



Inhibitors designed for the active site of dihydroorotase ☆

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Abstract

Four new compounds have been synthesized as potential inhibitors of dihydroorotase from *Escherichia coli*. NMR spectroscopy was used to show that 4,6-dioxo-piperidine-2(*S*)-carboxylic acid (**3**), exists in solution as a mixture of the hydrate (**7**), enol (**8**), and enolate (**9**) tautomeric forms. This compound was found to be a competitive inhibitor versus dihydroorotase and thio-dihydroorotase at pH values of 7–9. The K_i of 76 μ M was lowest at pH 7.0 where the ketone and hydrate forms of the inhibitor **3** predominate in solution. Compound **3** was reduced to the two diastereomeric 4-hydroxy derivatives (**4** and **5**) and then dehydrated to yield the alkene derivative, 1,2,3,6-tetrahydro-6-oxopyridine-2(*S*)-carboxylic acid (**6**). Compounds **4–6** were competitive inhibitors versus thio-dihydroorotase at pH 8.0 with K_i values of 3.0, 1.6, and 2.3 mM. Dihydroorotase was unable to dehydrate the 4-hydroxy derivative **4** or **5** to the alkene **6** or catalyze the reverse reaction.

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1. Introduction

Dihydroorotase (DHO¹) is a zinc metalloenzyme that catalyzes the reversible interconversion between *N*-carbamoyl-L-aspartate (**1**) and L-dihydroorotate (**2**) during the

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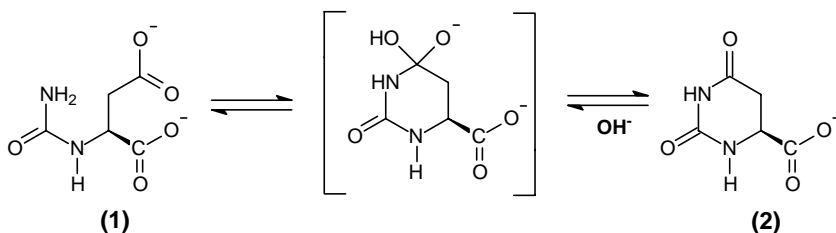
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¹ Abbreviations used: DHO, dihydroorotase; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TLC, thin-layer chromatography; Boc, *t*-butoxycarbonyl; Bn, benzyl; PMBn, *p*-methoxybenzyl.

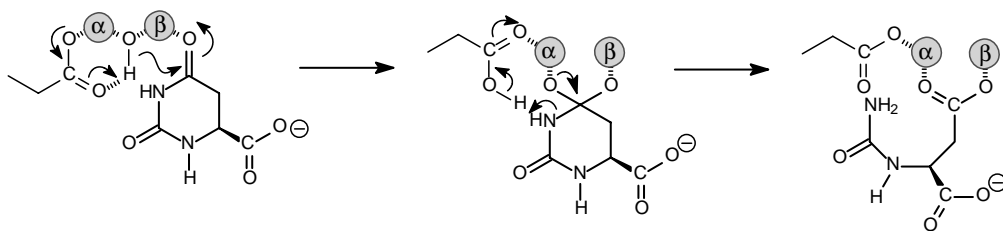
biosynthesis of pyrimidine nucleotides as shown in Scheme 1. This enzyme has been subdivided into two structural classes based upon differences in amino acid sequences [1]. The class I proteins are found in higher organisms and are bigger (~45 kDa) than their class II counterparts found in many bacteria and fungi. Members of the same class of DHO share more than 40% identity in their amino sequences, while less than 20% sequence identity is found for members between the two classes. The DHO from hamster, a member of class I, has been extensively characterized and studied by Christopherson [2–6]. A number of potential inhibitors, designed as transition-state or substrate analogues, have been synthesized and assayed against the hamster DHO because of the potential of these compounds as anticancer or antimalarial drugs [7–11].

The crystal structure of the DHO from *Escherichia coli* was determined in the presence of an equilibrium mixture of substrate and product [12]. The crystal structure revealed that the active site contains two zinc ions which are bridged by hydroxide. The structural study confirmed the prediction that DHO is a member of the amidohydrolase superfamily with a $(\beta\alpha)_8$ -barrel protein fold. The binuclear metal center at the active site is identical to those observed previously for phosphotriesterase and urease [12,13]. Based on these structural features, a mechanism that involves both zinc ions in the catalytic process has been proposed and subsequently support by mutagenesis, metal replacement, and kinetic measurements [14]. The proposed mechanism is presented in Scheme 2.

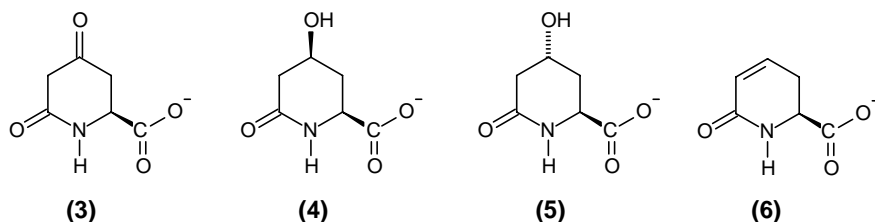
The class II dihydroorotases are generally found in bacteria and fungi and inhibitors of this enzyme are potential antimicrobial agents. Based on the proposed mechanism of action, 4,6-dioxo-piperidine-2-carboxylic acid (3) and the derivatives 4–6 are potential inhibitors of the reaction catalyzed by the bacterial dihydroorotase (Scheme 3). All four compounds can be considered as substrate analogues of dihydroorotate. In addition to acting as a substrate analogue of dihydroorotate, compound 3 may bind to the active site after the addition of water to form an analogue of the proposed tetrahedral intermediate



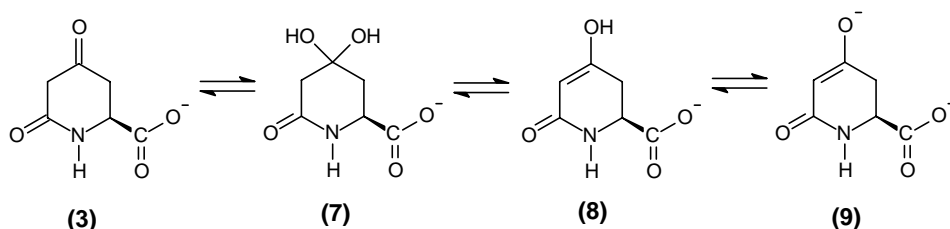
Scheme 1.



Scheme 2.



Scheme 3.



Scheme 4.

(7). Alternatively, ketone **3** may enolize to **8** or **9** (Scheme 4). In addition, compounds **4** and **5** may be dehydrated by DHO to **6** or compound **6** may be enzymatically hydrated to **4** or **5**. Here, we report the chemical synthesis and characterization of these compounds as potential inhibitors and substrates for the reaction catalyzed by dihydroorotase from *E. coli*.

2. Materials and methods

2.1. Inhibition assays

To evaluate the inhibitory properties of compounds **3–6**, the kinetic parameters for the DHO-catalyzed hydrolysis of dihydroorotate and 4-thio-dihydroorotate in the presence of these compounds were measured. For compound **3**, the equilibrium between the ketone and enol tautomeric forms are affected by pH in aqueous solution. Therefore, the kinetic parameters were measured at pH 7–9 for the inhibition by ketone **3**. The DHO used in these assays was a quadruple mutant (C221S/C263S/C265S/C268S) from *E. coli* and was purified as previously described [6]. This mutant is relatively stable to oxidation and has kinetic parameters consistent with the previously reported values for wild type DHO [15,16].

The assay methods for monitoring the enzymatic hydrolysis of dihydroorotate and thio-dihydroorotate were established previously [14,17,18]. The DHO-catalyzed hydrolysis of dihydroorotate or thio-dihydroorotate was monitored spectrophotometrically at 230 and 280 nm, respectively, using a SPECTRAmax (Molecular Device) plate reader. The assays were conducted in a 96-well quartz plate in a volume of 250 μ L. The buffer used in all the assays was 100 mM potassium phosphate. The concentration of dihydroorotate and thio-dihydroorotate was varied in the range of 50–200 and 10–100 μ M, respectively.

For data analysis, the initial rate for substrate hydrolysis in the presence of the inhibitor at different concentrations of the substrate was fitted to each of the following equations for competitive, uncompetitive, and noncompetitive inhibition [19]. In these equations v is the initial rate, A is the substrate concentration, I is the inhibitor concentration, k_{cat} is the turnover number, K_a is the Michaelis constant for the substrate, and K_{is} and K_{ii} are the slope and intercept inhibition constants.

$$v/E_t = (k_{\text{cat}}A)/(K_a(1 + I/K_{\text{is}}) + A) \quad (1)$$

$$v/E_t = (k_{\text{cat}}A)/(K_a + A(1 + I/K_{\text{ii}})) \quad (2)$$

$$v/E_t = (k_{\text{cat}}A/(K_a(1 + I/K_{\text{is}}) + A(1 + I/K_{\text{ii}}))). \quad (3)$$

2.2. 2-(*S*)-*tert*-Butoxycarbonylamino-3-oxo-pentanedioic acid 1-benzyl ester 5-ethyl ester (**11**)

Carbonyldiimidazole (9.2 g, 57 mmol) was added to a solution of *N*-Boc-*O*-Bn protected L-aspartic acid (**10**) (16.7 g, 52 mmol) in THF (250 ml). After the reaction mixture was stirred at room temperature for 8 h, into it was added potassium malonate mono ester (16.3 g, 57 mmol). The reaction mixture was stirred for another 24 h. The solvent was removed under reduced pressure and the residue partitioned between ethyl ether (250 ml) and an aqueous solution of hydrogen chloride (2 M, 250 ml). After separation, the aqueous phase was extracted with ethyl ether (2 × 100 ml). The ether solutions were combined and dried with sodium sulfate. Removal of solvent gave product **11** (19.9 g, 98%) as a colorless solid. ^1H NMR (ppm, CDCl_3): 7.38–7.30 (5H, m, aromatic); 5.5 (1H, NH, br); 5.16 (2H, s); 4.58 (1H, m); 4.18 (2H, q, $J = 7.1$ Hz); 3.42 (2H, s); 3.28 (1H, dd, $J_1 = 18.3$ Hz, $J_2 = 3.7$ Hz); 3.11 (1H, dd, $J_1 = 18.3$ Hz, $J_2 = 4.3$ Hz); 1.43 (9H, s); 1.26 (3H, t, $J = 7.1$ Hz). ^{13}C NMR (ppm, CDCl_3): 201.2; 171.2; 166.7; 155.7; 135.5; 128.8; 128.6; 128.4; 80.4; 67.7; 61.8; 49.8; 49.4; 45.0; 28.5; 14.3. MS: $\text{M} + \text{H}^+$, 394.2 (calcd), 394.2 (found).

2.3. 4-Oxo-2(*S*)-(pent-4-enoyl)-amino-hexanedioic acid 1-benzyl ester 6-ethyl ester (**12**)

To a solution of **11** (7.0 g, 17 mmol) in dichloromethane (10 ml) was added TFA (10 ml). The reaction mixture was stirred at room temperature for 1 h and then condensed to dryness under reduced pressure. The residue was dissolved in dichloromethane (40 ml) and into it was added 4-pentenoic anhydride (3.7 g, 1.2 equiv) and triethylamine (3.5 g, 2.0 equiv). The reaction mixture was stirred at room temperature for 8 h and then poured into 200 ml of ethyl ether. The solution was washed with water (3 × 20 ml), dried with anhydrous sodium sulfate, and condensed to about 50 ml and a colorless crystalline solid precipitated from the solution. After filtration and washing with cold ethyl ether, the solid was isolated after drying (3.6 g). The residue was chromatographed on silica gel to yield another portion of the product **12** (1.6 g) with a total yield of 86%. ^1H NMR (ppm, CDCl_3): 7.40–7.30 (5H, m, aromatic); 6.52 (1H, NH, d, br, $J = 7.82$ Hz); 5.84–5.74 (1H, m); 5.17 (2H, s); 5.08–4.95 (2H, m); 4.89–4.82 (2H, m); 4.18 (2H, q, $J = 7.1$ Hz); 3.41 (2H, s); 3.31 (1H, dd, $J_1 = 18.5$ Hz, $J_2 = 4.4$ Hz); 3.15 (1H, dd, $J_1 = 18.5$ Hz, $J_2 = 4.2$ Hz); 2.40–2.29 (4H, m); 1.27 (3H, $J = 7.1$ Hz). ^{13}C NMR (ppm, CDCl_3): 201.4, 172.4; 170.8; 166.7; 137.0; 135.4; 128.8; 128.7; 128.5; 115.9; 67.8; 61.8; 49.3; 48.3; 44.6; 35.7; 29.6; 14.3. MS: $\text{M} + \text{H}^+$, 376.2 (calcd), 376.2 (found).

2.4. 4-Hydroxy-2-(pent-4-enoyl)-amino-hexanedioic acid 1-benzyl ester 6-ethyl ester (**13a** and **b**)

A solution of ketone **12** (2.42 g) in a mixed solvent of methanol and THF (5 ml, 3:1 v/v) was cooled to 0 °C with an ice-water bath. To this solution was added sodium borohydride (0.12 g, 3.1 mmol) in three portions at intervals of 10 min. When the starting material was almost depleted (as monitored by TLC) the reaction mixture was poured onto ice and extracted with ethyl ether (3 × 100 ml). The solution of ethyl ether was dried with sodium sulfate and then condensed to dryness. The resulting residue was applied to a column of silica gel and eluted with a mixture of ethyl acetate and hexanes (1:4). Fractions containing each of the desired products were collected and condensed to dryness to yield the **13a** (4*S*, 2*S*, R_f = 0.32) and **13b** (4*R*, 2*S*, R_f = 0.25) diastereomers (0.85 g and 0.84 g, respectively) with a total yield of 70% in a ratio ~1:1. Compound **13a**: ^1H NMR (ppm, CDCl_3): 7.32–7.44 (5H, m, aromatic); 6.60 (1H, NH, d, br); 5.77–5.90 (1H, m); 5.20 (2H, s) 5.13–5.01 (2H, m); 4.89–4.83 (1H, m) 4.20 (1H, s, br); 4.16 (2H, q, J = 7.1 Hz); 4.03–3.96 (1H, m); 2.53 (1H, dd, J_1 = 15.9 Hz, J_2 = 8.3 Hz); 2.46–2.35 (5H, m); 1.99–1.91 (1H, m); 1.79–1.71 (1H, m); 1.27 (3H, t, J = 7.1 Hz). MS: $\text{M}+\text{H}^+$, 378.2 (calcd), 378.2 (found). Compound **13b**: ^1H NMR (ppm, CDCl_3): 7.42–7.32 (5H, m, aromatic); 6.54 (1H, NH, dd, J = 6.1 Hz, br); 5.89–5.78 (1H, m); 5.32 (1H, s); 5.25 (1H, d, J = 12.0 Hz); 5.19 (2H, d, J = 12.0 Hz); 5.13–4.49 (2H, m); 4.67 (1H, q, J = 5.62 Hz); 4.21–4.10 (3H, m); 2.50 (6H, m); 2.13–1.96 (2H, m); 1.28 (3H, CH_3 , J = 7.1 Hz). MS: $\text{M}+\text{H}^+$, 378.2 (calcd), 378.2 (found).

2.5. 4(*S*)-(4-Methoxy-phenoxy)-2(*S*)-(pent-4-enoyl)amino-hexanedioic acid 6-ethyl ester 1-(2-vinyl-but-2-enyl) ester (**14a**)

Lanthanum triflate (154 mg) was added to a solution of alcohol **13a** (1.8 g, 5.3 mmol) and *p*-methoxybenzyl trichloroacetimidate (3.0 g, 11 mmol) in toluene (20 ml). The reaction mixture was stirred at room temperature until the starting alcohol was depleted in about 4 h. After removal of the solvent at reduced pressure, the residue was applied to a silica gel column and eluted with a mixture of ethyl acetate and hexanes (1:3) to yield the product as a colorless oil (2.0 g, 80%). Compound **14a**: ^1H NMR (ppm, CDCl_3): 7.42–7.32 (5H, m, aromatic); 7.27 (2H, d, J = 8.2 Hz); 6.87 (2H, d, J = 8.2 Hz); 6.66 (1H, d, J = 8.1 Hz); 5.80–5.70 (1H, m); 5.22–5.16 (2H, m); 5.02–4.59 (2H, m); 4.83–4.77 (1H, m); 4.88 (1H, d, J = 10 Hz); 4.25 (1H, d, J = 10 Hz); 4.15 (2H, q, J = 7.1 Hz); 3.94–3.86 (1H, m); 3.80 (3H, CH_3 , s); 2.67 (1H, dd, J_1 = 15.4 Hz, J_2 = 12.6 Hz); 2.46 (1H, dd, J_1 = 15.4 Hz, J_2 = 7.3 Hz); 2.29–2.18 (2H, m); 2.14–1.98 (4H, m); 1.27 (3H, CH_3 , t, J = 7.1 Hz). ^{13}C NMR (ppm, CDCl_3): 172.4; 172.0; 171.1; 159.7; 137.2; 135.7; 115.5; 114.2; 74.3; 72.0; 67.4; 61.0; 55.5; 50.7; 39.5; 36.6; 35.7; 29.5; 14.2. MS: $\text{M}+\text{H}^+$, 498.2 (calcd), 498.2 (found).

2.6. 4(*R*)-(4-Methoxy-benzyloxy)-2(*S*)-(pent-4-enoyl)-amino-hexanedioic acid 6-ethyl ester 1-(2-vinyl-but-2-enyl) ester (**14b**)

This compound was obtained in the same manner as described above for **14a**. ^1H NMR (ppm, CDCl_3): 7.40–7.26 (5H, m, aromatic); 7.25 (2H, d, J = 8.9 Hz); 6.87 (2H, d, J = 8.9 Hz); 6.21 (1H, NH, d, J = 7.1 Hz, br); 5.86–5.70 (1H, m); 5.10–4.90 (3H, m);

4.63 (1H, q, $J = 6.0$ Hz); 4.47 (1H, d, $J = 10.1$ Hz); 4.28 (1H, d, $J = 10.1$ Hz); 4.14 (2H, q, $J = 7.1$ Hz); 3.96–3.90 (1H, m); 3.80 (3H, s, CH₃); 2.67 (1H, dd, $J_1 = 15.1$ Hz, $J_2 = 5.3$ Hz); 2.51 (1H, dd, $J_1 = 15.1$ Hz, $J_2 = 6.7$ Hz); 2.37–2.30 (2H, m); 2.22–2.13 (4H, m); 1.26 (3H, t, $J = 7.1$ Hz). MS: $M+H^+$, 498.2 (calcd), 498.2 (found).

2.7. 4(*S*)-(4-Methoxy-benzyloxy)-6-oxo-piperidine-2(*S*)-carboxylic acid benzyl ester (**15a**)

Compound **14a** (0.68 g) was dissolved in THF (10 ml). To this solution was added water (10 ml) immediately followed by addition of iodine (1.05 g). The reaction mixture was stirred vigorously for 30 min. Portions of sodium thiosulfate were added until the reaction mixture was colorless. The reaction mixture was concentrated to dryness and then resuspended in THF (10 ml). The solid was filtered and washed with THF (3 × 5 ml). The THF solutions were combined, dried with sodium sulfate, and condensed to dryness. The residue was applied to a silica gel column and eluted with a mixture of hexanes and ethyl acetate (2:1). The ninhydrin positive fractions were collected and condensed to dryness to yield the free amine. The amine was then dissolved in benzene (5 ml) and refluxed for 4 h. The reaction mixture was condensed to dryness and the residue applied to a column of silica gel and eluted with ethyl acetate. Fractions that were UV positive were collected and condensed to dryness to yield the cyclized product **15a** (0.15 g, 29%) as an oil. ¹H NMR (ppm, CDCl₃): 7.40–7.26 (5H, m, aromatic); 7.22 (2H, d, $J = 7.8$ Hz, aromatic); 6.87 (2H, d, $J = 8.7$ Hz, aromatic); 6.23 (1H, NH, s, br); 5.13 (1H, d, $J = 12.1$ Hz); 5.05 (1H, d, $J = 12.1$); 4.43 (1H, s); 4.13–4.08 (1H, m); 3.19–3.85 (1H, m); 3.80 (3H, CH₃, s); 2.66 (1H, dd, $J_1 = 17.3$ Hz, $J_2 = 5.0$ Hz); 2.25 (1H, dd, $J_1 = 17.3$, $J_2 = 6.4$ Hz); 2.40–2.33 (1H, m); 2.33–2.25 (1H, m). ¹³C NMR (ppm, CDCl₃): 171.2; 170.2; 159.5; 135.4; 130.1; 129.4; 128.8; 128.7; 128.6; 114.0; 70.3; 70.2; 67.7; 55.5; 52.1; 37.8; 30.0. MS: $M+H^+$, 370.2 (calcd), 370.2 (found).

2.8. 4(*R*)-(4-Methoxy-benzyloxy)-6-oxo-piperidine-2(*S*)-carboxylic acid benzyl ester (**15b**)

Compound **15b** was synthesized in the same manner as described for **15a** from **14b** with a yield of 35%. ¹H NMR (ppm, CDCl₃): 7.43–7.34 (5H, m, aromatic); 7.24 (2H, d, $J = 8.6$ Hz); 6.88 (2H, d, $J = 8.6$ Hz); 6.47 (1H, NH, s, br); 5.24 (1H, d, $J = 12.4$ Hz); 5.18 (1H, d, $J = 12.4$ Hz); 4.48 (1H, d, $J = 11.5$ Hz); 4.46 (1H, d, $J = 11.5$ Hz); 4.41 (1H, dd, $J_1 = 10.3$ Hz, $J_2 = 4.6$ Hz); 3.96 (1H, m); 3.81 (3H, s, CH₃); 2.64–2.57 (1H, m); 2.49 (1H, dd, $J_1 = 17.8$ Hz, $J_2 = 4.4$ Hz); 2.46–2.39 (1H, m); 1.94–1.85 (1H, m). ¹³C NMR (ppm, CDCl₃): 171.4; 169.9; 159.9; 135.2; 130.0; 129.4; 129.0; 128.9; 128.7; 114.1; 70.4; 69.5; 67.8; 55.5; 51.2; 36.9; 30.1. MS: $M+H^+$, 370.2 (calcd), 370.2 (found).

2.9. 4(*S*)-Hydroxy-6-oxo-piperidine-2(*S*)-carboxylic acid benzyl ester (**16a**)

Compound **15a** (0.38 g) was added to 5 ml of TFA and the resulting dark red colored solution was stirred at room temperature for 20 min and then condensed to dryness. The residue was applied to a column of silica gel and chromatographed. The second series of UV positive fractions were collected and condensed to dryness to yield product **16a** as a colorless solid (0.26 g, 99%). The product was recrystallized from chloroform and the single crystal

was used to determine the stereochemical configuration at the C4 position by X-ray crystallography. The crystal structure analysis showed that C4 has the *S*-configuration.

^1H NMR (ppm, CDCl_3): 7.4–7.3 (5H, m, aromatic); 6.91 (1H, NH, br); 5.21 (1H, d, $J = 12.1$ Hz); 5.17 (1H, d, $J = 12.1$ Hz); 4.24–4.17 (1H, m); 4.14–4.09 (1H, m); 3.24 (1H, OH, s, br); 2.62 (1H, dd, $J_1 = 17.7$ Hz, $J_2 = 4.7$ Hz); 2.32–2.25 (1H, m); 2.25–2.15 (1H, m). ^{13}C NMR (ppm, D_2O): 171.8; 171.1; 135.3; 128.9; 128.6; 67.8; 63.9; 52.0; 40.0; 32.5. MS: $\text{M} + \text{H}^+$, 250.1 (calcd), 250.1 (found).

2.10. 4(*R*)-Hydroxy-6-oxo-piperidine-2(*S*)-carboxylic acid benzyl ester (**16b**)

Compound **16b** was synthesized following the procedure used above for **16a** starting from compound **15b** and isolated as a colorless solid with a yield of 95%. ^1H NMR (ppm, CDCl_3): 7.41–7.31 (5H, m, aromatic); 6.88 (1H, NH, s, br); 5.21 (1H, d, $J = 12.4$ Hz); 5.17 (1H, d, 12.4 Hz); 4.48 (1H, dd, $J_1 = 10.27$ Hz, $J_2 = 5.0$ Hz); 4.31 (1H, m); 3.62 (1H, OH, s, br); 2.56–2.43 (2H, m); 2.39–2.13 (1H, m); 1.88–1.80 (1H, m). ^{13}C NMR (ppm, CDCl_3): 171.6; 171.2; 135.2; 128.95; 128.9; 128.6; 67.8; 62.9; 51.0; 39.5; 32.1. MS: $\text{M} + \text{H}^+$, 250.1 (calcd), 250.1 (found).

2.11. 4(*S*)-Hydroxy-6-oxo-piperidine-2(*S*)-carboxylic acid (**4**)

Compound **16a** (0.12 g) was dissolved in THF (5 ml) and to this solution was added the catalyst Pd/C (20 mg). The suspension was then stirred under hydrogen for 8 h. The catalyst was removed from the reaction mixture by filtration through a Celite pad (3 cm thick) and washed with THF. The filtrate and washing solution were combined and condensed to dryness under reduced pressure to yield product **4** as a colorless crystalline solid (64 mg) with a yield of 87%. ^1H NMR (ppm, D_2O): 4.16–4.08 (2H, m); 2.62 (1 H, ddd, $J_1 = 17.5$ Hz, $J_2 = 5.22$ Hz, $J_3 = 1.62$ Hz); 2.33–2.4 (1H, m); 2.27 (1H, dd, $J_1 = 17.5$ Hz, $J_2 = 7.5$ Hz), 1.99–1.90 (1H, m). ^{13}C NMR (ppm, D_2O): 173.8, 172.2, 63.8, 51.8, 39.4, 33.1. MS: $\text{M} + \text{H}^+$, 160.0 (calcd), 160.0 (found).

2.12. 4(*R*)-Hydroxy-6-oxo-piperidine-2(*S*)-carboxylic acid (**5**)

Compound **5** (0.058 g), a colorless crystalline solid was synthesized from compound **16b** (0.098 g) following the procedure described above for compound **4** with a yield 93%. ^1H NMR (ppm, D_2O): 4.21 (1H, dd, $J_1 = 8.3$ Hz, $J_2 = 5.21$ Hz), 4.12–4.07 (1H, m), 2.48 (1H, dd, $J_1 = 17.6$ Hz, $J_2 = 4.6$ Hz), 2.21 (1H, ddd, $J_1 = 17.6$ Hz, $J_2 = 4.8$ Hz, $J_3 = 1.5$ Hz), 2.13–2.06 (1 H, m), 1.95–1.88 (1 H, m). ^{13}C NMR (ppm, D_2O): 175.1, 173.3, 63.5, 52.1, 40.2, 33.4. MS: $\text{M} + \text{H}^+$, 160.0 (calcd), 160.0 (found).

2.13. 4,6-Dioxo-piperidine-2(*S*)-carboxylic acid (**3**)

Di-*tert*-butyl-(2*S*)-4,6-dioxo-1,2-piperidine dicarboxylate (1.54 g) was added to TFA (10 ml). The resulting solution was stirred at room temperature for 1 h and then condensed to dryness. After the residue was washed with ethyl ether (3×10 ml), the solid was dried under reduced pressure to yield the ketone **3** as a colorless solid. The ketone form dominates (67%) in aqueous solution at pH 2. The other component of the solution is the hydrate **7** (33%), which is characterized below. Compound **3**: ^1H NMR (ppm, D_2O): 4.4 (1H,

dd, $J_1 = 6.5$ Hz, $J_2 = 5.3$ Hz); 2.91 (1H, dd, $J_1 = 17.1$ Hz, $J_2 = 6.5$ Hz); 2.73 (1H, dd, $J_1 = 17.1$ Hz, $J_2 = 5.1$ Hz). ^{13}C NMR (ppm, D_2O): 206.6; 174.3; 172.1; 51.2; 46.6; 40.1. MS: $\text{M}-\text{H}^+$, 156.0 (calcd), 156.0 (found).

2.14. 4,4-Dihydroxy-6-oxo-piperidine-2(*S*)-carboxylic acid (7)

^1H NMR (ppm, D_2O): 4.16 (1H, dd, $J_1 = 6.8$ Hz, $J_2 = 6.3$ Hz); 2.25 (1H, dd, $J_1 = 13.7$ Hz, $J_2 = 6.3$ Hz); 2.17 (1H, dd, $J_1 = 13.8$ Hz, $J_2 = 6.8$ Hz). ^{13}C NMR (ppm, D_2O): 175.4; 172.9; 91.8; 52.2; 43.8; 35.9. MS: $\text{M}+\text{H}^+$, 176.0 (calcd), 176.0 (found).

2.15. Dianion of 1,2,3,6-tetrahydro-4-hydroxy-6-oxo-piperidine-2(*S*)-carboxylic acid (9)

Ketone **3** exits solely as the dianion after titration to pH 10 with potassium hydroxide (10 M in H_2O). ^1H NMR (ppm, $\text{D}_2\text{O}-\text{H}_2\text{O} = 1:4$): 6.01 (1H, br), 4.41 (1H, br), 3.9 (1H, t, $J = 7.5$ Hz), 2.44 (1H, dd, $J_1 = 16.5$ Hz, $J_2 = 6.8$ Hz), 2.35 (1H, dd, $J_1 = 16.5$, $J_2 = 8.8$ Hz). ^{13}C NMR (ppm, $\text{D}_2\text{O}-\text{H}_2\text{O} = 1:4$): 185.1; 179.6; 175.6; 88.4; 54.9; 36.2.

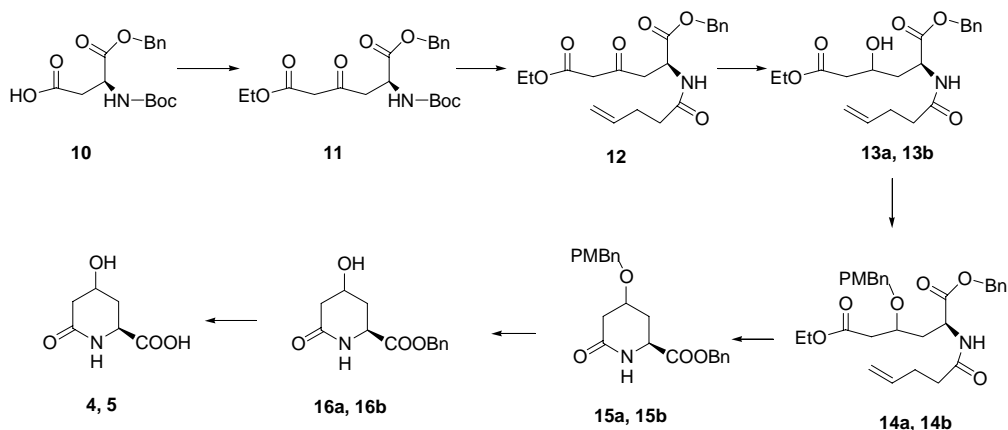
2.16. (*S*)-1,2,3,6-Tetrahydro-6-oxopyridine-2-carboxylic acid (6)

Neat trifluoroacetic acid (2 ml) was added to a solution of **20** (238 mg) in dichloromethane (2 ml). The resulting solution was stirred at room temperature for 2 h and then condensed to dryness under reduced pressure. The residue was washed with ethyl ether (3×10 ml) and dried under reduced pressure to yield the crystalline product **6** (106 mg, 94%). ^1H NMR (ppm, D_2O): 6.72–6.66 (1H, m), 5.8 (1H, dt, $J_1 = 10.7$ Hz, $J_2 = 1.77$ Hz), 4.24 (1H, t, $J = 6.6$ Hz), 2.81–2.67 (2H, m). ^{13}C NMR (ppm, D_2O): 173.2, 166.9, 140.7, 123.8, 52.0, 26.5. MS: $\text{M}+\text{H}^+$, 142.0 (calcd), 142.0 (found).

3. Results and discussion

3.1. Synthesis of inhibitors

The syntheses of alcohols **4** and **5** were carried out following the pathway shown in Scheme 5. Commercially available *N*-*tert*-butoxycarbonyl- α -*O*-benzyl-*S*-aspartic acid (**10**) was activated by reaction with carbonyl diimidazole (CDI) in THF and then reacted with the magnesium salt of ethyl hydrogen malonate to yield the β -ketone **11** [20,21]. The Boc group in **11** was changed to pent-4-enyl, which can be selectively cleaved under oxidation by I_2 [22]. This transformation gave **12** in 85% yield upon cleavage of the Boc protecting group with TFA and subsequent acylation with pent-4-enoic anhydride. Reduction of **12** with NaBH_4 in methanol provided the diastomeric mixture of alcohols **13a** and **b** in a ratio of about 1:1, and subsequently resolved from one another by silica gel chromatography. After the alcohols were protected to yield **14a** and **b** with a *p*-methoxybenzyl group [23,24], the pent-4-enyl group was selectively cleaved to yield the corresponding amines which partially cyclized during purification on silica gel. After the crude amine was refluxed in benzene for 8 h, the cyclization was complete to give the products **15a** and **b**. Treatment of **15a** and **b** in dichloromethane with TFA yielded the corresponding alcohols, **16a** or **b**, quantitatively. Reduction of **16a** or **b** by Pt-catalyzed hydrogenation yielded the desired product **4** or **5** in quantitative yield. A single crystal of **16a** was recrystallized from



Scheme 5.

chloroform and the structure solved by X-ray crystallography. The structure of **16a** is presented in Fig. 1. The structural analysis showed that C4 in **16a** has the *S*-configuration and thus C4 in **16b** is of the *R*-configuration.

A more convenient pathway to compounds **3**, **4**, and **6** starting from compound **17** was described by Marin et al. as shown in Scheme 6 [25]. Deprotection of **18–20** by neat TFA gave **3**, **4**, and **6** in quantitative yield. The NMR spectra of **4** made from **16a** are consistent with those of **4** made from **19**, and thus the stereochemistry at C4 is of the *S*-configuration. No racemization of the carbon at C2 was observed in any of the reactions described above.

The ^1H NMR spectrum of **3** in $\text{DMSO}-d_6$ shows that **3** exists as two tautomeric forms (Fig. 2A). The two doublets at 3.22 and 3.08 ppm are characteristic of the resonances for the two protons attached to C5 of ketone **3**. The other proton signals are assigned based on the integral ratio, chemical shifts, and splitting patterns. The signals at 4.79 and 10.48 ppm, which show coupling with none of the other protons, are assigned to the hydrogen at C5 and the hydroxyl of enol **8**, respectively. The ratio of ketone **3** to enol **8**

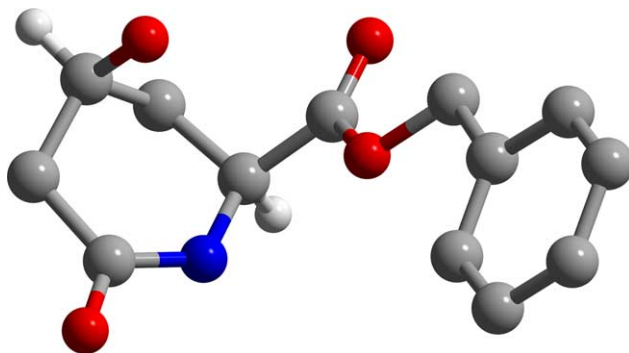
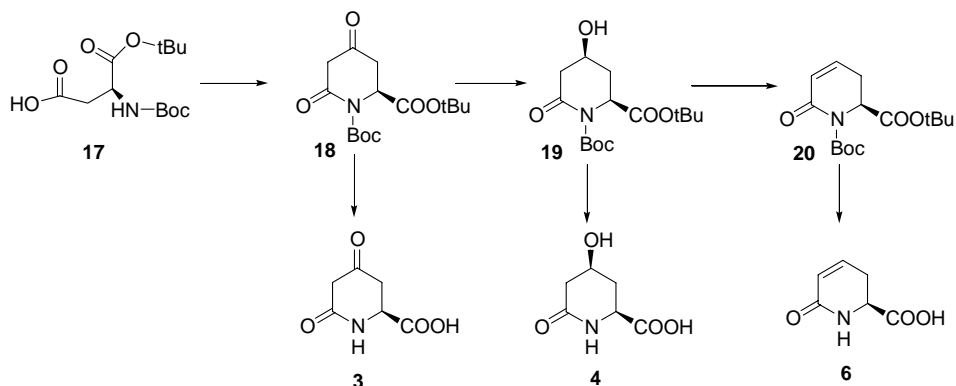


Figure 1. Crystal structure of compound **16a** with H atoms omitted except for those at C4 and C6. This structure confirms the *S*-stereochemistry at C4. The carbon, oxygen, nitrogen, and hydrogen atoms are presented as gray, red, blue, and white spheres, respectively. (For interpretation of the references to colors in this figure legend, the reader is referred to the web version of this paper.)



Scheme 6.

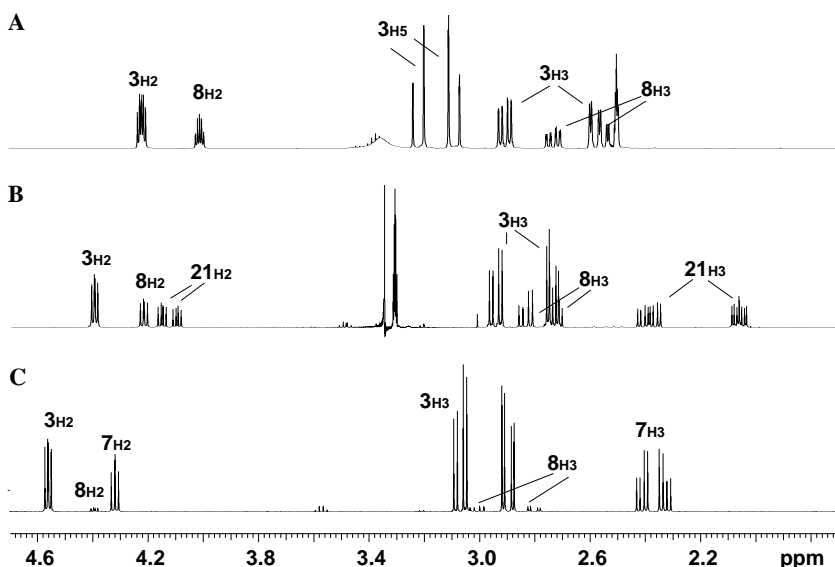


Figure 2. ^1H NMR of ketone **3** at 300 MHz. The solvent used in these spectra are (A) $\text{DMSO-}d_6$; (B) CD_3COD ; and (C) D_2O .

is about 2:1. The ^1H spectrum of **3** in deuterated methanol shows no signal for the C5 protons due to exchange between these protons and the deuterium of the solvent (Fig. 2B). However, the characteristic signals for the protons at C2 and C3 of the ketone **3** and enol **8** are clearly present. In addition to these signals, two more sets of resonances appear at 4.14, 4.08, 2.38, and 2.06 ppm and are assigned to the protons at C3 and C2 of the two diastereomers of hemiacetal **21**. The ^1H NMR spectrum of ketone **3** in D_2O at pH 1.8 indicates that ketone **3** and its hydrate **7** are the dominant species but some enol **8** is observed (Fig. 2C). The resonances at 4.15 ppm and those at 2.25 and 2.17 ppm are assigned to the C2 and C3 protons of the hydrate **7**.

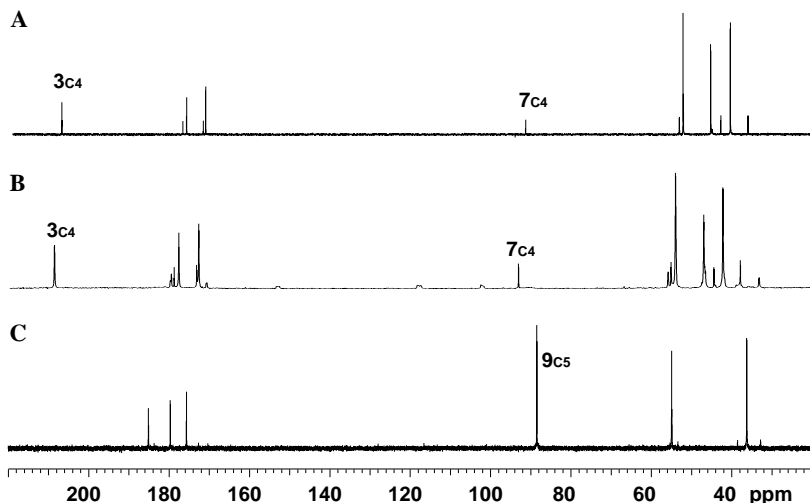


Figure 3. ^{13}C NMR spectra of ketone **3** in aqueous solution at different pH values. (A) 1.9; (B) 7.0; and (C) 10.

The identification of ketone **3** and hydrate **7** and enolate **9** is also confirmed by measurement of the ^1H and ^{13}C NMR spectra of ketone **3** in aqueous solutions at various values of pH (Fig. 3). The ^{13}C NMR spectra of ketone **3** show two sets of signals at pH 1.8 and 7.0 (Figs. 3A and B). The dominant species is ketone **3** and is indicated by the resonance at 207.8 ppm corresponding to the signal for the carbonyl carbon at C4 of ketone **3**. The other characteristic resonance at 92.4 ppm is assigned to C4 of hydrate **7**. This later assignment is also supported by the absence of this resonance in a proton-attached DEPT spectra obtained with ketone **3** in a mixture D_2O and H_2O . As the pH increases around 8, the relative amount of hydrate and ketone decrease and that of the enol or enolate increase. Both the ^1H NMR and ^{13}C NMR resonances of the ketone, enol, and enolate (which can be observed when the pH is higher than 7) are broadened at pH ~ 8 . The broadening of the NMR signals indicate an intermediate exchange rate between ketone **3** and enol **8** or enolate **9**. When the pH is greater than 8, the enolate dominates the distribution of species. When the pH is 10 only the enolate **9** can be observed in the ^1H and ^{13}C NMR spectra (Fig. 3C). The characteristic signal of C5 of enolate **9** appears at 88.4 ppm. This signal also appears in the DEPT spectra as one of the CH carbon atoms that confirms the identification of the enolate **9**. The UV spectrum of ketone **3** in aqueous solution varies with changes in pH. A new species appears with a maximum absorption at 277 nm when the pH is greater than 7 (Fig. 4). This species should be the enol or enolate as indicated by the ^1H and ^{13}C NMR spectra. The $\text{p}K_a$ for the absorbance change is 8.2.

3.2. Inhibition of dihydroorotase

Compounds **3–6** were tested as inhibitors of the reaction catalyzed by the dihydroorotase from *E. coli*. A summary of the kinetic constants are presented in Table 1. All of the compounds were found to be competitive inhibitors versus dihydroorotate and thio-dihydroorotate. Compounds **4–6** are relatively weak competitive inhibitors versus thio-dihydroorotate at pH 8. The K_i values varied from 1.6 to 3.0 mM. The variations in the

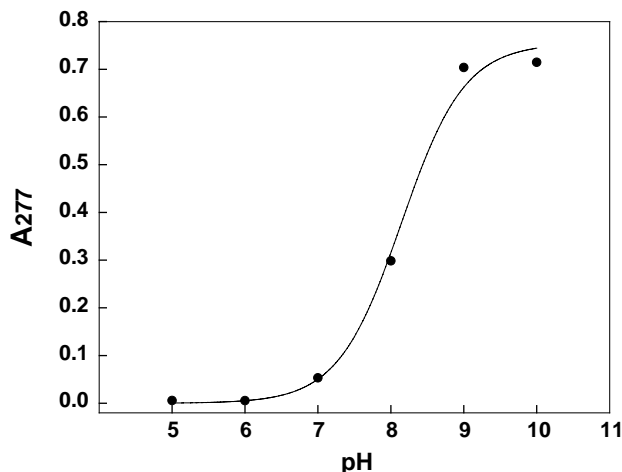


Figure 4. Change in absorbance at 277 nm of ketone **3** (27 μ M) as a function of pH.

Table 1
Inhibition of dihydroorotase by compounds **3–6**^a

Compound	3	3	3	4	5	6
pH	7.0	8.0	9.0	8.0	8.0	8.0
K_i						
Dihydroorotate	76 \pm 4	210 \pm 10	440 \pm 65	NA	NA	NA
Thio-dihydroorotate	96 \pm 7	120 \pm 5	320 \pm 32	3000 \pm 300	1600 \pm 340	2300 \pm 160

^a The data were fit to Eq. (2).

functional group attached to C4 do not contribute significantly to binding to the active site. It was anticipated that the attachment of a hydroxyl group at C4 would mimic and/or replace the hydroxide that bridges the two divalent cations within the active site of DHO. In the crystal structure of DHO complexed with dihydroorotate in the active site the bridging hydroxide is 2.8 Å away from the *re*-face of the amide group of the substrate [12]. This observation would predict that compound **5** should be a better inhibitor than the diastereomer **4**. In fact the K_i for **5** is approximately two-fold smaller than the K_i for **4**. If the hydroxyl group in **4** or **5** binds predominantly to the β -metal ion then one would also predict that **5** would be bound more tightly than **4**. The introduction of a double bond between carbons 4 and 5 in compound **6** did not significantly enhance or diminish the ability of this compound to bind to the active site of DHO. The relatively small competitive inhibitory activities exhibited by compounds **4–6** can largely be attributed to anchoring interaction of the free carboxylate group that has been determined to electrostatically interact with Asn-44, Arg-20, and His-254 in the enzyme [12].

The ketone **3** is the most active inhibitor among the four compounds prepared for this investigation. The ketone group at C4 can be hydrated in aqueous solution and the acidic protons at C5 can promote the formation of the enolic form of **3**. The composition of possible structures in solution is variable with pH. At low pH the NMR spectrum of ketone **3** indicates that it is a mixture of the ketone **3** and the hydrate **7**. The ratio of **3** to **7** at low

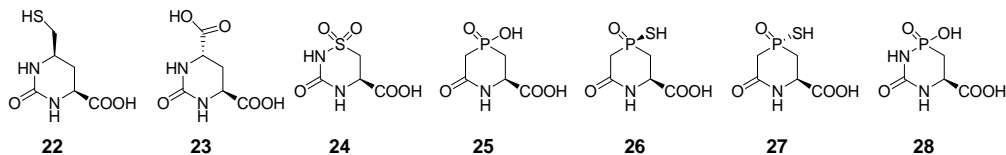
pH is 2:1. As the pH is raised to 7, ketone **3**, enol **8**, and hydrate **7** are the dominant species in the ratio of 1.0:1.0:0.6. At pH 8 the enol **8**, enolate **9**, and hydrate **7** are found in a ratio of 1.0:1.0:0.2. At pH 9, only enolate **9** is found in solution. In the inhibition experiments, ketone **3** showed the strongest inhibition at pH 7 and the apparent K_i is comparable to the K_m of the substrate dihydroorotate. As the pH of the medium increases the composite K_i for compound **3** also increases. These results indicate that ketone **3** and/or hydrate **7** are more inhibitory than the enol **8** and/or enolate **9**.

The best inhibitor of DHO reported to date is *cis*-4-carboxy-6-(mercaptomethyl)-3,4,5,6-tetrahydropyridin-2(1*H*)-one, **22**, with a K_i of 0.14 μ M [8]. The strong binding to the active center has been attributed to the strength of the interaction of Zn(II) to the thiol substituent in **22** relative to the same interaction with an oxygen in either a hydroxyl or carbonyl functional group. The carboxylate analogue, **23**, was found to bind to DHO about 30 times stronger than dihydroorotate [9]. The strong binding can be attributed to the chelation of the C4 carboxylate to either of the two zinc ions within the active site. Analogues designed to mimic potential transition-state structures such as **24** were found to have no significant inhibitory activity against DHO [9]. The three phosphinate mimics, **25**, **26**, and **27**, of the transition-state showed surprisingly weak inhibition against DHO from hamster with K_i values of 4.0, 2.9, and 3.1 mM, respectively [10,11]. A promising transition-state analogue inhibitor **28** has been suggested by Levenson and Meyer but this compound has thus far eluded synthesis [7]. The structures of **22–28** are presented in Scheme 7.

3.3. Substrate activity of 4–6

DHO has the demonstrated catalytic activity for the addition and release of water from dihydroorotate and carbamoyl aspartate. Therefore we felt that it may be possible for DHO to catalyze the hydration of alkene **6** to form either alcohol **4** or **5**. Alternatively, alcohols **4** and/or **5** could be dehydrated to **6**, depending on the thermodynamic equilibrium constant between the alkene and alcohol. To determine if DHO is able to catalyze the interconversion between alcohols **4** or **5** and the alkene **6**, each of these compounds was incubated with DHO for 40 h in aqueous solution at pH 8 buffered with phosphate. In these assays, the final concentration of the enzyme, substrate analogue (**4**, **5**, or **6**) and the buffer were 1.8×10^{-3} mM, 1.0 and 100 mM, respectively. The changes in the absorbance were monitored from 200–300 nm at various times up to 40 h. The alkene **6** shows a broad absorbance that occurs at 248 nm due to conjugation with the carbonyl group at C6. After 40 h we could obtain no evidence for enzymatic equilibration between **6** and either **4** or **5**. The estimated upper limit to the hydration/dehydration reaction is 0.1 s^{-1} .

In summary, compounds **3–6** have been synthesized and assayed for their inhibitory activity against dihydroorotase from *E. coli*. The inhibitory activity of ketone **3**, the hydrate **7** and the two tautomers, enol **8** and enolate **9**, were measured as a function of



Scheme 7.

pH. The inhibitory activities of these compounds and the tautomeric forms of **3** increases in the following order **4** < **6** < **5** < **8**, **9** < **3**, **7**. Compounds **3** and **7** may form transition-state like structures in the active site of DHO and provide the strongest inhibition among the compounds synthesized for this investigation. DHO does not catalyze the elimination of water from alcohols **4** or **5** or the hydration of ketone **6**.

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