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DESIGN & SYNTHESIS OF A NOVEL EPSP SYNTHASE INHIBITOR BASED ON ITS TERNARY COMPLEX WITH SHIKIMATE-3-PHOSPHATE AND GLYPHOSATE

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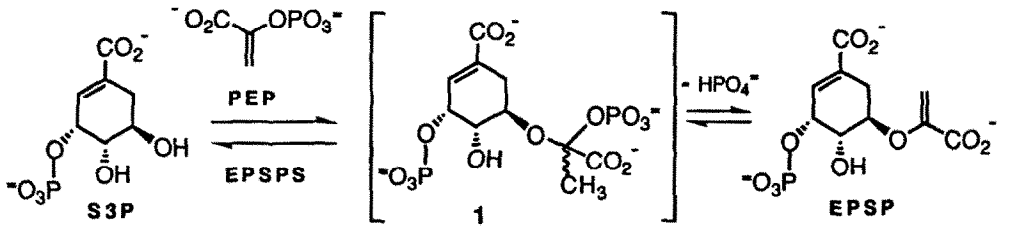
Abstract: A novel EPSP synthase inhibitor **4** has been designed and synthesized to define the conformational and configurational details of glyphosate recognition in its herbicidal ternary complex with enzyme and shikimate-3-phosphate (S3P).

The enzyme EPSP (5-enolpyruvoyl-shikimate-3-phosphate) synthase (EPSPS, E.C. 2.5.1.19) has generated considerable interest since it functions as the biological target for the commercially successful herbicide, glyphosate (PMG).[2] EPSPS catalyzes an unusual transfer reaction of the carboxyvinyl portion of phosphoenol-pyruvate (PEP) regiospecifically to the 5-OH of S3P forming EPSP and inorganic phosphate (P_i).[3] The enzyme exhibits a random kinetic mechanism[4] through a single, kinetically competent,[5] tightly bound,[6] tetrahedral intermediate, **1** (Scheme I). Glyphosate **2** has long been postulated to act as a transition state analog for the putative protonated PEP oxonium ion **3** formed transiently during catalysis (Scheme II).[7] Studies using kinetics,[7,8] fluorescence,[9] gel filtration,[10] ^{31}P-NMR[10] and Differential Scanning Calorimetry[11] demonstrate that glyphosate preferentially forms a stable ternary complex with enzyme and S3P (EPSPS•S3P•PMG). Here we report the first approach to a novel EPSPS inhibitor based on the bisubstrate analog **4** of this ternary complex.

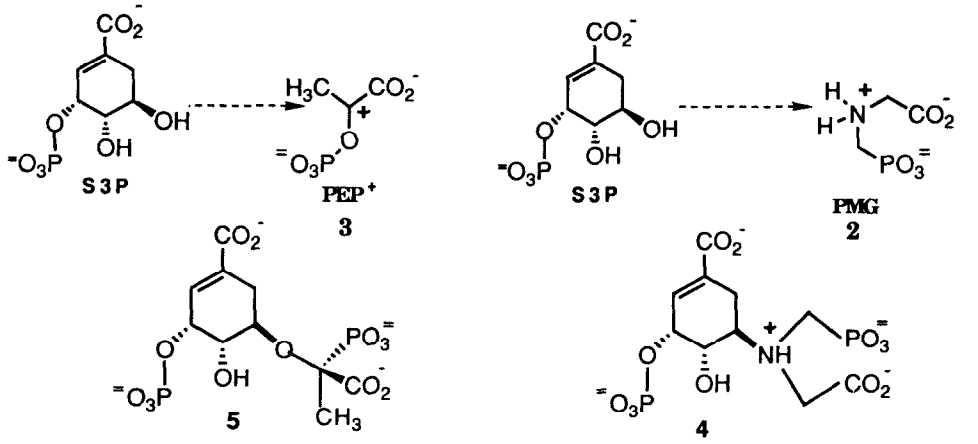
Scheme I



To function as a transition state inhibitor, glyphosate should overlap completely with **3**, even though it contains one extra atom between the anionic centers. The planar configuration of **3** requires that the ionic phosphonic and carboxylate functionalities of glyphosate be bound in a "pinched" conformation with each anionic group on the same side of the nitrogen atom. Molecular modeling studies indicate that this "pinched" conformation of glyphosate can be superimposed on PEP, without any energy penalty.[12] The flexibility of glyphosate allows it to adapt a conformation where each anionic group can be superimposed on PEP, with the methylene groups oriented above and below the plane. X-ray crystal structures of glyphosate have been reported

in both "pinched" and extended conformations.[13a,b] The calcium salt of glyphosate clearly permits such a "pinched" conformation where glyphosate acts as an internal bidentate chelating ligand for this metal ion.[13b]

Scheme II



Glyphosate acts as a competitive inhibitor versus PEP and an uncompetitive inhibitor versus S3P.[8] 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.[14] These amino acids all appear at the proposed active site interface of the two globular domains in this protein.[15] Fluorescence binding studies demonstrate that EPSP and **1** can cause a conformational change similar to that induced by S3P and glyphosate, but not by S3P, PEP or glyphosate alone.[6,9] Thus, the combined biochemical evidence strongly suggests that there is substantial overlap between the PEP and glyphosate binding domains within the EPSPS active site.

Recent microcalorimetry measurements indicate that S3P synergizes glyphosate binding much more effectively than PEP.[16] These results are consistent with the suggestion that the EPSPS•S3P•PMG ternary complex resembles the transition state leading to **1**. Since PMG binds nearly 75-fold more tightly to EPSPS than PEP,[9] appropriate bisubstrate analogs should be very potent EPSPS inhibitors. However, the exact spatial relationship between S3P and PMG in this ternary complex is not known. While the optimized orientation between glyphosate and S3P may require a more extended hybrid structure, the shortened bisubstrate inhibitor **4** was designed for direct comparison with the shortened R-phosphonate inhibitor **5** (K_d = 15 nM),[6] one of the most potent known EPSPS inhibitors.[17] The potency of **5** is believed to occur through a significant interaction at the PEP-phosphate site.[17] In addition, **4** fits well within the spatial constraints of a three dimensional active site model developed for EPSPS, when a "pinched" conformation of the glyphosate sidechains is employed (Figure 1).[18] The geminal proximity of the anionic centers at the tetrahedral center in **1** requires that the same "pinched" glyphosate conformation be employed to optimize overlap between **1** and **4**.

The synthesis of **4** was accomplished in eleven steps in ~1% overall yield starting from (-)-shikimic acid *via* the known epoxy alcohol **6**[19] as summarized in Scheme III. Ring opening of the epoxide with sodium azide[20] followed by acetonide formation[21] and hydrogenation using Lindlar's catalyst[22] produced the required protected

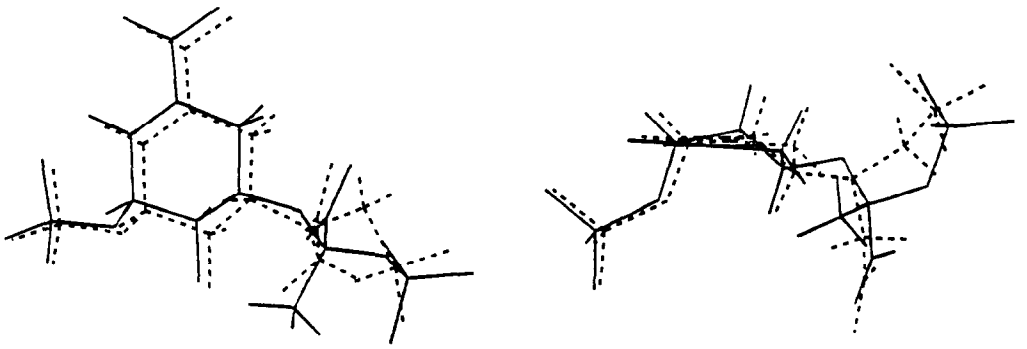


Figure 1. A molecular modeling comparison using SYBYL® of **4** (dashes) versus **1** (solid lines).

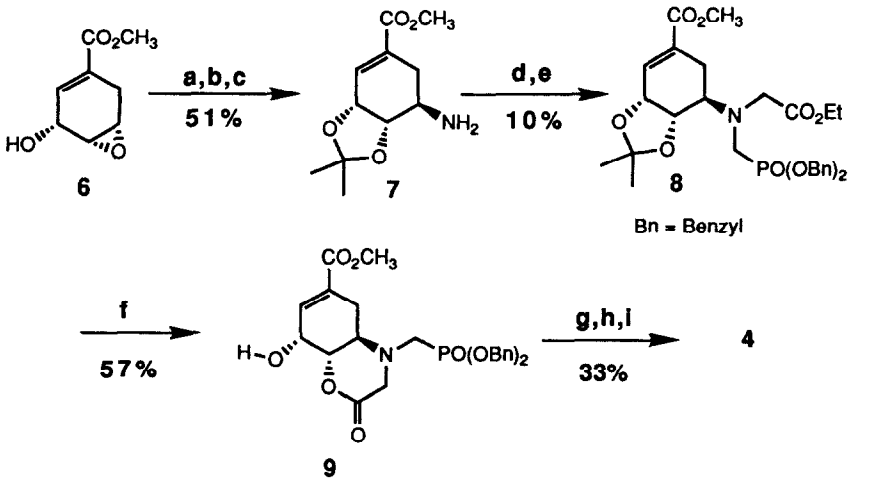
diol amine **7** in 51% overall yield. Sequential alkylation first with ethyl bromoacetate followed by dibenzyl-phosphonomethyltriflate[23] introduced the protected glyphosate functionality in 10% overall yield. A variety of methods were examined to effect the deprotection and lactonization of **8**. Most were plagued by multiple side products and/or product decomposition. These problems were circumvented using the modified deprotection procedure of Ho[24] (Dowex (H^+), CH_3CN-H_2O, reflux). Under these conditions, protection of the C_4-OH occurs through lactonization[17,25] to give the lactone **9** as analytically pure crystalline material in 57% yield. Phosphorylation[17,26] of **9** with tetrabenzylpyrophosphate[27] and removal of the six protecting groups by sequential treatment with TMSBr[28] and aqueous base followed by ion exchange chromatography gave **4** as a triethylammonium salt in 33% isolated yield from **9**. Purified **4** exhibited satisfactory microanalytical data. Spectral characterization using 2D-COSY, ^1H-, ^{13}C-, ^{31}P-NMR and mass spectra were all consistent with this structure.[29]

Hybrid **4** was evaluated as an inhibitor of EPSPS from *E. coli* using a continous spectrophotometric coupled assay monitoring loss of NADH for the EPSPS reverse reaction.[30] The kinetic evaluation (Figure 2) demonstrates that **4** displays mixed inhibition versus P_i with an apparent K_i of 13 ± 1 μM and competitive inhibition versus EPSP with a surprisingly weak apparent K_i of 7.4 ± 0.4 μM. The shortened tetrahedral intermediate mimic **5** exhibits significantly stronger 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*.[17] Phosphonate **5** is also reported to be competitive versus P_i with an apparent K_i of 0.11 ± 0.02 μM. Thus, a direct quantitative comparison indicates there is a significant difference in their potency and pattern of interaction with EPSPS. If PMG occupies exactly the same space as PEP when S3P is present, one would expect a dramatically more potent K_i for **4** versus EPSP and competitive kinetic patterns versus 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.[31] The observed competitive behavior for **4** versus EPSP (K_i = 7.4 ± 0.4 μM) suggests that **4** can occupy the S3P/EPSP site quite well, whereas the observed mixed inhibition pattern versus phosphate suggests incomplete overlap at the PEP-P_i site.

Hybrid **4** represents the first example of an *N*-alkyl glyphosate analog that exhibits a low micromolar interaction with EPSPS. A direct quantitative comparison is difficult given the uncompetitive nature of glyphosate inhibition versus S3P.[7,8] The steric and ionic limitations of the glyphosate binding site have been well characterized versus PEP.[32] As an EPSPS inhibitor class, modifications in glyphosate structure are not well

tolerated by this enzyme. Typically, *N*-substitution dramatically decreases the potency of glyphosate analog binding.[32] For example, N-*iso*-propyl-PMG exhibits dramatically reduced affinity (K_i > 200 μM). In such cases, the added steric interactions presumably decrease the overall stability of the glyphosate ternary complex with S3P; whereas with **4**, significantly stronger binding is observed.

Scheme III



a) NaN_3, NH_4Cl, H_2O-MeOH, reflux, (59%); b) 2,2-dimethoxypropane, PPTS; (88%);
c) H_2, MeOH, 5% Pd/C, (98%); d) $BrCH_2CO_2Et$, Et_3N, THF, (50%); e) $(BnO)_2POCH_2OTf$, CH_2Cl_2, sat. $NaHCO_3$, reflux, (20%); f) Dowex (H^+), H_2O-CH_3CN, reflux;
g) $(BnO)_2PO_2PO(OBn)_2$, $(Me_3Si)_2NNa$, THF, -78 °C; h) TMSBr;
i) aq. NaOH, Ion-Exchange Chromatography.

In summary, **4** represents the first example of a low micromolar EPSPS inhibitor based on the glyphosate ternary complex. Hybrid **4** binds competitively to EPSPS at the S3P/EPSP sites signficantly more tightly than 5-amino-S3P (K_i = 85 μM)[33] and at least as well as S3P (K_d = 8 μM).[9] Hybrid **4** has little, if any, interaction at the PMG-phosphonate or PEP-P_i sites. Such an interaction has been demonstrated to account for the observed potency of **5** as an EPSPS bisubstrate inhibitor. Hybrid **4** appears to be too short to reach the expected glyphosate phosphonate binding site. This would require a much more extended PMG conformation. A comparison of the kinetic properties of **4** versus **5** suggests that the two molecules display dramatically different binding properties. This difference provides the first evidence that glyphosate and PEP are not super-imposable in a shortened, "pinched" conformation covalently bound to S3P. This evidence therefore suggests that glyphosate may not be bound in a "pinched" conformation. *Therefore, glyphosate may not function at the molecular level as a transition state analog of the putative PEP oxonium ion as originally proposed.*[7] Efforts are underway to corrobroate these results by better defining the key interactions of **4** with enzyme, particularly at the PEP-P_i site.

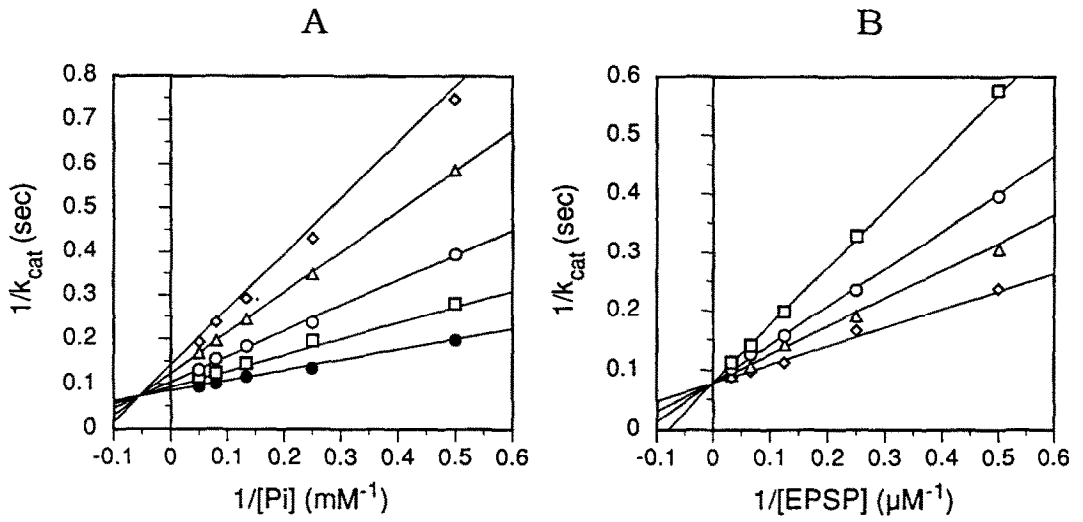


Figure 2: Lineweaver-Burke plot for inhibition of E. coli EPSP synthase by **4**. A) Mixed inhibition versus variable phosphate at fixed [EPSP] = 50 μM; [**4**] = 0 (●), 7.5 μM (□), 20 μM (○), 40 μM (△), & 60 μM (◇). B) Competitive inhibition versus variable EPSP at fixed [Pi] = 50 mM; [**4**] = 0 (◇), 4 μM (△), 8 μM (○), & 16 μM (□). Experiments were performed in a 1-cm pathlength rectangular cell for variable phosphate (total volume = 1.0 mL), and a 10-cm pathlength cylindrical cell for variable EPSP (total volume = 4 mL).

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