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# THE INHIBITION OF GLUTAMATE RACEMASE BY D-*N*-HYDROXYGLUTAMATE

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**Abstract:** We report that D-*N*-hydroxyglutamate is a competitive inhibitor of glutamate racemase ($K_I$ = 56 μM). The compound acts as an alternate substrate and is converted into α-ketoglutarate and ammonia ($K_M$ = 57 μM, $k_{cat}/K_M$ = 3.2 X $10^3$ $M^{-1}sec^{-1}$). An imine intermediate is likely the species causing the inhibition. L-*N*-hydroxyglutamate is a weak inhibitor and is slowly converted to α-ketoglutarate ($k_{cat}/K_M$ = 30 $M^{-1}sec^{-1}$).


## Introduction

The obvious health hazards presented by the emergence of antibiotic resistant bacteria have rekindled the efforts of scientists to search for entirely new classes of antibiotics.[1] Inhibitors that interfere with bacterial cell wall (peptidoglycan) construction are promising candidates since many of the clinically used antibiotics target this pathway and no similar pathway exists in humans.[2,3] The uncommon D-enantiomer of glutamic acid is a required component in the peptidoglycan of most bacteria and is produced from L-glutamate by the enzyme glutamate racemase (MurI). Despite the importance of this enzyme in peptidoglycan biosynthesis, only two reports of inhibitors of the enzyme have appeared to date.[4] In both cases the inhibition was irreversible and suffered either from an inefficiency of the process or an instability of the inhibitor. We wished to design mechanism-based reversible inhibitors of the enzyme and in particular demonstrate that compounds mimicking the D-enantiomer were effective. This is important since L-glutamate analogs could interfere with a variety of important biological processes.

Studies with recombinant glutamate racemase have been carried out using the enzymes from lactic acid bacterial sources[5] and on the enzyme from *E. coli*.[6] Glutamate racemase belongs to a subset of amino acid racemases and epimerases that operate in a cofactor-independent fashion.[5c,5d,7] Glutamate racemase uses a "two-base" mechanism (Figure 1) in which two cysteine thiol(ate)s serve as the general base/acid catalysts.[5c,5e,5f] An initial deprotonation event produces a resonance-stabilized carbanionic intermediate that is subsequently protonated on the opposite face to generate the enantiomeric product.

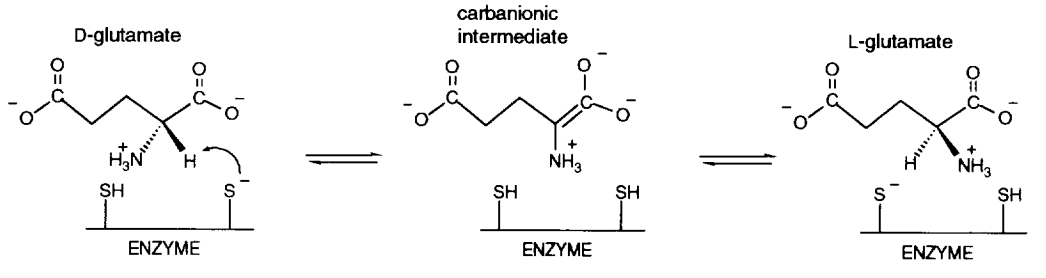



Figure 1

An excellent precedent for inhibitor design was found in the work of Vederas et al. who showed that *N*-hydroxydiaminopimelate was a potent inhibitor of the related enzyme, diaminopimelate epimerase.[8] The authors suggested that the enzyme catalyzes the elimination of water from the inhibitor to create an imine which is bound tightly in the active site. Few details were presented, however, presumably because the *N*-hydroxydiaminopimelate could only be prepared as a mixture of four diastereomers. We have therefore tested the enantiomers of *N*-hydroxyglutamate as inhibitors of the glutamate racemase from *Lactobacillus fermenti* and checked for the formation of α-ketoglutarate (Figure 2).

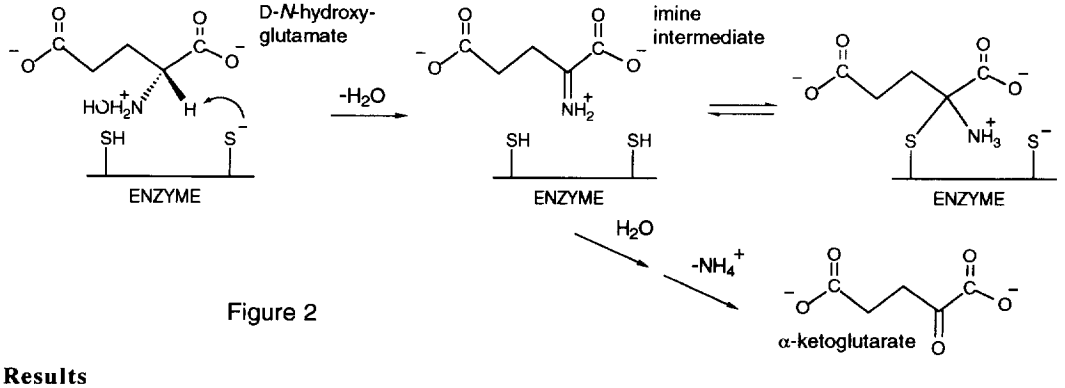



Figure 2

**Results**

Both enantiomers of *N*-hydroxyglutamate were synthesized from their respective glutamate precursors using a slight modification of the method of Polonski and Chimak.[9,10] In order to show that racemization did not occur during the synthetic procedure, the enantiomers were reduced back down to glutamic acid using hydrogenation over Pd/C.[9] These samples were assayed for L-glutamate using L-glutamate dehydrogenase and for total glutamate using glutamate racemase/L-glutamate dehydrogenase. In each case, the glutamate produced in this fashion had retained an e.e. of greater than 94% indicating that no significant degree of racemization had occurred at any point in the synthetic scheme.

The inhibition of glutamate racemase[11] by *N*-hydroxyglutamate was examined in the D-Glu-to-L-Glu direction using a coupled assay employing L-glutamate dehydrogenase, $NAD^+$, diaphorase, and $INT_{ox}$[12] (the final two components serve to regenerate $NAD^+$ and produce $INT_{red}$ with a corresponding change in $A_{500}$).[13] A potential problem with this strategy was that racemic *N*-hydroxyglutamate was a known inhibitor of the coupling enzyme.[14] The inhibition constants for each of the *N*-hydroxyglutamate enantiomers towards the dehydrogenase reaction were therefore determined under identical conditions to those of the glutamate racemase assay. The value of $K_I$ was found to be 28 μM for L-*N*-hydroxyglutamate and 0.19 mM for D-*N*-hydroxyglutamate. In both cases competitive inhibition patterns were observed. In the case of D-*N*-hydroxyglutamate the inhibition of the dehydrogenase was sufficiently weaker than that of racemase to permit the use of the coupled assay. In the case of L-*N*-hydroxyglutamate, however, inhibition of the coupling enzyme precluded the use of the assay.

D-*N*-Hydroxyglutamate was shown to be a competitive inhibitor of glutamate racemase with a $K_I$ value of 56 μM (Figure 3). This compares to a $K_M$ value of 0.3 mM for the racemization and suggests that the inhibitor is

acting as more than a simple substrate analogue. No signs of irreversible inhibition were observed, even during extended incubations with saturating amounts of D-*N*-hydroxyglutamate.

The observed inhibition may result from the enzymatic elimination of water from *N*-hydroxyglutamate. This would produce an imine that could mimic the normal carbanionic intermediate and thus be strongly bound by the racemase (Figure 2). Release of the imine into solution would be accompanied by a rapid hydration and a subsequent elimination of ammonia to produce α-ketoglutarate. In order to test for this process, the *N*-hydroxyglutamate enantiomers were incubated with relatively large amounts of glutamate racemase in deuterated buffer and the reaction was followed by $^1$H NMR spectroscopy.[15] In both cases, a clean conversion of *N*-hydroxyglutamate into α-ketoglutarate was observed (Figure 4). In the case of L-*N*-hydroxyglutamate, however, a much larger amount of racemase was required to bring about the conversion on a similar time scale. The $^1$H NMR experiment was also used to check if the enzyme is capable of catalyzing the racemization of *N*-hydroxyglutamate in a manner competitive with the elimination of water. It has been shown that during the racemization of the glutamate enantiomers each turnover results in the incorporation of a solvent derived deuterium atom at C-2.[5b,5c,5e] In the case of the *N*-hydroxyglutamate enantiomers, however, the disappearance of the signals due to the proton at C-2 were mirrored by the appearance of the signals due to α-ketoglutarate. This indicates that racemization was not competitive with the elimination reaction.

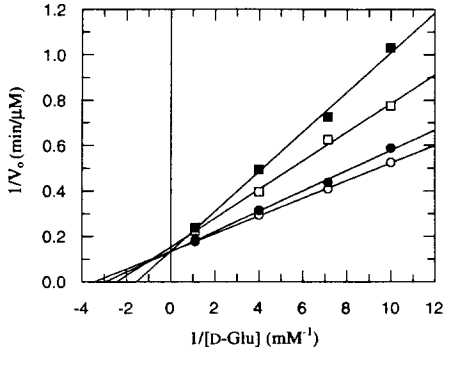



Figure 3. D-*N*-Hydroxyglutamate inhibition pattern. D-*N*-Hydroxyglutamate concentrations are: 0 μM (○), 5 μM (●), 20 μM (□), 60 μM (■).

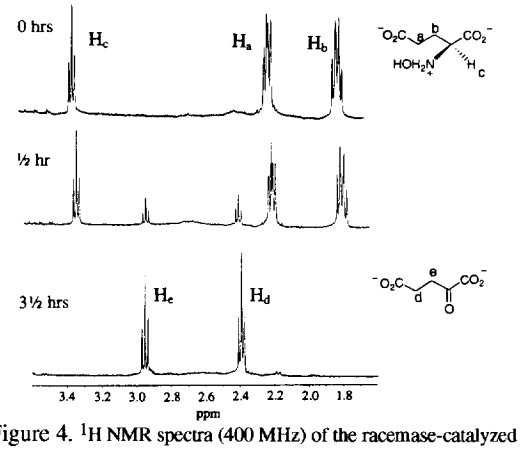



Figure 4. $^1$H NMR spectra (400 MHz) of the racemase-catalyzed conversion of L-*N*-hydroxyglutamate into α-ketoglutarate.

In order to obtain the kinetic parameters for the elimination reaction a coupled assay using L-glutamate dehydrogenase, ammonium, and NADH was employed (this assay is run in the thermodynamically favorable direction of α-ketoglutarate-to-L-Glu).[16] The kinetic parameters for α-ketoglutarate formation from D-*N*-hydroxyglutamate were found to be $k_{cat}$ = 0.18 sec$^{-1}$, $K_M$ = 57 μM, and $k_{cat}/K_M$ = 3.2 X 10$^3$ M$^{-1}$sec$^{-1}$. Problems with inhibition of the coupling enzyme once again prevented the determination of the $K_M$ value for the L-enantiomer (the problem was twofold in that it was a stronger inhibitor of the coupling enzyme and its $K_M$ value was higher). Instead, the complete course of the elimination reaction was followed at a low concentration of L-*N*-hydroxyglutamate (20 μM) using the coupled assay.[17] Under these conditions the L-*N*-hydroxyglutamate is consumed with a rate constant of $k_{obs} = [E]_o k_{cat}/K_M$. The time-dependent decrease in NADH concentration was analyzed as a first order exponential decay and a value of $k_{cat}/K_M$ = 30 M$^{-1}$sec$^{-1}$ was obtained for the elimination process with the L-enantiomer. A similar treatment with D-*N*-hydroxyglutamate (10

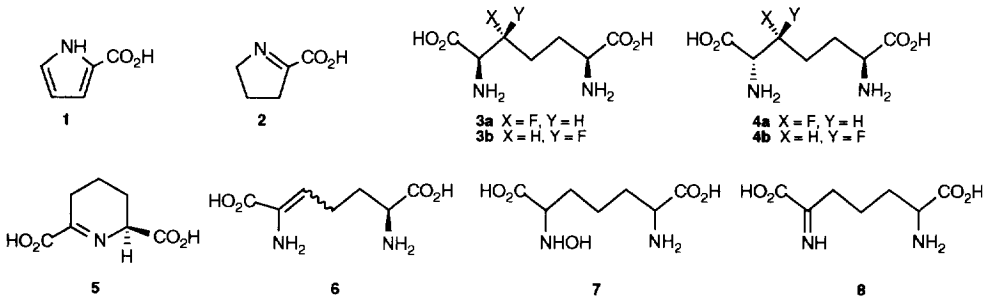
μM) gave a value of $k_{cat}/K_M$ = 3.7 X $10^3$ $M^{-1}sec^{-1}$, which agrees well with that obtained earlier. It is clear that the specificity of the racemase catalyzed elimination greatly favors the D-enantiomer.

A determination of the elimination kinetics provided a means to establish a direct link between *N*-hydroxyglutamate and the observed inhibition. A sample of D-*N*-hydroxyglutamate (1.2 mM) was treated with the racemase under conditions where 75% of it would be converted to α-ketoglutarate during the extent of the incubation (leaving approx. 0.3 mM D-*N*-hydroxyglutamate).[18] The reaction was quenched with acid, and the denatured enzyme was removed by ultrafiltration. A control reaction lacking enzyme was treated in a similar fashion. The samples were then neutralized and tested in an inhibition assay under conditions that resulted in a 20-fold dilution overall. The initial velocity of the enzyme-treated sample was 91% of that observed in a sample lacking inhibitor. This agrees well with the expected 12% reduction in velocity due to the presence of 0.015 mM D-*N*-hydroxyglutamate. The initial velocity of the control sample was 66% of that observed in a sample lacking inhibitor. This agrees well with the expected 35% reduction in velocity due to the presence of 0.06 mM D-*N*-hydroxyglutamate and indicates that the inhibitor was stable to the conditions of the experiment. The agreement between the rate of destruction of D-*N*-hydroxyglutamate and the loss of inhibition strongly supports the notion that the inhibition is not due to a minor impurity in the sample but is in fact due to the D-*N*-hydroxyglutamate itself.

## Discussion

The development of inhibitors for the cofactor-independent amino acid racemases and epimerases began with studies on proline racemase. Compounds **1** and **2** were found to be good inhibitors of the racemase presumably due to the $sp^2$-hybridization at C-2 that mimics the trigonal character of the transition state/intermediate in the racemization reaction.[19,20] The aromaticity of **1** and the cyclic structure of **2** serve to stabilize these inhibitors and allow for their synthesis and isolation. Most attempts to use a similar strategy in the design of inhibitors for enzymes that operate on acyclic amino acids are hampered by the instability of imine or eneamine compounds towards spontaneous hydrolytic reactions. A clever way in which this problem has been overcome is found in studies on the inhibitors of diaminopimelate (DAP) epimerase.[8,21,22] These inhibitors act as alternative substrates and undergo an enzyme-catalyzed elimination reaction that is thought to generate the eneamine or imine analogs at the active site. The best understood of these compounds are the 3-fluorodiaminopimelic acid isomers **3a,b** that mimic the *meso*-DAP epimer and **4a,b** that mimic the L,L-DAP epimer.[22] Compounds **3a** and **4a** were potent inhibitors ($IC_{50}$ 25 μM and 10 μM, respectively) and were found to undergo a facile enzyme-catalyzed elimination of HF, ultimately generating tetrahydrodipicolinic acid **5**. No epimerization at C-2 was observed. With analogy to the proline racemase inhibitor **1**, it seems reasonable to expect that enamine **6** was the species responsible for the strong binding. The compounds **3b** and **4b** were also found to be potent inhibitors of the epimerase ($IC_{50}$ 8 μM and 4 μM, respectively) however the elimination of HF occurred much more slowly and the compounds were rapidly interconverted via an epimerization at C-2. The authors concluded that with these isomers the intact 3-fluoro-DAP itself was responsible for the inhibition.

Potent inhibition of DAP epimerase by a mixture of the four possible *N*-hydroxy-DAP epimers **7** ($K_I$ = 5.6 μM) has also been reported.[8] The authors suggested that the imine **8** was formed by the enzyme-catalyzed




elimination of water and that this species was responsible for the potent inhibition (with analogy to the proline racemase inhibitor **2**). No direct evidence supporting the elimination reaction was presented, however.

The work described in this paper shows that D-*N*-hydroxyglutamate is a good competitive inhibitor ($K_I$ = 56 μM) of glutamate racemase from *Lactobacillus fermenti*. The observed formation of α-ketoglutarate indicates that the compound is actually acting as an alternate substrate and that the elimination of water produces an imine intermediate (Figure 2). The imine may be bound either non-covalently as a mimic of the normal reaction intermediate, or covalently through the reversible addition of a cysteine thiol. The agreement between the value of $K_M$ for this process (57 μM) and the value of $K_I$ is expected for this type of inhibition.[23] The low value of $k_{cat}$ for the elimination reaction (0.18 $sec^{-1}$ vs 70 $sec^{-1}$ for D-Glu racemization) ensures that the inhibitor persists at reasonable enzyme concentrations. These observations indirectly support the notion that imine formation is also involved in the inhibition of DAP-epimerase by **7**.

The epimerase showed high specificity with regards to catalyzing the elimination of water from the enantiomers of *N*-hydroxyglutamate. L-*N*-Hydroxyglutamate was a very poor substrate ($k_{cat}/K_M$ = 30 $M^{-1}$ $sec^{-1}$) with respect to the D-enantiomer ($k_{cat}/K_M$ = 3.2 X $10^3$ $M^{-1}sec^{-1}$) and was clearly a poor inhibitor as well. This points out the surprising degree of asymmetry that can be present in an active site that has evolved with an equal specificity for two enantiomers.

Glutamate racemase is also known to catalyze the elimination of HCl from both enantiomers of *threo*-3-chloroglutamate.[5f] It would be interesting to test whether these compounds are good inhibitors of the racemase with analogy to the halogenated DAP-analogs.

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10. The modifications included the use of $HONH_2$•HCl in the preparation of dimethyl *N*-hydroxyglutamate and the use of silica gel chromatography (25% $Et_2O$ in $CH_2Cl_2$) during its purification. The final product of *N*-hydroxyglutamate was purified by elution from a column of AG1-X8 resin (100-200 mesh, formate form) with 0.25 N formic acid. See also: Kelland, J. G.; Arnold, L. D.; Palcic, M. M.; Pickard, M. A.; Vederas, J. C. *J. Biol. Chem.* **1986**, *261*, 13216.
11. Glutamate racemase was purified from *Escherichia coli* DH5α carrying the glutamate racemase expression vector pKG3 as described in ref 5d.
12. The abbreviations used are: $INT_{ox}$, *p*-iodonitrotetrazolium violet in an oxidized form; $INT_{red}$, *p*-iodonitrotetrazolium violet in a reduced form; DAP, diaminopimelate; Trien, triethanolamine
13. This assay was performed under conditions identical to those described in ref 5d with the exception that 48 units of L-glutamate dehydrogenase was used and the pH was 8.0.
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15. L-*N*-Hydroxyglutamate (2 mg, 0.012 mmol) was dissolved in deuterated potassium phosphate buffer (250 mM, 0.5 mL, pD 8) containing 0.2 mM dithiothreitol (final pD of approx. 7 by pH indicator paper). Glutamate racemase (125 units in the same buffer) was added and the progress of the reaction was monitored at 37 °C using $^1H$ NMR spectroscopy (400 MHz). An analogous experiment with D-*N*-hydroxyglutamate was run using 45 units of glutamate racemase.
16. Assays were performed at 30 °C in 50 mM Trien-HCl buffer, pH 8.0, containing 0.08 mM NADH, 2.5 mM ADP, 2.0 mM dithiothreitol, 5.0 mM $NH_4Cl$, 12 units L-glutamate dehydrogenase and 3.2 units glutamate racemase (1 mL total volume). Initial velocities were measured by following the decrease in absorbance at 340 nm. The extinction coefficient of NADH at 340 nm was taken as 6220 $M^{-1}cm^{-1}$.
17. The assay was performed at 30 °C in 50 mM Trien-HCl buffer, pH 8.0, containing 0.1 mM NADH, 2.5 mM ADP, 2.0 mM dithiothreitol, 10 mM $NH_4Cl$, 12 units L-glutamate dehydrogenase and 280 units glutamate racemase (1 mL total volume). The reaction was followed to completion and first order rate constants describing the decrease in absorbance at 340 nm were obtained using the program GraFit.
18. D-*N*-Hydroxyglutamate (1.2 mM) was incubated for 12 min. at 30 °C in 0.45 mL of 50 mM Trien-HCl buffer, pH 8.0, containing 0.02 mM dithiothreitol and 14.5 units of glutamate racemase. A 0.20 mL aliquot of the reaction was quenched by the addition of 3 μL of conc. HCl to a final pH of 2 and the enzyme was removed by ultrafiltration through a Millipore Ultrafree-4 centrifugal filter. To a 50 μL aliquot of the filtrate was added 50 μL of Trien-HCl buffer (0.5 M, pH 8 (final pH of approx. 7 by pH indicator paper)). The sample was assayed for the presence of a racemase inhibitor with 0.3 mM D-glutamate. A control sample lacking racemase was prepared and analyzed in a similar fashion.
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