Determination of Physicochemical Parameters. Sulfonamide pK_a was determined in 50 mM phosphate buffer by monitoring changes in the UV absorption spectrum (300–210 nm) on a Beckman UV 5260 spectrophotometer. Fifteen to 25 separate absorption curves were run for each pK_a determination. Water solubilities were obtained by vortexing excess sulfonamide in 2 mL of H_2O for 2 h, filtering, and assaying for sulfonamide content. Longer vortexing times usually did not result in any additional solubilization of the sulfonamide. $CHCl_3$/buffer partition coefficients were determined by shaking 5-mL aliquots of a 0.1 mM sulfonamide solution in 50 mM phosphate buffer (pH 7.5) against 5 mL of $CHCl_3$ for 10 min and assaying both phases for sulfonamide content.

Design and Synthesis of Phosphonate Inhibitors of Glutamine Synthetase
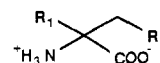
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Inhibitors 1–4 have been shown previously to undergo enzymatic phosphorylation by glutamine synthetase (GS). Phosphonates 6–9 were designed as chemically stable analogues of these phosphorylated inhibitors, incorporating either a tetrahedral sulfur group (6–8) (–S–, –SO–, –SO$_2$–) or phosphinate (9) adjacent to methylphosphonic acid. Phosphonates 6–8 resemble the transiently stable phosphorylated methionine sulfone (2), whereas 9 resembles phosphorylated 2-amino-4-phosphonobutyric acid (4). When tested as inhibitors of glutamine synthetase from bacteria, mammals, and plants, analogue 9 proved to be the most potent, with a K_i value of 7.5×10^{-5} M vs. the *Escherichia coli* enzyme. Analysis of the inhibition data for 6–9 suggests that a replacement of the oxygen bridging the tetrahedral sulfur (6–8) or phosphinate (9) and the terminal phosphate with a hydrophobic methylene drastically reduces the enzyme's affinity for inhibitors. Enhanced affinity of GS for phosphonate 9 may result from interaction of the negative charge on the phosphinate with Mn^{2+} at the active site.

Glutamine synthetase (GS) catalyzes the formation of glutamine, ADP, and P_i from glutamate, ATP, and ammonia, via a tetrahedral transition state resulting from nucleophilic attack of ammonia on the activated carboxylate of glutamate (Figure 1).[1-3] Among several known transition state analogue inhibitors of GS are L-methionine (*S*)-sulfoximine (1),[4-6] L-methionine sulfone (2),[1,2] phosphinothricin (3),[7,8] and 2-amino-4-phosphonobutyric acid (4).[9] Analogues 1–4 are all phosphorylated by *Escherichia coli* GS in the presence of ATP and metal ions.[10-13] L-Methionine (*S*)-sulfoximine phosphate (5) is stable and binds irreversibly with ADP at the active site of GS.[14-16] Meister et al.[17] synthesized L-methionine (*SR*)-sulfoximine phosphate (5) and showed that it rapidly inhibited ovine brain GS in the presence of ADP and metal ions. Recent studies indicate that phosphinothricin (3) may also be phosphorylated by *E. coli* GS in the presence of ATP and metal ions, resulting in irreversible binding at the active site.[18] Phosphorylation of methionine sulfone (2) and 2-amino-4-phosphonobutyric acid (4) by *E. coli* GS does not result in the formation of an irreversibly bound complex.[12,18] Although the phosphorylated sulfone is unstable and cyclizes,[12] it is not clear whether the cyclization occurs before or after dissociation from the active site of GS. Phosphorylated 2-amino-4-phosphonobutyric acid (4) does not irreversibly inactivate *E. coli* GS, but dissociates from the active site of *E. coli* GS after being phosphorylated.[13]

This paper describes the synthesis of phosphonates 6–9, designed to resemble phosphorylated transition-state analogues 1–4, plus inhibition data for analogues 6–9 obtained with *E. coli*, ovine brain, and pea seed GS. Phosphonates 6–8 resemble methionine sulfone phosphate,[12]

#	R1	R2	#	R1	R2
1	H-	-CH$_2$S(O)(NH)CH$_3$	7	CH$_3$-	-CH$_2$S(O)CH$_2$PO$_3$=
2	H-	-CH$_2$S(O)$_2$CH$_3$	8	CH$_3$-	-CH$_2$S(O)$_2$CH$_2$PO$_3$=
3	H-	-CH$_2$F(CH$_3$)O$_2^-$	9	H-	-CH$_2$F(O$_2^-$)CH$_2$PO$_3$=
4	H-	-CH$_2$PO$_3$=	10	H-	-CH$_2$C(O)CH$_2$PO$_3$=
5	H-	-CH$_2$S(O)(CH$_3$)NPO$_3$=	11	H-	-NHC(O)CH$_2$PO$_3$=
6	CH$_3$-	-CH$_2$SCH$_2$PO$_3$=			

whereas analogue 9 most closely resembles a stable analogue of phosphorylated 2-amino-4-phosphonobutyric acid.

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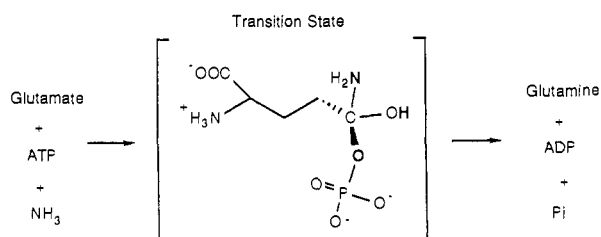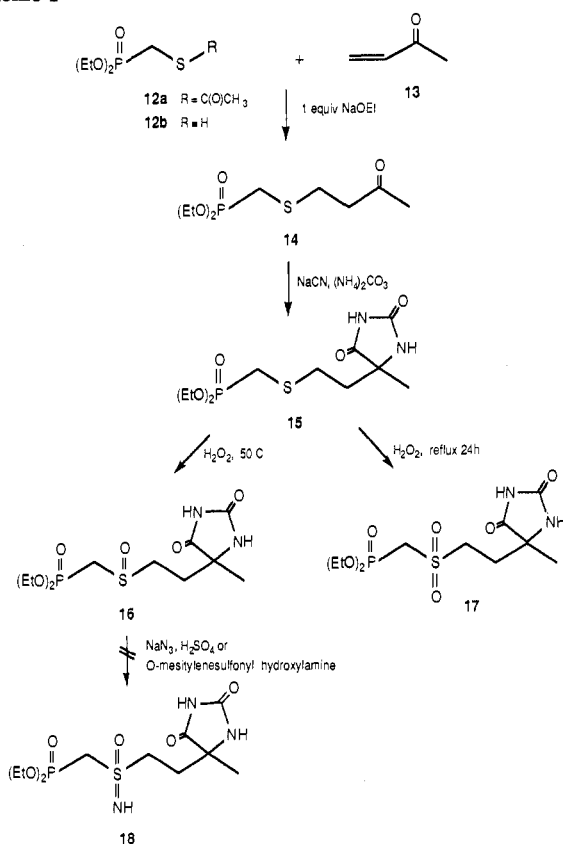[‡] Department of Molecular and Cellular Biology.

Transition State

Glutamate + ATP + NH₃ → Glutamine + ADP + Pi

Figure 1. Reaction catalyzed by glutamine synthetase, with the structure of the tetrahedral transition state.

Scheme I

12a R = C(O)CH₃
12b R = H

13

1 equiv NaOEt

14

NaCN, (NH₄)₂CO₃

15

H₂O₂, 50 C

H₂O₂, reflux 24h

16

17

NaN₃, H₂SO₄ or
O-mesitylenesulfonyl hydroxylamine

18

Scheme II

15

6 N HCl reflux 48h, Dowex 50 H+

6

1 equiv H₂O₂ room temp

H₂O₂, 10% HOAc reflux 18h

7

8

NaN₃, H₂SO₄

19

Scheme III

Al + CH₂Cl₂ → CH₂Br₂ reflux → [Cl₂Al—AlCl₂] → PCl₃ reflux → Cl₂P—PCl₂ → ROH + trimethylamine reflux

20

21

22a R = iPr
22b R = Et

23

1) neat 110 C
2) DMSO

24a R = iPr, R' = OiPr
24b R = Et, R' = OEt
24c R = R' = Et

1) 6N HCl reflux
2) Dowex 50 H+

9

## Chemistry

The synthesis of phosphonates 6–8 (Scheme I) utilized reagent 12, which was previously useful in the synthesis of phosphonate analogue inhibitors against aspartate transcarbamylase and threonine synthase.[19,20] The acetyl sulfide 12a was deblocked by stirring 1 h at room temperature with 1 equiv of sodium ethoxide in ethanol. The resulting mercaptan anion was added directly to methyl vinyl ketone (13) at room temperature. Alternatively, the reaction may be carried out in two steps with isolation of the intermediate mercaptan 12b; however, the overall yields are the same and mercaptan 12b is difficult to handle due to air oxidation and a potent stench. The resulting Michael addition product, β-keto sulfide 14 was isolated by extraction and then distillation. Ketone 14 was then converted to hydantoin 15 using the conditions of the
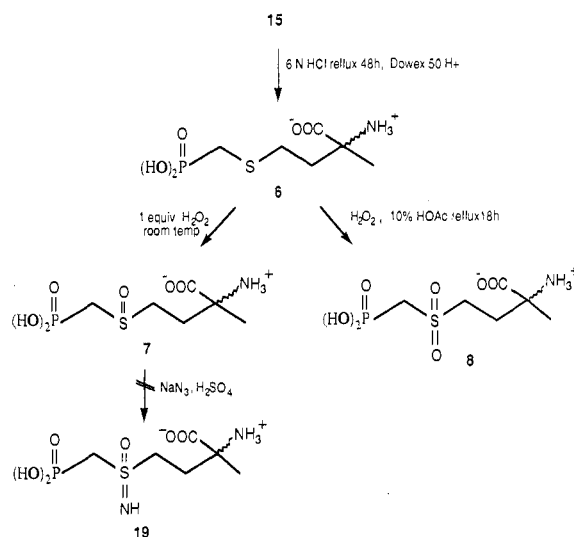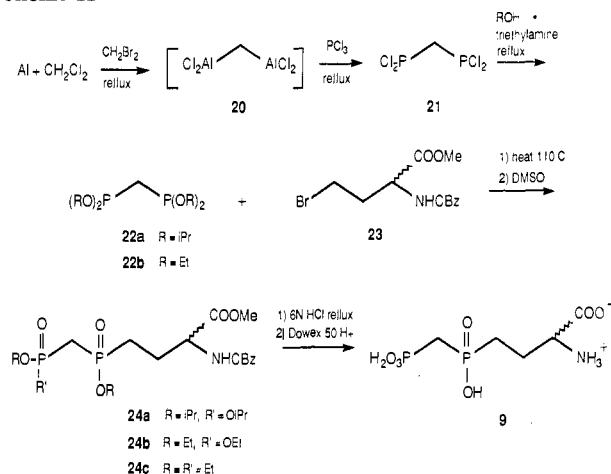
Strecker reaction. Sulfide 15 was then oxidized with either 1 equiv of hydrogen peroxide to sulfoxide 16 or by refluxing in the presence of excess hydrogen peroxide and acetic acid to sulfone 17 (Scheme II). Alternatively, hydantoin 15 could be hydrolyzed to free acid 6 by refluxing in 6 N HCl for 48 h. Purification of 6 was achieved by column chromatography with Dowex-50 H+. Oxidation of sulfide 6 with 1 equiv of hydrogen peroxide gave sulfoxide 7 and with an excess in the presence of acetic acid gave sulfone 8. Attempts to convert sulfoxides 7 and 16 to sulfoximines 19 and 18, respectively (utilizing the conditions employed in the Schmidt reaction or with the use of O-(mesitylenesulfonyl)hydroxylamine) were unsuccessful.[21-25]

The synthesis of phosphinate 9 is shown in Scheme III. Initial attempts to synthesize methylenebis[phosphorous dichloride] (21) by the method of Novikova et al.[26] was unsuccessful. However, when a catalytic amount of dibromomethane was included in the reaction as described by Lehmkuhl and Schafer,[27] the precursor methylenebis-

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Table I. Screening of Analogues 6–8 for Inhibition of GS Activity

no.	concn, mM	E. coli B[a]	E. coli T[b]	ovine brain B	ovine brain T	pea seed B	pea seed T
	0	1.0	1.0	1.0	1.0	1.0	1.0
6	0.1	1.0	1.1	1.0	1.0	1.0	1.0
	1.0	1.95	1.4	1.0	1.0	1.2	1.0
	10.0	1.25	1.5	1.2	1.1	0.5	1.0
7	0.1	1.2	1.0	1.2	1.0	1.1	0.9
	1.0	1.2	1.0	1.2	1.0	1.1	0.9
	10.0	0.95	1.5	1.0	1.2	0.75	1.0
8	0.1	1.1	1.1	1.2	0.95	1.1	0.8
	1.0	1.0	1.1	1.0	0.9	0.8	0.9
	10.0	1.0	1.0	1.0	0.8	0.85	0.9

[a] B = biosynthetic assay,[30] 50 mM Hepes (pH 7.0), 5 mM MgCl$_2$. [b] T = γ-glutamyltransferase assay,[29] 50 mM Hepes (pH 7.0), 5 mM MgCl$_2$.

Table II. K_i Values for Analogue 9 vs. L-Glutamate Determined with GS from Three Sources

	source of GS		
	E. coli	ovine brain	pea seed
$K_i,$[a] μM	75	750	250
type of inhibn	mixed NC[b]	C[c]	NC

[a] Biosynthetic assay of O'Neal and Joy.[30] [b] NC = noncompetitive. [c] C = competitive.

[aluminum dichloride] (20) was obtained. Compound 20 was not isolated, but was added directly to a mixture of methylene chloride and phosphorus trichloride to give methylenebis[phosphorous dichloride] (21). Tetraester phosphonite 22a or 22b was obtained by addition of 21 to dry isopropyl or ethyl alcohol and triethylamine at 0–10 °C and then refluxed for 4 h to complete the reaction. Arbuzov reaction of phosphonite 22b with alkyl bromide 23[28] gave phosphinates 24b and 24c. The ethyl phosphonate 24c resulted from the reaction of ethyl bromide, the product of the first Arbuzov reaction between 22b and 23, undergoing a second Arbuzov reaction with the terminal phosphonite prior oxidation with DMSO. The side product 24c is avoided by using the more sterically hindered isopropyl ester 22a; then the Arbuzov reaction yields only the desired triisopropyl ester 24a. The esters and carbobenzoxy group of 24a were removed by hydrolysis in 6 N HCl for 30 h to give free acid 9, which was then purified by column chromatography on Dowex 50 H+.

Inhibition Studies

Inhibition of GS by phosphonates 6–9 was investigated with enzymes isolated from three sources: bacteria (*E. coli*), plant (green pea) seeds, and mammalian (ovine) brain (Table I). The γ-glutamyl transferase assay of Stadtman et al.[29] and the biosynthetic assay of O'Neal and Joy[30] and were used in the initial screening for inhibitory activity of analogues 6–8 (Table I).[29,30] The maximum percent inhibition observed in only a few cases was 20%. *E. coli* GS exhibited weak activation by analogues 6 and 7, an effect observed previously observed with low levels of L-methionine (*R*)-sulfoximine (1).[31]

Phosphinate 9 was the most potent inhibitor, exhibiting mixed noncompetitive inhibition (vs. L-glutamate) with the *E. coli* and pea seed GS and competitive kinetics with

mammalian brain GS. K_i values were determined with analogue 9 for GS from each of these sources (Table II), utilizing the biosynthetic assay.[29] Synthetic analogues 6–9 were racemic (DL) mixtures; thus the actual effective inhibitory concentration for each analogue is half the determined value. The major structural difference between phosphinate 9 and analogues 6–8 is the negatively charged oxygen on the tetrahedral phosphinate.

Each of the three GS sources were also preincubated separately with analogues 6–9 to investigate the possibility of slow, tight binding to the active site of GS. No time-dependent inactivation by analogues 6–9 was observed either with or without added ADP.

Discussion of Structure–Activity Relationships

Computer modeling of the active site of ovine brain GS led Gass and Meister[32] to formulate a hypothesis regarding binding of 1 and 2 in the active site of ovine brain GS. Villafranca et al.[6] have examined the interaction of 1 and 2 with Mn(II) at the active site of *E. coli* GS by EPR. These investigations have provided insights important to the design of phosphonates 6–9 in terms of structural features that should provide favorable interactions with the active site of GS.

First, all four analogues have a tetrahedral sulfur (6–8) or phosphinate (9) that should structurally mimic the proposed tetrahedral adduct formed during the transition state (Figure 1).[2,3,32] *Second*, a methylphosphonic acid is incorporated, to mimic the phosphorylated transition state. Phosphorylation of L-methionine (*S*)-sulfoximine (1) has been shown to increase the binding affinity for 1 by at least fivefold.[6] *Third*, the size and charge of the tetrahedral moiety are important. In a comparison of methionine sulfone (2) with phosphonate 9, the methylphosphonate of 9 plus ADP replaces the sulfone of 2 plus ATP in the active site of GS,[32] and the methyl group of methionine sulfone (2) is replaced by an oxygen of sulfone 8 (Figure 1). *Fourth*, analogues 6–8 were designed with an α-methyl group in place of the α proton of methionine sulfone to prevent the enzymatic oxidative deamination of analogues 6–8 in vivo.[35] From studies with brain GS, the methyl group did not alter the binding of the L-isomers 1, 2 to the active site of GS,[2,32,36] but with rat liver GS, α-methyl-L-phosphinothricin exhibits a K_i value about sixfold lower than that of phosphinothricin.[9] *Fifth*, use of a methylene

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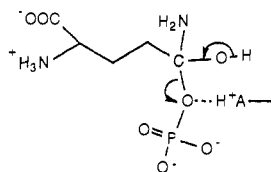
Figure 2. Proposed mechanism for breakdown of the transition state of the glutamine synthetase catalyzed reaction, including interaction of the anhydride oxygen with an acidic active-site group.

bridge between the tetrahedral sulfur or phosphorus and the terminal phosphonate has unknown implications for the binding of the inhibitors to the active site. 3-[(Phosphonoacetyl)amino]-L-alanine (**11**) and 4-(phosphonoacetyl)-L-α-aminobutyrate (**10**), both of which have a methylene bridging group, have been shown to inhibit both pea seed and *E. coli* GS.[33,34] Analogues **6–9** contain tetrahedral groups lacking in **10** and **11**.[6]

Conclusions

The data indicate that analogues **6–8**, each of which contain a methylphosphonate group adjacent to a tetrahedral sulfur moiety, are not optimal analogues of the phosphorylated transition-state analogues **1–5**. The binding of analogues **6–9** to the active site of GS may be adversely effected due to chemical and physical changes resulting from replacement of either the imino bridge of **5** or the oxygen bridge of phosphorylated inhibitors **2–4** with a methylene group, as in analogues **6–9**.

A comparison of bond angles and lengths of **6–9** with those expected for the P–O–P bonds in phosphorylated analogues **3** or **4** shows the P–C bond has a length[37–39] of 1.79 Å and S–C bond a length[40] of 1.82 Å whereas the P–O bond length is 1.63 Å.[38] However, the P–C–P and S–C–P bond angles are somewhat more acute, 117° and 119°, respectively, vs. 129° for P–O–P;[25,38] the overall P–P and S–P distances are essentially unchanged (3.05 Å for P–C–P, 3.06 Å for S–C–P, 2.94 Å for P–O–P). Thus the only *steric* constraint at the active site that could be responsible for the relative difference in the binding affinities of these analogues is the increase in size of the sulfur or phosphonite. The fact that methionine sulfoximine (**1**) and phosphinothricin (**3**) bind very tightly to GS argues that sulfur and phosphorus substitute favorably in place of the tetrahedral carbon adduct or group.

The affinity constants for Mg²⁺ and Mn²⁺ binding to AMP-PCP vs. ATP have been determined to be essentially identical.[38] Thus, the difference in the affinities of GS for **9** vs. phosphorylated phosphinothricin cannot be attributed to the inability of the phosphorus atoms of the P–C–P bridge to coordinate divalent metal ions.

The only other structural change that can account for the poor binding of analogues **6–8** to GS is the substitution of the hydrophobic methylene for the bridging anhydride oxygen that may be needed to interact with a critical active-site group. Considering the mechanism for breakdown of the tetrahedral intermediate (Figure 1), electrons must move from the O–H bond to form the carbonyl bond of glutamine, simultaneous with cleavage of the monophosphate ester bridge by transfer of the C–O–P electrons on to the oxygen of the phosphate, as shown in Figure 2.

This latter process could be assisted by electrostatic effects or donation of a proton from an active-site group to the anhydride oxygen. Substitution of a methylene group for an oxygen at this position would obviously be detrimental to this potentially important interaction. The interaction of the charged tetrahedral phosphinate of **9** with metal ions at the active site appears to overcome in part the repulsive effects of the hydrophobic methylene bridge. As the crystal structure of *Salmonella typhimurium* GS (analogous to *E. coli* GS) is brought to higher resolution and refinement,[41] the probability of this hypothesis will be better known.

Experimental Section

Enzymatic Assays. Enzyme activity with and without inhibitors was assayed with the transferase assay using the procedures of Stadtman et al. in which a standard 1-mL assay contained 5 mM MgCl₂, 20 mM KCl, 50 mM Hepes buffer (pH 7.0), 50 mM glutamine, 20 mM arsenate, and 50 mM hydroxylamine.[29] Alternatively, activity was determined by using the biosynthetic γ-glutamyl hydroxamate assay of O'Neal and Joy in which a standard 1-mL assay contained 25 mM Hepes (pH 7.0), 100 mM KCl, 25 mM MgCl₂, L-glutamate, ATP, and hydroxylamine, all initially present at concentrations twice their K_m values for each enzyme.[30,34] To test for inhibition, analogues **6–9** were included in the assay and concentrations were varied from 0.1 to 10 mM. The K_i values for phosphinate **9** were determined by using the biosynthetic assay and varying concentrations of L-glutamate from 2.5 to 100 mM; ATP and hydroxylamine were held at concentrations twice their K_m values for each enzyme.[34]

Preincubations were carried out with analogues **6–9** (0.1–10 mM) with 0.5-mL reaction mixtures containing *E. coli* GS (1.2 units), 5 mM MgCl₂, 20 mM KCl, 50 mM Hepes (pH 7.0), and ±5 mM ADP. After 15 min at 37 °C the synthetase assay was initiated by addition of 0.5 mL of an assay solution containing L-glutamate, ATP, and hydroxylamine to give final concentrations as above in a total volume of 1.0 mL. Enzyme substrates were obtained from Sigma Chemical Co. and were used without further purification.

Protein Purification. *E. coli* glutamine synthetase was a kind gift of Dr. S. G. Rhee. Ovine brain enzyme was provided by Dr. R. B. Denman. Pea seed glutamine synthetase was purified by a method adapted from the procedure of Elliot.[42] Pea seeds (Blue Bantum Dwarf variety, 100 g) were soaked overnight in 1.0 L of 0.1 N NaHCO₃, at 4 °C, and then placed in a blender and liquified for 2 min. The resulting solution was strained through four layers of cheese cloth. Polyvinylpyrolidone (150 g) was then added to the solution with sufficient 0.1 N NaHCO₃ to keep the mixture in solution. After being allowed to stand for 15 min, the solution was spun at 13000*g* and the supernatant collected. Yield 100%, 1367 units in 212 mL, containing 15.7 mg/mL of protein, sp act. 0.41 unit/mg of protein.

To this supernatant was added 20 mL of a 10% w/v streptomycin sulfate solution. The mixture was centrifuged at 13000*g* for 20 min and the supernatant collected. The total volume was 240 mL of which 60 mL was fractionated with ammonium sulfate as in stage 2 of Elliott's procedure.[42] The ammonium sulfate precipitate was collected by centrifugation for 20 min at 13000*g* and then redissolved in 20 mL of a 0.1 M Hepes buffer (pH 7.1).

The solution was then applied to Bio-Gel (0.5 m, 200–400 mesh, 2.5 × 65 cm) and eluted with 0.02 M imidazole (pH 7.2), 0.15 M NaCl, and 5 mM MgCl₂. Fractions 24–35 (5 mL each) contained the bulk of the activity and were pooled and used in the assays. Yield 88%, 300 units in 50 mL, 2.8 mg/mL, sp act. 2.0 units/mg.

Syntheses. Melting points were determined on a Thomas-Hoover oil submersion melting point apparatus and are uncorrected. ¹H NMR spectra were recorded on either a Varian EM-360-A spectrophotometer at 60 MHz or a Bruker WM 360 Fourier transform NMR at 360 MHz. The various splitting patterns are designated as follows: s, singlet; d, dodublet; t, triplet; q, quartet;

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m, multiplet. Chemical shifts are reported in parts per million (δ) downfield from Me$_4$Si or sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). Mass spectra were recorded on a KRATOS-MS9/50 spectrometer at 70 eV. Elemental analysis was preformed by Galbraith Laboratories Inc., Knoxville, TN. Chemical reagents and starting materials were available from Aldrich Chemical Co. or Alfa Products and were used without further purification unless otherwise noted. All trialkyl phosphites were distilled from sodium prior to use. Cation-exchange resin was Dowex 50W-X8 (100–200 mesh) from Bio-Rad Laboratories. Silica gel column purifications were performed by the flash chromatography method of Still et al.[43] with Baker silica gel for flash chromatography.

1-[[(Diethylphosphono)methyl]thio]butan-3-one (14). (Diethylphosphono)methyl acetyl sulfide (12; 15.3 g, 63 mM) was added to a solution of sodium ethoxide, freshly prepared from Na (1.6 g, 70 mM) dissolved in 50 mL of absolute ethanol.[19] The mixture was stirred 1 h at room temperature and then methyl vinyl ketone (13; 7.0 g, 100 mM) in benzene (35 mL) was added dropwise at room temperature. The solvent was rotoevaporated and the remainder distilled to give 13.2 g (77%) of a clear oil: bp 115 °C (0.03 mm); 60-MHz NMR (CDCl$_3$) δ 1.3 (t, 6 H, J = 6 Hz, OCH$_2$CH$_3$), 2.2 (s, 3 H, C(O)CH$_3$), 2.8 (d, 2 H, J_{H-P} = 13 Hz, SCH$_2$P), 2.8–3.0 (m, 4 H, CH$_2$CH$_2$S), 4.1 (dq, 4 H, J_{H-P} = 10 Hz, J = 6 Hz, POCH$_2$CH$_3$). Anal. (C$_9$H$_{19}$O$_4$PS).

5-Methyl-5-[2-[[(diethylphosphono)methyl]thio]ethyl]-hydantoin (15). Sodium cyanide (4.0 g, 82 mM), ammonium carbonate (27.0 g, 280 mM), and ketone 14 (20 g, 77 mM) were combined in 150 mL of ethanol and water (1:1). The reaction mixture was stirred vigorously and heated for 4 h at 60 °C, the solvents were then rotoevaporated, and water (100 mL) was added. This solution was extracted with methylene chloride (3 × 100 mL), and the extracts were combined and dried over Na$_2$SO$_4$, treated with Norit A charcoal, and filtered. Rotoevaporation of the solvent left an oil which was chromatographed on silica gel, eluted with methylene chloride and ethanol (20:1), to give 10 g (40%) of a clear oil which crystallized upon standing: mp 96–97 °C; 60-MHz NMR (acetone-d_6) δ 1.2 (t, 6 H, J = 6 Hz, OCH$_2$CH$_3$), 1.4 (s, 3 H, CH$_3$), 2.5–3.0 (m, 6 H, C(CO)(NH)CH$_2$CH$_2$SCH$_2$P), 3.9–4.4 (dq, 4 H, J_{H-P} = 10 Hz, J = 6 Hz, POCH$_2$CH$_3$); mass spectrum, *m/e* 324 (M$^+$). Anal. (C$_{11}$H$_{21}$O$_5$N$_2$PS).

5-Methyl-5-[2-[[(diethylphosphono)methyl]sulfinyl]-ethyl]hydantoin (16). Sulfide 15 (1.3 g, 4.0 mM) was added to methanol (10 mL) containing 54 μL (4.8 mM) of a 30% H$_2$O$_2$ solution. The mixture was heated to 50 °C for 3 h, and then the solvents were rotoevaporated. The resulting oil was crystallized from ethanol and ether to give 0.47 g (35%) of a white solid: mp 151–152 °C dec; 60-MHz NMR (DMSO-d_6) δ 1.2 (t, 6 H, J = 6 Hz, OCH$_2$CH$_3$), 1.5 (s, 3 H, CH$_3$), 1.8–2.2 (m, 2 H, C(NH)(CO)-CH$_2$CH$_2$), 2.6–3.0 (m, 2 H, CH$_2$CH$_2$S(O)CH$_2$P), 3.3 (s, 1 H, C-(NH)C(O)), 3.6 (d, 2 H, J_{H-P} = 13 Hz, S(O)CH$_2$P), 3.8–4.3 (dq, 4 H, J_{H-P} = 10 Hz, J = 6 Hz, OCH$_2$CH$_3$), 7.9 (s, 1 H, C(O)-CNHC(O)). Anal. (C$_{11}$H$_{21}$N$_2$O$_6$PS) C, H.

5-Methyl-5-[2-[[(diethylphosphono)methyl]sulfonyl]-ethyl]hydantoin (17). Sulfide 15 (0.83 g, 2.3 mM) was added to methanol (10 mL) containing (17.6 mM, 2.0 mL) of a 30% H$_2$O$_2$ solution. The mixture was refluxed for 24 h, and then the solvents were removed in the rotoevaporator. The resulting oil was crystallized from ethanol and ether to give 0.25 g (30%) of white solid: mp 233–234 °C dec; 60-MHz NMR (D$_2$O) δ 1.2 (t, 6 H, J = 6 Hz, OCH$_2$CH$_3$), 1.6 (s, 3 H, CH$_3$), 2.2–2.6 (m, 2 H, C(NH)-(CO)CH$_2$CH$_2$), 3.3–3.9 (m, 6 H, POCH$_2$CH$_3$, CH$_2$CH$_2$), 3.3–3.9 (m, 6 H, POCH$_2$CH$_3$, CH$_2$CH$_2$SCH$_2$P). Anal. (C$_{11}$H$_{21}$N$_2$O$_7$PS).

2-Amino-2-methyl-4-[(phosphonomethyl)thio]butanoic Acid (6). Hydantoin 15 (10.5 g, 32 mM) was refluxed in 6 M HCl (100 mL) for 48 h, the acid was then removed by rotoevaporation, and the remaining oil was diluted with water (25 mL). The solution was chromatographed on a column of Dowex-50-X8 (1.5 × 45 cm, H+ form), and the product was eluted with water at a flow rate of 1 mL/min. A total of 60 fractions (30 mL each) were collected. Fractions 20–50 that were acidic and exhibited a positive ninhydrin test for a free amine were pooled, and the water was rotoevaporated to give 4.5 g (60%) of white crystals.

The analytical sample was recrystallized from water: mp 244–245 °C dec; NMR (D$_2$O) δ 1.6 (s, 3 H, CH$_3$C(COOD)(ND$_3^+$)CH$_2$CH$_2$), 2.5–2.8 (m, 2 H, CH$_2$CH$_2$SCH$_2$P), 2.3 (t, 2 H, J = 6 Hz, CH$_3$C-(COOD)(ND$_3^+$)CH$_2$CH$_2$), 2.8 (d, 2 H, J_{H-P} = 13 Hz, SCH$_2$P). Anal. (C$_6$H$_{14}$NO$_5$PS) C, H.

2-Amino-2-methyl-4-[(phosphonomethyl)sulfinyl]butanoic Acid (7). Sulfide 6 (0.1 g, 0.4 mM) was dissolved in water (15 mL) containing (50 μL, 0.43 mM) of H$_2$O$_2$, and the mixture was stirred 3 h at room temperature, frozen, and lyophilized. The product was crystallized from acetone–water to give 80 mg (81%) of a solid: mp 152–153 °C; NMR (D$_2$O) δ 1.6 (s, 3 H, CH$_3$C-(COOD)(ND$_3^+$)CH$_2$CH$_2$), 2.3 (t, 2 H, J = 6 Hz, CH$_3$C(COOD)-(ND$_3^+$)CH$_2$CH$_2$), 2.9–3.3 (m, 2 H, CH$_2$S(O)CH$_2$P), 3.2 (d, 2 H, J_{H-P} = 13 Hz, S(O)CH$_2$P). Anal. (C$_6$H$_{14}$NO$_6$PS·H$_2$O) C, H, N.

2-Amino-2-methyl-4-[(phosphonomethyl)sulfonyl]butanoic Acid (8). Sulfide 6 (0.4 g, 1.6 mM) was refluxed for 18 h in 10% acetic acid (10 mL) containing (2.0 mL, 17.6 mM) of a 30% solution of H$_2$O$_2$. The aqueous acetic acid was rotoevaporated and the product was crystallized from water–acetone to give 0.25 g (55%) of white crystals: mp 239–240 °C dec; NMR (D$_2$O, NaOD) δ 1.6 (s, 3 H, CH$_3$C(ND$_2$)(COONa)), 2.3 (t, 2 H, J = 6 Hz, C(ND$_2$)(COONa)CH$_2$CH$_2$), 3.5 (d, 2 H, J_{H-P} = 13 Hz, SCH$_2$P), 3.2–3.5 (m, 4 H, CH$_2$S(O)$_2$CH$_2$P). Anal. (C$_6$H$_{14}$NO$_7$PS).

Methylenebis[phosphorus dichloride] (21). Aluminum (27 g, 1.0 M), dibromethane (5 mL, 71 mM), and dichloromethane (250 mL, 3.9 M) were refluxed under an argon atmosphere for 72 h. The resulting suspension of methylenebis[dichloroaluminum] (20) formed was then added dropwise with stirring to a mixture of phosphorus trichloride (138 g, 1.0 M) and methylene chloride (100 mL) at a rate such that the reaction mixture boiled uniformly. After the exothermic reaction was complete, the mixture was heated at 40–70 °C for 2 h, then phosphorus oxychloride (153 g, 1.0 M) and finely ground potassium chloride (74.5 g, 1.0 M) were added, and the mixture was heated again for 2 h at 40–70 °C. The solvent was then boiled off, and the compound was distilled from the reaction mixture under reduced pressure (5–15 mm). The resulting oil was redistilled to give 25.0 g (22%) of an air-sensitive clear oil: bp 80–82 °C (2.5 mm) (lit.[26] bp 48 °C (1.0 mm)); 60-MHz NMR (CDCl$_3$) δ 2.32 (t, 2 H, J_{H-P} = 16 Hz, PCH$_2$P). Anal. (CH$_2$Cl$_4$P$_2$).

Tetraisopropyl Methylenebis[phosphonite] (22a). Methylenebis[phosphorous dichloride] (20.4 g, 100 mM) in dry ether (20.0 mL) was added dropwise to a stirred and cooled mixture of triethylamine (11.1 g, 100 mM) and absolute 2-propanol (6.6 g, 110 mM) in ether (100 mL). The whitish mixture was stirred for 0.5 h under argon and then refluxed for an additional 4 h. The solution was cooled and then filtered to remove precipitated triethylammonium chloride. The filtrate was washed with dry pentane and ether (1:1, 2 × 25 mL) with minimal exposure to the atmosphere. Solvents were rotoevaporated, and the remaining oil was distilled under reduced pressure to give 25.5 g (80%) of a clear air-sensitive oil: bp 101–102 °C (1.0 mm) (lit.[26] bp 96 °C (1 mm)); 60-MHz NMR (CDCl$_3$) δ 1.3 (d, 24 H, J = 6 Hz, OCH(CH$_3$)$_2$), 2.1 (t, 2 H, J_{H-P} = 6 Hz, PCH$_2$P), 4.0–4.5 (m, 4 H, P(OCH(CH$_3$)$_2$)$_2$). Anal. (C$_{13}$H$_{30}$O$_4$P$_2$).

Methyl 2-[(Benzyloxycarbonyl)amino]-4-bromobutanoate (23).[28] To α-amino-γ-butyrolactone hydrobromide (25.0 g, 137 mM) was slowly added a 48% hydrobromic acid solution (200 mL) with stirring at ambient temperature. After the addition was complete, the mixture was heated to 60–65 °C in a sealed flask for 6 h, then cooled, and allowed to stand overnight. The reaction mixture was concentrated by rotoevaporation to a volume of 100 mL and upon cooling gave 30.0 g (82.0%) of 2-amino-4-bromobutanoic acid hydrobromide as a white solid. Dry HCl gas was bubbled into a solution of the carboxylic acid (26.3 g, 100 mM) in methanol (100 mL) for 3 h at 30–35 °C, refluxed for 4–5 h, and then left to stand overnight. The reaction mixture was concentrated by rotoevaporation to give methyl 2-amino-4-bromobutanoate hydrochloride (25.4 g, 100 mM) in quantitative yield. The amine hydrochloride (12.6 g, 0.05 mol) was then dissolved with stirring in water (100 mL) and cooled to 0 °C. To the cooled solution was added sodium bicarbonate (10.1 g, 120 mM), followed immediately by benzyl chloroformate (10.2 g, 60 mM). The reaction mixture was allowed to warm to room temperature over 0.5 h and then extracted with ether (2 × 100 mL). The ether fraction was dried over Na$_2$SO$_4$ and concentrated by rotoevapo-

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ration to give methyl 2-[(benzyloxycarbonyl)amino]-4-bromo-butanoate as an oily solid, which was crystallized from hexane–ether to give 14.3 g (80–82%) of a white powder: mp 87–88 °C (lit.[28] mp 87–89 °C); 60-MHz NMR (CDCl$_3$) δ 2.0–2.55 (m, 2 H, CH$_2$CH$_2$Br), 3.4 (t, 2 H, J = 7 Hz, CH$_2$Br), 3.75 (s, 3 H, COOCH$_3$), 4.25–4.70 (m, 1 H, (MeOOC)(CBz(H)N)CHCH$_2$), 5.10 (s, 2 H, OCH$_2$Ph), 7.35 (s, 5 H, aromatic H). Anal. (C$_{13}$H$_{16}$BrNO$_4$).

Methyl 4-[Isopropoxy[(diisopropylphosphono)methyl]-phosphinyl]-2-[(benzyloxycarbonyl)amino]butanoate (24a). Phosphonite **22** (1.0 g, 3.2 mM) and bromobutanoate **23** (1.1 g, 3.3 mM) were heated 110–112 °C for 1.5 h under argon. The evolution of a gas was observed, presumably isopropyl bromide. The reaction mixture was cooled and DMSO (25 mg, 3.3 mM) was added and the mixture heated to 60–65 °C for 2–3 h. The mixture was chromatographed on silica gel (methanol–ethyl acetate, 1:9) and gave 0.7 g (39%) of an oil: 360-MHz NMR (CDCl$_3$) δ 1.45–1.15 (m, 18 H, OCH(CH$_3$)), 1.84–2.45 (m, 6 H, CH$_2$CH$_2$PCH$_2$P), 3.7–3.78 (m, 3 H, POCH(CH$_3$)), 3.72 (s, 3 H,

OCH$_3$), 4.3–4.4 (m, 1 H, CH$_2$CH(NH)(COO)), 5.08 (s, 2 H, OCH$_2$Ph), 5.95–5.98 (m, 1 H, NH), 7.32 (s, 5 H, aromatic H); mass spectrum, m/e (relative intensity) 535 (1), 477 (17), 435 (20), 418 (100), 393 (11). Anal. (C$_{23}$H$_{39}$NO$_9$P$_2$).

2-Amino-4-[(phosphonomethyl)hydroxyphosphinyl]butanoic Acid (9). To **24** (1.0 g, 1.9 mM) was added 40 mL of 6 N HCl and the mixture was refluxed for 30 h. The solution was then rotoevaporated and residue chromatographed on a 1.5 × 30 cm Dowex-50 X8 H+ (100–200 mesh) column eluted with water. Seventy (5 mL each) fractions were collected and the acidic and ninhydrin positive fractions were combined and lyophilized to give 350 mg (64%) of a white hygroscopic solid: 360-MHz NMR (D$_2$O) δ 1.7–1.85 (m, 2 H, CHCH$_2$CH$_2$P), 1.95–2.2 (m, 4 H, CH$_2$CH$_2$P), 3.76 (t, 1 H, (DOOC)(D$_2$N)CHCH$_2$). Anal. (C$_5$H$_{13}$-NO$_7$P$_2$·H$_2$O) C, H, N.

A 500-MHz Proton Nuclear Magnetic Resonance Study of μ Opioid Peptides in a Simulated Receptor Environment

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The structure–activity relationship of several μ selective opioid peptides has been evaluated on the basis of both experimental and theoretical approaches. The conformations of Tyr-D-Ala-Phe-Gly-NH$_2$, the tetrapeptide N-fragment of dermorphin, and two analogues have been studied in solution by ^{1}H NMR spectroscopy. The physicochemical environment inside the receptor has been simulated by complexing the peptides with a crown ether and dissolving the complexes in chloroform. The family of conformations derived from the NMR data possesses most of the features previously proposed for μ agonists and is fully consistent with an original model of the μ receptor based on the structures of many rigid opiates. As a simple test of this model, the synthesis of a linear peptide with significant μ activity in spite of the absence of Tyr[1] is reported.

A huge amount of work has been devoted to the structure–activity relationship of flexible opioid agonists, notably opioid peptides.[1,2] This work has not been decisive for our knowledge of the opioid receptors owing to the intrinsic difficulty of identifying the so-called "biologically active conformation" of a flexible molecule and also because it has been largely directed to the search of similarities between the conformations of flexible molecules and the rigid structure of a single opioid, i.e., morphine.[3–5]

This approach is understandable if one considers the historical importance of morphine, but it is not justified, at least in the case of some endogenous opioids, since these peptides interact preferentially with a different receptor (δ for enkephalins vs. μ for morphine). It is the goal of this paper to interpret the SAR of a series of μ opioid peptides. Thus it is essential to refer their conformation to a reliable μ receptor model. Several important features of the μ receptor have been already identified through comparisons of the structures of many opioid molecules.[6–10] Once again, however, some of these comparisons are biased by the attempt to fit the structures of even the most potent molecules to the three-dimensional shape of morphine, in spite of the fact that this molecule is *not* one of the most potent agonists.

Thus it seems useful to reexamine all existing evidence on the μ receptor site starting from two elementary considerations: (i) the "molecular molds" used to infer the shape of the site can only be the most active ones and their completely inactive homologues, but not compounds with intermediate potency; (ii) it is essential to use only conformationally rigid molecules or at least compounds in which a substantial portion of the molecule has a fixed conformation.

The identification of a likely biologically active conformation for μ opioid peptides was based on the NMR study of Tyr-D-Ala-Phe-Gly-NH$_2$ (the tetrapeptide N-fragment of dermorphin) in a lipophilic environment. Dissolution in CDCl$_3$ was made possible by complexation of the NH$_3$+ group with a crown ether. This medium, although quite different from the natural receptor, is

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