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Dehydroquinate Synthase: The Use of Substrate Analogues To Probe the Early Steps of the Catalyzed Reaction[†]

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ABSTRACT: The early steps of the proposed mechanistic pathway for dehydroquinate synthase have been probed with a series of substrate analogues. These analogues, **3–9**, are structurally prohibited from undergoing the β-elimination of inorganic phosphate that represents the committed step in the conversion of the substrate 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (**1**) to dehydroquinate (**2**). In agreement with previous observations, the analogues that possess shortened side chains (**3, 5,** and **6**) bind more tightly to the enzyme than those (**4** and **7–9**) that are more nearly isosteric with the substrate. Two hitherto unrecognized factors that influence binding have been identified: (i) carbacyclic analogues bind 25–100 times more tightly than the corresponding oxacyclic materials (indeed, the carbacyclic phosphonate **5** has a K_i value of 8×10^{-10} M) and (ii) the side chain appears to be bound in a gauche conformation similar to the most stable conformation of the *cis*-vinylhomophosphonate **8**. These trends in binding can be rationalized by considering the behavior of the analogues in the first two chemical steps of the mechanism: NAD^+-mediated oxidation at C-5 and enolization at C-6 (the first part of the E1cB elimination of inorganic phosphate). Direct spectrophotometric determination of the equilibrium level of enzyme-bound NADH indicates that the carbacyclic analogues are more readily oxidized than the oxacyclic compounds, and this predictable difference in redox behavior is reflected in the observed differences in binding. The gauche conformation of the C-7 side chain appears to be required for proton abstraction from C-6, since only those analogues that can adopt this conformation undergo enzyme-catalyzed exchange of the C-6 proton with the solvent. This conformation positions one of the peripheral oxygens of the phosphate (or phosphonate) group close to the C-6 proton. Taken together with other data, these results suggest that the enzyme exploits this substrate base in the enolization, which occurs through an intramolecular proton transfer. The loss of P_i then completes the β-elimination.

In plants and microorganisms, aromatic amino acids are biosynthesized through the action of the enzymes of the shikimate pathway (Haslam, 1974; Weiss & Edwards, 1980). The second enzyme in the pathway, dehydroquinate synthase, catalyzes the conversion of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP,[1] **1**) to dehydroquinate (DHQ, **2**) (Srinivasan et al., 1963).

In this and the following paper, a number of substrate analogues are described that are processed by dehydroquinate

synthase to various stages along the reaction pathway illustrated in Scheme I. This approach allows the multistep mechanism to be dissected into its component transformations so that the existence of each step is verified and its mechanism is scrutinized. Furthermore, important characteristics of the enzyme mechanism become clear by examination of the trends observed for a given experimental parameter with a series of

[1] Abbreviations: ADPR, adenosine diphosphoribose; DAH, 3-deoxy-D-*arabino*-heptulosonate; DAHP, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate; DEAE, diethylaminoethyl; DHQ, dehydroquinate; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; MOPS, 3-(N-morpholino)propanesulfonate; NAD^+, nicotinamide adenine dinucleotide; NADH, reduced form of NAD^+; THF, tetrahydrofuran.

Scheme I: Mechanistic Pathway for the Reaction Catalyzed by Dehydroquinate Synthase
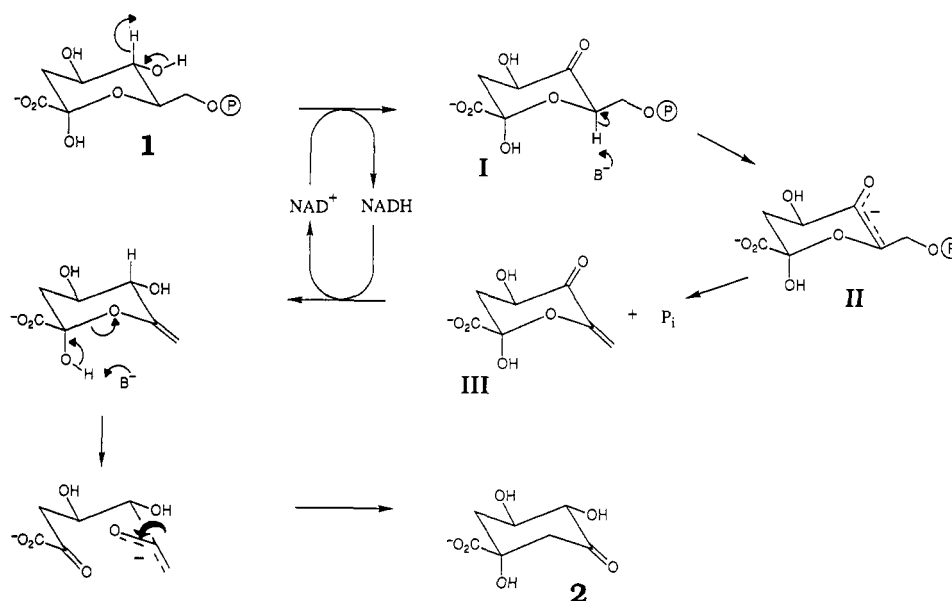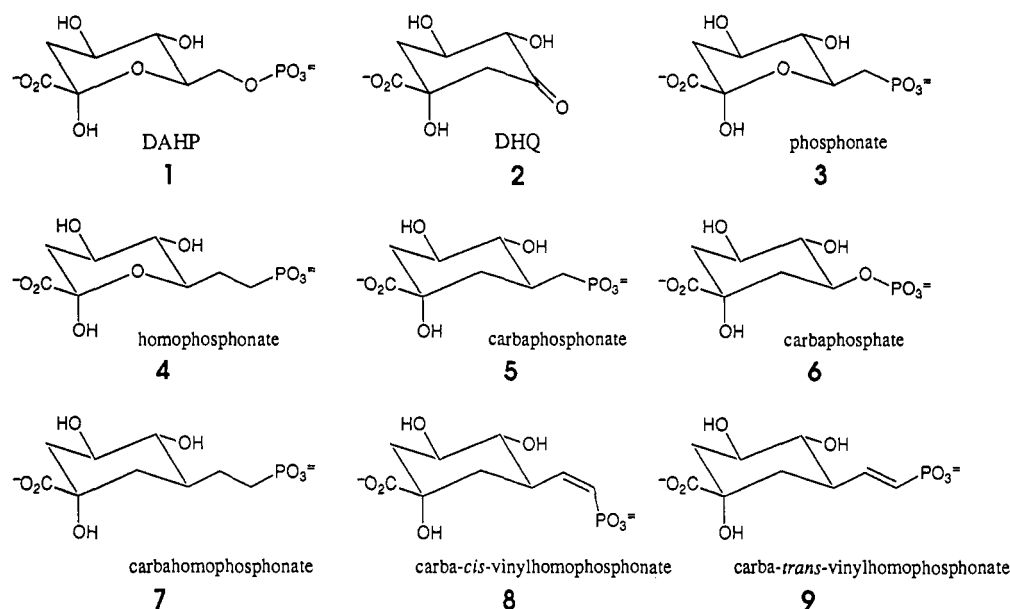

Chart I

related analogues. In this paper, the synthesis of substrate analogues that cannot undergo the β-elimination step is described, and the nature of the interaction of these compounds with the enzyme is discussed.

One of the difficulties in studying the mechanism of dehydroquinate synthase with the natural substrate is that the reaction appears to be essentially irreversible, and the unusually large number of enzyme-bound intermediates cannot be examined under equilibrium conditions. However, the steps that presumably occur prior to the β-elimination reaction (that is, substrate binding, substrate oxidation by enzyme-bound NAD$^+$, and substrate enolization) should be freely reversible, and analogues blocked at the elimination step should produce analogues of early enzyme-bound intermediates that may be studied in the absence of overall enzyme turnover.

There are several important unanswered questions concerning the early steps of the catalyzed reaction: binding, oxidation, and enolization. Does NAD$^+$ act as a redox cofactor in the mechanism as is implied in Scheme I? Is the open-chain form or a cyclic form of the substrate handled by the enzyme during these early steps? What is the conformation of the C-7

side chain bearing the phosphate leaving group when the substrate is bound at the active site? Is the elimination of P$_i$ stepwise (E1cB) or concerted (E2)? What is the identity of the base that accepts the proton in the β-elimination step? To answer these and other questions, analogues 3–9 (Chart I) have been synthesized. Two of these, 3 and 4, have been reported earlier. Thus, Le Maréchal and Azerad (1980) reported that the homophosphonate 4, although isosteric with DAHP, is a poor inhibitor of the synthase, whereas the phosphonate 3 is a potent competitive inhibitor. The new inhibitors studied here are cyclic carba-analogues of the isosteric and nonisosteric structural types defined by 3 and 4. Modeled after the phosphonate 3 are the carbacyclic phosphonate 5 and the carbacyclic phosphate 6, while the carbacyclic homophosphonate 7 and the Z- and E-vinylhomophosphonates 8 and 9 relate structurally to the homophosphonate 4. These carbacyclic analogues have several advantages: they are accessible synthetically from quinic acid, their redox potentials are more negative than those of the oxa counterparts, and there are no alternative forms (e.g., open chain or β-anomer) as are possible for the pyranoses 1, 3, and 4 due to the presence of

the anomeric center.

EXPERIMENTAL PROCEDURES

Materials. DAHP (**1**) and 3-deoxy-D-*arabino*-heptulosonate (DAH) were isolated from the growth medium of the auxotrophic strain of *Escherichia coli* JB-5, as previously described (Frost & Knowles, 1984). Dehydroquinate synthase was isolated and purified from *E. coli* RB791(pJB14) as described earlier (Frost et al., 1984). Dehydroquinate dehydratase (dehydroquinase) was purified from the overproducing strain of *E. coli* AB2848 (pKD201) according to the procedure of Coggins (Duncan et al., 1986), through the DEAE-Sephacel step to a specific catalytic activity of 175 units/mg. High-purity NAD^+ was grade I from Boehringer Mannheim (Indianapolis, IN). Adenosine diphosphoribose (ADPR) was obtained from Sigma (St. Louis, MO). Quinic acid, allyl-tri-*n*-butylstannane, and tetramethyl methylenediphosphonate, were from Aldrich (Milwaukee, WI).

[2R-(2α,4β,5α,6β)]-6-(Phosphonomethyl)-2,4,5-tri-hydroxytetrahydropyran-2-carboxylate (the Phosphonate Analogue **3**). To a solution of the methyl ester of DAH methyl pyranoside (Frost & Knowles, 1984) (150 mg, 0.64 mmol) in dry pyridine (2 mL) was added *p*-toluenesulfonyl chloride (150 mg, 0.77 mmol). The solution was stirred for 3 h at 0 °C, and acetic anhydride (0.25 mL) was then added. The solution was allowed to warm to room temperature over 30 min and was then concentrated under reduced pressure. The residue was purified by chromatography on silica, eluting with a gradient of dichloromethane to dichloromethane/ethyl acetate (19:1 v/v) to give the 7-*O*-tosylate diacetate (245 mg, 81%) as a viscous oil: ^1H NMR ($CDCl_3$, 300 MHz) δ 7.35 and 7.80 (2 d, 4 H, $J = 8$ Hz), 5.30 (ddd, 1 H, $J = 11$, 9, and 5 Hz), 4.90 (t, 1 H, $J = 10$ Hz), 4.15 (d, 2 H, $J = 4.5$ Hz), 3.92 (dt, 1 H, $J = 10$ and 4.5 Hz), 3.80 (s, 3 H), 3.23 (s, 3 H), 2.47 (s overlapping dd, 4 H), 2.00 and 2.02 (2 s, 6 H), 1.82 (dd, 1 H, $J = 13$ and 10.5 Hz).

A solution of the tosylate (245 mg, 0.52 mmol) with sodium iodide (0.80 g, 5.2 mmol) in dry acetone (4 mL) was heated at reflux for 3 h, during which time a precipitate formed. After cooling to room temperature, the mixture was partitioned between ethyl acetate/hexane (1:1 v/v) and dilute aqueous Na_2SO_3. The organic layer was washed sequentially with water and with brine, dried over Na_2SO_4, and concentrated. Purification of the residue by chromatography on silica afforded the C-7 iodide diacetate (203 mg, 88%) as a pale yellow oil: ^1H NMR ($CDCl_3$, 300 MHz) δ 5.32 (ddd, 1 H, $J = 11.5$, 9.4, and 5.4 Hz), 4.86 (t, 1 H, $J = 9.6$ Hz), 3.83 (s, 3 H), 3.74 (ddd, 1 H, $J = 9.7$, 8.8, and 2.5 Hz), 3.37 (s, 3 H), 3.35 (dd, 1 H, $J = 11$ and 2.5 Hz), 3.23 (dd, 1 H, $J = 11$ and 8.7 Hz), 2.50 (dd, 1 H, $J = 13$ and 5.4 Hz), 2.00 and 2.08 (2 s, 6 H), 1.86 (dd, 1 H, $J = 13$ and 10.5 Hz).

A solution of the iodide (203 mg, 0.45 mmol) in trimethyl phosphite (2 mL) was heated at reflux for 36 h. After cooling to room temperature, the solution was partitioned between dichloromethane and dilute aqueous H_2O_2. The aqueous layer was diluted with brine and the product extracted three times into dichloromethane. The combined organic layers were washed with brine, dried over Na_2SO_4, and concentrated. Purification of the residue by chromatography on silica gave a thick syrup, trituration of which with *tert*-butyl methyl ether/hexane provided the protected phosphonate (180 mg, 93%) as a white solid: ^1H NMR ($CDCl_3$, 300 MHz) δ 5.32 (ddd, 1 H, $J = 11.4$, 9.5, and 5.3 Hz), 4.83 (t, 1 H, $J = 9.7$ Hz), 4.13 (qd, 1 H, $J = 9.6$ and 3.1 Hz), 3.73 and 3.82 (2 d, 6 H, $J_{PH} = 11$ Hz), 3.80 (s, 3 H), 3.39 (s, 3 H), 2.55 (dd, 1 H, $J = 13$ and 5.4 Hz), 2.03–2.20 (m, 2 H), 2.00 and 2.06

(2 s, 6 H), 1.81 (dd, 1 H, $J = 13$ and 10.5 Hz).

To a solution of the C-7 dimethyl phosphonate diacetate (100 mg, 0.23 mmol) in dichloromethane (2 mL) was added trimethylsilyl bromide (0.2 mL). After 2 h, the solution was concentrated under reduced pressure, water (3 mL) was added to the residue, and the mixture was stirred for 1 h. The liquid supernatant was removed to give the phosphonic acid diacetate as a wet solid, which was then suspended in water (2 mL) at 0 °C. Dropwise addition of 2.5 N aqueous KOH (1 mL) gave a clear solution. After 15 min, this solution was passed through a column (5 mL) of Dowex 50 (H^+ form), and the eluant was concentrated to about 5 mL. A portion (1 mL) of 0.25 N aqueous HCl was added, and the solution was heated at 56 °C for 5 days. The solution was then cooled to room temperature, and the pH was adjusted to 4.5. The solution was applied to a column (15 mL) of AG1X8 (equilibrated with 0.20 N ammonium formate buffer, pH 4.5) and eluted with a linear gradient (200 mL plus 200 mL, 0.20–2.0 N) of ammonium formate buffer, pH 4.5. Fractions were analyzed with the thiobarbituric acid assay (Frost et al., 1984), and the active fractions were pooled and concentrated to dryness. The residue was dissolved in water, passed through a column of Dowex 50 (H^+ form), and concentrated again to give the phosphonate analogue **3** in high purity: ^1H NMR (free acid, D_2O, 300 MHz, referenced to HOD at 4.70 ppm) δ 3.90 (qd, 1 H, $J = 10.3$ and 3.3 Hz), 3.78 (ddd, 1 H, $J = 11.6$, 9.2, and 5.1 Hz), 3.09 (t, 1 H, $J = 9.4$ Hz), 2.24 (ddd, 1 H, $J = 19.9$, 15.6, and 2.4 Hz), 2.11 (dd, 1 H, $J = 13.1$ and 5.1 Hz), 1.87 (td, 1 H, $J = 15.6$ and 10.3 Hz), 1.70 (dd, 1 H, $J = 13$ and 11.7 Hz).

[2R-(2α,4β,5α,6β)]-6-(2-Phosphonoethyl)-2,4,5-tri-hydroxytetrahydropyran-2-carboxylate (the Homophosphonate Analogue **4**, *Scheme II*). To a solution of the lithium salt of DAH (300 mg, 1.3 mmol) in dry DMF (3 mL) was added benzyl bromide (0.35 mL, 3 mmol) that had been filtered through alumina. After 18 h, the solution was concentrated under reduced pressure to ~ 1 mL and then diluted with CH_2Cl_2 (5 mL). Chromatography on silica [35 g, eluting in three steps with 50%, 75%, and 100% (v/v) acetone/CH_2Cl_2] gave the benzyl ester (307 mg, 79%) as a thick syrup.

A solution of the benzyl ester (300 mg, 1 mmol) in dry pyridine (5 mL) was stirred and cooled at −50 °C as triethylsilyl triflate (1.13 mL, 5 mmol) was added dropwise. The reaction was allowed to warm from −50 °C to room temperature over 1 h, and then methanol (0.2 mL) was added. After 2 min, the reaction products were partitioned between CH_2Cl_2 in hexane (10% v/v) and water. The organic layer was washed sequentially with water, dilute aqueous $NaHSO_4$, and sodium phosphate buffer, pH 7. The washed organic layer was dried over anhydrous Na_2SO_4 and concentrated to give the ester **10** (690 mg, 92%, Scheme II), which was used without purification: ^1H NMR ($CDCl_3$, 500 MHz) δ 7.32–7.37 (m, 5 H), 5.16 and 5.22 (AB quartet, 2 H, $J = 12$ Hz), 3.95 (m, 1 H), 3.82 (dd, 1 H, $J = 11$ and 4.5 Hz), 3.74 (dd, 1 H, $J = 11$ and 0.5 Hz), 3.51–3.56 (m, 2 H), 2.16 (dd, 1 H, $J = 12.5$ and 4.5 Hz), 1.74 (dd, 1 H, $J = 12.5$ and 11.5 Hz), 0.90–1.00 (m, 36 H), 0.52–0.69 (m, 24 H).

To a solution of the unpurified ester **10** (690 mg) in THF/EtOH (25 mL, 3:2 v/v) at 0 °C was added 20 mM HCl (5 mL). After 90 min at 0 °C, pH 7 buffer was added. Aqueous workup, followed by chromatography on silica [eluting in three steps with ethyl acetate/hexane (10%, 25%, and 50% v/v)] provided the alcohol **11** (Scheme II, R = CH_2OH) (318 mg, 54%), the 4,7-diol (95 mg, 20%), and recovered **10** (77 mg, 11%): ^1H NMR of **11** (R = CH_2OH) ($CDCl_3$, 300 MHz) δ 7.32–7.37 (m, 5 H), 5.17 and 5.22 (AB

quartet, 2 H, J = 12 Hz), 4.00 (ddd, 1 H, J = 11, 8, and 4.5 Hz), 3.75 (br s, 2 H), 3.64 (dt, 1 H, J = 9 and 3.5 Hz), 3.50 (t, 1 H, J = 9 Hz), 2.21 (dd, 1 H, J = 12.5 and 4.5 Hz), 1.96 (br s, 1 H), 1.75 (dd, 1 H, J = 12.5 and 11.5 Hz), 0.89–1.00 (m, 27 H), 0.56–0.68 (m, 18 H).

Oxidation of the alcohol **11** (Scheme II, R = CH$_2$OH) (310 mg, 0.48 mmol) with oxalyl chloride (91 mg, 0.72 mmol), DMSO (124 mg, 1.6 mmol), and triethylamine (290 mg, 2.9 mmol) in dichloromethane according to the Swern protocol (Mancuso & Swern, 1981) afforded the corresponding aldehyde **11** (R = CHO) (309 mg, 100%).

To a solution of tetramethyl methylenediphosphonate (230 mg, 1 mmol) in THF (6 mL) at –78 °C was added *n*-butyllithium (0.33 mL of a 2.3 M solution in hexane). The solution was warmed briefly to 0 °C and recooled to –78 °C, and a solution of the unpurified aldehyde **11** (R = CHO, 309 mg, 0.48 mmol) in THF (3 mL) was then added by cannula. The mixture was allowed to warm to 0 °C over 30 min and then quenched with 1 M sodium phosphate buffer, pH 7. The reaction mixture was partitioned between hexane and water, and the organic layer was washed with brine, dried over anhydrous Na$_2$SO$_4$, and concentrated under reduced pressure. Purification of the residue by chromatography on silica [20 g, eluting with ethyl acetate/hexane (9:11 v/v)] gave the *E*-vinylhomophosphonate (307 mg, 86%) as a clear syrup: ^{1}H NMR (CDCl$_3$, 300 MHz) δ 7.32–7.37 (m, 5 H), 7.01 (ddd, 1 H, J = 22.9, 17.2, and 3.4 Hz), 6.01 (ddd, 1 H, J = 22.0, 17.2, and 2.0 Hz), 5.18 and 5.24 (AB quartet, 2 H, J = 12.3 Hz), 4.15–4.19 (m, 1 H), 4.01 (ddd, 1 H, J = 11.2, 8.4, and 4.6 Hz), 3.706 and 3.712 (2 d, 6 H, J = 11.1 Hz), 3.28 (t, 1 H, J = 9.0 Hz), 2.24 (dd, 1 H, J = 12.7 and 4.6 Hz), 1.77 (dd, 1 H, J = 12.7 and 11.2 Hz), 0.88, 0.95, and 0.96 (3 t, 27 H, J = 7.9 Hz), 0.52–0.69 (m, 18 H).

A mixture of the *E*-vinylhomophosphonate (100 mg, 0.13 mmol), triethylamine (22 mg, 0.22 mmol), and 10% Pd/C (20 mg) in ethanol (3 mL) was stirred under H$_2$ at 1 atm for 3 h. The catalyst was removed by filtration through Celite, and the filtrate was concentrated under reduced pressure. The residue was dissolved in ethyl acetate and concentrated to give the carboxylic acid, which was used without purification: ^{1}H NMR (CDCl$_3$, 300 MHz) δ 3.98 (ddd, 1 H, J = 10.5, 7.5, and 4.5 Hz), 3.75 and 3.77 (2 d overlapping m, 7 H, J = 11 Hz), 3.32 (dd, 1 H, J = 9 and 7.5 Hz), 2.18 (dd, 1 H, J = 13 and 4.5 Hz), 1.64–2.14 (m, 5 H), 0.97 (t, 18 H, J = 8 Hz), 0.60–0.70 (m, 12 H).

To a solution of the carboxylic acid from the above, with triethylamine (36 mg, 0.36 mmol) in dichloromethane (3 mL) at 0 °C, was added trimethylsilyl bromide (130 mg, 0.85 mmol). After 3 h, water (2 mL) was added, and the mixture was stirred vigorously for 2 h. The organic layer was removed, and the aqueous layer was passed through Dowex 50 (H$^+$ form) (1 mL). The pH of the eluate was adjusted to 4.5 with KOH, and the solution was concentrated under reduced pressure to give **4** as a white powder. The yield of **4** was 0.12 mmol (90%) as determined by quantitative analysis for inorganic phosphate after digestion with HClO$_4$. Ion-exchange chromatography on AG1X8 at pH 4.5 in formate buffer yielded pure material: ^{1}H NMR (D$_2$O, 500 MHz, pH 4.5, referenced to HOD at 4.70 ppm) δ 3.78 (ddd, 1 H, J = 11.5, 9, and 5 Hz), 3.61 (td, 1 H, J = 9 and 2.5 Hz), 3.16 (t, 1 H, J = 9 Hz), 2.07 (dd, 1 H, J = 13 and 5 Hz), 1.93–2.02 (m, 1 H), 1.45–1.78 (m, 4 H).

[*1R*-(*1α,3β,4α,5β*)]-5-(*Phosphonomethyl*)-*1,3,4-trihydroxycyclohexane-1-carboxylic Acid* (*the Carbaphosphonate Analogue* **5**, *Scheme III*). The protected hydroxymethyl

derivative synthesized in the following paper [methyl [*1R*-(1α,3β,4α,5β)]-5-(hydroxymethyl)-1,3,4-tris(benzoyloxy)cyclohexane-1-carboxylate (**11** in that paper)] (1.12 g, 2.5 mmol) was dissolved in THF (15 mL), and a mixture of triphenylphosphine (1.3 g, 5 mmol) and carbon tetrabromide (2.1 g, 6.25 mmol) was added. The deep yellow solution was stirred at room temperature for 2 h, after which time methanol (5 mL) was added and the mixture was stirred for a further 20 min. After removal of the solvent under reduced pressure, the residue was purified by chromatography on silica [eluting with ethereal dichloromethane (1:99 v/v)] to give the bromo compound (1.23 g, 96%) as a colorless oil: ^{1}H NMR (CDCl$_3$, 500 MHz) δ 8.20–7.29 (m, 15 H, Ar), 5.60 (m, 2 H), 3.78 (s, 3 H, OMe), 3.52 (d, 2 H, J = 4.3 Hz), 3.08 (ddd, 1 H, J = 3.1, 4.3, and 15.5 Hz), 2.83 (dt, 1 H, J = 3.3 and 14.7 Hz), 2.62 (m, 1 H), 2.26 (m, 2 H); ^{13}C NMR (CDCl$_3$, 75.6 MHz) δ 170.41, 165.57, 165.56, 164.78, 133.61, 133.05, 129.86, 129.59, 129.52, 129.22, 129.09, 128.56, 128.26, 128.22, 79.28, 73.96, 71.17, 52.79, 37.96, 36.14, 35.15, and 33.91.

The bromo compound [methyl [*1R*-(1α,3β,4α,5β)]-5-(bromomethyl)-1,3,4-tris(benzoyloxy)cyclohexane-1-carboxylate)] (0.23 g, 0.45 mmol) was dissolved in acetonitrile (10 mL), and sodium iodide (0.5 g, 3 mmol) in acetonitrile (5 mL) was added. After 5 min, the mixture was heated to 50 °C for 30 min. The solvent was then removed under reduced pressure, and the residue was dissolved in dichloromethane (100 mL). This solution was washed with aqueous sodium bisulfite (10% w/v), dried over magnesium sulfate, and concentrated to a yellow oil. This material was dissolved in trimethyl phosphite (20 mL), and the mixture was boiled under reflux for 24 h. After removal of the solvent, the product was purified by chromatography on silica (eluting with ethyl acetate). The protected phosphonate (80 mg, 63% after allowing for recovered iodo compound starting material) was obtained as a colorless oil.

The protected phosphonate [methyl [*1R*-(1α,3β,4α,5β)]-5-[(dimethoxyphosphinyl)methyl]-1,3,4-tris(benzoyloxy)cyclohexane-1-carboxylate)] (80 mg, 0.15 mmol, Scheme III) was dissolved in dichloromethane (5 mL), and trimethylsilyl bromide (1.16 g, 7.6 mmol) was added. The solution was stirred for 1 h, and the solvent was then removed under reduced pressure. Toluene (50 mL) was added and then removed under reduced pressure. THF (5 mL) was added and then water (2 mL), and the mixture was stirred vigorously for 10 min. After the addition of methanol (3 mL) and 1 N aqueous NaOH (2 mL), the solution was heated to 70 °C for 12 h. The solution was then cooled, water (15 mL) was added, and the mixture was passed through a short column of Amberlite IR 120 (H$^+$ form), followed by 2 column volumes of water. The combined aqueous fractions were washed with CHCl$_3$ (3 × 100 mL), the pH was raised to pH 8 with Na$_2$CO$_3$, and the solvent was removed under reduced pressure. The product phosphonate, **5** (0.15 mmol, 95%, Scheme III), had ^{1}H NMR (D$_2$O, 500 MHz) δ 3.59 (ddd, 1 H, J = 4.8, 9.2, and 12.1 Hz), 3.02 (t, 1 H, J = 9.6 Hz), 2.19 (ddd, 1 H, J = 2.7 and 15.6 Hz, J_{PH} = 20.3 Hz), 2.02 (dt, 1 H, J = 3.4 and 14.0 Hz), 1.94 (m, 2 H), 1.70 (dd, 1 H, J = 11.9 and 13.3 Hz), 1.60 (dd, 1 H, J = 12.9 and 13.8 Hz), 1.51 (ddd, 1 H, J = 7.1 and 15.6 Hz, J_{PH} = 18.2 Hz); ^{13}C NMR (D$_2$O, 75.6 MHz) δ 161.23, 71.96, 71.77, 67.05, 63.33, 32.67, 31.45, 26.29, 26.25, 22.28, and 17.49.

[*1R*-(*1α,3β,4α,5β*)]-*1,4,5-Trihydroxy-3-(phosphonooxy)cyclohexane-1-carboxylate* (*3-epi-Quinate 3-Phosphate* (**6**), *Scheme IV*). Methyl quinate (1.0 g, 5 mmol) and 1,8-bis-(dimethylamino)naphthalene (2.6 g, 12 mmol) were dissolved

in dry acetonitrile (15 mL) with warming to 50 °C. The solution was allowed to cool to 25 °C, and benzyl chloromethyl ether (1.5 mL, 1.7 g, 11 mmol) was added dropwise. After about 1 h, a precipitate began to form. The mixture was stirred for a total of 18 h at 25 °C and then partitioned between ethyl acetate/hexane (3:1 v/v) and 0.5 N aqueous NaHSO$_4$. The organic layer was washed with aqueous NaHSO$_4$ and then with brine, dried over Na$_2$SO$_4$, and concentrated. The residue was chromatographed on silica, eluting with increasing amounts (10%, 12%, 15% v/v) of acetone in dichloromethane. First to elute was the 3,4,5-tris[(benzoyloxy)methyl] ether, followed by the 3,5-bis ether, and then the desired 4,5-bis ether (450 mg, 20%): ^{1}H NMR (CDCl$_3$, 500 MHz) δ 7.27–7.39 (m, 10 H, Ar), 4.99–4.94 (2 H, q, J = 6.9 Hz), 4.90–4.84 (2 H, q, J = 6.9 Hz), 4.71–4.65 (2 H, q, J = 11.6 Hz), 4.66–4.60 (2 H, q, J = 11.7 Hz), 4.30–4.26 (m, 2 H), 4.15 (s, 1 H), 3.79 (s, 3 H, OMe), 3.68 (dd, 1 H, J = 2.9 and 9.5 Hz), 3.37 (d, 1 H, J = 5.0 Hz), 2.34 (ddd, 1 H, J = 3.2, 7.7 and 13.2 Hz), 2.16 (dt, 1 H, J = 3.3 and 14.7 Hz), 2.04 (dd, 1 H, J = 2.9 and 14.8 Hz), 1.89 (dd, 1 H, J = 11.5 and 13.1 Hz); ^{13}C NMR (CDCl$_3$, 75.6 MHz) δ 174.42, 137.80, 137.55, 128.47, 127.85, 127.78, 127.60, 94.62, 94.40, 80.67, 75.48, 72.05, 69.90, 69.67, 69.47, 52.86, 40.47, and 36.90.

To a suspension of chromium trioxide (0.1 g, 1 mmol) in dichloromethane (6 mL) was added pyridine (0.16 mL, 2 mmol). The mixture was stirred for 20 min, and Celite (1 g) was then added, followed by a solution of methyl 4,5-bis-[(benzyloxy)methyl]quinate (100 mg, 0.23 mmol) in dichloromethane (4 mL) and then acetic anhydride (100 mg, 1 mmol). The dark mixture was stirred for 20 min, and then ether (20 mL) was added. The mixture was filtered through a short column of silica, eluting with ethyl acetate. The filtrate was concentrated, and the residue was dissolved in ethyl acetate/hexane (1:1 v/v) and washed with 0.5 N aqueous NaHSO$_4$. The organic layer was then washed with 2 N sodium phosphate buffer (pH 7), dried over Na$_2$SO$_4$, and concentrated to give the ketone as a pale yellow oil that was used without further purification.

Acetic acid (0.6 mL) was *slowly* added to a suspension of NaBH$_4$ (76 mg, 2 mmol) in acetonitrile (4 mL). A portion (1 mL) of this solution of NaBH(OAc)$_3$ was added to a solution of the ketone obtained above in acetonitrile (2 mL) at 0 °C. After 1 h at 0 °C, an additional portion (0.5 mL) of the NaBH(OAc)$_3$ solution was added. The solution was stirred for 3 h at 25 °C and then partitioned between ethyl acetate/hexane (1:1 v/v) and 0.1 N aqueous NaHSO$_4$. The organic layer was washed with 2 N sodium phosphate buffer (pH 7), dried over Na$_2$SO$_4$, and concentrated. The residue was chromatographed on silica, eluting with ethyl acetate/hexane (55:45 v/v) to give the alcohol (86 mg, 82% for the two steps) as a colorless oil.

To a solution of the protected alcohol (140 mg, 0.3 mmol) in dry pyridine (2 mL) was added bis(2,2,2-trichloroethyl) phosphorochloridate (180 mg, 0.47 mmol). After 7 h at 25 °C, water (0.2 mL) was added. After 10 min, the reaction was partitioned between ethyl acetate/hexane (1:1 v/v) and 0.5 N aqueous NaHSO$_4$. The organic layer was washed with aqueous NaHCO$_3$ and then brine, dried over Na$_2$SO$_4$, and concentrated. Purification of the residue by chromatography on silica eluting with ethyl acetate/hexane (4:6 v/v) afforded the phosphate triester (160 mg, 65%) as a colorless syrup. The phosphate triester (160 mg, 0.2 mmol) was dissolved in methanol (3 mL), and Zn dust (200 mg, 3.65 mmol) was added. To this vigorously stirred solution was added acetic

acid (10 drops). The mixture was stirred for 1 h and the supernatant isolated by decantation. The solid residue was washed with methanol (5 mL), and the combined organic solutions were concentrated under reduced pressure. The residue was dissolved in dichloromethane (10 mL), and this solution was washed with 0.5 N sodium bisulfate (2 × 10 mL). The combined aqueous washings were reextracted with dichloromethane, and the combined organic extracts were dried over sodium sulfate and concentrated to a colorless syrup (103 mg, 96%).

To a solution of the unpurified phosphate from the above in methanol (3 mL) was added a portion (25 mg) of 10% Pd/C. The mixture was stirred for 14 h under H$_2$ (1 atm), and the catalyst was removed by filtration through Celite. The filtrate was concentrated, and the residue was dissolved in water (2 mL) to which 2.5 N aqueous KOH was added. After 2 h, the solution was passed through a column (5 mL) of Dowex 50 (H$^+$ form) resin. Concentration of the filtrate provided the product 6 in ∼90% purity (by ^{1}H NMR analysis). The yield of phosphate monoester was 0.14 mmol (70%), as determined by treatment of a portion with alkaline phosphatase followed by quantitative analysis for inorganic phosphate: ^{1}H NMR (D$_2$O, 500 MHz) δ 4.08 (m, 1 H), 3.69 (m, 1 H), 3.36 (t, 1 H, J = 9.2 Hz), 2.06 (ddd, 1 H, J = 2.7, 4.9, and 13.5 Hz), 1.87 (ddd, 1 H, J = 2.8, 4.9, and 13.4 Hz), 1.82 (dd, 1 H, J = 11.8 and 13.5 Hz), 1.75 (dd, 1 H, J = 11.9 and 13.3 Hz).

[1R-(1α,3β,4α,5β)]-5-(2-Phosphonoethyl)-1,3,4-trihydroxycyclohexane-1-carboxylic Acid (the Carbacyclic Homophosphonate Analogue 7, Scheme V). The carbacyclic allyl compound described in the following paper [methyl [1R-(1α,3β,4α,5β)]-[5-(3-propenyl)]-1,3,4-tris(benzoyloxy)-cyclohexane-1-carboxylate (9 in that paper)] (2.5 g, 5 mmol) was dissolved in methanolic dichloromethane (1:1 v/v), pyridine (5 drops) was added, and the solution was cooled to −78 °C. Ozone was bubbled through the solution until a blue color persisted. Oxygen was then bubbled through the mixture for 10 min, followed by nitrogen for 20 min. Sodium borohydride (2 g) was added in portions to the stirred solution over 1 h, and stirring was continued for a further hour at −78 °C. Acetic acid (4 mL) was then added, the solution was allowed to warm to room temperature, and the mixture was partitioned between water and ethereal dichloromethane (1:1 v/v). The organic phase was washed with saturated aqueous NaHCO$_3$ (100 mL), followed by brine (100 mL). The solution was dried over MgSO$_4$, and the solvent was removed under reduced pressure to give the corresponding protected hydroxyethyl compound. This material was purified by chromatography on silica [eluting with ethereal dichloromethane (1:4 v/v)] to give a colorless oil (2.2 g, 88%): ^{1}H NMR (CDCl$_3$, 500 MHz) δ 8.22–7.30 (m, 15 H, Ar), 5.49 (m, 1 H), 5.36 (dd, 1 H, J = 9.9 and 10.8 Hz), 3.68 (s, 3 H, OCH$_3$), 3.61 (m, 2 H, CH$_2$OH), 2.97 (ddd, 1 H, J = 3.1, 4.8, and 15.6 Hz), 2.69 (dt, 1 H, J = 3.3 and 14.6 Hz), 2.35 (m, 1 H), 2.14 (dd, 1 H, J = 11.8 and 13.7 Hz), 1.93 (dd, 1 H, J = 13.1 and 14.5 Hz), 1.80 (m, 1 H), 1.49 (m, 1 H); ^{13}C NMR (CDCl$_3$, 75.6 MHz) δ 170.93, 166.42, 165.71, 165.02, 133.55, 133.32, 133.07, 132.98, 129.93, 129.76, 129.64, 129.51, 129.33, 128.92, 128.58, 128.44, 128.39, 128.24, 79.64, 76.48, 71.55, 59.74, 36.29, 35.52, 33.69, and 33.40.

The protected hydroxyethyl compound [methyl [1R-(1α,3β,4α,5β)]-5-(2-hydroxyethyl)-1,3,4-tris(benzoyloxy)-cyclohexane-1-carboxylate] (1.37 g, 2.5 mmol) was converted to the bromo compound exactly as described for the hydroxymethyl homologue, above. The bromo compound (1.2 g,

97%) was obtained as a colorless oil: ^{1}H NMR (CDCl$_3$, 500 MHz) δ 8.20–7.29 (m, 15 H, Ar), 5.59 (ddd, 1 H, J = 4.9, 9.7, and 11.7 Hz), 5.42 (dd, 1 H, J = 9.8 and 10.8 Hz), 3.77 (s, 3 H, OCH_3), 3.50 (m, 1 H), 3.37 (m, 1 H), 3.08 (ddd, 1 H, J = 3.0, 4.8, and 14.8 Hz), 2.77 (dt, 1 H, J = 3.4 and 14.4 Hz), 2.54 (m, 1 H), 2.23 (dd, 1 H, J = 11.8 and 13.8 Hz), 2.17 (m, 1 H), 1.92 (dd, 1 H, J = 13.2 and 14.4 Hz), 1.85 (m, 1 H); ^{13}C NMR (CDCl$_3$, 75.6 MHz) δ 170.81, 166.27, 165.64, 133.57, 133.45, 133.36, 133.17, 133.02, 129.95, 129.83, 129.79, 129.66, 129.53, 129.44, 129.29, 128.58, 128.48, 128.41, 128.34, 128.26, 79.26, 75.97, 71.38, 52.77, 36.26, 34.87, 33.94, and 30.06.

The bromo compound [methyl [1R-(1α,3β,4α,5β)]-5-(2-bromoethyl)-1,3,4-tris(benzoyloxy)cyclohexane-1-carboxylate] (1.0 g, 1.66 mmol) was converted into the protected homophosphonate (0.8 g, 76%) as described above for the lower homologue, except that only 12 h under reflux was required.

The protected homophosphonate [methyl [1R-(1α,3β,4α,5β)]-5-[2-(dimethoxyphosphinyl)ethyl]-1,3,4-tris(benzoyloxy)cyclohexane-1-carboxylate] (450 mg, 0.79 mmol) was deprotected exactly as described above for the lower homologue to give the carbacyclic homophosphonate 7 in 95% yield: ^{1}H NMR (D$_2$O, 500 MHz) δ 3.59 (ddd, 1 H, J = 4.8, 9.1, and 11.8 Hz), 3.05 (t, 1 H, J = 9.7 Hz), 1.97 (ddd, 1 H, J = 3.1, 4.7, and 13.4 Hz), 1.86 (m, 1 H), 1.77–1.52 (m, 5 H), 1.50 (t, 1 H, J = 13.4 Hz), 1.38 (m, 1 H); ^{13}C NMR (D$_2$O, 75.6 MHz) δ 183.36, 82.19, 79.02, 75.48, 44.53, 42.52, 42.29, 41.71, 28.94, 28.65, 28.60, and 27.15.

[1R-(1α,3β,4α,5β)]-5-[(Z)-(2-Phosphonoethenyl)]-1,3,4-trihydroxycyclohexane-1-carboxylic Acid (the Carbacyclic (Z)-Vinylhomophosphonate Analogue 8). Trimethylsilyl methyl dimethylphosphonate (0.59 g, 3 mmol) (Savignac & Mathey, 1982) was dissolved in THF (4 mL) and cooled to –78 °C. n-Butyllithium (1 mL of a solution 2.5 N in hexane, 2.5 mmol) was added, and the solution was stirred at –78 °C for 4 h. The aldehyde tribenzoate methyl ester described in the following paper [methyl [1R-(1α,3β,4α,5β)]-5-formyl-1,3,4-tris(benzoyloxy)cyclohexane-1-carboxylate (10 in that paper)] (0.89 g, 2 mmol) was added at –78 °C, and the solution left at –78 °C for 20 min before being allowed to warm to room temperature. Acetic acid (4 mL) was then added, and the solvent was removed under reduced pressure. Flash chromatography on silica (eluting with ethyl acetate) gave the protected (Z)-vinylhomophosphonate as a colorless oil (0.4 g, 36%): ^{1}H NMR (CDCl$_3$, 500 MHz) δ 8.23–7.32 (15 H, Ar), 6.43 (ddd, 1 H, J = 10.2 and 12.9 Hz, J_{PH} = 54.9 Hz), 5.70–5.62 (m, 2 H), 5.51 (t, 1 H, J = 10.4 Hz), 4.03 (m, 1 H), 3.77 (s, 3 H, OMe), 3.49 (d, 3 H, OMe, J_{PH} = 11.2 Hz), 3.45 (d, 3 H, OMe, J_{PH} = 11.2 Hz), 3.07 (ddd, 1 H, J = 3.1, 4.9, and 13.8 Hz), 2.62 (dt, 1 H, J = 3.5 and 14.3 Hz), 2.24 (dd, 1 H, J = 11.8 and 13.9 Hz), 2.14 (dd, 1 H, J = 13.0 and 14.5 Hz).

The protected (Z)-vinylhomophosphonate (0.22 g, 0.4 mmol) was dissolved in dichloromethane (5 mL), and trimethylsilyl bromide (1.16 g, 7.6 mmol) was added. The mixture was stirred for 1 h, and the solvent was then removed by evaporation under reduced pressure. Toluene (50 mL) was added, and the solvent was again removed under reduced pressure. THF (5 mL) was then added, followed by water (2 mL), and the solution was stirred for 10 min. After the addition of methanol (3 mL) and 1 N NaOH (3 mL), the solution was heated for 12 h at 70 °C. The cooled solution was then diluted with water (15 mL) and filtered through a column of Amberlite IR 120 (H$^+$ form). The column was washed with water, and the combined eluates were washed with CHCl$_3$ (3

× 100 mL) before the pH was adjusted to pH 8 with sodium carbonate. The solvent was then removed under reduced pressure. The deprotected product 8 had ^{1}H NMR (D$_2$O, 500 MHz) δ 5.78 (dd, 1 H, J = 13.0 and 15.0 Hz), 5.65 (ddd, 1 H, J = 9.7 and 12.8 Hz, J_{PH} = 42.1 Hz), 3.66 (ddd, 1 H, J = 4.8, 9.2, and 11.6 Hz), 3.23 (m, 1 H), 2.98 (t, 1 H, J = 9.8 Hz), 1.83 (m, 1 H), 1.69 (t, 1 H, J = 12.0 Hz), 1.65–1.57 (m, 2 H).

[1R-(1α,3β,4α,5β)]-5-[(E)-(2-Phosphonoethenyl)]-1,3,4-trihydroxycyclohexane-1-carboxylic Acid (the Carbacyclic (E)-Vinylhomophosphonate Analogue 9). Tetramethyl methylenediphosphonate (0.33 g, 3 mmol) was dissolved in THF (5 mL) and cooled to –78 °C. n-Butyllithium (1 mL of a solution 2.5 N in hexane, 2.5 mmol) was added over 5 min, and the solution was stirred at –78 °C for 30 min. The aldehyde (10 in the following paper) was added, and the solution was stirred at –78 °C for 20 min before being allowed to warm to room temperature. Acetic acid (2 mL) was then added, and the solvent was removed by evaporation under reduced pressure. The residue was dissolved in dichloromethane (100 mL) and washed successively with saturated aqueous sodium bicarbonate (2 × 100 mL) and with water (100 mL). Solvent was removed from the dried solution by evaporation to give the protected (E)-vinylhomophosphonate (1.15 g, 97%): ^{1}H NMR (CDCl$_3$, 500 MHz) δ 8.15–7.30 (m, 15 H, Ar), 6.66 (ddd, 1 H, J = 8.1 and 17.1 Hz, J_{PH} = 21.6 Hz), 5.75 (ddd, 1 H, J = 0.7 and 17.1 Hz, J_{PH} = 18.1 Hz), 5.64 (ddd, 1 H, J = 4.8, 9.9, and 11.7 Hz), 5.50 (t, 1 H, J = 9.9 Hz), 3.75 (s, 3 H, OMe), 3.54 (d, 3 H, OMe, J_{PH} = 11.1 Hz), 3.45 (d, 3 H, OMe, J_{PH} = 11.1 Hz), 3.07–2.98 (m, 2 H), 2.73 (dt, 1 H, J = 3.4 and 14.7 Hz), 2.23 (t, 2 H, J = 12.0 Hz).

The protected (E)-vinylhomophosphonate was then deprotected exactly as described for the Z isomer, above, to give the product 9: ^{1}H NMR (CDCl$_3$, 500 MHz) δ 5.95 (dt, 1 H, J = 8.3 and 17.0 Hz), 5.74 (t, 1 H, J = 16.6 Hz), 3.62 (ddd, 1 H, J = 4.9, 9.5, and 11.8 Hz), 3.22 (t, 1 H, J = 9.9 Hz), 2.29 (m, 1 H), 1.85 (ddd, 1 H, J = 2.8, 4.7, and 13.1 Hz), 1.72 (m, 2 H), 1.56 (dt, 1 H, J = 3.3 and 14.0 Hz).

Methods. Spectrophotometric assays were made on a Hewlett-Packard 8452A diode array spectrophotometer or on a Kontron Uvikon spectrophotometer. Assay solutions (1.00 mL) for dehydroquinate synthase were made up in deionized, glass-distilled water containing 50 mM MOPS buffer, pH 7.75, CoSO$_4$ (50 μM), NAD$^+$ (10 μM), DAHP (0.50 mM), and dehydroquinase (1 unit). After incubation at 20 °C for 5 min, dehydroquinate synthase (10–50 milliunits) was added, and the production of dehydroshikimate was monitored spectrophotometrically at 234 nm. The initial reaction rate was calculated from a linear least-squares fit of the first 30 s of the progress curve. The effective extinction coefficient for the coupled assay under these conditions was 9850 M^{-1} cm^{-1} (Bender et al., 1989). One unit of enzyme activity catalyzes the consumption of 1 μmol of DAHP/min at 20 °C.

Determination of the Rate Constant for the Dissociation of 5 from Dehydroquinate Synthase. A solution of the complex of dehydroquinate synthase with 5 was prepared by adding 5 (20 μL of a 0.12 mM solution, 2.4 nmol) to a solution of dehydroquinate synthase (2.25 units, 0.75 nmol) in 100 mM MOPS buffer, pH 7.5 (1 mL), containing NAD$^+$ (20 μM) and CoSO$_4$ (100 μM). A portion (10 μL) of this solution was assayed for catalytic activity in the usual way (see above) except that the DAHP concentration was 700 μM and the pH was 7.0. The first 13 min of the progress curve was fitted to the expression: absorbance = $at + be^{-kt} + c$. The value of k_{off}, the first-order rate constant for the dissociation of 5, was

found to be 3.4×10^{-3} s^{-1}. The dissociation rate constant at pH 7.5 was determined similarly, except that in the solution of the complex of **5** and the enzyme, the total enzyme concentration was 0.5 μM, and the total concentration of **5** was 0.75 μM. The first 25 min of the progress curve was fitted to give $k_{off} = 8.3 \times 10^{-4}$ s^{-1}.

Determination of the Rate Constant for the Association of **5** *with Dehydroquinate Synthase.* Assays were conducted as usual (see above) except that the substrate solution also contained the phosphonate **5**. The early part of the progress curve was fitted to a simple exponential to obtain the first-order rate constant for loss of activity, k_{obsd}. The use of an exponential expression was justified since the residual reaction velocity at long times was <10% of the initial velocity. The association rate, k_{on}, was then obtained from

$$k_{obsd} = k_{on} [5]/(1 + [DAHP]/K_m) + k_{off}$$

Since the concentration of DAHP was much greater than its K_m, this expression simplifies to

$$k_{on} = (k_{obsd} - k_{off})[DAHP]/K_m[5]$$

The value of k_{obsd} was shown to be independent of the concentration of DAHP or of **5**. The mean values of k_{on} from these experiments were 1.0×10^6 M^{-1} s^{-1} at pH 7.5 and 2.7 $\times 10^6$ M^{-1} s^{-1} at pH 7.0, by use of $k_{off} = 8.3 \times 10^{-4}$ s^{-1} and $K_m = 4.0$ μM at pH 7.5 and $k_{off} = 3.4 \times 10^{-3}$ s^{-1} and $K_m = 1.2$ μM at pH 7.0.

Determination of the Concentration of Enzyme-Bound NADH in the Presence of Inhibitors. A solution (0.70 mL) of dehydroquinate synthase at a known concentration between 10 and 20 μM in 50 mM MOPS buffer, pH 7.9, containing CoSO$_4$ (50 μM) and NAD$^+$ (10 μM) was incubated at 20 °C in a quartz cuvette. Spectra (250–600 nm) were acquired at a rate of 10/min, and the difference in absorbance at 338 and 420 nm was monitored. After a few scans to establish the base-line absorbance, a small portion (2–20 μL) of a solution of the inhibitor was added such that the inhibitor concentration was at least 10 times its K_i (and greater than the enzyme concentration). The pH of the solution was varied by additions (4 μL) of 1.5 M aqueous acetic acid. The change in absorbance from the base line was determined at each pH, and the concentration of enzyme-bound NADH was calculated by using an extinction coefficient of 5.4×10^3 M^{-1} cm^{-1} (see Results and Discussion).

Determination of the Rate Constant for the Dissociation of NAD$^+$ in the Presence of Substrate Analogues. The complex of enzyme, oxidized substrate analogue, and NADH was formed as described above and then added at pH 7.5 to buffer solution containing ADPR (100 μM). The decrease in the absorbance at 338 nm with time was fitted to an exponential expression to obtain the first-order rate constant for the dissociation of NAD$^+$ from the complex of enzyme and substrate analogue. For the phosphonate **3** and the homophosphonate **4**, thio-NAD$^+$ (50 μM) was used instead of ADPR as the displacing ligand. In this case, the absorbance at 400 nm (corresponding to thio-NADH) increased as the absorbance at 338 nm decreased.

Deuterium Exchange at C-6 of the Homophosphonate **4** *Catalyzed by Dehydroquinate Synthase.* A solution of buffer in D$_2$O was prepared by lyophilizing 50 mM Tris–trifluoroacetate buffer, pH 7.5, and dissolving the residue in D$_2$O. A concentrated solution of dehydroquinate synthase in D$_2$O was prepared by repeatedly concentrating the enzyme solution by ultrafiltration and rediluting with deuteriated buffer containing CoSO$_4$ (100 μM) and NAD$^+$ (20 μM). The catalytic activity of dehydroquinate synthase in deuteriated buffer was ap-

proximately 70% of the activity observed with the same buffer in H$_2$O.

To a solution of the homophosphonate (15 μmol) in 50 mM Tris–trifluoroacetate buffer, pH 7.5 (3 mL), in D$_2$O containing CoSO$_4$ (100 μM) and NAD$^+$ (20 μM) at 20 °C was added dehydroquinate synthase (15 units) in the same buffer. At timed intervals, portions (0.5 mL) were quenched into 20% aqueous formic acid (0.1 mL). The solutions were passed through a small column (3 mL) of Dowex 50 (H$^+$ form), and the eluate was concentrated under reduced pressure, redissolved in D$_2$O, and then lyophilized. The residue was then analyzed by ^{1}H NMR (500 MHz), and the extent of deuterium incorporation at C-6 was estimated by the difference in the integration of the multiplet for the C-6 proton (at 3.55 ppm) relative to the multiplet for the proton at C-4 (at 3.75 ppm). The fraction of ^{1}H remaining at C-6 as a function of time gave good first-order plots, from which the rate constant for ^{2}H exchange was obtained. Deuterium exchange experiments for other analogues were conducted analogously, except that the amount of enzyme was increased to achieve observable exchange rates (or to set a lower limit on the exchange rate).

RESULTS AND DISCUSSION

Synthesis of Substrate Analogues. The phosphonate analogue **3** of the natural substrate DAHP (**1**) was prepared from DAH by a slight modification of the method of Reimer et al. (1986), in which the Arbusov formation of the P–C bond was achieved by reaction of trimethyl phosphite with the appropriately protected primary iodide. DAH was also used as the starting material in the preparation of the homophosphonate analogue **4**, but a more versatile protection scheme was developed for this application, and this is illustrated in Scheme II. Treatment of the lithium salt of DAH with benzyl bromide in DMF provided the benzyl ester of DAH, persilylation of which with triethylsilyl triflate in pyridine proceeded smoothly to give the protected derivative **10**. The primary alcohol **11** (R = CH$_2$OH) was obtained by monodesilylation in aqueous HCl/THF, Swern oxidation of which provided the corresponding aldehyde. This aldehyde was subjected to Horner–Emmons olefination with the lithium salt of tetramethyl methylenediphosphonate. Hydrogenation and deprotection then provided the desired homophosphonate analogue **4**.

The carbacyclic phosphonate **5** was synthesized as shown in Scheme III. The protected hydroxymethyl derivative of quinic acid was converted via the bromo compound to the iodomethyl derivative. The iodo compound was then allowed to react with boiling trimethyl phosphite in the Arbusov transformation to give the dimethyl phosphonate ester in 50% yield. Deprotection with trimethylsilyl bromide and saponification then produced the carbacyclic phosphonate **5**. The carbacyclic homophosphonate **7** was synthesized analogously from the allylated derivative of quinic acid reported in the following paper, as summarized in Scheme V.

The carbacyclic phosphate **6** was prepared from commercially available quinic acid as shown in Scheme IV. Direct etherification of methyl quinate with benzyl chloromethyl ether afforded a mixture of regioisomers from which the desired 4,5-bis(benzyloxymethyl) ether was readily isolated by flash chromatography. The remaining secondary alcohol (at C-3) was oxidized with Collins reagent, and the required inversion of stereochemistry at C-3 was then achieved by a hydroxyl-directed delivery of hydride from sodium triacetoxyborohydride (Evans et al., 1988). This highly stereoselective reaction appears to be the first reported example of the use of a tertiary hydroxyl group to direct this reagent. The inverted alcohol was then converted to the phosphate monoester, deprotection

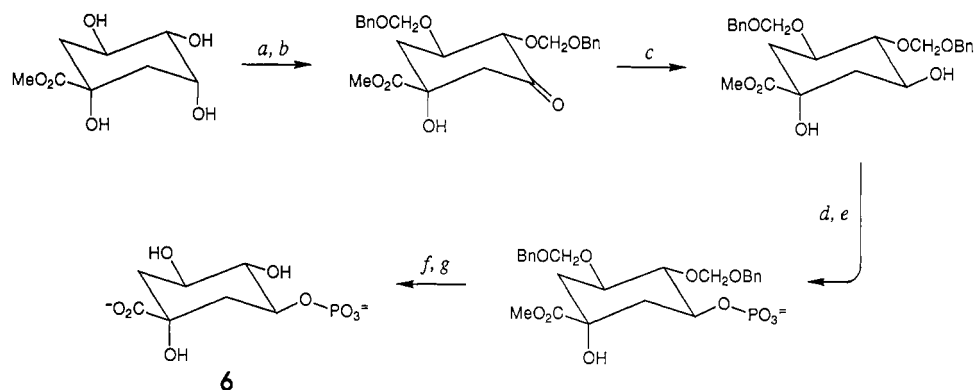Scheme II: Synthetic Route to the Homophosphonate Analogue **4**
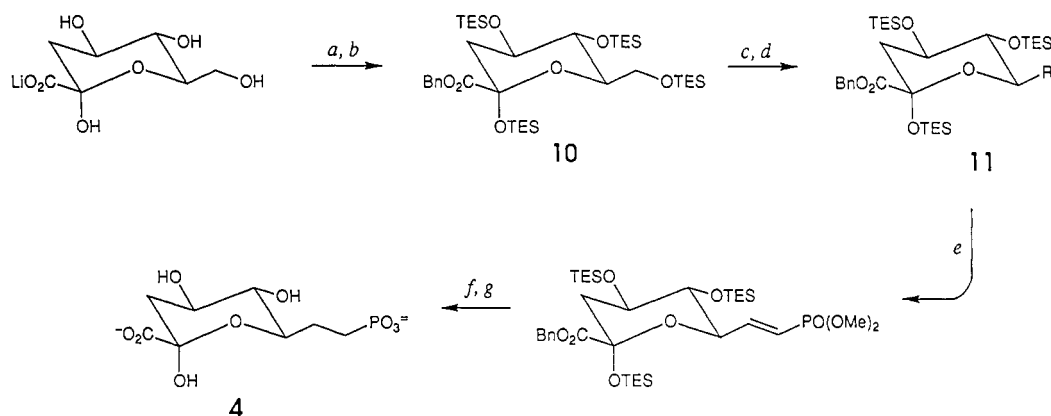
a Benzyl bromide, DMF. *b* Triethylsilyl triflate. *c* HCl (to **11**, R = CH₂OH). *d* Oxalyl chloride, DMSO, triethylamine (to **11**, R = CHO). *e* Tetramethyl methylenediphosphonate, *n*-butyllithium. *f* H₂, Pd/C, EtOH. *g* Trimethylsilyl bromide.

Scheme III: Synthetic Route to the Phosphonate Analogue **5**
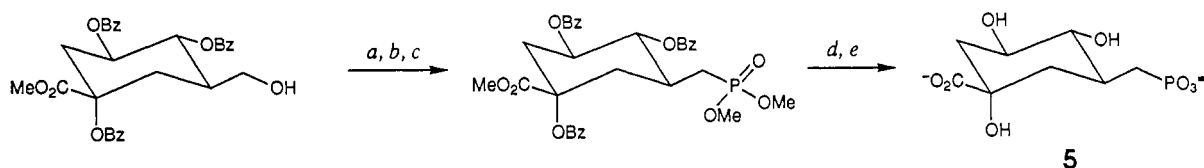
a CBr₄, Ph₃P, THF. *b* NaI, acetonitrile. *c* Trimethyl phosphite. *d* Trimethylsilyl bromide. *e* NaOH.

Scheme IV: Synthetic Route to 3-*epi*-Quinate 3-Phosphate (**6**)

a Benzyl chloromethyl ether. *b* CrO₃, pyridine. *c* NaBH(OAc)₃. *d* Bis(2,2,2-trichloroethyl) phosphorochloridate, pyridine. *e* Zn, MeOH. *f* H₂, Pd/C. *g* KOH.

Scheme V: Synthetic Route to the Carbacyclic Homophosphonate Analogue **7**
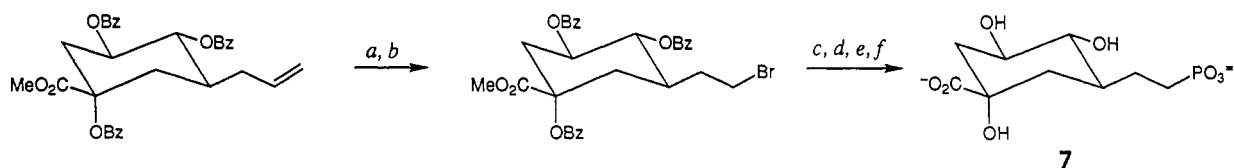
a O₃, then NaBH₄. *b* CBr₄, Ph₃P, THF. *c* NaI, acetonitrile. *d* Trimethyl phosphite. *e* Trimethylsilyl bromide. *f* NaOH.

of which provided 3-*epi*-quinic acid 3-phosphate (**6**).

The carbacyclic *cis*- and *trans*-vinylhomophosphonates **8** and **9**, respectively, were produced from the protected aldehyde (**10** under Experimental Procedures of the following paper). The Horner–Emmons reaction using tetramethyl methylenediphosphonate gave good yields of the *trans*-vinylhomophosphonate, though none of the *cis*-vinyl isomer was detected. The *cis*-vinylhomophosphonate was therefore synthesized by a Peterson-type olefination reaction. It was hoped that, because the addition to carbonyl is irreversible, the stereochem-

istry of the resulting double bond would be determined by the stereochemistry of the addition reaction (rather than by the greater thermodynamic stability of the trans isomer). This expectation was fulfilled, and the contaminating trans isomer was easily removed by chromatography on silica. Deprotection of each isomer was achieved without isomerization by using trimethylsilyl bromide followed by saponification of the esters.

Interaction of Substrate Analogues with the Enzyme. The seven substrate analogues (**3–9**) described in this paper were designed to probe the early steps in the mechanistic pathway

Table I: Inhibition Constants of the Substrate Analogues 3–9 with Dehydroquinate Synthase

inhibitor	K_m/K_i		
	pH 7.0	pH 7.5	pH 8.0
3	10	54	180
4	ND[a]	0.07	ND
5	1000	5000	ND
6	ND	2	ND
7	ND	2	ND
8	3	25	80
9	ND	0.16	ND

[a] ND, not determined.

that appears to be followed by dehydroquinate synthase and which is outlined in Scheme I. In the discussion that follows, we shall examine each step of the enzyme reaction in turn and describe the behavior of each analogue as it bears upon the nature of the step under scrutiny.

Binding to the Enzyme. Each of the analogues (3–9) was found to inhibit the synthase. For all except the carbacyclic phosphonate 5, the inhibition constants could be measured from initial velocity studies, and inhibition was found to be competitive. Compound 5 is a slow binding inhibitor, however, and is discussed separately below. The inhibition constants are listed in Table I as the ratio of the K_m value for the substrate DAHP to the K_i value of the inhibitor. Uniformly, the binding affinity of these analogues is enhanced as the pH increases. Since the phosphonates undergo their second ionization in the pH range studied, this trend suggests that it is the phosphonate *di*anion that is bound to the enzyme.

In agreement with previous work (Le Maréchal et al., 1980), the phosphonate 3 is a much more potent inhibitor than the homophosphonate 4, by nearly 3 orders of magnitude. Thus, although the homophosphonate 4 is a closer structural analogue of the substrate DAHP than is the phosphonate 3, it is evident that subtle structural alterations can have dramatic consequences in analogue binding affinity. For instance, replacement of the ring oxygen in the homophosphonate 4 with a methylene group to form the carbacyclic species 7 results in a 25-fold decrease in the K_i. This trend is repeated for the phosphonates, and the carbacyclic phosphonate 5 binds 100 times more tightly than the simple phosphonate analogue of the pyranose 3. This material, 5, is the most potent inhibitor of dehydroquinate synthase known. Compound 5 is a slow binding inhibitor, the K_i of which cannot be measured from initial velocity data. To obtain the dissociation constant, the rate constants for association to and dissociation from the enzyme were measured independently. The association rate constant was determined to be 1.0×10^6 M^{-1} s^{-1} from the progress curve obtained when the enzyme was mixed with substrate DAHP in the presence of 5 according to the method of Williams and Morrison (1979). The dissociation rate was found to be 8×10^{-4} s^{-1} from the progress curve that is observed when the preformed complex of enzyme with 5 was added to a high concentration of substrate. A sample curve is shown in Figure 1. From these rate constants, K_i was calculated to be 0.8 nM at pH 7.5. This can be compared to the K_m for DAHP of 4 μM under these conditions.

The *cis*- and *trans*-vinylhomophosphonates 8 and 9 probe another aspect of substrate binding, namely, the side-chain conformation. Molecular mechanics calculations [using the MM2 force field in the MACROMODEL version 2.0 software package (W. C. Still et al., Columbia University)] suggest that, as expected intuitively, the lowest energy conformation of 8 has the proton at C-6 in the plane of the double bond, as shown in Figure 2. In contrast, the trans isomer 9 has much greater
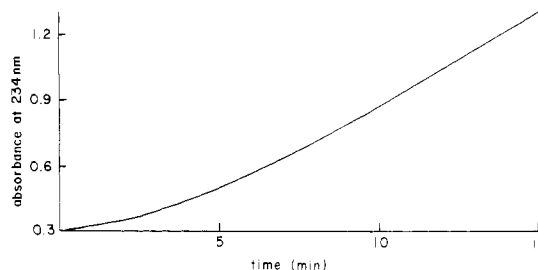

FIGURE 1: Dissociation of the carbaphosphonate 5 from dehydroquinate synthase. Carbaphosphonate 5 (2.4 μM) was incubated with dehydroquinate synthase (0.75 μM) and then diluted 100-fold into assay buffer at pH 7.0 containing substrate DAHP (0.70 mM). The absorbance was monitored at 234 nm.
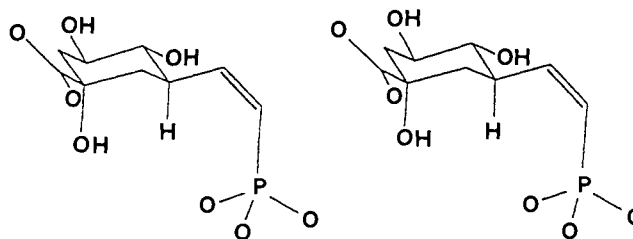

FIGURE 2: Stereoview of the favored conformation calculated for the *cis*-vinylhomophosphonate 8.

rotational freedom about the exocyclic single bond (that between C-6 and C-7) and can adopt several extended conformations. The inhibition data for these two analogues show that the cis isomer 8 binds about 2 orders of magnitude more tightly than the trans isomer 9. The *cis*-vinylhomophosphonate even binds 10 times more tightly than the unconstrained analogue 7. These inhibition results are consistent with the preferred binding mode to the active site having a side-chain conformation similar to that of the most stable conformation of 8. For the rotationally unrestricted structures such as DAHP (1) and analogues 4 and 7, this gauche conformation is sterically disfavored relative to the extended conformations that predominate in free solution, thus reducing the binding affinity of these unconstrained derivatives relative to the preorganized species, 8. The low but significant affinity of the trans isomer 9 may indicate that the more highly populated extended conformations of the substrate DAHP initially bind with low affinity and subsequently rearrange to the tightly bound conformation that is mimicked by 8. As will be seen later, a preferred binding mode like that adopted by 8 is supported by the proton-exchange behavior of analogues 3, 4, 8, and 9.

The First Chemical Step: Oxidation of Substrate at C-5. The first chemical step in the mechanism outlined in Scheme I is the oxidation at C-5 of the substrate, 1, by NAD^{+}. With the natural substrate DAHP, the resulting NADH would be expected to be present at some steady-state concentration that is less than the total enzyme concentration and that corresponds to the fraction of enzyme having intermediates I, II, or III bound to it. A search for enzyme-bound NADH during the enzyme-catalyzed reaction of the natural substrate DAHP was, however, in vain. No transient absorbance at 340 nm could be detected, nor was NADH found by fluorescence spectroscopy of the reaction at the steady state. It seems that either the fluorescence of the transiently formed NADH is quenched by the protein or that the steady-state levels of intermediates I–III (Scheme I) are too low to allow their detection by these methods. [We should note that the trapping of I by tritiated sodium borohydride has been reported (Maitra & Sprinson, 1978), and it was suggested that 15% of the
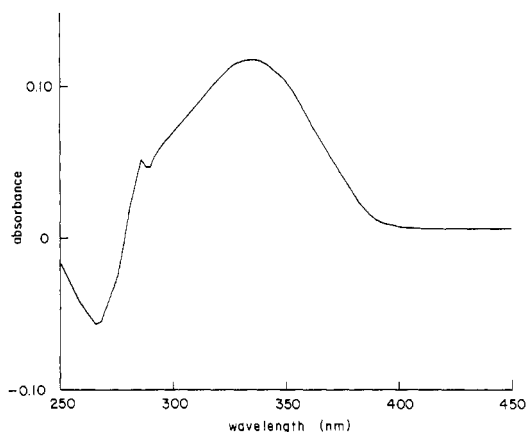
FIGURE 3: Ultraviolet difference spectrum for the carbahomophosphonate **7** bound to dehydroquinate synthase. Carbahomophosphonate **7** (100 μM) was incubated with Co(II)–dehydroquinate synthase (20 μM) in assay buffer at pH 7.5. The spectrum of the enzyme alone has been subtracted.

FIGURE 4: Dependence of the ΔA_{340nm} for analogues **3–8** bound to dehydroquinate synthase, as a function of pH.

enzyme-bound species exists as intermediate I. However, the fact that the enzyme was of much lower specific catalytic activity than that used here (Frost et al., 1984), added to the likelihood of a substantial isotopic discrimination, makes this estimate improbably high.] In contrast to the use of DAHP itself, none of the substrate analogues **3–9** is turned over by the enzyme, yet each of them is susceptible, in principle at least, to redox chemistry at C-5. Incubation of any of these analogues with the synthase should result in the establishment of an equilibrium mixture of bound states. High enzyme concentrations may thus be employed, allowing the search for enzyme-bound NADH by simple absorption measurements.

When the carbacyclic homophosphonate **7** is added (at a concentration 20 times that of its K_i) to dehydroquinate synthase, a new absorbance at 340 nm is observed. The difference spectrum shown in Figure 3 indicates that the new absorbance at 340 nm is accompanied by the loss of absorbance at 260 nm, which is entirely consistent with the transformation of NAD$^+$ to NADH. Addition of lactate dehydrogenase and pyruvate to the solution containing the complex of synthase with **7** did not change the absorbance at 340 nm, indicating that the NADH that we presume gives rise to this 340-nm absorbance is tightly bound to DHQ synthase.

The intensity of the absorbance at 340 nm may be used to quantitate the extent of cofactor reduction at equilibrium in the presence of the various enzyme-bound substrate analogues. As shown in Figure 4, and as expected for a redox equilibrium involving the NAD$^+$/NADH couple, the substrate is more fully oxidized (and the cofactor more fully reduced) at high pH. From the results for analogues **5**, **8**, and **7**, we can presume that the limiting absorbance at pH 8 represents the complete reduction of bound cofactor, and it is gratifying to find that, in absolute terms, this absorption corresponds to a stoichiometry of 0.85 mol of NADH/mol of enzyme. On the basis that the enzyme-bound NAD$^+$ is completely reduced to NADH, the molar extinction coefficient of bound NADH does not differ much from the value for free NADH (6300 M^{-1} cm^{-1}) used in the above calculation.

A number of interesting conclusions can be drawn from the NADH levels shown in Figure 4. First, both the homophosphonate analogues **4**, **7**, and **8**, and the shorter inhibitors **3**, **5**, and **6** undergo oxidation on the enzyme, suggesting that the orientation of all of them in the active site is similar (at least with respect to the nicotinamide cofactor and the appropriate catalytic groups). Second, the carbacycles **5** and **7** are oxidized to a greater extent than their oxacyclic coun-
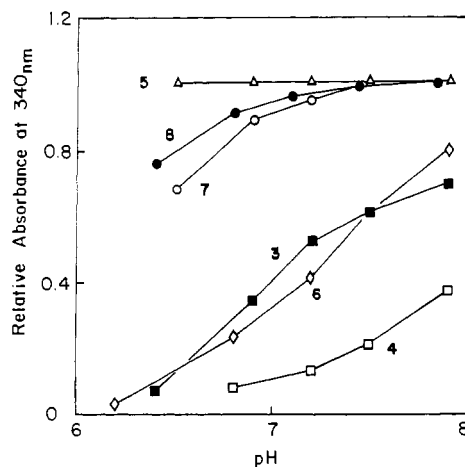
terparts **3** and **4**, which may well contribute to the increased binding affinity observed in the carbacyclic series. The change in the redox potential at C-5 for the different analogues can be ascribed to electronic effects. Thus, the α-heteroatom in **3** and **4** inductively destabilizes the oxacyclic ketones relative to the carbacyclic ketones that are formed from the corresponding analogues **5** and **7**. In agreement with this view, the NADH level observed for the analogue **6** is also low, consistent with the existence of an α-heteroatom in this molecule, also. [It is well-known that ketones possessing α-heteroatoms are less easily oxidized than those without. For example, the ratios of the overall redox equilibrium constants for such pairs as lactate/pyruvate and glycerate/hydroxypyruvate, or acetol/propanediol and dihydroxyacetone/glycerol, are between 25 and 50 (Barman, 1969).] In summary, those species with *no* heteroatom α to the oxidized center at C-5 (**5**, **7**, and **8**) are all more readily oxidized on the enzyme than are those that possess such a functionality (**3**, **4**, and **6**) (see Figure 4). These observations also suggest a possible strategy for the design of tight binding inhibitors of dehydroquinate synthase. The incorporation of features into an inhibitor that favor oxidation at C-5 will favor binding to the enzyme, because dehydroquinate synthase binds the intermediate NADH (along with the intermediate ketone) very tightly. It may be noted, however, that the fact that the redox equilibrium is sensitive to pH (Figure 4) suggests that the proton "released" in the oxidation of substrate equilibrates with the medium, even though the oxidized substrate analogue and the reduced nicotinamide cofactor do not.

NAD$^+$ Exchange. As has been reported in the previous paper, the cofactor NAD$^+$ is only slowly lost from the enzyme in the absence of substrate. When substrate is present, however, the cofactor dissociates much more rapidly. The same phenomenon is evident when a substrate analogue is present, and the cofactor off-rate can readily be monitored from the rate of loss of A_{340nm} (most easily for those analogues that drive a reasonable proportion of the enzyme into the form enzyme–NADH–oxidized substrate). In the presence of adenosine diphosphoribose (ADPR), which binds tightly to free enzyme, the fall in A_{340nm} precisely reflects the loss of NAD$^+$. These rates are listed in Table II. The rough correlation between the dissociation rate for NAD$^+$ (k_{off}) from the ternary complexes with enzyme and substrate analogue, the binding constant of the substrate analogue to the enzyme in the presence of cofactor, and the proportion of the ternary complex in the form containing oxidized analogue and reduced cofactor points to the interrelationships among these processes.

Table II: Dissociation Rates of NAD$^+$ from Dehydroquinate Synthase in the Presence of Substrate Analogues

| substrate analogue	k_{off} (NAD$^+$) (min^{-1})	K_m/K_i (pH 7.5)	NADH level (% of enzyme)
3	0.17	54	60
4	3.5	0.07	20
5	<0.01	5000	>95
6	0.05	2	60
7	<0.01	2	>95
8	ND^a	19	>95
9	ND	0.13	<10

^{a} ND, not determined.
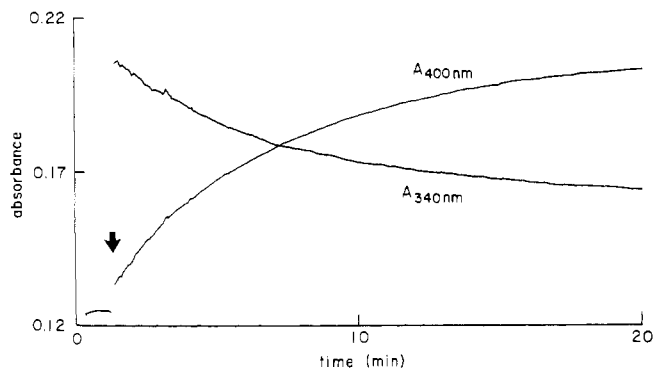
FIGURE 5: Exchange of enzyme-bound NADH for enzyme-bound thio-NADH on dehydroquinate synthase in the presence of the phosphonate analogue 3. The analogue 3 was added at the point marked by the arrow.

Further confirmation that enzyme-bound NADH is responsible for the absorbance observed at 340 nm when substrate analogues are incubated with enzyme in the presence of NAD$^+$ was obtained by exploiting the more rapid NAD$^+$ dissociation that occurs in the presence of substrate and substrate analogues. Thus, addition of the phosphonate 3 to dehydroquinate synthase containing bound NAD$^+$ results in the instantaneous appearance of the band at 340 nm that corresponds to enzyme-bound NADH. If the cofactor analogue thio-NAD$^+$ is also present, the A_{340nm} decays exponentially, with the simultaneous formation of a new band centered at 400 nm (Figure 5). This wavelength is the absorbance maximum of thio-NADH, and the data in Figure 5 are consistent with rapid formation of a complex enzyme–NADH–oxidized 3, in equilibrium with the complex of enzyme–NAD$^+$–reduced 3 from which NAD$^+$ dissociates in a first-order process. The resulting complex of enzyme with reduced 3 then binds thio-NAD$^+$, and the resulting ternary complex then undergoes the internal redox reaction. From these initial experiments, it appears that the internal redox equilibrium is not shifted markedly by using thio-NAD$^+$ in place of NAD$^+$. Considering the fact that the redox potentials of thio-NAD$^+$ and NAD$^+$ are different, further work is needed to understand the nature of thio-NAD$^+$ bound to DHQ synthase.

Involvement of the Metal Cation. During the course of the studies on enzyme-bound NADH, we observed weak bands at 460 and 560 nm in the difference spectra (Figure 6). These absorbances are induced by addition of substrate analogues to Co(II)–dehydroquinate synthase, but are not observed when the Mn(II)–enzyme is used. Addition of substrate analogues to Co(II)–containing buffer in the absence of enzyme does not give rise to these absorbances, and it seems that the bands at 460 and 560 nm derive from changes in the environment of the enzyme-bound cobalt(II) ion when the analogues bind. In accordance with literature precedent, the observed absorbance changes can be tentatively ascribed to the generation of five-coordinate cobalt(II) having nitrogen and oxygen ligands
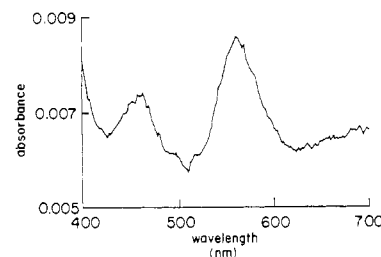
FIGURE 6: Visible difference spectrum for Co(II)–dehydroquinate synthase (20 μM) in the presence of the *cis*-vinylhomophosphonate 8 (100 μM).
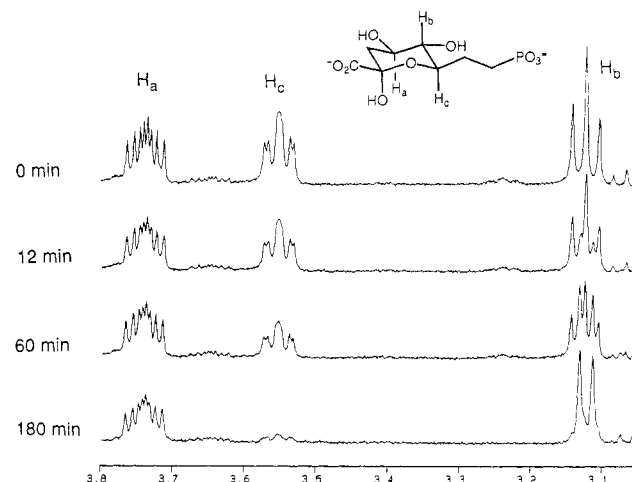
FIGURE 7: Exchange of the proton at C-6 of the homophosphonate 4, catalyzed by dehydroquinate synthase. The solution contained Co(II)–dehydroquinate synthase (5.6 units) in the presence of the homophosphonate 4 (5 μM) in 25 mM imidazole–trifluoroacetate buffer, pH 7.5, in D$_2$O.

(Bertini et al., 1983; Bränden & Eklund, 1978). Whether the changes that are observed are due to the direct ligation of substrate analogue to the cobalt(II) ion or whether they come from a protein conformation change consequent upon the binding of substrate analogue cannot yet be answered.

The Second Step: Enolization of the Substrate Ketone. In the proposed mechanism (Scheme I), oxidation at C-5 is followed by the elimination of inorganic phosphate across C-6 and C-7. Recently, we have shown that the stereochemical course of this elimination step is syn (Widlanski et al., 1987), which has often been taken to indicate a stepwise process. Now, although the analogues reported in this paper were designed to block the elimination step (in that none of the phosphonates or homophosphonates can lose P$_i$), the first half of a notional stepwise elimination, the enolization step, is still structurally allowed. Provided that the base that is responsible for proton abstraction can exchange its proton(s) with solvent, we could expect that incubation of the analogues with dehydroquinate synthase in D$_2$O could result in enzyme-catalyzed exchange of the proton at C-6 with solvent deuterium. This exchange reaction is conveniently monitored by ^{1}H NMR, and the results of such an experiment for the homophosphonate 4 are shown in Figure 7. The enzyme catalyzes deuterium exchange at C-6 of 4, at a rate that is about 1% that of the V_{max} for the normal substrate, 1. Similar enzyme-catalyzed deuterium exchange is seen at C-6 of the homophosphonate 7 and the *cis*-vinylhomophosphonate 8. Although the carbacyclic analogues 7 and 8 are more fully oxidized on the enzyme than is 4 (Table II), the exchange rate is about 10-fold slower for these species. Two factors that may be responsible for this difference are the steric effect of the adjacent methylene group and the acidifying effect of the electronegative
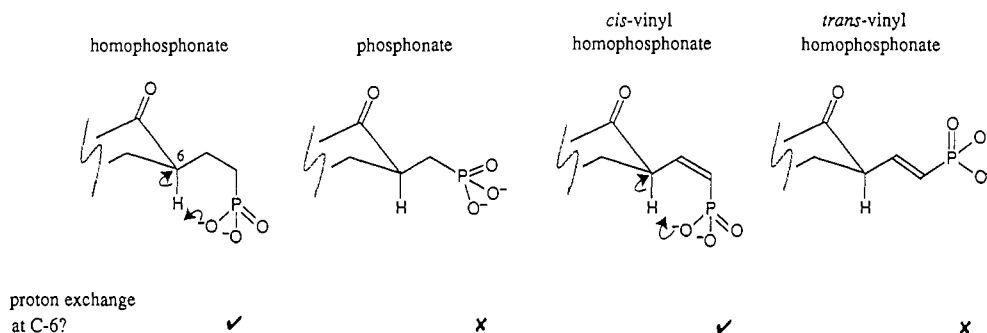
FIGURE 8: Proton exchange at C-6 for several substrate analogues, catalyzed by dehydroquinate synthase.

ring oxygen in the oxacycle **4** (Matthews et al., 1975). Since the observed exchange rates represent a *minimum* for the rates of enolization, the relatively rapid exchange (relative to V_{max} for DAHP) observed with analogue **4** supports the view that the normal sequence for the substrate DAHP follows a stepwise path via the intermediate formation of the enol(ate) species II (Scheme I). In contrast to the exchanges observed with **4**, **7**, and **8**, the nonisosteric phosphonate **3** and carbaphosphate **6** show *no* detectable exchange. The C-6 protons in these molecules are certainly available for enolization, and both analogues readily undergo the oxidation step (which we may presume is a prerequisite for proton removal). These facts suggest that while the orientation of **3** and **6** at the active site is appropriate for hydride transfer from C-5, the alignment of these species for proton abstraction from C-6 by an enzymic base is somehow improper.

An alternative view, however, is that the enolization is effected not by an *enzymic* base but rather by a base provided by the substrate, namely, one of the phosphate (or phosphonate) peripheral oxygens (see Figure 8). This type of intramolecularly catalyzed enolization is well precedented [e.g., in the solution reactions of 3-hydroxypropionaldehyde phosphate (Gallopo & Cleland, 1979), of the two triosephosphates (Richard, 1984), or of a derivative of one of them (Motiu-DeGrood et al., 1979)] and has been suggested for other enzymic reactions (Periana et al., 1980; Gouaux et al., 1987). The present proposal is consistent with the following. The elimination of phosphate is a syn process (Widlanski et al., 1987), which is, of course, necessary if the phosphate oxygen is to serve as the proton abstractor. Further, the fact that the *cis*-vinylhomophosphonate binds more tightly to the enzyme than the corresponding trans isomer suggests the possibility that the conformation of bound substrate is analogous to that shown in Figure 2. Indeed, models of this conformation show that the proton at C-6 is shielded by the oxygen(s) of the phosphonate group, making access (and proton abstraction) by an *enzymic* base difficult. Yet the deuterium-exchange reaction proceeds readily with **8**, and the nearest peripheral phospho group oxygen is the obvious choice for the recipient base. Furthermore, the evident lack of enolization with analogues **3** and **6** is entirely consistent with this model, since the oxygen base in these compounds cannot achieve the preferred orientation with respect to the C–H bond at C-6. The tight binding of these compounds may be due to their ability to interact well with the phosphate binding site, which must force the substrate (and the analogues **4** and **7**) to adopt a folded conformation similar to that proposed for **8** (Figure 2). Finally, it should be pointed out that direct transfer of the proton on C-6 to the phosphate oxygen in the substrate DAHP would provide a catalytic advantage: protonation of the phosphate group improves its effectiveness as a leaving group in the

subsequent elimination step.

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Dehydroquinate Synthase: The Use of Substrate Analogues To Probe the Late Steps of the Catalyzed Reaction[†]

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ABSTRACT: The later steps of the proposed mechanistic pathway for the reaction catalyzed by dehydroquinate synthase have been probed by using three substrate analogues. Each of these analogues is structurally prohibited from undergoing the ring-opening reaction that necessarily precedes the carbon–carbon bond-forming step in the overall conversion of the substrate 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (1) to dehydroquinate (2). Two of the analogues (the 2-deoxy cyclic compound 3 and the carbacyclic material 4) are locked into a cyclic form, mimicking the pyranose form of the substrate DAHP. The third analogue, 5, contains no carbonyl group at C-2 and may thus resemble the open-chain form of DAHP. Analogues 3 and 4 each bind to the enzyme and are competitive inhibitors having K_i values of 35 and 0.12 μM, respectively. More importantly, however, incubation of these analogues with the enzyme leads to the catalytic production of P_i along with the corresponding exomethylene compounds that are analogous to the enol ether IV postulated for the normal synthase reaction. In contrast to these results, the acyclic analogue 5 is neither a substrate nor an inhibitor of the enzyme. These data suggest that the enzyme recognizes and acts upon the α-pyranose form of the natural substrate. The ready release of the exomethylene products from the processing of analogues 3 and 4 is consistent with the suggestion of Bartlett and his group that the enzyme may release the enol ether intermediate IV into solution, where the ring opening and cyclization occur nonenzymically. The use of 3 stereospecifically labeled with deuterium at C-7 allows the stereochemical course of the β-elimination of phosphate to be established. This step proceeds with syn stereochemistry, which fits the pattern of enzyme-catalyzed elimination from substrates where the proton is lost from a position α to a ketone, an aldehyde, or a thiolester. Since the overall stereochemical course of the transformation mediated by dehydroquinate synthase had been shown to be inversion, the present finding of a syn elimination suggests that the transition state for the subsequent intramolecular aldol reaction has a chairlike geometry.

The second enzyme of the shikimate pathway, dehydro-quinate synthase, catalyzes the conversion of the seven-carbon keto acid 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP,[1] 1) to dehydroquinate (DHQ, 2), the first carbocyclic metabolite in the pathway (Haslam, 1974; Weiss & Edwards, 1980). The enzyme-catalyzed reaction is known to require the presence of NAD⁺, but because the overall conversion is redox neutral, a pathway involving five steps has been proposed (Srinivasan et al., 1963). This suggestion is illustrated for the cyclic form of DAHP (1) in Scheme I. According to this scheme, the secondary alcohol at C-5 is first oxidized by bound NAD⁺ to the ketone, thereby facilitating the β-elimination of inorganic phosphate across C-6 and C-7. The enzyme-bound NADH then reduces C-5 back to the alcohol, and after ring opening, an intramolecular aldol reaction yields the product dehydroquinate (2).

This mechanistic pathway—while being chemically reasonable and accommodating a number of observations—is extraordinary in that a relatively small monomeric protein

Chart I
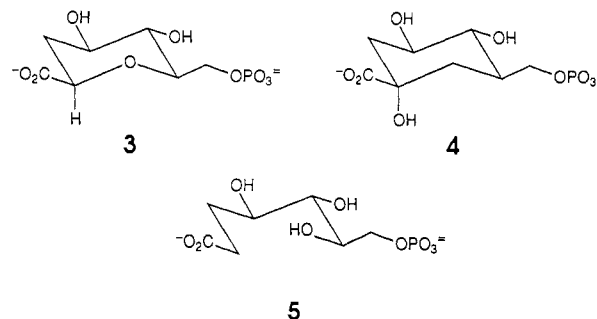
(Frost et al., 1984) catalyzes a reaction sequence involving four different types of chemical transformations: redox reactions, a β-elimination, a pyranose ring opening, and an intramolecular aldol reaction. None of the putative enzyme-bound intermediates has been isolated, and we report here the use

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[1] Abbreviations: DAHP, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate; DBU, diazabicycloundecene; DHQ, dehydroquinate; DMSO, dimethyl sulfoxide; NAD⁺, nicotinamide adenine dinucleotide; NADH, reduced form of NAD⁺; THF, tetrahydrofuran.