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5-Enolpyruvylshikimate 3-Phosphate Synthase: Chemical Synthesis of the Tetrahedral Intermediate and Assignment of the Stereochemical Course of the Enzymatic Reaction

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Abstract: A chemical synthesis of both diastereomers of the tetrahedral intermediate involved in 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS) catalysis has been accomplished. Combination of methyl dibromopyruvate with a protected shikimic acid derivative, phosphorylation, and lactonization afforded the intermediates (*S*)-**15** and (*R*)-**15**, whose configurations were assigned by NMR. After introduction of the 3-phosphate group and deprotection, photoinitiated radical debromination of the dibromo analogues (*S*)-**5** and (*R*)-**5** was accomplished with tributyltin hydride in mixed aqueous solvents in the presence of surfactant to give the pyruvate ketal phosphates (*R*)-TI and (*S*)-TI, respectively. These compounds are stable at high pH, but decompose at pH 7 with a half-life of ca. 10 min. (*R*)-TI proved to be inert to EPSPS, while (*S*)-TI was converted by the enzyme to a mixture of 5-enolpyruvylshikimate 3-phosphate, shikimate 3-phosphate, and phosphoenolpyruvate. The demonstration that the enzymatic intermediate possesses the *S*-configuration at the ketal center confirms the mechanism as an *anti* addition followed by a *syn* elimination. Furthermore, it appears that the *syn* stereochemistry of the second step requires the phosphate leaving group to serve as the base in catalyzing its own elimination.

Introduction

Few biosynthetic sequences are as replete with unusual chemical transformations as the shikimate−chorismate pathway.[1] The novelty of its reactions and its key position in primary metabolism and as a herbicide target have stimulated great interest in elucidating the chemical mechanism and steric course for each of the enzymes involved.[2] By 1990, all of the stereochemical questions had been answered, except for one: the mechanism of one of the most important reactions of all, that catalyzed by 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS).

Sprinson's original suggestion[3] that the transfer of a carboxyvinyl group from phosphoenolpyruvate (PEP) to shikimate 3-phosphate (S3P) to form 5-enolpyruvylshikimate 3-phosphate (EPSP) proceeds via an addition−elimination mechanism was confirmed by Anderson and co-workers at Monsanto by isolating the tetrahedral intermediate (TI) from the enzyme (Figure 1).[4] In an experiment employing equimolar amounts of enzyme and S3P with excess PEP and P_i, equilibrium was established between the enzyme complexes of S3P and PEP, TI, and EPSP and P_i. Under these conditions, the TI constitutes up to 33% of all enzyme-bound species, with the balance primarily the EPSP and P_i complex. This mixture was denatured with neat triethylamine, and the TI was isolated and characterized by ^{1}H, ^{13}C, and ^{31}P NMR.[4,5] However, the configuration of the ketal stereocenter could not be assigned. Anderson et al. noted the instability of the TI at neutral pH, where it decomposes rapidly to S3P, pyruvate, and P_i. The relevance of the isolated intermediate was further demonstrated by reexposure to EPSPS,[6] which catalyzed its decomposition to EPSP and P_i as major products and S3P and PEP as minor products, consistent with

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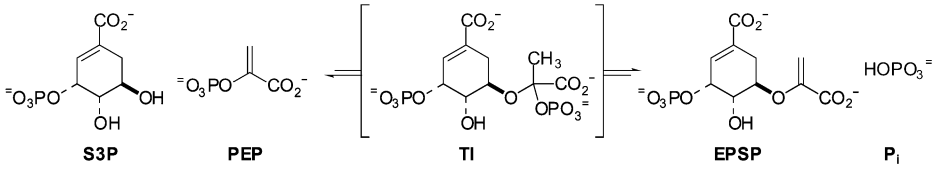

Figure 1. Addition−elimination mechanism for the transfer of a carboxyvinyl group catalyzed by EPSP synthase.

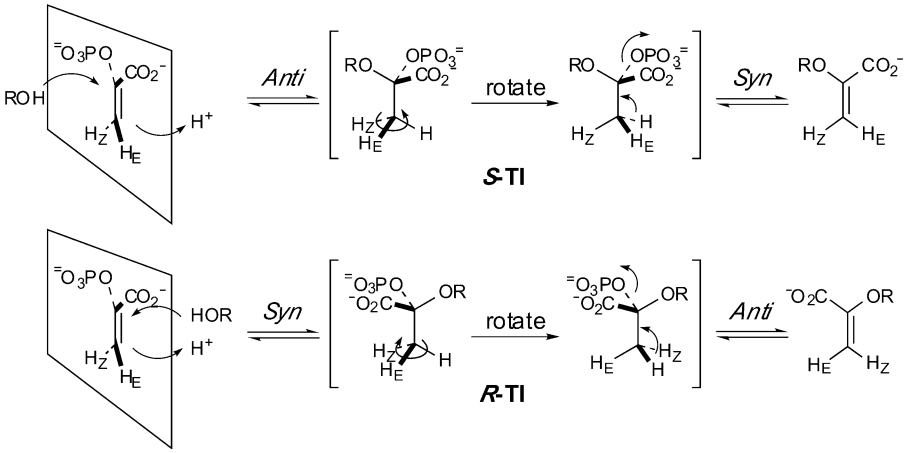

Figure 2. Remaining stereochemical pathways for EPSP synthase involving *si* face protonation of PEP and overall retention of the double bond configuration.

the partition ratio predicted from the forward and reverse rate constants determined previously.[7]

Two paths may be followed for each of three different steps in the formation and decomposition of the TI, resulting in eight possibilities for the detailed stereochemical mechanism of the overall transformation. In the first step, the proton may be added to either the *re* or *si* face of the PEP double bond. In the second, the 5-hydroxyl of S3P may add *syn* or *anti* to this hydrogen. And in the third, the elimination of phosphate may also occur in a *syn* or *anti* sense. Three independent pieces of information are therefore required to define completely the stereochemical course of the process.

The first piece of information came from the Knowles and Floss groups, who used stereoisotopically labeled PEP (^{3}H and ^{2}H) to show that the double bond of EPSP is generated with the same geometry as that in the PEP substrate.[2e,f] This finding meant that the addition and elimination steps proceed with opposite stereochemistries; i.e., one is *syn* and one is *anti*. The Walsh group supplied the second piece of information by studying the EPSPS-catalyzed addition of S3P to (*E*)- and (*Z*)-fluoro-PEP in deuterated water.[2g] Trapping the fluorinated TI and determining the configuration of the chiral fluoromethyl group (CHDF) allowed them to conclude that the proton is added to the *si* face of PEP in the first step of the EPSPS reaction.

These experiments left only two possible stereochemical mechanisms to be distinguished (Figure 2): *anti* addition to form an *S*-configured ketal phosphate, followed by *syn* elimination, or the opposite, *syn* addition/*anti* elimination via an (*R*)-ketal phosphate. To resolve this question required that the absolute configuration of the ketal phosphate stereocenter in the TI be determined.

Assignment of the ketal phosphate configuration has been confounded by the elusive nature of the TI. It can only be isolated in small quantities from the enzymatic reaction (ca. 300 μg from an equilibrium mixture with substrate in the presence of 1 g of enzyme), and it is highly unstable, with a reported half-life of about 45 min at neutral pH.[4,6] Thus, although Anderson et al. could establish the identity of the isolated TI by NMR and through its chemical and enzymatic transformations, they were unable to determine the configuration of the ketal center.

The structure of a decomposition product, bicyclic ketal **1**, suggested the *S*-configuration for the TI, on the assumption that cyclization to **1** occurs with inversion at the ketal center.[5,8] Stable analogues of the TI have provided mixed signals: the greater affinity of the (*R*)-phosphonate **2** in comparison to the *S*-isomer first led us to suggest that the TI also has the *R*-configuration.[9] However, similar affinity for the trifluoro-TI diastereomers **3** and greater affinity for the opposite diastereomer of the difluoro analogue **4** support the assignment as *S* (note that fluorine inverts the Cahn−Ingold−Prelog prioritization among the ketal substituents).[10] More recently, the crystal structure of the complex of EPSPS with S3P and glyphosate ($^-O_2CCH_2\ NH_2^+CH_2PO_3^{2-}$) has been determined (Figure 3).[11] With the assumption that the active site orientation of PEP is the same as that of glyphosate, Schönbrunn et al. proposed that Glu-341 serves as the proton donor in catalyzing *anti* addition; the consequence of such a process would be formation of a TI with the *S*-configuration. Finally, there are strong similarities in structure and in chemical mechanism between EPSPS and MurA, which catalyzes the transfer of a carboxyvinyl group from PEP to an *N*-acetylglucosamine hydroxyl group as a step in bacterial cell wall biosynthesis. The demonstration by Walsh and co-workers that the MurA reaction proceeds with *anti* addition to an *S*-tetrahedral

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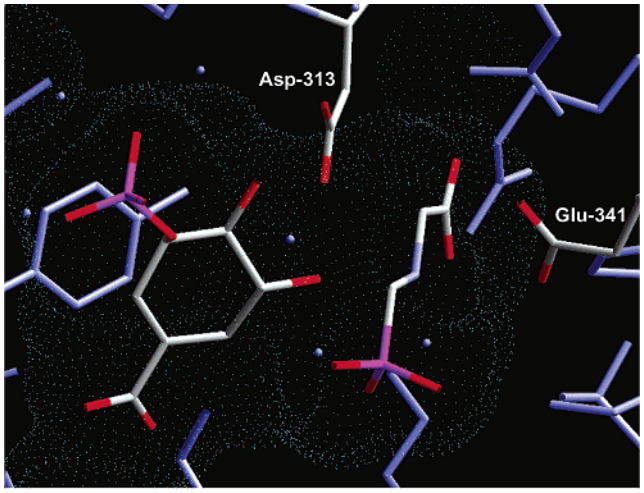
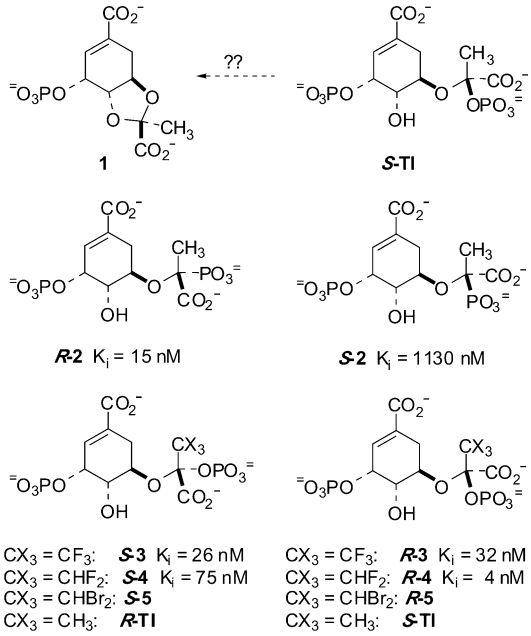


Figure 3. Active site of EPSP synthase occupied by S3P and glyphosate.[11]

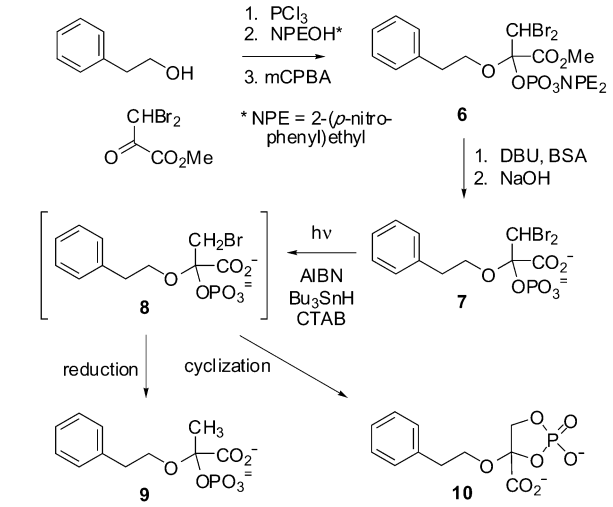
intermediate provided further circumstantial evidence for the same reaction course for EPSPS.[12]



We have now removed any remaining ambiguity as to the mechanism of EPSPS through the synthesis and stereochemical assignment of both diastereomers of the TI itself. The observation that only one of them is processed by the enzyme defines the stereochemical course of the EPSPS reaction and provides the last piece in the fascinating puzzle of the shikimate−chorismate pathway.

Synthetic Strategy. Acyclic hemiacetal and hemiketal phosphates were apparently unknown prior to the isolation of the EPSPS TI. Direct synthesis by phosphorylation of a hemiketal hydroxyl group poses two challenges: first, in the bimolecular equilibrium with alcohol and ketone, the hemiketal is usually disfavored, and second, the product is exceptionally prone to decomposition if the phosphate group is esterified or protonated. The first problem is overcome in the cyclic analogues (for example, the anomeric phosphates of furanoses and pyranoses)

Scheme 1



because the hemiketal or hemiacetal is favored in the intramolecular equilibrium with the acyclic forms. Both problems are mitigated by electron-withdrawing substituents in the ketone, which stabilize the hemiketal adduct relative to the ketone and disfavor mechanisms for decomposition of the ketal phosphate that involve loss of phosphate and formation of an oxocarbonium ion. We took advantage of these effects in synthesizing the fluorine-substituted TI analogues **3** and **4**, which are exceptionally stable.[10] Our strategy for the synthesis of the TI was patterned on the synthesis of the stabilized analogues **3** and **4**, employing bromine in place of fluorine for stabilization of the intermediates.[10] We anticipated that the carbon−bromine bonds could be reduced as the last step in the synthesis, which would involve the diastereomeric dibromo-TI analogues **5** as the immediate precursors to the TI and its stereoisomer.

Model System. To evaluate conditions that would effect debromination of a highly charged species and allow purification of an acid-labile product, we first explored a relatively simple model substrate. The ketal phosphate **7** was prepared in a straightforward manner from 2-phenethyl alcohol and methyl dibromopyruvate as shown in Scheme 1. Methyl dibromopyruvate was prepared by heating pyruvic acid with bromine in refluxing chloroform, followed by Fischer esterification in methanol and vacuum distillation from P_2O_5.[13] In a one-pot process, the hemiketal formed on combination of the alcohol and ketone was treated successively with PCl_3, 2-(*p*-nitrophenyl)ethanol, and *m*-chloroperbenzoic acid to generate the protected derivative **6**. The phosphate esters were cleaved by elimination of *p*-nitrostyrene, induced by DBU in the presence of bis(trimethylsilyl)acetamide (BSA).[10] As noted previously,[10] inclusion of BSA silylates the anionic intermediates and ensures that elimination of the second NPE ester from each phosphate group is as rapid as that of the first. The intermediate di(trimethylsilyl)phosphate esters are hydrolyzed along with the lactone and carboxyl ester on subsequent exposure to 1 N NaOH.

A variety of reductive conditions were assessed in aqueous or alcoholic solutions. Dissolving metal reagents (e.g., with Na, Li, Ca, Mg, and Zn), chromous ion, borohydrides, and catalytic hydrogenation conditions were all ineffective. Radical reduction by photolysis in the presence of AIBN and the water-soluble

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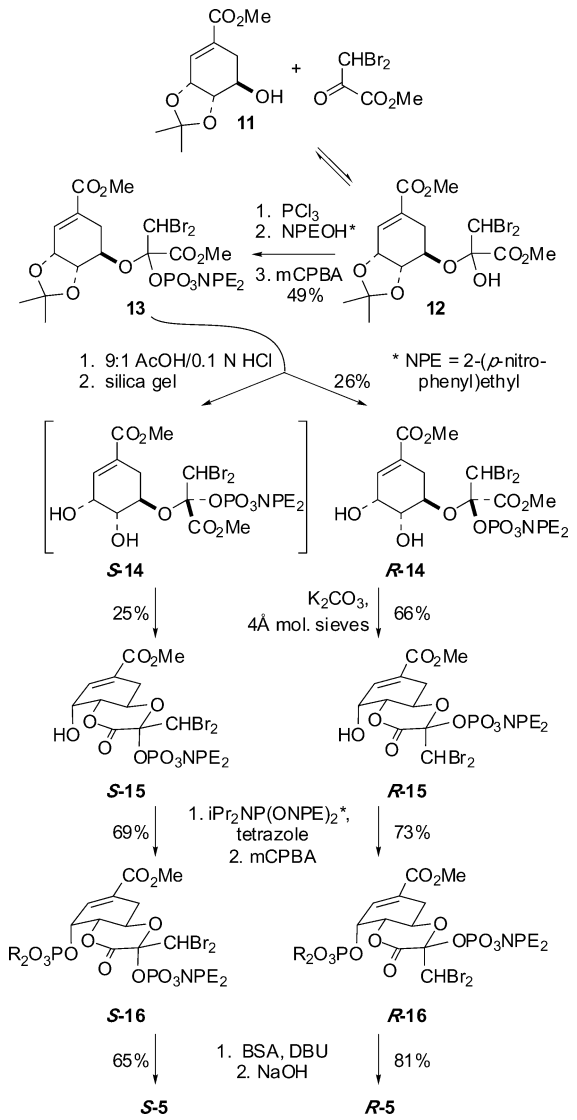
tris(3-(2-methoxyethoxy)propyl)tin hydride reagent introduced by Breslow was also evaluated.[14] The photoinitiated process proved to be the most successful using the conventional tributyltin hydride reagent in combination with cetyltrimethylammonium bromide (CTAB) as a solubilizing detergent. An emulsion of the substrate, tin hydride, detergent, and AIBN in a $NaHCO_3/Na_2CO_3$ buffer was stirred under irradiation with a mercury vapor lamp. At the end of the reaction, the detergent and neutral reagents were adsorbed onto a cation-exchange resin and readily separated from the anionic product and byproducts. The desired pyruvate ketal phosphate **9** was detected by the characteristic methyl singlet at δ 1.73 ppm in the ^{1}H NMR spectrum, which is replaced by the singlet of pyruvic acid at δ 2.43 ppm on acidification.

A major byproduct in this reduction, formed in varying amounts, proved to be the cyclic ketal phosphate **10**, arising from cyclization of the monobromo intermediate **8** in competition with the second reductive step. Two modifications to the cocktail used to solubilize the reagents and substrate were found that reduced the amount of cyclic product. The first was the inclusion of zinc ion, in the form of $ZnCl_2$, which was identified from a series of metal salts screened for their potential chelation by the phosphate−carboxylate trianion. We reasoned that such a chelate would reduce the nucleophilicity of the phosphate moiety and slow cyclization. Indeed, when 10 equiv of $ZnCl_2$ was included in the photolysis mixture, nearly quantitative conversion to the desired ketal phosphate **9** was observed. A more straightforward method for reducing competition from cyclization was to carry out the reduction in methanol. In this solvent, a homogeneous solution was obtained even in the absence of a detergent (tetrabutylammonium iodide was substituted for CTAB), and the photochemically induced radical debromination was more rapid. Unfortunately, it turned out that neither of these modifications could be applied when it came time to reduce the TI precursors **5** (see below), but recognizing the need for a homogeneous process turned out to be significant.

Synthesis of Diastereomeric Dibromo Ketal Phosphates. The ketal phosphate moiety of the EPSPS tetrahedral intermediate was assembled in the same one-pot process employed for the model compound **7** (Scheme 2). A 2.2 M solution of methyl shikimate 3,4-acetonide **11**[15] and 8.2 M methyl dibromopyruvate[13] in CH_2Cl_2 was stirred with molecular sieves. Such high concentrations of starting materials and the excess pyruvate were required to favor formation of hemiketal **12**. After successive treatments with PCl_3, 2-(*p*-nitrophenyl)ethanol, and mCPBA, the phosphate **13** was isolated in 49% overall yield as a ~1:1 mixture of *R*/*S*-diastereomers. The efficiency of this four-step, one-pot sequence may be limited by the intrinsic equilibrium between hemiketal **12** and the starting materials, because the yield was reduced to 29% when only 1 equiv of the dibromopyruvate was used.

The next step, selective cleavage of the acetonide ketal in the presence of the ketal phosphate, put our stabilization strategy to the test and required considerable optimization. Some mild conditions were ineffective (e.g., iodine in methanol), while others resulted in decomposition or complex product mixtures (e.g., methanolic solutions in the presence of sulfonic acid resins, toluenesulfonic acid, 1 N HCl, or $FeCl_3$ on silica). Ready

Scheme 2



lactonization of one of the product diastereomers on silica gel chromatography further complicated the optimization process. Fortunately, we eventually found that treatment of the mixture of acetonides **13** in 9:1 AcOH/0.1 N HCl affords the diols **14** reproducibly in greater than 80% yield.

In the subsequent lactonization step, the markedly different reactivity of the two ketal phosphate diastereomers became apparent. One lactone diastereomer, eventually shown to be *S*, not only forms more readily but also decomposes more rapidly. If separation of the diol isomers **14** was attempted directly, much of the *S*-isomer converted to the lactone **15** on chromatography. However, if lactonization of the mixture of diols **14** was driven to completion prior to separation, the proportion of (*S*)-**15** in the product was significantly reduced. These problems were circumvented by adsorbing the diol mixture onto silica gel to effect lactonization of the *S*-isomer; elution then afforded the (*S*)-lactone (*S*)-**15** in 25% yield and the (*R*)-diol (*R*)-**14** in 26% yield, based on acetonide **13**. Subsequently, diol (*R*)-**14** was lactonized with K_2CO_3 and molecular sieves in acetonitrile to afford lactone (*R*)-**15** in 66% yield.

The remarkable dichotomy in chemical reactivity between the stereoisomers is ascribed to the different anomeric effects in the (*S*)- and (*R*)-lactones. The shikimate 5-O in (*S*)-lactone

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(*S*)-**15** shares its axial lone pair electrons with the σ^* antibonding orbital of the antiperiplanar pseudoaxial C−O ketal phosphate bond. While anomeric stabilization favors formation of this lactone in comparison to the *R*-isomer, it also lowers the barrier to its decomposition. Thus, (*S*)-lactone (*S*)-**15** is at once thermodynamically more stable and kinetically less stable than (*R*)-lactone (*R*)-**15**.[16]

The (*S*)- and (*R*)-lactones **15** were carried forward in parallel to the TI diastereomers (Scheme 2). The 3-hydroxyl groups were phosphitylated with di-2-(4-nitrophenyl)ethyl-*N*,*N*-diisopropylphosphoramidite catalyzed by tetrazole.[10] The resulting phosphites were oxidized in situ with mCPBA to give the (*S*)- and (*R*)-bis(phosphates) **16** in 69% and 73% yields, respectively. The bisphosphates **16** (of MW 1172!) include in protected form all the components of the TI (MW 404). In the subsequent deprotection steps, the anionic phosphates of the lactones **16** were unveiled first. The *p*-nitrophenethyl groups were eliminated as described for the model compound **6** by stirring a solution of the starting material in dry acetonitrile containing BSA and DBU;[10] subsequent exposure to 1 N NaOH then hydrolyzed the phosphate silyl esters along with the lactone and carboxyl ester. The fully deprotected dibromo hexaanions **5** were isolated and purified via anion exchange chromatography, eluting with a gradient of triethylammonium bicarbonate. A small amount of 1 M aqueous NaOH was added to each fraction before lyophilization to prevent acidification of the product caused by removal of triethylamine. Although the hexasodium salts were isolated in good overall yields (65−81%), they were contaminated with comparable amounts of Na_2CO_3 and $NaHCO_3$ as a consequence of this basification step.

Assignment of Ketal Configuration. The crucial stereochemical assignment of the ketal phosphate configurations in the bicyclic lactones **15** and **16** was accomplished in two ways: by direct NOE analysis and by NMR comparison to related structures whose configurations had been determined unambiguously by proton−fluorine NOE experiments involving both isomers.[9,10] In the (*R*)-hydroxylactone (*R*)-**15**, a weak nuclear Overhauser effect is observed between the dibromomethyl hydrogen and the shikimate H5 hydrogen, reflecting their *cis* relationship. No comparable NOE (e.g., to shikimate H4) is observed for the other diastereomer, which we attribute to the pseudoequatorial orientation of the dibromomethyl group in (*S*)-**15**.

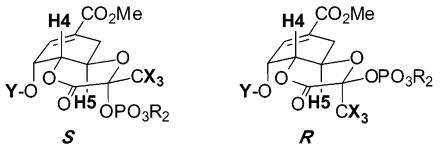
The relative ^{1}H NMR chemical shifts of the H4 and H5 hydrogens in the lactone derivatives are strikingly different, depending upon the configuration of the ketal stereocenter. In structures as divergent as the methyl phosphonates **17** and the difluoromethyl, trifluoromethyl, and bromofluoromethyl phosphates **18**−**20**, the H5 hydrogen resonates downfield of H4 when the phosphorus moiety is axial (see Table 1). Their relative positions are reversed when the methyl or halomethyl substituent is axial. Assignment of the *S*-isomers in the dibromomethyl series is clear-cut by this criterion, although in the case of the *R*-isomers there is little separation between these hydrogens.

Radical Debromination. As noted above, smooth reduction of the dibromo ketal phosphate depends on a homogeneous reaction medium. If the two reduction steps are not reasonably rapid, the monobromo intermediate undergoes intramolecular displacement to form the cyclic phosphate (e.g., **10**). The challenge of solubilizing both the hydrophobic tributyltin hydride reagent and the charged dibromo ketal phosphate model substrate **7** could be solved with a cationic surfactant in aqueous solution or by using methanol as the solvent. However, these conditions were not effective for the hexaanionic substrates **5**. In the water−CTAB medium, a complex mixture was obtained in which the major product appeared to be the cyclic phosphate **21**; addition of zinc chloride to this mixture simply led to an insoluble precipitate. Methanol could not be used directly as solvent, since it does not dissolve the hexaanions either. However, we were able to achieve a homogeneous solution by dissolving the substrate in a small amount of water, diluting with an equal volume of methanol, and then adding this solution to a nearly saturated solution of CTAB in methanol. In this way, a 2.4 mM solution of the dibromo ketal phosphate (*S*)-**5** in 95:5 methanol/water could be prepared. After addition of tributyltin hydride and AIBN, photolysis, and isolation by anion exchange, the desired product, (*R*)-TI, was obtained in 60% yield. The diastereomeric substrate, (*R*)-**5**, was typically isolated from the column fractions with a larger amount of sodium carbonate and bicarbonate salts; as a consequence, homogeneous solutions of this material proved to be more difficult to achieve. However, we ultimately succeeded with a 50:45:5 methanol/DMSO/water combination. A 0.5 mM solution of (*R*)-**5** in this mixture afforded the (*S*)-TI product in 50% yield, contaminated with a small amount of the cyclic phosphate (*S*)-**21**.[17]

***S*-21**

The stability of the TI diastereomers is highly pH dependent, as expected. At pH 10.5, (*R*)-TI has a half-life on the order of days in aqueous solution at room temperature. When the pH is

Table 1. Chemical Shift Trends among Ketal Phosphate Lactones in $CDCl_3$ Solvent



S-isomer (δ ppm) *R*-isomer (δ ppm)

			S-isomer (δ, ppm)		*R*-isomer (δ, ppm)	
compd	Y	CX_3	H4	H5	H4	H5
17[a]	PO_3Bn_2	CH_3	4.45	4.88	4.46	3.99
18[b]	PO_3NPE_2[c]	CF_3	4.65	5.14	4.95	4.46
19[b]	H	CHF_2	4.44	5.02	4.92	4.68
20a[d]	H	CHBrF	4.45	5.1	4.92	4.70
20b[d]	H	CHBrF	4.56	5.1	5.05	4.98
15	H	$CHBr_2$	4.65	5.10	4.98−5.17[e]	
16	PO_3NPE_2[c]	$CHBr_2$	4.75	5.15[e]	4.92−5.25[f]	

[a] Reference 9; phosphonate (PO_3Bn_2) in place of the ketal phosphate moiety; stereochemical designation (CIP) reversed. [b] Reference 10. [c] NPE = 2-(*p*-nitrophenyl)ethyl. [d] **20a** and **20b** are diastereomers due to the CHBrF stereocenter (configuration unknown): D. Alberg and U. Neidlein, unpublished results. [e] H3 and H5 not clearly distinguished. [f] H4 and H5 not clearly distinguished.

(16) We observed similar differences in the rates of lactone formation in our earlier work with the trifluoro and difluoro analogues.[10]

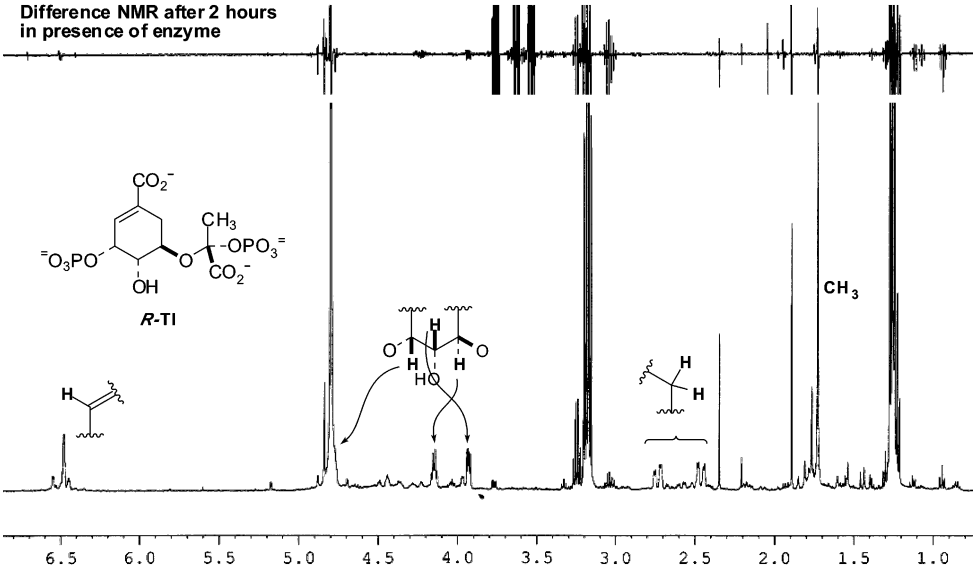


Figure 4. ^{1}H NMR spectrum of (*R*)-TI and difference spectra in the presence of EPSPS.

adjusted to 7.0, (*R*)-TI decomposes to S3P and pyruvate with a half-life of approximately 10 min.

Assignment of the Configuration of the EPSP Synthase Tetrahedral Intermediate. Comparison of the published ^{1}H NMR spectrum of the TI isolated from the enzymatic reaction[4] with those of the individual *R*- and *S*-diastereomers provided the first suggestion that the natural isomer possesses the *S*-configuration. However, a definitive assignment from a direct correspondence in chemical shifts was not possible in view of the different counterions and buffer solutions employed.

Conclusive identification of the enzymatic TI as the (*S*)-ketal phosphate came from incubation of the synthetic diastereomers with EPSPS to determine which one is susceptible to enzymatic conversion. (*R*)-TI (4 μmol) and (*S*)-TI (3 μmol) were each incubated with 0.16 unit of EPSPS in D_2O at pD 9 in an NMR tube, and the ^{1}H NMR spectra were recorded at 30 min intervals at room temperature. Reaction progress was conveniently monitored by subtracting the first spectrum from each subsequent spectrum, thereby emphasizing the changes and minimizing the peaks due to solvent, buffer, and counterions. Although cancellation of the ancillary signals is imperfect, it is apparent that (*R*)-TI is stable in the presence of the enzyme; none of the peaks for this compound disappear, and no new peaks appear (Figure 4). In contrast, in the presence of the enzyme, the peaks for (*S*)-TI disappear to be replaced by those of S3P, PEP, and EPSP (Figure 5, Table 2). The peaks for EPSP grow in more prominently than those for S3P and PEP because the enzyme−TI complex partitions to EPSP in preference to S3P and PEP (Figure 6). A 3:1 preference can be determined most readily from the peaks for the enolpyruvyl hydrogens of PEP (δ 5.17 and 5.38) and EPSP (δ 4.70 and 5.20). Indeed, the observed ratio of these products agrees well with the 5:1 ratio of forward and reverse rate constants determined by Anderson and Johnson and observed in their treatment of the natural TI with EPSPS.[6,7]

(17) Although we worked hard to minimize formation of the cyclic ketal phosphate (*S*)-**21** as a byproduct in the synthesis of the (*S*)-TI, this stable analogue of the TI may be of interest as a potent inhibitor of EPSPS. Modeling suggests that this structure would mimic very closely the bound conformation predicted for the (*S*)-TI (see Figure 7c). Although we did not pursue this idea directly, we noted that enzymatic conversion of (*S*)-TI to its products was slowed in the presence of (*S*)-**21** as a contaminant.

Table 2. ^{1}H NMR Chemical Shifts in an Enzyme−Buffer Mixture[a]

hydrogen	S3P	PEP	(*S*)-TI	EPSP
H2	6.47		6.46	6.52
H4	(3.8)		3.93	4.06
H5	4.0		4.25	4.45
H6α	2.7		2.69	2.90
H6β	2.20		2.23	2.23
CH_3			1.76	
Hz		5.17		4.70
He		5.38		5.20

[a] $NaHCO_3/Na_2CO_3$ buffer, pD 9.

Integrated Stereochemical Mechanism of EPSP Synthase. Assignment of the *S*-configuration to the ketal stereocenter in the EPSPS TI completes the stereochemical definition of the reaction catalyzed by this enzyme. The TI is formed by *si* face addition of the proton to the PEP double bond coupled with *anti* addition of the S3P hydroxyl oxygen; the TI then collapses by *syn* elimination of the methyl proton and phosphate (see Figure 2). In combination with the recently reported crystal structure of the complex of EPSPS with S3P and glyphosate,[11] this stereochemical insight helps to define the chemical mechanism of the addition−elimination reaction and the active site residues that play a key role.

The (*S*)-TI can be readily modeled onto the structures of S3P and glyphosate in their bound conformations. The position of glyphosate in the active site (Figure 3) is indicative of the orientation of the carboxyl and phosphate moieties of PEP (Figure 7a) and, in turn, of the ketal phosphate side chain of the TI (Figure 7b). As Schönbrunn et al. have concluded from their X-ray analysis of the EPSPS−S3P−glyphosate complex, the roles of the active site residues, Asp-313 and Glu-341, are apparent.[11,28] Glu-341 is positioned above the *si* face of the double bond of PEP, opposite the 5-OH group of S3P, and serves as the proton donor in the *anti* addition step leading to the TI. Asp-313 is located adjacent to the 5-OH group of S3P

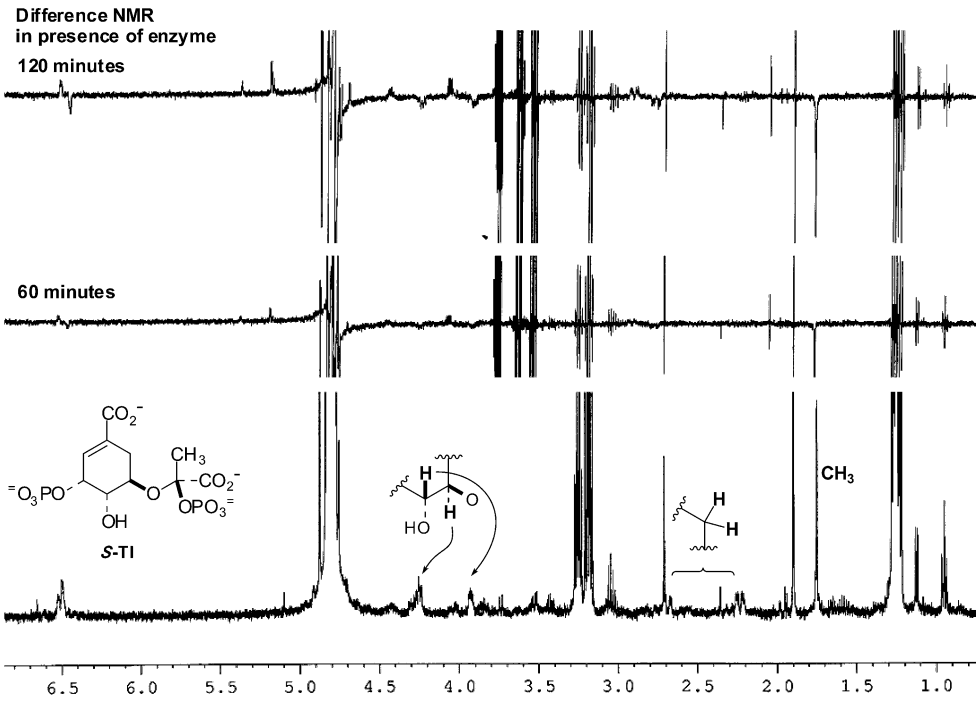


Figure 5. [1]H NMR spectrum of (*S*)-TI and difference spectra in the presence of EPSPS.

and can thus serve as the proton acceptor as the carbon−oxygen bond is formed.

Specific roles for enzymatic residues in the *syn* elimination of phosphate from the TI are not evident. The most logical enzymatic base is the original proton donor, Glu-341. However, this carboxylate is oriented 90° to the C−O bond that must be broken (Figure 7b); to bring the TI and Glu-341 into alignment for *syn* elimination would require a drastic and probably impossible reorientation of the enzyme−TI complex. No other active site residues are in a position to effect *syn* elimination. The base in the most advantageous site to remove the methyl proton *syn* to the phosphate C−O bond is the phosphate itself; we suggest that the elimination is thus intramolecular, without direct action by any enzymatic group. This possibility has also been raised by Walsh et al. from their analysis of the related MurA mechanism.[12]

Self-elimination of phosphate has also been proposed as a key step in the mechanism of dehydroquinate synthase.[2c] Phosphate derivatives in general are not particularly unstable, but there are structural features in the intermediates of both DHQ synthase and EPSPS which make these reactions possible. Interestingly, quite opposite effects are at play in making these substrates prone to elimination (Figure 8). In the case of DHQ synthase, cleavage of the C−H bond likely precedes cleavage of the C−O bond, with the adjacent carbonyl group stabilizing the partial negative charge that builds up in the transition state. In contrast, the ether oxygen in the EPSP TI stabilizes an adjacent positive charge, so that C−O cleavage precedes C−H cleavage in loss of phosphate from this intermediate. In the active site orientation proposed for the TI (Figure 7b), an electron lone pair on the shikimate 5-oxygen is antiperiplanar to the carbon−phosphate bond, and thus in a position to provide anchimeric assistance in its cleavage. The proposal that phosphate induces its own elimination not only follows from the stereochemical constraints, but is also consistent with evidence that the transition state for the elimination step is more ionic than that for the addition step. While the fluoromethyl-TI can

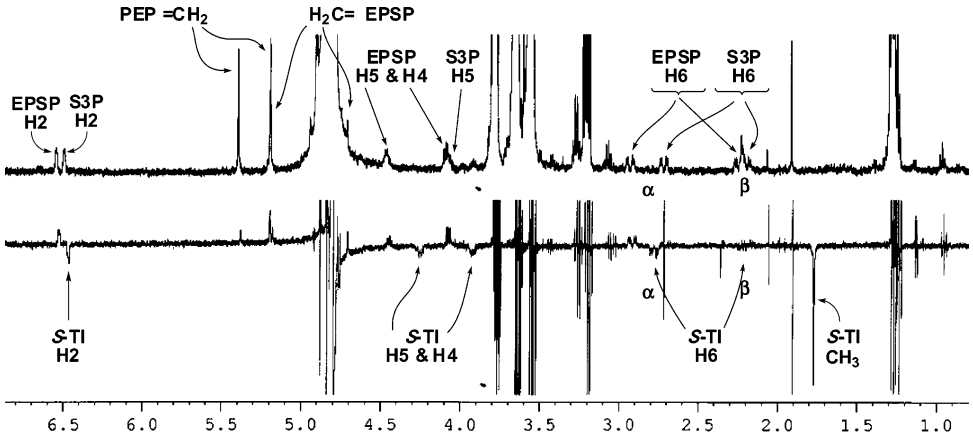


Figure 6. Difference spectrum from treatment of (*S*)-TI with EPSPS (bottom) and reference spectrum of an equimolar mixture of S3P, PEP, and EPSP (top).

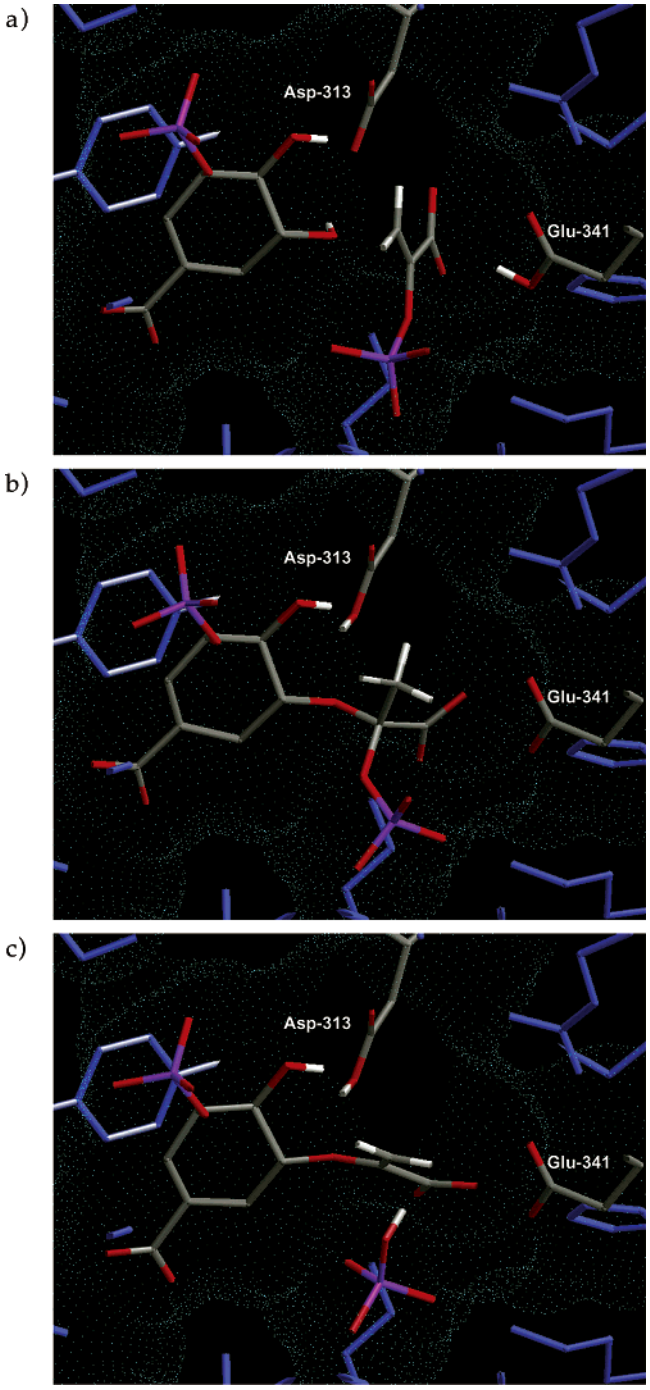


Figure 7. Model of the EPSPS active site with bound substrates: (a) S3P and PEP; (b) (*S*)-TI; (c) EPSP and P_i.

be generated enzymatically from fluoro-PEP,[18] it is not interconverted with fluoro-EPSP.[19]

The conversion of S3P and PEP to EPSP and P_i as catalyzed by EPSPS is fully reversible.[7] If phosphate elimination is indeed self-induced in the forward direction, the principle of microscopic reversibility dictates that phosphate itself provides the proton to promote its addition to EPSP to form the TI in the reverse direction. Wibbenmeyer et al. demonstrated that enzyme-catalyzed solvent exchange of the enolpyruvyl hydrogens on

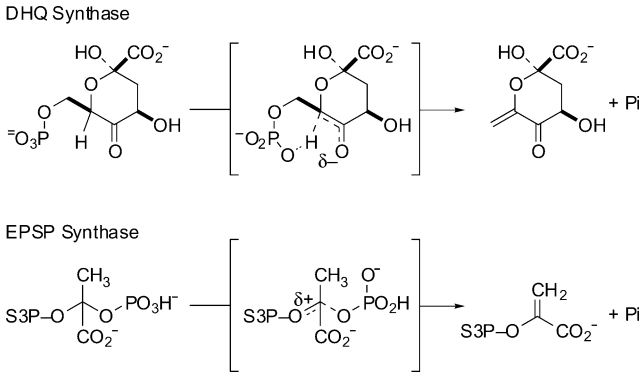


Figure 8. Contrasting mechanisms for self-elimination of phosphate.

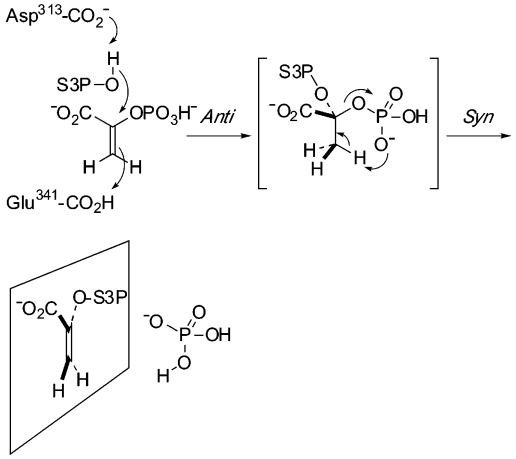


Figure 9. Acids and bases involved in EPSPS-catalyzed synthesis, illustrated in the forward direction.

EPSP requires phosphate ion,[20] in contrast to hydrogen exchange on PEP, which the enzyme is able to catalyze in the presence of 4,5-dideoxy-S3P.[21] These results were first thought to be inconsistent, with the latter observation offered in support of an enzyme-bound PEP adduct and the former as evidence for the noncovalently bound TI. In fact, these results are fully in accord with expectation if an enzymatic acid/base is responsible for the half-reaction interconverting S3P and PEP with the TI and if phosphate is the acid/base responsible for its addition/elimination from the TI (Figure 9).[22]

For the elimination step, it appears that the enzyme simply serves as a template to complement the steric and electronic characteristics of the transition state. This role mirrors that now ascribed to other enzymes of the shikimate−chorismate pathway, namely, DHQ synthase and chorismate mutase. For DHQ synthase, direct involvement of the enzyme side chains and the

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(22) The protonation state of the phosphate moiety involved in the elimination step is thus of interest. It is clear that the TI is quite stable in basic solution, since the phosphate trianion is a poor leaving group. Viewed from the other direction, HPO_4^{2-}, with a pK_a of 12.3, is probably not acidic enough to protonate the EPSP double bond. Thus, a reasonable assumption is that the ketal phosphate moiety of the TI carries a single proton and a single negative charge, eliminating $H_2PO_4^-$ (pK_a = 7.2) on formation of EPSP. However, the crystal structure of the EPSPS complex with S3P and glyphosate shows that each of the phosphonate oxygens of glyphosate is within 3 Å of a positively charged nitrogen on Lys-22, Lys-411, or Arg-124.[11] Thus, the charged residues in the active site may stabilize the ketal phosphate anion of the TI sufficiently to enable PO_4^{3-} to serve as the leaving group in the forward direction and HPO_4^{2-} as the acid in the reverse direction.

NAD cofactor appears to be required only for the steps involving oxidation and reduction; the phosphate elimination and final aldol cyclization may occur spontaneously in the active site.[23,24] For the chorismate mutases, which accelerate the formal [3,3]-sigmatropic rearrangement of chorismate to prephenate, catalysis results simply from binding the substrate in the reactive, diaxial conformation and from stabilization of the charge that builds up briefly in the asymmetric transition state.[25−27]

Conclusion

The chemical synthesis and stereochemical assignment of the tetrahedral intermediate of EPSPS not only defines the mechanism of this enzyme, but completes the stereochemical assignment of all of the enzymatic steps of the shikimate−chorismate pathway. The roles proposed previously[11] for the catalytic residues Gly-341 and Asp-313 in the *anti* addition step are fully consistent with the (*S*)-TI configuration.[28] Moreover, the apparent absence of any enzymatic residues that could serve as proton acceptors for the *syn* elimination step suggest that it is substrate-induced. EPSPS thus joins two of its clever companions in the shikimate pathway that get their substrates to do some of the work.

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Supporting Information Available: Experimental procedures for the synthesis and characterization of the tetrahedral intermediates (*R*)-TI and (*S*)-TI. This material is available free of charge via the Internet at http://pubs.acs.org.

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(28) After the preparation of our paper, Mizyed et al. reported the effects of active site mutations on the partitioning of enzymatically synthesized TI to PEP and EPSP.[29] All 14 residues within 5 Å of a reactive atom from the TI were mutated individually; while many mutations reduced the catalytic activity significantly, none produced a large change in the partition ratio. Mizyed et al. thus concluded that the active site residues play similar acid/base roles in the breakdown of the TI, regardless of the direction. They proposed Glu-341 and Lys-22 as the dual-role acid/base catalysts; that is, Glu-341 would be the proton donor in formation of the TI from S3P and PEP *and* the proton acceptor in elimination of the TI to EPSP and P_i. Lys-22 would serve as the proton acceptor from S3P in the first step and the general-acid catalyst for phosphate elimination in the second. This interpretation contrasts with our mechanistic proposal and those offered previously,[11,12] on the basis of the orientation of the TI in the active site.

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