Inhibition of Chorismate Synthase by (6*R*)- and (6*S*)-6-Fluoro-5-enolpyruvylshikimate 3-Phosphate

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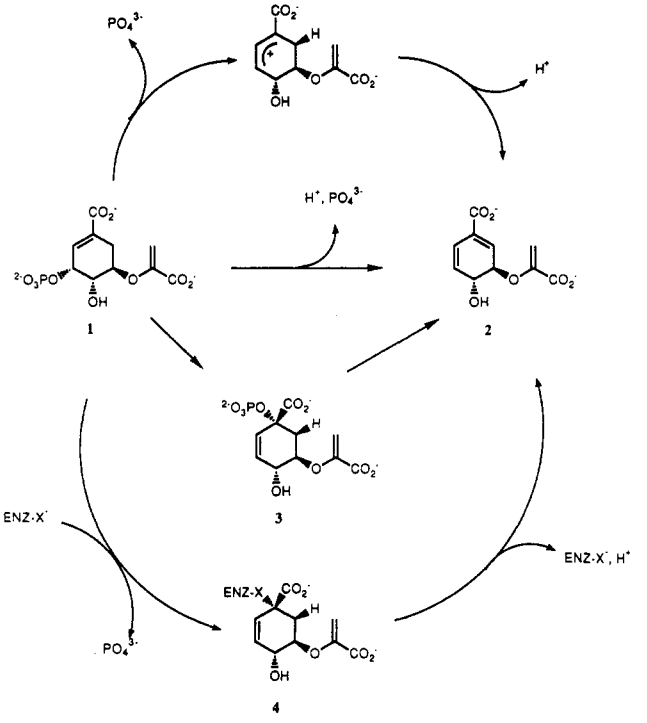
The seventh step of the shikimate pathway,[1] mediated by chorismate synthase (EC 4.6.1.4), is the conversion of 5-enolpyruvylshikimate 3-phosphate (**1**, EPSP) to chorismate (**2**) (Scheme I). This unusual enzymatic transformation proceeds by an overall trans-1,4-elimination of phosphate with abstraction of the C-6 *pro*-R hydrogen.[2,3] The mechanism of this reaction is unknown. Experiments with model systems[4] and arguments based on molecular orbital considerations[5] have been used to discount a concerted E2′ elimination. Some of the other postulated mechanisms are summarized in Scheme I.[1-3,6] The rearrangement mechanism[1] proceeding via **3** is unlikely as this compound is a competitive inhibitor but not a substrate for the enzyme.[7] It has also been shown that phosphate loss is not a fast step prior to the rate-determining step of the reaction.[6] More recently we have shown that the reaction proceeds with an associated primary kinetic isotope effect at C-6 on V and V/K.[8]

Modification of the reactivity of EPSP by stereospecific substitution of hydrogen by fluorine at C-6 gives compounds which could potentially discriminate between the mechanisms in Scheme I. These 6-fluoro-EPSPs (**7b** and **7c**) could act as substrates, suicide inhibitors, or competitive inhibitors of chorismate synthase. We report the synthesis of (6*R*)-6-fluoro-EPSP (**7b**) and (6*S*)-6-fluoro-EPSP (**7c**) and their interaction with chorismate synthase.

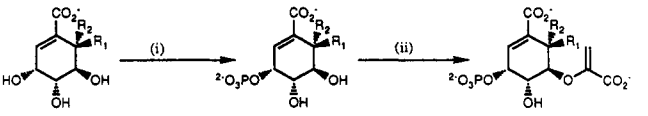
6-Fluoro-EPSPs were synthesized from the corresponding 6-fluoroshikimates (**5b** and **5c**)[9,10] following our protocol for the enzymatic transformation of shikimate (**5a**) to EPSP (**7a**) via shikimate 3-phosphate (**6a**).[10,11] The transformations were performed sequentially in deuteriated buffer using shikimate kinase and EPSP synthase, each isolated from overexpressing strains of *Escherichia coli* (Scheme II).[12,13] The reaction was monitored by ^{1}H NMR spectroscopy in which the C-2 vinyl proton resonance of each compound was clearly visible. Both 6-fluoroshikimates **5b** and **5c** are good substrates for shikimate kinase and were transformed at rates comparable to that of shikimate under the same conditions. (6*R*)-6-Fluoroshikimate 3-phosphate (**6b**) was produced quantitatively, and (6*S*)-6-fluoroshikimate 3-phosphate (**6c**) was produced in greater than 85% yield (by ^{1}H NMR spectroscopy). On addition of EPSP synthase, **6b** and **6c** were each transformed at a rate which was about an order of magnitude slower than that of shikimate 3-phosphate under the same conditions. (6*R*)-6-Fluoro-EPSP (**7b**) was produced quantitatively, and (6*S*)-6-fluoro-EPSP (**7c**) was produced in approximately 85% yield. The final reaction mixtures were treated with apyrase (Sigma, grade VII) to degrade ATP and ADP, which facilitated purification of **7b** and **7c** by ion-exchange chromatography on Dowex 1X8.[11,14] Both purified analogues were isolated as the dibarium salts in 30-40% overall yield.

To explore the possibility that either **7c** or, more likely, **7b** is a substrate for chorismate synthase, each was incubated with the purified *Neurospora crassa* enzyme under V_{max} conditions.[15] UV spectroscopy was used to detect the appearance of a diene chromophore by monitoring changes in absorbance in the region 240-300 nm.[16] No diene formation was detected for either

Scheme I. Postulated Mechanisms for the Chorismate Synthase Catalyzed Reaction



Scheme II[a]



5a $R_1 = R_2 = H$
5b $R_1 = F$, $R_2 = H$
5c $R_1 = H$, $R_2 = F$

6a $R_1 = R_2 = H$
6b $R_1 = F$, $R_2 = H$
6c $R_1 = H$, $R_2 = F$

7a $R_1 = R_2 = H$
7b $R_1 = F$, $R_2 = H$
7c $R_1 = H$, $R_2 = F$

[a] (i) Shikimate kinase, 1.8 units, 24 h. (ii) EPSP synthase, 0.22 units, 24 h. Transformations i and ii were carried out sequentially on 50 mM (6*R*)- and (6*S*)-6-fluoroshikimic acid in 1.0 mL of D_2O, pD 7.1 (Tris·DCl, 300 mM), at 25 °C, containing 50 mM $MgCl_2$, 50 mM ATP, and 50 mM phosphoenol pyruvate, and were followed by ^{1}H NMR spectroscopy.

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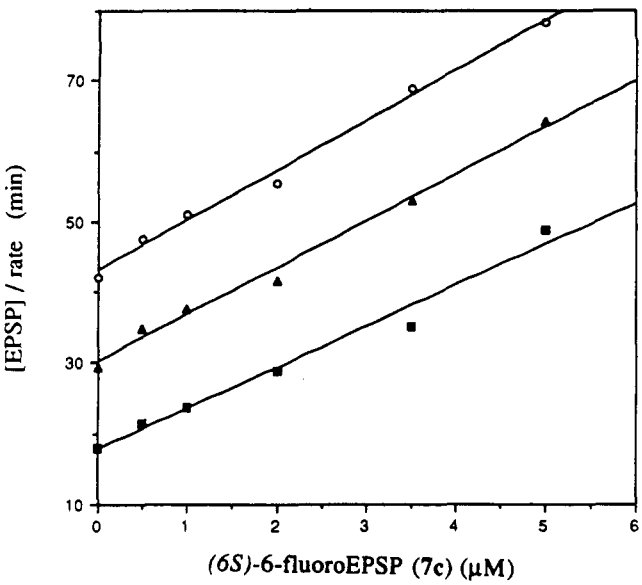

Figure 1. Cornish-Bowden plot showing competitive inhibition of chorismate synthase by (6*S*)-6-fluoro-EPSP (**7c**). UV assays (275 nm) were carried out at 25 °C, pH 7.0 (triethanolamine hydrochloride, 50 mM), and included 1.2 milliunts of chorismate synthase, 20 μM NADPH, 10 μM FMN, 50 mM KCl, and 2.5 mM $MgCl_2$ in addition to substrate EPSP (**7a**) and inhibitor (6*S*)-6-fluoro-EPSP (**7c**) in a final volume of 1 mL. Inhibitor concentrations were 0, 0.5, 1, 2, 3.5, and 5 μM, and substrate concentrations were (■) 20 μM EPSP, (▲) 35 μM EPSP, and (○) 50 μM EPSP.

compound under conditions which would have easily detected a turnover rate 0.2% that of EPSP itself.[17]

Competition experiments were performed in which chorismate synthase was assayed at various fixed concentrations of EPSP in the presence of a range of concentrations of **7b** or **7c**. Figure 1 shows a Cornish-Bowden plot[18] of the data obtained for (6*S*)-6-fluoro-EPSP (**7c**). The parallel plots clearly signify a competitive mode of inhibition. The inhibition constant K_i was determined from a Dixon plot.[19] It is found that both fluoro-EPSPs show clean competitive inhibition with **7c** having an affinity an order of magnitude greater than **7b**: K_i ((6*S*)-6-fluoro-EPSP) = 0.2 ± 0.1 μM, K_i ((6*R*)-6-fluoro-EPSP) = 3.0 ± 0.3 μM. These values compare with K_i (iso-EPSP **3**) = 8.7 μM,[7] and K_m (EPSP) = 2.2 μM.[8] The lack of irreversible inhibition by either compound was confirmed by incubation of *N. crassa* chorismate synthase with 50 μM of each inhibitor at 25 °C. Over a period 1 h, no loss of enzyme activity was observed relative to a control which lacked inhibitor.

The lack of irreversible inhibition is inconsistent with a mechanism involving a covalent enzyme–intermediate adduct such as **4**. While the observation that both 6-fluoro-EPSPs are potent competitive inhibitors does not itself support or preclude any of the other mechanisms in Scheme I, it does provide a useful tool for future mechanistic studies of the enzyme.

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Supplementary Material Available: Preparation of **7b,c** from **5b,c**, spectroscopic characterization of **5b,c** and **7b,c**, and Dixon plot showing inhibition of chorismate synthase by **7c** (4 pages). Ordering information is given on any current masthead page.

(16) Chorismate formation is normally monitored by the appearance of the diene chromophore which has its λ_{max} at 275 nm. In these experiments, absorbance was monitored in the range 240–300 nm in order to accommondate a possible shift in the absorbance maximum due to a fluorine substituent.

(17) This experiment does not rigorously preclude either compound being a substrate but puts an upper limit on their turnover rate.

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Modulation of Physical and Chemical Properties of η-H_2 Complexes of Osmium Ammines by Facile Substitution

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Since the discovery of the first dihydrogen complex by Kubas et al.,[1] many dihydrogen complexes have been synthesized. In 1971,[2] the preparation in our laboratory of $[Os(en)_2H_2]^{2+}$ as the chloride salt was reported. It was described as a dihydride and was assigned a cis configuration on the strength of ^1H NMR results which revealed two sets of amine protons in equal number. Our investigation of the analogous species $[Os(NH_3)_4H_2]^{2+}$, not heretofore reported, throws new light on that structural assignment and, as well, provides ready access to a series of complexes arrived at by the simple addition of a variety of ligands to these 16e$^-$ moieties.

When $[Os(NH_3)_4H_2](B(C_6H_5)_4)_2$[3] (**1**) is dissolved in $(CD_3)_2CO$, the ^1H NMR spectrum reveals only two kinds of protons ascribable to the cation, in the abundance ratio 6:1 at δ = 3.82 ppm and −11.37 ppm, respectively. For the purposes of species differentiation, the value of T_1 for the coordinated hydrogen was also measured (T_1 = 572 ms, 20 °C), as it was for the other species to be dealt with. When a trace of acid, for example, HO_3SCF_3, is present, slow H/D exchange between the solvent and coordinated hydrogen ensues, and, in a partially exchanged sample, J_{HD} was measured as 4.0 Hz. When any of a large number of solutes is added in excess, among them acetonitrile (AN), pyridine (Py), imidazole (Im), I^-, Cl^-, D_2O, and Br^-, δ (ppm) J_{HD} (Hz), and T_1 (ms, 400 MHz) change and new characteristic values are registered. (See Table I.) In every case except with D_2O and $(CD_3)_2CO$ as addend, the corresponding solid salt was also prepared,[5] and dissolved, with no discernible differences in the ^1H NMR signals. Because the solute level is low (0.010 M), we can conclude, at least in the case of the labile systems I^-, Br^-, or Cl^- as addend, that in acetone the affinity of the osmium center for the ligand is very high. As expected, it is much reduced in D_2O as solvent.

Of potential anionic ligands, the only one among those we have introduced which does not change the values of δ, J_{HD}, and T_1 is PF_6^- (even $CF_3SO_3^-$ produces a set of characteristic values). This indicates that neither $B(C_6H_5)_4^-$ nor PF_6^- enters the coordination sphere of the osmium complex, a supposition which, in the case of the former at least, is reasonable and, in view of the bulk and almost spherical shape of PF_6^-, is reasonable for it also. However, it leaves open the question of whether $(CD_3)_2CO$ also is a ligand when the $B(C_6H_5)_4^-$ and PF_6^- salts are dissolved. That this is in fact the case is indicated by observations made for $[Os(en)_2H_2]^{2+}$, and it is therefore likely also the case for $[Os(NH_3)_4H_2]^{2+}$.

In preparing $[Os(en)_2H_2]^{2+}$, we followed the literature procedure,[2] but with the difference that, instead of Cl^-, $B(C_6H_5)_4^-$ was

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(3) $[Os(NH_3)_4H_2](B(C_6H_5)_4)_2$ was made by the folllowing procedure: $Os(NH_3)_4(O_3SCF_3)_3$[4] (100 mg) in 15 mL of H_2O was reduced by Zn/Hg (3 g) for 3 h, and then 15 mL of 0.2 M $NaB(C_6H_5)_4$ solution was added. The resulting precipitate was dried under vacuum. Microanal. Calcd for $[Os(NH_3)_4H_2](B(C_6H_5)_4)_2 \cdot 2H_2O$: C, 61.67; H, 6.25; N, 5.99. Found: C, 61.50; H, 6.20; N, 5.80. Yield: >70%.

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(5) The preparation of the pyridine adduct is typical of the others. The compound $[Os(NH_3)_4(H_2)\cdot Py][B(C_6H_5)_4]_2$ (**2**) was prepared by dissolving **1** (100 mg) in pyridine (5 mL); after 1 h, ether was added to cause precipitation. The precipitate was collected, washed with ether, and dried. Yield: 90%. Microanal. Calcd for $[Os(NH_3)_4(H_2)\cdot Py][B(C_6H_5)_4]_2 \cdot 2H_2O$: C, 62.81; H, 5.82; N, 6.91. Found: C, 62.76; H, 6.03; N, 6.64. ^1H NMR in $(CD_3)_2CO$ (ppm): 8.83 (d, 2 H, Py), 8.14 (t, 1 H, Py), 7.75 (t, 2 H, Py), 7.40–6.70 (m, 40 H, C_6H_5), 3.74 (s, br, 12 H, 4 NH_3), −7.44 (s, 2 H, OsH_2).

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