Substrate Analogs as Mechanistic Probes for the Bifunctional Chorismate Synthase from *Neurospora crassa*†

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ABSTRACT: Analogs of EPSP (**4**−**8**) have been prepared, and their activity as substrates for the chorismate synthase from *Neurospora crassa* has been characterized kinetically. The enzyme appears to show strict discrimination against substitution at the *Z*-position of the enol ether side chain as well as against substitution at the *S*-position of the reduced analogs. Both the glycolyl and (*R*)-lactyl analogs **4** and (*R*)-**5** are good substrates, with (*R*)-**5** having a higher *V* value than the natural substrate. Three substrates, including EPSP, have been found to show significant substrate inhibition with this enzyme, which at present can be explained by a noncompetitive model involving formation of a catalytically incompetent, ternary ES_2 complex. A significant secondary kinetic isotope effect on *V* of 1.10 ± 0.02 has been observed at C-3 with EPSP, indicating that C−O bond cleavage is kinetically significant at saturating substrate concentration; this effect is severely depressed at limiting substrate, with $^{D}(V/K) = 0.97 \pm 0.02$. A similar effect is found for the primary deuterium isotope effect at C-6*R*, as observed previously [Balasubramanian, S., Davies, G. M., Coggins, J. R., & Abell, C. (1991) *J. Am. Chem. Soc. 113*, 8945−8946]. The primary isotope effects at C-6*R* with reduced analogs (*R*)-**5** and (*S*)-**6** are significantly larger than those with EPSP. The larger values of *V* and ^{D}V for (*R*)-**5**, when compared to EPSP, are evidence that release of chorismate is partially rate-limiting under saturating conditions. Incubation of the enzyme with reduced 5-deazaFMN does not result in any observable formation of chorismate, consistent with previous results indicating that reduced flavin is chemically involved in the synthesis of chorismate from EPSP [Ramjee, M. N., Balasubramanian, S., Abell, C., Coggins, J. R., Davies, G. M., Hawkes, T. R., Lowe, D. J., & Thorneley, R. N. F. (1992) *J. Am. Chem. Soc. 114*, 3151−3153].

Chorismate synthase (EC 4.6.1.4) catalyzes the 1,4-elimination of phosphate from 5-enolpyruvylshikimate 3-phosphate (EPSP)[1] to form the 1,3-cyclohexadiene moiety of chorismate (Figure 1). This reaction comprises the last common step of the shikimic acid pathway, a biosynthetic sequence which provides the aromatic amino acids as well as a number of other aromatic metabolites in bacteria, fungi, and higher plants (Haslam, 1993; Dewick, 1993; Bentley, 1990; Weiss & Edwards, 1987). The overall stereochemistry of the elimination reaction is 1,4-*trans*, with removal of the *pro-R* hydrogen at C-6 (Hill et al., 1969; Onderka & Floss, 1969; Floss et al., 1972). This result was provocative in light of theoretical predictions (Anh, 1968; Fukui, 1965) and

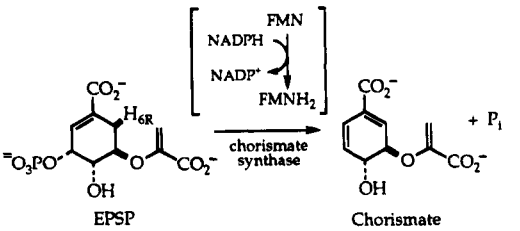

FIGURE 1: Reactions catalyzed by the bifunctional chorismate synthase from *N. crassa*.

experimental work (Hill et al., 1978; Toromanoff, 1980) suggesting that concerted 1,4-elimination reactions should favor the *syn* stereochemistry. As a result of this discrepancy, a number of alternative, stepwise processes have been proposed (Ganem, 1978; Bartlett et al., 1988). One such mechanism involving prior [3,3]-rearrangement of the allylic phosphate of EPSP to give *iso*-EPSP has been ruled out by the finding that *iso*-EPSP is not a competent substrate with the enzyme from *Neurospora crassa* (Bartlett et al., 1986). Single turnover experiments with the enzyme from *Escherichia coli* have shown that there is no "phosphate burst" as might be observed in an E1-type mechanism (Hawkes et al., 1990). Recently, biomimetic studies with an EPSP model compound (Giese & Almstead, 1994) have provided support for a proposed radical mechanism involving hydrogen atom abstraction at the C-6 position (Bartlett et al., 1989).

An intriguing property of chorismate synthase is a requirement for reduced flavin, since the reaction of EPSP to chorismate involves no net oxidation or reduction. How the various chorismate synthases obtain the reduced flavin differs

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[1] Abbreviations: AS, anthranilate synthase; ATP, ADP, and AMP, adenosine tri-, di-, and monophosphate; BisTris, 2,2-bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol; BSA, bis(trimethylsilyl)acetamide; 5-deazaFAD, 5-deazaflavin adenine dinucleotide; 5-deazaFMN and 5-deazaFMNH$_2$, oxidized and reduced forms of 5-deazaflavin mononucleotide; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DDQ, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone; DTT, dithiothreitol; EPSP, 5-enolpyruvylshikimate 3-phosphate; FMN, flavin mononucleotide; *iso*-EPSP, 5(*R*)-(1-carboxyethenyloxy)-4(*R*)-hydroxy-1(*R*)-phosphoryloxy-2-cyclohexenecarboxylate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high pressure liquid chromatography; mCPBA, *m*-chloroperoxybenzoic acid; NADPH, reduced form of nicotinamide adenine dinucleotide phosphate; NMR, nuclear magnetic resonance; NPE, 2-(*p*-nitrophenyl)ethyl; S-3-P, shikimate 3-phosphate; TAPS, 3-[[tris(hydroxymethyl)methyl]amino]-1-propanesulfonic acid; THF, tetrahydrofuran; TMSBr, trimethylsilyl bromide; Tris, tris(hydroxymethyl)aminomethane; *p*TsOH, *p*-toluenesulfonic acid.

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from organism to organism. The enzymes from *N. crassa* (Welch et al., 1974; Cole et al., 1975) and *Bacillus subtilis* (Hasan & Nester, 1978) are bifunctional, with covalently attached or associated flavin reductases, respectively, that utilize NADPH to produce reduced flavin. In contrast, the chorismate synthases from *E. coli* (Morell et al., 1967), *Pisum sativum* (Mousdale & Coggins, 1986), and *Corydalis sempervirens* (Schaller et al., 1990) are monofunctional and must be supplied with exogenous reduced flavin under anaerobic conditions. Single turnover experiments with recombinant enzyme from *E. coli* have shown that a unique, enzyme-bound flavin species accumulates during turnover (Ramjee et al., 1991). Recently, a stabilized enzyme-bound flavin semiquinone has been observed when enzyme from *E. coli* is incubated with the competitive inhibitor (6*R*)-F-EPSP (Ramjee et al., 1992). Thus, it is likely that reduced flavin is directly involved chemically in the conversion of EPSP to chorismate.

A significant deuterium kinetic isotope effect has been demonstrated in the enzymatic reaction of (6*R*)-EPSP-*d* (Balasubramanian et al., 1991). The primary isotope effect on V ($^{D}V = 2.7$) at C-6*R* is diminished for V/K [$^{D}(V/K) = 1.6$]. This reduction reflects a significant forward commitment for EPSP, with other, isotopically insensitive, steps partially rate-determining at limiting substrate. A standard approach for increasing the kinetic significance of the chemical step in an enzyme-catalyzed reaction is to use alternative substrates with V/K values lower than that of the natural substrate (Northrup, 1978). Such analogs should be processed with a chemical step that is more rate-determining, due either to an increased rate of ligand dissociation or to a slower chemical step. To address this issue and to provide mechanistic probes for the chorismate synthase reaction, we prepared EPSP analogs **4**–**8** (Table 1) and evaluated them as substrates with chorismate synthase from *N. crassa*.

MATERIALS AND METHODS

Materials. Chorismate synthase from *N. crassa* was a generous gift from Prof. C. Abell (University of Cambridge). 5-Deazariboflavin was a gift from Prof. C. T. Walsh (Harvard Medical School), and FAD synthetase (bifunctional enzyme from *Brevibacterium ammoniagenes*) was a gift of Prof. V. Massey (University of Michigan). Shikimate kinase from *E. coli* (crude extract) and EPSP synthase (recombinant from *Petunia hybrida*) were generous gifts from Dr. J. Sikorski and Dr. G. Kishore (Monsanto). Apyrase (grade VII) and alkaline phosphatase (calf intestinal), FMN, and NADPH were obtained from Sigma and used without further purification. 5-DeazaFMN was prepared by *Naja naja* phosphodiesterase I treatment of 5-deazaFAD, prepared by the literature method (Fisher et al., 1976) and purified by reverse phase HPLC (Marletta & Walsh, 1980). Substrate analogs **4**–**7** were prepared as previously described (Alberg et al., 1992) and were judged homogeneous by ^{1}H and ^{31}P NMR and anion exchange HPLC (Mono Q, Pharmacia). NMR spectra were recorded in the indicated solvent on Bruker AM-400, AMX-400, or AM-500 instruments.

Synthesis of (−)-(3R)-EPSP-d. (A) (−)-3-Dehydroshikimic Acid (DHS). This compound was prepared using a modification of the literature procedure (McKittridge & Ganem, 1985). To a slurry of 1.4 g (8.1 mmol) of (−)-shikimic acid in 80 mL of THF was added 1.85 g (8.1 mmol) of 2,6-dichloro-3,5-dicyanoquinone (DDQ), and the mixture was refluxed overnight. After 12 h, an additional 1.85 g of DDQ was added, and after 5 h more, 0.92 g of DDQ was added and the mixture was refluxed for a total of 36 h. The solvent was removed under reduced pressure, and the residue was partitioned between 50 mL each of ether and H_2O. The aqueous layer was removed, and the organic layer was extracted with three 50-mL portions of H_2O. The aqueous extracts were combined and diluted to 400 mL, and the pH was adjusted to 7.5 with 1 N NaOH. The mixture was then applied to an anion exchange column (Dowex AG-1 X4, formate form), which was washed with 200 mL of H_2O and eluted with a linear gradient (500 mL) of 0−6 N formic acid. Fractions absorbing at 240 nm (diluted 100-fold due to formate absorbance) were combined and lyophilized to give 640 mg (46%) of 3-dehydroshikimic acid as a white fluffy solid. The purified material showed no contamination by shikimic acid in the ^{1}H NMR spectrum. ^{1}H NMR (D_2O) δ 6.64 (d, 1, J = 3.4, H-2), 4.16 (d, 1, J = 11.0, H-4), 3.89 (m, 1, H-5), 3.05 (dd, 1, J = 5.4, 18.2, H-6), 2.52 (ddd, 1, J = 3.4, 10.0, 18.2, H-6′). ^{13}C NMR (D_2O) δ 204.0, 172.7, 151.6, 132.9, 80.6, 72.9, 35.4.

(B) (−)-(3R,S)-Shikimic Acid-d. To 8 mL of cold acetic acid-d_4 was added 10 mg (0.23 mmol) of sodium borodeuteride (Aldrich, 98 atom % *d*) in small portions. The mixture was allowed to warm to 21 °C while stirring for 3 min, and 50 mg (0.29 mmol) of 3-dehydroshikimic acid was added in one portion. After stirring for 48 h, the solvent was removed by lyophilization, and the residue was dissolved in 100 mL of water and neutralized with NaOH. This solution was then loaded onto an anion exchange column (Dowex AG-1 X2 formate form), which was eluted with a linear gradient of 0−6 N formic acid (400 mL total volume). Fractions absorbing at 240 nm (10-μL aliquots diluted to 1 mL) were pooled and lyophilized to give 43 mg (85%) of a 60:40 *R*:*S* ratio of shikimic acid-*3-d*. Although it was possible to achieve a limited separation of the diastereomers, the mixture was carried on without further purification.

(C) (−)-(3R)-EPSP-d. The conversion of deuterated shikimic acid to shikimate 3-phosphate (S-3-P) was achieved essentially according to the reported procedure (Wibbenmeyer et al., 1988) using a crude extract of shikimate kinase from *E. coli*. The reaction mixture contained 100 mM HEPES, pH 7.5, 50 mM KCl, 10 mM DTT, 10% v/v glycerol, 0.2 mM $NaWO_4$, 20 mM shikimic acid, 40 mM ATP, and 200 μL of kinase extract, in a total volume of 5 mL. The reaction was followed by HPLC on a Mono Q 5/5 anion exchange column using a linear gradient (flow rate = 1 mL/min, gradient time = 10 min) of 0−1 M triethylammonium bicarbonate, pH 8.2, monitoring at 240 nm. Aliquots of the reaction mixture were treated with apyrase (Sigma, grade VII) prior to injection, in order to degrade ATP and ADP to AMP, which enabled separation of the S-3-P peak (Balasubramanian & Abell, 1991). The total reaction time was 48 h. S-3-P isolated from multiple injections on a Mono Q 10/10 column was concentrated by coevaporation of the solvent with methanol to give the pure triethylammonium salt by ^{1}H NMR.

Conversion of S-3-P to EPSP was achieved using EPSP synthase from *P. hybrida*. The reaction mixture typically contained 50 mM Tris-HCl, pH 7.5, 15 mM S-3-P, 30 mM PEP, and 2.5 milliunits of EPSP synthase. The reaction was

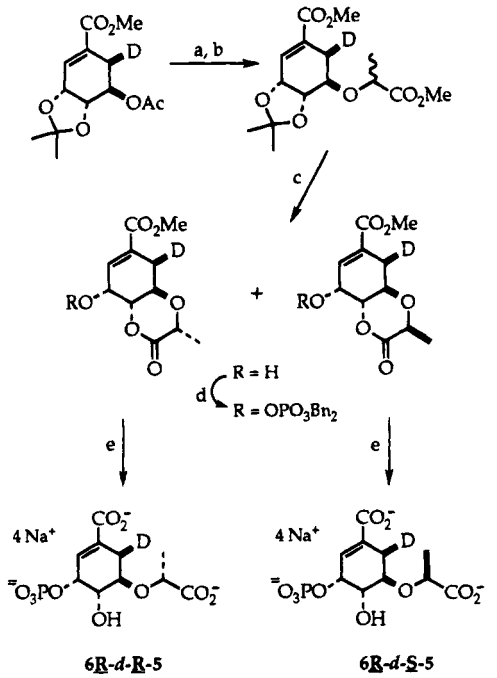

FIGURE 2: Synthesis of 6(*R*)-deuterio (*R*)- and (*S*)-dihydro analogs of EPSP, (6*R*)-(*R*)-**5**-*d* and (6*R*)-(*S*)-**5**-*d*. (a) K_2CO_3, MeOH, 100%; (b) N_2CHCO_2Et, $Rh(OAc)_2$, benzene, 86%; (c) Dowex 50W-X8, MeOH, 60 °C; *p*TsOH, benzene, Δ, 81%; (d) $iPr_2NP(OBn)_2$, 1*H*-tetrazole, THF; mCPBA, CH_2Cl_2; (e) TMSBr, pyridine, $CHCl_3$; aq NaOH, 47−65%; see Alberg et al. (1992).

complete in 8 h. The reaction mixture was treated with excess apyrase, and the EPSP was isolated as described above for S-3-P. The triethylammonium salt of EPSP was converted to the sodium salt by passage through a short column of Dowex cation exchange resin (Na^+ form). All of the isotopically labeled EPSP analogs were judged >95% pure by ^1H and ^{31}P NMR and by anion exchange HPLC.

Synthesis of (−)-(6R)-EPSP-d. This was prepared from (±)-(6*R*)-shikimic acid-*d* according to the previously reported method (Balasubramanian et al., 1991). (−)-(6*R*)-Shikimic acid-*d* was prepared as described previously (Bartlett et al., 1988) with modifications. Methyl *cis*-3-deuterioacrylate was prepared from methyl 3-deuteriopropiolate by the literature method (Hill & Newkome, 1969). The deuterated propiolate was prepared by the literature method for the corresponding ethyl ester (Cristol & Noreen, 1976).

Synthesis of (6R,8R)-8,9-Dihydro-EPSP-d [(6R)-(R)-5-d] and the 8S Isomer [(6R)-(S)-5-d]. These were prepared from (−)-(6*R*)-shikimic acid-*d* as shown in Figure 2 [see Alberg et al. (1992)].

Preparation of Phosphorothioate Analog **8**. *(A) Methyl 6(R)-[4aR-(4aβ,8α,8aα)]-2,3,4a,5,8,8a-Hexahydro-2-oxo-3-methylene-8-[[bis[2-(4-nitrophenyl)ethoxy]thiophosphinyl]-oxy]-4-benzodioxin-6-carboxylate-d (***10***).* A mixture of 50 mg (0.21 mmol) of hydroxy lactone **9** (Alberg et al., 1992), 120 mg (0.26 mmol) of *N,N*-diisopropylbis(*p*-nitrophenethyl)phosphoramidite (Uhlmann & Engels, 1986), 44 mg (0.63 mmol) of 1*H*-tetrazole, and 20 mg of 4 Å molecular sieves in 1 mL of THF was stirred under argon for 1 h; 2 mL of pyridine was then added via syringe, followed by 77 mg of elemental sulfur. The resulting mixture was stirred for 2.5 h, at which time ^{31}P NMR showed two peaks at δ 67 and 56 ppm in an 85:15 ratio, respectively, with no evidence of phosphite species at >100 ppm. The mixture was filtered through a plug of glass wool into 10 mL of ethyl acetate, the resulting solution was washed with saturated $NaHCO_3$ and dried ($MgSO_4$), and the solvent was removed to give a light yellow solid. The crude product was purified by chromatography on a short column of silica using CH_2Cl_2 as eluant to give 234 mg (89%) of phosphorothioate **10** as a pale yellow, hygroscopic solid. IR ($CHCl_3$) 3010 (s), 1740, 1730, 1605, 1520 (s), 1450, 1350, 1315, 1255, 1210 (s), 1020, 975, 750 (s), 670 cm^{-1}. ^1H NMR ($CDCl_3$) δ 8.14 (d, 4, J = 8.7, Ar-H), 7.38 (dd, 4, J = 8.7, 12.5, ArH), 6.75 (dd, 1, J = 2.4, 5.5, H-2), 5.71 (d, 1, J = 1.4, H-9), 5.28 (m, 1, H-3), 5.13 (d, 1, J = 1.4, H-9), 4.47 (ddd, 1, J = 1.4, 4.1, 10.6, H-5), 4.16−4.38 (m, 5, H-4 and OCH_2), 3.81 (s, 3, OCH_3), 3.15 (dd, 1, J = 6.4, 18.4, H-6), 3.07 (m, 4, CH_2Ar), 2.37 (ddd, 1, J = 2.8, 9.5, 18.4, H-6′). ^{13}C NMR ($CDCl_3$) δ 165.1, 158.3, 146.8 (d, J = 5.3), 146.0, 144.9, 144.7, 132.7, 130.5 (d, J = 2.3), 129.9, 129.8, 129.3, 123.5, 105.3, 76.7 (d, J = 5.9), 69.6 (d, J = 4.6), 67.9 (d, J = 4.7), 67.7, 67.4 (d, J = 4.9), 52.5, 35.9, 35.8, 30.0. ^{31}P NMR ($CDCl_3$) δ 68.6. Anal. Calcd for $C_{27}H_{27}N_2O_{12}PS$: C, 51.11; H, 4.29; N, 4.41; P, 4.88; S, 5.05. Found: C, 51.13; H, 4.14; N, 4.35; P, 5.35; S, 5.02.

*(B) (−)-5-Enolpyruvylshikimate 3-Phosphorothioate (***8***).* A solution of 100 mg (0.16 mmol) of **10** in 2 mL of dry acetonitrile was transferred via cannula under argon to an NMR tube equipped with a septum. To this mixture was added 117 μL (0.48 mmol) of *N,O*-bis(trimethylsilyl)-acetamide (BSA) followed by 71 μL (0.48 mmol) of DBU via syringe, and the reaction was followed by ^{31}P NMR. After 3 h, the mixture was partitioned between 5 mL of H_2O and 5 mL of $CHCl_3$ at 0 °C. The aqueous layer was removed, and the organic layer was extracted with two 1-mL portions of cold H_2O. The combined aqueous layer was made basic by the addition of 400 μL of 1 N NaOH, and the mixture was allowed to stand at 0 °C for 1 h. The mixture was then diluted to 50 mL and loaded onto an anion exchange column (DEAE Sephadex A25−120), which was eluted with a linear gradient of 0−0.8 M triethylammonium bicarbonate, pH 8.2. Fractions absorbing at 240 nm were combined, and the solvent was coevaporated with methanol to give the tetra-(triethylammonium) salt. This material was passed through a short column of cation exchange resin (Dowex, X8, Na^+ form) to give 55 mg (82%) of the tetrasodium salt of **8**. ^1H NMR (D_2O) δ 6.51 (dd, 1, J = 2.1, 2.1, H-2), 5.16 (d, 1, J = 2.5, H-9), 5.02 (m, 1, H-3), 4.68 (d, 1, J = 2.5, H-9′), 4.42 (m, 1, H-5), 4.09 (dd, 1, J = 4.2, 8.4, H-4), 2.89 (dd, 1, J = 18.2, H-6), 2.21 (dd, 1, J = 6.5, 18.2, H-6′). ^{13}C NMR (D_2O) δ 175.9, 172.0, 154.6, 136.1, 131.5 (d, J = 3.5), 94.0, 74.3, 71.0, 70.3, 29.5. ^{31}P NMR (D_2O) δ 47.2.

Determination of Substrate and Inhibitor Concentrations. The concentrations of EPSP and analog **8** were determined by acid hydrolysis (0.1 N HCl, 90 °C, 1 h) and end-point analysis of the pyruvate released using lactate dehydrogenase and NADH. The concentrations of analogs **4**−**7** were determined by treatment with alkaline phosphatase and quantitation of the phosphate released using a malachite green assay (Lanzetta et al., 1979). The values from the phosphate release assay were compared to the value obtained for a known amount of EPSP using the same method, as well as to a standard curve with inorganic phosphate.

Enzyme Assays: General. The determination of kinetic constants for EPSP and substrate analogs and the measurement of isotope effects were carried out under the following

conditions. Assays were performed at 25 °C using a UVIKON 860 spectrophotometer with 1-cm path length cells. The assay mixture contained 50 mM BisTris, pH 7.0, 50 mM KCl, 2.5 mM $MgCl_2$, 20 μM NADPH, 10 μM FMN, and varying amounts of substrate or inhibitor and enzyme in a total volume of 1 mL. Chorismate production was followed at 274 nm using a $\Delta\epsilon_{274}$ of 2600 M^{-1} cm^{-1}. The $\Delta\epsilon_{274}$ values for reaction of substrate analogs **4** and (*R*)-**5** were measured and found to be essentially identical to that for the conversion of EPSP to chorismate. Individual rates were measured by analysis of the data with the program ENZFITTER (Leatherbarrow, 1987), using the linear portion of the first 10−15% of the reaction. Kinetic parameters for substrates were obtained by fitting data to eqs 1 and 2 using the HYPER and SUBIN programs, respectively (Cleland, 1979). Inhibition constants were obtained by fitting data to the COMP or NCOMP programs of Cleland (eqs 3 and 4, respectively; Cleland, 1979). The best fit was determined by the lowest σ value with each equation.

$$v = \frac{VS}{K + S} \quad (1)$$

$$v = \frac{VS}{K + S(1 + S/K_{ses})} \quad (2)$$

$$v = \frac{VS}{K(1 + I/K_i) + S} \quad (3)$$

$$v = \frac{VS}{K(1 + I/K_{is}) + S(1 + I/K_{ii})} \quad (4)$$

Determination of Isotope Effects for Labeled EPSP and Dihydro Analogs. These measurements were carried out by direct comparison of initial rates for the labeled and unlabeled substrates. Reaction mixtures were prepared as described in the general section except for the determination of isotope effects on labeled EPSP, in which a fixed concentration (60−100 μM) of the competitive inhibitor *iso*-EPSP was added to raise K_m values to conveniently measured levels (V_{app} = 10−20 μM). Data were fit to eq 5 using the ISOVKV program of Cleland, which assumes independent isotope effects on *V* and *V/K* (Cleland, 1978). The term F_i represents the fractional isotopic purity of the substrate, while E_V and $E_{V/K}$ are the isotope effects minus one on *V* and *V/K*, respectively. Because of observed substrate inhibition, substrate concentrations were kept below $10K_m$.

$$v = \frac{VS}{K(1 + F_i/E_{V/K}) + S(1 + F_i/E_V)} \quad (5)$$

*Determination of pH Profiles for EPSP and (R)-***5**. Reaction mixtures were prepared as described above, and the pH was measured using a glass pH electrode with the following buffers: BisTris (pH 6.0−7.5), Tris (pH 7.5−8.5), and TAPS (pH 8.5−9.5). The concentration of buffer was 50 mM. Data for determining *V* and *V/K* with (*R*)-**5** as substrate were fit to eq 2 using the SUBIN program of Cleland. Apparent pK_a values were obtained by fitting to eq 6.

$$\log Y = \log \frac{C}{1 + H/K_1 + H/K_2} \quad (6)$$

RESULTS

Preparation of Substrate Analogs **4**−**8**. The synthesis of analogs **4**−**7** has been described previously and follows the general strategy for the synthesis of EPSP from (−)-shikimic acid (Teng et al., 1985; Chouinard & Bartlett, 1985). The modified side chains are introduced via rhodium(II)-catalyzed insertion (Ganem et al., 1982) of α-diazoesters to give the corresponding ethers at C-5. Lactonization protects the hydroxyl at C-4 so that phosphorylation at C-3 can be achieved selectively, using phosphoramidite methodology (Beaucage & Carruthers, 1981; Perich & Johns, 1986) and oxidation to the phosphate. Deprotection of the phosphate followed by basic hydrolysis gives the substrate analogs as the tetrasodium salts after ion exchange chromatography.

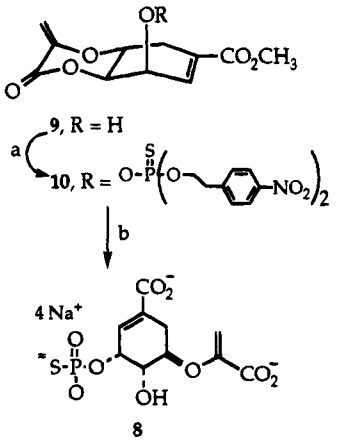

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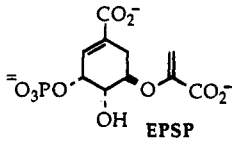
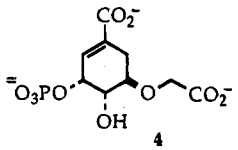
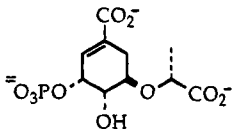
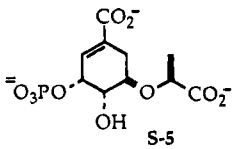
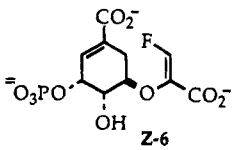
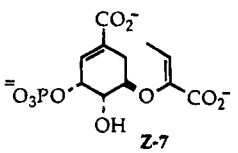
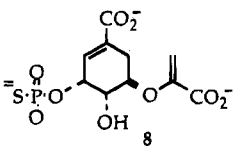
FIGURE 3: Synthesis of phosphorothioate analog **8**. (a) $iPr_2NP(OpNPE)_2$, 1*H*-tetrazole, THF; S_8, pyridine, 89%; (b) DBU, BSA, CD_3CN; aq NaOH, 82%.

The synthesis of phosphorothioate **8** (Figure 3) is achieved by phosphitylation of known hydroxy lactone **9** (Chouinard & Bartlett, 1986) followed by oxidation of the bis(4-nitrophenethyl) (NPE) phosphite (Uhlmann & Pfleiderer, 1981) with elemental sulfur (Burgers & Eckstein, 1978; Wu & Orgel, 1991). Deprotection of the NPE groups using DBU with *N,O*-bis(trimethylsilyl)acetamide (BSA) (Alberg et al., 1992) gives the bis(trimethylsilyl) phosphorothioate, which is hydrolyzed with aqueous NaOH to give analog **8** in 65% yield from **9**.

Characterization of Substrate Analogs. Table 1 shows the kinetically derived parameters for EPSP analogs **4**−**8** with the bifunctional chorismate synthase from *N. crassa*. The higher K_m values for the alternative substrates **4** and **5** allowed the course of the reaction to be monitored directly at 274 nm, as a result of formation of the cyclohexadiene chromophore ($\Delta\epsilon$ = 2600 M^{-1} cm^{-1}). Because fluoro analog (*Z*)-**6** is a relatively poor substrate (*V* = 2% of EPSP by direct measurement at 274 nm), K_m determination was not practical using this method. In this case, a coupled assay was used with antranilate synthase (AS) and the product anthranilate was monitored fluorimetrically (Zalkin & Hwang, 1971). Successful use of this assay implies that the initial product, (*Z*)-9-fluorochorismate, is a good substrate for anthranilate synthase (Figure 4). In the coupled reaction with (*Z*)-**6** using the same amount of coupling enzyme (AS) as in reactions with EPSP, no lag in product formation was observed. The corresponding chorismate analogs of **4** and **5** have been shown previously to be substrates of anthranilate synthase (Walsh et al., 1987). In contrast to (*Z*)-**6**, these analogs could not be used in the coupled assay because they required large amounts of anthranilate synthase to overcome a significant lag period. The fact that the reduced analogs of chorismate are processed by AS less efficiently than (*Z*)-**6**

Table 1: EPSP Analogs as Alternative Substrates of the Chorismate Synthase from *N. crassa*[a]

	K_m (μM)	$(V)_{rel}$	$(V/K)_{rel}$
 EPSP	2.7	1.00	1.00
 4	6.6	0.52	0.22
 R-5	17.5	1.18	0.18
 S-5	162	0.08	0.0013
 Z-6	12	0.02	0.0045
 Z-7	K_i = 234	<0.005	
 8	K_i = 0.6	<0.005	

[a] Rates are relative to EPSP; errors in K_m and V_{rel} are ±10%.

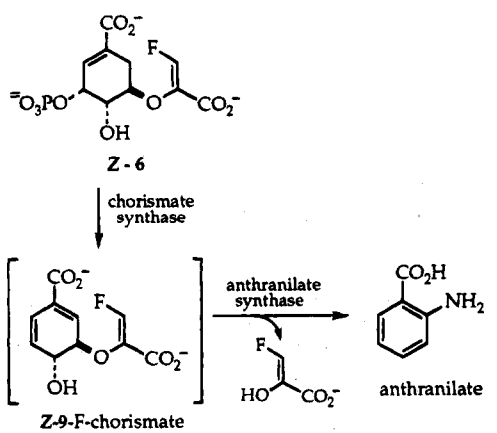


FIGURE 4: Coupled assay for measuring the activity of (*Z*)-**6** using anthranilate synthase; anthranilate is measured fluorimetrically.

is not surprising, in view of the fact that fluoropyruvate is a better leaving group than glycolate, lactate, or pyruvate itself, and since elimination of the side chain is thought to be the rate-determining step for the AS reaction with the lactyl and glycolyl analogs of chorismate (Walsh et al., 1987).

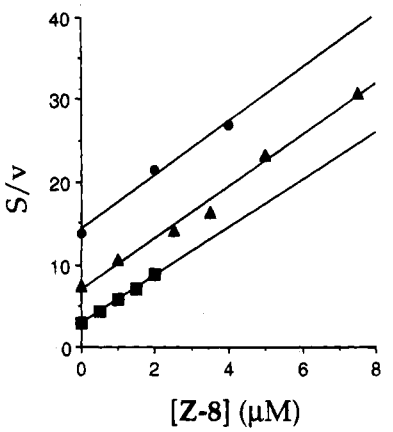


FIGURE 5: Cornish-Bowden plot of inhibition of chorismate synthase by **8**, under conditions in which total ligand concentration [S + I] is less than 25 μM. [EPSP] = 3 μM (●), 10 μM (▲), and 20 μM (■).

Glycolyl and (*R*)-lactyl analogs **4** and (*R*)-**5** are good alternative substrates for chorismate synthase, with *V/K* values that are 20% of EPSP itself. The enzyme shows signficant discrimination in *V/K* between the two lactyl isomers by over 2 orders of magnitude, indicating that proper orientation of the side chain in the active site is important for catalysis. Such steric requirements are even more evident for substitution at the *Z*-position of the enolpyruvyl methylene carbon. The fluorine substituent in (*Z*)-**6** causes nearly a 5-fold drop in binding affinity (from K_i = 13 μM) and a 50-fold drop in V_{max}, while substitution of a methyl group in this position (data not shown) drops activity below an observable level (V_{rel} <0.5%, K_i ~250 μM). Significantly, the (*R*)-lactyl analog (*R*)-**5** is actually turned over faster than EPSP under saturating conditions. This rate effect could be due to faster product release in the reaction with (*R*)-**5**, while for the normal substrate EPSP, release of chorismate may be partially rate-limiting at high substrate concentration.

The phosphorothioate analog **8** does not show detectable substrate activity for chorismate synthase (V_{rel} <0.5%), but it binds tightly to the enzyme with a K_i value 4-fold lower than the K_m value for EPSP. The inhibition is competitive at concentrations below 20 μM, but becomes mixed at higher ligand concentrations, perhaps due to binding with the ES complex. A Cornish-Bowden plot for inhibition by **8** shows curvature at total ligand concentration (S + I) over 25 μM, although at lower concentrations the lines appear to be parallel, indicating competitive inhibition (Figure 5). This kinetic behavior is consistent with apparent substrate inhibition at concentrations greater than $10K_m$ (see below), indicating that a second substrate or inhibitor molecule may bind to the initial ES or EI complex. There was no evidence of irreversible inhibition by this analog.

Substrate Inhibition. Inhibition by excess substrate is a general phenomenon and has been examined in a number of enzyme-catalyzed reactions [for some recent investigations, see Poncz (1988), Schullek and Wilson (1989), and Gruys et al. (1993)]. We have found that chorismate synthase from *N. crassa* is significantly inhibited by its substrate EPSP at concentrations greater than 10 times the K_m value. Plots of *v* vs *S* for EPSP and alternative substrates **4** and (*R*)-**5** are shown in Figure 6, and show significant deviation from normal hyperbolic behavior at these substrate

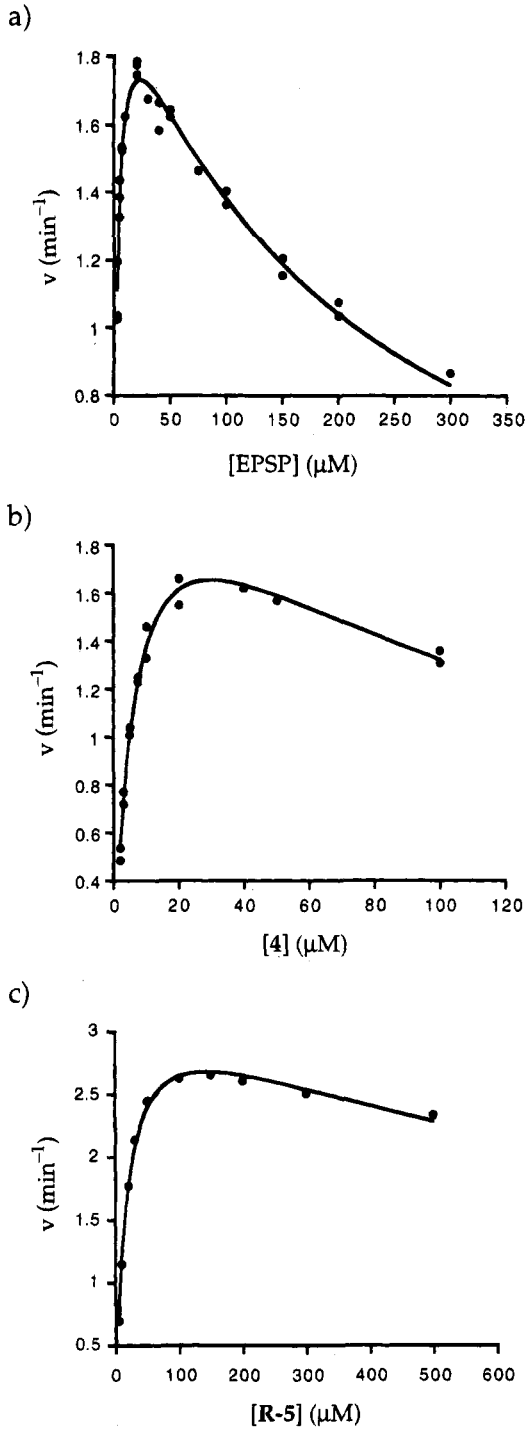
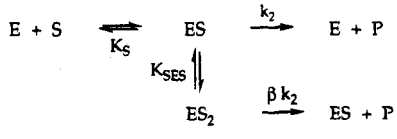


FIGURE 6: Plot of V vs S for (a) EPSP, (b) **4**, and (c) (*R*)-**5**. The curves were generated by fitting the data to eq 2.

Scheme 1



concentrations. The simplest case of substrate inhibition can be described by a noncompetitive model, involving the formation of a catalytically incompetent, ternary ES_2 complex (Scheme 1, $\beta = 0$). In this model, the substrate can act as an inhibitor only by binding to the productive ES complex. Kinetic data were fit to both the normal hyperbolic equation (eq 1) and an equation that describes substrate inhibition of

Table 2: Kinetically Determined Parameters for Substrate Inhibition for Chorismate Synthase from *N. crassa*[a]

substrate	K_m (μM)	S_m (μM)	K_{ses} (μM)
EPSP	2.7	20	190
4	6.6	30	136
(*R*)-**5**	17.5	150	1180

[a] Calculated using eq 2. Errors in K_m and S_m are ±10%. Errors in K_{ses} are ±15%.

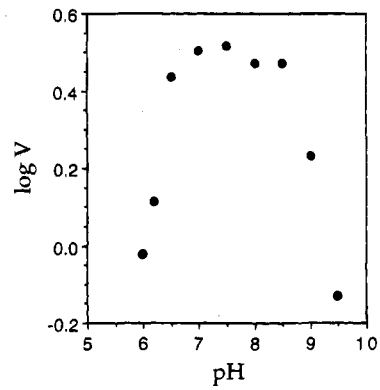


FIGURE 7: pH profile for EPSP as substrate with chorismate synthase at constant [S] = 20 μM.

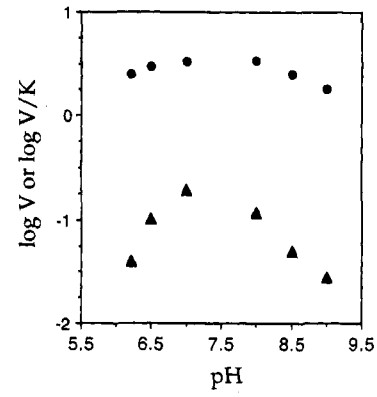


FIGURE 8: pH profile for V (●) and V/K (▲) for the (*R*)-dihydro analog of EPSP, (*R*)-**5**, with chorismate synthase (V and V/K calculated using eq 2).

the type shown by Scheme 1 (eq 2). Data for EPSP and analogs **4** and (*R*)-**5** showed significantly better agreement with eq 2. Table 2 shows the values for the optimal substrate concentration (S_m) and for the binding constant (K_{ses}) of the second substrate molecule to ES. For EPSP and (*R*)-lactyl analog (*R*)-**5**, the values for S_m and K_{ses} are larger than K_m by approximately the same degree (ca. 70-fold); in contrast, for the sterically smaller glycolate, these values are closer to the K_m value. With (*R*)-**5**, substrate inhibition was observed throughout the pH range of 6.2−9.0. The effect of ionic strength was not investigated.

pH Studies. The pH profile was determined for the chorismate synthase reaction with both EPSP and (*R*)-**5**. Only the V profile could be measured for EPSP because of the low K_m value of this substrate; V was estimated at a single concentration of [S] = S_m (20 μM). Both the V and V/K profiles could be measured for (*R*)-**5**, and a plot of S_m rate vs pH gave a profile similar to that calculated for V (using eq 2). Therefore, it is likely that the V profile for (*R*)-**5** reflects qualitatively the shape of the actual V−pH profile for EPSP. Both of the V plots (Figures 7 and 8) show a bell shape with slopes lower in magnitude than +1 and −1 for

Table 3: Isotope Effects for EPSP and Alternative Substrates with Chorismate Synthase from *N. crassa* Determined at pH 7.0 (BisTris)

substrate	^{D}V	$^{D}(V/K)$
(6*R*)-EPSP-*d* (1°)	2.64 ± 0.02	1.22 ± 0.04
EPSP-*3-d* (2°)	1.10 ± 0.02	0.97 ± 0.02
(6*R*)-(*R*)-**5**-*d*	3.8 ± 0.4	1.32 ± 0.02
	3.6 ± 0.1[a]	1.43 ± 0.09[a]
(6*R*)-(*S*)-**5**-*d*	12.6 ± 1.0[b]	nd[c]

[a] Determined at pH 8.5 (Tricine), one determination. [b] Estimated at substrate concentration of 0.5 mM ($K_{m,protio}$ = 162 μM). [c] Not determined.

the acidic and basic arms of the curve, respectively. When the data for EPSP are fit to eq 6 (see Materials and Methods section), apparent pK_a values of 6.44 ± 0.07 and 8.86 ± 0.06 for the two ionizable groups are obtained. The *V/K* curve for (*R*)-**5** suggests that there are two ionizable groups involved in substrate binding and catalysis. By conducting measurements at a pH away from the optimum, it may be possible to measure increased isotope effects in *V/K*, if the suppression of these effects has an external (ligand binding) component. There was no evidence of enzyme inactivation in the pH range examined, or of a significant effect of the buffers on reaction rate.

*Isotope Effects with EPSP and Substrate Analogs (R)-***5** *and (S)-***5**. (−)-(6*R*)-EPSP-*d* was prepared according to previous methods with modifications (see Materials and Methods section) to give a level of 95% deuteration, as measured by ^{1}H NMR. (−)-EPSP-*3-d* was prepared by reduction of 3-dehydroshikimate (Bartlett et al., 1988; McKittridge & Ganem, 1985). Sodium triacetoxyborodeuteride (Saksena & Mangiaracina, 1983; Evans et al., 1988) was used for this reduction because it gave significantly higher selectivity, leading to 60% of the natural isomer of shikimate, while sodium borohydride only gives 22% of the desired product. A possible explanation for this selectivity stems from the known coordinating ability of acetoxyborohydride with 1,3-keto alcohols, to give *syn*-1,3-diols. The 60:40 *R*:*S* mixture was carried on using the enzymes shikimate kinase and EPSP synthase (Balasubramanian & Abell, 1991) to give only the natural isomer of (−)-EPSP-*3-d*, as determined by ^{1}H NMR. (−)-3,6*R*-d_2-labeled EPSP was also prepared by DDQ oxidation of (±)-(6*R*)-shikimic acid-*d* and reduction with sodium (triacetoxy)borodeuteride.

Deuterium kinetic isotope effects (Table 3) were measured with chorismate synthase by direct comparison with EPSP by following 1,4-diene formation directly at 274 nm. Because of the low K_m value of EPSP, we used a known competitive inhibitor, *iso*-EPSP (Maitra et al., 1986), to raise the apparent value of K_m. This method has been used successfully by Stone and Morrison in their studies with dihydrofolate reductase (Stone & Morrison, 1991). The concentration of *iso*-EPSP does not effect the isotope effect determined for *V/K*, since the observed K_m is increased for both the labeled and nonlabeled substrates by the same factor (1 + I/K_i), and since *V* is unchanged.

There is a significant secondary isotope effect at saturating substrate, as indicated by ^{D}V = 1.10. However, the large, forward commitment for EPSP obscures this effect at low substrate concentration, giving $^{D}(V/K)$ = 0.97; this observation is consistent with the diminished value of $^{D}(V/K)$ at C-6. This commitment may have an external component, signifying that EPSP is a sticky substrate, or it may reflect a large internal commitment, such as a rate-limiting conformational change. Attempts were made to measure the effects of double labeling at both C-6*R* and C-3, but the results are inconclusive for $^{D}(V/K)$ due to the kinetic insignificance of the secondary effect at C-3. As mentioned above, the use of substrate analogs with lower *V/K* values may reveal suppressed isotope effects by increasing the rates of substrate association and product dissociation steps. Because the lactyl analogs (*R*)-**5** and (*S*)-**5** have widely different kinetic properties with chorismate synthase from *N. crassa*, we chose to prepare these analogs labeled with deuterium at the 6*R*-position.

The isotope effects for the (*R*)- and (*S*)-lactyl analogs were measured by direct comparison using the UV assay at 274 nm, with data fit to the Fortran program ISOVKV (Cleland, 1978), which describes the isotope effects on *V* and *V/K*. The results shown in Table 3 indicate that the isotope effects for the analogs are significantly larger than for EPSP. The (*R*)-dihydro analog (*R*)-**5** displays a larger ^{D}V than EPSP, which may indicate that product release is partially rate-limiting for the reaction with the normal substrate EPSP. This conclusion is supported by the fact that *V* for (*R*)-**5** is also larger than that for EPSP. However, the $^{D}(V/K)$ value remains significantly suppressed, indicating either that this substrate remains sticky or that there is a significant internal commitment for both substrates. Initial results indicate that $^{D}(V/K)$ increases as the pH is increased, while the effect on *V* is unchanged. Under these conditions, the value of K_m for the protio substrate increases 2-fold to 30 μM. Together, these results suggest that the (*R*)-dihydro analog is a sticky substrate and may indicate that EPSP is also sticky.

The isotope effects for the (*S*)-dihydro analog (*S*)-**5** were difficult to measure due to the low activity of this compound as a substrate and the apparent large isotope effect. Rough estimation of the isotope effect on *V* at a substrate concentration of 0.5 mM (three times K_m) gives a value of 12.6. If the intrinsic isotope effect is not significantly different for the dihydro analogs, the large effect observed for the *S*-isomer indicates that the isotope effects for EPSP are severely depressed by other rate-limiting steps. There is ample precedent for suppression of this magnitude. For example, the reaction catalyzed by *p*-hydroxybenzoate hydroxylase shows an observed ^{D}V = 2.5, while the intrinsic isotope effect is 10 ± 2 (Ryerson et al., 1982).

Use of 5-DeazaFMN/5-DeazaFMNH$_2$ as Cofactor Analog. Both 5-deazaFAD and 5-deazaFMN were prepared enzymatically as described above and purified by reverse phase HPLC. The reduced analogs were prepared by adding a small amount of borohydride, quenching with acetone, and adjusting the pH of the solution to ~5 with acetic acid (Spencer et al., 1976). UV spectra were identical to the previously published spectra.

At concentrations up to 20 μM (K_m of FMN = 1 μM), 5-deazaFMNH$_2$ does not lead to detectable activity (V_{rel} <0.1%) for the chorismate synthase reaction with enzyme from *N. crassa*. Activity was determined spectrophotometrically at 274 nm with EPSP (20 μM) as substrate. This result is consistent with previous work, which has shown that reduced flavin is involved chemically in the chorismate synthase reaction (Ramjee et al., 1991), and it is inconsistent with the hypothesis that binding of the cofactor alone is sufficient for turnover. In addition, oxidized 5-deazaFMN does not produce detectable activity for formation of chorismate by this enzyme. Utilization of the oxidized form of

these analogs requires reduction by the flavin reductase using NADPH as reducing agent, which may occur in this case, but is likely to be much slower than the reaction with FMN. Even with prolonged preincubation (>1 h), there was no evidence of chorismate production.

The measurement of a binding constant for the reduced 5-deaza analogs to the synthase binding site did not prove to be possible by direct competition experiments with FMN, because of rapid oxidation of the reduced deaza analogs by the natural flavins (FMN acts catalytically to transfer electrons from 5-deazaFMNH$_2$ to O$_2$). We found, however, that oxidized 5-deazaFMN is a good inhibitor of chorismate synthesis by the enzyme from *N. crassa*. At an FMN concentration of 10 μM (10-fold higher than the reported K_m value), the I_{50} value for 5-deazaFMN is 4 μM. Comparison of reaction rates in the presence of an excess of either FMN or 5-deazaFMN indicates that the deaza analog is better able to inhibit the coupled reaction. This inhibition may reflect tighter binding of the oxidized deaza analog relative to FMN for the FMNH$_2$ binding site of the synthase domain, the binding affinity of which is unknown.

DISCUSSION

Substrate analogs of EPSP were prepared to provide structural and mechanistic probes for the enzyme that catalyzes the unusual 1,4-elimination reaction leading to chorismic acid. Although the structural modifications are for the most part in the side chain of these compounds, remote from the site where bond cleavage takes place, we anticipated that they could serve as useful probes of the steric characteristics of the active site and that their kinetic behavior might provide valuable mechanistic information. The binding data reveal that the active site is closely contoured to the enolpyruvyl side chain, in spite of the peripheral location of this group. Substitution at the *Z*-position of the methylene carbon [(*Z*-**7**)], as well as at the *pro-S* position of the reduced analog [(*S*)-**5**], is deleterious for both binding and catalysis. Presumably, proper positioning of the side chain is required for orienting the substrate in the active site or inducing the reactive conformation of the cyclohexene ring for 1,4-elimination. On the other hand, the glycolyl and (*R*)-lactyl analogs [**4** and (*R*)-**5**] are good substrates for this enzyme, indicating that reduction in the size of the side chain, or introduction of steric bulk at the *R*-position of the ether carbon, has only a small effect on *V*/*K*.

Interestingly, the alterations that produce the greatest change in electronic characteristics, fluorine substitution on the side chain [(*Z*)-**6**] and sulfur incorporation into the phosphate leaving group (**8**), have modest effects on binding, but lead to significant reduction in reactivity. Both the enhanced binding affinity (4-fold) and the reduced reactivity of **8** (V_{rel} <0.5%) are readily rationalized; thiophosphate is more hydrophobic than phosphate, on the one hand, but larger and less readily activated by protonation, on the other. The 200-fold reduction in *V*/*K* for the fluorine analog (*Z*)-**6** is less easily explained.

The observation that the reduced analog (*R*)-**5** is turned over faster than EPSP under saturating conditions, in spite of weaker binding as a substrate, suggested that product release, rather than the chemical elimination step, is rate-limiting. Evidence in support of this interpretation was provided by measurement of the isotope effect on *V* from deuterium substitution at C-6. The larger effect found for (*R*)-**5** in comparison with EPSP under saturating conditions is strong evidence that a isotopically insensitive step is less rate-limiting with the analog than with the normal substrate. Dissociation of chorismate, for example, or a conformational change permitting its release, may be candidates for such a step. This result, together with the suppression of isotope effects on *V*/*K* relative to *V* for both EPSP and (*R*)-**5**, indicates that isotopically insensitive steps are partially rate-determining for both substrate binding and product release. The primary effect on *V*/*K* is larger for (*R*)-**5** than for EPSP, but it is still severely depressed relative to ^{D}V. The relative rates of isotopically insensitive steps before and after the chemical step may not be affected to the same extent by the steric change in (*R*)-**5**, which may simply reflect different orientations of the side chain in substrate and product complexes. Preliminary results indicate that isotope effects with the poorer substrate (*S*)-**5** are quite large for *V*. If these effects turn out to be equally expressed for *V*/*K*, then this analog will be valuable for measuring the intrinsic isotope effects of the reaction, as well as for use in multiple isotope experiments to determine the reaction mechanism.

Observation of a significant secondary deuterium isotope effect on *V* at C-3 with EPSP indicates that both C−H and C−O cleavage are partially rate-determining. The depression of the secondary effect on *V*/*K* again indicates a slow step prior to C−O cleavage and precludes analysis of reaction mechanism based on double isotope effects with doubly labeled EPSP. Thus, the substrate analogs used in this study may provide mechanistic information otherwise unattainable with the natural substrate.

In characterizing these derivatives kinetically, we observed significant substrate inhibition for all analogs, including EPSP. Since the compounds were purified to apparent homogeneity, in some instances by different methods, it is unlikely that this inhibition is due to the presence of a contaminant. Biosynthetic reactions that are essentially irreversible, as elimination to chorismate is likely to be, are prime targets for regulation (Stryer, 1988), and recent work with mutant bacterial strains indicates that chorismate synthase is one of the rate-limiting enzymes of the common pathway (Dell & Frost, 1993). Additional experiments are needed to determine the mechanism of substrate inhibition, and to determine if it has any *in vivo* significance.[2]

Finally, the inability of 5-deazaFMNH$_2$ to induce any observable activity in the *N. crassa* chorismate synthesis provides further support for an active role for flavin in this reaction. We have earlier proposed a radical process for this reaction based on the formation of a stabilized cation-radical intermediate (Bartlett et al., 1988), and the chemical viability of this mechanism has been demonstrated recently in a model

[2] Conceivably, substrate inhibition may act like a servo-mechanism: if transient buildup of chorismate (for example, as a result of feedback inhibition of the downstream enzymes) slows chorismate synthase, this inhibition could be enhanced and continued by the resulting buildup of EPSP. This admittedly speculative mechanism for regulation could be particularly appropriate when the product of the reaction is both unstable thermally and a branch point metabolite in high demand for a number of subsequent biosynthetic pathways, since allosteric binding sites for the different metabolites would be avoided. However, it is possible that this phenomenon is strictly an *in vitro* effect and that the enzyme is not normally exposed to concentrations of EPSP much greater than K_m.

system (Giese & Almstead, 1994). The recent observation of a flavin semiquinone in the presence of a unreactive substrate analog suggests that a radical species may indeed be involved in the enzymatic reaction, although the nature of this species remains unclear. It would be interesting to observe the effects of 1-deazaFMNH$_2$ in this case, since, unlike the 5-deaza derivative, this analog can readily form the semiquinone species by donation of an electron, albeit at a potential different than that of the natural flavin.

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REFERENCES

Alberg, D. G., Lauhon, C. T., Nyfeler, R., Fassler, A., & Bartlett, P. A. (1992) *J. Am. Chem. Soc. 114*, 3535−3546.

Anh, N. G. (1968) *J. Chem. Soc., Chem. Comm.*, 1089−1090.

Balasubramanian, S., & Abell, C. (1991) *Tetrahedron Lett. 32*, 963−966.

Balasubramanian, S., Davies, G. M., Coggins, J. R., & Abell, C. (1991) *J. Am. Chem. Soc. 113*, 8945−8946.

Bartlett, P. A., Maitra, U., & Chouinard, P. M. (1986) *J. Am. Chem. Soc. 108*, 8068−8071.

Bartlett, P. A., McLaren, K. L., Alberg, D. G., Fässler, A., Nyfeler, R., Lauhon, C. T., & Grissom, C. B. (1989) *Proc. Soc. Chem. Ind. Pesticides Group Meeting*, BCPC Monograph 42, pp 155−170, British Crop Protection Council, London.

Beaucage, S., & Caruthers, M. (1981) *Tetrahedron Lett. 37*, 3557.

Bentley, R. (1990) *Crit. Rev. Biochem. Mol. Biol. 25*, 6827−6835.

Burgers, P. M. J., & Eckstein, F. (1978) *Tetrahedron Lett.*, 3835−3838.

Cleland, W. W. (1978) in *Isotope Effects in Enzyme-Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., & Northrup, D. E., Eds.) pp 261−270, University Park Press, Baltimore.

Cleland, W. W. (1979) *Methods Enzymol. 63*, 103−138.

Chouinard, P. M., & Bartlett, P. A. (1986) *J. Org. Chem. 52*, 75−77.

Cole, K. W., & Gaertner, F. H. (1975) *Biochem. Biophys. Res. Commun. 67*, 170−175.

Dell, K. A., & Frost, J. W. (1993) *J. Am. Chem. Soc. 115*, 11581−11589.

Dewick, P. M. (1988) *Nat. Prod. Rep. 5*, 73−97.

Evans, D. A., Chapman, K. T., & Carreira, E. M. (1988) *J. Am. Chem. Soc. 110*, 3560−3578.

Fisher, J., Spencer, R., & Walsh, C. T. (1976) *Biochemistry 15*, 1054−1064.

Floss, H. G., Onderka, D. K., & Carroll, M. (1972) *J. Biol. Chem. 247*, 736−744.

Fukui, K. (1965) *Tetrahedron Lett.*, 2427−2432.

Ganem, B., Ikota, N., Muralidharan, V. B., Wade, W. S., Young, S. D., & Yukimoto, Y. J. (1982) *J. Am. Chem. Soc. 104*, 6787−6788.

Giese, B., & Almstead, N. G. (1994) *Tetrahedron Lett. 35*, 1677−1680.

Gruys, K. J., Walker, M. C., & Sikorski, J. A. (1992) *Biochemistry 31*, 5534−5544.

Hasan, N., & Nester, E. W. (1978) *J. Biol. Chem. 253*, 4993−4998.

Haslam, E. (1993) *Shikimic Acid Metabolism and Metabolites*, Wiley, New York.

Hawkes, T. R., Lewis, T., Coggins, J. R., Mousdale, D. M., Lowe, D. J., & Thorneley, R. N. F. (1990) *Biochem. J. 265*, 899−902.

Hill, R. K., & Newkome, G. R. (1969) *J. Am. Chem. Soc. 91*, 5893−5894.

Hill, R. K., & Bock, M. G. (1978) *J. Am. Chem. Soc. 100*, 637−639.

Lanzetta, P. A., Alvarez, L. J., Reinbach, P. S., & Candia, O. A. (1979) *Anal. Biochem. 100*, 95−97.

Leatherbarrow, R. J. (1987) *ENZFITTER*, Elsevier Science, Amsterdam.

Levin, J. G., & Sprinson, D. B. (1963) *J. Biol. Chem. 239*, 1142.

McKittridge, B. A., & Ganem, B. (1985) *J. Org. Chem. 50*, 5897−5898.

Morell, H., Clark, M. J., Knowles, P. F., & Sprinson, D. B. (1967) *J. Biol. Chem. 242*, 82−90.

Mousdale, D. M., & Coggins, J. R. (1986) *FEBS Lett. 205*, 328−332.

Onderka, D. K., & Floss, H. G. (1969) *J. Am. Chem. Soc. 91*, 5894−5896.

Poncz, L. (1988) *Arch. Biochem. Biophys. 266*, 508−515.

Ramjee, M. N., Coggins, J. R., Hawkes, T. R., Lowe, D. J., & Thorneley, R. N. F. (1991) *J. Am. Chem. Soc. 113*, 8566−8567.

Ramjee, M. N., Balasubramanian, S., Abell, C., Coggins, J. R., Davies, G. M., Hawkes, T. R., Lowe, D. J., & Thorneley, R. N. F. (1992) *J. Am. Chem. Soc. 114*, 3151−3153.

Ryerson, C. C., Ballou, D. P., & Walsh, C. (1982) *Biochemistry 21*, 1144−1151.

Saksena, A. K., & Mangiaracina, P. (1983) *Tetrahedron Lett. 24*, 273−276.

Schaller, A., Windhofer, V., & Amrhein, N. (1990) *Arch. Biochem. Biophys. 282*, 437−442.

Schullek, J. R., & Wilson, I. B. (1989) *Peptides 10*, 431−434.

Shea, P., Nelson, S. D., & Ford, G. P. (1983) *J. Am. Chem. Soc. 105*, 5451.

Stryer, L. (1988) *Biochemistry*, W. H. Freeman, San Francisco.

Teng, C.-Y., Yukimoto, Y., & Ganem, B. (1985) *Tetrahedron Lett. 26*, 21−24.

Toromanoff, E. (1980) *C. R. Hebd. Seances Acad. Sci., Ser. C 290*, 81−84.

Uhlmann, E., & Pfleiderer, W. (1981) *Helv. Chim. Acta 64*, 1688.

Uhlmann, E., & Engels, J. (1986) *Tetrahedron Lett. 27*, 1023−1026.

Weiss, U., & Edwards, J. M. (1987) *The Biosynthesis of Aromatic Compounds*, Wiley, New York.

Welch, G. R., Cole, K. W., & Gaertner, F. H. (1974) *Arch. Biochem. Biophys. 165*, 505−518.

Wibbenmeyer, J., Brundage, L., Padgette, S. R., Likos, J. J., & Kishore, G. M. (1988) *Biochem. Biophys. Res. Commun. 153*, 760−766.

Wu, T., & Orgel, L. (1991) *J. Mol. Evol. 32*, 274−277.

Zalkin, H., & Hwang, L. H. (1971) *J. Biol. Chem. 246*, 6899.