Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 7, No. 4, pp. 457-462, 1997

0960-894X/97 $17.00 + 0.00

PII: S0960-894X(97)00042-5

MECHANISM-BASED INACTIVATION OF L-PIPECOLATE OXIDASE BY A SULFUR-CONTAINING SUBSTRATE ANALOG, 5-THIA-L-PIPECOLIC ACID

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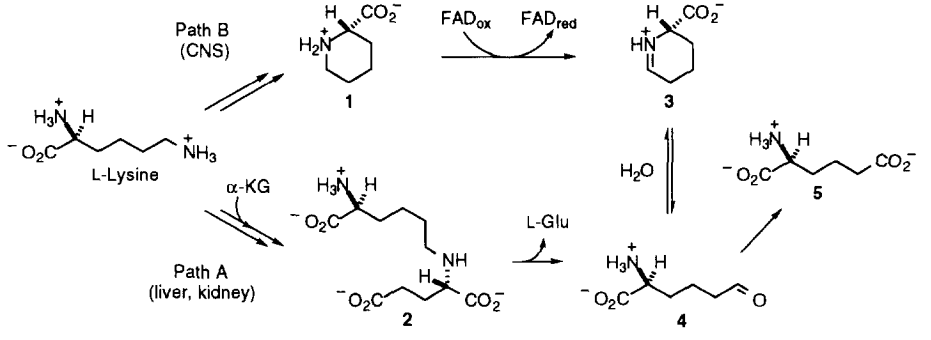
Abstract. 5-Thia-L-pipecolic acid, (*S*)-1,3-thiazane-4-carboxylic acid (**6**), was synthesized and found to serve as an excellent substrate for Rhesus monkey liver L-pipecolate oxidase (L-PO) and also to cause time-dependent, irreversible inactivation of the enzyme. Data are presented demonstrating **6** is a mechanism-based inactivator of L-PO and one of the enzyme turnover products is identified as homocysteine.

L-Pipecolic acid (L-PA, **1**) is a cyclic, six-carbon, nonproteinogenic amino acid found widely distributed in nature. L-PA is an intermediate of lysine metabolism in numerous organisms, is a key structural element in many biologically active natural products, and is a minor product of lysine metabolism in mammals. In most mammalian tissues the principal route of L-lysine degradation proceeds via L-saccharopine (**2**) (Scheme 1).[1,2] However, the saccharopine pathway is not operative in the central nervous system (CNS), where the major pathway for L-lysine catabolism occurs through L-PA (Scheme 1).[3] In tissues such as liver and kidney, both saccharopine and pipecolic acid are produced although D-lysine serves as the precursor to L-PA.[4,5]

Scheme 1: Mammalian L-Lysine Degradation.



The first step in the degradation of pipecolic acid is a flavin-dependent oxidation forming L-Δ^1-piperideine-6-carboxylate (Δ^1-P6C, **3**). This is a species-dependent process carried out by a dehydrogenase associated with the electron transport components in the bacterium *Pseudomonas putida*[6] and non-primates,[7] and by an oxidase requiring molecular oxygen and generating H_2O_2 in primates[7,8] and the red yeast *Rhodotorula glutinis*.[9] The imine **3** hydrolyzes nonenzymatically to L-α-aminoadipate-δ-semialdehyde (L-ASA, **4**) the direct product of L-

saccharopine oxidation (Scheme 1).[1,2] Thus, the saccharopine and pipecolate paths converge at L-ASA, which is further oxidized to L-α-aminoadipate (**5**).[10] To date, only the rat has been shown to possess both types of enzymes.[11]

The tissue-specific metabolism of lysine and identification of a specific degradative pathway for L-PA prompted suggestions that L-PA might function as a neuromodulator.[12] This is supported by studies demonstrating that L-PA can affect the release and uptake of the inhibitory neurotransmitter γ-aminobutyric acid (GABA).[13,14] L-PA can also delay the onset of chemically-induced seizures at high doses[15] and increase the effectiveness of the anticonvulsive agent phenobarbital, a compound that acts by modifying central GABA-mediated neurotransmission.[16] Thus, compounds that block pipecolate degradation and increase the synaptic concentrations of L-PA may be useful pharmacological tools and serve as leads for new agents to treat convulsive disorders.

Primate pipecolate oxidase (L-PO; EC 1.5.3.7) has been isolated and characterized from Rhesus monkey liver.[17] L-PO is a membrane-associated 46 kDa monomeric peroxisomal oxidase possessing a covalent flavin cofactor. The chemical mechanisms of flavin-dependent amine oxidases are well established as proceeding via radical intermediates which are proposed to arise from either a single-electron transfer mechanism,[18] or through a direct hydrogen abstraction mechanism.[19] In designing compounds which could serve as selective inactivators of L-PO, we have focused on substrate analogs able to exploit the reactivity of transient radical species resulting in a relocated or stabilized radical.[20] Carbon-centered radicals have been shown to be stabilized by adjacent sulfur atoms in both chemical studies[21] and enzyme systems.[22] Molecular orbital calculations suggest that this stabilization can be attributed to the interaction of non-bonding orbitals of the sulfur atom with the singly occupied p-orbital of the carbon atom.[23] In this report, the sulfur-containing pipecolate analog 5-thia-L-pipecolic acid ((*S*)-1,3-thiazane-4-carboxylic acid, **6**), is shown to cause time-dependent, irreversible inactivation of primate pipecolate oxidase.

5-Thia-L-pipecolic acid (**6**) was prepared following a published procedure.[24] Frozen Rhesus monkey liver was obtained from the Oregon Regional Primate Research Center and L-PO was isolated as described by Mihalik et al.[17] The enzyme was judged to be >90% homogeneous by SDS-PAGE and exhibited a K_M of 3.5 mM and a k_{cat} of 7.8 min^{-1} with L-PA as substrate. Routine enzyme activity measurements were performed using a spectrophotometric assay linking the production of H_2O_2 by L-PO to the oxidation of *o*-dianisidine catalyzed by horseradish peroxidase.[17]

Initial evaluation of **6** as an inhibitor of L-PO using the peroxidase-coupled assay resulted in observed reaction rates greater than those seen for controls containing only L-PA. When L-PA was omitted from the assays, there was no significant reduction in the rate of peroxide formation, establishing **6** as an alternate substrate for L-PO. A plot of the effect of 5-thia-L-PA concentration on the rate of the reaction demonstrated saturation kinetics and

estimates for the kinetic parameters K_M and k_{cat} of 0.94 mM and 5.1 min^{-1}, respectively, were calculated from a Lineweaver-Burk plot.

When 5-thia-L-PA was incubated with L-PO for extended periods the resulting progress curves were nonlinear, suggesting an apparent time-dependent loss of activity. Confirmation of the progressive inhibition came from preincubation/dilution experiments. Figure 1 (panel A) illustrates the time- and concentration-dependent inhibition of L-PO by **6**, a Kitz and Wilson plot (Figure 1, panel B) correlates the dependence of the observed rate of inactivation (k_{obs}) with the concentration of **6**, and was used to calculate values for K_I and k_{inact} of 36 mM and 0.06 min^{-1}, respectively.[25] A partition ratio of 85 can be calculated from the ratio k_{cat}/k_{inact}. To ensure the observed loss of activity was not due to inactivation of the coupling enzyme in the spectrophotometric assay, experiments establishing the time-dependence of the inactivation were also conducted using a radioassay.[26] The values obtained for K_I and k_{inact} were in good agreement with those obtained using the spectrophotometric assay. Additionally, 5-thia-D-PA was prepared and evaluated as an inhibitor of L-PO. There was no evidence of time-dependent inhibition from preincubation experiments with 5-thia-D-PA, indicating the inactivation is stereospecific.

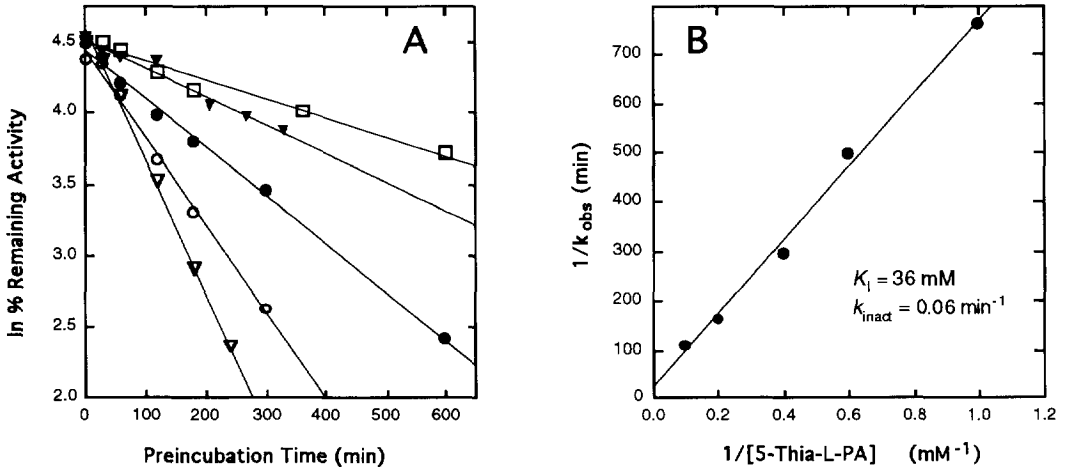


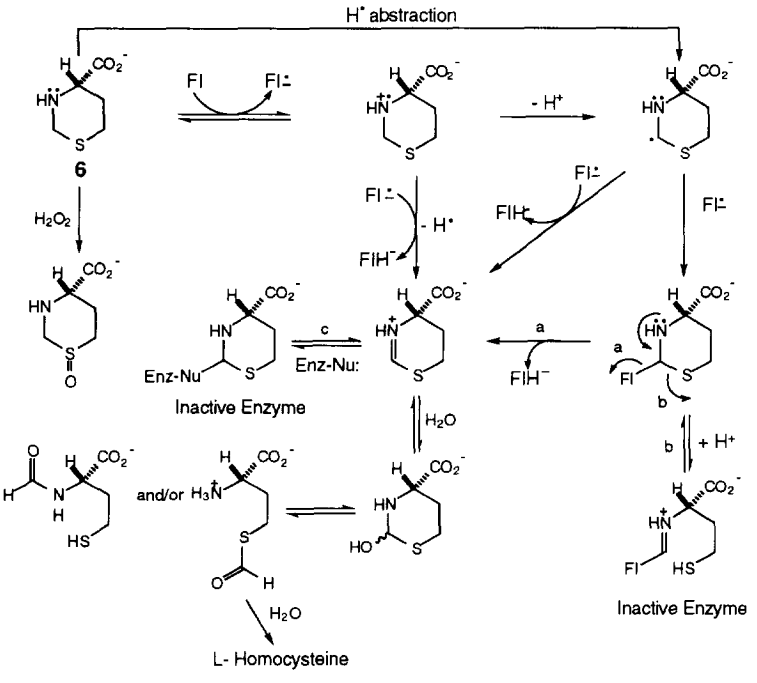
Figure 1. **A**: Time- and concentration-dependent inactivation of L-PO by **6**. L-PO was incubated with various concentrations of **6** in 40 mM Tris, 80 mM KCl and 0.8 mM EGTA, pH 8.5 buffer in a total volume of 250 µL, at 25 °C in the dark. At the times indicated, 25 µL aliquots were removed and diluted into 475 µL of 37 °C assay cocktail (40 mM Tris, 80 mM KCl and 0.8 mM EGTA, pH 8.5 buffer containing 20 mM L-PA, 320 µM *o*-dianisidine and 1.8 U horseradish peroxidase) and the activity monitored by the increase in absorbance at 460 nm. Concentration of **6**: 1.0 mM (□); 1.7 mM (▼); 2.5 mM (●); 5.0 mM (○); and 10 mM (▽). **B**: Kitz and Wilson plot of the dependence of the rate of inactivation of L-PO on the concentration of **6**.

The inactivation was shown to be irreversible by treating L-PO with 20 mM **6** at 25 °C for 4 h at which point there was no measurable L-PO activity. The remaining enzyme solution was dialyzed at 4 °C for 28 h to remove unbound and reversibly-bound **6**. At 7, 13, and 28 h aliquots of the dialyzed enzyme solution were removed

and assayed for recovered enzyme activity. At each time point only 12-14% of the activity had been recovered relative to a control treated in a similar manner. Confirmation that **6** acts at the active site of the enzyme came from substrate protection experiments. L-PO was incubated with 5 mM **6** and various concentrations of L-PA at 25 °C in the dark. At incubation times of 1.5 and 3 h, aliquots were removed and the percent remaining activity determined relative to a similarly treated control sample. The degree of remaining enzyme activity was directly proportional to the L-PA concentration and confirms that **6** competes with substrate for the active site.

Further evidence that **6** is a mechanism-based inactivator of L-PO came from experiments to detect if the release of a highly reactive species results in indiscriminate inactivation the enzyme. The inactivation of a sample of L-PO with 15 mM **6** at 37 °C was monitored with the spectrophotometric assay until there was no detectable activity. A second aliquot of L-PO was added to the assay mixture and an immediate increase in H_2O_2 production resulted, followed by a progressive loss of activity with approximately the same rate constant as the first aliquot (k_{obs} = 2.40 x 10^{-3} min^{-1} and 2.55 x 10^{-3} min^{-1}, respectively). Together with evidence that the coupling enzyme is not inactivated by **6**, this confirms that inactivation does not occur in a topologically random manner or from increasing concentration of a metabolite of **6**.

Scheme 2: Possible Mechanisms for the Inactivation of L-PO by **6**.



Scheme 2 outlines proposed mechanisms for the observed inactivation of L-PO by 5-thia-L-pipecolate (**6**). One possibility is that hydrogen peroxide generated during flavin reoxidation oxidizes **6** to the corresponding sulfoxide which acts as the true inactivator. Attempts to prepare the sulfoxide were unsuccessful owing to the instability of the compound. However, when 1000 units of catalase were added to an incubation mixture containing **6** and L-PO there was no protection from inactivation. Another route leading to leading to inactivation could result from radical combination between an active site radical (such as the flavin semiquinone) and the α-amino radical form of **6**. Heterolytic decomposition of this adduct could lead to reduced cofactor and oxidized product (Path a) or

cleavage of the carbon-sulfur bond yielding inactivated enzyme (Path b). The observed inactivation and relatively high partition ratio can also be explained if an enzyme nucleophile attacks the product iminium ion one out of every 85 turnover events to form a stable adduct (path c).

Inactivation could also result from thiol reduction of the flavin cofactor and/or disulfide bonds. To evaluate if L-homocysteine, a possible turnover product (Scheme 2), is responsible for the loss of activity, L-PO was incubated with 5 μM L-homocysteine for 1 h which resulted in a 12% reduction in activity compared to a control sample. To determine if L-homocysteine is actually produced in the reaction, L-PO was incubated with 10 mM **6** for 14 h followed by treatment of the reaction mixture with phenylisothiocyanate (PITC). Analysis by C_{18} reverse-phase HPLC (0.1% trifluoroacetic acid:MeCN, 7:3; UV detection at 270 nm) revealed a single peak which coeluted with an authentic sample of L-homocysteine phenylthiohydantoin (Figure 2). High resolution electron impact mass spectrometry supported the identity of the derivatized enzyme product (*m/z* 252.0391; calcd for $C_{11}H_{12}N_2OS_2$: 252.0391). Incubating L-PO for 1 h with 5 μM L-cysteine produced no inactivation. Thus, while homocysteine causes some inhibition and is a product of 5-thia-L-PA oxidation, it seems unlikely that inactivation is due solely to a thiol metabolite, particularly in light of data suggesting that inactivation does not result from metabolite build up and occurs prior to release of an activated species.

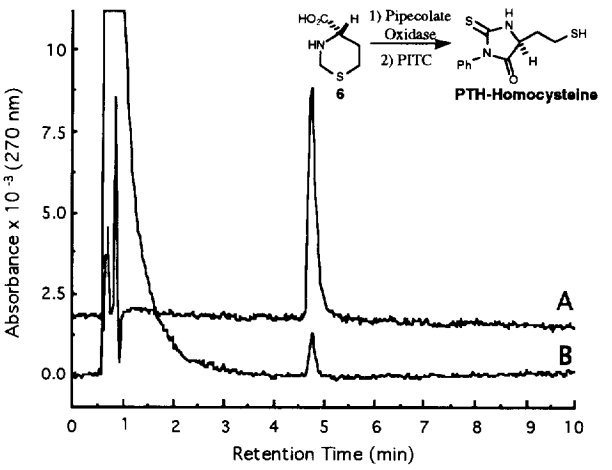


Figure 2. **A**: HPLC chromatogram of authentic phenylthiohydantoin derivative of L-homocysteine.
B: Chromatogram of the derivatized product from the pipecolate oxidase catalyzed oxidation of **6**.

In summary, 5-thia-L-pipecolate (**6**) is shown to serve as an excellent substrate for primate L-pipecolate oxidase and act as a moderate, irreversible mechanism-based inactivator of the enzyme. Further experiments to determine if the inactivator or a metabolite covalently modifies L-PO are ongoing.

Acknowledgment: This work is supported by grants from the National Institute for Neurological Disorders and Stroke (NS 32421) and the Medical Research Foundation of Oregon. Primate tissue used in these studies was obtained from the Oregon Regional Primate Research Center which is supported by NIH Grant RR 00163.

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(*Received in USA* 19 *December* 1996; *accepted* 13 *January* 1997)