacked to give adduct **11**. Compound **10** also may be attack on by the active-site nucleophile to result in adduct **11** prior to its release from the active site.[8] Pan et al. also reported that (1s, 3s)-3-amino-4-methylenyl-1-cyclopentanoic acid could be catalyzed by GABA-T to yield an α,β-unsaturated ketone, which may return to the active site of the enzyme and become covalently attached to the enzyme, leading to the enzyme's inactivation.[9] By comparisons of the structures between **2** and **7**, and between **10** and **8**, it was concluded that **8** is similar to **10** in structure. Thus, like **10**, **8** is a potent electrophile and a Michael acceptor, so that it may be attacked on by the active-site nucleophile to result in formation of adduct **13** after **8** reacted with the coenzyme to form Schiff base **12**. Since **10** may be converted into adduct **14** in the presence of β-mercaptoethanol,[17] it is obvious that **8** can be trapped by the nucleophile via a Michael addition. The conjugative effect of the benzene ring may result in low K_I value of **8**, according to **7**, but decrease the electrophilic reactivity of **8**, thus leading to low k_{inact} value of **8**. We will further investigate its metabolites to confirm the presumed mechanism.

It should be noted that based on the present and previous results, the phenol moiety may mimic the carboxylic acid functionality in the case of α-ketoglutarate or GABA binding to GABA-T. Introduction of a phenol moiety may not only result in a stronger affinity for GABA-T, but also increase lipophilicity and rigidity of drug candidates. GABA is a small polar and hydrophilic molecule and does not cross the blood–brain barrier, but GABA-T inhibitors that are slightly more lipophilic than GABA and thus do cross the blood–brain barrier, such as vigabatrin, may elevate GABA concentration in brain. Our results suggest that introduction of a phenol moiety might be very useful to design GABA-T inhibitors that could be more effective at crossing the blood–brain barrier. Moreover, design of conformationally rigid analogues of vigabatrin has been an active area of research in recent years because vigabatrin was found to be associated with the irreversible visual field defects[18] and is catalyzed by GABA-T to produce a potent electrophile.[8] Apparently, introduction of the phenol moiety may increase the conformational rigidity of its inhibitors. Substitution of a phenol moiety for the carboxylic acid of vigabatrin is probably an interesting and worthy work.

GABA analogues inactivating GABA-T could potentially inhibit pyridoxal 5′-phosphate (PLP)-dependent glutamate decarboxylase (GAD) and thus inhibit synthesis of GABA. Gabaculine, for example, an irreversible inactivator of GABA-T,[19] also inactivates potently GAD.[20] Therefore, structural analogues of succinic semialdehyde (2) are useful as selective inactivators of GABA-T. So far, however, only two analogues of succinic semialdehyde, 3,5-dioxocyclohexanecarboxylic acid[21] and 5-fluoro-4-oxopentanoic acid,[22] were found to inactivate only the PMP form of the enzyme. Although **8** has some potentially side and toxic effects as a Michael acceptor, these results may give a clue to the design of a novel class of GABA-T inhibitors that inactivate only the PMP form of the enzyme.

In summary, 4-acryloylphenol was found to inactivate potently the enzyme in a time-dependent manner. The inactivation was protected by α-ketoglutarate, indicating that it is active site-directed. The presumed mechanism involving a Michael addition was proposed to elucidate the inactivation. The present work is the first successful attempt to design GABA-T inactivator using 4-hydroxybenzaldehyde as a lead compound. The results also suggested future directions for the design of more potent GABA-T inhibitors.

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