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MECHANISM-BASED INACTIVATION OF *E.COLI* γ-GLUTAMYLCYSTEINE SYNTHETASE BY PHOSPHINIC ACID- AND SULFOXIMINE-BASED TRANSITION-STATE ANALOGUES

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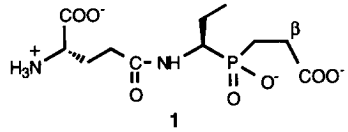
Abstract: Phosphinic acid- and sulfoximine-based transition state analogues having a carboxyl group at the β-carbon to the hetero atom exhibited significantly higher potency as mechanism-based inhibitors of *E. coli* γ-glutamylcysteine synthetase as compared with L-buthionine-*SR*-sulfoximine. The enhanced inhibition potency is evidenced by both tight binding of the inhibitor and slow enzyme reactivation. Copyright © 1996 Elsevier Science Ltd

Glutathione biosynthesis is mediated consecutively by two mechanistically related ligases, γ-glutamylcysteine synthetase (γ-GCS, EC 6.3.2.2) and glutathione synthetase (EC 6.3.2.3). The design and synthesis of specific inhibitors of these two physiologically relevant enzymes are of critical importance not only for use as mechanistic and physiological probes but also for therapeutic agents.[1] We recently reported that the phosphinic acid-derived transition state analogue **1** caused a mechanism-based inactivation of *E. coli* glutathione synthetase, in which a carboxyl group attached to the β-carbon to the phosphorus atom was found to serve as a major recognition element by the enzyme in the transition state, thereby exerting a decisive effect on the inhibitor potency.[2]



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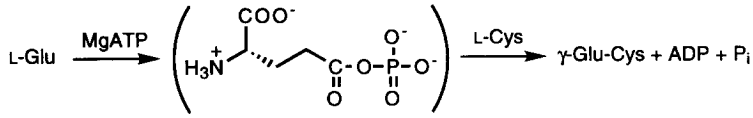
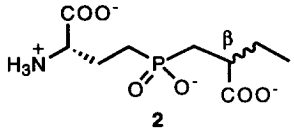
Considering that the reaction catalyzed by γ-GCS is mechanistically related to that by glutathione synthetase and is thought to proceed through the initial formation of an acyl phosphate intermediate followed by the nucleophilic attack of cysteine (Scheme I),[1,2] we applied the same rationale for the development of potent inhibitors of *E. coli* γ-GCS and designed the phosphinic acid- and sulfoximine-based transition state analogues

Scheme I.





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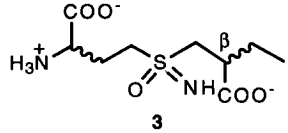

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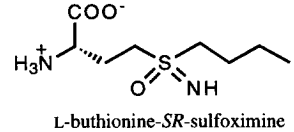


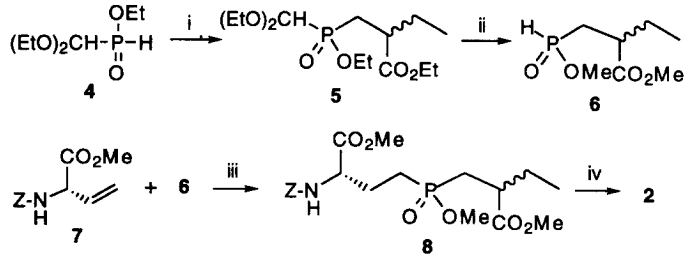
L-buthionine-*SR*-sulfoximine

2 and **3** having a carboxyl function at the β-carbon to the central hetero atom. This moiety represents the carboxyl group of the attacking cysteine in the transition state, and the incorporation of this function is expected to conform more to the transition state, thereby enhancing the potency of inhibitors such as buthionine sulfoximine, a well-known mechanism-based inactivator of γ-GCS.[3]

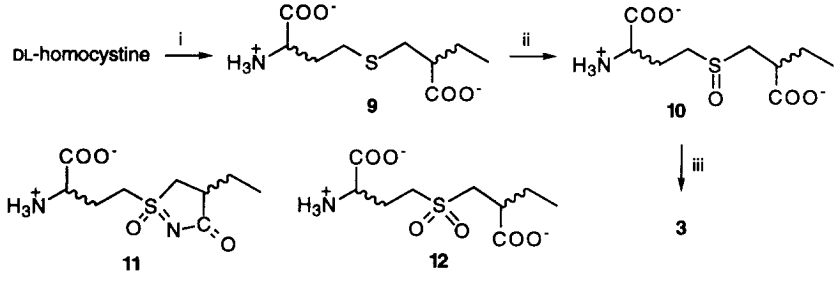
In this article, we report a remarkably effective and time-dependent inactivation of γ-GCS from *E. coli* B[4] by compounds **2** and **3**, and discuss the inhibition potency in terms of inhibition constant and the extent of recovery of enzyme activity as compared with buthionine sulfoximine.

Synthesis

The phosphinate **2** and sulfoximine **3** were prepared as shown in Scheme II and III, respectively. Ethyl (diethoxymethyl)phosphinate **4**[5] was subjected to base-catalyzed conjugate addition to ethyl 2-ethylacrylate[6] to yield **5**. Acid hydrolysis of diethoxymethyl group of **5**[7] and methylation with diazomethane gave the hydrogen phosphinate **6**. Compound **6** was added to the vinyl glycine derivative **7**[8] under radical conditions[9] to give the fully protected phosphinate **8**. Alkaline hydrolysis followed by hydrogenolysis afforded the phosphinate **2** as a 1:1 mixture of diastereomers.



Scheme II. Synthesis of phosphinate **2**
(i) ethyl 2-ethylacrylate, NaOEt, 75%; (ii)(a) conc. HCl, reflux 4 h; (b) CH_2N_2, 60%; (iii) *tert*-butyl peroxybenzoate, xylene, 110°C, 22%; (iv)(a) NaOH, (b) H_2/10% Pd-C, (c) Dowex 50W (H^+), eluted with H_2O, 80%.



Scheme III. Synthesis of sulfoximine **3**
(i)(a) DTT, NaOH, EtOH-H_2O, (b) ethyl 2-ethylacrylate, (c) NaOH, 25°C, (d) Dowex 50W (H^+), eluted with 3% NH_4OH, 90%; (ii) $NaIO_4$, 100%; (iii)(a) NaN_3, H_2SO_4, $CHCl_3$, (b) Dowex 50W (H^+), eluted with 1*N* HCl, then 3% NH_4OH, 22%.

For the synthesis of sulfoximine **3**, DL-homocystine was reduced by dithiothreitol (DTT, 1.1 eq) in aqueous EtOH in the presence of NaOH. The conjugate addition of the resulting DL-homocysteine di-sodium salt to ethyl 2-ethylacrylate and subsequent hydrolysis afforded the sulfide **9** in 90% overall yield after purification with Dowex 50W. Stepwise oxidation[10] of the sulfide **9** via sulfoxide **10** gave the sulfoximine **3** as a mixture of eight diastereomers (ratios not determined). The sulfoximine **3** was prone to cyclize and afforded crystalline **11** quantitatively when heated in AcOH (50°C, 3 h). The sulfone **12** was synthesized by oxidation of the sulfide **9** with 30% H_2O_2 in AcOH (50°C, 7 h, 23% yield).

Enzyme assay

Enzyme activity was determined by measuring ADP formation with a pyruvate kinase (PK)-lactate dehydrogenase (LDH) coupled enzyme assay.[11] The second order rate constants for time-dependent inhibition (k_{inact}/K_i) were obtained from a plot of pseudo first-order inhibition rates (k_{obs}) vs. [I] by regression to $k_{obs} = k_{inact}[I]/(K_i + [I])$ or $k_{obs} = (k_{inact}/K_i)[I]$ when saturation or no saturation was observed, respectively. The values of k_{obs} were calculated from the progress curves[12] in which the reaction was initiated by adding the enzyme to an assay solution containing varying concentrations of the inhibitor. The overall dissociation constants for slow binding (K_i^*) were obtained from a plot of the steady state inhibited velocities (v_s) vs. [I] by nonlinear least-squares analysis. The values of v_s were obtained by incubating the enzyme in an inhibition mixture containing ATP and varying concentrations of the inhibitors at 37°C for 30 min to establish the binding equilibrium, followed by adding L-glutamic acid to start the assay for enzyme activity.

Results and discussion

Both phosphinate **2** and sulfoximine **3** were found to serve as a potent inactivator of *E. coli* γ-GCS. Treatment of γ-GCS with varying concentrations of **2** or **3** in the presence of ATP caused a time-dependent inactivation of the enzyme (Figure 1).

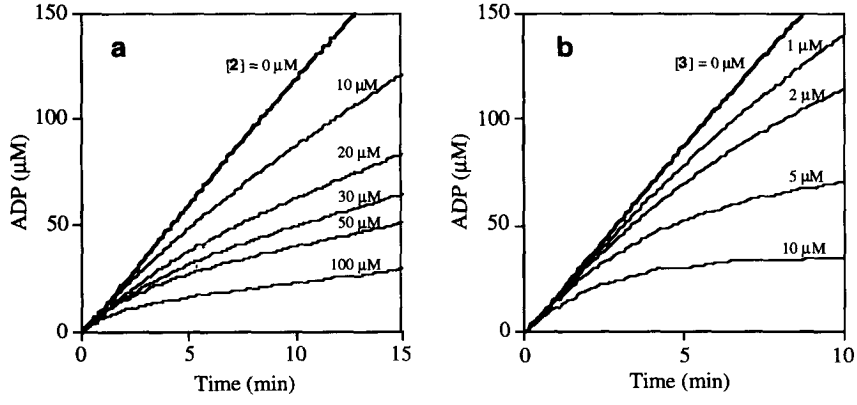


Figure 1. Progress curves for the inactivation of γ-GCS by (a) phosphinate **2**, and (b) sulfoximine **3**. The reaction was initiated by adding the enzyme (20-31 nM) to a standard assay mixture [0.1 M Tris-HCl buffer (pH 7.5), 37°C] containing 0.75 mM L-glutamic acid, 150 mM L-2-aminobutyric acid, 1 mM ATP, 10 mM $MgSO_4$, 0.1 M KCl, 1 mM phosphoenolpyruvate, 0.24 mM NADH, 25 units of LDH, 10 units of PK and varying concentrations of the inhibitor as indicated.

The shape of the progress curves represents a typical slow binding inhibition as observed with the inhibition of rat kidney γ-GCS by a series of *S*-alkyl homocysteine sulfoximines.[3] A large difference, however, was noted in its potency. Table 1 depicts the extent of inhibition, the second order rate constants for time-dependent inhibition (k_{inact}/K_i),[13] and the overall dissociation constants (K_i*) obtained from the steady-state reaction rates after the binding equilibrium was reached. For comparison, the inhibition by L-buthionine-*SR*-sulfoximine (L-BSO) and sulfone **12** was also examined. The overall binding of phosphinate **2** and sulfoximine **3** is one and two orders of magnitude greater than that of L-BSO, respectively. In particular, sulfoximine **3** acted as an extremely powerful inactivator: although the sulfoximine **3** is a mixture of eight diastereomers, it inhibited γ-GCS about 126 times more effectively than did L-BSO. Assuming that the sulfoximine **3** contains equal amounts of diastereomers and only one stereoisomer inhibits the enzyme, it could be more than 500 times as effective as an active diastereomer of L-BSO.[14] Both phosphinate **2** and sulfoximine **3** required ATP for enzyme inactivation: Non-hydrolyzable ATP analogue such as 5'-adenylylimidodiphosphate (AMPPNP) failed to cause the inactivation of the enzyme, suggesting a mechanistic scheme involving phosphorylation of the inhibitors by ATP.[2,3] This is also supported by the fact that sulfone **12** served as a simple reversible inhibitor and the cyclized sulfoximine **11** was much less active (7 % inhibition at 5 μM). Although sulfone **12** did not inactivate the enzyme, its inhibition potency was more than five times higher than that of L-BSO as measured by the inhibition constant. The difference is still underestimated because the initial inhibition constant of sulfone **12** (K_i = 9.2 μM) is compared with the overall inhibition constant of L-BSO (K_i* = 49 μM) where the ATP-dependent tight binding equilibrium was established.

Table 1. Inhibition of *E. coli* γ-glutamylcysteine synthetase

Compound	Conc. [μM]	% Inhibition[a]	k_{inact}/K_i [$M^{-1}sec^{-1}$]	K_i* [μM]
Phosphinate **2**	5.7	35	436 ± 33	4.95 ± 0.27
Sulfoximine **3**	5.1	98	1206 ± 117	0.39 ± 0.11
L-BSO[b]	25.0	9.7	ND[c]	49.3 ± 7.40
Sulfone **12**	16.3	32	—[d]	9.23 ± 1.76[e]

[a] As measured by steady state inhibited velocities (v_s) (see text); [b] L-Buthionine-*SR*-sulfoximine (Sigma); [c] Not determined; [d] No time-dependent inhibition was observed; [e] Initial inhibition constant (K_i).

Another criterion relevant to inhibitor potency is the duration of enzyme inactivation. We therefore measured the extent of recovery of enzyme activity upon 1000-fold dilution after the enzyme was completely inactivated with sufficient concentrations of **2** or **3** and ATP. Under a standard assay condition, no regain of enzyme activity was observed with sulfoximine **3**, whereas a significant enzyme reactivation ($t_{1/2}$ < 1 min) was noted with phosphinate **2** as evidenced by a concave upward progress curve (Figure 2). Although the inhibition by sulfoximine **3** was virtually irreversible within the time scale of assay, a very slow regain of enzyme activity was observed ($t_{1/2}$ = 3.9 day) when the inactivated enzyme was gel filtered and incubated in the absence of ATP.[15] Under the same conditions, the enzyme inactivated by L-BSO regained almost 40% of activity immediately after gel filtration (data not shown). Thus, the incorporation of a carboxyl group at the β-carbon to the sulfoximine

sulfur atom not only enhanced the inhibitor binding, but also increased the duration of enzyme inactivation significantly.

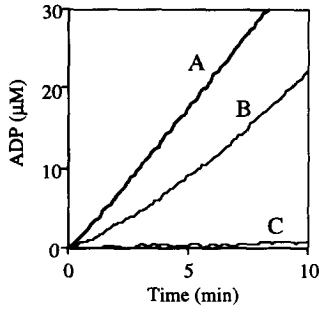


Figure 2. Regain of enzyme activity. The enzyme (10 μM) was inactivated by phosphinate **2** (1 mM, curve B) or sulfoximine **3** (0.1 mM, curve C) in the presence of ATP (5 mM) at 25°C for 30 min in 50 mM Tris-HCl (pH 7.5), and the enzyme reaction was initiated after 1000-fold dilution by adding a standard assay solution. Curve A (Control) : no inhibitor added.

A question may arise here why the enzyme regains activity so quickly with phosphinate **2**, but not with sulfoximine **3**, although both compounds are structurally analogous. If the phosphorylation of phosphinate oxygen or sulfoximine nitrogen is prerequisite for enzyme inactivation, then the chemical stability of the phosphorylated species may account for the difference. Thus, the putative *N*-phosphorylated sulfoximine is expected to be hydrolytically stable since a similar species was actually isolated from glutamine synthetase inactivated by methionine sulfoximine and ATP.[16] On the other hand, the presumed phosphorylated phosphinate, a phosphinic-phosphoric anhydride, is potentially unstable and is still capable of transferring the phosphoryl group either to water (hydrolysis) or to ADP (reverse reaction) to release the free phosphinate **2**. So far we favor the latter possibility, because no ADP formation was detected when the enzyme was incubated with ATP and phosphinate **2** under a standard assay condition, under which the enzyme regains activity quickly as shown in Figure 2.

The phosphinate **1** and **2** are designed by the same rationale as a transition-state analogue inhibitor of *E. coli* glutathione synthetase and γ-GCS, respectively. However, a large difference was observed in the rate of enzyme reactivation: glutathione synthetase was inhibited by **1** almost irreversibly (regain $t_{1/2}$ = 53 h),[2] while γ-GCS inactivated by **2** regained the activity rather quickly ($t_{1/2}$ < 1 min). The difference may reflect the active site geometry of both enzymes which share the same chemistry. Since high resolution X-ray structure of *E. coli* glutathione synthetase has been determined,[2,17] an answer to this question awaits the elucidation of the crystal structure of γ-GCS complexed with phosphinate **2**.

In conclusion, the incorporation of a carboxyl function at the β-carbon to the hetero atom dramatically increased the inhibition potency, suggesting that the active site of *E. coli* γ-GCS is furnished with a recognition site for the carboxyl group of the attacking cysteine in the transition state. These results emphasize the importance of inhibitor design considering this point. The stereoselective synthesis of a series of sulfoximine-based inhibitors is in progress and will be reported in due course.

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13. A plot of k_{obs} vs. [I] with phosphinate **2** gave a saturation curve, thereby furnishing a value of 28.9 μM for the initial inhibition constant (K_i) and 0.0126 sec^{-1} for the inactivation rate constant (k_{inact}). No saturation, however, was observed with sulfoximine **3** at concentrations up to 10 μM, beyond which rapid inactivation precluded the measurement of initial reaction rate by conventional methods.
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15. The enzyme (10 μM) was inactivated with sulfoximine **3** (0.1 mM), ATP (0.5 mM) and $MgCl_2$ (1.0 mM) in 50 mM Tris-HCl (pH 7.5) at 25°C for 30 min. The inactivated enzyme (residual activity < 0.1%) was isolated by gel filtration and was incubated at 25°C in 20 mM Tris-HCl (pH 7.3) containing $MgCl_2$ (5 mM) and 10% (v/v) glycerol. An aliquot of the enzyme solution was taken periodically and was assayed for enzyme activity.
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