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# Inhibition of glutamate racemase by substrate–product analogues

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ABSTRACT

D-Glutamate is an essential biosynthetic building block of the peptidoglycans that encapsulate the bacterial cell wall. Glutamate racemase catalyzes the reversible formation of D-glutamate from L-glutamate and, hence, the enzyme is a potential therapeutic target. We show that the novel cyclic substrate–product analogue (*R*,*S*)-1-hydroxy-1-oxo-4-amino-4-carboxyphosphorinane is a modest, partial noncompetitive inhibitor of glutamate racemase from *Fusobacterium nucleatum* (*Fn*GR), a pathogen responsible, in part, for periodontal disease and colorectal cancer ($K_i$ = 3.1 ± 0.6 mM, cf. $K_m$ = 1.41 ± 0.06 mM). The cyclic substrate–product analogue (*R*,*S*)-4-amino-4-carboxy-1,1-dioxotetrahydro-thiopyran was a weak inhibitor, giving only ~30% inhibition at a concentration of 40 mM. The related cyclic substrate–product analogue 1,1-dioxo-tetrahydrothiopyran-4-one was a cooperative mixed-type inhibitor of *Fn*GR ($K_i$ = 18.4 ± 1.2 mM), while linear analogues were only weak inhibitors of the enzyme. For glutamate racemase, mimicking the structure of both enantiomeric substrates (substrate–product analogues) serves as a useful design strategy for developing inhibitors. The new cyclic compounds developed in the present study may serve as potential lead compounds for further development.



The rise of antibiotic resistance in pathogenic organisms has led to an increasing need to develop antibacterial agents and to identify new drug targets.[1–3] One such bacteria-specific target is glutamate racemase (GR).[4–9] This cofactor-independent enzyme catalyzes the reversible conversion of L-glutamate to D-glutamate.[10–12] D-Glutamate is a key component of the peptidoglycan layer, which encapsulates the bacterial cell wall in a number of pathogenic organisms and protects them against osmotic lysis.[13–15] Previously, we described the overexpression, kinetic properties, and quaternary structure of GR from the opportunistic pathogen *Fusobacterium nucleatum* (*Fn*GR).[16] This gram-negative, obligate anaerobe[17] promotes the onset of periodontal disease by facilitating the co-aggregation of different bacterial species in oral biofilms, leading to the permanent establishment of pathogenic strains within dental plaque and periodontal disease.[18,19] *F. nucleatum* is also associated with extraoral disease such as intrauterine infections associated with pregnancy complications[20–22] and colorectal cancer.[23,24] Recently, it was established that *F. nucleatum* promotes colorectal cancer by adhering to, invading, and inducing oncogenic and inflammatory responses to stimulate the growth of colorectal cancer cells.[25] Consequently, *Fn*GR is a potential therapeutic target for development of drugs directed against periodontal disease and colorectal cancer.

GR catalyzes the stereoinversion of L-glutamate and D-glutamate via a two-base mechanism wherein one enantiospecific Brønsted base abstracts the proton from L-glutamate and the conjugate acid of a second enantiospecific Brønsted base protonates the intermediate to form D-glutamate, and vice versa (Scheme 1A). For GRs, two cysteine residues serve as the Brønsted acid/base catalysts within the active site.[26–29]

The development of inhibitors for GRs has been particularly challenging.[6,9,30] Amino acid derivatives including L-serine-*O*-sulfate,[31] D-*N*-hydroxyglutamate,[32] aziridino glutamate,[33] (2*R*,4*S*)-4-substituted D-glutamate analogues,[4] and boron- and imide-containing glutamate analogues[34] have been reported as GR inhibitors. A number of non-amino acid inhibitors have also been reported including 9-benzyl purines,[35] 8-benzyl pteridinediones,[5] pyrazolopyrimidinediones,[12,36,37] and benzodiazepine amines.[7] However, many of these compounds suffered from poor water solubility and low bioavailability.[9] Some large-molecule[38,39] and peptide-based inhibitors[40] have also been reported. More recently, Spies and co-workers[8,41,42] employed in silico screening against a 'transition state conformation' of GR to identify several cyclic inhibitors bearing anionic groups (vide infra).

Previously, we reported that the substrate–product analogue benzilate is a competitive inhibitor of mandelate racemase (mechanistically similar to GRs), binding with an affinity slightly better than that observed for the substrate mandelate ($K_i$ = 0.67 mM vs $K_m$ = 1.0 mM).[43] Similarly, Ohtaki et al.[44] reported that citric acid

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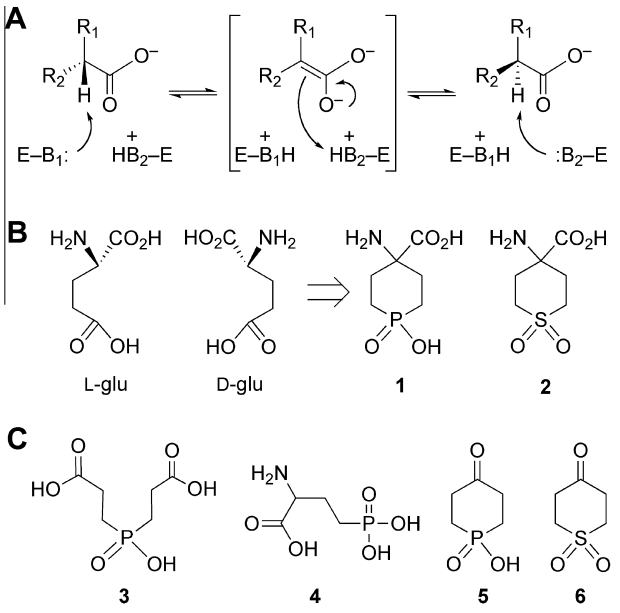


**Scheme 1.** (A) Two-base racemization mechanism of cofactor-independent racemases. For glutamate racemase, $R_1 = NH_3^+$ and $R_2 = CH_2CH_2CO_2^-$; for mandelate racemase, $R_1$ = OH and $R_2$ = Ph; and for aspartate racemase, $R_1 = NH_3^+$ and $R_2 = CH_2CO_2^-$. The (*S*)- and (*R*)-enantiospecific Brønsted acid/base catalysts are labeled generically as $B_1$ and $B_2$, respectively. The proposed cyclic substrate–product analogues (**1**, **2**) are shown in (B); and the related linear chain molecules (**3**, **4**) and cyclic (**5, 6**) analogues are shown in (C).

was a substrate–product analogue for aspartate racemase, acting as a competitive inhibitor ($K_i$ = 7.4 mM vs $K_m$ = 0.7 mM). Harty et al.[45] showed that the substrate–product analogues α-(hydroxymethyl)serine and tetrahydro-1*H*-pyrrolizine-7*a*(5*H*)-carboxylate were modest inhibitors of serine racemase and proline racemase, respectively. Figure 1 shows the superposition of the X-ray crystal structures of the active sites of GR from *Enterococcus faecalis* with bound D- and L-glutamate.[12] Unlike mandelate racemase and aspartate racemase, wherein one of the groups attached to the stereogenic center moves during catalysis (i.e., the phenyl ring of mandelate[46] and apparently the $CH_2CO_2^-$ side chain of aspartate,[44] respectively), GR holds the α-$CO_2^-$, γ-$CO_2^-$, and α-$NH_3^+$ groups of glutamate firmly in place by multiple H-bonds. Therefore, we anticipated that only the two methylene groups of the side chain might move within the active site during racemization as suggested by the crystal structure (Fig. 1). Hence, the alternative binding positions of the methylene groups of D- and L-glutamate might be mimicked by cyclic structures (substrate–product analogues) such as **1** and **2** (Scheme 1B). Indeed, crystallographic and computational studies have suggested that GR is quite flexible and therefore likely to accommodate cyclic substrate–product analogues within its active site.[8,26,47–49] Herein, we report that these compounds and related cyclic structures (Scheme 1C) are modest inhibitors of *Fn*GR, binding with affinities between 2- and 13-fold less than that of the substrate.

The synthesis of 1-hydroxy-1-oxo-4-amino-4-carboxy-phosphorinane (**1**) was accomplished in a 6-step sequence (Scheme 2) starting from $H_3PO_2$. The intermediate 1-methoxy-1-oxo-4-phosphorinanone (**10**) was synthesized using a route similar to that described by Wróblewski and Verkade.[51] The Michael-type addition of the methyl hypophosphite P–H unit, generated in situ by reaction of concd hypophosphorous acid with trismethoxy(propyl)-silane, to methyl acrylate gave **7** in 62% yield.[52] Subsequent Michael-type addition of the other P–H unit in **7** to methyl acrylate followed by Dieckmann cyclization of **8** gave enol **9**, which after decarboxylation with 1 M HCl afforded **10** in 35% yield. We did not hydrolyze the methyl ester in **10** at this stage to avoid solubility issues in subsequent steps. Using the Strecker synthesis,[53] **10** was converted to a 1:2 mixture of diastereomers of **11**, as determined using NMR spectroscopy (Figs. 13S–15S). Treatment of **11** with 6 M HCl gave a yellow crude mixture containing **1** and $NH_4Cl$. The $NH_4Cl$ was removed by precipitation from methanol/$CHCl_3$ (10:90) and filtration to yield white crystals of **1**. Optimization of the $NH_4Cl$ removal and crystallization conditions permitted preparation of the target racemic cyclic phosphorinane **1** on a scale sufficient for enzyme assays.

The cyclic analogue, 4-amino-4-carboxy-1,1-dioxo-tetrahydrothiopyran (**2**), was prepared by deprotection of the commercially available 4-*N*-Boc-amino-4-carboxy-1,1-dioxo-tetrahydrothiopyran using TFA/$CH_2Cl_2$.[54]

To test the inhibitory properties of the cyclic substrate–product analogues **1** and **2**, we purified *Fn*GR as a fusion protein bearing an N-terminal hexahistidine ($His_6$) tag as described previously.[16] The $(His)_6$-tagged enzyme was used for all inhibition studies since it had previously been shown that removal of the tag did not significantly alter the kinetic properties of the enzyme.[16] Compound **1** inhibited *Fn*GR with an $IC_{50}$ value of 24.1 ± 3.8 mM (Table 1; Fig. 44S). Interestingly, we could not achieve 100% inhibition, even with inhibitor concentrations up to 37 mM—the highest inhibitor concentration compatible with the circular dichroism (CD)-based

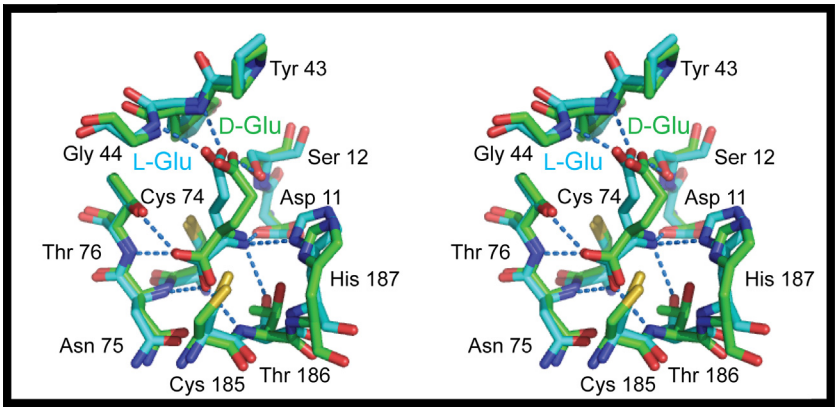


**Figure 1.** Stereoview (wall-eyed) showing putative motion of the glutamate side chains during racemization. Active site residues of GR from *Enterococcus faecalis* (PDB 2JFO)[12] corresponding to the A and B chains with bound L-glu (green carbons) and D-glu (blue carbons), respectively, are superposed. Oxygen, nitrogen, and sulfur are colored red, blue, and yellow, respectively. H-bonds securing the α-$COO^-$, α-$NH_3^+$, and γ-$COO^-$ groups are indicated by the blue dashed lines. The L- and D-enantiospecfic Brønsted acid/base catalysts are Cys 185 and Cys 74, respectively. This figure was prepared using MacPyMOL.[50]