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Aromatic Inhibitors of Dehydroquinate Synthase: Synthesis, Evaluation and Implications for Gallic Acid Biosynthesis

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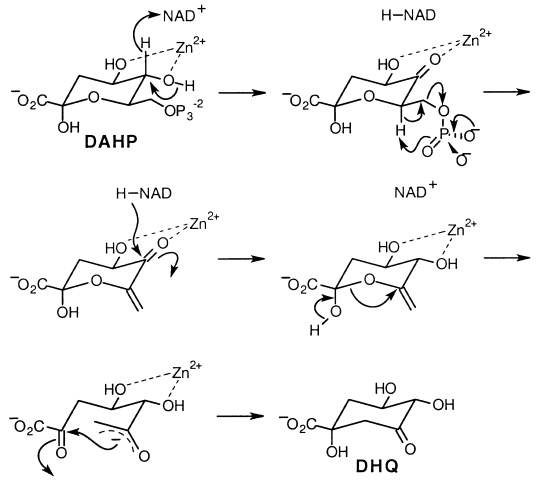
Abstract—The role of the active site metal in determining binding to 3-dehydroquinate synthase has been examined. Protocatechuic acid, catechol, and derivatives of these aromatics were synthesized that shared the common element of an *ortho* dihydroxylated benzene ring. Inhibition constants were determined for each aromatic as well as the variation of this inhibition as a function of whether Co^{+2} or Zn^{+2} was the active site metal ion.

3-Dehydroquinate synthase is an enzyme in the common pathway of aromatic amino acid biosynthesis that catalyzes the conversion of 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP, Scheme 1) into 3-dehydroquinic acid (DHQ, Scheme 1).[1,2] This reaction assembles the carbocyclic ring that ultimately becomes the benzene ring of L-phenylalanine, L-tyrosine, L-tryptophan, coenzyme Q, folic acid, and a spectrum of aromatic secondary metabolites. Protocatechuic acid, catechol, and derivatives of these aromatics have been synthesized and their inhibition examined of both the Co^{+2}-form and the Zn^{+2}-form of 3-dehydroquinate synthase. In addition to illustrating the importance of the metal ion as a determinant of binding to the active site of 3-dehydroquinate synthase, the *ortho* dihydroxylated benzene ring of these inhibitors represents a fundamental departure from all previously reported inhibitors of this enzyme.[2b,c,3]



Scheme 1.

3-Dehydroquinate synthase is mechanistically distinguished by its catalytic use of cofactor NAD^+. Reduction of NAD^+ to NADH during the first step of the enzyme-catalyzed reaction is followed by reoxidation of NADH to NAD^+ at a later enzyme-catalyzed step prior to release of 3-dehydroquinic acid from the active site (Scheme 1). Increasing attention has recently been paid to the catalytic role of the metal at the active site.[2a,4] 3-Dehydroquinic acid is a metalloenzyme, which utilizes either Co^{+2} or Zn^{+2} as its metal cofactor. A crystal structure of the Zn^{+2}-form of 3-dehydroquinate synthase isolated from *Aspergillus nidulans* has been solved with a carbaphosphonate analogue of DAHP bound at the active site.[5] The active site Zn^{+2} is positioned in close proximity to the backbone vicinal hydroxyl groups with metal to C-4 and C-5 oxygen bond distances of 2.3 Å and 2.2 Å, respectively. There has been disagreement as to whether the native form of 3-dehydroquinate found in nature binds Co^{+2} or Zn^{+2} at its active site. The Co^{+2}-form of the enzyme has been reported to be more stable and has a higher specific activity relative to the Zn^{+2}-form.[4] However, the bioavailability of Zn^{+2} in nature is much greater than Co^{+2}.[6]

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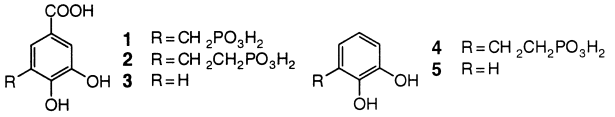
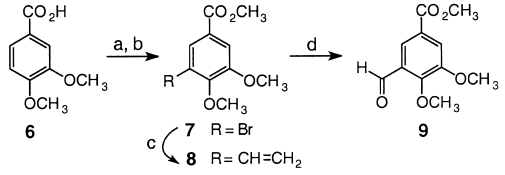
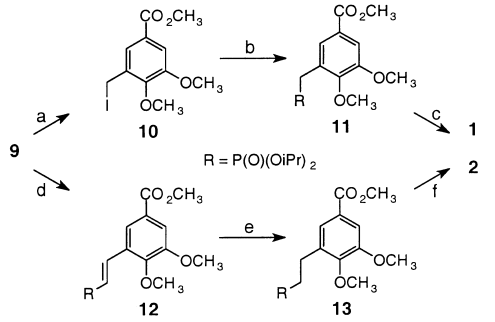
To explore the role of the divalent ion as a determinant of active site binding, several derivatives of protocatechuic acid were prepared to exploit the potential complexation of the *ortho* hydroxyl groups of these putative inhibitors with the active site metal ion. Both the Co^{+2}-form and the Zn^{+2}-form of 3-dehydroquinate synthase were also prepared. These two avenues facilitated exploration of how active site binding as measured by inhibition constant (K_i) varied as a function of inhibitor structure and enzyme-bound metal cofactor.



A trianionic ionization state in substrate analogues has been demonstrated to be important for binding to the active site of 3-dehydroquinate synthase.[3c] This was the ionization state for both phosphonomethyl protocatechuate **1** and phosphonoethyl protocatechuate **2**. The phospho-ethyl ring substituent of **2** is isosteric while the phosphonomethyl substituent of **1** is non-isosteric to the phosphate monoester of substrate DAHP. More potent inhibition of 3-dehydroquinate synthase is often, but not always, observed for non-isosteric phosphate monoester analogues.[3] For this reason, it is generally advisable to synthesize both phosphonomethyl and phosphonoethyl substrate analogues when a new class of 3-dehydroquinate synthase inhibitor is examined. A precursor synthesized (Scheme 2) from commercially available 3,4-dimethoxybenzoic acid was used for the synthesis (Scheme 3) of both phosphonomethyl protocatechuate **1** and phosphonoethyl protocatechuate **2**.



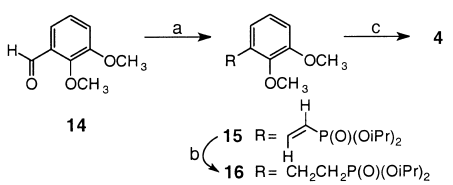
Scheme 2. (a) MeOH, H_2SO_4, 98%; (b) Br_2, AcONa, AcOH, 70 °C, 97%; (c) $Bu_3Sn(CH{=}CH_2)$, $Pd(PPh_3)_4$, HMPA, 65 °C, 63%; (d) O_3, CH_2Cl_2, −78 °C, 91%.



Scheme 3. (a) $[(CH_3)_2HSi]_2O$, TMSCl, NaI, AcCN, reflux; (b) $P(OiPr)_3$, toluene, reflux, 23%; (c) (i) BBr_3, CH_2Cl_2, −78 °C to rt; (ii) concd HCl, 62%; (d) *n*-BuLi, $[(iPrO)_2P(O)]_2CH_2$, THF, −78 °C to rt, 34%; (e) H_2, 10% Pd/C, MeOH, 76%; (f) (i) BBr_3, CH_2Cl_2, −78 °C to rt; (ii) concd HCl, 88%.

Inhibitors **3**–**5** facilitated a stepwise dissection of the role of complexation with the active site metal as a determinant of binding to 3-dehydroquinate synthase. Phosphonoethyl catechol **4**, which was synthesized (Scheme 4) from commercially available 2,3-dimethoxy benzaldehyde, lacks a carboxylic acid group. Active site binding for **4** must then be derived from active site interactions of the phosphonoethyl group and the metal binding of the *ortho* hydroxyl groups. Protocatechuic acid **3** is missing a substituent mimicking the phosphate monoester of DAHP. Its binding to 3-dehydroquinate synthase must therefore be derived from active site interactions with its carboxylate and metal complexation to the *ortho* hydroxyl groups. Differences in the inhibition of 3-dehydroquinate synthase for phosphonoethyl catechol **4** and protocatechuic acid **3** can be used to infer the importance of the carboxylate relative to the phosphate monoester analogue to active site binding. Catechol **5** constitutes the minimized set of active site binding capabilities. The only apparent basis for binding to 3-dehydroquinate synthase is complexation between the *ortho* hydroxyl groups of **5** and the active site metal.

3-Dehydroquinate synthase purified from *Escherichia coli* was employed for all inhibition studies.[7] Purification of the Co^{+2}-form of 3-dehydroquinate synthase, generation of *apo*enzyme and final reconstitution with Zn^{+2} resulted in an unstable enzyme preparation. A stable Zn^{+2} form of 3-dehydroquinate synthase was obtained when the 250 μM $CoCl_2$ normally present in all buffers beginning with cell lysis and extending through each step of the enzyme purification process was replaced with 75 μM $ZnSO_4$. Inhibition constants (K_i) were obtained from Dixon plots of the reciprocal initial velocities plotted against varying inhibitor concentrations in the presence of a fixed concentration of substrate. For phosphonomethyl protocatechuate **1**, phosphonoethyl protocatechuate **2**, phosphonoethyl catechol **4**, and protocatechuic acid **3**, replots of the Dixon data gave a line passing through the origin indicating competitive inhibition. Lineweaver–Burke and Hanes–Woolf plots also gave results consistent with competitive inhibition. Inhibition by catechol **5** was estimated from an I_{50} determination where reciprocal initial velocities were plotted against inhibitor concentrations using a concentration of substrate equal to the K_m (18 μM) for the Co^{+2}-form or the K_m (16 μM) for the Zn^{+2}-form of 3-dehydroquinate synthase. One half of the value of the *x*-intercept was taken as an estimate of K_i.



Scheme 4. (a) *n*-BuLi, $[(iPrO)_2P(O)]_2CH_2$, THF, −78 °C to rt, 95%; (b) H_2, 10% Pd/C, MeOH, 75%; (c) (i) BBr_3, CH_2Cl_2, −78 °C to rt; (ii) concd HCl, 97%.

Table 1. Inhibition constants (μM) for binding to the Co^{+2} and Zn^{+2} forms of 3-dehydroquinate synthase[a]

Metalloform	Inhibitor				
	1	**2**	**3**	**4**	**5**
Co^{+2}	21	5	44	200	880
Zn^{+2}	0.35	1.7	65	90	630

[a]Conditions: DHQ synthase activity was assayed in a 1.0 mL solution of 3-(*N*-morpholino)propanesulfonate (MOPS) buffer (50 mM, pH 7.5) containing either 50 μM $CoCl_2$ or 75 μM $ZnSO_4$ depending on the form of enzyme being studied, NAD^+ (10 μM), dehydroquinase (1 unit), and varying concentrations of DAHP and inhibitor. After equilibration at rt, purified DHQ synthase (0.024 units) was added, and the increase in absorbance at 234 nM was monitored over time. Initial rates were determined by linear square fits of the progress curves and were used to determine inhibition constants.

A pronounced difference in the pattern of inhibition of the Co^{+2}-form relative to the Zn^{+2}-form of 3-dehydroquinate synthase was observed (Table 1). Protocatechuate **2**, whose phosphonoethyl substituent is isosteric to a phosphate monoester, was the most potent inhibitor of the Co^{+2}-form (Table 1) of the enzyme. By contrast, nonisosteric phosphonomethyl protocatechuate **1** was the most potent inhibitor of the Zn^{+2}-form (Table 1) of 3-dehydroquinate synthase. The Zn^{+2}-form of 3-dehydroquinate synthase bound phosphonomethyl protocatechuate **1** more tightly than the Co^{+2}-form of the enzyme by a factor of 60-fold (Table 1). Binding of the carboxylate and the phosphonoethyl substituents by the Zn^{+2}-form of the enzyme was approximately the same based on the similar extent to which enzyme inhibition was reduced for phosphonoethyl catechol **4** and protocatechuic acid **3** relative to phosphonoethyl protocatechuate **2** (Table 1). The Co^{+2}-form of 3-dehydroquinate, by comparison, displayed a more pronounced difference (Table 1) in binding of carboxylate and phosphonoethyl substituents based on the different extent to which enzyme inhibition was reduced for phosphonoethyl catechol **4** and protocatechuic acid **3** relative to phosphonoethyl protocatechuate **2**. Although both the Co^{+2}-form and the Zn^{+2}-form of 3-dehydroquinate synthase were inhibited (Table 1) only weakly by catechol **5**, the fact that this structurally simple aromatic was an inhibitor at all attests to the important role that the active site metal ion plays in substrate binding.

A valuable dividend of the inhibition of 3-dehydroquinate synthase by *ortho* dihydroxylated aromatics is the ability to now explain the accumulation of 3-deoxy-D-*arabino*-heptulosonic acid as a major contaminant in the biocatalytic synthesis of gallic acid.[8d] Because it is known to be an impediment to the flow of carbon through the common pathway of aromatic amino acid biosynthesis, expression of *aroB*-encoded 3-dehydroquinate synthase is routinely amplified in hydroaromatic- or aromatic-synthesizing *Escherichia coli* biocatalysts. However, even with amplified expression of *aroB*-encoded 3-dehydroquinate synthase in gallate-synthesizing recombinant *E. coli*, accumulation of 3-deoxy-D-*arabino*-heptulosonic acid has been observed. This can now be explained by the intermediacy of protocatechuic acid **3** in the microbe-catalyzed conversion of glucose to gallic acid. Apparently, the protocatechuic acid **3** is inhibiting 3-dehydroquinate synthase. DAHP accumulating in the cytoplasm due to inhibition of 3-dehydroquinate synthase is dephosphorylated, which results in export and accumulation of 3-deoxy-D-*arabino*-heptulosonic acid as a major contaminant in fermentation broths. Given that protocatechuic acid **3** is an intermediate in numerous biocatalytic syntheses, inhibition of 3-dehydroquinate synthase by this *ortho* dihydroxylated aromatic constitutes a significant impediment to the elaboration of high-yielding microbial syntheses of *cis*,*cis*-muconic acid, catechol, vanillic acid, and gallic acid.[8]

Acknowledgements

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^1H NMR spectra were recorded on a 300 MHz spectrometer. Chemical shifts for ^1H NMR spectra are reported (in parts per million) relative to internal tetramethyl silane (Me_4Si, $\delta = 0.0$ ppm) with $CDCl_3$ as the solvent, and to sodium 3-(trimethylsilyl) propionate-2,2,3,3-d_4 (TSP, $\delta = 0.0$ ppm) when D_2O was the solvent. ^{13}C NMR were recorded at 75 MHz. Chemical shifts for ^{13}C NMR spectra are reported (in parts per million) relative to $CDCl_3$ ($\delta = 77.0$ ppm) or internal acetonitrile (CH_3CN, $\delta = 3.69$ ppm) in D_2O. FAB and CI mass spectra were obtained on a double-focusing mass spectrometer. Elemental analysis were performed by Atlantic Microlab Inc. (Norcross, GA).

5-Bromo-3,4-dimethoxy methybenzoate 7. ^{1}H NMR ($CDCl_3$) δ 3.90 (s, 3H), 3.92 (s, 6H), 7.10 (s, 1H), 7.42 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 165.8, 151.9, 147.7, 122.7, 116.8, 114.1, 113.8, 56.2, 56.1, 52.2. Anal. calcd for $C_{10}H_{11}BrO_4$: C, 43.84; H, 4.05. Found: C, 43.94; H, 4.07.

5-Vinyl-3,4-dimethoxy methyl benzoate 8. ^{1}H NMR ($CDCl_3$) δ 3.90 (s, 3H), 3.92 (s, 3H), 3.96 (s, 3H), 5.30 (dd, $J=1$, 11 Hz, 1H), 5.57 (dd, $J=1$, 17 Hz, 1H), 7.03 (s, 1H), 7.43 (s, 1H), 7.54 (dd, $J=11$, 17 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 167.1, 151.8, 147.8, 135.8, 134.2, 120.2, 114.9, 112.6, 109.2, 55.8, 55.7, 51.8. Anal. calcd for $C_{12}H_{14}O_4$: C, 64.86; H, 6.35. Found: C, 64.79; H, 6.33.

3-Formyl-4,5-dimethoxy methyl benzoate 9. ^{1}H NMR ($CDCl_3$) δ 3.97 (s, 3H), 3.99 (s, 3H), 4.01 (s, 3H), 7.47 (s, 1H), 7.51 (s, 1H), 10.65 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 191.2, 166.2, 152.3, 151.9, 131.2, 125.9, 112.5, 109.5, 56.3, 56.2, 52.6. Anal. calcd for $C_{11}H_{12}O_5$: C, 58.96; H, 5.40. Found: C, 58.57; H, 5.49.

Phosphonate intermediate 10. ^{1}H NMR ($CDCl_3$) δ 3.92 (s, 3H), 3.93 (s, 3H), 3.94 (s, 3H), 4.96 (s, 2H), 6.86 (s, 1H), 7.48 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 166.4, 152.0, 148.0, 135.4, 120.0, 113.7, 113.3, 56.02, 52.03, 5.2.

Phosphonate intermediate 11. ^{1}H NMR ($CDCl_3$) δ 1.17 (d, $J=6$ Hz, 6H), 1.27 (d, $J=6$ Hz, 6H), 3.77 (d, $J=2.3$ Hz, 2H), 3.89 (s, 3H), 3.91 (s, 3H), 3.93 (s, 3H), 4.61 (m, 2H), 6.95 (d, $J=2.4$ Hz, 1H), 7.46 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 167.2, 151.3, 146.9, 127.8, 121.6, 114.3, 113.2, 70.2 (d, $J=7.0$ Hz), 55.8, 51.7, 31.4 (d, $J=138$ Hz), 24.0, 23.8, 23.7, 23.6. Anal. calcd for $C_{17}H_{27}O_7P$: C, 54.54; H, 7.27. Found: C, 54.40; H, 7.34.

Protocatechuic phosphonate 1. ^{1}H NMR (D_2O) δ 3.5 (d, $J=22$ Hz, 2H), 6.85 (s, 1H), 7.38 (s, 1H); ^{13}C NMR (D_2O) δ 174.4, 150.8, 145.1, 130.9 (d, $J=10$ Hz), 124.8 (d, $J=6$ Hz), 121.9, 121.4, 34.9 (d, $J=130$ Hz); HRMS (FAB) calcd for $C_8H_8PO_7$ 247.0008, found 246.9998.

Homophosphonate intermediate 12. ^{1}H NMR ($CDCl_3$) δ 1.38 (s, 12H), 3.91 (s, 3H), 3.95 (s, 3H), 3.96 (s, 3H), 4.76 (m, 2H), 6.12 (dd, $J=17$, 18 Hz, 1H), 7.03 (s, 1H), 7.46 (s, 1H), 8.23 (dd, $J=17$, 22 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 166.6, 151.8, 149.3, 146.0 (d, $J=8$ Hz), 131.0 (d, $J=25$ Hz), 122.0, 117.2 (d, $J=191$ Hz), 112.8, 109.3, 70.6 (d, $J=6$ Hz), 70.5, 56.0, 52.1, 24.1, 24.0, 23.9, 23.8. Anal. calcd for $C_{18}H_{27}O_7P$: C, 55.95; H, 7.05. Found: C, 55.40; H, 6.83.

Homophosphonate intermediate 14. ^{1}H NMR ($CDCl_3$) δ 1.34 (s, 12H), 2.06 (m, 2H), 3.20 (m, 2H), 3.89 (s, 3H), 3.91 (s, 3H), 3.93 (s, 3H), 4.72 (m, 2H), 6.78 (s, 1H), 7.48 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 166.9, 151.9, 146.8, 137.7 (d, $J=18$ Hz), 120.4, 113.5, 113.4, 69.9 (d, $J=6$ Hz), 28.8 (d, $J=123$ Hz), 27.8 (d, $J=11$ Hz), 23.9. Anal. calcd for $C_{18}H_{29}O_7P$: C, 55.66; H, 7.53. Found: C, 55.03; H, 7.50.

Protocatechuic homophosphonate 2. ^{1}H NMR (D_2O) δ 2.00 (m, 2H), 3.02 (m, 2H), 6.76 (s, 1H), 7.37 (s, 1H); ^{13}C NMR (D_2O) δ 172.9, 151.3, 144.7, 140.3 (d, $J=19$ Hz), 122.3, 121.7, 120.6, 31.1 (d, $J=131$ Hz), 29.9; HRMS (FAB) calcd for $C_9H_{10}PO_7$ 261.0164, found 261.0158.

Catechol homophosphonate intermediate 15. ^{1}H NMR ($CDCl_3$) δ 1.33 (s, 3H), 1.35 (s, 3H), 1.37 (s, 3H), 1.39 (s, 3H), 3.86 (s, 3H), 3.87 (s, 3H), 4.72 (m, 2H), 6.35 (dd, $J=18$, 19 Hz, 1H), 6.93 (dd, $J=2$, 8 Hz, 1H), 7.06 (dd, $J=8$, 8 Hz, 1H), 7.13 (dd, $J=2$, 8 Hz, 1H), 7.78 (dd, $J=18$, 23 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 153.0, 148.0, 142.1 (d, $J=8$ Hz), 129.1 (d, $J=23$ Hz)), 124.0, 118.8, 117.1 (d, $J=190$ Hz), 113.5, 70.3 (d, $J=6$ Hz), 61.1, 55.8, 24.1, 24.0, 23.9, 23.8. Anal. calcd for $C_{16}H_{25}O_5P$: C, 58.53; H, 7.68. Found: C, 57.91; H, 7.70.

Catechol homophosphonate intermediate 16. ^{1}H NMR ($CDCl_3$) δ 1.32 (s, 3H), 1.33 (s, 3H), 1.34 (s, 3H), 1.35 (s, 3H), 2.01 (m, 2H), 2.90 (m, 2H), 3.84 (s, 3H) 3.85 (s, 3H), 4.72 (m, 2H), 6.79 (m, 2H), 6.99 (dd, $J=8$, 8 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 152.6, 146.9, 135.0 (d, $J=18$ Hz), 123.9, 121.4, 110.6, 69.9 (d, $J=6$ Hz), 60.5, 55.6, 27.9 (d, $J=138$ Hz), 23.9, 23.6 (d, $J=4$ Hz). Anal. calcd for $C_{16}H_{27}O_5P$: C, 58.17; H, 8.24. Found: C, 57.56; H, 8.24.

Catechol homophosphonate 4. ^{1}H NMR (D_2O) δ 2.10 (m, 2H), 2.90 (m, 2H), 6.82 (m, 3H); ^{13}C NMR (D_2O) δ 147.2, 144.6, 132.2 (d, $J=17$ Hz), 123.8 (d, $J=27$ Hz), 117.0, 29.5 (d, $J=132$ Hz), 25.768; HRMS (FAB) calcd for $C_8H_{12}PO_5$ 219.0422, found 219.0416.