An EPSP Synthase Inhibitor Joining Shikimate 3-Phosphate with Glyphosate: Synthesis and Ligand Binding Studies

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Received September 7, 1995; Revised Manuscript Received December 18, 1995[⊗]

ABSTRACT: A novel EPSP synthase inhibitor **4** has been designed and synthesized to probe the configurational details of glyphosate recognition in its herbicidal ternary complex with enzyme and shikimate 3-phosphate (S3P). A kinetic evaluation of the new 3-dephospho analog **12**, as well as calorimetric and ^{31}P NMR spectroscopic studies of enzyme-bound **4**, now provides a more precise quantitative definition for the molecular interactions of **4** with this enzyme. The very poor binding, relative to **4**, displayed by the 3-dephospho analog **12** is indicative that **4** has a specific interaction with the S3P site. A comparison of $K_{i(calc)}$ for **12** versus the $K_{i(app)}$ for **4** indicates that the 3-phosphate group in **4** contributes about 4.8 kcal/mol to binding. This compares well with the 5.2 kcal/mol which the 3-phosphate group in S3P contributes to binding. Isothermal titration calorimetry demonstrates that **4** binds to free enzyme with an observed K_d of 0.53 ± 0.04 μM. As such, **4** binds only 3-fold weaker than glyphosate and about 150-fold better than *N*-methylglyphosate. Consequently, **4** represents the most potent *N*-alkylglyphosate derivative identified to date. However, the resulting thermodynamic binding parameters clearly demonstrate that the formation of EPSPS·**4** is entropy driven like S3P. The binding characteristics of **4** are fully consistent with a primary interaction localized at the S3P subsite. Furthermore, ^{31}P NMR studies of enzyme-bound **4** confirm the expected interaction at the shikimate 3-phosphate site. However, the chemical shift observed for the phosphonate signal of EPSPS·**4** is in the opposite direction than that observed previously when glyphosate binds with enzyme and S3P. Therefore, when **4** occupies the S3P binding site, there is incomplete overlap at the glyphosate phosphonate subsite. As a glyphosate analog inhibitor, the potency of **4** most likely arises from predominant interactions which occur outside the normal glyphosate binding site. Consequently, **4** is best described as an S3P-based substrate−analog inhibitor. These combined results corroborate the previous kinetic model [Gruys, K. J., Marzabadi, M. R., Pansegrau, P. D., & Sikorski, J. A. (1993) *Arch. Biochem. Biophys. 304*, 345−351], which suggested that **4** interacts well with the S3P subsite but has little, if any, interaction at the expected glyphosate phosphonate or phosphoenolpyruvate−P_i subsites.

Glyphosate (*N*-phosphonomethylglycine, **1**) is an effective broad spectrum herbicide which exhibits many of the characteristics desired in an environmentally friendly herbicide (Franz, 1985; Franz et al., 1996). Over the last 2 decades a number of physiological, biochemical, and genetic experiments have demonstrated that glyphosate acts by inhibiting a single enzyme, EPSP[1] (5-enolpyruvoylshikimate 3-phosphate) synthase (EPSPS, EC 2.5.1.19). EPSPS is a key enzyme involved in plant and microbial aromatic amino acid biosynthesis (Amrhein et al., 1980). This biochemical mode of action is unique for glyphosate-based herbicides. No other classes of commercial herbicides are known to inhibit this enzyme. The selective and specific interaction of glyphosate with EPSPS has led to the proposal that glyphosate functions as a transition-state inhibitor (Anton et al., 1983; Steinrücken & Amrhein, 1984).

EPSPS catalyzes an unusual transfer reaction of the carboxyvinyl portion of phosphoenolpyruvate (PEP) regiospecifically to the 5-OH of shikimate 3-phosphate (S3P) forming EPSP and inorganic phosphate (P_i) [for reviews see Sikorski et al. (1991) and Anderson and Johnson (1990a)]. The *Escherichia coli* enzyme exhibits a random kinetic mechanism (Gruys et al., 1992, 1993) through a single, kinetically competent (Anderson et al., 1988a,b), tightly bound (Anderson & Johnson, 1990b), tetrahedral intermediate, **2** (Scheme 1). Various studies using enzyme kinetics (Boocock & Coggins, 1983; Steinrücken & Amrhein, 1984), fluorescence (Anderson et al., 1988c), gel filtration (Castellino et al., 1989), solution (Castellino et al., 1989), and solid-state NMR (Christensen & Schaefer, 1993), as well as equilibrium titration (Ream et al., 1992) and differential scanning calorimetry (Merabet et al., 1993), demonstrate that glyphosate preferentially forms a stable ternary complex with enzyme and S3P (EPSPS·S3P·glyphosate). This ternary complex most likely represents the actual enzyme-bound

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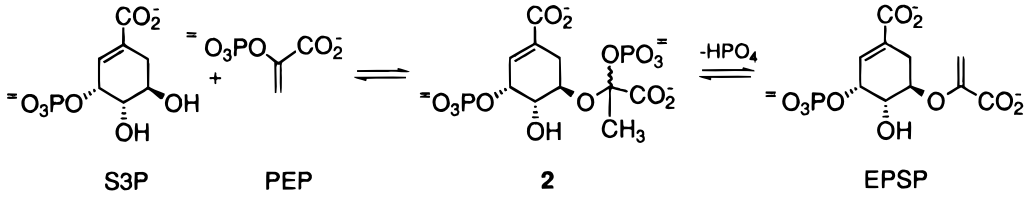
[⊗] Abstract published in *Advance ACS Abstracts,* March 1, 1996.

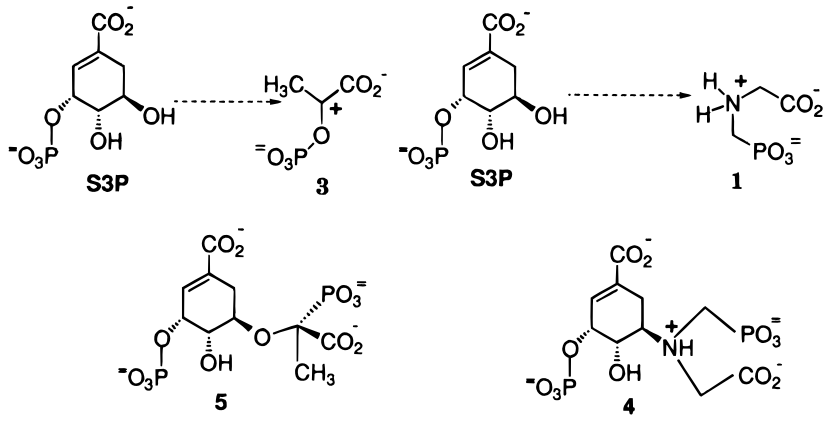
[1] Abbreviations: ADP, adenosine 5′-diphosphate; Bn, benzyl; EDTA, ethylenediaminetetraacetic acid; EPSPS, 5-enolpyruvoylshikimate 3-phosphate synthase; EPSP, 5-enolpyruvoylshikimate 3-phosphate; Et, ethyl; glyphosate, *N*-phosphonomethylglycine; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; LDH, lactate dehydrogenase; PEP, phosphoenolpyruvate; P_i, inorganic phosphate; PK, pyruvate kinase; S3P, shikimate 3-phosphate; TEAB, triethylammonium bicarbonate.

0006-2960/96/0435-4199$12.00/0

Scheme 1: Enzyme Reaction Catalyzed by EPSPS



Scheme 2



form of glyphosate which is responsible for its herbicidal activity *in planta*. Glyphosate has been postulated (Anton et al., 1983; Steinrücken & Amrhein, 1984) to act as a transition-state analog of the putative protonated PEP oxonium ion **3** formed transiently during catalysis (Scheme 2). The transient production of this PEP oxonium ion during EPSPS catalysis is suggested from the observation that the combination of S3P and enzyme will induce deuterium exchange from D_2O into PEP (Bondinell et al., 1971). A similar deuterium exchange has also been demonstrated with 4,5-dideoxy-S3P and enzyme (Anton et al., 1983). More recently, the tetrahedral geometry at the quaternary center in **2** and its tight interaction with enzyme led to the proposal (Anderson & Johnson, 1990a) that the EPSPS·S3P·glyphosate ternary complex may actually mimic the enzyme-bound tetrahedral intermediate (EPSPS·**2**). Either scenario implies that glyphosate binds within the EPSPS active site and has direct interactions with those amino acid residues intimately involved in PEP binding and catalysis.

Much of the available biochemical evidence accumulated since 1980 strongly suggests that there is substantial overlap between the PEP and glyphosate binding domains within the EPSPS active site. For example, glyphosate acts as a competitive inhibitor versus PEP ($K_{i(app)} = 0.16\ \mu M$) and an uncompetitive inhibitor versus S3P (Boocock & Coggins, 1983). Therefore, the resulting EPSPS·S3P·glyphosate ternary complex was thought to resemble the EPSPS·S3P·PEP ternary complex required for catalysis. A number of active site amino acid residues are protected from inactivation by either EPSP alone or S3P plus glyphosate, but not by glyphosate, PEP, or S3P alone (Huynh et al., 1988; Huynh, 1988, 1991). These amino acids all appear at the proposed active site interface of the two globular domains in this protein (Stallings et al., 1991). Fluorescence binding studies demonstrate that EPSP, **2**, and **5** can cause a conformational change similar to that induced by S3P and glyphosate, but not by S3P, PEP, or glyphosate alone (Anderson et al., 1988c; Anderson & Johnson, 1990b). Microcalorimetry measurements indicate that stronger binding synergism is observed between S3P and glyphosate (Ream et al., 1992) than exists between S3P and PEP. These results are consistent with the proposal that the EPSPS·S3P·glyphosate ternary complex mimics the transition state leading to **2**.

However, several recent observations are less consistent with this model of glyphosate action. Both planar analogs of glyphosate (Ream et al., 1988) and more compact cyclic spatial glyphosate mimics (Anderson et al., 1995) designed to fit well within the spatial constraints of the presumably planar PEP oxonium ion are all ineffective ($K_{i(app)} \geq 1$ mM) EPSPS inhibitors. Moreover, kinetic and ligand binding studies (Sammons et al., 1995) demonstrate that glyphosate can also form a reasonably tight ternary complex (EPSPS·EPSP·glyphosate) with enzyme and EPSP. Thus, the carboxyvinyl group in EPSP does not preclude glyphosate from binding. Furthermore, glyphosate was unexpectedly found to be a mixed inhibitor versus P_i in the reverse reaction, suggesting the formation of an EPSPS·EPSP·P_i·glyphosate quaternary complex under steady-state conditions.

These results pointed to the need for additional experiments to more completely characterize glyphosate's molecular mode of action. Since glyphosate binds nearly 75-fold more tightly than PEP to EPSPS·S3P (Anderson et al., 1988b,c), appropriate analogs which combine key structural features of S3P and glyphosate into a single molecule should be extremely potent EPSPS inhibitors. Here we report an optimized synthesis and more complete biochemical evaluation of a novel EPSPS inhibitor **4** and its 3-dephospho derivative **12** to help probe the spatial relationships between S3P and glyphosate in the EPSPS·S3P·glyphosate ternary complex.

An earlier preparation (Marzabadi et al., 1992) of hybrid **4** and its kinetic evaluation (Gruys et al., 1993) as an inhibitor of the *E. coli* EPSPS reverse reaction have been reported. The competitive inhibition pattern observed for **4** versus EPSP ($K_{i(app)} = 7.4 \pm 0.4\ \mu M$) and its mixed inhibition versus P_i ($K_{i(app)} = 13 \pm 1\ \mu M$) indicated that **4** interacts about as well as S3P ($K_d = 7\ \mu M$; Anderson et al., 1988c) with the S3P subsite. The kinetic results also suggested that **4** has little, if any, interaction at the expected glyphosate phosphonate or phosphoenolpyruvate−P_i subsites. A series of

analog kinetic evaluations and calorimetric and ^{31}P NMR spectroscopic studies now provide a more precise quantitative definition for the molecular interactions of **4** with this enzyme.

MATERIALS AND METHODS

Enzyme Purification. EPSPS was isolated from a cloned *E. coli* strain which overproduces the enzyme (Rogers et al., 1983) and was purified as described previously (Castellino et al., 1989). The enzyme concentration was determined using an extinction coefficient of 35 200 M^{-1} cm^{-1} at 280 nm or by the bicinchoninic acid (BCA) procedure of Pierce. A molecular weight of 46 000 was used for calculation of enzyme active site concentration and k_{cat} values.

Enzyme Substrates. S3P was synthesized enzymatically by treating shikimic acid (Sigma) with shikimate kinase (Millar et al., 1986) and purified using ion-exchange HPLC techniques (Castellino et al., 1991). EPSP was enzymatically synthesized from S3P and PEP (Sigma) or [2-^{14}C]PEP (New England Nuclear), respectively, using catalytic amounts of EPSPS and purified as previously described (Anderson et al., 1988b). The EPSP used in these studies was analytically pure (≥98%) and contained ≤1% S3P by HPLC analyses. Analytically pure (>99%) glyphosate was obtained from Monsanto internal stocks. HEPES, NADH, ADP, lactic dehydrogenase (LDH), and pyruvate kinase (PK) were purchased from Sigma Chemical Co. All other reagents were of highest commercial purity. Barnstead Nanopure water was used for all enzyme assay solutions.

Kinetic Assays. Kinetic studies for the inhibition of EPSPS by the 3-dephospho analog **12** were performed by monitoring the EPSPS reverse reaction spectrophotometrically following the exact protocol as described previously (Gruys et al., 1993). The relatively weak interaction of **12** with the enzyme, however, made a full kinetic evaluation difficult. This was due to limited amounts of compound and the assay requirements for a 10 cm path-length cell with a volume of 4 mL per reaction. Therefore, the apparent K_i was calculated using eq 1 by assuming that **12** was competitive with variable

$$K_{i(calc)} = K_m I/(V_m S/v - K_m - S) \quad (1)$$

EPSP. The reported $K_{i(calc)}$ and error limits result from the average of six assays (duplicates of three different substrate concentrations). Phosphate was fixed at 50 mM, just as in the original analysis of **4** with variable EPSP (Gruys et al., 1993).

The individual terms define v, the measured velocity; V_m, maximal velocity (13.5 s^{-1} for the reverse reaction); K_m, the apparent Michaelis constant (1.6 μM for EPSP); S, the variable substrate concentration (2, 4, and 8 μM EPSP); I, the inhibitor concentration (4 mM); and $K_{i(calc)}$, the calculated apparent inhibitor constant.

Microcalorimetry. All microcalorimetry experiments for **4** were carried out in binding buffer (50 mM HEPES/KOH, 50 mM potassium chloride, pH 6.8) degassed by stirring under vacuum, as previously described (Ream et al., 1992). Purified EPSPS (1 mM) was exchanged into binding buffer containing β-mercaptoethanol (5 mM), through repeated concentration by ultrafiltration (Amicon, YM10 membrane) and resuspension in binding buffer. The enzyme concentration was adjusted as desired using the A_{280} and an extinction coefficient of 35 200 M^{-1} cm^{-1}. Both titrant and enzyme samples were prepared in exactly the same buffer at the same pH and degassed immediately before analysis to reduce errors associated with these factors.

Ligand binding to EPSPS was analyzed in a Microcal Omega titration calorimeter equilibrated to 27 °C. To correct for ligand heats of dilution, a control experiment was also performed using similar conditions with solutions containing buffer but no enzyme. After subtracting the contribution from the heat of dilution for each injection, the sum of the heat evolved was plotted against the total ligand concentration to produce the binding isotherm. Binding constants, heats of binding, and stoichiometry were determined by fitting the binding isotherm against the binding equations (Wiseman et al., 1989; Friere et al., 1990) provided in the Omega instrument software.

^{31}P NMR Spectroscopic Studies. NMR spectra were accumulated on a Varian XL-300 FT spectrometer equipped with a Nalorac 4-nucleus Z-spec probe for 5 mm diameter NMR tubes, as previously described (Gruys et al., 1992). Samples were prepared in a buffered solution containing 50 mM HEPES, 50 mM KCl, 0.05 mM EDTA, and 5 mM β-mercaptoethanol, at pH = 7.25, containing 25% (v/v) D_2O. β-Mercaptoethanol served to stabilize the enzyme, while EDTA was included to avoid signal broadening due to potential trace amounts of paramagnetic metals. ^{31}P NMR spectra were acquired at 7 °C with a one-pulse sequence using a 98° pulse width, with delay and acquisition times of 1.0 s each. Spectra were Waltz decoupled and subject to 1−5 Hz line broadening. Spectra were referenced to 0.0 ppm using a 1 M D_3PO_4 insert. A minimum of 3000 transients were required for experiments with enzyme.

General Synthetic Methods. All reactions were performed under an inert nitrogen atmosphere and the reagents, neat or in appropriate solvents, were transferred to the reaction vessel *via* syringe or cannula techniques. Unless stated otherwise all solvents were AR grade and used as supplied. Anhydrous solvents were purchased from Aldrich Chemical Co. and used as received. Triethylamine, used in the preparation of the ion-exchange chromatographic buffer, was distilled prior to use. Triethylammonium bicarbonate (TEAB) was prepared by bubbling CO_2 into an ice-cold mixture of 520 mL of triethylamine in 3.2 L of water until a pH of 7.40−7.50 was attained.

Flash chromatography (Still et al., 1978) on silica gel (mesh size 230−400) and a Waters Prep-500 HPLC were used for preparative chromatographic separations of the products. In some cases the silica used in the flash chromatographic separations was neutralized by triethylamine as the slurry was prepared. Thin-layer chromatography and a Waters HPLC (reverse phase and ion exchange) were used for analytical analysis of the reaction mixtures. Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. ^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded on Varian (300 and 400 MHz) and Brüker (300 and 360 MHz) instruments, and the spectra were normally calibrated by the lock signal of the deuterated solvent. Signals in the ^{1}H NMR spectra are described as s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; and b, broad. Spectral characterization using 2D-COSY NMR confirmed the indicated stereochemistry of products over the entire synthetic sequence. Elemental analyses were performed by Atlantic Microlab Inc., Norcross, GA.

Methyl 5-Azido-3,4-dihydroxy-1-cyclohexene-1-carboxylate (3α,4α,5β). A solution of 2.96 g of shikimate epoxide

7 (McGowan & Berchtold, 1981) (17.4 mmol), 5.65 g of NaN_3 (86.9 mmol), and 2.05 g of NH_4Cl (35.2 mmol) in 215 mL of H_2O−MeOH (25:190) was heated at reflux for 22 h under N_2. The reaction mixture was concentrated *in vacuo*, and the residue was washed with dichloromethane until TLC of the washings did not show the presence of the product. The combined dichloromethane extracts were applied to 200 g of silica packed with 70% ether−petroleum ether and eluted with the same solvent system to give 2.60 g (70%) of the desired 5-azidoshikimate methyl ester. ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 6.84 (m, 1H), 4.40 (t, J = 4.29 Hz, 1H), 3.87−3.79 (m, 1H), 3.74 (s, 3H), 3.69 (dd, J = 4.22, 9.16 Hz, 1H), 3.35 (bs, 2H), 2.86 (d, J = 5.32,18.10 Hz, 1H), 2.24 (d, J = 8.17, 19.07 Hz, 1H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ: 166.02, 135.59, 129.76, 70.76, 65.18, 57.86, 51.85, 28.43. Anal. Calcd for $C_8H_{11}N_3O_4$: C, 45.07; H, 5.20. Found: C, 45.16; H, 5.22.

*Methyl 7-Amino-3a,6,7,7a-tetrahydro-2,2-dimethyl-1,3-benzodioxole-5-carboxylate (3aα,7β,7aα) (***8***).* A mixture of 2.45 g of the azide product from above (11.5 mmol), 10.0 mL of 2,2-dimethoxypropane, and 210 mg of pyridinium *p*-toluenesulfonate (PPTS) in 200 mL of dry dichloromethane was stirred at room temperature for 15 h under N_2, at which time TLC of the reaction mixture showed no remaining starting material (EtOAc/silica). The reaction mixture was washed with saturated $NaHCO_3$ solution (3 × 30 mL) dried (Na_2SO_4), and the solvent was removed *in vacuo*. The crude acetonide product (2.88 g, quantitative) was used in the next experiment without further purification. ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 6.75 (m, 1H), 4.52 (m, 1H), 3.95 (dd, J = 6.15, 7.90 Hz, 1H), 3.60 (s, 3H), 3.54−3.46 (m, 1H), 2.62 (dd, J = 4.71, 17.57 Hz, 1H), 2.05 (td, J = 1.85, 8.81, 17.65 Hz, 1H), 1.28 (s, 3H), 1.22 (s, 3H).

The crude acetonide was dissolved in 30 mL of dry methanol containing 500 mg of Lindlar's catalyst and hydrogenated in a Parr apparatus at 35 psi for 1.5 h and at 50 psi for 2 h. The reaction mixture was monitored hourly by TLC (EtOAc/silica), and only a trace of the starting material could be seen after 3.5 h. The reaction mixture was filtered through a pad of Celite 545, and the residue was washed with 3 × 10 mL of methanol. The combined filtrates were concentrated *in vacuo*, and the crude product was distilled (Kügelrohr, bp 120−140 °C (0.1 Torr)) to give 2.47 g (96% over two steps) of the desired acetonide-protected 5-aminoshikimate ester **8** as a colorless viscous oil, identical in all respects to that reported previously (Corey et al., 1993). ^{1}H NMR ($CDCl_3$, 360 MHz) δ: 6.94 (m, 1H), 4.30 (m, 1H), 3.63 (t, J = 5.22 Hz, 1H), 3.36 (s, 3H), 2.85 (m, 1H), 2.64 (dd, J = 3.80, 14.41 Hz, 1H), 1.92 (qt, J = 1.58, 6.85, 14.44 Hz, 1H), 1.31 (s, 3H), 1.25 (s, 3H), 0.86 (bs, 2H).

*Methyl 7-[(2-Ethoxy-2-oxoethyl)amino]-3a,6,7,7a-tetrahydro-2,2-dimethyl-1,3-benzodioxole-5-carboxylate (3aα,7β,7aα) (***9***).* Triethylamine (1.67 g, 16.5 mmol) was added in one portion to a stirred solution of 2.47 g of **8** (11.0 mmol) in 100 mL of dry THF followed by the addition of ethyl bromoacetate (2.76 g, 16.5 mmol) in one portion at room temperature under N_2. Copious amounts of a white precipitate could be seen after 15 h. Since TLC of the reaction mixture (silica/EtOAc) showed some starting material remaining, additional triethylamine (1.15 mL) and ethyl bromoacetate (0.98 mL) were sequentially added, and the reaction mixture was stirred for another 24 h. The TLC of the reaction mixture showed only traces of the starting material. The reaction mixture was quenched with 50 mL of aqueous saturated $NaHCO_3$ solution and 50 mL of dichloromethane, the layers were separated, and the aqueous layer was extracted with 3 × 20 mL of dichloromethane. The combined dichloromethane extracts were dried (Na_2SO_4), and the solvent was removed *in vacuo*. The crude product was chromatographed on 200 g of silica packed with Et_3N−EtOAc−hexanes (1:49:50) eluting with 50% ethyl acetate−hexanes to give 2.76 g (80%) of the desired alkylated glycinate ester product **9** as a colorless viscous oil. ^{1}H NMR (C_6D_6, 360 MHz) δ: 6.95 (m, 1H), 4.28 (t, J = 4.87 Hz, 1H), 3.88 (q, J = 7.13 Hz, 1H), 3.82 (dd, J = 6.29, 7.70 Hz, 1H), 3.36 (dd, J = 17.33 Hz, 1H), 3.34 (s, 3H), 3.26 (dd, J = 17.35 Hz, 1H), 2.85 (m, 1H), 1.96 (qt, J = 1.88, 8.41, 16.94 Hz, 1H), 1.42 (s, 3H), 1.24 (s, 3H), 0.89 (t, J = 7.14 Hz, 3H). ^{13}C NMR (C_6D_6, 90 MHz) δ: 165.80, 159.94, 128.04, 124.96, 102.84, 72.00, 65.72, 53.88, 48.89, 45.04, 42.51, 21.67, 21.55, 19.50, 7.69. Anal. Calcd for $C_{15}H_{23}NO_6$: C, 57.50; H, 7.40. Found: C, 57.41; H, 7.42.

*Methyl 7-[[[Bis(phenylmethoxy)phosphinyl]methyl](2-ethoxy-2-oxymethyl)amino]-3a,6,7,7a-tetrahydro-2,2-dimethyl-1,3-benzodioxole-5-carboxylate (3aα,7β,7aα) (***10a***).* A solution of 3.75 g of dibenzylphosphonomethyltriflate (8.51 mmol) (Phillion & Andrew, 1986) in 4 mL of dichloromethane was added in one portion to a stirred mixture of 1.62 g of Na_2CO_3 and 2.22 g of the acetonide-protected *N*-carboethoxymethyl 5-shikimate amine product **9** from above (7.09 mmol) in 16 mL of CH_2Cl_2−H_2O (1:1). The resulting mixture was stirred at 40 °C for 1 h. The TLC of the reaction mixture (EtOAc/silica) showed that approximately 50% of the starting material (R_f = 0.40) had not reacted and a new spot (R_f = 0.53) had appeared. Additional triflate (3.75 g, 8.51 mmol), Na_2CO_3 (1.62 g), 4 mL of CH_2Cl_2, and 8 mL of water were added, and then the reaction mixture was heated at reflux for 2 h. The TLC of the reaction mixture then showed no remaining starting material. The reaction mixture was cooled, the layers were separated, and the aqueous layer was extracted with CH_2Cl_2 (5 × 5 mL). The combined CH_2Cl_2 extracts were dried (Na_2SO_4), and the solvent was removed *in vacuo* (8.77 g). The crude product was applied to 200 g of silica packed with Et_3N−EtOAc−hexanes (1:29:70) and eluted with 1000 mL of 30%, 1000 mL of 45%, and 1000 mL of 50% EtOAc−hexanes to give 4.12 g (99%) of the desired phosphonomethylated product **10a** as a colorless viscous oil. ^{1}H NMR ($CDCl_3$) δ: 7.32−7.19 (m, 10H), 6.79 (bm, 1H), 5.09−4.94 (ABX, 4H), 4.58 (dd, J = 3.5, 5.8 Hz, 1H), 4.14 (dd, J = 6.8, 7.9 Hz, 1H), 4.05 (q, J = 7.2 Hz, 3H), 3.73 (s, 3H), 3.70 (AB q, 2H), 3.28 (d, J = 9.3 Hz, 2H), 3.05 (dt, J = 4.3, 8.9 Hz, 1H), 2.70 (dd, J = 4.5, 17.5 Hz, 1H), 1.34 and 1.27 (2 s, 6H), 1.17 (t, J = 7.2 Hz, 3H). ^{13}C NMR ($CDCl_3$) δ: 171.59, 171.57, 166.33, 136.50, 136.46, 136.44, 136.40, 134.24, 131.41, 128.52, 128.32, 128.31, 128.02, 127.97, 109.01, 75.45, 72.32, 67.77, 67.70, 67.62, 60.35, 60.01, 59.90, 53.88, 52.01, 48.27, 46.43, 27.75, 25.87, 25.59, 14.21. ^{31}P NMR ($CDCl_3$) δ: 26.11. Anal. Calcd for $C_{30}H_{38}N_1O_9P_1$ (+0.6H_2O): C, 60.22; H, 6.60; N, 2.34. Found: C, 60.20; H, 6.40; N, 2.34.

*Methyl 4-[[Bis(phenylmethoxy)phosphinyl]methyl]-3,4,-4a,5,8,8a-hexahydro-8-hydroxy-2-oxo-2H-1,4-benzoxazine-6-carboxylate (4aα,8β,8aβ) (***11***).* Dowex 50W-X8 resin (28.6 g, 5 mol equiv) was washed with 1.5 M HCl and then with water before using in the following procedure. The mixture of resin and 2.86 g of **10a** (4.87 mmol) in 500 mL

of acetonitrile−water (1:1) was heated at 75 °C for 2 h. The TLC of the reaction mixture showed incomplete reaction. The reaction mixture was then heated at reflux for 2.5 h. The TLC of the reaction mixture (silica/EtOAc) showed mainly one product. The reaction mixture was cooled and filtered, and the residue was washed with water (2 × 100 mL) and then with dichloromethane (4 × 50 mL). The organic layer was separated, and the aqueous layer was extracted with 3 × 50 mL of dichloromethane. The combined organic extracts were dried (Na_2SO_4), and the solvent was removed *in vacuo*. At this stage, small amounts of crystalline material could be seen in the flask. Further crystallization was induced by trituration of the crude product with ether. The material was collected by filtration and air-dried to give 1.70 g (60%) of the desired lactone product **11** as a white solid: mp 108.5−109.5 °C. ^{1}H NMR ($CDCl_3$) δ: 7.42−7.30 (m, 10H), 6.88 (dd, J = 2.8, 5.2 Hz, 1H), 5.04 (ABX, J = 9.4 Hz, 4H), 4.46 (apparent t, J = 4.6 Hz, 1H), 4.09−4.04 (m, 2H), 3.76 (s, 3H), 3.43 (dd, J = 2.4, 18.0 Hz, 1H), 3.11 (apparent t, J = 16.0 Hz, 1H), 3.03−2.92 (m, 1H), 2.69 (dd, J = 5.6, 15.6 Hz, 1H), 1.85 (ddd, J = 2.6, 7.2, 14.4 Hz, 1H), 1.61 (b, 1H). ^{13}C NMR ($CDCl_3$) δ: 166.46, 166.02, 135.99, 135.82, 135.75, 134.24, 131.30, 128.74, 128.71, 128.34, 81.49, 68.24, 58.15, 67.77, 67.68, 64.11, 55.10, 52.26, 51.69, 51.52, 48.66, 46.58, 29.31. ^{31}P NMR ($CDCl_3$) δ: 22.50. Anal. Calcd for $C_{25}H_{28}N_1O_8P_1$: C, 59.88; H, 5.63; N, 2.79. Found: C, 59.98; H, 5.62; N, 2.77.

*3-(Phosphonooxy)-4-hydroxy-5-[N-(phosphonomethyl-2-oxoethyl)amino]-1-cyclohexene-1-carboxylic Acid (3α,4α,5β), Compound with Diethylethanamine (***4***).* A solution of 0.30 mmol of sodium bis(trimethylsilyl)amide in 300 μL of THF was added dropwise to a stirred solution of 100.3 mg of **11** (0.20 mmol) and 161.5 mg of tetrabenzylpyrophosphate (0.30 mmol) in 2 mL of dry THF at −78 °C under N_2. The TLC of the reaction mixture (EtOAc/silica) after 10 min showed no starting material (R_f = 0.28) and a new spot had appeared (R_f = 0.37). The reaction mixture was diluted with 20 mL of ether. A white crystalline precipitate appeared which was filtered and washed with THF−ether (50:50). On the basis of spectral and microanalyses, this precipitate was identified as sodium dibenzylphosphate: mp >250 °C. ^{1}H NMR (D_2O) δ: 7.50−7.40 (m, 10 H), 4.89 (d, J = 7.4 Hz, 4H). ^{31}P NMR (D_2O) δ: 6.26. Anal. Calcd for $C_{14}H_{14}O_4P_1Na_1$(+0.18H_2O): C, 55.40; H, 4.77. Found: C, 55.39; H, 4.66.

The filtrate was diluted with 20 mL of dichloromethane and 20 mL of aqueous saturated $NaHCO_3$ solution. The organic layer was separated, and the aqueous layer was extracted with 10 mL of dichloromethane. The combined organic extracts were dried (Na_2SO_4), and the solvent was removed *in vacuo*. The resulting crude product (^{31}P NMR (CD_3CN) δ: 32.70, 6.75) was carried forward without further purification. The crude product was dissolved in 10 mL of CD_3CN and was sequentially treated with triethylamine (307 μL, 2.20 mmol) and bromotrimethylsilane (147 μL, 1.11 mmol). The reaction mixture was stirred at room temperature for 0.5 h and then cooled in an ice bath. Triethylamine (460 μL) and TMSBr (220 μL) were added, and the reaction mixture was stirred for an additional 1 h. The reaction mixture was quenched with 5 mL of MeOH and 5 mL of water and concentrated to a small volume (∼5 mL). The residue was treated with 2.5 M NaOH (pH = 13). The resulting solution was washed with ether (2 × 5 mL), and the traces of ether were removed under reduced pressure. The resulting aqueous solution was stirred at room temperature for 2 h and then at 0−5 °C for 8 h. The reaction mixture was diluted with water to a volume of 2 L, applied to a DEAE Sephadex A-25 ion-exchange column and eluted with two 6 L gradients of triethylammonium bicarbonate buffer (3 L of 0.01−0.50 M and then 3 L of 0.5 M−1 M buffer). The product eluted at a buffer concentration of ∼0.5−0.6 M. The fractions containing the product were combined and concentrated to give 58.4 mg (55%) of residue which was eluted through a small C_{18} column to remove the remaining slight yellow impurities to give **4** as an extremely hygroscopic white crystalline solid (mixture of triethylammonium salts) that made measuring the melting point impossible. ^{1}H NMR (D_2O) δ: 6.49 (dd, J = 1.8, 5.7 Hz, 1H), 4.78 (ddd, J = 3.3, 5.7, 8.1 Hz, 1H), 4.08 (d, J = 16.4 Hz, 1H), 3.90 (dd, 1 J = 3.3, 10.9 Hz, 1H), 3.81 (ddd, 1 H, J = 10.9, 6.6, 4.1 Hz, 1H), 3.74 (dd, J = 7.0 Hz, 1H), 3.61 (d, 1H), 3.12 (dd, J = 7.0 Hz, 1H), 3.10 and 2.97 (2 q, J = 7.5 Hz), 2.75 (dd, J = 14.7, 4.1 Hz, 1H), 2.34 (ddd, J = 1.8, 6.6, 14.7 Hz, 1H), 1.18 (t, J = 7.5 Hz). ^{13}C NMR (D_2O) δSPCLN 176.65, 173.18, 138.58, 132.45, 71.64, 65.51, 64.05, 61.73, 58.24, 57.83, 49.47, 45.73, 45.03, 27.57, 13.56, 11.22. ^{31}P NMR (D_2O) δ: 6.10, 2.76. Anal. Calcd for $C_{10}H_{17}N_1O_{12}P_2$ (+3.3Et_3N + 4.5H_2O): C, 43.64; H, 9.28; N, 7.34. Found: C, 43.41; H, 8.91; N, 7.79.

The sodium salt of **4** was prepared by a slow elution of the triethylammonium salts through a short column of Dowex H^+ that had been sequentially washed with 1 N NaOH and then water. The product elutes at the solvent front. ^{1}H NMR (D_2O) δ: 6.38 (m, 1H), 4.80 (m, 1H), 4.17 (d, J = 15.9 Hz, 1H), 4.00 (dd, J = 3.7, 13.4 Hz, 1H), 3.86 (bm, 1H), 3.68 (d, J = 15.9 Hz, 1H), 3.02 (d, J = 11.0 Hz, 2H), 2.87 (ddm, J = 4.8, 14.7 Hz, 1H), 2.41 (ddm, J = 12.2, 14.7 Hz, 1H). ^{31}P NMR (D_2O) δ: 13.82, 10.71.

*Methyl 7-[[[(Diethoxy)phosphinyl]methyl](2-ethoxy-2-oxymethyl)amino]-3a,6,7,7a-tetrahydro-2,2-dimethyl-1,3-benzodioxole-5-carboxylate (3aα,7β,7aα) (***10b***).* A solution of 0.566 g of diethylphosphonomethyltriflate (2.11 mmol) (Phillion & Andrew, 1986) in 1 mL of dichloromethane was added in one portion to a stirred mixture of 0.40 g of Na_2CO_3 and 0.550 g of the acetonide-protected *N*-carboethoxymethyl 5-shikimate amine **9** from above (1.76 mmol) in 4 mL of CH_2Cl_2−H_2O (1:1). The resulting mixture was stirred at 45 °C for 2 h. Additional triflate (0.566 g, 2.11 mmol), Na_2CO_3 (0.40 g), 2 mL of CH_2Cl_2, and 2 mL of water were added, and then the reaction mixture was heated at reflux for 2 h. The TLC of the reaction mixture showed no starting material. The reaction mixture was cooled, and the aqueous layer was extracted with CH_2Cl_2 (2 × 2 mL). The combined CH_2Cl_2 extracts were dried (Na_2SO_4), and the solvent was removed *in vacuo*. The crude product was applied to 30 g of silica packed with EtOAc−hexanes (1:1) and eluted with 200 mL of 50%, 200 mL of 60%, and 200 mL of 70% EtOAc−hexanes to give 780 mg (96%) of the desired N-alkylated product **10b** as a colorless viscous oil. ^{1}H NMR ($CDCl_3$) δ: 6.80 (apparent bt, J = 2.3 Hz, 1H), 4.65 (dd, J = 2.3, 6.7 Hz, 1H), 4.20 (dd, J = 6.7, 7.6 Hz, 1H), 4.14−3.99 (m, 6H), 3.68 (s, 3H), 3.66 (AB q, 2H), 3.15 (d, J = 10.1 Hz, 2H), 2.96 (apparent dt, J = 4.5, 9.1 Hz, 1H), 2.68 (dd, J = 4.5, 17.4 Hz, 1H), 2.05 (ddt, J = 17.4, 9.6, 2.3 Hz, 1H), 1.42 and 1.35 (2 s, 6H), 1.23 (dt, J = 1.16, 5.5 Hz, 6H), 1.18 (t, J = 7.1 Hz, 3H). ^{13}C NMR ($CDCl_3$) δ: 171.92, 171.90, 166.33, 133.07, 131.51, 109.19, 75.01,

72.35, 62.35, 62.25, 60.58, 60.35, 60.28, 60.22, 53.83, 53.79, 52.02, 48.03, 45.82, 27.58, 25.38, 25.21, 16.39, 16.31, 14.11. ^{31}P NMR ($CDCl_3$) δ: 25.01. Anal. Calcd for $C_{20}H_{34}N_1O_9P_1$: C, 51.83; H, 7.40; N, 3.02. Found: C, 51.94; H, 7.49; N, 3.03.

*3,4-Dihydroxy-5-[N-(dihydroxyphosphinylmethyl)-N-(2-oxoethyl)amino]-1-cyclohexene-1-carboxylic Acid (3*α*,4*α*,5*β*), Compound with Diethylethanamine (***12***).* Bromotrimethylsilane (1.39 g, 9.06 mmol, 1.68 mL) was added to a stirred solution of 1.40 g of **10b** (3.02 mmol) and 1.22 g of triethylamine (12.08 mmol, 1.68 mL) in 50 mL of CD_3CN at 0−5 °C under N_2. After 1 h another aliquot each of triethylamine and then TMSBr was added, and the reaction mixture was stirred for an additional 2 h. The reaction mixture was quenched with 20 mL of methanol and 20 mL of water and concentrated to a small volume, followed by the addition of 50 mL of water. This solution was washed with ether (2 × 20 mL), and traces of ether were removed under reduced pressure. The aqueous extract was adjusted to pH 13.4 by the addition of 50% NaOH solution. The resulting yellow solution was stirred at room temperature for 48 h, and then the pH of the reaction mixture was lowered to about 2 (pH paper) by addition of Dowex H^+ to the reaction mixture. This mixture was stirred at room temperature for 24 h and then filtered, and the residue was washed with water (2 × 50 mL) and then saturated triethylamine in water (2 × 50 mL). The combined aqueous extracts were concentrated *in vacuo* to remove the excess triethylamine, and water was added until the conductivity of the solution was 30 (∼3.5 L). This solution was applied to a DEAE Sephadex A-25 ion-exchange column (150 g) and eluted with a 5.5 L gradient of triethylammonium bicarbonate (3.5 L of 0.01−0.35 M and then 2 L of 0.35−0.7 M). The product eluted at ∼0.4−0.5 M buffer. Fractions containing the product were combined and concentrated to give a slightly yellow oil which was filtered through a C_{18} filter column to give 1.35 g (84%) of pure **12** as a colorless glassy solid. ^{1}H NMR (D_2O) δ: 6.57 (dd, J = 2.2, 5.2 Hz, 1H), 4.35 (dd, J = 4.2, 5.2 Hz, 1H), 4.00 (d, 1H), 3.95 (dd, J = 4.2, 11.2 Hz, 1H), 3.68 (d, 1H), 3.35 (dd, J = 14.4, 11.1 Hz, 1H), 3.21 (t, J = 14.4 Hz, 1H), 3.09 (q, J = 7.6 Hz), 2.96 (q, J = 7.2 Hz), 2.87 (dd, J = 5.2, 17.2 Hz, 1H), 2.38 (ddd, J = 2.2, 11.6, 17.2 Hz, 1H), 1.17 (tt, J_{major} = 7.6 Hz). ^{13}C NMR (D_2O) δ: 175.22, 172.62, 136.64, 134.87, 69.52, 68.37, 63.41, 64.93, 578.56, 49.58, 45.72, 45.15, 27.10, 13.57, 11.26. ^{31}P NMR (D_2O) δ: 6.76. Anal. Calcd for $C_{10}H_{16}N_1O_9P_1$ (+1.5Et_3N + 3.0H_2O): C, 42.97; H, 8.45; N, 6.59. Found: C, 43.07; H, 8.42; N, 6.73.

RESULTS AND DISCUSSION

Design of Hybrid Inhibitor **4**. A number of possible structures might be considered to probe the orientation of glyphosate relative to S3P in the EPSPS·S3P·glyphosate ternary complex. The design of **4** arose from a consideration of the three-dimensional spatial restrictions imposed by the transition-state inhibitor model. To function optimally as a transition-state inhibitor, glyphosate should overlap as completely as possible with **3**, even though it contains one extra atom between the anionic centers. While glyphosate and PEP vary somewhat in their chemical and functional group composition, these two molecules are reasonably similar in three-dimensional space. However, a special relationship exists between glyphosate and PEP for recognition to occur with EPSPS, since other aminoalkylphosphonates of similar size, shape, and functionality exhibit little significant interaction with this enzyme (Franz et al., 1996). As shown in Figure 1, a three-dimensional representation of the known (Knuuttila & Knuuttila, 1979) extended conformation of glyphosate resembles the general overall shape of PEP quite well. However, optimum overlap between this extended glyphosate conformation and PEP at the critical anionic recognition centers is somewhat precluded by the extra atom that glyphosate contains between the phosphonate and carboxylate groups.

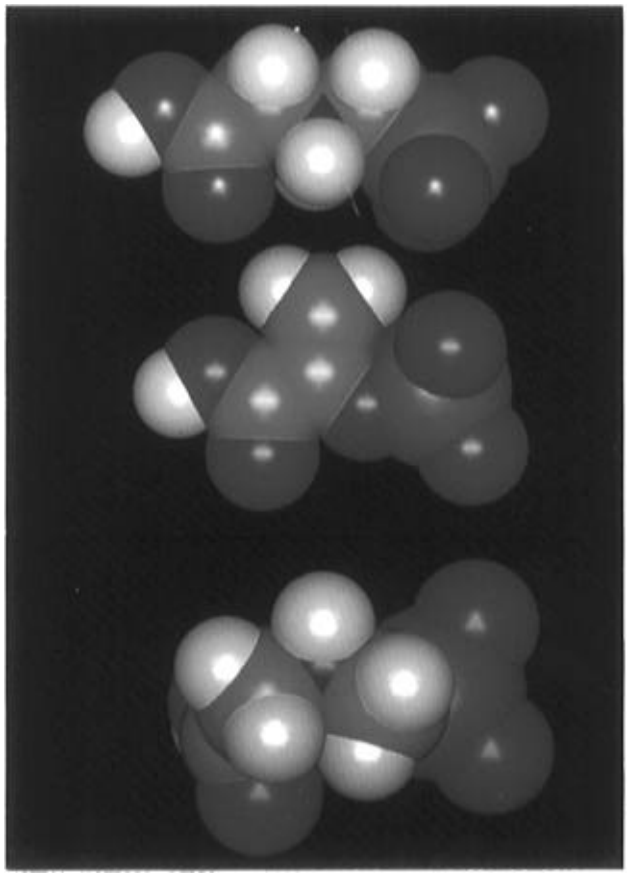


FIGURE 1: Three-dimensional comparison of the known extended conformation of glyphosate from X-ray crystallography (top) versus PEP (middle) and a representative pinched conformation of glyphosate (bottom) using space-filled models generated from INSIGHT. Atoms are color-coded as follows: carbon = green, oxygen = red, nitrogen = blue, hydrogen = white, and phosphorus = magenta.

One might also assume that the tetrahedral geometry at the glyphosate nitrogen atom might preclude an optimum fit with the planar configuration of **3**. The planar configuration of this oxonium ion also requires that the phosphonate and carboxylate functionalities in glyphosate be bound in a more "pinched" conformation with each anionic group on the same side of the protonated nitrogen atom. As depicted in Figure 1, the resulting distance between the glyphosate phosphonate and carboxylate groups is reduced considerably in this pinched conformation and more closely matches the distance between the carboxylate and phosphate groups in PEP. An even better three-dimensional fit (data not shown) can be obtained when the methylene group α to the phosphonate center in glyphosate is localized below the plane of PEP and the methylene group α to the carboxylate center is arranged above this plane. The protonated nitrogen can be subsequently localized slightly above the PEP carbon−carbon bond. A nearly planar triad can be formed between

the two anionic centers and the protonated amine group, and the resulting glyphosate conformation is virtually superimposable with PEP at the key recognition centers.

Precise three-dimensional details of the enzyme-bound glyphosate conformation are incomplete. X-ray crystal structures of glyphosate have been reported in both extended (Knuuttila & Knuuttila, 1979) and pinched (Krawczyk & Bartczak, 1993) conformations. The reported (Castellino et al., 1989) ^{31}P NMR solution characterization of the EPSPS·S3P·glyphosate ternary complex supports the smaller N−C−P bond angle in the bound conformation of glyphosate, but a similar conclusion could not be made for the N−C−C bond angle to the carboxylate center. More sophisticated semiempirical charge calculations (J. A. Sikorski, J. P. Snyder, and S. Rao, unpublished results) suggest that the smaller N−C−P bond angle in glyphosate is preferred even in aqueous solution and that the overall electrostatic charge distribution in three-dimensional space for the glyphosate dianion also closely resembles that calculated for **3**.

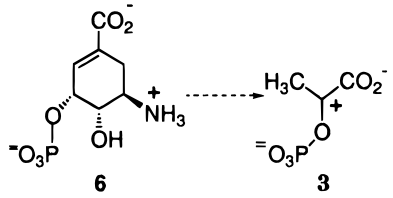
The crystal structures of several glyphosate salts have now been reported which indicate that certain metals can also induce a more pinched glyphosate conformation. This effect has been observed in the bidentate glyphosate calcium complex (Smith & Raymond, 1988). The structures of the corresponding tridentate platinum (Appleton et al., 1994) and cobalt (Heineke at al., 1994) complexes with glyphosate demonstrate that a more planar orientation can exist between the carboxylate, phosphonate, and nitrogen centers. While no evidence has ever been presented implicating a metal requirement for EPSPS catalysis, it is reasonable to assume that the enzyme might provide the appropriate interactions required to induce this more compact conformation.

Two other difficulties immediately arise concerning the design of an appropriate S3P·glyphosate hybrid. First, the exact spatial relationship between S3P and glyphosate is not known. Second, the high structural specificity of the glyphosate skeleton makes any attachment point difficult. The addition of a single methyl group to any atom in glyphosate dramatically reduces enzyme affinity (Knowles et al., 1993). However, since N-alkylation exhibits the least detrimental effect, the glyphosate nitrogen atom was selected for the point of attachment.

Additionally, the design of **4** arose for direct comparison with the known (Alberg et al., 1992) shortened R-phosphonate inhibitor **5**, one of the most potent (K_d = 15 nM; Anderson & Johnson, 1990b) EPSPS inhibitors. The potency of **5** is believed to occur through a significant interaction at the PEP−phosphate site. Presumably, the glyphosate phosphonate must also interact with this site, if the transition-state comparison with **3** or the analogy with **2** is valid. In addition, **4** fits well within the spatial constraints of a three-dimensional active site model developed for EPSPS, when a pinched conformation of its glyphosate side-chain components is employed (Marzabadi et al., 1992). The geminal proximity of the anionic centers at the quaternary center in **2** requires that the same pinched glyphosate conformation be employed to optimize overlap between **2** and **4**.

Furthermore, the observation that 5-amino-S3P **6** will slowly catalyze the exchange of deuterium into PEP (Pansegrau et al., 1991) indicates that the 5-amino substituent permits the transient production of **3** at the enzyme active site *via* the ternary complex depicted in Scheme 3. As such, **4** presumably accomplishes the proper overlap of the glyphosate moiety with **3** and thus provides the first direct comparison of glyphosate with PEP in S3P-based inhibitors. A complete biochemical characterization of **4** should permit, for the first time, a direct comparison of the binding properties of glyphosate versus PEP in these bisubstrate inhibitors and thus provide a test of the transition-state and intermediate mimic models.

Scheme 3

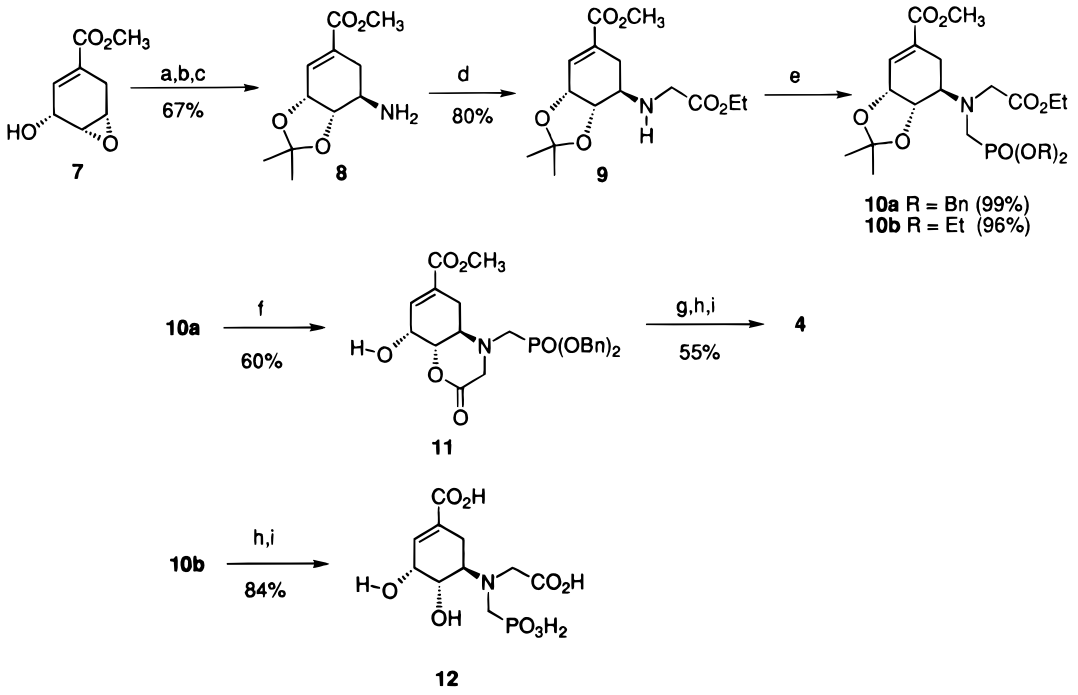


Synthesis of Hybrid Inhibitors **4** *and* **12**. An optimized synthesis of **4** was accomplished in 11 steps in ∼18% overall yield starting from (−)-shikimic acid *via* the known (McGowan & Berchtold, 1981) epoxy alcohol **7** as summarized in Scheme 4. Ring opening of the epoxide with sodium azide (Caron & Sharpless, 1985) followed by acetonide formation (Greene, 1981) and hydrogenation using Lindlar's catalyst (Ohme & Zubek, 1972) produced the required protected diol amine **8** in 67% overall yield. Alkylation first with ethyl bromoacetate gave the glycinate ester **9** in 80% yield. Subsequent treatment of **9** with dibenzylphosphonomethyltriflate (Phillion & Andrew, 1986) to introduce the protected phosphonate functionality gave **10a** in 99% yield. A variety of methods were examined to effect the deprotection and lactonization of **10a**. Most were plagued by multiple side products and/or product decomposition. These problems were circumvented using Dowex (H^+) in aqueous acetonitrile at reflux (Ho, 1978). Under these conditions, protection of the C_4-OH occurs through lactonization to give the lactone **11** as analytically pure crystalline material in 60% yield. Subsequent phosphorylation of **11** with tetrabenzyl pyrophosphate (Khorana & Todd, 1953) was accomplished *via* known methods (Alberg et al., 1992; Vacca et al., 1987). Removal of the six protecting groups by sequential treatment with TMSBr (McKenna & Schmidhauser, 1979) and aqueous base, followed by ion-exchange chromatography, gave **4** as a mixture of triethylammonium salts in 55% isolated yield from **11**. The spectral characterizations of purified **4** using 2D-COSY, ^{1}H, ^{13}C, ^{31}P NMR, and mass spectra were all consistent with this structure.

Similarly, alkylation of **9** with diethylphosphonomethyltriflate produced the diethyl phosphonate analog **10b** in 96% yield. Full deprotection of this material under standard conditions gave the 3-dephosphoshikimate analog **12** in 84% yield.

Kinetic Evaluation of **12**. The 3-phosphate group in S3P is known to be critical for substrate binding and catalysis (Gruys et al., 1992). The specific interaction of **4** with the S3P site should also be highly dependent upon this functionality, and the removal of the 3-phosphate group would be expected to dramatically reduce the overall potency of **4**. The new 3-dephospho analog **12** was found to be a very weak inhibitor of the EPSPS reverse reaction, which precluded a full kinetic evaluation. However, an apparent K_i was calculated using eq 1 by assuming that **12** was competitive with variable EPSP. This appeared to be valid on the basis of the results which gave a mean value with a respectable error for the calculated K_i of 3.5 ± 0.5 mM. One can calculate (Fersht, 1985) from a comparison of this $K_{i(calc)}$

Scheme 4: Synthetic Scheme Leading to EPSPS Inhibitors **4** and **12**[a]



[a] Reagents: (a) NaN_3, NH_4Cl, $H_2O-MeOH$, reflux (70%); (b) 2,2-dimethoxypropane, PPTS (98%); (c) H_2, MeOH, 5% Pd/C (98%); (d) $BrCH_2CO_2Et$, Et_3N, THF; (e) $(RO)_2POCH_2OTf$, CH_2Cl_2, saturated $NaHCO_3$, reflux; (f) Dowex (H^+), H_2O-CH_3CN, reflux; (g) $(BnO)_2PO_2PO(OBn)_2$, $(Me_3Si)_2NNa$, THF, −78 °C; (h) TMSBr; (i) aqueous NaOH, ion-exchange chromatography.

for **12** versus the $K_{i(app)}$ for **4** (7.4 ± 0.4 μM) that the 3-phosphate group in **4** contributes about 4.8 kcal/mol to binding. This compares quite well with the 5.2 kcal/mol which the 3-phosphate group contributes to binding in S3P, as calculated from the ratio of apparent K_m's for shikimic acid turnover versus S3P (Gruys et al., 1992).

These results suggest that the potency of **4** arises largely from binding at the S3P site. As such, the enzyme appears well able to tolerate all of the accompanying functionalities in the *N*-alkylated amine at the 5-position without seriously impeding recognition. This was surprising based upon the well-known steric limitations exhibited by the glyphosate binding site (Steinrücken & Amrhein, 1984; Knowles et al., 1993). However, such kinetic data are frequently subject to multiple interpretations, and few other biophysical techniques have been used to corroborate reliably subsite localization for multisite inhibitors. In order to test this kinetic model, we sought a more precise quantitative definition for the molecular interactions of **4** with EPSPS through a series of calorimetry-based and spectroscopic measurements.

Microcalorimetry Experiments. Isothermal titration calorimetry measurements take advantage of changes in heat flow that accompany ligand binding. The amount of heat released or absorbed during the addition of ligand to enzyme is directly proportional to the overall change in the bound ligand concentration. In a typical experiment, fixed volumes of the ligand solution are sequentially injected into a stirring cell containing the enzyme sample, and the heat evolved from each injection is measured. Such measurements have previously been used to characterize substrate and inhibitor binding with EPSPS (Ream et al., 1992). To the best of our knowledge, the characterization of bisubstrate inhibitors has been largely limited to kinetic evaluations, and few other biophysical techniques have been applied to this area. Since the previously determined thermodynamic binding parameters distinguished localized interactions of small molecules

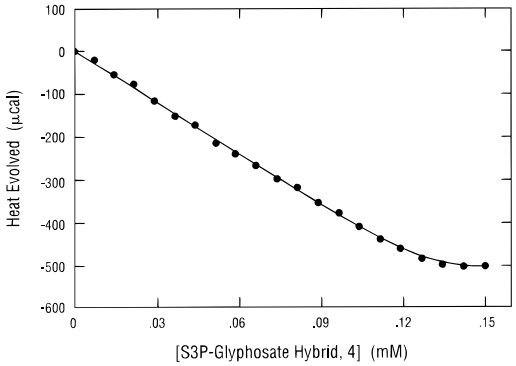


FIGURE 2: Binding curve showing total heat evolved at 27 °C versus cumulative concentration of ligand **4** following the addition of 5-μL aliquots of 2.0 mM **4** to a buffered solution of EPSPS.

with this enzyme at the S3P site from those occurring at either the PEP or glyphosate subsites, it appeared that a calorimetric evaluation of hybrid **4** could also define the specific location responsible for the primary interaction of this potential bisubstrate inhibitor.

The titration binding curve for hybrid **4** is depicted in Figure 2. Aliquots of **4** were injected into a stirring cell containing EPSPS until little heat was detected at a high total concentration of **4**. Overall, a very good calorimetric time response was observed following each injected aliquot. From this single binding curve, the dissociation constant (K_d) and number of binding sites (stoichiometry, *n*), as well as the enthalpic and entropic contributions to the Gibbs free energy of association for **4**, can be determined ($\Delta G = \Delta H - T\Delta S$), as summarized in Table 1. These data indicate that **4** forms a simple 1:1 binary complex with enzyme (EPSPS·**4**) with an observed K_d of 0.53 ± 0.04 μM, in reasonable agreement with that calculated previously (K_d = 1.2 ± 0.1 μM) using steady-state kinetic data fitted to a random kinetic mechanism (Gruys et al., 1993). This observed K_d represents about an order of magnitude increase in the potency for **4** relative to

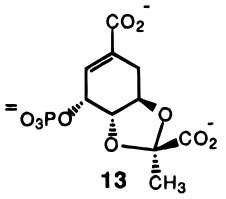
Table 1: Characterization of EPSPS·Ligand Complexes by Microcalorimetry at 27 °C[a]

complex		K_d (μM)	stoichiometry (n)	ΔH (kcal/mol)	ΔS [cal/(mol·K)]
(a) Enz[b]	+**4**	0.53 ± 0.04	1.21 ± 0.02	−2.9 ± 0.1	19.1 ± 0.4
(b) Enz[c]	+S3P	10 ± 0.7	1.03 ± 0.02	−5.2 ± 0.1	5.4 ± 0.6
(c) Enz[c]	+**13**	24 ± 8	1.21 ± 0.02	−1.4 ± 0.01	16.4 ± 0.8
(d) Enz[c]	+PEP	390 ± 15	0.69 ± 0.10	−4.9 ± 0.3	−0.6 ± 0.8
(e) Enz[c]	+glyphosate	12,000 ± 2,000	1.00 ± 0.0[d]	−13.7 ± 1.6	−36.7 ± 5.5
(f) Enz·S3P[c]	+glyphosate	0.15 ± 0.03	0.99 ± 0.02	−19.0 ± 0.4	−31.9 ± 1.8

[a] Values in Table 1 represent the average values from at least duplicate titration experiments performed using the same sample solutions on the same day. Titrant (on the right side of the complex column) was injected sequentially into stirring enzyme sample solution (on the left side of the complex column). [b] The sample cell contained 0.106 mM EPSPS (Enz) in buffer solution which was titrated with fixed aliquots of 2.0 mM **4**. [c] Values taken from Ream et al. (1992). [d] The stoichiometry was fixed at 1.00 to begin data analysis.

the apparent K_i and thus demonstrates the need to use K_d's whenever possible for accurate comparisons between inhibitors of this system.

As an S3P mimic, the dissociation constant observed for the formation of the EPSPS·**4** binary complex compares quite favorably to the observed dissociation constant for EPSPS·EPSP ($K_d = 1.0 \pm 0.01$ μM; Anderson et al., 1988c) and is significantly stronger than observed previously (Ream et al., 1992) for the association of 5-amino-S3P ($K_d = 17 \pm 1$ μM; Gruys et al., 1993), the EPSP ketal **13** ($K_d = 24 \pm 8$ μM;



13

Ream et al., 1992), or S3P ($K_d = 7 \pm 1.2$ μM; Anderson et al., 1988c) with free enzyme. Thus, the *N*-carboxymethyl group in **4** may provide some small additional interaction with enzyme, comparable to that provided by the EPSP carboxyvinyl group. On the other hand, the mixed inhibition pattern previously observed for **4** versus phosphate suggests incomplete overlap at the PEP−P_i site.

As a glyphosate analog inhibitor, **4** forms a reasonably tight binary complex with enzyme, and its potency approaches that of glyphosate ($K_d = 0.15 \pm 0.03$ μM; Ream et al., 1992) in the formation of the EPSPS·S3P·glyphosate ternary complex. Similarly, the enzyme affinity for **4** also compares quite favorably to that of *N*-aminoglyphosate ($K_d = 0.50 \pm 0.03$ μM; Knowles et al., 1993). *N*-Aminoglyphosate is the most potent known glyphosate analog inhibitor and exhibits many of the same binding characteristics as glyphosate. As such, hybrid **4** represents the first example of an *N*-alkylglyphosate analog that exhibits a sub-micromolar interaction with enzyme.

A direct quantitative comparison of **4** with glyphosate is difficult. Whereas **4** prefers to bind to free enzyme, glyphosate and its analogs prefer to bind to the EPSPS·S3P binary complex (Boocock & Coggins, 1983). The steric and ionic limitations of the glyphosate binding site have been well characterized (Ream et al., 1988) and compared with those observed for PEP (Walker et al., 1991). As an EPSPS inhibitor class, modifications in glyphosate structure are generally not well tolerated. Typically, N-substitution dramatically decreases the potency of glyphosate analog binding (Ream et al., 1988; Knowles et al., 1993). For example, the relatively small change resulting from the introduction of a single *N*-methyl group reduces inhibitor potency by about 500-fold (*N*-methylglyphosate, $K_{i(app)} =$ 78 μM). This effect becomes even more dramatic as the size of the nitrogen substituent increases. Thus, *N*-isopropylglyphosate ($K_{i(app)} > 200$ μM) exhibits very weak affinity with enzyme and S3P. In such cases, the added steric interactions presumably decrease the overall stability of the glyphosate ternary complex with S3P. In contrast, **4** exhibits significantly stronger binding than *N*-isopropylglyphosate, even though **4** contains the more extensive secondary alkylamine substituent provided by the shikimate ring. As such, hybrid **4** represents one of the most potent glyphosate analog inhibitors identified to date.

As shown in Table 1, the thermodynamic binding parameters observed for formation of the EPSPS·**4** binary complex demonstrate that the enzyme recognition of **4** is clearly entropically driven. The observed values agree remarkably well with those observed previously (Ream et al., 1992) for formation of either EPSPS·S3P or EPSPS·**13**. The recognition of **4** is even more entropically favored than either S3P or **13**. The accompanying ΔH term exhibited by **4** is nearly midway between those observed previously for S3P or **13**. This again suggests that the shikimate 3-phosphate and 1-carboxylate groups in **4** contribute significantly to its interaction with enzyme. However, the hindered tertiary amine functionality at the 5-position in **4** may significantly reduce the hydrogen-bonding interactions with enzyme mediated through either the 4-OH or protonated 5-amine groups.

The energetics of S3P recognition have previously been shown to involve a favorable positive entropy effect which is somewhat modulated by a negative enthalpy term. Electrostatic interactions with EPSPS are expected to predominate through desolvation and salt-bridge formation at the charged S3P anionic centers. The shikimate 3-phosphate group is known to contribute more than 8 kcal/mol to recognition at the S3P site (Gruys et al., 1992). However, both S3P and enzyme can also contribute hydrogen-bonding interactions through the alcohol and heteroatom centers to help stabilize this complex. Such hydrogen-bonding interactions usually contribute about 2 kcal/mol to the enthalpy term for each hydrogen bond with an accompanying decrease in entropy (Eftink & Biltonen, 1980).

Similarly, the formation of the EPSPS·**13** binary complex was previously shown to be entropically favored with an accompanying ΔH near zero. This is typical for an association governed largely by a change in solvation state through release of bound water from either the enzyme or the ligand to the bulk solvent as a result of either electrostatic or hydrophobic interactions (Eftink & Biltonen, 1980). Presumably, the 3-phosphate and dual carboxylate groups in **13** contribute significantly to its interaction with enzyme. However, the hindered cyclic ketal functionality should

dramatically reduce the hydrogen-bonding interactions with enzyme mediated through either the 4- or 5-OH groups. As a result, the recognition of **13** is more entropically favored than S3P.

The close agreement in thermodynamic binding parameters observed for binary enzyme complexes containing **4**, **13**, or S3P stand in sharp contrast to the differences in binding parameters observed previously (Ream et al., 1992) for binary enzyme complexes containing PEP or glyphosate. Recognition of PEP was largely enthalpy driven with an essentially zero entropy term. The enthalpy terms observed for both S3P and PEP binding are quite similar, but PEP recognition is attenuated by a more negative entropy effect. The binding of glyphosate to free enzyme was much more enthalpy favored than PEP (Ream et al., 1992), but this effect was largely counterbalanced by a dramatic negative entropy term. This large negative entropy term suggests that there is a substantial degree of complementarity between glyphosate and enzyme, even in this binary complex. However, the thermodynamic binding parameters observed for **4** demonstrate that many of these favorable enthalpic interactions are missing. Like S3P, the binding of **4** to free enzyme is largely entropy driven, and these characteristics are fully consistent with a primary interaction localized at the S3P subsite. These data also indicate that there is a relatively poor overlap between **4** and the glyphosate binding site.

The thermodynamic parameters determined earlier (Ream et al., 1992) for the formation of the EPSPS·S3P·glyphosate ternary complex are also summarized in Table 1. Addition of glyphosate to the preformed EPSPS·S3P binary complex was much more favored by enthalpy than was the association of glyphosate with native enzyme. However, a fairly substantial negative entropy term effectively counteracted this effect. The thermodynamic properties observed for the formation of the EPSPS·S3P·glyphosate ternary complex are quite consistent with the ligand-induced change in macromolecular conformation, as observed previously through fluorescence binding studies (Anderson et al., 1988c). The large negative entropy term suggests that glyphosate fits very well within its binding site either alone with enzyme or in the EPSPS·S3P·glyphosate ternary complex. The high complementarity indicated by these parameters is consistent with a significant loss in motional freedom in either complex. Presumably, a series of dipole−dipole, hydrogen-bonding, and electrostatic interactions severely restrict the rotational freedom of glyphosate and several amino acid side chains in these complexes. While significant desolvation of the enzyme and ligands may also accompany glyphosate binding, any overall positive entropy contributions which result from desolvation must be completely masked by the restrictions in motional freedom induced by glyphosate. This implies that the dead-end EPSPS·S3P·glyphosate ternary complex is fairly rigid compared to native enzyme. Similarly, the large enthalpy change accompanying glyphosate addition to the EPSPS·S3P binary complex versus that observed with just inorganic phosphate (Ream et al., 1992) suggests that the interactions between glyphosate and enzyme reach far beyond this phosphate binding site. A significant change in interactions between S3P and enzyme as well as between S3P and glyphosate may occur in the EPSPS·S3P·glyphosate ternary complex.

^{31}P NMR Spectroscopic Studies. Since ^{31}P NMR has been used previously (Castellino et al., 1989) to demonstrate the complementarity between the S3P and glyphosate binding sites in the EPSPS·S3P·glyphosate ternary complex, we sought complementary ^{31}P NMR data for the formation of EPSPS·**4** to corroborate these microcalorimetry and kinetic results. The potency of **4** could potentially be attributed to a weak interaction which occurs simultaneously at the S3P and glyphosate binding sites. The observed competitive behavior for **4** versus EPSP suggests that **4** can occupy the S3P/EPSP site quite well, whereas the observed mixed inhibition pattern versus P_i suggests incomplete overlap at this site, which is known to be critical for tight binding by structural mimics of **2** (Alberg et al., 1992). To further explore this issue, we used ^{31}P NMR to probe the recognition of **4** at the enzyme sites responsible for binding to either the S3P 3-phosphate or glyphosate phosphonic acid moieties.

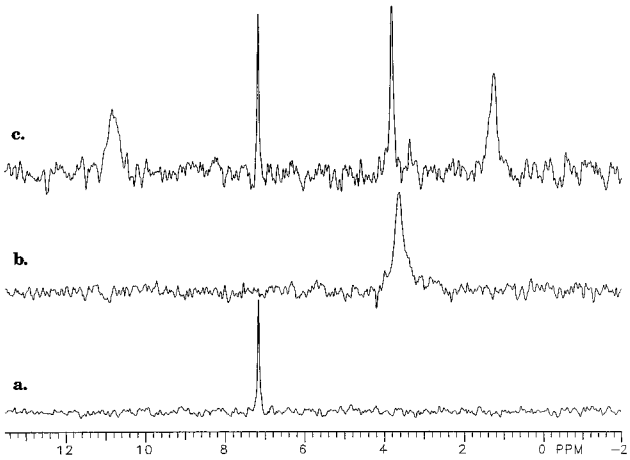


FIGURE 3: ^{31}P NMR spectra of (a) 1.0 mM glyphosate in buffered solution at pH = 7.25, (b) 1.0 mM S3P and 0.7 mM EPSPS at pH = 7.25, and (c) 1.0 mM glyphosate with 1.0 mM S3P and 0.7 mM EPSPS at pH = 7.25.

The formation of the EPSPS·S3P·glyphosate ternary complex has been characterized previously by ^{31}P NMR (Castellino et al., 1989). As shown in Figure 3 at pH = 7.25, the signals from both the glyphosate phosphonate (δ = 11.0 ppm) and S3P 3-phosphate (δ = 1.6 ppm) groups are broadened and shifted in this ternary complex. Formation of the ternary complex produces a consistent, well-defined upfield shift ($\Delta\delta$ = 2.2 ppm) for the S3P 3-phosphate moiety relative to that of the EPSPS·S3P binary complex (δ = 3.8 ppm). An even more dramatic downfield chemical shift ($\Delta\delta$ = 3.9 ppm) is observed for the bound glyphosate phosphonate group relative to its solution spectrum (δ = 7.1 ppm). Thus, the binding of glyphosate influences the environment surrounding the 3-phosphate moiety in S3P. The affinity of glyphosate in a ternary complex is significantly reduced using shikimic acid in place of S3P under these conditions (Gruys et al., 1992). Therefore, the S3P 3-phosphate group not only contributes significantly to substrate binding and catalysis but also plays an important role in stabilizing the EPSPS·S3P·glyphosate ternary complex.

As shown in Figure 4, the addition of **4** to excess enzyme at pH = 7.25 produces significant line broadening of both the 3-phosphate and phosphonate signals, relative to the corresponding solution spectrum. The shikimate 3-phosphate resonance in EPSPS·**4** (δ = 3.2 ppm) is shifted slightly upfield ($\Delta\delta$ = 0.7 ppm), indicating that at least some recognition at the S3P site has occurred. The phosphonate signal in EPSPS·**4** (δ = 5.4 ppm) is also shifted slightly upfield ($\Delta\delta$ = 0.7 ppm), indicating that the interaction desired with the phosphonate site in the EPSPS·S3P·

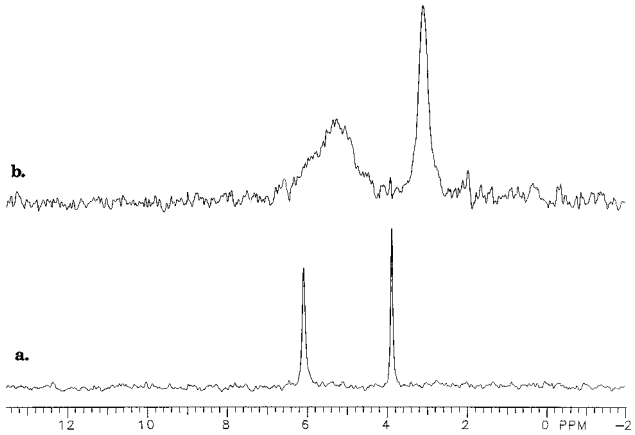


FIGURE 4: ^{31}P NMR spectra of 1.0 mM **4** (a) in buffered solution at pH = 7.25 and (b) with 1.4 mM EPSPS at pH = 7.25.

glyphosate ternary complex is largely inaccessible in this binary complex. Similarly, the heterogeneous line broadening of the bound phosphonate signal is indicative of a more highly disordered recognition at this center. Presumably, this lack of significant interaction at the phosphonate site also reduces the overall upfield chemical shift observed for the 3-phosphate group. These spectroscopic results confirm that the complementarity observed between the S3P and glyphosate binding sites in the EPSPS·S3P·glyphosate ternary complex is lacking in the EPSPS·**4** binary complex. The combined kinetic and ligand binding results clearly demonstrate that the interaction of **4** with free enzyme predominantly occurs at the S3P binding site and that there is little, if any, interaction at the glyphosate phosphonate site. Consequently, even though **4** is the most potent *N*-alkylglyphosate analog identified to date, it can best be characterized as an S3P-based substrate−analog inhibitor of EPSPS.

Implications to the Transition-State Model for Glyphosate Action. The results presented above clearly demonstrate that **4** does not represent the optimum three-dimensional orientation between S3P and glyphosate in the EPSPS·S3P·glyphosate ternary complex. A comparison of the kinetic and ligand binding properties of **4** versus **5** suggests that the two molecules display dramatically different binding patterns. While **4** exhibits competitive inhibition versus EPSP, it displays mixed inhibition versus P_i, indicating that when **4** occupies the S3P binding site, there is incomplete overlap at the P_i site. Interaction at the PEP−P_i subsite has been previously shown to account for the observed potency of **5** as an EPSPS bisubstrate inhibitor. Compared to **4**, the shortened tetrahedral intermediate mimic **5** exhibits significantly greater potency with an observed apparent K_i of 15 ± 1 nM, clearly competitive with EPSP, when measured for the reverse reaction utilizing enzyme from *Petunia hybrida* (Alberg et al., 1992). Phosphonate **5** is also reported to be a potent, competitive inhibitor versus P_i with an apparent K_i of 0.11 ± 0.02 μM. A comparison of apparent K_i's, using enzymes from two different sources, indicates that **4** binds about 500−1200-fold less tightly to enzyme than **5**. However, a direct comparison of observed K_d's using *E. coli* EPSPS now indicates that **4** binds about 33-fold less tightly to enzyme than **5** (Anderson & Johnson, 1990b). In either case, there is a significant difference in both the potency and pattern of interaction of these two inhibitors with enzyme. If glyphosate occupies the same space as PEP when S3P is present, one would logically expect a dramatically more potent K_d for **4** and competitive kinetic patterns versus both EPSP and P_i. Competitive inhibition versus P_i is expected for tight binding bisubstrate inhibitors based on the proposed random kinetic mechanism for the EPSPS reverse reaction (Gruys et al., 1993).

Thus, the combined kinetic, spectroscopic, and microcalorimetry measurements confirm that the interaction of **4** with free enzyme is localized predominantly at the S3P binding site. The interaction at the PEP−P_i subsite, which was so important for the observed potency of the tetrahedral intermediate mimic **5**, is completely lacking in **4**. Therefore, an EPSPS bisubstrate inhibitor linking S3P with PEP displays different properties from one covalently combining S3P with glyphosate, even though both molecules fit well within the spatial constraints imposed by the tetrahedral intermediate. This difference implies that glyphosate and PEP are not superimposable in a shortened, pinched conformation covalently bound to S3P and suggests that glyphosate must be bound in a more extended conformation in the EPSPS·S3P·glyphosate ternary complex, as recently reported by solid-state REDOR NMR experiments (Christensen & Schaefer, 1993). However, an extended bound glyphosate conformation does not provide an optimal match with the spatial requirements imposed by the planar PEP oxonium ion. These results combined with the recent observation that glyphosate can still bind to enzyme when the active site is occupied by both EPSP and P_i (Sammons et al., 1995) provide additional evidence that the transition-state inhibitor and intermediate mimic models are inadequate to explain glyphosate's molecular mode of action.

Conclusions. In summary, compound **4** represents the first example of a potent EPSPS inhibitor based on the herbicidally active EPSPS·S3P·glyphosate ternary complex. A more precise quantitative definition for the molecular interactions of **4** with this enzyme was developed through a series of analog kinetic evaluations as well as calorimetry-based and ^{31}P NMR spectroscopic measurements. The specific interaction of **4** with the S3P site was corroborated by the very poor binding displayed by the new 3-dephospho analog **12**.

Isothermal titration calorimetry demonstrated that **4** binds to free enzyme with an observed K_d of 0.53 ± 0.04 μM. As an S3P mimic, **4** binds to free enzyme about as well as EPSP. As a glyphosate analog inhibitor, **4** binds nearly as well as glyphosate. With a sub-micromolar K_d, **4** represents the most potent *N*-alkylglyphosate derivative identified to date. However, the resulting thermodynamic binding parameters demonstrate that the formation of EPSPS·**4** is entropy driven like S3P, and the binding characteristics of **4** are fully consistent with a primary interaction localized at the S3P subsite.

Furthermore, ^{31}P NMR studies of enzyme-bound **4** confirm the expected interaction at the shikimate 3-phosphate site. However, the heterogeneous line broadening observed for the phosphonate signal indicates that the concomitant recognition of the phosphonate group in **4** is fairly disordered and induces a chemical shift in the opposite direction than that observed previously when glyphosate binds with enzyme and S3P. The complementarity previously observed by ^{31}P NMR studies of the EPSPS·S3P·glyphosate ternary complex is lacking in the EPSPS·**4** binary complex. The correct spatial orientation between S3P and glyphosate is certainly not achieved in hybrid **4**. When **4** occupies the S3P binding site, there is clearly incomplete overlap at the glyphosate phosphonate subsite. Thus, as a glyphosate analog inhibitor, the potency of **4** arises from predominant interactions which occur outside the normal glyphosate binding site, and

consequently **4** is best characterized as an S3P-based substrate−analog inhibitor.

ACKNOWLEDGMENT

The authors thank Dr. Henry E. Dayringer of G. D. Searle and Co. for his assistance in generating the color figure comparing the two conformations of glyphosate with PEP. The authors gratefully acknowledge Dr. Claude R. Jones of Monsanto Co. for his assistance in acquiring the ^{31}P NMR spectral data and Dr. Michael J. Miller of Ceregen for his helpful commentary and review of the manuscript.

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BI9521349