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STRUCTURE–ACTIVITY RELATIONSHIPS OF PYRIMIDINES AS DIHYDROOROTATE DEHYDROGENASE INHIBITORS

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Abstract—The activity of dihydroorotate dehydrogenase (DHO-dehase) has been reported to decrease both *in vitro* and *in vivo* in hepatocellular carcinomas. DHO-dehase, the fourth enzyme of the *de novo* pyrimidine biosynthetic pathway, is a mitochondrial enzyme which is both a potential rate-limiting reaction in the *de novo* pyrimidine biosynthetic pathway and a potential therapeutic target for tumor inhibitors. This paper reports results on a series of pyrimidine analogs of dihydroorotate (DHO) and orotic acid (OA) as inhibitors of DHO-dehase. The enzyme test results established that the intact amide and imide groups of the pyrimidine ring and the 6-carboxylic acid are required for significant enzyme inhibition. The testing of several functional groups similar in characteristics to that of the carboxylic acid, such as sulfonamide, tetrazole and phosphate, indicated that the carboxylic acid group is preferred by the enzyme. Using various 5-substituted OA and DHO derivatives, it was shown that there is a steric limitation of a methyl group at this position. The compound D,L-5-*trans*-methyl DHO (7) (K_i of 45 μM) was both an inhibitor and a weak substrate for the enzyme, demonstrating that mechanism-based enzyme inhibitors should be effective. The testing results further suggest that a negatively charged enzyme substituent may be present near the 5-position of the pyrimidine ring and that there may be an enzyme-substrate metal coordination site near the N-1 and carboxylic acid positions of the pyrimidine ring. The combined testing results were then used to define both conformational and steric substrate enzyme binding requirements from which a model was proposed for the binding of DHO and OA to the DHO-dehase active site.

Dihydroorotate dehydrogenase (DHO-dehase, EC 1.3.3.1), the fourth enzyme of the *de novo* pyrimidine biosynthetic pathway, catalyzes the oxidation of dihydroorotic acid (DHO) to orotic acid (OA) (Fig. 1). The enzyme is an integral membrane protein on the outer surface of the inner mitochondrial membrane and is linked to the electron transport system [2, 3]. A hypothetical model for electron transfer from DHO-dehase to the electron transport system has been proposed and includes a ubiquinone cofactor for the enzyme [3–5].

In contrast to the adjacent *de novo* pyrimidine enzyme complexes A and U, both of which have elevated activities in neoplastic tissue, the activity of DHO-dehase has been found to decrease in hepatocarcinoma *in vitro* [6, 7] and during hepatocarcinogenesis *in situ* [8]. The decrease in DHO-dehase activity which was observed in neoplastic tissues would therefore provide a potential rate-limiting reaction in the *de novo* pyrimidine pathway and a potential site for selective tumor inhibition. The naphthoquinone antibiotics, lapachol and dichloroallyllawsone, which have received clinical trial, act via inhibition of DHO-dehase through interruption of the transfer of electrons [9–11]. DHO-dehase inhibitors also have potential as selective antimalarial agents, as malaria parasites lack the salvage pathway and thus are dependent upon the *de novo* biosynthetic pathway for pyrimidines [12].

To establish the basis for rational design of mechanism-based inhibitors of bacterial, rat and bovine enzymes, we and others have studied the mechanism of DHO-dehase. Analysis of the course of the reaction by NMR [13] and MS [14] has established that the stereochemical course in mammals is identical to that in bacteria. Steric requirements for activity also were refined in order to establish appropriate areas for modification of the dihydro derivatives at carbon-5.

The structure–activity relationships for inhibitors of the bacterial DHO-dehase have been summarized [15]. Studies on the mammalian non-human enzyme have been limited but have involved the testing of several pyrimidines and all of the important intermediates of pyrimidine biosynthesis [1, 2, 5, 16]. These studies also determined that L-DHO is the enzyme substrate and that the D-enantiomer is not a substrate and only a weak competitive inhibitor. The product of the reaction, orotic acid, is a competitive inhibitor. Inhibitors of human spleen DHO-dehase have also been examined and several inhibitor binding requirements described [17]. Barbituric acid and related derivatives were examined as inhibitors of enzymes in both the pyrimidine *de novo* and salvage

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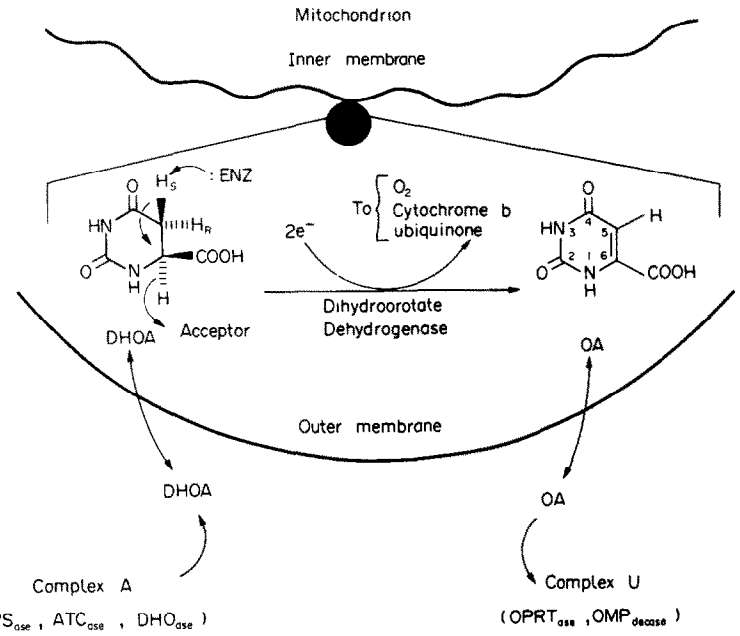


Fig. 1. Cellular location of an orotic acid pathway as adapted from Jones [1]. Abbreviations: CPSase, carbamoyl phosphate synthetase; ATCase, aspartate transcarbamoylase; DHOase, dihydroorotase; OPRTase, orotate phosphoribosyltransferase; and OMPdecase, orotate monophosphatedecarboxylase.

pathways [18]. A series of orotic acid analogs were also tested against the enzyme isolated from mouse reticulocytes and the protozoan, *Plasmodium berghei* [12]. Through these inhibitor studies, it was found that both enzymes tolerate some bulk in the 5-position of substituted orotic acids and that the 6-carboxyl could be replaced by a 6-thiol group. The analog 5-aza-5,6-dihydroorotate was found to inhibit both the mammalian [12, 17, 19] and the protozoan enzyme [12]. *In vitro* and *in vivo* antineoplastic activities for most of the aforementioned compounds have been determined [20].

This paper reports results on a series of more than forty pyrimidine analogs of DHO and OA that were tested as inhibitors of rat liver DHO-dehase. Results were used to establish structure–activity relationships for inhibitors of DHO-dehase and to define both conformational and steric substrate binding requirements.

MATERIALS AND METHODS

General procedures. Nuclear magnetic resonance (^{1}H NMR) spectra were obtained on an 80 MHz Varian FT80 spectrometer and at the Purdue University Nuclear Magnetic Resonance Laboratories using a 470 MHz Nicolet spectrometer. Chemical shift values are expressed in δ values (parts per million). Infrared spectra (IR) were obtained on a Beckman IR33 spectrometer. Mass spectrometry was performed on a Finnegan model 4023 mass spectrometer. Microanalyses were performed at the Purdue University Microanalysis Laboratories and are within ±0.4% for each element analyzed. All reagents were reagent grade. Melting points are uncorrected.

L-Dihydroorotate and dichlorophenolindophenol (DCIP) were purchased from Sigma. Menadione was purchased from ICN Biochemicals. Lapachol was purchased from Aldrich and dichloroallyllawsone was a gift from the National Cancer Institute. *In vitro* cell culture assays were performed by the Cell Culture Laboratory of the Purdue Cancer Center.

(cis)-5,6-*Dihydro-5-methylorotic acid* (**4**). Compound **4** was prepared from 5-methylorotic acid (**13**) as described [21, 22]. Yield (38%), m.p. = 238.5–239.5° (dec.); ^{1}H NMR (DMSO-d_6), δ 10.03 (bs, 1H, 3-NH), δ 7.77 (bs, 1H, 1-NH), δ 3.94 (dd, $J_{1,6}$ = 3.8 Hz, $J_{5,6}$ = 6.6 Hz, 1H, 6-CH), δ 2.89 (dq, $J_{5,6}$ = 6.6 Hz, $J_{5,7}$ = 7.0 Hz, 1H, 5-CH), δ 1.03 (d, J = 7.0 Hz, 3H, CH_3); CIMS *m/e* (relative intensity), 173 ($M^+ + 1$, 100); IR (KBr, cm^{-1}), 3430, 3210, 3000, 1690, 1655, 1610.

(trans)-5,6-*Dihydro-5-methylorotic acid* (**7**). A mixture containing 250 ml of anhydrous ethanol and 0.33 g (14.5 mmol) of sodium was reacted and then 0.5 g (2.9 mmol) of (*cis*)-5,6-dihydro-5-methylorotic acid (**4**) was added and the solution was refluxed for 4 days. The ethanol was then concentrated to 50 ml, and the white suspension was acidified with 5 N HCl to pH 1.0. After cooling overnight, the white precipitate was filtered and recrystallized from water yielding 0.389 g (77%), m.p. 227–228°; it then resolidified and melted at 245–246° (dec.). An analytical sample was prepared by recrystallization from ethanol. The NMR indicated that the solid contained 84% of the *trans* and 16% of the *cis* isomers. ^{1}H NMR (DMSO-d_6), δ 10.09 (bs, 1H, 3-NH), δ 7.81 (bs, 1H, 1-NH), δ 3.78 (t, $J_{5,6}$ = 3.1 Hz, $J_{1,6}$ = 3.1 Hz, 1H, 6-CH), δ 2.68 (dq, $J_{5,6}$ = 3.1 Hz, $J_{5,7}$ = 7.2 Hz, 1H, 5-CH), δ 1.22 (d, $J_{5,7}$ = 7.2 Hz, 3H, CH_3); CIMS, *m/e* (relative intensity), 127 (12), 173 ($M^+ + 1$, 100); IR (KBr, cm^{-1}), 3350, 3260, 2980, 1740, 1720, 1695, 1665, 1655. Elemental analysis for $C_6H_8N_2O_4$.

1,5 - *Dihydrofuro*[3,4 - *d*]*pyrimidine* - 2,4,7(3*H*)-*trione* (**6**) (cis-5,6-*dihydro*-5-*hydroxymethylorotic acid lactone*). Recrystallized 5-hydroxymethylorotic acid lactone (**15**) [23] (2.0 g, 11.9 mmol) was added to a solution of 800 ml of distilled water and 50 ml of glacial acetic acid in a hydrogenation bottle. The mixture was heated and stirred until dissolved. After the solution cooled, 0.96 g of 5% Rh/Al catalyst was added. The hydrogenation bottle was attached to a Parr hydrogenation apparatus and filled with hydrogen (40 psi). After 36 hr, the reaction was terminated and the catalyst filtered. The filtrate was evaporated *in vacuo* and the resulting white precipitate dissolved in 125 ml of anhydrous methanol (any insoluble material was filtered off). This slightly yellow solution was decolorized with activated carbon and the methanol removed *in vacuo*. The white powder was recrystallized from methanol. Yield: 1.53 g (76%); m.p. = 245–247° (dec.); ^{1}H NMR (DMSO-d_6), δ 3.58 (bq, $J_{5a,5b}$ = 9.2 Hz, $J_{4a,5b}$ = 5.7 Hz, 2H, 5-H_2), δ 4.45 (q, $J_{4a,5a}$ = 5.8 Hz, $J_{4a,7a}$ = 9.1 Hz, 1H, 4a-H), δ 4.57 (q, $J_{7a,1}$ = 4.5 Hz, $J_{6,5}$ = 9.1 Hz, 1H, 7a-H), δ 8.46 (bd, $J_{1,7a}$ = 4.5 Hz, 1H, 1-H), δ 10.6 (bs, 1H, 3-NH); CIMS, *m/e* (relative intensity), 171 (M^+ + 1, 100); IR (KBr, cm^{-1}): 3300, 3160, 2830, 1780, 1720, 1609, 1420. Elemental analysis for $C_6H_6N_2O_4$.

5-*Aza*-5,6-*dihydrouracil* (**18**). Oxonic acid (1.0 g, 5.12 mmol) was dissolved with heating in a solution of 400 ml of water and 25 ml of glacial acetic acid in a hydrogenation bottle. The catalyst was added (0.43 g of 5% Rh/Al or PtO_2), and the hydrogenation bottle was attached to a Parr hydrogenation apparatus and filled with hydrogen (40 psi). After 36 hr the reaction was terminated, the catalyst was removed by filtration, and the solution was evaporated *in vacuo*. The resulting white precipitate was recrystallized from distilled water and characterized. Yield: 0.70 g (90%); m.p. = 290–292° (dec.). Physical and spectroscopic data confirmed the structure [24].

L-*DHO-methyl ester* (**16**). To a suspension of 0.5 g (3.16 mmol) of L-DHO in 15 ml of CH_3OH and 4 ml of water was added a 20% Cs_2CO_3 solution until the pH was 7.0 (~3.6 ml), resulting in a clear solution. The solution was rotary evaporated to dryness yielding a white solid. To this solid was added 2 × 10 ml of anhydrous *N,N*-dimethylformamide (DMF) (distilled from CaH_2) and, after each addition, the solution was evaporated to dryness. To the white solid was then added 60 ml of anhydrous DMF and 2.5 g (17.66 mmol) of methyl iodide, and this mixture was stirred at ambient temperature for 48 hr. The solvent was then removed yielding a yellow semi-solid which was placed on a flash column of silica gel and eluted with $CHCl_3/CH_3OH$ (9:1). Fractions of R_f = 0.22 on silica gel TLC plates developed in $CHCl_3/CH_3OH$ (9:1), visualized by starch/KI, were recombined and evaporated to dryness. The resulting yellow solid was decolorized with carbon and recrystallized in ethanol yielding white needles (0.152 g, 28% yield), m.p. = 192–192.5° (lit. m.p. = 180°) [24]; $[\alpha]_D^{24}$ = +60.4 (DMF, C = 0.506). Physical and spectroscopic data confirmed the structure [25].

L-*DHO-benzyl ester* (**17**). This procedure was identical to that for the methyl ester (**16**) with benzyl bromide and benzyl alcohol as reactants. After the white suspension was heated in benzyl alcohol at 45° for 45 hr, 20 ml of water was added causing the product to solidify. The product was filtered yielding a light yellow solid which was decolorized with carbon and recrystallized in ethanol. White needles (0.405 g, 51% yield) were obtained, m.p. = 196.5–197.5°; $[\alpha]_D^{24}$ = +54.7° (DMF, C = 0.506). ^{1}H NMR (DMSO-d_6) δ 10.17 (bs, 1H, 3-NH), δ 7.96 (bd, J = 3.3 Hz, 1H, 1-NH), δ 7.37–7.33 (m, 5-H, phenyl), δ 5.16 (s, 2H, CH_2), δ 4.27 [p (ddd), J = 7.5, 3.3, 1.8 Hz, 1H, 6-H], δ 2.94 (dd, J = 7.5, 16.9 Hz, 1H, 5-H), δ 2.59 (dd, J = 16.9, 1.8 Hz, 1H, 5-H); CIMS, *m/e* (relative intensity), 91 (100), 159 (9), 249 (M^+ + 1, 53); IR (KBr, cm^{-1}) 3240, 1745, 1710, 1695, 1680, 1670. Elemental analysis for $C_{12}H_{12}N_2O_4$.

Uracil-6-*phosphonate* (**25**). The synthesis of 2,4-dimethoxyuracil-6-diethoxyphosphonate was analogous to the procedure reported for uracil-5-phosphonate [26]. A solution of 5.61 g (30.0 mmol) of 2,4-dimethoxy-6-chloropyrimidine in 100 ml of anhydrous tetrahydrofuran was stirred under an argon gas flow, while 19.3 ml (1.5 equiv.) of 2.5 M *n*-butyl lithium in tetrahydrofuran was added dropwise. This mixture was stirred for 1 hr and then cooled to −78° while a solution of diethyl chlorophosphate (12.34 ml, 14.7 g, 85.0 mmol, 2.64 equiv.) in 36 ml of dried tetrahydrofuran was added slowly. The reaction was stirred for 30 min and then quenched with a saturated ammonium formate solution (30 ml) followed by 6 ml of concentrated ammonium hydroxide. The layers were separated, and 100 ml of tetrahydrofuran was used to back extract the aqueous layer. The organic layers were then combined, dried over anhydrous magnesium sulfate, and evaporated *in vacuo* to an oil. The oil was purified by flash chromatography (chloroform/ethyl acetate, 4:1) to give 2.1 g of a yellow oil (29% yield). The physical and spectroscopic data for **25** confirmed the structure [27].

The 2,4-dimethoxyuracil-6-diethoxyphosphonate was deprotected as described to produce **25** [26, 27].

3-n-*Propyl orotic acid* (**32**). A general literature procedure was followed in which 3-*n*-propyl-5-carboethoxymethylidene hydantoin was first prepared [28]. All glassware was flame dried three times while flushing with argon. A mixture of 9.35 g (0.05 mmol) of diethylaminofumerate, 0.1 g of $AlCl_3$ and 8.51 g (0.1 mol) of *n*-propylisocyanate was heated at 120° for 2.5 hr. After cooling, the orange solid was recrystallized in ethanol yielding white needles, 4.8 g (42%), m.p. = 92.5–94°; ^{1}H NMR (DMSO-d_6) δ 5.58 (s, 1H, vinyl), δ 4.20 (q, J = 7.0 Hz, 2H, OCH_2), δ 3.42 (t, J = 7.0 Hz, 2H, NCH_2), δ 1.56 (q, J = 7.0 Hz, 2H, 9-CH_2), δ 1.24 (t, J = 7.0 Hz, 3H, 10-CH_3), δ 0.93 (t, J = 7.0 Hz, 3H, ester CH_3); CIMS, *m/e* (relative intensity), 181 (38), 227 (M^+ + 1, 100); IR (KBr, cm^{-1}), 3250, 1790, 1735, 1700, 1675. Elemental analysis for $C_{10}H_{14}N_2O_4$.

A mixture of 4.0 g (17.7 mmol) of the 3-*n*-propyl-5-carboethoxymethylidene hydantoin, 17.7 ml of 2 N KOH, and 8.8 ml of ethanol was refluxed for 2.5 hr. After cooling, the solution was acidified with 2 N HCl, cooled overnight, and filtered. The white solid was recrystallized from ethanol, yielding 3.0 g (86%)

of **32**, m.p. = 233°; ^{1}H NMR (DMSO-d_6), δ 6.12 (s, 1H, vinyl), δ 3.73 (t, J = 7.0 Hz, 2H, NCH_2), δ 1.52 (q, J = 7.0 Hz, 2H, 9-CH_2), δ 0.85 (t, J = 7.0 Hz, 3H, CH_3); CIMS, *m/e* (relative area), 114 (14), 199 ($M^+ + 1$, 100); IR (KBr, cm^{-1}), 1750, 1705, 1650, 1600. Elemental analysis for $C_8H_{10}N_2O_4$.

3-*Methyl orotic acid* (**31**). A general literature procedure used for **32** was followed [28]. The yield of 3-methyl-5-carboethoxymethylidene hydantoin was 73% as white needles, m.p. = 141–143°; (lit. m.p. = 139.5°) [29].

The base hydrolysis of 3-methyl-5-carboethoxymethylidene hydantoin yielded 72% of **31** as a white solid, m.p. = 289–293° (dec.); [lit. m.p. = 306–311° (dec.)] [30]. Physical and spectroscopic data confirmed the structure [30].

3-*Benzyl orotic acid* (**33**). The general literature procedure used for **32** was followed [28]. The yield of 3-benzyl-5-carboethoxymethylidene hydantoin was 45% as yellow needles, m.p. = 134.5–136°; (lit. m.p. = 133–135°) [29].

Base hydrolysis of the hydantoin yielded 49% of 3-benzylorotic acid (**33**), as white needles, m.p. = 227–229°; (lit. m.p. = 226–227.5°) [30]. Physical and spectroscopic data were as reported previously [30].

3-*Phenyl orotic acid* (**34**). The general literature procedure used for **32** was followed [28]. The yield of 3-phenyl-5-carboethoxymethylidene hydantoin was 37%; m.p. = 195–196°; (lit. m.p. = 191°) [28]. Physical and spectroscopic data confirmed the structure of **34**.

Base hydrolysis of the hydantoin yielded 38% of **34**, m.p. = 288–290 (dec.); [lit. m.p. = 280° (dec.)] [28].

Enzyme preparation and assay. The DHO-dehase was isolated as a partially purified inner mitochondrial membrane fraction [31] from the liver of Cox (SD) outbred male rats following literature procedure [18]. The assay used to measure DHO-dehase activity was based on previously published assays which utilized menadione-cytochrome *c*, phenazine methosulfate, DCIP, or ubiquinone-Q6-DCIP as electron acceptors [2, 4, 5, 7, 16]. In the present assay menadione was used to accept the electrons from the enzyme and to pass these subsequently to DCIP which produced a color change that was monitored at 600 nm. To make certain that all electrons were transferred from menadione to DCIP and not to the electron transport system, KCN was included in the assay. A typical incubation consisted of 67 mM Tris–HCl (pH 7.2), 1 mM KCN, 0.67 mM menadione, 0.05 mM DCIP, 1 mM L-DHO, and enzyme in a final volume of 1.5 ml at 25°. The decrease in absorbance of DCIP at 600 nm in the presence and absence of L-DHO was followed. All compounds tested as inhibitors were dissolved in 100 mM Tris buffer and adjusted to pH 7.2 or dissolved in DMF as specified (see Table 2). Incubation mixtures were thermally equilibrated and the reaction was started by the addition of enzyme preparation. Specific activity was calculated from slopes of the absorbance change taken 3 min from the

Table 1. ^{1}H NMR spectral data of solvent effects on *cis*- and *trans*-5-methyl DHOs*

Compound†	Solvent	Chemical shifts (coupling constants, J, Hz) H_6	H_5	H_7	H_1	H_3
4	DMSO-d_6‡	3.94 dd ($J_{5,6}$ = 6.6, $J_{1,6}$ = 3.8)	2.89 p ($J_{5,6}$ = 6.6, $J_{5,7}$ = 7.0)	1.03 d ($J_{5,7}$ = 7.0)	7.77 bs	10.03 bs
7	DMSO-d_6‡	3.78 t ($J_{5,6}$ = $J_{1,6}$ = 3.1)	2.68 dq ($J_{5,6}$ = 3.1, $J_{5,7}$ = 7.2)	1.22 d ($J_{5,7}$ = 7.2)	7.81 bs	10.09 bs
4	D_2O§ (phosphate, 0.1 M, pH 7.8)	4.15 d ($J_{5,6}$ = 5.6)	2.98 dq ($J_{5,6}$ = 5.6, $J_{5,7}$ = 7.2)	1.15 d ($J_{5,7}$ = 7.2)	SE‖	SE
7	D_2O§ (phosphate, 0.1 M, pH 7.8)	3.78 d ($J_{5,6}$ = 4.9)	2.90 dq ($J_{5,6}$ = 4.9, $J_{5,7}$ = 7.3)	1.32 d ($J_{5,7}$ = 7.3)	SE	SE
4	CD_3OD‡	4.23 d ($J_{5,6}$ = 6.0)	3.03 dq ($J_{5,6}$ = 6.0, $J_{5,7}$ = 7.2)	1.24 d ($J_{5,7}$ = 7.2)	SE	SE
7	CD_3OD‡	3.94 d ($J_{5,6}$ = 3.2, $J_{5,6}$ = 5.6)	2.95 dq ($J_{5,6}$ = 5.6, $J_{5,7}$ = 7.1)	1.42 d ($J_{5,7}$ = 7.1)	SE	SE
4	Pyridine-d_5¶	4.69 dd ($J_{1,6}$ = 2.7, $J_{5,6}$ = 5.6)	3.32 dq ($J_{5,6}$ = 5.6, $J_{5,7}$ = 7.1)	1.53 d ($J_{5,7}$ = 7.1)	9.26 bs	12.17 bs
7	Pyridine-d_5¶	4.40 t ($J_{5,6}$ = 3.9, $J_{1,6}$ = 3.9)	3.48 dq ($J_{5,6}$ = 3.9, $J_{5,7}$ = 7.2)	1.56 d ($J_{5,7}$ = 7.2)	9.41 bs	12.30 bs

* Performed on a 470 MHz Nicolet ^{1}H NMR at 23°.
† There are 3 mg of compound dissolved in 0.5 ml of solvent.
‡ Referenced to solvent.
§ Referenced to DSS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate).
‖ SE, solvent exchange.
¶ Referenced to TMS (tetramethylsilane).

time of enzyme addition. Absorbance measurements were with a Cary 17 double beam spectrometer or an AMINCO rotochem IIC. Using zero order kinetics, the optimum pH was 7.2 [32] and the optimum ionic strength 67 mM (Tris·HCl). Saturating concentrations of menadione, the proximal electron acceptor, and DCIP, the final electron acceptor, were used. The enzyme assay remained linear up to 1 mg of protein per 1.5 ml reaction volume. Routinely, 0.7 to 0.9 mg of protein in 1.5-ml incubations was used for the inhibition studies. Saturating L-DHO concentration was 0.2 mM, and the K_m was found to be 12.5 ± 0.25 μM. Under these standard conditions, the assay was linear with respect to time for at least 10 min. The catalytic properties and the kinetic data obtained with this assay were in close agreement with previously reported assays [2, 4, 5, 7, 16, 33]. Protein was determined by a modification of the Lowry procedure [34].

RESULTS AND DISCUSSION

The *cis*- and *trans*-5-methyl derivatives **4** and **7** respectively, were synthesized to study the steric enzyme binding requirements for the pyrimidines in the 5-position. Although **7** has been examined in NMR conformation studies [21], its synthesis had not been reported. Compound **7** was therefore synthesized by a base catalyzed isomerization of **4**. The reaction mixture containing 5 equivalents of $NaOC_2H_5$ in CH_3CH_2OH was refluxed for 4 days and produced a 77% yield of a *trans* enriched, *cis*/*trans* mixture. The conversion of *cis* to *trans* as determined from the integration of the 470 MHz ^1H NMR spectrum produced 84% of the *trans* isomer **7** and 16% of the *cis*. The isomerization, however, did proceed using ambient temperatures or less base but at a much slower rate. Coupling constants of **7** for $J_{5,6}$ in DMSO-d_6 were found to be small, 3.1 Hz, while **4** (*cis*) has a $J_{5,6}$ of 6.6 Hz (Table 1). These values are in close agreement with literature values [21] and, as can also be seen in Table 1, the coupling constants and chemical shifts are solvent dependent. This was not unexpected since the conformations of other dihydropyrimidine ring systems have been shown to be solvent dependent [13, 21, 35, 36].

The enzyme assay for DHO-dehase was developed by a modification of literature procedures [2, 16]. A K_m value of 12.5 μM (±0.25) for L-DHO (**1**) was obtained and is in good agreement with literature values of 5, 40, and 80 μM using similar assays (Table 2) [2, 5, 6, 16]. D-DHO (**2**) was neither a substrate nor a very good competitive inhibitor with a K_i of 2.4 mM. Orotic acid (**8**), the product of the enzyme reaction, was an excellent competitive inhibitor with a K_i of 13 μM (Fig. 2).

The most potent competitive inhibitor tested was 5-aza-DHO (DHOX, **3**) which had a K_i of 9 μM. DHOX has been shown to be a specific inhibitor of DHO-dehase and is active against L1210 and other cells *in vitro* [20]. This compound only moderately decreased the growth of L1210 and P388 murine tumors *in vivo* and increased the lifespan of the tumor bearing animals by less than 40%. In contrast, **7** was found to be inactive or only weakly active in all *in vitro* systems tested.

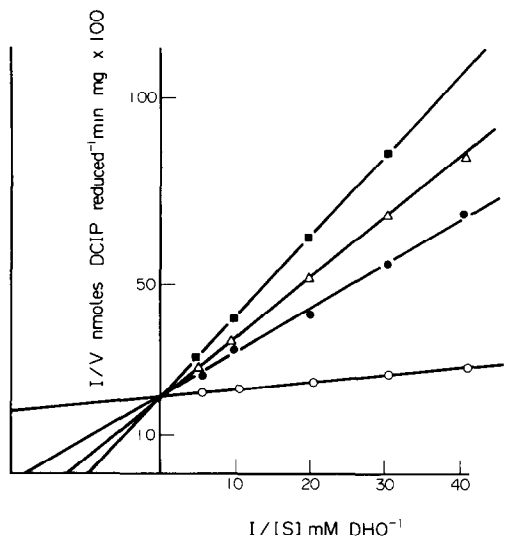


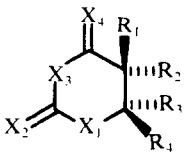
Fig. 2. Double-reciprocal plot of the inhibition of dihydroorotate dehydrogenase in the presence of orotic acid (**8**) with L-DHO as the variable substrate. Key: (○) no inhibitor, (●) 5×10^{-5} M, (△) 7.5×10^{-5} M, and (■) 1×10^{-4} M inhibitor. The reaction mixture contained 67 mM Tris buffer (pH 7.2), 1 mM KCN, 0.67 mM menadione, 0.05 mM DCIP and 1.2 mg of enzyme protein.

All of the (±)-*cis*-5-alkyl derivatives (**4–6**) were very poor enzyme inhibitors. The best of these competitive inhibitors (**4**) only had a K_i of 694 μM. The D,L-*trans*-5-methyl DHO (**7**) was comparable in activity to **4** (K_i = 684 μM) and only a weak substrate for the enzyme.

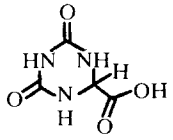
The 5-methyl OA (**13**) was an excellent competitive inhibitor with a K_i of 18 μM, whereas 5-ethyl OA (**14**) was a very poor enzyme inhibitor. Compound **15** was also a poor enzyme inhibitor. Since the compound exists as a lactone, however, the poor inhibition may be partially due to the lack of a negatively charged functionality in the 6-position. The combined results of the *cis*- and *trans*-5-substituted DHOs and the 5-substituted OAs, therefore, indicate an enzyme steric constraint of a methyl group at the 5-position of the pyrimidine. Similar 5-position steric requirements of bovine DHO-dehase have been observed with **4** and **13** [5].

The 5-amino derivative (**12**) produced a 46% inhibition of the enzyme at a concentration of 1 mM. In comparison, 5-nitro OA (**11**) only inhibited the enzyme 39% at a concentration of 5 mM, whereas **9** and **10**, despite the large Van der Waals' radii of the halogens and the observed steric effects at the 5-position, were both good inhibitors. The results of **9–12** suggest that, in addition to the steric constraints at the 5-position and the necessity of a negative charge at the 6-position, there are several additional enzyme–inhibitor interactions. A study of both the inductive and resonance effects of 5-substituents with the pyrimidine ring for **9–12** has been reported [45], which suggests that bromine, in **9**, and iodine, in **10**, possess partial positive charges, whereas the nitro group (**11**) bears a partial negative charge. These

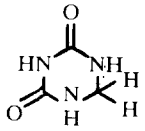
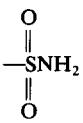
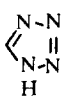
Table 2. Inhibition of dihydroorotate dehydrogenase by dihydroorotate and orotate analogs



A. C_5-substituents (X_1,X_3 = NH; X_2,X_4 = 0)

No.	R_1	R_2	R_3	R_4	% Inhibition[a] (mM inhibitor)	K_i (type of inhibition)[b]
1	H	H	H	COOH		K_m = 12.5 μM (±0.25)[c]
2[d]	H	H	COOH	H	54 (3)[e]	2.4 mM (C)
3					100 (1)	9 μM (C)
4[f,g]	CH_3	H	H	COOH	19 (1)[h]	694 μM (C)
5[f,g]	C_2H_5	H	H	COOH	80 (5)[h]	3.9 mM (C)
6[f]	CH_2O—	H	H	—C— (as lactone with R_1)	72 (5)[h]	915 μM (C)
7[f]	H	CH_3	H	COOH	ND[i]	684 μM (C)[j]
8[k]	H (C_5,C_6 =			COOH	86 (1)	13 μM (C)
9[k]	Br			COOH	84 (1)	56 μM (C)
10[k]	I			COOH	86 (1)	
11[k]	NO_2			COOH	39 (5)	
12[k]	NH_2			COOH	46 (1)	296 μM (C)
13[l]	CH_3			COOH	82 (1)	18 μM (C)
14[l]	C_2H_5			COOH	42 (5)	
15[m]	CH_2O—			—C— (as lactone)	22 (1)	

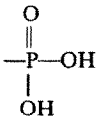
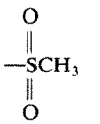
B. C_6-substituents (X_1,X_3 = NH; X_2,X_4 = 0)

No.	R_1	R_2	R_3	R_4	% Inhibition[a] (mM inhibitor)	K_i (type of inhibition)[b]
16	H	H	H	$COOCH_3$		K_m = 984 μM[n]
17	H	H	H	$COOCH_2C_6H_5$		K_m = 878 μM[n]
18					0 (5)	
19[o]	H	H	H	$CONH_2$	15 (5)[p]	
20[q]	H	(C_5,C_6 =		$CONH_2$	14 (2)	
21[r]	H				71 (1)	
22[s]	H				37 (1)	
23[t]	H			CN	33 (2)	
24[k]	H			OH	63 (1)	

continued

Table 2—*continued*

B. C_6-substituents (X_1,X_3 = NH; X_2,X_4 = 0)

No.	R_1	R_2	R_3	R_4	% Inhibition[a] (mM inhibitor)	K_i (type of inhibition)[b]
25	H				0 (1)	
26[u]	H				12 (1)[n]	
27[u]	H			CH_2OH	0 (1)[n]	
28[k]	H			NH_2	0 (1)[n]	

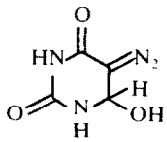
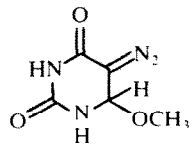
C. N_1 and N_3 substituents (R_1 = H; X_2,X_4 = 0; R_4 = COOH)

No.	R_2	R_3	X_1	X_3	% Inhibition[a] (mM inhibitor)	K_i (type of inhibition)[b]
29[v]	H	H	CH_2	NH	90 (5)[h]	632 μM (C)
30[w]	(C_5,C_6 =		NH	CH_2	34 (5)	
31			NH	NCH_3	0 (5)	
32			NH	NC_3H_7	11 (5)	
33			NH	$NCH_2C_6H_5$	8 (5)	
34			NH	NC_6H_5	8 (5)	

D. C_2 and C_4 substituents (R_1 = H; X_1,X_3 = NH)

No.	R_2	R_3	R_4	X_2	X_4	
35[x]	H	H		O	H_2	0 (1)[n]
36[k]	(C_5,C_6 =		OH	NH_2	O	35 (5)
37[k]			COOH	NH_2	O	35 (5)
38[k]			OH	S	O	23 (1)[n]
39[k]			COOH	S	O	14 (1)

E. Miscellaneous

No.			
40[y]		34 (1)	NC[b]
41[y]		100 (1)[n]	
41	Lapachol	0 (1)[n]	
43	Dichloroallyllawsone	100 (1)[n]	

[a] The concentration of L-DHO was 0.025 mM.
[b] C = competitive; NC = non-competitive.
[c] Standard deviation as determined from four separate experiments.
[d] Synthesized according to Ref. 37.
[e] Not a substrate at 10 mM inhibitor.
[f] Tested as an enantiomeric mixture.
[g] See Ref. 21 for synthesis.
[h] Not a substrate at 5 mM inhibitor.
[i] ND = not determined.
[j] Tested as a mixture of 84% *trans* **7** and 16% *cis* **4**.
[k] Purchased from Aldrich.
[l] Synthesized according to Ref. 38.
[m] See Ref. 23 for synthesis.
[n] Dissolved in DMF (*N*,*N*-dimethylformamide).
[o] See Ref. 39 for synthesis.
[p] Not a substrate at 1 mM inhibitor.
[q] Synthesized according to Ref. 40.
[r] See Ref. 41 for synthesis.
[s] Synthesized according to Refs. 42 and 43.
[t] See Ref. 43 for synthesis.
[u] Purchased from Sigma.
[v] Synthesized according to Ref. 44.
[w] See Ref. 45 for synthesis.
[x] Gift from Dr. G. Marc Loudon, Purdue University.
[y] See Ref. 46 for synthesis.

charge densities may affect enzyme–inhibitor interactions and, from the results, suggest the possibility of a negatively charged enzyme substituent near the 5-position.

The partial charges of **9–12**, however, are probably not the only factors affecting enzyme–inhibitor binding. A study of the metal coordination abilities of **9–12** has shown that the 5-substituents affect both the pK_a and the metal coordination abilities of the N-1 and carboxylic acid positions [47]. The abilities of pyrimidines to coordinate metals have been shown to decrease in the order $NH_2 > I > Br > NO_2$, while enzyme testing results of **9–12** have suggested a similar pattern for enzyme inhibition of $I \simeq Br > NH_2 \gg NO_2$. The possibility of an enzyme metal-coordination site may not be unreasonable since DHO-dehase does contain several iron and zinc metal centers in which one of the iron centers has been proposed to be involved in the enzyme catalytic process [4, 16, 48]. Further testing is in progress to determine which of these effects are important for enzyme binding.

In the series of compounds in which the carboxylic acid was modified, the only analogs that were enzyme inhibitors were those possessing an ionizable functionality with the appropriate pK_a in the 6-position of the pyrimidine. These compounds, barbituric acid (**24**) and 6-sulfonamide uracil (**21**), both contain 6-position substituents which, under physiological conditions, possess a negative charge. In fact, barbituric acid (**24**) is known to be a DHO-dehase inhibitor [2, 18]. Various 5-substituted barbiturates were also reported as possible DHO-dehase inhibitors but were devoid of inhibitory activity [18], supporting our findings that steric bulk in the 5-position cannot be tolerated. The 6-tetrazole uracil analogs, (**22**) and 6-phosphitidic acid uracil (**25**), were devoid of enzyme inhibitor activity, probably due also to the large size of the tetrazole and phosphate moieties.

All carboxylic substituents which were neutral in net charge (**18–20**, **23**, **26**, and **27**) or which contained a positively charged substituent in the 6-position (**28**) were all inactive as enzyme substrates or inhibitors. In addition, both the L-DHO methyl and benzyl esters (**16** and **17**) were found to be poor enzyme substrates with K_m values of 984 and 878 μM respectively. The importance of the 6-carboxylic acid group is apparent in the comparison of **3** and its decarboxylated analog, **18**. The methyl (**16**) and benzyl (**17**) esters, however, have been shown to be excellent substrates for bovine DHO-dehase [5]. This indicates a major difference in binding requirements between the two mammalian enzymes.

Substitution of the N-3 nitrogen or replacement with methylene, **30–34**, resulted in complete loss of DHO-dehase binding. These results showed that the N-3 proton is necessary for substrate binding and that steric alterations at this position are not tolerated by the enzyme. In addition, replacement of the N-1 nitrogen with carbon, as in compound **29**, resulted in a tremendous loss of enzyme inhibitory activity, also suggesting that the N-1 proton is necessary for enzyme binding.

Several derivatives (**35–39**) replacing the 2-oxygen were also tested. From these results, it can be seen that none of these alterations were tolerated by the enzyme. However, compounds **36** and **37** contain tautomeric forms in which either the N-1 or the N-3 proton would be missing. This may partially explain their lack of inhibition since it was shown that both amide protons are necessary for enzyme inhibitory activity. Compounds **38** and **39**, both of which contain a sulfur with a larger Van der Waals' radius than oxygen but exist predominantly in the thione form [49], were also poor enzyme inhibitors, suggesting that there is a size limitation in the 2-position.

An unusual class of DHO derivatives was also tested. The 5-diazo-6-substituted uracils (**40** and **41**) were both found to be good enzyme inhibitors, with **40** being a non-competitive inhibitor. Whether this inhibition is an irreversible interaction at or near the active site of the enzyme or due to binding to an allosteric site is currently under investigation.

The two quinone antineoplastic agents dichloroallylawsone (DCL, **43**) and lapachol (LAP, **42**) were also tested in the enzyme system. It was reported previously [11] that both compounds inhibit DHO-dehase as their major antineoplastic effect in assays in which the activity of DHO-dehase is coupled to the electron transport chain. Compound **42** is considerably less active than **43** in reducing intracellular UMP concentrations [11]. However, whether these compounds inhibit DHO-dehase directly or indirectly through the electron flow some place removed from the enzyme *per se* is not clear. Since KCN was present in the assay used by us and by others [33], electrons were inhibited from flowing through the electron transport system but only reduced the added menadione. Furthermore, it has been shown [4, 48] that there are two separate pools of ubiquinone in the inner mitochondria, one for DHO-dehase and the other for generalized electron transport. It was not surprising, therefore, that only **43** inhibited the DHO-dehase in the assay used herein. Naphthoquinone cytotoxic agents related to LAP and DCL have been shown to inhibit ubiquinone reduction of electron transport at the cytochrome *b*-Cl region [11, 50, 51]. As can be seen in Fig. 1, electron transport inhibition by these compounds at the cytochrome *b*-Cl region would not be observed by the present enzyme assay despite their known inhibition of the electron transport system and antineoplastic activity through DHO-dehase inhibition [11]. From the enzyme inhibition results of LAP and DCL with DHO-dehase, it can be inferred that **42** inhibits the electron transport system at a point different than the location of the enzyme DHO-dehase, in agreement with the observation

Table 3. Compounds active in 9PS cytotoxicity tests

Compound	LD_{50}* (μg/ml)	LC_{50} (μM)
5-Aza-5,6-dihydroorotic acid, **3**	2×10^{0}	12.6
6-Sulfonamide uracil, **21**	7×10^{-1}	3.7
6-Methylsulfone uracil, **26**	9×10^{-1}	4.7
2,4-Dimethoxy-6-sulfeneamine uracil†	5×10^{0}	23.3
Lapachol, **42**	7×10^{-1}	3.3
Dichloroallyllawsone, **43**	9×10^{-1}	3.2

* The dose that is lethal to 50% of the cell population.
† Intermediate obtained during the synthesis of **21**.

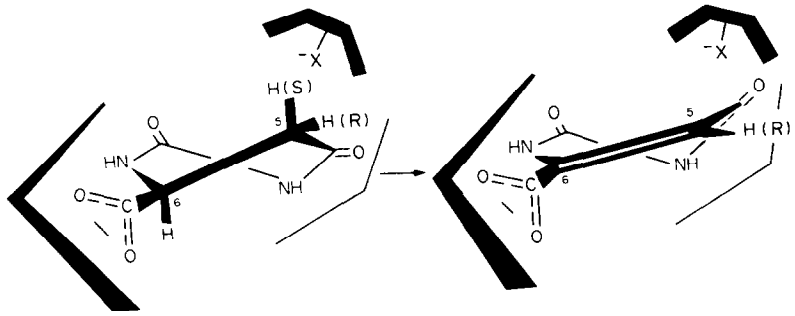


Fig. 3. Proposed model of binding of substrate to dihydroorotate dehydrogenase active site.

[52] that **42** uncouples and inhibits oxidative phosphorylation. DCL, however, appears to be capable of inhibiting either the reduction of DCIP by menadione or of DHO-dehase directly. The latter result would suggest that the ubiquinone binding specificity of DHO-dehase is different than that of other enzymes of the electron transport system, and the selective inhibition of DHO-dehase via quinone analogs may be possible.

All compounds and synthetic intermediates were screened for *in vitro* cellular cytotoxicity against both PS (mouse leukemia) and KB cell systems. The active compounds are shown in Table 3. In the PS assay, all of the compounds shown were marginally active. None of the compounds was cytotoxic against 9KB. Selected inhibitors are also under test as potential antimalarial agents.

Structure–activity relationships among DHO-dehase substrates are summarized below and incorporated into a proposed model for substrate and product binding to the enzyme (Fig. 3).

1. Substitution at N-3 or replacement of N-1 or N-3 with carbon results in a decrease in enzyme inhibitory activity.
2. Modifications of the carbonyl oxygens at the 2 and 4 positions result in a decrease in enzyme inhibitory activity.
3. Modification of the carboxylic acid moiety can be accommodated when the functionalities both contain a negative charge and are not too large. However, the methyl and benzyl esters of L-DHO are poor substrates.
4. 5-Substituted orotic acids exhibit decreased enzyme inhibitory activity as the 5-substituent increases in size ($CH_3 > C_2H_5$; CH_2OH). The presence of a negatively charged substituent or pocket on the enzyme to accommodate the substituent at the 5-position is suggested, and the possible presence of a metal coordination site for the N-1 and carboxylic acid moieties is indicated.
5. 5-Substituted dihydroorotic acids have the following properties:
   a. All *cis* isomers are inactive as enzyme inhibitors.
   b. The dihydro *trans*-5-methyl DHO (**7**) is a weak inhibitor and a poor substrate.
   c. D-Dihydroorotic acid is neither an inhibitor nor a substrate.
   d. DHOX (**3**) is the most potent inhibitor.

Only the configurations of L-DHO need be considered for binding to DHO-dehase since D-DHO was not an inhibitor. The amide protons of both OA and L-DHO would exist in the lactam form since both amide protons are necessary for enzyme binding. In fact, at physiological pH, both L-DHO and OA predominantly exist in the tautomeric lactam form [21, 49].

In our model, the configuration of L-DHO would be in a chair form, placing the carboxylic acid in an equatorial position. This configuration would be preferred for two reasons. First, the carboxylic acid of L-DHO would be in a position similar to that of OA after enzyme catalysis. This configuration would also place the 5-alkyl functionalities of the *trans*-5-alkyl DHO derivatives in positions similar to the alkyl groups of 5-substituted OAs. Second, the 5-pro S and 6 protons would be in a *trans* diaxial position which would energetically favor elimination of these protons. In fact, the *trans* 5-pro S and 6 protons have been shown to be the protons removed during enzyme catalysis by the rat and bovine enzyme [13, 14].

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