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Design and Synthesis of Potent Inhibitors of Glutamine Synthetase

1. Cyclic Analogs of Phosphinothricin

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A series of γ-substituted and cyclic phosphinothricin analogs have been synthesized, and their interaction with mung bean glutamine synthetase has been characterized. The kinetics of inactivation and reactivation were determined for these compounds and the measured rate constants were then used to estimate their dissociation constants. A cyclopentane phosphinothricin analog is as effective as L-phosphinothricin itself, whereas the cyclohexane analog binds nearly 700-fold less tightly to the enzyme. Another analog, γ-hydroxy-L-phosphinothricin, inactivates the enzyme 50% faster than L-phosphinothricin. On the basis of these results, a heterocyclic analog was designed and synthesized. The L-*cis* isomer of this analog binds 2.3-fold less tightly than L-phosphinothricin, but is a 4.4-fold faster inactivator. The results demonstrate that both the inactivation and the reactivation kinetics must be measured to fully characterize inhibitor binding. In addition, the results indicate that there is significant tolerance for the design of novel, mechanism-based inhibitors of glutamine synthetase.

L-Phosphinothricin is the active component of the herbicides glufosinate (Hoechst) and bialaphos (Meiji Seika) (*1, 2*). Scheme 1 illustrates that glutamine synthetase catalyzes the phosphorylation of L-phosphinothricin by ATP to yield a structure that mimics a high energy intermediate (formed by addition of ammonia to γ-glutamyl phosphate) along the reaction pathway (*3–7*). In plants, inhibition of glutamine synthetase causes a 30- to 100-fold increase in the level of free ammonia, which is the actual phytotoxic species (*1, 2*). L-Phosphinothricin and other natural product inhibitors of glutamine synthetase (methionine sulfoximine and tabtoxinine-β-lactam) represent some of the earliest known examples of mechanism-based enzyme inhibitors. Colanduoni and Villafranca and others (*7–12*) characterized the interaction of phosphinothricin with glutamine synthetase. Logusch and others (*13–17*) synthesized analogs of phosphinothricin and characterized their interaction with the enzyme. The earliest work in this area was carried out by Meister and co-workers. They reported extensively on the inactivation of glutamine synthetase by methionine sulfoximine and also mapped the active site utilizing a series of glutamate analogs (*3, 18–23*). In this paper, we build on those results and report the preparation and testing of a series of phosphinothricin

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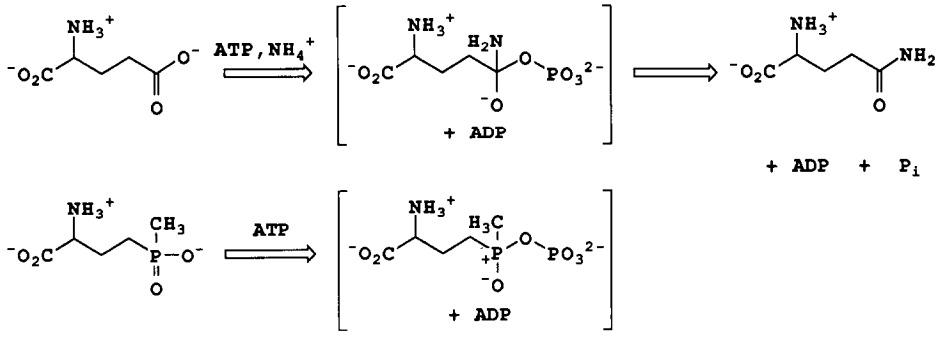


SCHEME 1

analogs. Included in this series are some compounds reported by Logusch *et al.* (*13*), as well as more potent carbocyclic and heterocyclic analogs.[1]

The isolation of L-phosphinothricin (**1** in Table 1) and the synthesis of γ-hydroxy-L-phosphinothricin, **3**, and the heterocyclic analog, **6**, will be described elsewhere. The ^1H and ^{13}C NMR spectra indicated that **3** was a 2 : 1 mixture of the *erythro* and *threo* isomers, although which isomer predominated was not established. Compound **6** was a mixture of *cis* and *trans* amino acids in a one to four ratio as shown by ^1H and ^{13}C NMR spectroscopy. Compounds **2**, **4**, and **5** were prepared in a manner analogous to that described by Logusch *et al.* (*13*).

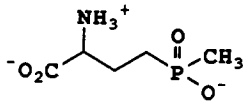
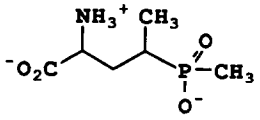
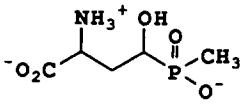
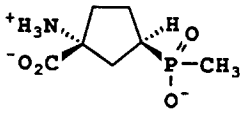
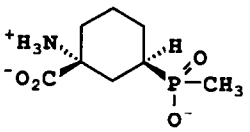
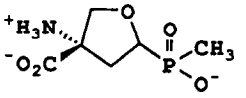
Glutamine synthetase was partially purified from mung beans. The enzyme was assayed at pH 7.0 using the pyruvate kinase/lactate dehydrogenase coupling system. The inactivation kinetics were measured by rapid addition of the inhibitor to an assay mixture containing 2.0 mM L-glutamate at 20°C with MgATP and NH_4^+ at saturating concentrations. Inactivation rate constants (k_{obs}) were obtained by fitting the data to the equation $\text{Abs} = Ae^{-k(\text{obs})^* t} + B^* t + C$ (24). The k_{inact} and K_{iapp} values for each inhibitor were estimated using a nonlinear least-squares hyperbolic fit of the k_{obs} and inhibitor concentration data. The K_i values (the dissociation constants which characterize the rapidly reversible interaction of the inhibitors and MgATP with the enzyme *prior* to the inactivation event) were then calculated using the relationship $K_i = K_{iapp}/(1 + [\text{Glu}]/K_s)$. The reactivation rate constants, k_{react}, were determined by measuring the recovery of enzymatic activity following inactivation of the enzyme with the inhibitors. Samples of inactivated enzyme were diluted sufficiently to allow reactivation and portions were removed at various times to determine the recovered activity. A linear least-squares fit of $\log(\text{activity}_{control}) - \log[(\text{activity}_{control}) - (\text{activity}_{sample})]$ versus time was used to determine k_{react} (*25*). The K_i^* values (the dissociation constants that characterize the interaction of the inhibitors and MgATP with the enzyme and include the inactivation and reactivation reactions) were calculated using the relationship $K_i^* = K_i \times k_{react}/k_{inact}$ (*26, 27*).[2]

[1] Some of these results were reported at the Eleventh Enzyme Mechanisms Conference in St. Petersburg Beach, FL, January 6–8, 1989.

[2] Details of enzyme purification, assay conditions, and data analysis will be published elsewhere.

TABLE 1

Inhibition of Glutamine Synthetase by Phosphinothricin Analogs

	COMPOUND	k_{inact}/K_i[a] ($mM^{-1}min^{-1}$)	k_{react}[b] (min^{-1})	K_i* (nM)
1.[c] (L)		45±4	$1.0\pm0.15X10^{-5}$	0.22±0.05
2. (D,L)		1.9±0.2	$2.6\pm0.3X10^{-4}$	140±30
3.[c] (L)		66±6	$3.0\pm0.1X10^{-3}$ [d,e,f] $5\pm0.3X10^{-5}$ [d,e,f]	45±6 0.8±0.1
4.[g] (D,L)		21±2	$9.8\pm1.7X10^{-6}$	0.47±0.12
5.[g] (D,L)		3.6±0.2	$1.1\pm0.1X10^{-3}$ [e]	310±20
6.[h] (D,L)		20±2 100±10 [i]	$1.0\pm0.2X10^{-4}$	5.0±1.5 1.0±0.3 [i]

[a] Values determined at 20°C without glycerol present. Glycerol (20%) has no effect on this ratio.

[b] Values determined at 20°C with 20% glycerol present. Glycerol (20%) decreases the reactivation rate.

[c] This compound is the L isomer. The remaining amino acids are D,L mixtures.

[d] The reactivation kinetics were biphasic.

[e] This value was determined without 20% glycerol and was corrected for the effect of glycerol (factor = 3.6).

[f] This value determined at 30°C and corrected to 20°C (factor = 3.7).

[g] This compound is the *cis* isomer.

[h] This compound is a mixture of 20% *cis* and 80% *trans* isomers.

[i] This is the calculated value for the *cis* isomer assuming that the *trans* isomer is not active.

Table 1 summarizes the results of experiments with glutamine synthetase and the phosphinothricin analogs. The k_{inact}/K_i ratio measures the relative effectiveness of each inhibitor as an inactivator. The design of potent glutamine synthetase inhibitors depends on both maximizing the k_{inact}/K_i ratio and minimizing k_{react} (however, for herbicidal activity, the k_{inact}/K_i ratio is more important if the half-life of reactivation is sufficiently long for phytotoxicity to be irreversible). γ-Methylphosphinothricin, **2,** and the cyclohexane analog, **5,** are the slowest inactivators listed. The cyclopentane analog, **4,** is equal in effectiveness to L-phosphinothricin, **1,** if only the L isomer is active.[3]

The k_{react} values reported here vary by more than three orders of magnitude. Glutamine synthetase, inhibited with L-phosphinothricin, reactivates with a half-life of 48 days under these conditions. The differences in the reactivation rates for γ-methylphosphinothricin, **2,** the cyclopentane analog, **4,** and the cyclohexane analog, **5,** indicate that both size and geometry greatly influence the reactivation rate. γ-Hydroxy-L-phosphinothricin, **3,** exhibits biphasic reactivation kinetics, perhaps reflecting different reactivation rates for the *erythro* and *threo* isomers.

The K_i^* values listed in the table characterize the binding of the inhibitors and MgATP with the enzyme. The cyclopentane analog, **4,** binds 300-fold more tightly to the enzyme than γ-methylphosphinothricin, **2,** and almost 700-fold more tightly than the cyclohexane analog, **5.** This illustrates that small steric and geometric differences have large effects on inhibitor binding.

On the basis of the observation that **4** binds as well as L-phosphinothricin and that **3** is a faster inactivator of glutamine synthetase, the heterocyclic analog, **6,** was designed and synthesized. Although compound **6** (L-*cis* isomer) binds 2.3-fold less tightly (K_i^*) than L-phosphinothricin, it nevertheless is a 4.4-fold faster inactivator (k_{inact}/K_i).

The substrate analogs of **3,** *erythro-* and *threo*-γ-hydroxy-L-glutamate, are excellent substrates for sheep brain glutamine synthetase. The V_{max}/K_m ratio for the *threo* and *erythro* isomers are, respectively, 1.6 and 0.57 times the V_{max}/K_m ratio of L-glutamate (*18*). In the present studies, the k_{inact}/K_i ratio for the *threo* and *erythro* mixture of γ-hydroxy-L-phosphinothricin, **3,** is 1.5 times the k_{inact}/K_i ratio of L-phosphinothricin, **1.** Likewise, the V_{max}/K_m ratio for *threo*-γ-methyl-L-glutamate is 0.29 times the V_{max}/K_m ratio of L-glutamate (*18*) and in the present studies the k_{inact}/K_i ratio for γ-methylphosphinothricin is 0.17 times the ratio for L-phosphinothricin.[4] On the other hand, the correlation between the V_{max}/K_m ratio of the glutamate analogs and the k_{inact}/K_i ratio of the phosphinothricin analogs does not hold with the cyclic structures. The V_{max}/K_m ratio for the cyclohexane glutamate analog is approximately 3 times the value for the cyclopentane analog (*22*). This

[3] The k_{inact}/K_i ratio for D,L-phosphinothricin is approximately one-half the value for L-phosphinothricin.

[4] The k_{inact}/K_i ratio used in this comparison for γ-methylphosphinothricin was calculated by assuming that only one of the four isomers present in the sample was active. This is a reasonable assumption since only *threo*-γ-methyl-L-glutamate is a substrate for glutamine synthetase (*3*).

contrasts with the lower k_{inact}/K_i ratio observed for the cyclohexane phosphinothricin analog, **5**, when compared with that for the cyclopentane analog, **4**.[5]

Gass and Meister developed a computer model for the active site of glutamine synthetase (*20, 21*). In studies with the cyclopentane glutamate analog (*22*), they found that some conformations of the phosphorylated tetrahedral intermediate derived from the cyclopentane analog of glutamate fit into the model's active site better than the tetrahedral intermediate derived from L-glutamate. The results in Table 1, indicating that the carbocyclic, **4**, and heterocyclic, **6**, cyclopentyl analogs are particularly effective glutamine synthetase inhibitors, are consistent with those computer modeling studies.

The results reported in this paper demonstrate the critical importance in fully characterizing the kinetics of inhibition (i.e., both inactivation and reactivation kinetics) when evaluating slowly reversible inhibitors such as phosphinothricin and its analogs. An understanding of the steric and electronic features that are important in maximizing the k_{inact}/K_i ratio and minimizing k_{react} should lead to the design of more potent inhibitors. Likewise, a better understanding of the reactivation reaction is also essential to inhibitor design. It is not known if reactivation proceeds by reversal of the inactivation reaction with resynthesis of ATP or if the phosphorylated inhibitor is released intact (followed by rapid hydrolysis to the original inhibitor and phosphate). Experiments to be published will address this question. Finally, these results demonstrate that there is significant tolerance for the design of novel, mechanism-based inhibitors of the glutamine synthetase.

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[5] Ammonia concentrations greatly influence the measured kinetic constants with the cyclic glutamate analogs (*22*). This difference in behavior between the cyclic glutamate analogs and the cyclic phosphinothricin analogs may reflect the effect of ammonia or possibly a difference in the sheep brain and mung bean enzymes.

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