

Srinivasan, P. R., Rothschild, J., & Sprinson, D. B. (1963) *J. Biol. Chem.* 238, 3176-3182.
 Weiss, U., & Edwards, J. M. (1980) *The Biosynthesis of Aromatic Compounds*, Wiley, New York.

Widlanski, T. S., Bender, S. L., & Knowles, J. R. (1987) *J. Am. Chem. Soc.* 109, 1873-1875.
 Williams, J. W., & Morrison, J. F. (1979) *Methods Enzymol.* 63, 437-467.

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

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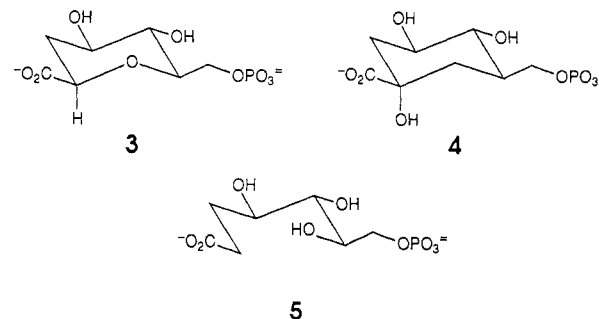
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ABSTRACT: The later steps of the proposed mechanistic pathway for the reaction catalyzed by dehydroquinase synthase have been probed by using three substrate analogues. Each of these analogues is structurally prohibited from undergoing the ring-opening 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 dehydroquinase (**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 thioester. Since the overall stereochemical course of the transformation mediated by dehydroquinase 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, dehydroquinase synthase, catalyzes the conversion of the seven-carbon keto acid 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP,¹ **1**) to dehydroquinase (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 dehydroquinase (**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



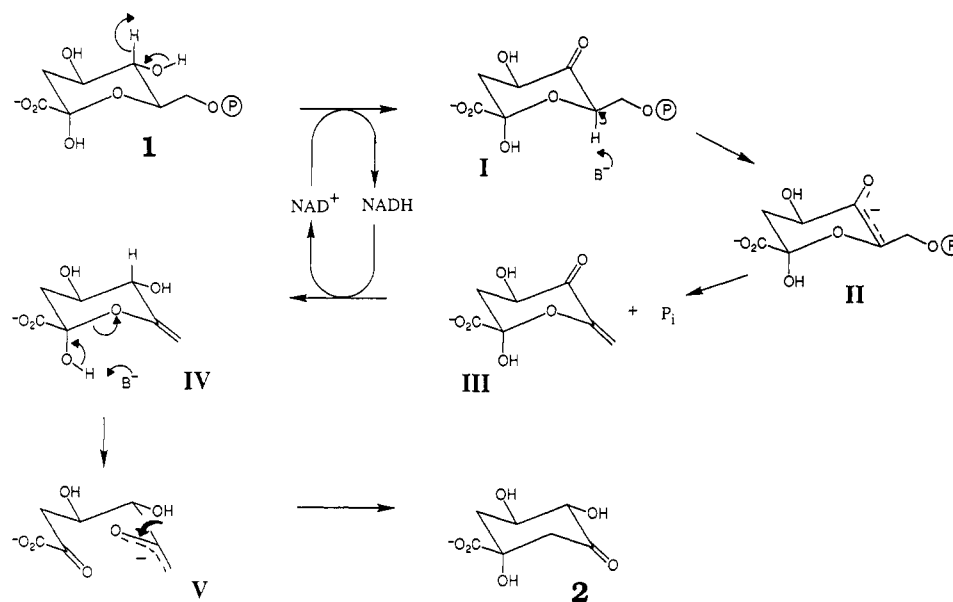
(Frost et al., 1984) catalyzes a reaction sequence involving four different types of chemical transformations: redox reactions, a β -elimination, a pyranose ring opening, and an intramolecular aldol reaction. None of the putative enzyme-bound intermediates has been isolated, and we report here the use

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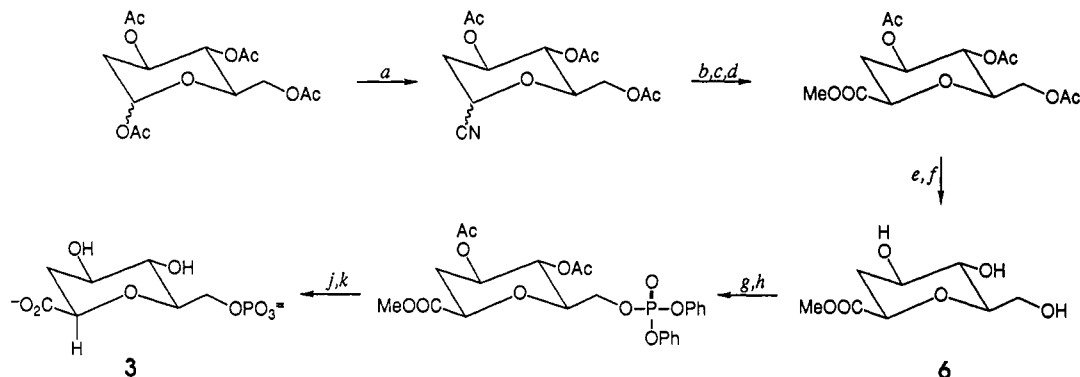
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¹ Abbreviations: DAHP, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate; DBU, diazabicycloundecene; DHQ, dehydroquinase; DMSO, dimethyl sulfoxide; NAD^+ , nicotinamide adenine dinucleotide; NADH, reduced form of NAD^+ ; THF, tetrahydrofuran.

Scheme I: Proposed Mechanistic Pathway for Dehydroquinate Synthase



Scheme II: Synthetic Route to the 2-Deoxy Substrate Analogue 3



^aTrimethylsilyl cyanide, BF_3 etherate, nitromethane. ^bKOH, THF. ^c CH_2N_2 . ^d Ac_2O , pyridine. ^eKOH. ^f CH_2N_2 . ^gDiphenyl phosphorochloridate, pyridine. ^h Ac_2O , pyridine. ⁱ H_2 , Pt. ^kKOH.

of substrate analogues to probe the nature of these intermediates and the timing of the chemical steps involved.

The use of substrate analogues that cannot complete a sequence of enzymic steps may provide information about the nature of fleeting intermediates in enzyme-catalyzed reactions. To investigate the mechanism of DHQ synthase, we have synthesized a series of analogues that cannot interconvert between the cyclic hemiketal and the acyclic keto form of DAHP. In the overall conversion of DAHP to DHQ, the cyclic hemiketal (IV, Scheme I) must open to allow the ultimate formation of the new carbon-carbon bond (in 2, Scheme I). To probe the events leading up to this ring-opening step, we have made three substrate analogues, the 2-deoxy cyclic analogue 3, the carbacyclic analogue 4, and the 2-deoxy acyclic compound 5 (Chart I). Analogues 3 and 4 are locked into a cyclic structure mimicking the predominant pyranose form of DAHP, while 5 is locked into an acyclic form. These substrate analogues were designed to address four questions: (i) When in the reaction sequence does ring opening occur? (ii) What is the structure of the intermediate that undergoes the aldol reaction? (iii) What is the stereochemical course of the β -elimination? (iv) Given that inversion at C-7 occurs in the overall conversion of DAHP to DHQ, what is the transition-state geometry of the aldol reaction?

EXPERIMENTAL PROCEDURES

Materials. Enzymes and substrate were obtained as described in the first paper of this series (Bender et al., 1989a). In all the synthetic work described, reactions were performed under dry argon, unless otherwise noted.

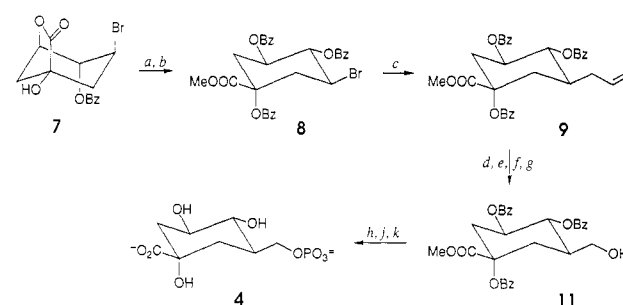
(2R,4R,5S,6R)-(2-Carboxy-4,5-dihydroxytetrahydropyran-6-yl)methyl Phosphate [2-Deoxy-DAHP (3), See Scheme II]. 2-Deoxy-D-glucose tetraacetate (1 g, 3 mmol) was dissolved in anhydrous nitromethane (20 mL); trimethylsilyl cyanide (2 mL) was then added, followed by boron trifluoride etherate (8 drops). The mixture was stirred at room temperature for 1 h, and the solvent was then evaporated under reduced pressure. Residual trimethylsilyl cyanide was removed by repeated additions and evaporations of portions (20 mL) of toluene. The resulting oil was dissolved in THF (10 mL), and methanol (10 mL) and NaOH (1 g, dissolved in 5 mL of water) were added. This mixture was heated to 80 °C for 12 h, after which time it was cooled and diluted with water (20 mL). The solution was passed through a short column of Dowex 50 (H^+ form), which was then washed well with methanol. The combined eluates were evaporated under reduced pressure, and the residue was dissolved in methanol and treated with ethereal diazomethane. This solution was evap-

orated to dryness, and the resulting solid was dissolved in pyridine (10 mL). Acetic anhydride (10 mL) was added, and the mixture was stirred for 3 h. After dilution with ethereal dichloromethane (200 mL, 2:1 v/v), the solution was washed successively with 3 N HCl (2 × 100 mL), water (100 mL), saturated aqueous sodium bicarbonate (2 × 100 mL), and brine (100 mL). The solution was dried over magnesium sulfate, and the solvent was removed to give a brown oil, chromatography of which on silica [eluting with ethyl acetate/hexane (6:4 v/v)] gave the carbomethoxy triacetate as a white solid (0.76 g, 76%). Thin-layer chromatography on silica [eluting with ethyl acetate/hexane (6:4 v/v)] gave $R_f = 0.55$: ^1H NMR (CDCl_3 , 500 MHz) δ 5.06–4.98 (m, 2 H), 4.28 (dd, 1 H, $J = 5.0$ and 12.4 Hz), 4.12 (dd, 1 H, $J = 2.2$ and 12.2 Hz), 4.11 (dd, 1 H, $J = 2.1$ and 12.4 Hz), 3.74 (s, 3 H, OMe), 3.63 (ddd, 1 H, $J = 2.1$, 5.0, and 9.5 Hz), 2.49 (ddd, 1 H, $J = 2.2$, 4.7, and 12.8 Hz), 2.07 (s, 3 H, OAc), 2.03 (s, 3 H, OAc), 2.02 (s, 3 H, OAc), 1.95 (ddd, 1 H, $J = 12.3$, 12.6, and 12.8 Hz); ^{13}C NMR (CDCl_3 , 75.6 MHz) δ 170.01, 169.57, 169.12, 168.78, 75.96, 73.78, 71.20, 68.83, 62.27, 51.86, 33.06, 20.27, 20.16, and 20.10.

The carbomethoxy triacetate (0.76 g, 2.3 mmol) was dissolved in methanolic THF (20 mL, 2:1 v/v), and water (10 mL) and 1 N NaOH (14 mL) were then added. The mixture was stirred at room temperature for 1 h and then passed through a column of Dowex 50 (H^+ form) that was washed with methanol. After removal of the solvent from the combined eluates under reduced pressure, the residue was dissolved in methanol and treated with ethereal diazomethane. Evaporation of the solvent gave the ester **6**, which was recrystallized from methanol/ether (440 mg, 85%): ^1H NMR (D_2O , 500 MHz) δ 4.20 (dd, 1 H, $J = 2.2$ and 12.2 Hz), 3.79 (dd, 1 H, $J = 2.2$ and 12.5 Hz), 3.67 (s, 3 H, OMe), 3.63 (ddd, 1 H, $J = 5.9$, 9.7, and 12.5 Hz), 3.62 (dd, 1 H, $J = 5.9$ and 12.5 Hz), 3.24 (ddd, 1 H, $J = 2.2$, 5.9 and 9.7 Hz), 3.18 (t, 1 H, $J = 9.5$ Hz), 2.27 (ddd, 1 H, $J = 2.3$, 5.7, and 12.9 Hz), 1.49 (ddd, 1 H, $J = 12.2$, 12.5, and 12.9 Hz); ^{13}C NMR (D_2O , 75.6 MHz) δ 182.00, 88.93, 82.89, 80.59, 79.00, 70.23, 62.09, and 44.71.

The ester **6** (206 mg, 1 mmol) was dissolved in pyridine (5 mL) at 0 °C, and diphenyl phosphorochloridate (0.32 g, 1.2 mmol) was added. The mixture was stirred at 0 °C for 1 h, acetic anhydride (5 mL) was then added, and the solution was stirred at 0 °C for a further 3 h. The mixture was diluted with ethereal dichloromethane (100 mL, 2:1 v/v) and then washed successively with 2 N HCl (2 × 100 mL), water (100 mL), saturated aqueous sodium bicarbonate (2 × 100 mL), and brine (100 mL). The solution was dried over magnesium sulfate, and the solvent was removed to give a yellow oil, chromatography of which on silica [eluting with hexane/ethyl acetate (6:4 v/v)] gave the protected phosphorylated ester diacetate. This material was deprotected by dissolution in methanol (15 mL) followed by hydrogenolysis (50 psi, 12 h) over PtO_2 (100 mg). After filtration, the solvent was removed under reduced pressure, and the residue was dissolved in methanolic THF (10 mL, 2:1 v/v). Water (4 mL) and 1 N NaOH (3 mL) were added, and the solution was stirred for 1 h at room temperature. After filtration through a column of Dowex 50 (H^+ form), the column was washed with water, and the combined eluates were neutralized with triethylamine. Ion-exchange chromatography on AG1X8 (100–200 mesh) eluted with a linear gradient (100–600 mM) of triethylammonium bicarbonate, pH 7.0, produced the 2-deoxy phosphate **3** (0.36 mmol, 36%): ^1H NMR (D_2O , 500 MHz) δ 3.90 (m, 2 H), 3.84 (dd, 1 H, $J = 12.1$ and 2.1 Hz), 3.63

Scheme III: Synthetic Route to the Carbacyclic Substrate Analogue **4**



^a MeOH-TsOH. ^b PhCOCl, pyridine. ^c Allyltributyltin, α, α' -azobis(isobutyronitrile), benzene, 50 °C. ^d O_3 . ^e Triethylsilyl triflate, NEt_3 . ^f O_3 . ^g NaBH₄. ^h Bis(trichloroethyl) phosphorochloridate. ^j Zn, MeOH. ^k KOH.

(ddd, 1 H, $J = 5.1$, 9.1, and 11.4 Hz), 3.34 (t, 1 H, $J = 9.3$ Hz), 3.27 (ddd, 1 H, $J = 2.2$, 3.8, and 9.5 Hz), 2.20 (ddd, 1 H, $J = 2.1$, 5.1, and 12.9 Hz), 14.3 (q, 1 H, $J = 12.1$ Hz).

[1*S*-(1 α ,3 β ,4 α ,5 β)]-5-[(Phosphonoxy)methyl]-1,3,4-trihydroxycyclohexane-1-carboxylate (the Carbacyclic Analogue **4**, See Scheme III). The bromo lactone monobenzoate **7** (12.3 g, 36 mmol) (Bartlett et al., 1986) was dissolved in anhydrous methanol (100 mL) containing trimethyl orthoformate (5 mL). *p*-Toluenesulfonic acid monohydrate (100 mg) was added, and the solution was boiled under reflux for 12 h. After the mixture was cooled, pyridine (2 mL) was added and the solvent removed under reduced pressure. The resulting white solid was repeatedly dissolved in hot toluene (100 mL), which was then removed under reduced pressure. The resulting solid, now free of methanol, was dissolved in anhydrous pyridine (100 mL), and the solution was cooled on ice as benzoyl chloride (24 g, 170 mmol) was added over 10 min. After being stirred at 0 °C for 3 h, the solution was allowed to warm to room temperature, and stirring was continued for a further 7 h. The mixture was cooled to 0 °C, and water (5 mL) was added. After being stirred for 15 min, the solution was evaporated to dryness. The residue was dissolved in dichloromethane (100 mL) with ether (100 mL) and petroleum ether (10 mL). This organic solution was washed successively with water (200 mL), saturated aqueous sodium bicarbonate (2 × 200 mL), and saturated aqueous sodium bisulfate (200 mL). After being dried over magnesium sulfate, the solvent was removed to yield the protected bromo compound **8** [methyl [1*R*-(1 α ,3 β ,4 α ,5 β)]-3-bromo-1,4,5-tris(benzoyloxy)cyclohexane-1-carboxylate] (17.6 g, 98%) which was recrystallized from methanol: ^1H NMR (CDCl_3 , 500 MHz) δ 8.18–7.32 (m, 15 H, Ar), 5.87 (t, 1 H, $J = 10.1$ Hz), 5.60 (ddd, 1 H, $J = 4.7$, 9.9, and 11.7 Hz), 4.47 (ddd, 1 H, $J = 4.4$, 10.7, and 12.8 Hz), 3.78 (s, 3 H, OMe), 3.31 (ddd, 1 H, $J = 3.0$, 4.5, and 14.5 Hz), 3.15 (ddd, 1 H, $J = 2.8$, 4.3, and 13.7 Hz), 2.78 (dd, 1 H, $J = 13.0$ and 14.5 Hz), 2.38 (dd, 1 H, $J = 11.9$ and 13.7 Hz); ^{13}C NMR (CDCl_3 , 75.6 MHz) δ 169.34, 165.37, 165.27, 164.59, 134.27, 133.68, 133.09, 133.02, 130.32, 129.78, 129.56, 129.49, 129.19, 129.02, 128.78, 128.68, 128.54, 128.21, 127.85, 79.64, 76.48, 69.99, 52.81, 44.43, 40.74, and 36.04.

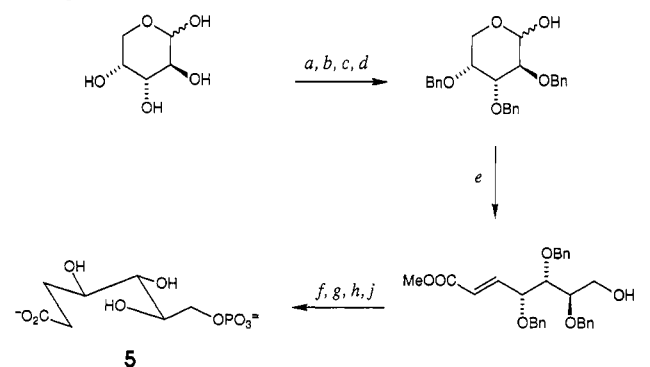
The bromo compound **8** (5 g, 10 mmol) was dissolved in hot anhydrous benzene (50 mL), and allyltributyltin (6.6 g, 20 mmol) was added. The solution was degassed with a stream of argon for 30 min, and α, α' -azobis(isobutyronitrile) (200 mg) was added. The solution was heated at 50 °C for 12 h, a further portion (100 mg) of the initiator being added after 6 h. The solvent was then removed from the cooled mixture, and the residue was dissolved in acetonitrile (200 mL). This solution was washed with hexane (3 × 100 mL). Removal

of the solvent gave **9** as an oil (3.95 g, 8.7 mmol, 86%), which was used without purification in the next step. The allyl compound **9** was dissolved in dichloromethane (20 mL) and cooled to -78°C . Ozone was bubbled through the solution until a blue color persisted. The mixture was then sparged with oxygen, and triethylamine (3 mL) was added. The reaction mixture was allowed to warm to room temperature and then washed with 1 M sodium phosphate buffer, pH 7 (2×100 mL). After being dried over magnesium sulfate, the solvent was removed under reduced pressure. The crude aldehyde product was dissolved in benzene (25 mL), and triethylamine (3.4 g, 35 mmol) and triethylsilyl triflate (4.5 g, 17.4 mmol) were added. The solution was heated at reflux for 1 h and then cooled and washed at 0°C with 0.5 N sodium phosphate buffer, pH 7 (3×50 mL). The solution was dried over sodium sulfate and the solvent removed. The residue was dissolved in dichloromethane (20 mL), and the ozonolysis was repeated as described above, except that the ozonide was reduced with dimethyl sulfide (2 mL, 12 h, 0°C). The sodium containing the aldehyde **10** was washed successively with water (5×100 mL) and brine (100 mL), and the solvent was removed from the dried solution under reduced pressure. The product was then crystallized from ether/hexane (3.2 g, 81%): ^1H NMR (CDCl_3 , 500 MHz) δ 9.68 (d, 1 H, $J = 1.1$ Hz), 8.20–7.28 (m, 15 H, Ar), 5.80 (t, 1 H, $J = 10.6$ Hz), 5.68 (m, 1 H), 3.77 (s, 3 H, OMe), 3.16 (m, 1 H), 3.07 (dt, 1 H, $J = 4.0$ and 13.8 Hz), 2.84 (dt, 1 H, $J = 3.1$ and 14.7 Hz), 2.31 (t, 1 H, $J = 14.4$ Hz), 2.40 (t, 1 H, $J = 13.4$ Hz).

The aldehyde **10** (1 g, 2.2 mmol) was dissolved in methanolic THF (15 mL, 2:1 v/v), and sodium borohydride (0.37 g, 10 mmol) was added over 5 min at 0°C . After 20 min, glacial acetic acid (3 mL) was added cautiously, and the solvent was then removed under reduced pressure. The residue was dissolved in dichloromethane (200 mL), and the solution was washed successively with saturated aqueous sodium bicarbonate (2×100 mL) and brine (100 mL). After the solution was dried over magnesium sulfate, the solvent was removed by evaporation to give the protected alcohol **11** (1.1 g, 100%): ^1H NMR (CDCl_3 , 500 MHz) δ 8.13–7.29 (m, 15 H, Ar), 5.75 (ddd, 1 H, $J = 4.9$, 10.6, and 13.6 Hz), 5.40 (dd, 1 H, $J = 10.5$ and 10.6 Hz), 3.77 (s, 3 H, OMe), 3.72 (dd, 1 H, $J = 2.8$ and 12.1 Hz), 3.53 (m, 1 H), 3.06 (ddd, 1 H, $J = 3.0$, 3.3, and 14.1 Hz), 2.94 (s, 1 H), 2.57 (dt, 1 H, $J = 3.4$ and 14.7 Hz), 2.44 (t, 1 H, $J = 14.7$ Hz); ^{13}C NMR (CDCl_3 , 75.6 MHz) δ 170.76, 167.62, 165.69, 164.98, 133.56, 133.48, 133.09, 129.91, 129.83, 129.76, 129.48, 129.34, 129.22, 128.85, 128.56, 128.43, 128.38, 128.29, 128.22, 79.94, 73.93, 70.79, 69.12, 68.78, 62.56, 61.23, 52.71, 39.13, 36.02, 34.71, 33.78, and 29.72.

The alcohol **11** (0.45 g, 1 mmol) was dissolved in pyridine (10 mL) and cooled to 0°C . Bis(2,2,2-trichloroethyl) phosphorochloridate (0.76 g, 2 mmol) was added, and the mixture was stirred for 1 h at 0°C . Water (2 mL) was then added, and after 15 min, a mixture of dichloromethane, ether, and petroleum ether (200 mL, 1:1:1 v/v/v) was added. This mixture was washed successively with saturated aqueous sodium bisulfate (3×100 mL) and saturated sodium bicarbonate (2×100 mL). The dried solution was evaporated to dryness, and the residue was dissolved in dichloromethane. This solution was filtered through silica and the solvent removed by evaporation. The product was dissolved in methanol (15 mL), and zinc dust (1 g, 15 mmol) and acetic acid (3 drops) were added. The mixture was stirred for 2 h and then filtered through Celite. The Celite was washed with methanol, and the solvent was removed from the combined filtrate and

Scheme IV: Synthetic Route to the Acyclic 2-Deoxy Substrate Analogue **5**



^a Allyl alcohol, HCl. ^b Benzyl bromide, NaH. ^c *t*-BuOK, DMSO. ^d H_3O^+ . ^e (Carbomethoxymethylene)triphenylphosphorane. ^f Bis(trichloroethyl) phosphorochloridate. ^g Zn, MeOH. ^h H_2 , Pd. ⁱ KOH.

washings. The resulting solid was dissolved in a mixture of water, methanol, and THF (30 mL, 1:1:1 v/v/v), and 1 N NaOH (5 mL) was added. This solution was heated at 70°C for 12 h. After cooling, water (15 mL) was added and the solution passed through a short column of Amberlite IR120 (H^+ form). The column was washed with water, and the combined filtrate and washings were washed with chloroform (3×100 mL). The aqueous mixture was then brought to pH 8 with sodium carbonate, and the solvent was removed under reduced pressure. The product **4** (0.95 mmol, 95%) was assayed by acid hydrolysis and quantitative assay for inorganic phosphate: ^1H NMR (D_2O , 500 MHz) δ 3.98 (m, 1 H), 3.88 (ddd, 1 H, $J = 3.9$, 5.4, and 10.0 Hz), 3.66 (ddd, 1 H, $J = 4.8$, 9.3, and 14.1 Hz), 3.29 (t, 1 H, $J = 9.3$ Hz), 1.99 (dd, 1 H, $J = 4.6$ and 13.3 Hz), 1.90 (m, 1 H), 1.79 (d, 2 H, $J = 8.9$ Hz), 1.72 (t, 1 H, $J = 12.0$ Hz).

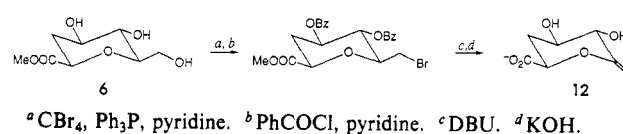
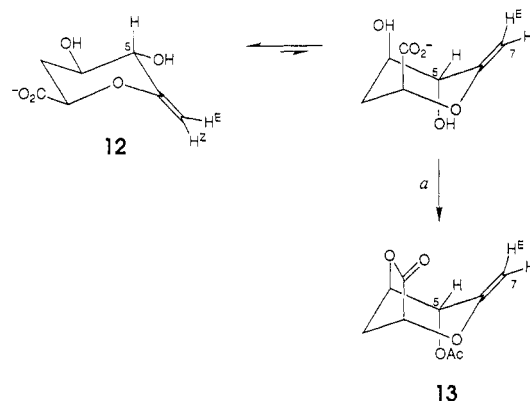
(4*R*,5*S*,6*S*)-7-(Phosphonooxy)-4,5,6-trihydroxyheptanoic Acid (the Acyclic 2-Deoxy Analogue **5**, See Scheme IV). D-Arabinose (15 g, 0.1 mol) was suspended in allyl alcohol (500 mL), and acetyl chloride (0.5 mL) was added slowly. The mixture was then refluxed for 30 min, after which time the solution was concentrated by evaporation to about 50 mL and hot anhydrous benzene (50 mL) was added. On cooling, the allyl pyranoside (6.5 g, 35%) was deposited from solution: ^1H NMR (D_2O , 500 MHz) δ 5.88 (dddd, 1 H, $J = 5.60$, 6.21, 10.32, and 17.25 Hz), 5.27 (ddt, 1 H, $J = 1.33$, 2.64, and 17.25 Hz), 5.16 (ddt, 1 H, $J = 1.32$, 2.64, and 10.32 Hz), 4.87 (d, 1 H, $J = 4.34$ Hz), 4.11 (ddt, $J = 1.31$, 5.60, and 12.81 Hz), 3.96 (ddt, 1 H, $J = 1.34$, 6.21, and 12.81 Hz), 3.89 (br s, 1 H), 3.80 (dd, 1 H, $J = 1.1$ and 12.68 Hz), 3.77–3.71 (m, 2 H), 3.54 (dd, 1 H, $J = 2.15$ and 12.77 Hz); ^{13}C NMR (D_2O , 75.6 MHz) δ 141.89, 126.34, 106.17, 77.06, 77.02, 76.88, 76.39, and 70.98.

1-*O*-Allylarabinopyranoside (3 g, 16.6 mmol) was suspended in THF (20 mL) at 0°C , and sodium hydride (as an oil dispersion, 3.2 g, 66 mmol) was added over 5 min. Anhydrous dimethylformamide (20 mL) was added over 10 min at 0°C , and the mixture was then allowed to warm to room temperature over 30 min with stirring. The solution was then cooled to 0°C , and benzyl bromide (9.9 g, 58 mmol) was then added over 5 min. This mixture was then stirred overnight at room temperature. The solution was then cooled to 0°C , and methanol (10 mL) was added over 10 min. After being stirred at room temperature for 1 h, the solution was mixed with ether (500 mL), and the mixture was washed successively with water (500 mL) and brine (500 mL). After being dried over magnesium sulfate, the solvent was removed, and the residue was

chromatographed on silica (eluting with dichloromethane). The resulting tribenzyl ether (7.2 g, 16 mmol) was dissolved in anhydrous DMSO (20 mL), and potassium *tert*-butoxide (3.4 g, 32 mmol) was added. This mixture was heated at 100 °C for 20 min. After being cooled, ether (200 mL) was added, and the solution washed with water (5 × 100 mL). The dried ethereal solution was concentrated, and THF (30 mL) was added, followed by 2 N HCl (2 mL). This mixture was heated at 50 °C for 2 h. After being cooled, dichloromethane (200 mL) was added, and the solution was washed with saturated aqueous sodium bicarbonate. The dried solution was concentrated, and the product 2,3,4-tri-*O*-benzyl-D-arabinose (6.5 g, 92%) crystallized from ether/hexane. A portion (0.84 g, 2 mmol) of this tri-*O*-benzylarabinose was dissolved in dimethoxyethane (10 mL), and benzoic acid (50 mg) was added, followed by (carbomethoxymethylene)triphenylphosphorane (1 g, 3 mmol). The mixture was then heated at 90 °C for 4 h. After removal of the solvent by evaporation, the residue was purified by chromatography on silica [eluting with ethereal dichloromethane (1:9 v/v)] to give a mixture of *E* and *Z* olefins [methyl (4*S*,5*R*,6*R*)-4,5,6-tris(benzyloxy)-7-hydroxyhept-2-enoates] in 3:2 ratio (0.67 g, 71%): ¹H NMR (*E* isomer) (CDCl₃, 500 MHz) δ 7.36–7.19 (m, 15 H, Ar), 6.99 (dd, 1 H, *J* = 6.39 and 15.84 Hz), 6.13 (dd, 1 H, *J* = 1.15 and 15.84 Hz), 4.70–4.64 (q, 2 H, *J* = 14.3 Hz), 4.50–4.44 (q, 2 H, *J* = 12.8 Hz), 4.35–4.26 (q, 2 H, *J* = 13.9 Hz), 4.23 (ddd, 1 H, *J* = 1.15, 2.23, and 6.41 Hz), 3.86–3.78 (m, 2 H), 3.76 (s, 3 H, OMe), 3.76–3.68 (m, 2 H); ¹³C NMR (CDCl₃, 75.6 MHz) δ 166.23, 145.79, 137.61, 128.41, 128.36, 128.26, 128.14, 128.04, 127.85, 127.77, 127.70, 122.90, 80.47, 78.78, 78.27, 74.86, 71.82, 71.60, 60.57, and 51.58; ¹H NMR (*Z* isomer) (CDCl₃, 500 MHz) δ 7.36–7.20 (m, 15 H, Ar), 6.34 (dd, 1 H, *J* = 8.8 and 11.2 Hz), 5.86 (dd, 1 H, *J* = 0.9 and 11.2 Hz), 5.33 (ddd, 1 H, *J* = 0.9, 3.1 and 8.8 Hz), 4.75–4.65 (q, 2 H, *J* = 13.9 Hz), 4.58 (d, 1 H, *J* = 13.7 Hz), 4.52 (d, 1 H, *J* = 13.2 Hz), 4.42 (d, 1 H, *J* = 13.2 Hz), 4.33 (d, 1 H, *J* = 13.7 Hz), 3.90–3.80 (m, 2 H), 3.75–3.79 (m, 2 H), 3.68 (s, 3 H, OMe); ¹³C NMR (CDCl₃, 75.6 MHz) δ 166.21, 148.34, 137.65, 128.40, 128.35, 128.26, 128.14, 128.04, 127.78, 127.63, 121.70, 81.01, 79.30, 78.25, 75.06, 71.75, 71.30, 60.85, and 51.55.

The mixture of *Z* and *E* olefins above (0.67 g, 1.4 mmol) was phosphorylated as described for the phosphorylation of **11**, above, by using bis(trichloroethyl) phosphorochloridate. The phospho group was deprotected with zinc dust as described. The three benzyl ether protecting groups were removed by hydrogenolysis (55 psi, 1 h) in methanol over Pd/C (10% w/w). The methyl ester was saponified by the addition of 1 N NaOH (8 mL) with stirring at room temperature for 1 h. The solution was then brought to pH 8 by the addition of Amberlite IR120 (H⁺ form). After removal of the resin by filtration, the solvent was evaporated, and the residue dissolved in water. Quantitative assay for phosphate esters gave a yield of product **5** of 1.2 mmol (82%). The material was stored in aqueous solution at pH 7: ¹H NMR (D₂O, 500 MHz) δ 3.88 (ddd, 1 H, *J* = 2.62, 6.60, and 11.53 Hz), 3.82 (m, 1 H), 3.76 (ddd, 1 H, *J* = 1.61, 4.88, and 8.75 Hz), 3.69 (ddd, 1 H, *J* = 2.47, 5.20, and 11.43 Hz), 3.43 (dd, 1 H, *J* = 1.72 and 8.69 Hz), 2.27–2.13 (m, 2 H), 1.8–1.64 (m, 2 H); ³¹P NMR (D₂O, 121.56 MHz) δ 5.40 (t, *J* = 6.96 Hz).

(2*R*,4*R*,5*S*)-4,5-Dihydroxy-6-methylenetetrahydropyran-2-carboxylate (the Enol Ether **12**, See Scheme V). The triol ester **6** (0.21 g, 1 mmol) was dissolved in pyridine (5 mL) at 0 °C, and a mixture of triphenyl phosphine (0.52 g, 2 mmol) and carbon tetrabromide (0.83 g, 2.5 mmol) was added. The

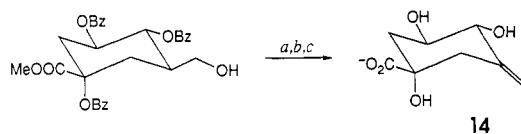
Scheme V: Synthetic Route to the Cyclic Enol Ether **12**Scheme VI: Ring Inversion and Lactonization of the Enol Ether **12** to the Bicyclic Lactone **13**

^a Ac₂O, pyridine.

solution was stirred overnight at room temperature, and methanol (5 mL) was then added. Stirring was continued for 20 min, and the solvent was then removed under reduced pressure. Toluene (20 mL) was added and the solution then evaporated to dryness. Anhydrous pyridine (10 mL) was added, followed by benzoyl chloride (2 mL), and the mixture was stirred at room temperature for 2 h. Water (2 mL) was then added and the mixture stirred for a further 15 min. The solvent was removed by evaporation under reduced pressure, and the residue was dissolved in ether (200 mL). After successive washes with water (200 mL), saturated aqueous sodium bicarbonate (2 × 200 mL), and saturated aqueous sodium bisulfate (200 mL), the solution was dried over magnesium sulfate. Flash chromatography on silica gave the bromodibenzoate ester (0.45 g, 94%). Thin-layer chromatography on silica (eluting with dichloromethane) gave *R_f* = 0.6.

This bromo compound (0.45 g, 0.94 mmol) was dissolved in benzene (10 mL), and diazabicycloundecene (0.28 g, 1.9 mmol) was added. After being heated at 60 °C for 3 h, the solution was cooled and washed with 1 M sodium phosphate buffer, pH 7, at 0 °C. The mixture was dried over magnesium sulfate and the solvent removed. Chromatography of the resulting oil on silica (eluting with dichloromethane) gave the protected enol ether, which was dissolved in methanolic THF (10 mL, 1:1 v/v) with water (5 mL) and 1 N NaOH (3 mL). After the solution was stirred overnight at room temperature, ammonium chloride (1 equiv. to NaOH) was added, and the solution was evaporated to dryness under reduced pressure to yield the enol ether **12**, along with 2 mol equiv of sodium benzoate: ¹H NMR (D₂O, 500 MHz) δ 4.60 (t, 1 H, *J* = 1.7 Hz), 4.57 (t, 1 H, *J* = 1.7 Hz), 3.87 (dd, 1 H, *J* = 12.3 and 2.4 Hz), 3.76 (dt, 1 H, *J* = 9.5, 1.7, and 1.7 Hz), 3.53–3.58 (m, 1 H), 2.26 (ddd, 1 H, *J* = 13.1, 4.9, and 2.4 Hz), 1.60 (q, 1 H, *J* = 12.2 Hz).

The enol ether **12** was converted into the cyclic lactone **13** (see Scheme VI) as follows. To the enol ether **12** (approximately 50 μmol) was added pyridine (3 mL), and acetic anhydride (0.4 mL) was added dropwise with stirring at 0 °C over 30 min. The mixture was stirred at room temperature for 2 h and then partitioned between water and ethyl ace-

Scheme VII: Synthetic Route to the Elimination Product, **14**, from the Carbacyclic Substrate Analogue **4**

^aCBr₄, Ph₃P, THF. ^bAgF, pyridine. ^cKOH.

tate/hexane (3:1 v/v). The organic layer was washed with water and phosphate buffer, pH 7, dried over Na₂SO₄, and concentrated. The residue was purified by chromatography on silica, eluting with ethyl acetate/hexane (2:3 v/v) to give the lactone **13** (52% from **3** via **12**): ¹H NMR (CDCl₃, 500 MHz) δ 5.57 (dd, 1 H, *J* = 4.3 and 1.4 Hz), 4.90 (d, 1 H, *J* = 1.8 Hz), 4.87 (br t, 1 H, *J* ≈ 5 Hz), 4.72 (d, 1 H, *J* = 1.8 Hz), 4.48 (br d, 1 H, *J* = 3.3 Hz), 2.71 (dd, *J* = 13.3 and 0.9 Hz), 2.23 (dddd, *J* = 13.3, 5.5, 3.3, and 1.5 Hz), 2.12 (s, 3 H).

[1*R*-(1*α*,3*β*,4*α*)]-5-Methylene-1,3,4-trihydroxycyclohexane-1-carboxylate (the Olefin **14**, See Scheme VII). The bromo compound, methyl [1*R*-(1*α*,3*β*,4*α*,5*β*)]-5-(bromomethyl)-1,3,4-tris(benzoyloxy)cyclohexane-1-carboxylate [the synthesis of which is described in the previous paper (0.51 g, 1 mmol)], was dissolved in pyridine (5 mL), and silver fluoride (0.25 g, 2 mmol) was added. The mixture was stirred in the dark overnight. Ether (100 mL) was then added, and the solution was washed successively with water (100 mL), 2 N HCl (2 × 100 mL), saturated aqueous sodium bicarbonate (100 mL), and brine (100 mL). The solvent was removed from the dried solution, and the resulting oil was dissolved in methanolic THF (10 mL, 1:1 v/v); 1 N NaOH (5 mL) was then added, and the mixture was heated at 70 °C for 12 h. Water (15 mL) was then added, and the solution was passed through a column of Amberlite IR120 (H⁺ form). The column was washed with water, and the combined eluates were washed with CHCl₃ (3 × 100 mL). The solution was brought to pH 8 with sodium bicarbonate, and the solvent was removed under reduced pressure to give **14**: ¹H NMR (D₂O, 500 MHz) δ 5.01 (s, 1 H), 4.84 (s, 1 H), 3.85 (d, 1 H, *J* = 9.3 Hz), 3.48 (ddd, 1 H, *J* = 4.8, 9.3, and 11.5 Hz), 2.53 (d, 1 H, *J* = 14.2 Hz), 2.35 (dd, 1 H, *J* = 3.2 and 14.3 Hz), 2.01 (ddd, 1 H, *J* = 3.2, 4.8, and 13.5 Hz), 1.87 (dd, 1 H, *J* = 11.5 and 13.5 Hz).

Enzyme-Catalyzed Elimination of Phosphate from 2-Deoxy-DAHP (3). To a portion (2.40 mL) of 80 mM imidazole-trifluoroacetate buffer, pH 7.0, containing 2-deoxy-DAHP (**3**) (50 μmol), CoSO₄ (4 μmol), and NAD⁺ (0.4 μmol) at 15 °C was added DHQ synthase (27.5 units) in phosphate buffer. The course of the reaction was monitored by removal of small portions (5–20 μL) for phosphate analysis. Approximately 40 μmol of phosphate was released after 20 h. After 24 h, 2.5 M KOH (50 μL) was added, and the solution was concentrated under reduced pressure. This material was either subjected to ¹H NMR analysis or cyclized directly to the lactone **13** as described above.

RESULTS

The synthesis of the three substrate analogues and the details of their interaction with dehydroquinase synthase are discussed in turn.

The cyclic 2-deoxy analogue **3** of the natural substrate DAHP (**1**) was synthesized from 2-deoxyglucose tetraacetate as shown in Scheme II. Treatment of an anomeric mixture

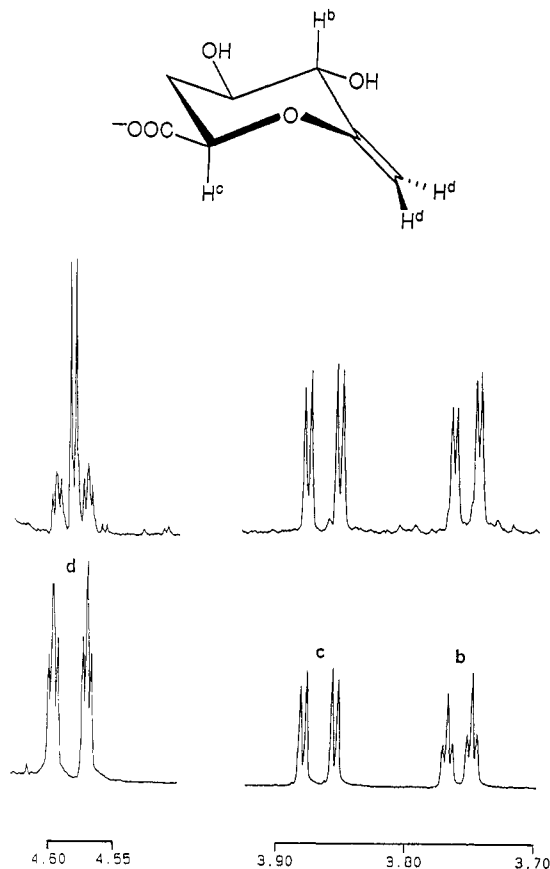
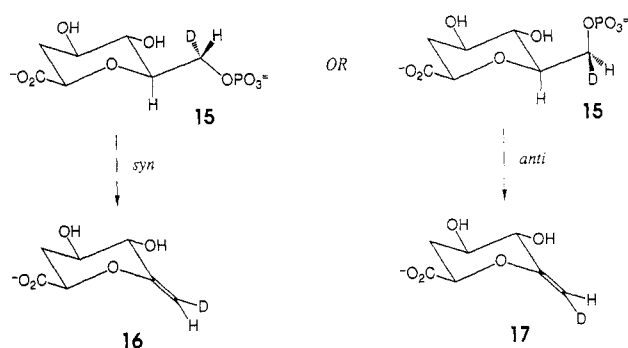


FIGURE 1: Partial ¹H NMR spectra of the enol ether produced by the action of dehydroquinase synthase on the 2-deoxy substrate analogue. (Upper spectrum) **16**, produced from the 7*S*-²H-labeled 2-deoxy analogue **15** (see Scheme VIII). (Lower spectrum) **12**, produced from the unlabeled 2-deoxy analogue **3**.

of 2-deoxyglucose tetraacetate with trimethylsilyl cyanide/BF₃·etherate in nitromethane gives a mixture of the *α* and *β* epimers of the nitrilo triacetate in almost quantitative yield (de las Heras & Fernandez-Reza, 1982). Basic hydrolysis followed by esterification and reacylation affords the desired *β*-carbomethoxy triacetate accompanied by some of the *α*-anomer and trace amounts of triacetyl glucal. The desired ester is readily isolated and is converted to the crystalline triol ester **6**. Selective phosphorylation of the primary hydroxyl group with diphenyl phosphorochloridate followed by diacetylation gives the protected phosphate triester, which is hydrogenolyzed and saponified to give 2-deoxy-DAHP (**3**). This product was purified by ion-exchange chromatography. It was later found that selective phosphorylation with bis-(trichloroethyl) phosphorochloridate proceeds in higher yield.

Incubation of **3** with dehydroquinase synthase in the presence of Co(II) and NAD⁺ results in the liberation of inorganic phosphate. The rate of P_i release is about 2% of that observed with the natural substrate DAHP under saturation conditions. The analogue **3** is a competitive inhibitor of the enzyme and has a *K_i* (35 μM) that is about 10 times the *K_m* for DAHP. The sole carbohydrate product from incubation of **3** with the enzyme was identified as the cyclic enol **12**. This identification was based upon the ¹H NMR spectrum (part of which is shown in Figure 1). Authentic **12** was obtained from the ester **6** as outlined in Scheme V.

If the enol ether **12** (Scheme V) is produced by a sequence of enzymic steps analogous to that which produces intermediate IV (Scheme I) from DAHP, then determination of the position (*E* or *Z*) of a deuterium label at C-7 in the product **12** that derives from stereospecifically monodeuterated

Scheme VIII: Products from Syn (16) and Anti (17) Elimination from Specifically Deuterated [7S-²H]-2-Deoxy-DAHP (15)

[7-²H]-3 will allow the stereochemical sense of the elimination to be defined. The two possibilities are illustrated in Scheme VIII. Appropriately deuterated material was therefore synthesized, as shown in Scheme IX. First β -D-[6S-²H]-glucose pentaacetate was prepared according to the method of Ohrui et al. (1983). This material was then converted to α -D-deoxyglucose tetraacetate by rearrangement of the radical derived from the corresponding glycosyl bromide, according to the method of Giese et al. (1987). Further transformation as for the unlabeled material yielded 15, the 7S-²H-labeled analogue of 2-deoxy DAHP (3). Incubation of the deuterated material 15 together with a small amount of the unlabeled compound 3 with the enzyme produced a sample of specifically deuterated 12, the ¹H NMR spectrum of which is shown in Figure 1. Depending on whether the enzyme-catalyzed elimination of phosphate proceeds with syn or anti stereochemistry, this product is either 16 or 17, respectively (Scheme VIII). The ¹H NMR spectrum illustrated in Figure 1 shows the resonances from the pair of vinylic protons (labeled d) from the small amount of unlabeled material, and one dominant doublet from the single vinylic proton of the monodeuterated material, shifted upfield somewhat by the presence of the geminal deuterium. The remaining vinylic proton in 16 or 17 is therefore that which gives rise to the downfield resonance in the unlabeled material. The spectrum of the deuterated material in Figure 1 also shows the expected simplification of the resonances for the proton at C-5 (labeled b).

It is clear from the ¹H NMR spectrum in Figure 1 that the enzyme has processed the monodeuterated sample of 3 stereospecifically. To determine the stereochemical course of the elimination (Scheme VIII), however, the *E* and *Z* proton resonances must be assigned. Unfortunately, these resonances (d) are only 0.03 ppm apart (see Figure 1) and show essentially equal long-range coupling to the allylic proton (b) on C-5. In

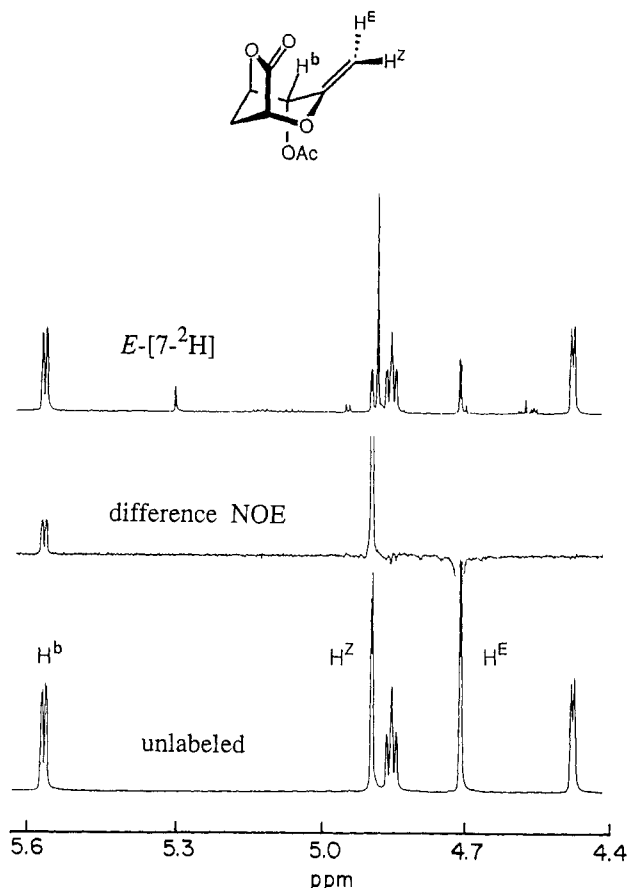
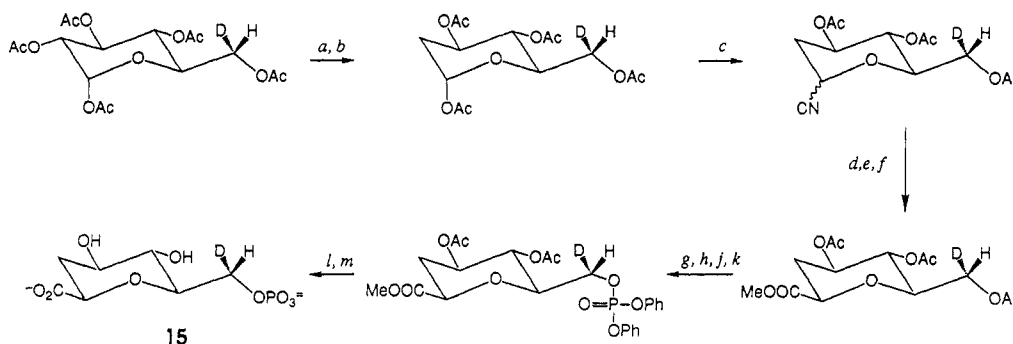


FIGURE 2: Partial ¹H NMR spectra of lactone acetate derived from the enol ether product of the dehydroquinase synthase reaction. (Bottom spectrum) Unlabeled lactone acetate 13, derived from the unlabeled enol ether 12. (Middle spectrum) Difference nuclear Overhauser enhancement spectrum after irradiation of the resonance labeled H^E. (Top spectrum) Labeled lactone acetate derived from the labeled enol ether 16, which is produced by the action of the synthase on the 7S-²H-labeled 2-deoxy analogue 15 (see Scheme VIII).

addition, we were unable to distinguish the two C-7 protons by nuclear Overhauser experiments with the proton at C-5, presumably because of the large intermolecular distance between the protons in the favored chair conformation of 12 (see Scheme VI). In the alternate chair conformer in which all substituents are axial, however, molecular models suggest that the C-5 proton is much closer to the *E* (rather than the *Z*) vinyl proton on C-7. Fortunately, lactonization of 12 locks the molecule in this conformation, and the unlabeled enol ether 12 was therefore converted into the corresponding monoacetyl lactone 13 (Scheme VI). Irradiation of the upfield vinyl proton

Scheme IX: Synthetic Route to the 7S-²H-Labeled 2-Deoxy Substrate Analogue 15

^a HBr, AcOH. ^b Tri-*n*-butyltin hydride. ^c Trimethylsilyl cyanide, BF₃ etherate, nitromethane. ^d KOH. ^e CH₂N₂. ^f Ac₂O, pyridine. ^g KOH. ^h CH₂N₂. ⁱ Diphenyl phosphorochloridate, pyridine. ^j Ac₂O. ^k H₂, Pt. ^l KOH.

resonance at 4.72 ppm (Figure 2) now results in a $\sim 3\%$ enhancement of the doublet at 5.57 ppm, allowing the assignment of this upfield resonance to the *E* vinyl proton in the lactone **13**. Similar treatment of the enzymic product from the 7S monodeuteriated sample of **3** (that is, **15**) showed that the product had deuterium in the *E* position. The enzymic product from **15** was therefore **16** and not **17** (see Scheme VIII), thus establishing that DHQ synthase catalyzes the syn elimination of inorganic phosphate from 2-deoxy-DAHP.

The conclusion that DHQ synthase catalyzes a syn elimination is confirmed by the synthesis of an authentic sample of the labeled enol **16** by a chemical route of predictable stereochemical course. Treatment of the 7S- ^2H -labeled analogue of **3** with carbon tetrabromide/triphenylphosphine (Whistler & Anisuzzaman, 1980) followed by perbenzoylation gave the [7S- ^2H]-7-bromo compound, treatment of which with DBU in boiling benzene effected the elimination of HBr (Scheme V). Since we can assume inversion for the bromination and an anti elimination with DBU, the product of this chemical sequence will have the same stereochemistry as material resulting from a syn elimination in the enzymic reaction. Basic hydrolysis of the protected enol ether gives authentic **16**, the ^1H NMR spectrum of which is identical with that produced by incubation of **15** with the enzyme. This identity provides independent evidence that the enzyme mediates an elimination the stereochemical course of which is syn.

The carbacyclic analogue **4** of DAHP was derived from quinic acid. The readily available bromolactone **7** (Bartlett et al., 1986) (Scheme III) was first converted into the monocyclic bromoester **8**. Radical alkylation of **8** with allyltributyltin (Keck et al., 1985) gave the allyl derivatives in good yield, containing the β and α epimer in 97:3 ratio. Although this selectivity can be ascribed to steric shielding of the α face of the molecule by the axial benzoate at C-2, stereoelectronic effects may also play a role. Giese and co-workers have reported that oxygenated substituents in equatorial positions (such as that at C-5) tend to give equatorial adducts in free radical trapping reactions (Giese & Gröning, 1984). [In an effort to extend this methodology, we subjected the bromolactone **7** directly to radical allylation, and to our surprise, the product after methanolysis and perbenzoylation was the β epimer **9** (Scheme III). We had expected that the radical would be trapped from the less hindered exo face of the bicyclic lactone system. A possible explanation for this result is that the vicinal axial benzoate exerts a stereoelectronic effect, both stabilizing the axial antiperiplanar radical and making the radical electrophilic by overlap of the singly occupied orbital of the radical with the σ^* orbital of the benzoate group. This effect could enhance the reactivity of the radical toward electron-rich olefins such as allyltributyltin. Trapping with an electrophilic olefin might therefore be expected to give the opposite stereochemistry. Consistent with this view, trapping of the radical with acrylonitrile gives the α and β epimers in 7:3 ratio.] The β -allyl ester **9** was therefore produced either directly from **7** or via **8** (Scheme III). Conversion of the allyl side chain into a hydroxymethyl group was accomplished by ozonolysis to the chain-shortened aldehyde, which was then converted to the triethylsilyl enol ether. This enol ether was ozonized, and the resulting aldehyde was reduced with sodium borohydride to produce the hydroxymethyl derivative **11** (Scheme III). Phosphorylation of the primary hydroxyl group followed by deprotection and basic hydrolysis gave the desired carbacyclic analogue **4**.

Incubation of saturating levels of **4** with dehydroquinase synthase results in the liberation of inorganic phosphate at a

rate that is about 0.3% of that observed with the natural substrate DAHP under saturation conditions. This decrease in V_{max} is compensated by the tighter binding of **4** to the enzyme: the K_i for **4** is 25-fold smaller than the K_m for DAHP. The organic product from the reaction of **4** with the enzyme was identified as **14** from its ^1H NMR spectrum. Authentic **14** was synthesized in three steps from the protected hydroxymethyl derivative as shown in Scheme VII.

The acyclic 2-deoxy substrate analogue **5** was derived from D-arabinose as shown in Scheme IV. The allyl glycoside was subjected to perbenzoylation, and 2,3,4-tri-*O*-benzyl D-arabinose obtained in good yield after removal of the aglycon (Tejima & Fletcher, 1963). This material was then allowed to react with (carbomethoxymethylene)triphenylphosphorane to yield the appropriately protected seven-carbon ester. Phosphorylation at C-7 and deprotection completed the synthesis of **5**.

When the acyclic analogue **5** was incubated with dehydroquinase synthase, no P_i release could be detected. Moreover, even at a concentration of **5** of 10 mM (which is more than 1000 times the K_m for DAHP), no inhibition of the enzyme could be discerned.

DISCUSSION

The three substrate analogues that are described in this paper were designed to resolve several questions concerning the proposed mechanism of dehydroquinase synthase shown in Scheme I.

First, it appears that the enzyme does not tolerate acyclic substrates. Thus, while the cyclic 2-deoxy substrate analogue **3** binds to the enzyme and is catalytically processed by it, the corresponding acyclic 2-deoxy substrate analogue **5** does not bind to the active site. These results are in agreement with earlier indications from the laboratories of Sprinson and Coggins. Thus, Sprinson and co-workers (Maitra & Sprinson, 1978) have reported that borohydride reduction of the substrate DAHP gives a mixture of the two epimeric 2-dihydro analogues of DAHP and that these materials are neither substrates nor inhibitors of dehydroquinase synthase. Further, Coggins and his group (Lambert et al., 1985) have shown that the yeast synthase accepts the cyclic form of DAHP, though it is true that these workers did not show that the acyclic form is *not* a substrate. Our results show that the enzyme will *process* molecules that cannot ring open, thus providing some evidence that the nature and timing of the early chemical events depicted in Scheme I are correct.

When the cyclic 2-deoxy substrate analogue **3** is incubated with the enzyme, inorganic phosphate and the enol ether **12** are produced catalytically. Clearly, compound **12** is the 2-deoxy analogue of the enol ether that is intermediate IV in the normal reaction (see Scheme I). This result shows that the enzyme will release intermediate analogues into solution, even those that have not (because of the existence of a structural block) undergone the last two suggested steps in the mechanism of Scheme I (the ring opening and the internal aldol reaction). This fact is consistent with the suggestion of Bartlett's group that dehydroquinase synthase might not catalyze these last two steps at all. For, as Bartlett and Satake (1988) have elegantly shown, when intermediate IV is generated in neutral aqueous solution in the absence of enzyme, only dehydroquinone (**2**) is detected. In summary, while the involvement of the synthase in the conversion of intermediate IV (Scheme I) to dehydroquinone (**2**) remains conjectural, all the data are consistent with the sequence of transformations illustrated in Scheme I.

The 2-deoxy analogue **3** has a higher K_m than the natural substrate DAHP (**1**), and the overall rate of reaction of **3** with

Table I: Enzyme-Catalyzed Elimination of HX

enzyme	stereochemical course	proton α to
aconitase ^a	anti	acid
adenylosuccinase ^a	anti	acid
argininosuccinase ^a	anti	acid
aspartase ^a	anti	acid
citraconate hydratase ^a	anti	acid
enolase ^b	anti	acid
fumarase ^a	anti	acid
hydroxystearate dehydratase ^a	anti	alkyl
isopropylmalate dehydratase ^c	anti	acid
maleate hydratase ^a	anti	acid
mesaconate hydratase ^a	anti	acid
<i>S</i> -adenosylhomocysteine hydrolase ^d	syn	ketone
dehydroquinase dehydratase ^a	syn	ketone
dehydroquinase synthase ^e	syn	ketone
dehydroshikimate dehydratase ^f	syn	eneone
β -hydroxyacyl-CoA dehydratases		
β -hydroxybutyryl- ^g	syn	thiolester
β -hydroxydecanoyl- ^h	syn	thiolester
β -hydroxy- β -methylglutaryl- ⁱ	syn	thiolester
β -hydroxy- α -methylbutyryl- ^j	syn	? thiolester
UV endonuclease V ^k	syn	aldehyde

^a Arigoni and Eliel (1969). ^b Cohn et al. (1970). ^c Cole et al. (1973).

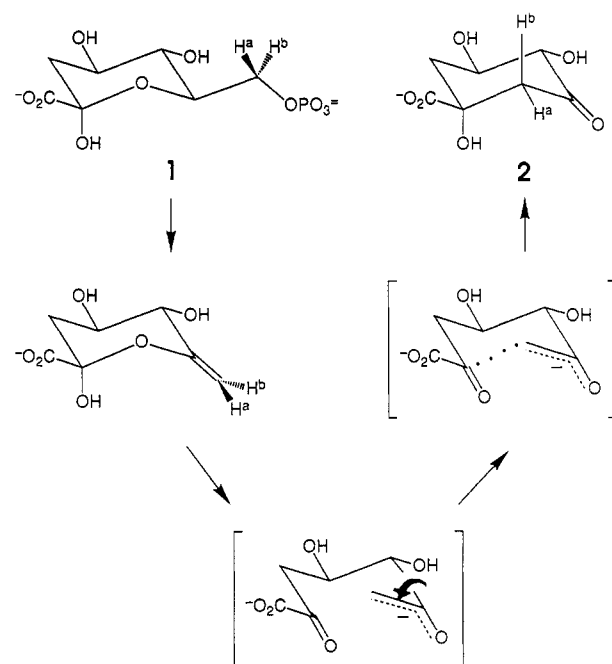
^d Parry and Askonas (1985). ^e This work. ^f Scharf et al. (1971).

^g Willarden and Eggerer (1975); Sedgwick et al. (1978). ^h Schwab et al. (1986). ⁱ Messner et al. (1975). ^j Aberhart and Tann (1979). ^k J. Gerlt, private communication.

the enzyme is, at saturating substrate levels, about 50-fold slower than that of substrate **1**. The cause of this difference cannot yet be defined, and it is clearly possible either that one or more of the steps leading up to the analogue of intermediate IV (i.e., enzyme-bound **12**) is slowed by the improper fit of **3** in the active site or that the release of **12** into the medium is rate-limiting overall. This uncertainty is, however, peripheral to the more important question of the stereochemical course of the elimination step.

As is described under Results, the loss of phosphate from **3** that is catalyzed by the synthase occurs with syn geometry. This fact alone suggests strongly that the elimination reaction is a stepwise process, and mechanistic precedent would indicate an E1cB pathway. This view is strengthened by the observation that the homophosphonate analogues of DAHP (which cannot, of course, lose P_i) exchange their C-6 proton with the solvent in the presence of enzyme (see previous paper). The implication is that these analogues can proceed up to and including the formation of the substrate enolate, but (being phosphonates) are unable to complete the two-step elimination process. Presuming that the natural substrate proceeds analogously, we find that the observed syn elimination of P_i catalyzed by dehydroquinase synthase fits the stereochemical pattern of β -eliminations (of water and of P_i) in enzymology. Thus, as listed in Table I, all enzymes that catalyze β -elimination reactions for substrates where the proton is lost from a carbon atom α to a carboxylate group, or to an alkyl group, proceed with anti stereochemistry. In all other cases, the abstracted proton is α to the carbonyl group of a ketone, an enone, an aldehyde, or a thiolester, and all these reactions go with syn stereochemistry. We must beware of being overzealous in searching for functional distinctions, since the two classes of enzymes could simply derive from an ancestral pair of catalysts, one that followed anti and one that followed syn stereochemistry. Yet there is a suggestion of a functional difference, since those substrates having the more acidic protons follow the syn pathway. More important for the present discussion is the fact that the cryptic stereochemical course of the elimination of phosphate that is buried in the

Scheme X: Stereochemical Relationships for the Reaction Catalyzed by Dehydroquinase Synthase



middle of the dehydroquinase synthase reaction sequence obeys the stereochemical correlation established for the simpler dehydration catalysts listed in Table I.

Since it has been known for many years that the overall transformation catalyzed by dehydroquinase synthase inverts the configuration at C-7 (Scheme X) (Rotenberg & Sprinson, 1970; Turner et al., 1975), knowledge of the stereochemical course of the phosphate elimination step limits the possible geometry of the transition state for the subsequent aldol reaction. There are only three transition states that satisfy the stereochemical requirements; two of boatlike and one of chairlike geometry. That which is illustrated in Scheme X can be achieved simply by a 180° rotation of one bond (that between C-5 and C-6). In contrast, the two transition states having boatlike geometry that are consistent with the stereochemical facts are each only reached by substantial reorganization of the molecular geometry in the complex containing the enolate. On the basis of minimal motion, therefore, we favor a transition state for the internal aldol reaction that has chairlike geometry. It is also worth noting that the chair transition state leads directly to the more stable conformation of the product dehydroquinase.

In most respects, the carbacyclic analogue **4** behaves similarly to the 2-deoxy species **3**. Each compound can proceed through the chemical steps outlined in Scheme I until P_i is lost. In both cases the corresponding olefin (**12** or **14**) is formed and is released from the enzyme, allowing true (if slow) turnover. The significant difference is observed in binding. Whereas (on the basis only of the values of K_m) the 2-deoxy analogue binds 10-fold less strongly to the synthase than the natural substrate **1**, the carbacyclic analogue **4** binds 25-fold more tightly. The existence of a hydroxyl group at C-2 is presumably important, but a part of this increase may derive from the fact that **4** is more readily oxidized at C-5 than **1** and that we observe the accumulation of the ternary complex enzyme-oxidized substrate-reduced cofactor. Whatever the reason, this behavior suggested the synthesis of yet more potent inhibitory molecules, as have been discussed in the previous paper.

Mechanism of the Enzyme-Catalyzed Reaction. DHQ

synthase appears to catalyze a remarkably complex reaction sequence, the understanding of which requires answers to two questions. First, what is the sequence of chemical events and reaction intermediates by which the overall transformation of DAHP to DHQ takes place? Second, how does a relatively small monomeric enzyme actually bring these chemical changes about? The starting point for our work was the chemically seductive and beautifully logical pathway first suggested by Sprinson and his collaborators (Srinivasan et al., 1963). This formulation is shown in Scheme I, using (consistent with our inhibitor binding experiments) the cyclic α -pyranose form of DAHP in the early steps. In the light of what follows, it is useful first to describe the logical sense of this pathway. Having collected the substrate, the enzyme-bound cofactor NAD^+ (possibly, as is well preceded for many dehydrogenases, in concert with the essential divalent cation) oxidizes DAHP at C-5. This is a logical first step: elimination of P_i from DAHP itself will be much less facile than from intermediate I, where the elimination is partly driven by formation of the eneone III (Scheme I). Ring opening will also be much less easy directly from the substrate **1** than it becomes later in IV, when the leaving group is an enol(ate) rather than an alcohol(ate). Having produced the C-5 ketone (I), enolization to II and loss of inorganic phosphate to III are now facile. This puts the first step into the class of an oxidative activation. The choice from the eneone intermediate III is either reduction to IV or ring opening to the terminal enol(ate) of the α -diketone. The reduction of the eneone III will not be as favorable as that of the ketone I, and the notionally competitive reaction (the facile ring opening of III) makes the formulation of IV as the next intermediate more a matter of preference than of necessity. [This preference notwithstanding, Maitra and Sprinson (1978) have presented evidence for the transient formation of a carbonyl group at C-5, but not for any at C-6.] Having reduced the substrate at C-5 to give IV, the last two steps, ring opening to V and reclosure in the aldol reaction to dehydroquinase (2), could follow uneventfully.

Although the sequence of events presented above has many attractive features, and although much that is known about the catalyzed reaction is consistent with this formulation, there are troubling aspects of Scheme I. Not least among these is the question of how all the necessary and appropriate acidic and basic catalytic groups, as well as NAD^+ and (presumably) a divalent cation with most of its ligation sphere, can possibly be assembled at a single active site. Many enzymes, after all, have as their *sole* function the catalysis of redox chemistry with NAD^+ , the β -elimination of P_i , the ring opening of a pyranose, or an aldol reaction. While the existence of such monofunctional enzymes does not deny the possibility of fitting in more catalytic groups to mediate other processes, the idea that a single active site could be assembled that would specifically recognize and stabilize the six transition states of Scheme I is disquieting. We are confronted, then, with the dilemma of evaluating an elegantly rational chemical pathway that puts seemingly impossible structural demands upon the active site of a single enzyme.

The experiments reported in this paper, along with recent work from Bartlett's group (Bartlett & Satake, 1988), provide some resolution to the problem. Thus, it appears that while the overall pathway shown in Scheme I may be correct, the enzyme's involvement in the catalysis of some of the six steps is peripheral at best. In the previous paper we have suggested that the basic group that abstracts the proton from C-6 in step 2 (Scheme I) may be the phospho group of the substrate DAHP itself. This proposal enjoys several advantages: (i)

a strong base (a phosphate monoester) is used for proton abstraction; (ii) the expected steric hindrance associated with the removal of a proton from a tertiary center that is 1,3-diaxial to a hydroxyl group is avoided; (iii) an enzymic base does not have to be brought close to the charged phospho ester group; and (iv) proton transfer to the phospho group makes this a better leaving group, thus avoiding the need for any general acid assistance from the enzyme in step 3 (Scheme I). If, then, the enzyme merely binds substrate in the gauche-gauche conformation that puts a peripheral phospho group oxygen near the C-6 proton, then after the oxidation at C-5 (step 1), enolization and phosphate loss (steps 2 and 3) may follow with no more enzymic involvement.

The eneone intermediate III (Scheme I) is now reduced by the enzyme-bound NADH to give the enol pyranose IV. At this point, IV may be lost from the enzyme to rearrange rapidly and smoothly [as demonstrated by Bartlett and Satake (1988)] to dehydroquinase (2). Our findings that the 2-deoxy substrate analogue **3** is processed by the enzyme as far as the reduced product IV, and that this product is then released from the enzyme, are entirely consistent with the idea that the enzyme takes no part in catalyzing the last two steps (steps 5 and 6) of Scheme I. The idea that not all the elementary steps of every metabolic transformation are enzyme-catalyzed is not new, though experimentally well-established examples are rare, and we must emphasize that the present case of intermediate IV to dehydroquinase (2) (Scheme I) is still unproved. One early suggestion that has some analogy with the present proposal is that of the enol generated by aldonic acid dehydrases such as 6-phosphogluconate dehydrase (Meloche & Wood, 1964). Here it appears that the enolic product is released from the enzyme and ketonizes in free solution.

By the arguments presented above, the catalytic role of dehydroquinase synthase is reduced to that of a simple oxidoreductase. It may only be necessary that the enzyme catalyze the oxidation at C-5 (after which the β -elimination is inevitable) and then the reduction (when, after the release of IV, ring opening and stereoselective aldol ring closure are necessary consequences). In this way, nature seems neatly to have exploited the normal chemical behavior of reasonably labile reaction intermediates in achieving an overall transformation of impressive complexity.

REFERENCES

- Aberhart, D. J., & Tann, C.-H. (1979) *J. Chem. Soc., Perkin Trans. 1*, 939-942.
- Arigoni, D., & Eliel, E. L. (1969) *Top. Stereochem.* **4**, 127-243.
- Bartlett, P. A., & Satake, K. (1988) *J. Am. Chem. Soc.* **110**, 1628-1630.
- Bartlett, P. A., Maitra, U., & Chouinard, P. M. (1986) *J. Am. Chem. Soc.* **108**, 8068-8071.
- Bender, S. L., Mehdi, S., & Knowles, J. R. (1989a) *Biochemistry* (first of three papers in this issue).
- Bender, S. L., Widlanski, T., & Knowles, J. R. (1989b) *Biochemistry* (second of three papers in this issue).
- Cohn, M., Pearson, J. E., O'Connell, E. L., & Rose, I. A. (1970) *J. Am. Chem. Soc.* **92**, 4095-4098.
- Cole, F. E., Kalyanpur, M. G., & Stevens, C. M. (1973) *Biochemistry* **12**, 3346-3350.
- de las Heras, F. G., & Fernández-Resa, P. (1982) *J. Chem. Soc., Perkin Trans. 1*, 903-907.
- Frost, J. W., Bender, J. L., Kadonaga, J. T., & Knowles, J. R. (1984) *Biochemistry* **23**, 4470-4475.
- Giese, B., & Gröninger, K. (1984) *Tetrahedron Lett.* **25**, 2743-2746.

- Giese, B., Gröninger, K. S., Witzel, T., Korth, H.-G., & Sustmann, R. (1987) *Angew. Chem., Int. Ed. Engl.* 26, 233-234.
- Haslam, E. (1974) *The Shikimate Pathway*, Wiley, New York.
- Keck, G. E., Enholm, E. J., Yates, J. B., & Wiley, M. R. (1985) *Tetrahedron* 41, 4079-4094.
- Lambert, J. M., Boocock, M. R., & Coggins, J. R. (1985) *Biochem. J.* 226, 817-829.
- Maitra, U. S., & Sprinson, D. B. (1978) *J. Biol. Chem.* 253, 5426-5430.
- Meloche, H. P., & Wood, W. A. (1964) *J. Biol. Chem.* 239, 3505-3510.
- Messner, B., Eggerer, H., Cornforth, J. W., & Mallaby, R. (1975) *Eur. J. Biochem.* 53, 255-264.
- Ohrui, H., Horiki, H., Kishi, H., & Meguro, H. (1983) *Agric. Biol. Chem.* 47, 1101-1106.
- Parry, R. J., & Askonas, L. J. (1985) *J. Am. Chem. Soc.* 107, 1417-1418.
- Rotenberg, S. L., & Sprinson, D. B. (1970) *Proc. Natl. Acad. Sci. U.S.A.* 67, 1669-1672.
- Rotenberg, S. L., & Sprinson, D. B. (1978) *J. Biol. Chem.* 253, 2210-2215.
- Scharf, K. H., Zenk, M. H., Onderka, D. K., Carroll, M., & Floss, H. G. (1971) *J. Chem. Soc., Chem Commun.*, 765-766.
- Schwab, J. M., Klassen, J. B., & Habib, A. (1986) *J. Chem. Soc., Chem. Commun.*, 357-358.
- Sedgwick, B., Morris, C., & French, S. J. (1978) *J. Chem. Soc., Chem. Commun.*, 193-194.
- Srinivasan, P. R., Rothschild, J., & Sprinson, D. B. (1963) *J. Biol. Chem.* 238, 3176-3182.
- Tejima, S., & Fletcher, H. G., Jr. (1963) *J. Org. Chem.* 28, 2999-3004.
- Turner, M. J., Smith, B. W., & Haslam, E. (1975) *J. Chem. Soc., Perkin Trans. 1*, 52-55.
- Weiss, U., & Edwards, J. M. (1980) *The Biosynthesis of Aromatic Compounds*, Wiley, New York.
- Whistler, R. L., & Anisuzzaman, A. K. M. (1980) *Methods Carbohydr. Chem.* 8, 227-231.
- Willarden, P., & Eggerer, H. (1975) *Eur. J. Biochem.* 54, 247-252.

Effect of the Distal Histidine Modification (Cyanation) of Myoglobin on the Ligand Binding Kinetics and the Heme Environmental Structures

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ABSTRACT: The kinetics of carbon monoxide (CO) binding to myoglobin (Mb) modified at the distal histidine (His) by cyanogen bromide (BrCN) has been studied. The CO association and dissociation rates of BrCN-modified Mb were obtained as $1.8 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ and 0.13 s^{-1} , respectively (20 °C and pH 7.0). Thermodynamic parameters were obtained as well. These values are notable, compared with those for other hemoproteins, the slowest association and the fastest dissociation rates among various hemoproteins examined so far. On the basis of the available structural data obtained from the absorption, ^1H NMR, and IR spectral measurements, these unique kinetic and thermodynamic properties were reasonably explained in terms of the steric restriction at the modified distal side.

We previously reported that the distal histidine (His E7) of sperm whale myoglobin (Mb) is specifically modified by reaction with an equimolar amount of cyanogen bromide (BrCN) (Shiro & Morishima, 1984), where the imidazolyl N-H of the distal His is displaced by the substituted N-CN. This distal His modification caused drastic ^1H NMR and absorption spectral changes of aquometMb, which suggested that the heme environmental structure of the BrCN-modified Mb (BrCN-Mb) is very different from that of native Mb. Quite different features of the hyperfine-shifted ^2H NMR resonances for meso-deuterated porphyrin containing native and BrCN-modified Mb's in the aquomet state indicated that the water molecule coordinated to the heme iron at the sixth

site is expelled upon BrCN modification (Morishima et al., 1985). It was also noted that ferric BrCN-Mb cannot combine with external ligands such as CN^- and imidazole which potentially bind to native Mb. These findings suggest that BrCN-Mb has unique ligand binding properties, due to the substantial structural changes in the heme distal site.

The importance of the distal His in controlling the heme iron reactivities of Mb and hemoglobin (Hb) has long been suggested on the basis of several biochemical and physicochemical investigations. In this connection, structural factors such as steric restriction and hydrogen-bonding interaction have been pointed out on the basis of X-ray and neutron diffraction studies of CO and O_2 complexes of Mb and Hb (Norvell et al., 1975; Baldwin, 1980; Phillips, 1980; Phillips & Schoenborn, 1981; Hanson & Schoenborn, 1981; Kuriyan et al., 1986). The distal His effects on ligand binding are pronouncedly manifested in the kinetic properties of mutant Mb and hB, in which the distal His is replaced by other amino acid residues (Giacometti et al., 1980; Parkhurst et al., 1980;

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