Derailing Dehydroquinate Synthase by Introducing a Stabilizing Stereoelectronic Effect in a Reaction Intermediate

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Received July 17, 1997

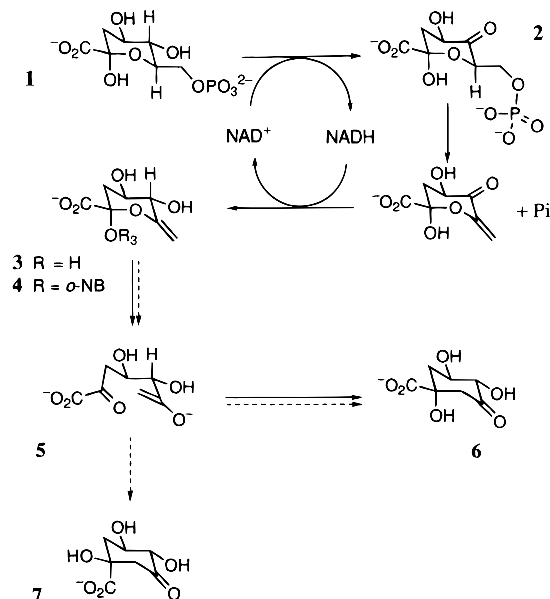
3-Dehydroquinate synthase (E.C. 4.6.1.3) catalyses the conversion of 2-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP) (**1**) into 3-dehydroquinate **6**. This is the second step on the shikimate pathway to the aromatic amino acids.[1] The mechanism of this transformation (Scheme 1) was first proposed by Sprinson[2] and substantially confirmed by Knowles.[3]

The complexity of the dehydroquinate synthase mechanism raised the question of how the oxidation and reduction, phosphate cleavage, and aldol ring closure reactions could be catalyzed by a small monomeric enzyme. A series of elegant studies suggested that the inherent reactivity of the various enzymic intermediates reduced the role of the enzyme to little more than that of an "internal cycling dehydrogenase".[4] For example, evidence was presented to show that the base responsible for the deprotonation of the intermediate **2** at C-6 was the substrate phosphate dianion.[5]

The involvement of the enzyme in the conversion of the enol pyranose **3** to dehydroquinate **6** has also been questioned. Photochemical removal of the *o*-nitrobenzyl protecting group from **4** was initially reported to result in complete conversion of **3** to dehydroquinate in the absence of enzyme.[6] Furthermore, the cyclization was shown to proceed through the same chairlike transition state as had been established for the enzyme-catalyzed aldol step. This led to the suggestion that the enolpyranose **3** was the product of the enzyme reaction. Upon subsequent reinvestigation of this nonenzymatic reaction, it was found that the reaction of **3** was not entirely stereospecific with 2.5−4% of 1-*epi*-dehydroquinate **7** also being formed.[7] As this product is not observed in the enzymatic reaction, the role of the enzyme in guiding the conversion of **3** to **6** was reinstated. We now show that a modification of the enol pyranose intermediate by putting an axial fluorine next to the hemiketal center (e.g., in **12**) promotes the dissociation of the enol pyranose intermediate from the enzyme, so that it cyclizes in solution.

We have previously developed an enzymatic synthesis of 6-fluoroshikimates[8] as part of a study of their antibiotic properties.[9] Using a mixture of (3*R*)- and (3*S*)-3-fluoroDAHP (**8** and **11**) generated by DAHP synthase,[10] we observed that the (3*R*)-isomer was very rapidly converted to (6*R*)-6-fluorodehydroquinate (**10**) before the (3*S*)-3-fluoroDAHP was slowly converted to (6*S*)-6-fluorodehydroquinate (**13**) and an unknown product. We now report on a detailed study of this reaction and identify the unknown product as (6*S*)-1-*epi*-6-fluorodehydroquinate (**14**). The formation of **13** and **14** appears to be from a common intermediate **12** *after* its release from the enzyme (Scheme 2).

Scheme 1. Mechanism for the Dehydroquinate Synthase Catalyzed Reaction[a]



[a] When DAHP **1** is the substrate only dehydroquinate **6** is formed, and the intermediate enolpyranose **3** is cyclized on the enzyme. When **3** is generated by deprotection of **4**, a mixture of **6** and **7** is formed nonenzymatically.[7] The solid arrows indicate enzymatic reactions, and the dotted arrows are nonenzymatic transformations.

(3*R*)-3-FluoroDAHP (**8**) and (3*S*)-3-fluoroDAHP (**11**) were separately synthesized enzymatically from erythrose 4-phosphate and *(E)*-3-fluoroPEP and *(Z)*-3-fluoroPEP, respectively, using DAHP synthase.[11] The fluoroDAHPs were then used in kinetic studies as substrate analogues for dehydroquinate synthase. The kinetic constants were determined in a coupled spectrophotometric assay by using an excess of *E. coli* dehydroquinase to convert the 6-fluorodehydroquinates to the corresponding 6-fluorodehydroshikimates,[12,13] after first confirming that (6*R*)- and (6*S*)-6-fluorodehydroquinate are good substrates for dehydroquinase.

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(1) Bentley, R. *CRC Crit. Rev. Biochem.* **1990**, *25*, 307−384.
(2) Sprinson, D. B.; Rothschild, M.; Sprecher, M. *J. Biol. Chem.* **1963**, *238*, 3170−3175.
(3) Knowles, J. *Aldrichim. Acta* **1989**, *22*, 59−66.
(4) Bender, S. L.; Mehdi, S.; Knowles, J. R. *Biochemistry* **1989**, *28*, 7555−7560.
(5) Widlanski, T.; Bender, S. L.; Knowles, J. R. *J. Am. Chem. Soc.* **1989**, *111*, 2299−2300.
(6) Bartlett, P. A.; Satake, K. *J. Am. Chem. Soc.* **1988**, *110*, 1628−1630.
(7) Bartlett, P. A.; Mclaren, K. L.; Marx, M. A. *J. Org. Chem.* **1994**, *59*, 2082−2085.
(8) Duggan, P. J.; Parker, E.; Coggins, J.; Abell, C. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 2347−2352.
(9) Davies, G. M.; Barrettbee, K. J.; Jude, D. A.; Lehan, M.; Nichols, W. W.; Pinder, P. E.; Thain, J. L.; Watkins, W. J.; Wilson, R. G. *Antimicrob. Agents Chemother.* **1994**, *38*, 403−406.
(10) Pilch, P. F.; Somerville, R. L. *Biochemistry* **1976**, *15*, 5315−5320.
(11) Details of the preparation and purification of **8** and **11**, the kinetic data for the enzymatic conversions, and ^{19}F NMR spectra of **10**, **13**, and **14** are available as Supporting Information.
(12) Dehydroquinate synthase was purified according to the method of: Frost, J. W.; Bender, J. L.; Kadonaga, J. T.; Knowles, J. R. *Biochemistry* **1984**, *23*, 4470−4475.
(13) However, these findings are at variance with the work of Le Marechal who reported that the K_M^{app} for **8** and **11** was in the range 40−60 μM and the V_{max} values were in the order **1** >**11** > **8**. Le Marechal, P.; Froussios, C.; Azerad, R. *Biochimie* **1986**, *68*, 1211−1215.

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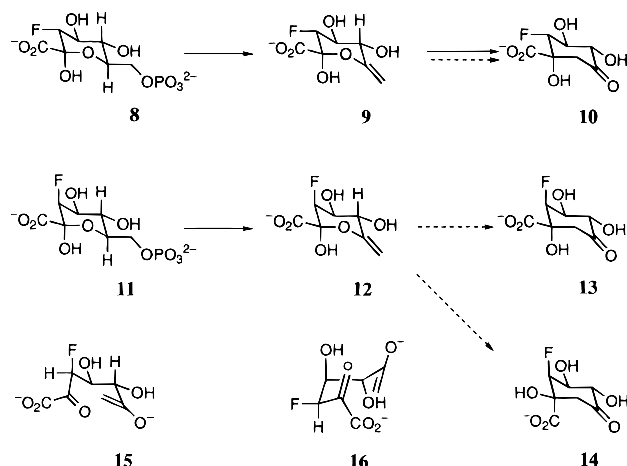
Scheme 2. Formation of (6*S*)-6-Fluorodehydroquinate (10) when (3*R*)-3-FluoroDAHP 8 Is Used as a Substrate for Dehydroquinate Synthase[a]



[a] However, when (3*S*)-3-fluoroDAHP (**11**) is the substrate a 2:1 mixture of (6*S*)-6-fluorodehydroquinate (**13**) and (6*S*)-1-*epi*-6-fluorodehydroquinate (**14**) is formed. The solid arrows indicate enzymatic reactions, and the dotted arrows are possible nonenzymatic steps.

Table 1. Steady-State Kinetic Parameters for DAHP, (3*R*)-3-FluoroDAHP (8), and (3*S*)-3-FluoroDAHP (11) with DHQ Synthase

	K_M^{app} (μM)	$k_{cat.}$ (s^{-1})	$k_{cat.}/K_M^{app}$ (s^{-1} M^{-1}) × 10^6
DAHP (**1**)	5.7 ± 0.2	50 ± 3	8.8
(3*R*)-3-fluoroDAHP (**8**)	1.6 ± 0.3	7.7 ± 0.6	4.8
(3*S*)-3-fluoroDAHP (**11**)	21 ± 2	0.9 ± 0.1	0.04

Incubation of (3*R*)-3-fluoroDAHP (**8**) with dehydroquinate synthase results in the rapid and quantitative formation of (6*R*)-6-fluorodehydroquinate (**10**). Although the $k_{cat.}$ is reduced approx 7-fold relative to DAHP, K_M^{app} is also smaller and the specificity constant ($k_{cat.}/K_M^{app}$) for (3*R*)-3-fluoroDAHP is similar to that for the natural substrate (Table 1).

By contrast, incubation of dehydroquinate synthase with (3*S*)-3-fluoroDAHP (**11**) results in the much slower formation of two products (6*S*)-6-fluorodehydroquinate (**13**) and (6*S*)-1-*epi*-6-fluorodehydroquinate (**14**) in a 2:1 ratio (Figure 1). The specificity constant is 100 times lower than for (3*R*)-3-fluoroDAHP (**8**), consistent with our observation that (3*R*)-3-fluoroDAHP is converted into product first when a mixture of isomers is present.[8,13]

The conversion of (3*S*)-3-fluoroDAHP (**11**) to a mixture of **13** and **14** goes to completion when sufficient enzyme is used for the reaction to take place in 20−30 min. If less enzyme is used, so that the reaction proceeds over several hours, the reaction becomes strongly inhibited and addition of more enzyme has no observable effect. This inhibition has been ascribed to decomposition products of NAD^+ that reversibly inhibit dehydroquinate synthase.[4]

The two products **13** and **14** were separated by HPLC and their identities confirmed spectroscopically. The ^{1}H NMR spectra of (6*S*)-6-fluorodehydroquinate (**13**) and (6*S*)-1-*epi*-6-fluorodehydroquinate (**14**) are very similar (Figure 2) with almost identical J_{H-H} and J_{H-F} coupling constants, consistent with the relative configuration and conformation of the fluorine and the hydrogens on the ring being the same. This similarity extends to the COSY

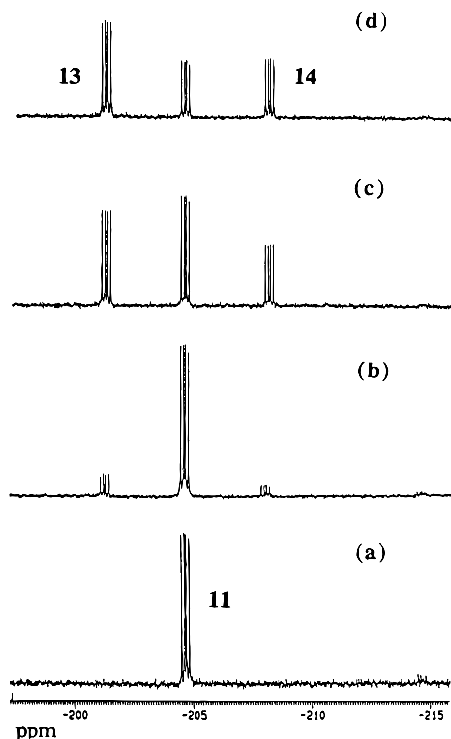


Figure 1. ^{19}F NMR spectra (235 MHz) showing the conversion of (3*S*)-3-fluoroDAHP (**11**) into a 2:1 mixture of (6*S*)-6-fluorodehydroquinate (**13**) and (6*S*)-1-*epi*-6-fluorodehydroquinate (**14**) (a) before addition of dehydroquinate synthase, (b) t = 20 min, (c) t = 80 min, (d) t = 180 min. Spectra were run in H_2O containing 10−20% D_2O (total volume 550 μL) and were referenced to CCl_3F at 0 ppm. [Dehydroquinate synthase] = 10 μM (18 units), [(3*S*)-3-fluoroDAHP] = 45 mM, [NAD^+] = 30 μM, pH =7.0, Co^{2+} 50 μM, T = 26 °C. The weak signal at −215 ppm is due to the C-1 epimer of **11**.

spectra, and NOEs observed with both compounds. In addition, the HRMS of the sodium salts of **10**, **13**, and **14** prove that they are all isomeric.[11]

The significant differences between **13** and **14** are in the chemical shift of the fluorine signal and the stability of the two compounds. The chemical shift of the fluorine in **14** comes at −209 ppm, compared to −202 ppm for **13** and −201 ppm for **10**. Significantly, the chemical shift for the fluorine in (3*S*)-3-fluoroDAHP is −205, while that of its C-1 anomer, where the carboxyl is also antiperiplanar to the fluorine, comes at −215 ppm. Like 1-*epi*-dehydroquinate **7**,[7] (6*S*)-1-*epi*-6-fluorodehydroquinate **14** is not a substrate or inhibitor for *E. coli* dehydroquinase.

(6*S*)-1-*epi*-6-Fluorodehydroquinate (**14**) decomposes over several hours at room temperature but can be stored at −20 °C. The decomposition was first seen as a steady reduction in the signal-to-noise of the ^{1}H NMR spectrum of a sample in D_2O, with no other signals appearing. The decomposition product is 1,2,4-trihydroxybenzene, which becomes fully deuterated and hence is not detected directly in the ^{1}H NMR spectrum. The mechanism for formation of this product is presumed to be by antiperiplanar elimination of carbon dioxide and fluoride followed by dehydration of the resulting diketo diol.

The formation of two products **13** and **14** from the enzymatic conversion of **11** contrasts with the enzymatic formation of only **6** from **1** but is reminiscent of the nonenzymatic formation of a mixture of **6** and **7** from **3**.[7,14] It is unlikely that dehydroquinate synthase can catalyze the alternative aldol reactions to form **13** and **14** and

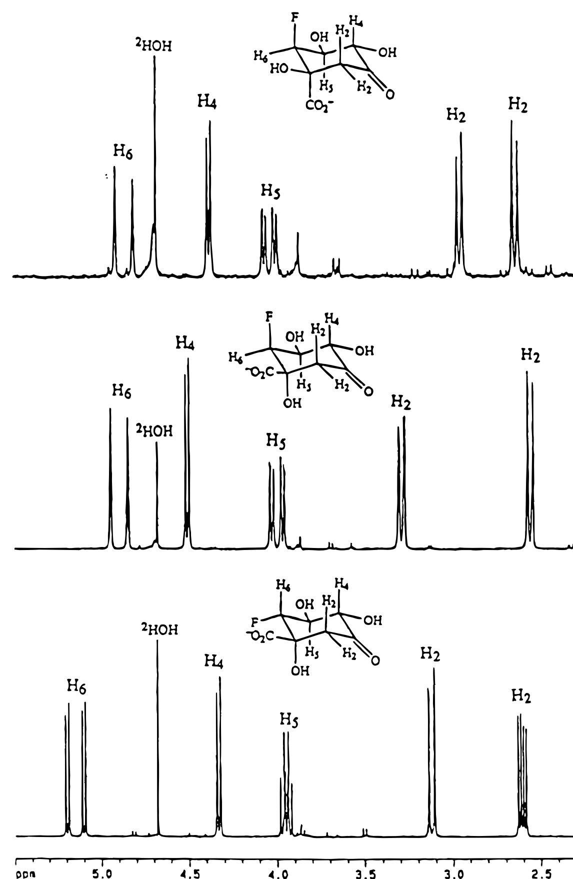


Figure 2. ^{1}H NMR spectra (500 MHz) of (a) (6*R*)-6-fluorodehydroquinate (**10**), (b) (6*S*)-6-fluorodehydroquinate (**13**), and (c) (6*S*)-1-*epi*-6-fluorodehydroquinate (**14**). Spectra were recorded at 26 °C in D_2O with presaturation on the HOD signal and were referenced to HOD at 4.67 ppm.

suggests that at least **14** and perhaps both compounds are formed off the enzyme. This could occur if the effect of the fluorine atom at C-3 is to increase the rate of dissociation of the enol pyranose intermediate **12** from the enzyme or decrease the rate of ring opening of **12** on the enzyme or both.[15] Neighboring fluorine atoms are known to favor hemiketals and hydrates relative to the corresponding ketones. Once in solution, ring opening of **12** and subsequent cyclization could proceed via the chairlike conformation **15** to form **13**. Labeling studies have shown that the formation of **6** enzymatically from **1**[16] and nonenzymatically from **3** proceeds through the intermediate in a chair conformation **5**.[7] However, the Felkin−Ahn model for attack on a carbonyl adjacent to a chiral center predicts that the attack would be favored when the electronegative fluorine atom is othogonal to and behind the ketone group.[17] Cyclization via the chairlike conformation **16** may account for the increased proportion of the epimeric product **14** relative to **13** (compared to the ratio of **7** to **6** formed nonenzymatically from **3**).[7]

The equatorial fluorine atom at C-3 might have a similar effect on the partitioning of the enol pyranose intermediate **9**, which could also dissociate from the enzyme. However, the conformation for cyclization in solution favored by the Felkin−Ahn rule would lead to **10**. This is the same cyclization as would be expected on the enzyme, and so it is impossible to conclude from the exclusive formation of **10** whether this cyclization has occurred on or off the enzyme.

The steady-state kinetic parameters determined for the three substrates give limited insight to what is happening on the enzyme. The $k_{cat.}/K_M$ for reaction of **11** is 200 times slower than for DAHP (**1**) and about 100 times slower than for (3*R*)-3-fluoroDAHP (**8**). However, if the elimination of phosphate from **2** is accompanied by release of inorganic phosphate (P_i) from the enzyme, then this step should become effectively irreversible. If this is so, then the differences in $k_{cat.}/K_M$ must be explained by differences in the rates of steps before phosphate release and cannot be explained by differences in the rates of the processing of the enol pyranose intermediate.

The $k_{cat.}$ for the conversion of **11** is also slower than for **1** and **8** (by factors of 50 and 7, respectively) and also slower than the very fast nonenzymatic conversion of **3** to a mixture of **6** and **7**.[7] This could be due to slowing of steps either before or after phosphate release. One possibility is that it reflects release of enolpyranose **12** from the enzyme at a rate that is slower than the rate for further processing of **3** (and **9**) on the enzyme. This interpretation would imply that the fluorine substitution in **12** is indeed stabilizing the enol pyranose form as expected and not simply enhancing its dissociation from the enzyme.

It has previously been concluded that dehydroquinate synthase plays some role in the cyclization of **3** to **6**, perhaps more as a template than as a catalyst.[7] The evidence for the role of the enzyme was the exclusive formation of **6**. By the simple expedient of introducing a fluorine to stabilize the enol pyranose intermediate long enough to allow it to dissociate from the enzyme we have highlighted how subtle the role of the enzyme must be.

Experimental Section

Kinetic Studies Using DAHP and (3*R*)- and (3*S*)-3-FluoroDAHPs as Substrates for Dehydroquinate Synthase. All kinetic parameters were determined in "bis-tris" propane·HCl buffer (50 mM) containing NAD^+ (35 μM), $MnSO_4$ (50 μM), and $CoSO_4$ (20 μM) at pH 7.5 and 25 °C. Solutions also contained *E. coli* dehydroquinase (1.5 units). Reactions were initiated by adding 10 μL of a solution containing dehydroquinate synthase (0.6 μM for DAHP, 1.4 μM for (3*R*)-fluoroDAHP and 7 μM for (3*S*)-fluoroDAPH). The absorbance increase was then monitored spectrophotometrically at 234 nm for **1** and 230 nm for **8** and **11**. The extinction coefficients at these wavelengths for the corresponding dehydroshikimates were determined to be 1.2×10^4 M^{-1} cm^{-1}, 3.4×10^3 M^{-1} cm^{-1}, and 9.6×10^3 M^{-1} cm^{-1}, respectively. Initial rates were recorded, and the data were fitted to the Michaelis−Menton equation using Enzfitter.

HPLC Conditions Used To Purify the 6-Fluorodehydroquinates. HPLC was performed on an LKB HPLC system using a BioRad Aminex ion-exclusion HPX-87H organic acid semipreparative column (300 × 16 mm). The compounds were eluted using 50 mM formic acid as eluant at a flow rate of 1.2 mL per min with continuous detection at 277 nm. Under these conditions, dehydroquinate elutes after 18.1 min.

Enzymatic Conversion of (3*R*)-Fluorodehydroquinate into (6*R*)-6-Fluorodehydroquinate. To an unbuffered aqueous solution (600 μL) containing (3*R*)-3-fluorodehydroquinate (45 mM), NAD^+ (30 μM), and cobalt sulfate (50 μM) at pH 7.0 was

(14) It is considered unlikely that the formation of the **14** is due to dehydroquinate synthase using the C-1 epimer of the (6*S*)-6-fluoroDAHP as a substrate. Less than 4% of this compound is present in the starting material (see Figure 1).

(15) Analogous to the increase in stability seen when a fluorine is introduced next to the anomeric center in 2-deoxy-2-fluorosugars. Street, I. P.; Rupitz, K.; Withers, S. G. *Biochemistry* **1989**, *28*, 1581−1587.

(16) Widlanski, T. S.; Bender, S. L.; Knowles, J. R. *J. Am. Chem. Soc.* **1987**, *109*, 1873−1875.

(17) Anh, N. T.: Eisenstein, O. *Nouv. J. Chem.* **1977**, *1*, 61−70.

added dehydroquinate synthase (0.5 units). The reaction was incubated at 26 °C for 3 h. The enzyme was then removed by filtration through an Amicon Centricon concentrator and the filtrate purified by HPCL. (6*R*)-6-Fluorodehydroquinate (**10**): δ_H (500 MHz, D_2O) 5.15 (dd, J = 47.7, 9.5 Hz, H6), 4.33 (d, J = 9.8 Hz, H4), 3.95 (ddd, J = 12.8, 9.8, 9.5 Hz, H5), 3.13 (d, J = 14.8 Hz, $H2_{ax}$), 2.61 (J = 14.8, 7.6 Hz, $H2_{eq}$); δ_F (235 MHz, D_2O) −200.9 (ddd, J = 47.7, 12.7, 7.6 Hz); COSY (500 MHz, D_2O) correlations between H6 and H5, H5 and H4, $H2_{ax}$ and $H2_{eq}$; m/z (−ve ESMS) 207 ($M - H^-$), 208 ($M - 2H + D^-$); found (+ve ESMS) $M + Na^+$ 231.027 28, $C_7H_9O_6FNa$ requires 231.028 12, $M - H + D + Na^+$ 232.034 87, $C_7H_8DO_6FNa$ requires 232.034 39; HPLC retention time (organic acids column, 50 mM formic acid) 15.5 min.

Enzymatic Conversion of (3*S*)-3-Fluorodehydroquinate into (6*S*)-6-Fluorodehydroquinate and (6*S*)-6-Fluoro-1-epidehydroquinate. To an unbuffered aqueous solution (600 μL) containing (3*R*)-3-fluorodehydroquinate (15 mM), NAD^+ (30 μM), and cobalt sulfate (50 μM) at pH 7.3 was added dehydroquinate synthase (45 units). The reaction was incubated at 26 °C for 30 min. The enzyme was then removed by filtration through an Amicon Centracon concentrator and the filtrate purified by HPLC. (6*S*)-6-Fluorodehydroquinate (**13**): δ_H (500 MHz, D_2O) 4.85 (dd, J = 48.9, 2.5 Hz, H6), 4.51 (d, J = 10.1 Hz, H4), 4.0 (ddd, J = 29.6, 10.2, 2.5 Hz, H5), 3.29 (d, J = 14.8 Hz, $H2_{ax}$), 2.53 (d, J = 14.8 Hz, $H2_{eq}$); δ_F (235 MHz, D_2O) −201.7 (dd, J = 48.9, 29.6 Hz); m/z (−ve ESMS) 207 ($M - H^-$); COSY (500 MHz, D_2O) correlations between H6 and H5, H5 and H4, $H2_{ax}$ and $H2_{eq}$; found $M + Na^+$ (+ve ESMS) 231.026 43, $C_7H_9O_6FNa$ requires 231.028 12; HPLC retention time (organic acids column, 50 mM formic acid) 16.3 min. (6*S*)-6-Fluoro-1-epidehydroquinate (**14**): δ_H (500 MHz, D_2O) 4.86 (dd, J = 50.8, 2.4 Hz, H6), 4.38 (d, J = 9.9 Hz, H4), 4.03 (ddd, J = 30.0, 9.9, 2.4 Hz, H5), 2.95 (d, J = 14.7 Hz, $H2_{ax}$), 2.64 (d, J = 14.7 Hz, $H2_{eq}$): δ_F (235 MHz, D_2O) −209.0 (dd, J = 50.8, 30.0 Hz); m/z 207 ($M - H^-$); COSY (500 MHz, D_2O) correlations between H6 and H5, H5 and H4, $H2_{ax}$ and $H2_{eq}$; found $M + Na^+$ (+ve ESMS) 231.027 21, $C_7H_9O_6FNa$ requires 231.028 12; HPCL retention time (organic acids column, 50 mM formic acid) 14.6 min.

Acknowledgment. We thank the Association of Commonwealth Universities for a studentship to E.J.P. and Dr. F. J. Leeper for helpful discussions.

Supporting Information Available: Methods for preparation of DAHP, (3*R*)- and (3*S*)-3-fluoroDAHP, data for the enzymatic conversion of **1**, **8**, and **11**, and ^{19}F NMR spectra of **10**, **13**, and **14** (9 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

JO9713060