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The Synthesis and Stability of Aziridino-glutamate, an Irreversible Inhibitor of Glutamate Racemase

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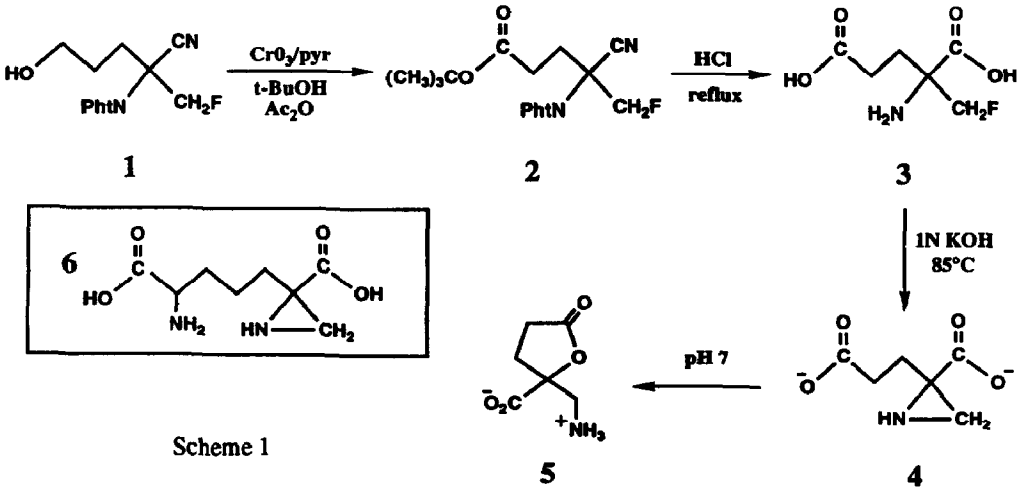
Abstract: Aziridino-glutamate (2-(2-carboxyethyl)aziridine-2-carboxylic acid, (±)4) was synthesized by heating α-fluoromethylglutamate in base. In neutral solution, 4 was shown to cyclize to the γ-lactone 5 with a half life of 4 minutes. Aziridino-glutamate was shown to irreversibly inactivate glutamate racemase by alkylating an active site cysteine residue. Electrospray mass spectrometry was used to establish that a covalent bond had formed and that this bond protects one of the enzyme's two cysteine residues from reacting with iodoacetate under denaturing conditions.

Analogs of glutamic acid are attractive synthetic targets because of the importance of this amino acid in a variety of biological processes.[1] We are interested in the inhibition of the bacterial enzyme, glutamate racemase, that provides a source of the "unnatural" D-enantiomer of glutamate for use in cell wall biosynthesis.[2,3] Since many bacteria are known to incorporate D-glutamate into their cell walls, inhibitors of this enzyme may serve as broad spectrum antibiotics.[4] Furthermore, analogs that mimic the D-configuration would not be expected to interfere with other L-glutamate handling biomolecules. Previous work by Higgins *et al.*,[5] demonstrated that the aziridino analog of diaminopimelate, **6**, is an irreversible inhibitor of the related enzyme, diaminopimelate epimerase. We now report the synthesis of aziridino-glutamate (±)4 (2-(2-carboxyethyl)aziridine-2-carboxylic acid) and demonstrate that it acts as an irreversible inhibitor of *Lactobacillus* glutamate racemase.

The synthesis (Scheme 1) begins with the known alcohol **1** which was prepared in three steps from 3-benzyloxypropyl magnesium chloride and fluoroacetonitrile.[5a] The *t*-butyl ester **2** was then prepared in a



1 2 3

4 5

Scheme 1

single step (48% yield) by oxidation with CrO_3/pyridine in the presence of *t*-butanol/acetic anhydride.[6] Hydrolysis in refluxing HCl afforded racemic α-fluoromethylglutamate (±)**3** in 86% yield.[7] Heating **3** in 1N KOH at 85°C for 1.5 hours converts it cleanly into a new compound to which we have assigned the structure (±)**4**.[8] This synthetic strategy had previously been used to synthesize the aziridino analogue of diaminopimelate(DAP), **6**.[5a] The structural assignment of **4** is based on its ^{1}H-NMR spectrum that displays two weakly coupled upfield signals indicative of the aziridine methylene protons (in agreement with the reported spectrum of **6**), and on the compound's chemical reactivity. Aziridino-glutamate **4** is stable for several weeks when stored in 1N KOH, however, it readily converts to **5** in neutral solution. Lowering the pH protonates the aziridine and results in the cyclization to γ-lactone **5**, as opposed to the disfavored endo cyclization[9] which would give the δ-lactone. This reactivity is unlike that of aziridino-DAP, **6**, (which is indefinitely stable in solution at neutral pH) because of the differences in the length of the side chain. When a solution of **4** in KOD/D_2O was neutralized by the addition of KD_2PO_4/D_2O and the rate of cyclization was immediately monitored by ^{1}H-NMR spectroscopy, a half-life of approximately 4 minutes (at 25°C) was observed. For this reason, the inhibitor was prepared as a 0.1N solution in 1N KOH, and then diluted into buffer and immediately added to an enzyme sample in all inhibition studies.

Glutamate racemase from *Lactobacillus* (EC 5.1.1.3, monomer of M_r 28 kD) belongs to a subset of the amino acid racemases that operate in a cofactor-independent fashion (no pyridoxal phosphate requirement) and use two active site cysteine residues.[2,5,10] One of the cysteines serves to deprotonate the C-2 carbon of the amino acid, and the other cysteine protonates the resulting intermediate from the opposite face (Figure 1a). Since the enzyme places the C-2 of glutamate proximal to a nucleophilic thiol functionality, it should likewise position the aziridine inhibitor **4** appropriately for alkylation to occur (Figure 1b).

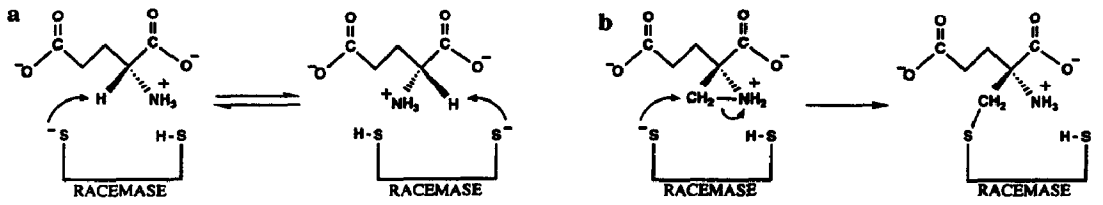


Figure 1. a) Proposed mechanism of glutamate racemization. b) Proposed mechanism of inactivation by **4**.

Incubation of glutamate racemase with 10^{-4} M concentrations of (±)**4** leads to a time-dependent irreversible loss of enzyme activity (one-half of the racemase activity was lost during a two minute incubation with 0.4 mM inhibitor at 25°C).[11,12] The rate of inactivation was decreased by the presence of substrate as expected for an active-site directed process. Samples of (±)**4** rapidly lost the ability to irreversibly inhibit the racemase when kept at neutral pH and room temperature. This is consistent with the proposal that cyclization to the lactone **5** destroys the inhibition activity and rules out the possibility that the inhibition is due to a stable, minor impurity. The instability of aziridino-glutamate prevented the accurate determination of the inhibition constants.

Further evidence for covalent attachment was obtained by electrospray mass spectrometry.[13] Samples of glutamate racemase were incubated with and without 1 mM (±)**4** and then subjected to mass spectral analysis (Figure 2). The unlabeled enzyme has a mass of 28,309 ±4 daltons whereas the inhibitor-treated sample shows

a single peak at 28,472 ±4 daltons (the difference of 163 ±8 corresponds well with the 160 dalton mass of 4).[14] No unlabeled enzyme was visible in the inhibitor-treated sample.

The proposed mechanism of action suggests the inhibitor is covalently tagging an active site cysteine (the racemase has only two cysteine residues, both in the active site). This was investigated[13,15] by incubating samples of the racemase with and without (±)**4**, denaturing the samples, treating them with iodoacetate (a reagent that reacts rapidly with cysteine residues), and analyzing the masses of the resulting proteins. The inhibitor-treated sample shows a single peak (Figure 3) with a mass that corresponds to the combined masses of the racemase, the inhibitor, and a single acetate adduct (28,528 ±4 daltons). The predominant peak in the control sample has a mass corresponding to that of the racemase with two associated acetate units (28,430 ±4 daltons). Peaks with masses corresponding to the unmodified (28,312 ±4 daltons) and the singly-acetate labeled (28,370 ±4 daltons) peaks were also observed in the control sample. These may have been due to a fraction of the enzyme being oxidized to a thermodynamically stable internal disulfide form[16] during the incubation period (with low thiol concentration) and then gradually being reduced by dithiothreitol after denaturation. Since the inactivated sample shows only a single acetate adduct and since the covalently bound inhibitor would not be expected to shield an unmodified cysteine residue in the denatured protein, it is reasonable to assume that the inhibitor is attached to one of the two cysteine side chains.

Aziridino-glutamate has therefore been shown to act as an active site-directed irreversible inactivator of glutamate racemase. Future studies will involve the synthesis and resolution of constrained analogs of aziridino-glutamate that cannot cyclize.

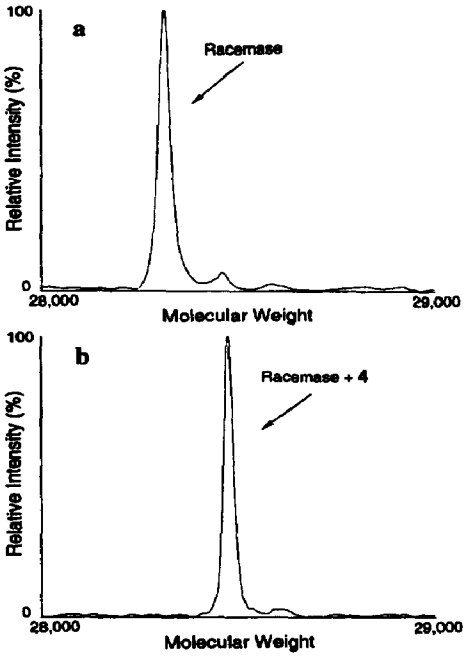


Figure 2.
a) Electrospray MS of racemase.
b) Electrospray MS after treatment with (±)**4**.

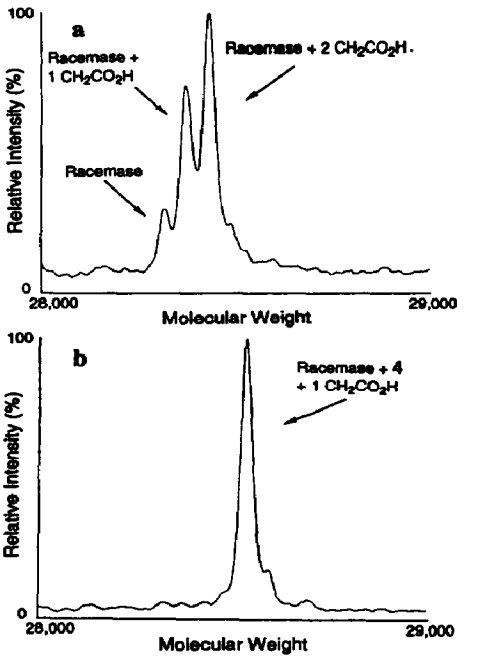


Figure 3.
a) Electrospray MS of racemase after treatment with iodoacetate
b) Electrospray MS after treatment with (±)**4**, then iodoacetate.

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8. (±)4 and (±)5. A solution of (±)3 in 0.5 N KOD/D_2O was heated at 85°C for 90 min. The resulting ^1H NMR spectrum showed a clean conversion to aziridino-glutamate (±)4: δ 2.07 (m, 3H, CH_2CH_2), 1.76, 1.46 (2s, 2H, aziridine CH_2), 1.27 (m, 1H, CH_2CH_2). To this sample was added KD_2PO_4/D_2O which neutralized the solution. Several ^1H NMR spectra were immediately collected and the conversion of (±)4 to the lactone (±)5 was observed with a half-life of approx. 4 minutes at 25°C: δ 3.39 (d, J = 13.8 Hz, 1H, CH_2N), 3.16 (d, J = 13.8 Hz, 1H, CH_2N), 2.54 (m, 2H, CH_2CO), 2.19 (m, 2H, CH_2C). Spectra taken in D_2O were referenced to HOD at 4.65 ppm.

   To further characterize the lactone, a solution of (±)3 in 1N NaOH was heated at 85°C for 1.5 hrs. HCl was added to give a final pH of 7. After 30 min at 25°C the solution was lyophilized. The resulting solid gave an identical ^1H NMR spectrum (D_2O) to that obtained in deuterated buffer. The FTIR spectrum (KBr disc) showed a strong band at 1779 cm^{-1}, consistent with a γ-lactone. High Res. LSIMS (3-NOBA matrix), 160.0608 ($M+H^+$, 100%); Calcd. for $C_6H_9NO_4+H^+$, 160.0609.
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11. Recombinant *Lactobacillus* glutamate racemase was overexpressed in *E. coli* and purified as described previously.[2b] The coupled enzyme assay[2b] for racemase activity was employed in all kinetics measurements.
12. Inhibition studies. A 0.1 M stock solution of (±)4 in 1N KOH was prepared by heating a solution of (±)3 at 85°C for 1.5 hrs. This was diluted into triethanolamine-HCl, pH 8, along with an equivalent amount of 1N HCl (final pH 8, final conc. 2 mM) immediately before use. The inhibitor solution was added to enzyme samples in Trien-HCl, pH 8, containing 2mM dithiothreitol at 25°C and incubated for timed intervals. The reaction was stopped by diluting aliquots directly into cuvettes. One-half of the racemase activity was lost during a two minute incubation with 0.4 mM inhibitor. When the same incubation was carried out in the presence of 1 mM D,L-glutamate only 15% of the activity was lost.
13. Electrospray Mass Spectra. A 1 mg/mL sample of racemase in phosphate buffer, pH 7, containing 1 mM dithiothreitol was prepared. To one-half of this solution was added a solution of (±)4 in 1N KOH, and to the other half was added 1N KOH. The final pH of these was solutions was about 8. The solutions were incubated at 37°C for 30 min and then analyzed.

   Iodoacetate-treated samples were prepared as follows. A solution of racemase (100 μL, 6 mg/mL) in phosphate buffer, pH 7.6, containing 0.7 mM D,L-glutamate and 0.25 mM dithiothreitol was prepared. To one half of this sample was added a solution of (±)4 (neutralized immediately before use) and to the other half was added water. The samples were incubated at 37°C for 45 min. To each of the samples was added 6 M guanidine hydrochloride in a phosphate buffer, pH 7.3, containing 1.0 mM sodium iodoacetate. The samples were incubated in the dark at 25°C for 75 min and quenched by the addition of excess dithiothreitol.
14. The calculated mass of the enzyme based on the published sequence data is 28,295 daltons.[2b] The observed mass was 28,309 ±4 daltons. The discrepancy (14 daltons) is likely due to a sequencing error that missassigned a single amino acid residue.
15. An initial attempt was made to quantify the number of free thiols with the use of Ellman's reagent (Ellman, G.L. *Arch. Biochem. Biophys.* **1959**, *82*, 70-77). A low value of 0.9 thiol groups per enzyme (vs. the expected 2.0) was obtained presumably because of intramolecular disulfide bond formation[16] occuring in the absence of added dithiothreitol. Treatment with 4 reduced this value to 0.5 thiol groups per enzyme, which is consistent with alkylation of one of the cysteines.
16. This has been suggested to occur with the related enzyme proline racemase.[10b,c]

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