252.0998, found 252.100 ± 0.001.

The third compound to elute was compound **16a**. It was obtained as 6 mg (3%) of fairly pure oil: ^{1}H NMR ($CDCl_3$) δ 7.20 (H_a, t, J_{ae} = 7.5 Hz, J_{af} = 7.5 Hz), 5.86 (H_b, d, J_{bd} = 3.8 Hz), 5.06 (H_c, dd, J_{cd} = 12 Hz, J_{cg} = 5.6 Hz), 4.52 (H_d, dd, J_{cd} = 12 Hz, J_{bd} = 3.8 Hz), 3.82 (3 H, s, carbomethoxy), 3.75 (3 H, s, carbomethoxy), 2.47 (H_e, m, J_{ae} = 7.5 Hz, also coupled to H_f), 2.20 (H_f and H_g, m, J_{af} = 7.5 Hz, J_{cg} = 5.6 Hz, also coupled to $H_{e,i,j}$), 2.02 (H_h, m), 1.76 (H_i, m, coupling was observed to $H_{e,f,g}$), 1.57 (H_j, m, coupling was observed to $H_{e,f,g}$).

Diels–Alder adduct **17a** was next to elute and was obtained as 74 mg (34%) of oil: IR (neat) 2960, 1740, 1715, 1635, 1440, 1285, 1270, 1250, 1200, 1105, 1065 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 6.86 (1 H, dd, J = 6.1, 1.8 Hz), 4.48 (1 H, t, J = 6.8 Hz), 3.83 (3 H, s), 3.78 (3 H, s), 3.59 (1 H, ddd, J = 6.8, 5.9, 1.8 Hz), 3.19 (1 H, m), 2.11 (1 H, dd, J = 9.8, 5.9 Hz), 2.07–1.91 (2 H, m), 1.68 (1 H, d, J = 9.8 Hz), 1.47–1.26 (2 H, m); ^{13}C NMR ($CDCl_3$) δ 172.2 (s), 165.5 (s), 140.0 (d), 139.4 (s), 81.3 (s), 74.1 (d), 52.6 (q), 52.0 (q), 45.6 (t), 39.8 (d), 39.1 (d), 24.0 (t), 18.6 (t); mass spectrum, m/e (rel intensity) 252 (M^+, 26.4), 221 (16.1), 220, (36.7), 213 (19.8), 195 (43.6) , 193 (34.8), 163 (26.5), 161 (66.2), 149 (47.4), 133 (55.6), 119 (27.2), 118 (30.8), 105 (96.6), 91 (100), 84 (88.3), 77 (46.0). Anal. Calcd for $C_{13}H_{16}O_5$: C, 61.90; H, 6.39. Found: C, 61.90; H, 6.50.

The final compound to elute was Diels–Alder adduct **18a**, and it was obtained as 53 mg (25%) of a white powder. An analytically pure sample was prepared by sublimation (50–60 °C, 0.1 Torr): mp 109–110 °C; IR ($CHCl_3$) 3010, 2955, 1740, 1440, 1290, 1270, 1205 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 6.76 (1 H, d, J = 9.3 Hz), 6.51 (1 H, dd, J = 9.3, 7.6 Hz), 4.75 (1 H, t, J = 5.4 Hz), 3.76 (3 H, s), 3.73 (3 H, s), 2.64 (1 H, m), 2.55 (1 H, dd, J = 11, 5.4 Hz), 2.23 (1 H, dd, J = 12, 3.6 Hz), 2.06 (1 H, d, J = 11 Hz), 1.98 (1 H, dt, J = 14, 5.4 Hz), 1.80 (1 H, dm, J = 12 Hz), 1.54 (1 H, dm, J = 14 Hz); ^{13}C NMR ($CDCl_3$) δ 173.4 (s), 172.1 (s), 137.0 (d), 134.5 (d), 88.1 (s), 79.3 (d), 56.5 (s), 52.4 (q), 52.3 (q), 47.9 (t), 41.1 (t), 29.7 (t), 29.3 (d); mass spectrum, m/e (rel intensity) 252 (M^+, 11.8), 221 (10.8), 220 (27.0), 193 (9.3), 151 (13.3), 150 (41.0), 133 (21.5), 119 (25.7), 118 (26.1), 105 (53.9), 91 (100). Anal. Calcd for $C_{13}H_{16}O_5$: C, 61.90; H, 6.39. Found: C, 62.11; H, 6.49.

Thermolysis of 4b. Diacid **4b** was heated at 80 °C in DMSO-d_6 under a N_2 atmosphere. This gave a complex mixture of products that could not be separated. The ^{1}H NMR spectrum of the crude reaction mixture suggested the formation of products analogous to those formed from the thermolysis of **4a**. The half-life for disappearance of **4b** under these conditions was crudely estimated between 11 and 16 h.

Thermolysis of 4c. Disodium salt **4c** (15.7 mg, 0.059 mmol) was dissolved in DMSO-d_6 (1 mL) and placed in a NMR tube under a N_2 atmosphere. Complete conversion to double-bond isomer **15c** was observed after heating the sample at 70 °C for 11 h: ^{1}H NMR (DMSO-d_6) δ 6.73 (1 H, t, J = 5.5 Hz), 6.04 (1 H, s), 5.16 (1 H, s), 4.50 (1 H,s), 2.44 (2 H, t, J = 5.5 Hz), 2.30 (2 H, q, J = 5.5 Hz), 1.82 (2 H, quintet, J = 5.5 Hz).

Disodium 1-(2-Carboxylato-2-oxoethyl)cyclohepta-2,6-diene-1-carboxylate (5c). Cold 1.0 M NaOH (0.064 mL, 0.064 mmol, 3.0 equiv) was added to a solution of diester **5a** (5.4 mg, 0.021 mmol) in THF/H_2O (2:1, 1.5 mL) at ~0 °C (ice/H_2O). The yellow solution was stirred for 5 h, and the pH was adjusted to 6.8 with Amberlite IR-120 (plus) acidic resin. The resin was removed by suction filtration, and the filtrate was concentrated to give 7.0 mg (>100%) of hygroscopic disodium salt **5c** as an off-white solid: ^{1}H NMR (CD_3OD) δ 5.74 (4 H, m), 3.18 (2 H, br s), 2.25 (4 H, br m).

A sample of diacid **5b** (pale yellow solid) was prepared as indicated above, except that the pH was adjusted to ~4 before filtration: IR (neat) 3400–3000, 3027, 2926, 1773, 1719, 1636, 1437, 1186 cm^{-1}; ^{1}H NMR (acetone-d_6) δ 5.90 (2 H dt, J = 11, 5.4 Hz), 5.63 (2 H, d, J = 11 Hz), 3.33 (4 H, br s, acid + methylene protons), 2.46 (2 H, m), 2.19 (2 H, m); mass spectrum, m/e (rel intensity) 224 (M^+, 0.6), 180 (8.9), 151 (17.4), 150 (10.6), 149 (10.2), 136 (29.9), 135 (25.1), 133 (25.3), 105 (64.2), 91 (100).

Acknowledgment. We are grateful to the National Institutes of Health, Grant GM 31958, for financial support. We thank Professor John F. Morrison for a generous supply of chorismate mutase–prephenate dehydrogenase from *E. coli* JFM30. We acknowledge Dr. John J. Delany and Dr. Robert A. Aleksejczyk for performing inhibition studies with chorismate mutase–prephenate dehydrogenase.

Synthesis of α- and γ-Alkyl-Substituted Phosphinothricins: Potent New Inhibitors of Glutamine Synthetase

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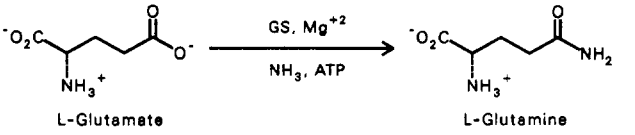
Considerations of substrate structural variability for the enzyme glutamine synthetase (GS) have led to the design of α- and γ-substituted analogues of the naturally occurring GS inhibitor phosphinothricin (PPT). The novel cyclic inhibitor D,L-cyclohexanephosphinothricin (CHPPT) was prepared via conjugate addition of diethyl methylphosphonite to 2-cyclohexenone, followed by stereospecific Bucherer–Bergs amino acid synthesis. CHPPT stereochemistry was determined by COSY and NOESY 2D NMR techniques. The substituted phosphinothricins function as active site probes useful for elucidating the mechanism of GS inhibition by PPT.

Introduction

The enzyme glutamine synthetase (GS, EC 6.3.1.2) catalyzes a reaction of central importance in nitrogen metabolism, the conversion of L-glutamate to L-glutamine.[1] The amide functionality of glutamine is the ultimate source of nitrogen introduced into α-amino acids via transaminase-catalyzed reactions and also provides nitrogen for the urea cycle and pyrimidine biosynthesis.[2]



(1) (a) Meister, A. In *The Enzymes*; Boyer, P. D., Ed.; Academic: New York, p 699. (b) Stadtman, E. R.; Ginsburg, A. *Ibid.*; p 755.

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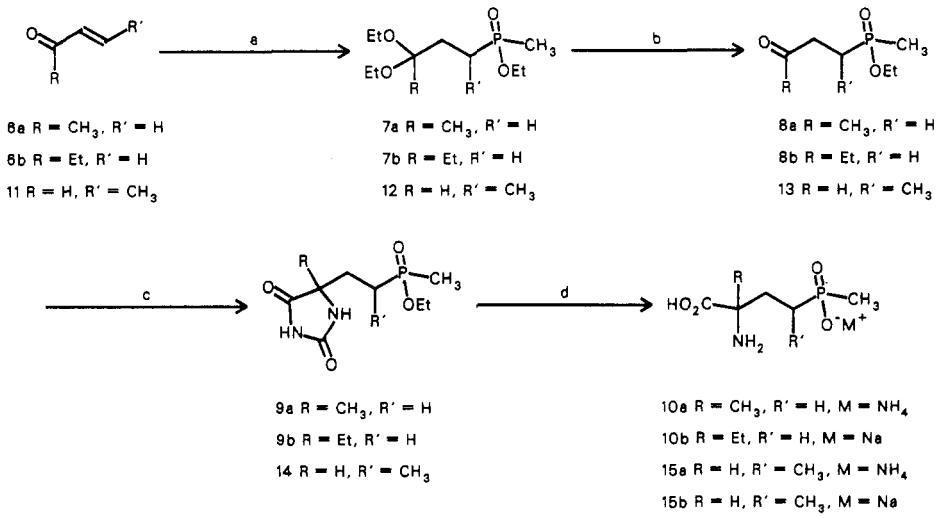
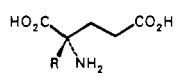
Scheme I[a]

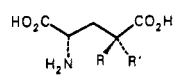


[a] Reagents: (a) $CH_3P(OEt)_2$, EtOH; (b) 2 N HCl; (c) KCN, $(NH_4)_2CO_3$, aqueous EtOH, 55 °C; (d) $Ba(OH)_2$, H_2O, reflux; Dowex 50 (NH_4OH); 1 equiv NaOH.

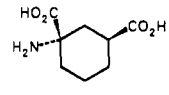
While L-glutamic acid (**1**) is the natural substrate for glutamine synthetase, substituted glutamic acids are also known to function as substrates.[3] These include the α- and γ-alkyl-substituted analogues **2–4**, all of which display substrate K_m values comparable to that of glutamic acid itself. Enzymes recognizing a variety of substrates for reaction catalysis may be subject to inhibition by variously substituted transition state analogue inhibitors. The correlation between substrate efficacy of a congeneric series of substrates and the potency of analogously substituted inhibitors has recently been advanced as a fundamental criterion for transition state analogue inhibition.[4]



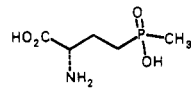
1 R = H
2 R = CH_3



3a R = CH_3, R' = H
3b R = H, R' = CH_3



4



5

Phosphinothricin (PPT) (**5**) is a phosphinic acid mimic of L-glutamic acid produced by various streptomycete species.[5] It is a potent inhibitor of glutamine synthetase[6] and an effective herbicide against a broad range of plant species.[7] We have been engaged in the design of analogues of phosphinothricin,[8] with the aim of obtaining a better understanding of its mechanism of inhibition, and have been interested in compounds bearing α- and γ-substituents by analogy with substrates of types **2–4**. We describe herein a number of such substituted phosphinothricins, which are potent new inhibitors of glutamine synthetase. These analogues were prepared by phosphonite 1,4-additions to appropriate carbonyl precursors, followed by Bucherer–Bergs amino acid synthesis. The stereochemistry of the cyclic analogue was unambiguously determined by a combination of COSY and NOESY 2D NMR techniques. The substituted phosphinothricins display unique enzyme inhibitory properties and are useful for defining conformational and steric requirements for phosphinothricin binding at the active site of glutamine synthetase.[9]

Results and Discussion

Treatment of methyl vinyl ketone (**6a**) and ethyl vinyl ketone (**6b**) with diethyl methylphosphonite in absolute ethanol afforded the acetal phosphinates **7a** and **7b**, acid hydrolysis of which gave the ketones **8a** and **8b** (Scheme I).[10] The latter were converted to the hydantoins **9a** and **9b** under Bucherer–Bergs conditions (KCN and ammonium carbonate in refluxing aqueous methanol). Hydrolysis of **9a** and **9b** with refluxing aqueous barium hydroxide, followed by ion-exchange purification, furnished the ammonium salt of D,L-α-methylphosphinothricin (AMPPT) (**10a**) and the sodium salt of D,L-α-ethylphosphinothricin (AEPPT) (**10b**). Repetition of this sequence, commencing with crotonaldehyde **11**, provided the D,L-γ-methylphosphinothricin (GMPPT) as a 57:43 mixture of diastereomers in either ammonium (**15a**) or sodium (**15b**) salt forms.[11]

Enzymatic assay of the α- and γ-alkyl-substituted phosphinothricins **10a** (AMPPT) and **15b** (GMPPT) demonstrated their ability to inhibit mammalian glutamine synthetase (see below). Our next goal was to connect the α- and β-substituents in a conformationally restricted carbocyclic ring analogue of the cyclic glutamic acid sub-

(2) Miflin, B. J.; Lea, P. J. *Annu. Rev. Plant Physiol.* **1977**, *28*, 299.
(3) Meister, A. *Harvey Lect.* **1969**, *63*, 139.
(4) (a) Bartlett, P. A.; Marlowe, C. K. *Biochemistry* **1983**, *22*, 4618. (b) Bartlett, P. A.; Marlowe, C. K. *Science (Washington, D.C.)* **1987**, *235*, 569.
(5) (a) Bayer, E.; Gugel, K. H.; Hägele, K.; Hagenmeier, H.; Jessipow, S.; König, W. A.; Zähner, H. *Helv. Chim. Acta* **1972**, *55*, 224. (b) Ogawa, Y.; Tsuruoka, T.; Inoue, S.; Niida, T. *Meiji Seika Kenkyu Nempo* **1973**, *No. 13*, 42; *Chem. Abstr.* **1974**, *81*, 37806r. (c) Omura, S.; Hinotozawa, K.; Imamura, N.; Murata, M. *J. Antibiot.* **1984**, *37*, 939.
(6) Colanduoni, J. A.; Villafranca, J. J. *Bioorg. Chem.* **1986**, *19*, 163.
(7) (a) Ridley, S. M.; McNally, S. F. *Plant Sci. (Limerick, Irel.)* **1985**, *39*, 31. (b) Acaster, M. A.; Weitzman, P. D. J. *FEBS Lett.* **1985**, *189*, 241.
(8) Logusch, E. W. *Tetrahedron Lett.* **1986**, *27*, 5935.
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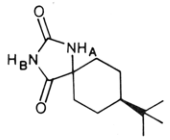
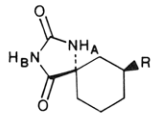
(10) (a) Harvey, R. G. *Tetrahedron* **1966**, *22*, 2561. (b) Worms, K. H.; Schmidt-Dunker, M. In *Organic Phosphorus Compounds*; Kosolopoff, G. M., Meier, L., Eds.; Wiley: New York, 1976; Vol. 7, Chapter 18.
(11) Scheme I experimental details included as supplementary material.

Table I. Spiro Hydantoin N–H Proton Shifts[a]

compound	H_A	H_B
		
23 (trans)	7.80	10.55
24 (cis)	8.62	10.65
		
25, R = CO_2H	8.43	10.60
19, R = P(O)(Me)OH	8.47	10.67

[a] Determined in DMSO-d_6.

strate **4**. Since the isomer of **4** possessing trans carboxyl groups is inactive as a substrate,[12] the C-3 methylphosphinic acid analogue of **4** having both acid groups cis was needed in order to function as an effective inhibitor of glutamine synthetase. Studies with conformationally defined cyclohexanones have demonstrated that the Strecker synthesis (via amino nitriles) gives amino acids with an axial carboxyl group, whereas the Bucherer–Bergs reaction (via hydantoins) proceeds with largely the opposite stereochemistry.[13] We hoped that a C-3 methylphosphinyl group would exert sufficient conformational control to permit the synthesis of a cyclic amino acid having the proper stereochemistry.

2-Cyclohexenone (**16**) was converted to the methylphosphinyl spiro hydantoin **19**, which consisted of a 92:8 mixture of diastereomers after purification (Scheme II). Hydrolysis of **19**, followed by ion-exchange purification, gave the sodium salt of D,L-cyclohexanephosphinothricin (CHPPT) (**20**), in 96% diastereomeric purity. During structure assignment, **20** was transformed to the amide **22** by a sequence of diazomethane treatment, acylation, and phosphinate deesterification with bromotrimethylsilane.

Studies on the stereochemistry of the Strecker and Bucherer–Bergs reactions permit the interpretation of NMR spectra of spiro hydantoins similar to **19** (Table I). The hydantoin **23**, obtained from 4-*tert*-butylcyclohexanone and having an axial 4′-carbonyl group (Strecker product), was reported to display a proton resonance for H_A of 7.80 ppm.[13c] The equatorial carbonyl isomer **24** (Bucherer–Bergs product) shows an H_A resonance of 8.62 ppm, shifted downfield due to axial compression. We reprepared the spiro hydantoin **25**, from which amino acid **4** is formed upon base hydrolysis,[12] and observed a proton resonance for H_A of 8.43 ppm. Finally, the methylphosphinyl-substituted hydantoin **19** displays an H_A resonance of 8.47 ppm, consistent with the presence of the desired equatorial 4′-carbonyl group.

However, **19** was converted to the diacid **20** under fairly drastic conditions, making it desirable to establish the configuration of the latter compound as well. Since **20** could not be crystallized satisfactorily, the derivative **22** was prepared (Scheme II). The intermediate diester **21**, which consisted of inseparable phosphinate ester diastereomers, crystallized under all conditions as thin stacked plates unsuitable for X-ray crystallographic analysis. The

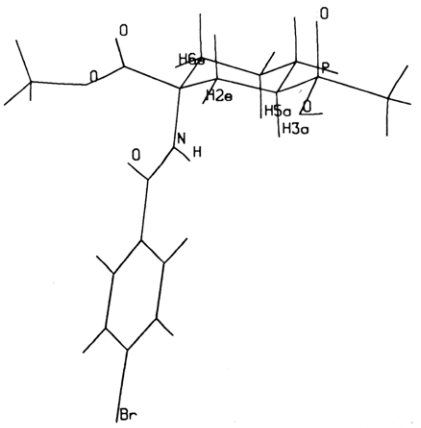


Figure 1. Energy-minimized structure of **22**. The calculated internuclear distances are as follows: NH–H6e, 2.51 Å; NH–H2e, 3.50 Å; NH–H5a, 2.14 Å; NH–H3a, 2.92 Å.

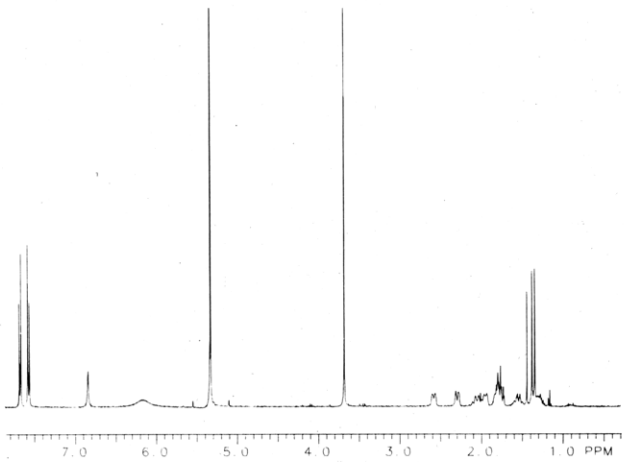


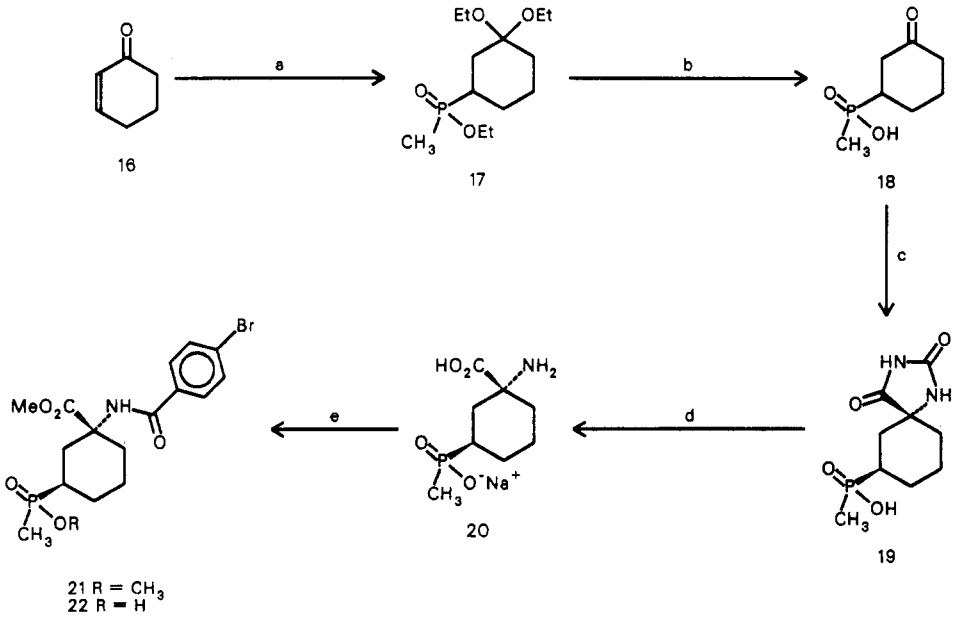
Figure 2. ^1H NMR spectrum (400 MHz) of PPT analogue **22** in CD_2Cl_2.

monoester **22** could not be crystallized. However, a rigorous structural determination of **22**, corroborating the cis stereochemistry assigned to the acid groups, was possible with the use of 2D NMR techniques.

To aid in interpreting NMR experiments, a model of **22** was constructed by using SYBYL software[14] on a VAX network. Standard functional group fragments were utilized, and the conformational energy of the overall structure was minimized with the MAXIMIN molecular mechanics routine.[15] The resulting low-energy conformer of **22** is depicted in Figure 1. ^1H NMR (400 MHz) and two-dimensional COSY spectra of **22** are shown in Figures 2 and 3. From the coupling pattern of Figure 3 it is evident that the multiplet at 2.06 ppm corresponds to H3a, the axial proton adjacent to phosphorus. Four geminal pairs of methylene protons can be distinguished, the equatorial proton in each case appearing downfield from the axial. Long-range W-type coupling is seen between the equatorial protons H2e and H6e. The two-dimensional NOESY spectrum[16] of **22** shows cross-peaks between the amide proton at 6.85 ppm and four other protons. These are the equatorial protons H2e and H6e and the axial protons H3a and H5a all of which are located near the amide NH (Figure 1).[17] The observation of a NOE between the

(12) Gass, J.; Meister, A. *Biochemistry* **1970**, *9*, 842.
(13) (a) Sacripante, G.; Edwards, J. T. *Can. J. Chem.* **1982**, *60*, 1982. (b) Trigo, G. G.; Avendano, C.; Santos, E.; Edward, J. T.; Wong, S. C. *Ibid.* **1979**, *57*, 1456. (c) Edward, J. T.; Jitrangsri, C. *Ibid.* **1975**, *53*, 3339. (d) Maki, Y.; Masugi, T. *Chem. Pharm. Bull.* **1973**, *21*, 685.
(14) Molecular modeling software purchased from Tripos Associates, Inc., 6548 Clayton Road, St. Louis, MO 63117.
(15) Labanowski, J.; Motoc, I.; Naylor, C. B.; Mayer, D.; Dammkoehler, R. A. *Quant. Struct.–Act. Relat.* **1986**, *5*, 138.
(16) Jeener, J.; Meier, B. H.; Bachmann, P.; Ernst, R. R. *J. Chem. Phys.* **1979**, *79*, 4546.

Scheme II[a]



[a] Reagents: (a) $CH_3P(OEt)_2$, EtOH; (b) 6 N HCl, reflux; (c) KCN, $(NH_4)_2CO_3$, aqueous EtOH, 55 °C; (d) $Ba(OH)_2$, H_2O, reflux; Dowex 50 (NH_4OH); 1 equiv of NaOH; (e) CH_2N_2, MeOH; *p*-BrPhCOCl, pyridine, DMAP, CH_2Cl_2; TMSBr, CH_2Cl_2.

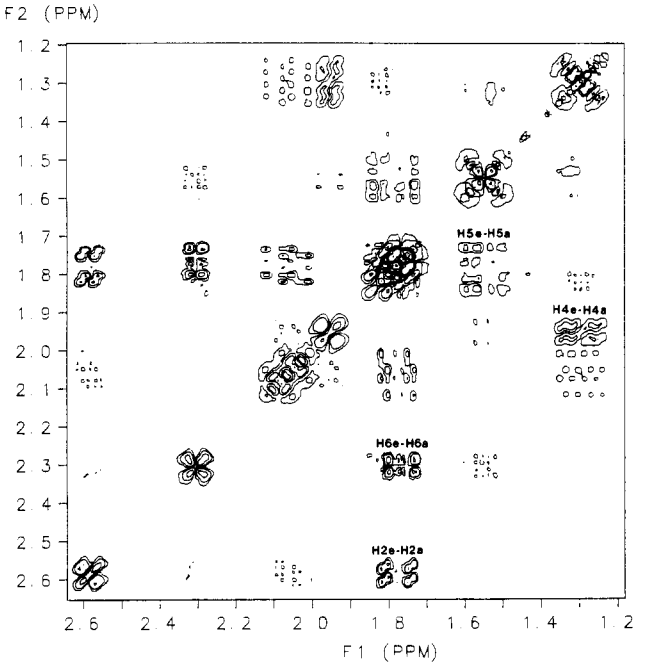


Figure 3. Contour plot of 300-MHz DQ-COSY spectrum of the high-field region of PPT analogue **22** in CD_2Cl_2 with the four geminal connectivities indicated.

amide NH and the proton adjacent to phosphorus confirms the cis relationship between the acid groups in **22**, as well as the configuration previously inferred for diacid **20**. D,L-Cyclohexanephosphinothricin (**20**), as expected, was an effective inhibitor of glutamine synthetase.

α- and γ-Substituted Analogues of Phosphinothricin as Inhibitors of Glutamine Synthetase. A competitive inhibitor's potency under steady-state conditions is characterized by the inhibition constant K_i, which can be determined from graphical treatment of enzyme activity in the presence of varying concentrations of both substrate and inhibitor.[18] The phosphinothricin analogues prepared in this study displayed competitive inhibition of sheep brain glutamine synthetase. The experimentally determined K_i values are listed in Table II, together with reported substrate K_m values for the corresponding glutamic acids. The α-alkylated and cyclic analogues (entries 2–4) gave similar values for K_i and were somewhat weaker inhibitors than D,L-phosphinothricin[8] (entry 1). The γ-methyl-substituted analogue GMPPT displayed significantly reduced inhibition (entry 5), in part because of the presence of both the *threo* and *erythro* diastereomers. Inhibition is expected only for the former isomer, since the *erythro*-γ-methylglutamic acid (**3b**) is not a GS substrate to any measurable extent.[3]

Table II. Substituted Phosphinothricin K_i's and Corresponding Glutamate K_m's

entry	compound	K_i,[a] μM	K_m,[b] mM
1	**5**, PPT	25.2	3.9
2	**10a**, AMPPT	125	6.7
3	**10b**, AEPPT	111	c
4	**20**, CHPPT	125	5.1
5	**15b**, GMPPT	407	3.6[d]

[a] Relative standard deviations are ≤15%. [b] As reported in ref 3. [c] Not available. [d] The *threo* isomer.

While the substituted phosphinothricins showed some differences in steady-state kinetic properties, the use of time-dependent kinetic methods revealed striking variations in the interaction of these compounds with mammalian GS. Figure 4 illustrates time-course experiments in which sheep brain GS was incubated for 15 min with ATP, $MgCl_2$, and 5 × K_i concentrations of inhibitor, followed by 50-fold dilution and continuous assay of enzyme activity. Under these conditions, recovery of GS activity was observed for all compounds with the exception of AMPPT. Inhibition with D,L-phosphinothricin resulted in slight but measurable recovery of activity.

(17) The amide NH would adopt an equatorial position in the chair conformation of the isomer of **23** having acid groups trans, making it impossible to observe a NOE with the axial protons H3a and H5a (calculated internuclear distances: 4.48 and 4.88 Å respectively).

(18) Fersht, A. *Enzyme Structure and Mechanism*; W. H. Freeman: New York, 1985.

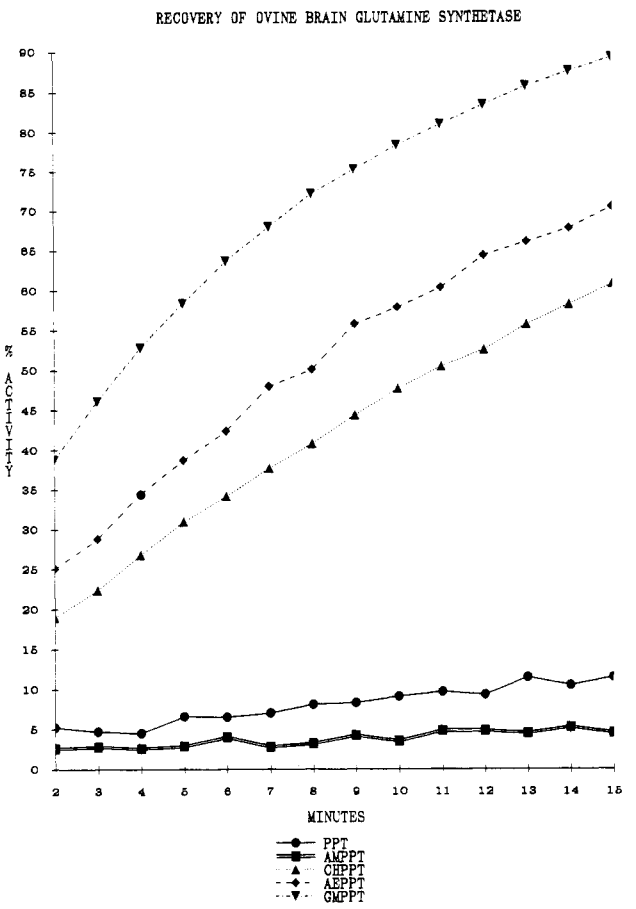
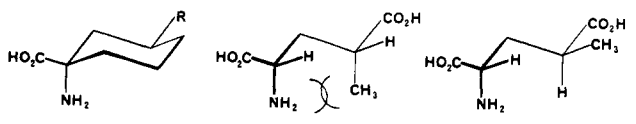


Figure 4. Recovery curves of PPT (**5**), AMPPT (**10a**), AEPPT (**10b**), GMPPT (**15b**), and CHPPT (**20**) with ovine brain GS.

Inhibitor α-alkyl substitution is evidently better accommodated by the mammalian enzyme than γ-alkyl substitution. Connection of α- and γ-alkyl substituents in a cyclohexane ring causes a decrease in the measured K_i value as well as reduced time-dependent enzyme recovery relative to γ-alkyl substitution. This effect is likely to have a conformational basis, since CHPPT presents its functional groups to the enzyme in a locked, fully staggered array (**26b**), whereas acyclic analogues must adopt such a conformation from among many possibilities, and suggests that phosphinothricin itself must adopt a fully staggered conformation. Relevant to this interpretation is the failure of *erythro*-γ-methylglutamate (**27**) to act as a GS substrate, in contrast with the reported *threo* substrate **28**.[3] In the former case, a 1,3 steric interaction prevents adoption of the staggered conformation. The tolerance of mammalian GS for α-alkyl groups in phosphinothricin is striking and suggests an enhanced ability of the enzyme active site to accommodate steric volume at this position, in contrast to the γ-position. In a related observation, both AMPPT and AEPPT are potent herbicides in vivo, whereas GMPPT and CHPPT are devoid of phytotoxicity. The enzyme's accommodation of inhibitor diversity does not extend to the relative distance adopted by the terminal acid functionalities. Addition or removal of a single intervening methylene carbon results in a complete loss of inhibitory activity.[19]



26a R = CO_2H
26b R = P(O)(Me)OH

27 28

Both $MgCl_2$ and ATP are required for enzyme inhibition and recovery, i.e., interaction of the phosphinothricins with GS occurs only under phosphorylation conditions. This result strongly suggests that such compounds undergo enzyme-mediated phosphorylation as do the glutamic acids, generating a close analogue **29** of the presumed biosynthetic reaction intermediate **30**. Variable recovery of GS activity may thus be a consequence of the differing extent to which substituted analogues of **30** can fit within the active site of the enzyme. Studies exploring these ideas are in progress and will be described in greater detail elsewhere.

29 30

Conclusion

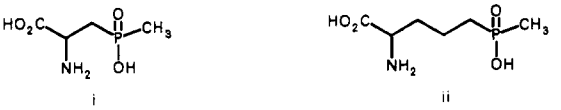
We have demonstrated that considerations of substrate variability for the enzyme glutamine synthetase provide a rationale for the design of novel inhibitory analogues of phosphinothricin. These substituted analogues are attractive steric probes for the active site of glutamine synthetase and can be used to study the mechanism of inhibition of glutamine synthetase by phosphinothricin. They can also be used to explore subtle interspecies variations in active-site structure. For example, we have found that preliminary steady-state kinetic evaluation of D,L-α-methylphosphinothricin (AMPPT) as an inhibitor of *Escherichia coli* glutamine synthetase provides an inhibition K_i value of 5.7 μM, very close to the K_i value of 1.1 μM displayed by D,L-phosphinothricin under the same conditions. Non-steady-state kinetic methods also show promise as tools for studying the mechanistic effects of GS inhibitor substitution. Finally, it is worthwhile to note that the ongoing X-ray structure determination of bacterial (*Salmonella typhimurium*) glutamine synthetase[20] offers the possibility of direct observation of the dynamic effects of bound phosphinothricin analogues on the enzyme conformation and active-site geometry.

Experimental Section

General Methods. All reactions were run under a nitrogen atmosphere. Preparative thin-layer chromatography was performed on 12-cm-diameter circular silica gel plates (PF-254/$CaSO_4$) by using a Harrison Research Model 7924 chromatotron. Melting points are uncorrected. ^1H NMR chemical shifts are reported in units of δ relative to internal tetramethylsilane (TMS) or the sodium salt of 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid. ^{13}C NMR chemical shifts are reported in units of δ relative to TMS, using the deuteriated solvent as the standard or relative to the internal instrument lock on D_2O. ^{31}P NMR chemical shifts are reported in units of δ relative to external H_3PO_4.

(2,4-Dioxo-1,3-diazospiro[4.5]dec-7-yl)methylphosphinic Acid (19). To a solution of cyclohexenone (5.78 g, 60 mmol) in dry ethanol (60 mL) at 0 °C was added diethyl methylphosphonite (9.0 mL, 60 mmol). The reaction mixture was allowed to warm

(19) Logusch, E. W.; McDonald, J. F., unpublished results. D,L-Norphosphinothricin i has been reported previously (ref 9c). D,L-Homophosphinothricin ii was prepared in the present study by alkylation of diethyl acetamidomalonate with ethyl (3-bromopropyl)methylphosphinate (see supplementary material).



i ii

(20) Almassy, R. J.; Janson, C. A.; Hamlin, R.; Xuong, N.-H.; Eisenberg, D. *Nature (London)* **1986**, *323*, 304.

to room temperature and was stirred overnight. The solution was concentrated, and the crude acetal **17** was taken up in 6 N HCl (100 mL) and heated to reflux for 6.5 h. The solution was concentrated (50 °C) to afford the crude keto phosphinic acid **18** as an orange oil.

The crude ketone was dissolved in aqueous methanol (50%, 200 mL), and ammonium carbonate (28.9 g, 0.3 mol) and potassium cyanide (4.29 g, 66 mmol) were added. The solution was heated at 55 °C for 18 h and at 100 °C for an additional 1.5 h. Half of the crude reaction solution was concentrated and acidified with 2 N HCl. The aqueous solution was saturated with NaCl and extracted with 2-butanol. The organic layer was washed with brine, dried (Na_2SO_4), and concentrated to afford a tan foam (5.40 g, 73%). Formation of the sodium salt or the cyclohexylamine salt of the phosphinic acid did not give analytically pure material. A portion of the cyclohexylamine salt (1.98 g, 5.74 mmol) in ethanol was passed through Dowex 50×8-100 ion-exchange resin. The ethanol solution was concentrated, taken up in water, and lyophilized to a fluffy white solid (0.85 g, 60%) of the hydantoin phosphinic acid: mp 255–259 °C dec; ^{1}H NMR (Me_2SO-d_6, 400 MHz) δ 1.24 (d, 3, J = 12.9 Hz), 1.12–1.32 (m, 2), 1.43–1.98 (series of m, 7), 4.72 (br s, 1), 8.47 (br s, 1), 10.67 (br s, 1); ^{13}C NMR (Me_2SO-d_6, 100.6 MHz, coupled) δ 12.8 (dq, $^1J_{CP}$ = 90.4 Hz), 20.7 (dt, $^3J_{CP}$ = 14.4 Hz), 23.9 (dt, $^2J_{CP}$ = 3.2 Hz), 32.7 (t), 32.9 (t), 33.6 (dd, $^1J_{CP}$ = 97.2 Hz), 62.2 (d, $^3J_{CP}$ = 13.6 Hz), 156.6 (t, J_{CH} = 7.0 Hz, $W_{1/2}$ = 14.0 Hz); ^{31}P NMR (Me_2SO-d_6, 40.3 MHz) δ 49.7 (92%), 50.8 (8%); high-resolution FAB MS (glycerol matrix) calcd for $C_9H_{16}N_2O_4P$ (MH^+) 247.0848, found 247.0896.

1-Amino-3-(hydroxymethylphosphinyl)cyclohexanecarboxylic Acid, Monosodium Salt (20). The remaining half of the crude reaction solution containing **19** was concentrated and dissolved in water (250 mL), barium hydroxide (octahydrate, 37.9 g, 0.12 mol) was added, and the suspension was heated to reflux for 56 h. Ammonium carbonate (16.35 g, 0.17 mol) was added, and reflux was continued for 2 h. The hot suspension was filtered, concentrated, and desalted on Dowex 50×8-100 ion-exchange resin (150 g) eluting with 3 N NH_4OH. The aqueous solution was concentrated to afford the ammonium salt of the phosphinic acid as a light yellow solid (5.50 g, 77% overall, mp 225–230 °C dec). The ammonium salt (1.92 g, 8.1 mmol) was dissolved in methanol (25 mL), and 2.5 N NaOH (3.2 mL, 8.0 mmol) was added. The sodium salt was precipitated by slow addition of acetone. The salt was filtered, dried under high vacuum, taken up in water, concentrated, and dried again to afford 1.53 g (78%) of a white solid: mp 322–324 °C dec; ^{1}H NMR (D_2O, 60 MHz) δ 1.17 (d, 3, J = 14 Hz), 1.10–3.20 (m, 9); ^{13}C NMR (D_2O, 90.5 MHz) δ 13.4 (d, $^1J_{CP}$ = 91.0 Hz), 20.8 (d, $^3J_{CP}$ = 13.8 Hz), 25.0, 32.5 (d, $^3J_{CP}$ = 24.1 Hz), 34.6 (d, $^1J_{CP}$ = 94.6 Hz), 61.1 (d, $^3J_{CP}$ = 12.2 Hz), 179.8; ^{31}P NMR (D_2O, 40.3 MHz) δ 44.5 (96%), 45.1 (4%).

Anal. Calcd for $C_8H_{15}NaNO_4P \cdot 1.5H_2O$: C, 35.56; H, 6.72; N, 5.18. Found: C, 35.65; H, 6.44; N, 5.13.

***trans*-3-(Methoxymethylphosphinyl)-1-[[(*p*-bromophenyl)carbonyl]amino]cyclohexanecarboxylic Acid, Methyl Ester (21).** To a solution of the ammonium salt of **20** (1.00 g, 4.20 mmol) in methanol (30 mL) was added an ethereal solution of diazomethane generated from *N*-nitroso-*N*-methylurea (5.07 g, 49 mmol). The yellow solution was stirred for 5 min, quenched with acetic acid, and concentrated. The crude product was purified on a 4-mm chromatotron plate (10% MeOH (1.5 M NH_3)/$CHCl_3$) to afford a light yellow oil (0.42 g, 40%): ^{1}H NMR ($CDCl_3$, 60 MHz) δ 1.39 (d, 3, J = 13.4 Hz), 1.15–2.80 (m, 11), 3.31 (d, 3, J = 11.0 Hz), 3.67 (s, 3); ^{31}P NMR ($CDCl_3$, 40.3 MHz) δ 58.5 (48%), 58.8 (52%).

The amine (0.42 g, 1.69 mmol) in dry CH_2Cl_2 (15 mL) was treated with *p*-bromobenzoyl chloride (0.41 g, 1.86 mmol), pyridine (0.15 mL, 1.85 mmol), and (dimethylamino)pyridine (20 mg). The reaction mixture was stirred overnight, diluted with CH_2Cl_2, washed with 2 N HCl and saturated $NaHCO_3$, dried ($MgSO_4$), and concentrated to a white solid (0.70 g). The product was purified on a 4-mm chromatotron plate (5% MeOH/$CHCl_3$) to afford the amide as a white solid (0.58 g, 79%): mp 191–195 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.40 (d, 1.5, J = 13.2 Hz), 1.42 (d, 1.5, J = 13.2 Hz), 1.50–1.60 (m, 1), 1.77–2.10 (m, 5), 2.23–2.35 (m, 1), 2.62–2.79 (m, 1), 3.63 (d, 1.5, J = 10.8 Hz), 3.70 (d, 1.5, J = 11.0 Hz), 3.74 (s, 1), 6.42 (br s, 1), 7.57–7.69 (m, 4); ^{31}P NMR ($CDCl_3$, 40.3 MHz) δ 59.7, 59.8; CI MS (MH^+) 432, 434.

***trans*-3-(Hydroxymethylphosphinyl)-1-[[(*p*-bromophenyl)carbonyl]amino]cyclohexanecarboxylic Acid, Methyl Ester (22).** A solution of the phosphinate ester **21** (0.31 g, 0.72 mmol) in dry CH_2Cl_2 (5 mL) at 0 °C was treated with bromotrimethylsilane (0.19 mL, 1.44 mmol) and was allowed to warm to room temperature overnight. The reaction mixture was concentrated, taken up in CH_2Cl_2, washed with H_2O, dried ($MgSO_4$), and concentrated to afford a white solid (0.26 g, 86%): mp 126–138 °C; ^{1}H NMR (CD_2Cl_2, 400 MHz) δ 1.30 (m, 1, H4a), 1.35 (d, 3, J = 13.8 Hz), 1.54 (m, 1, H5a), 1.77 (m, 3, H2a, H5e, H6a), 11.94 (br d, 1, H4e, J = 12.1 Hz), 2.06 (m, 1, H3a), 2.29 (br d, 1, H6e, J = 12.4 Hz), 2.57 (br d, 1, H2e, J = 13.0 Hz), 3.68 (s, 3), 6.18 (br s, 1), 6.84 (s, 1, NH), 7.59 (d, 2, J = 2.7 Hz), 7.69 (d, 2, J = 2.7 Hz); ^{13}C NMR (CD_3OD, 100.6 MHz) δ 12.5 (d, $^1J_{CP}$ = 92.2 Hz), 22.1 (d, $^3J_{CP}$ = 15.1 Hz), 25.3 (d, $^2J_{CP}$ = 3.4 Hz), 31.2, 33.0, 35.2 (d, $^1J_{CP}$ = 97.5 Hz), 53.1, 60.5 (d, $^3J_{CP}$ = 14.4 Hz), 127.3, 130.6 (2 C), 132.7 (2 C), 134.7, 169.8, 176.1; high-resolution FAB MS (glycerol matrix) calcd for $C_{16}H_{22}NO_5PBr$ (MH^+) 418.0419, found 418.0409.

Acknowledgment. We thank Professor Joseph J. Villafranca (Pennsylvania State University) for many helpful discussions and for a generous gift of *E. coli* glutamine synthetase.

Supplementary Material Available: Synthesis procedures for the acyclic phosphinothricin analogues, a discussion of quantitative 2D NMR routines used in structure elucidation of compound **22**, and glutamine synthetase assay methods (9 pages). Ordering information is given on any current masthead page.

Asymmetric Induction in Nitrone Cycloadditions: A Total Synthesis of Acivicin by Double Asymmetric Induction

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A total synthesis of acivicin, (αS,5*S*)-α-amino-3-chloro-4,5-dihydroisoxazole-5-acetic acid, has been achieved in 39% overall yield from a derivative of (2*S*)-vinylglycine. The required diastereomer was obtained in an extremely efficient manner (≥19:1) through double asymmetric induction in the reaction of a (2*S*)-vinylglycine derivative with a chiral, nonracemic *N*-glycosylnitrone derived from D-ribose. The origin of the asymmetric induction with respect to the nitrone has been investigated by using semiempirical (MOPAC) computational methods.

Acivicin (AT-125) is an antitumor antibiotic that was isolated in 1973 at the UpJohn Company[1] from fermentation broths of the soil bacterium *Streptomyces sviceus*. The subsequent determination of structure and absolute

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