Mercaptan and Dicarboxylate Inhibitors of Hamster Dihydroorotase†

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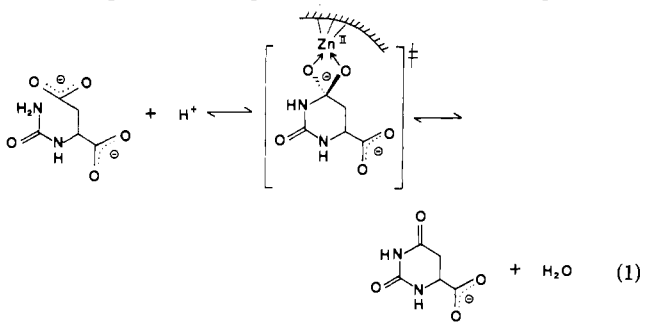
ABSTRACT: In mammals, dihydroorotase is part of a trifunctional protein, dihydroorotate synthetase, which catalyzes the first three reactions of de novo pyrimidine biosynthesis. Dihydroorotase catalyzes the formation of a peptide-like bond between the terminal ureido nitrogen and the β-carboxyl group of *N*-carbamyl-L-aspartate to yield heterocyclic L-dihydroorotate. A variety of evidence suggests that dihydroorotase may have a catalytic mechanism similar to that of a zinc protease [Christopherson, R. I., & Jones, M. E. (1980) *J. Biol. Chem. 255*, 3358–3370]. Tight-binding inhibitors of the zinc proteases, carboxypeptidase A, thermolysin, and angiotensin-converting enzyme have been synthesized that combine structural features of the substrates with a thiol or carboxyl group in an appropriate position to coordinate a zinc atom bound at the catalytic site. We have synthesized (4*R*)-2-oxo-6-thioxohexahydropyrimidine-4-carboxylate (L-6-thiodihydroorotate) and have found that this analogue is a potent competitive inhibitor of dihydroorotase with a dissociation constant (K_i) in the presence of excess Zn^{2+} ion of 0.17 ± 0.02 μM at pH 7.4. The potency of inhibition by L-6-thiodihydroorotate in the presence of divalent metal ions decreases in the order $Zn^{2+} > Ca^{2+} > Co^{2+} > Mn^{2+} > Ni^{2+}$; L-6-thiodihydroorotate alone is less inhibitory and has a K_i of 0.85 ± 0.14 μM. 6-Thioorotate has a K_i of 82 ± 8 μM which decreases to 3.8 ± 1.4 μM in the presence of Zn^{2+}. Zn^{2+} alone is a moderate inhibitor of dihydroorotase and does not enhance the potency of other inhibitors. We have synthesized 2-oxo-1,2,3,6-tetrahydropyrimidine-4,6-dicarboxylate as a "transition-state analogue" for the reaction catalyzed by dihydroorotase and have found it to be a potent inhibitor with a K_i value of 0.74 ± 0.10 μM. By contrast, a cyclic sulfone (5*R*)-1,1,3-trioxothiomorpholine-5-carboxylate with structural resemblance to the proposed transition state had no inhibitory effect upon dihydroorotase. L-Cysteine and iminodiacetate activate dihydroorotase at low concentrations (0.1 μM) but are effective inhibitors at higher concentrations with apparent K_i values of 550 μM and 200 μM, respectively, for the proposed interaction of a second inhibitor molecule with the enzyme–inhibitor complex. 5-Fluoroorotate is a noncompetitive inhibitor with a dissociation constant for interaction with the free enzyme of 15 ± 4 μM, while orotate is a competitive inhibitor with a K_i of 81 ± 9 μM.

Dihydroorotase catalyzes the third reaction of de novo pyrimidine biosynthesis and in mammals is part of a trifunctional protein (Jones, 1980) called dihydroorotate synthetase.[1] This reaction involves formation of a peptide-like bond between the terminal ureido nitrogen and the β-carboxyl group of carbamyl aspartate to yield heterocyclic dihydroorotate (eq 1). Christopherson and Jones (1980a) proposed



(1)

that mammalian dihydroorotase contains a bound zinc atom at the active site essential for catalytic activity, a proposal confirmed directly by Kelly et al. (1986). The catalytic role proposed by Walsh (1979) for zinc bound at the active site of bacterial dihydroorotase was to stabilize the transition state of the reaction catalyzed by formation of an inner-sphere coordination complex with the two oxygens at carbon 6 of the dihydropyrimidine ring (eq 1). A variety of evidence suggests that dihydroorotase may have a catalytic mechanism similar to that of a zinc protease (Christopherson & Jones, 1980a). The rapid inactivation of dihydroorotase by L-cysteine and the slower effect of 2-mercaptoacetate (Christopherson & Jones, 1980a) are very similar to their effects on bovine carboxypeptidase A (Coombs et al., 1962). The pH–activity profile for ring cleavage of dihydroorotate (Christopherson & Jones, 1979) is similar to those for carboxypeptidase A (Auld & Vallee, 1970). The pH–activity profiles for the biosynthetic and degradative reactions of dihydroorotase from *Clostridium oroticum* also resemble those for mouse dihydroorotase, and the bacterial enzyme contains 1 mol of zinc/subunit, probably at the active site (Taylor et al., 1976). For carboxypeptidase A, it has been suggested from X-ray crystallographic data and other studies that the carbonyl oxygen of the terminal peptide bond of the substrate becomes a ligand of the active-site zinc, resulting in polarization of the C=O bond. The carbonyl

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[1] Dihydroorotate synthetase is the trifunctional protein containing the first three enzymic activities of the pyrimidine pathway: carbamyl phosphate synthetase (EC 2.7.2.9), aspartate transcarbamylase (EC 2.1.3.2), and dihydroorotase (EC 3.5.2.3).

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carbon is then susceptible to nucleophilic attack by two possible mechanisms. The γ-carboxylate of glutamate 270 can function as a general-base catalyst, generating hydroxide ion which attacks the polarized C=O bond, or glutamate 270 can act directly as a nucleophile, generating an anhydride intermediate (Walsh, 1979). Many potent inhibitors have now been synthesized for zinc proteases such as angiotensin-converting enzyme (Cushman et al., 1977), carboxypeptidases A and B (Ondetti et al., 1979), thermolysin (Nishino & Powers, 1979), and enkephalinase (Roques et al., 1982). These analogues gain their inhibitory potency either by their resemblance to the proposed transition state of the reaction or by containing functional groups with appropriate stereochemistry for chelation of the zinc atom bound at the active site of the protease. Ondetti and his co-workers have synthesized (D-3-mercapto-2-methylpropanoyl)-L-proline (SQ 14225, Captopril), a potent competitive inhibitor of angiotensin-converting enzyme (K_i = 1.7 nM; Cushman et al., 1977); DL-2-benzyl-3-mercaptopropanoate, a competitive inhibitor of carboxypeptidase A (K_i = 1.1×10^{-8} M; Ondetti et al., 1979); and DL-2-(mercaptomethyl)-5-guanidinopentanoate, an inhibitor of carboxypeptidase B ($K_i = 4.2 \times 10^{-10}$ M; Ondetti et al., 1979). These three mercaptan inhibitors are highly specific for their respective target enzymes with negligible inhibitory activity against the other zinc proteases. Nishino and Powers (1979) have synthesized (3-mercapto-2-benzylpropanoyl)-L-alanylglycinamide, a potent inhibitor of the bacterial zinc protease thermolysin (K_i = 0.75 μM). X-ray crystallographic studies by Monzingo and Matthews (1982) have shown that this mercaptan inhibitor binds to thermolysin with the sulfur, presumably in the anionic form, tetrahedrally coordinated to the zinc and displacing a water molecule bound to the native enzyme.

Transition-state analogues have been synthesized for angiotensin-converting enzyme, carboxypeptidase A, and thermolysin where the tetrahedral arrangement of two oxygens in the transition state with a negative charge (eq 1) is mimicked by a carboxylate group on the inhibitor. Patchett et al. (1980) synthesized *N*-[1(*S*)-carboxy-3-phenylpropyl]-L-alanyl-L-proline (MK-421, Enalapril), a slow, tight-binding inhibitor of angiotensin-converting enzyme with an overall inhibition constant (K_i^*) of 5×10^{-11} M (Shapiro & Riordan, 1984). Benzylsuccinate is a potent competitive inhibitor of carboxypeptidase A ($K_i = 5 \times 10^{-7}$ M) considered to be a multisubstrate analogue (Byers & Wolfenden, 1973; Palmer et al., 1982), and *N*-(1-carboxy-3-phenylpropyl)-L-leucyl-L-tryptophan (CLT) is a potent inhibitor of thermolysin (K_i = 5×10^{-8} M; Maycock et al., 1981). The K_i values listed above are all apparent values determined at a particular pH; true dissociation constants describing the interaction between active forms of inhibitor and enzyme in appropriate states of ionization will be lower [e.g., Palmer et al. (1982)].

The tight-binding inhibitors of zinc proteases described above combine structural features of their respective substrates with a thiol or carboxyl group in an appropriate position to coordinate a zinc atom bound at the catalytic site. Using similar principles, we have now prepared two new inhibitors, and we find both analogues to be potent inhibitors of dihydroorotase.

Experimental Procedures

The following were purchased from Sigma Chemical Co.: $ZnCl_2$, $CaCl_2(H_2O)_2$, $CoCl_2(H_2O)_6$, $MnCl_2$, and $NiCl_2(H_2O)_6$; $MgCl_2$ was obtained as a 4.9 M solution. L-Cysteine, L-cysteic acid, L-cysteinesulfinic acid, iminodiacetic acid, and orotic acid were also from Sigma. 5-Fluoroorotic acid was purchased from P-L Biochemicals. All other chemicals were of analytical reagent grade.

Purification and Assay of Dihydroorotase. Trifunctional dihydroorotate synthetase was purified from a mutant hamster cell line (165–23) by the procedure of Coleman et al. (1977) with the following modifications. All buffers were purged with nitrogen gas; confluent cells were removed from plastic culture dishes with PBS (9.7 mM sodium phosphate–137 mM NaCl, pH 7.4) containing 0.1 mM EDTA; the hypotonic buffer used to swell the cells prior to homogenization contained the additional components 0.3 mM UTP and 1 mM EDTA; cells were disrupted by 5 strokes in a glass tissue homogenizer, and then glycerol was mixed in to a final concentration of 30% (v/v) for a further 15 strokes. Purified dihydroorotate synthetase (0.272 mg of protein/mL) had a specific activity for dihydroorotase of 1180 pmol of dihydroorotate formed/(min·μg of protein) and a ratio for the three enzymic activities (carbamyl phosphate synthetase):(aspartate transcarbamylase):(dihydroorotase) of 1.0:104:9.1 at pH 7.4 according to procedures described by Christopherson and Jones (1980b). Assay mixtures for determination of dihydroorotase activity in the degradative direction contained the following in a total volume of 25 μL: 50 mM K·Hepes, pH 7.4, 5% (v/v) glycerol, L-[^{14}C]dihydroorotate (47.1 Ci/mol), and inhibitor at the indicated concentrations. The reaction was initiated by addition of purified dihydroorotate synthetase as indicated in the figure legends, and three samples of 7 μL were withdrawn at appropriate times and applied to the origin of a poly(ethylenimine)–cellulose thin-layer chromatogram (Macherey-Nagel, Doren, Germany). ^{14}C-Labeled carbamylaspartate and dihydroorotate were separated by ascending chromatography with 0.34 M LiCl and quantified as previously described (Christopherson et al., 1978). Initial reaction velocities were determined by linear regression for product formed versus time. At pH 7.4, the equilibrium between carbamylaspartate and dihydroorotate favors the former by a ratio of 16.6:1 (Christopherson & Jones, 1979). Thus, the interactions between analogues and dihydroorotase reported here have been characterized by measuring inhibition of the reverse reaction where considerably more product (carbamylaspartate) can be formed at a constant initial reaction velocity.

Synthesis of L-[^{14}*C*]*Dihydroorotate.* [^{14}C]Bicarbonate (Amersham Australia) was converted enzymically to [^{14}C]-carbamylaspartate and [^{14}C]dihydroorotate. The conversion mixture contained the following in a volume of 2.38 mL: 25 mM K·Hepes (pH 7.4), 10% (v/v) glycerol, 4.0 mM dithiothreitol, 8 mM [^{14}C]bicarbonate (1 mCi, 47.1 Ci/mol), 4.0 mM L-glutamine, 10 mM K_4ATP, 15 mM $MgCl_2$, 10 mM phosphoenolpyruvate, 0.6 unit of pyruvate kinase (rabbit muscle, Sigma Chemical Co.), 0.10 mM 5-phosphoribose 1-pyrophosphate, 5.0 mM L-aspartate, and dihydroorotate synthetase with a total dihydroorotase activity of 12.5 nmol/min. The mixture (pH 7.4) was incubated for 16 h in a sealed tube at 37 °C, and then K·Mes (pH 6.0) buffer was added to a final concentration of 200 mM with additional dihydroorotase activity (2.5 nmol/min). The incubation was continued for a further 24 h at pH 6.0, where the equilibrium ratio carbamylaspartate:dihydroorotate is 0.66 (Christopherson & Jones, 1979), and 2-μL samples were analyzed by chromatography on poly(ethylenimine)–cellulose thin-layer chromatograms with 0.34 M LiCl as the developing solvent (Christopherson et al., 1978). The entire reaction mixture was then loaded onto a column of DEAE-Sephacel (39 × 1.6 cm) equilibrated with 0.2 M ammonium bicarbonate (pH 7.8). [^{14}C]Carbamylaspartate and [^{14}C]dihydroorotate were eluted

at a flow rate of 4.0 mL/h, and fractions of 0.58 mL were collected. Fractions 21–23 were pooled for [^{14}C]dihydroorotate, and fractions 27–31 were pooled for [^{14}C]-carbamylaspartate. These two pools were lyophilized several times to remove ammonium bicarbonate and then separately rechromatographed under the same conditions. Final yields were 500 μL each of [^{14}C]carbamylaspartate (7.65 mM, 99.9% radiochemically pure) and [^{14}C]dihydroorotate (11.1 mM, 99.8% pure).

Synthesis of Pyrimidine Analogues. Melting points were determined thermoelectrically on a Reichert hot stage melting point apparatus and are uncorrected. Microanalyses were performed by the Australian Microanalytical Service AMDEL, Melbourne. Infrared spectra were recorded on a Perkin-Elmer 221 or a Bio-Rad FTS-20 FTIR spectrometer as KBr plates, unless otherwise stated. ^{1}H NMR spectra were recorded on a Joel JNM FX-100 or otherwise on a Varian wide-bore XL-400 spectrometer. Spectra were determined by using 5–10% (w/v) solutions in deuterium oxide unless otherwise stated, with sodium 3-(trimethylsilyl)-2,2,3,3-tetradeuteriopropionate (1 mg) as an internal reference. Each signal was recorded in terms of chemical shift (ppm), multiplicity, coupling constants, and assignment. Mass spectra were recorded on an AEI MS-902 spectrometer at 70 eV. Thin-layer chromatography was performed on Merck Kieselgel HF 254+366 (type 60). Flash chromatography (Still et al., 1978) was performed on Merck silica gel 60 (230–400 mesh). High-pressure liquid chromatography (HPLC) was performed with a Whatman Partisil 10 SAX anion-exchange column, initially equilibrated with buffer A (7.0 mM KH_2PO_4, pH 3.0), and eluted at a flow rate of 2.0 mL/min with a concave gradient (Waters curve 7) from buffer A to buffer B (250 mM KH_2PO_4, 500 mM KCl, pH 3.8) over 45 min. Instantaneous whole spectra from 190–370 nm of the eluate were recorded by an LKB diode array ultraviolet detector and stored on the 20-MB hard disk of an IBM XT microcomputer.

S-(Ethylcarboxymethyl)-L-cysteine. A modification of the procedure of Ozawa (1963) was used. Ethyl bromoacetate (20.0 g, 120 mmol) and aqueous potassium hydroxide (10 M, 12 mL) were added simultaneously to a suspension of L-cysteine (14.5 g, 120 mmol) in water (24 mL) over a period of 20 min. The reaction mixture was stirred for 2 h and cooled and the product filtered to give *S*-(ethylcarboxymethyl)-L-cysteine (15.6 g, 63%).

(5R)-3-Oxothiomorpholine-5-carboxylic Acid. A modification of the procedure of Ozawa (1963) was used. *S*-(Ethylcarboxymethyl)-L-cysteine (1.5 g, 7.2 mmol) was dissolved in warm water (15 mL), and the solution was refluxed for 7 h, cooled, and concentrated to 4 mL and the thiomorpholine collected (0.7 g, 58%).

(1R,5R)- and (1S,5R)-1,3-Dioxothiomorpholine-5-carboxylic Acid. The diastereomeric mixture of sulfoxides was prepared by a procedure similar to that used by Levenson and Meyer (1984). To a stirred suspension of (5*R*)-3-oxothiomorpholine-5-carboxylic acid (490 mg, 3.0 mmol) in water (40 mL) at 5 °C was added a solution of sodium metaperiodate (716 mg, 3.4 mmol) in water (15 mL); the mixture was stirred at room temperature for 20 h. Lead nitrate (570 mg, 1.72 mmol) in water (10 mL) was added, the precipitate filtered, and the filtrate lyophilized to give the mixture of diastereomeric sulfoxides. The residue when dissolved in water (7 mL) and cooled to 2 °C for 1 h gave one diastereoisomer of 1,3-dioxothiomorpholine-5-carboxylic acid [probably (1*R*,5*R*)-] (180 mg, 33%), as colorless crystals, mp 182–183 °C dec. Anal. Calcd for $C_5H_7NO_4S\cdot H_2O$: C, 30.8; H, 4.7; N, 7.2; S, 16.4. Found: C, 30.9; H, 4.8; N, 7.3; S, 16.6. ^{1}H NMR (100 MHz) δ 3.41 (dd, $J_{6ax,6eq}$ = 14.4, $J_{6ax,5}$ = 7.6 Hz, H6ax), 3.61 (dd, $J_{6eq,6ax}$ = 14.4, $J_{6eq,5}$ = 6.2 Hz, H6eq), 4.03 (ABq, J = 15.1 Hz, H2), 4.35 (dd, $J_{5,6ax}$ = 7.6, $J_{5,6eq}$ = 6.2 Hz, H5); ν_{max} (KBr) 1600, 1025 cm^{-1}; $[\alpha]^{25}_D$ +41.2° (*c* 0.5, H_2O).

The filtrate from above was evaporated to a small volume, applied to a Sephadex G-10 column, and eluted with water. Small fractions were collected and monitored by thin-layer chromatography (cellulose, 2-propanol:water = 7:3) and the ninhydrin-positive fractions combined to give the other diastereoisomer of 1,3-dioxothiomorpholine-5-carboxylic acid [probably (1*S*,5*R*)-] (110 mg, 20%), as colorless crystals from water, mp 166–167 °C dec. ^{1}H NMR (100 MHz) δ 3.50 (dd, $J_{6ax,6eq}$ = 15.2, $J_{6ax,5}$ = 7.3 Hz, H6ax), 3.61 (dd, $J_{6eq,6ax}$ = 15.2, $J_{6eq,5}$ = 4.8 Hz, H6eq), 4.06 (ABq, J = 15.2 Hz, H2), 4.39 (dd, $J_{5,6ax}$ = 7.3, $J_{5,6eq}$ = 4.8 Hz, H5); ν_{max} (KBr) 1705, 1008 cm^{-1}; $[\alpha]^{25}_D$ −31.2° (*c* 0.5, H_2O).

(5R)-1,1,3-Trioxothiomorpholine-5-carboxylic Acid. Ammonium molybdate tetrahydrate (250 mg) was dissolved in water (15 mL), perchloric acid (2 mL, 60%) was added slowly, and the mixture was refluxed for 10 min, cooled, and filtered. Hydrogen peroxide (1.13 mL, 100 volumes) was added dropwise to a stirred solution of (5*R*)-3-oxothiomorpholine-5-carboxylic acid in the ammonium molybdate/perchlorate solution (1.08 mL) at 4 °C. The mixture was stored at 4 °C for 24 h and the colorless crystalline product filtered and washed with water followed by methanol. Recrystallization from water gave (5*R*)-1,1,3-trioxothiomorpholine-5-carboxylic acid (430 mg, 72%), as colorless crystals, mp 175–176 °C dec. Anal. Calcd for $C_5H_7NO_5S\cdot H_2O$: C, 28.4; H, 4.3; N, 6.6. Found: C, 28.4; H, 4.4; N, 6.6. ^{1}H NMR (100 MHz) δ 3.90 (dd, $J_{6ax,5eq}$ = 15.3, $J_{6ax,5}$ = 8.9 Hz, H6ax), 4.16 (dd, $J_{6eq,6ax}$ = 15.3, $J_{6eq,5}$ = 3.2 Hz, H6eq), 4.46 (dd, $J_{5,6ax}$ = 8.9, $J_{5,6eq}$ = 3.2 Hz, H5), proton H2 exchanged immediately with D_2O; ν_{max} (KBr) 1307, 1137 cm^{-1}.

(4R)-2-Oxo-6-thioxohexahydropyrimidine-4-carboxylic Acid (L-6-Thiodihydroorotic Acid). 6-Thioorotic acid, DL-6-thiodihydroorotic acid, and L-6-thiodihydroorotic acid (TDHO) were synthesized by the methods described in a complete patent specification (Christopherson et al., 1987). (4*R*)-2-Oxo-6-thiohexahydropyrimidine-4-carboxylic acid was obtained as a pale yellow solid, mp 226–230 °C dec. Anal. Calcd for $C_5H_6N_2O_3S$: C, 34.5; H, 3.5; N, 16.1. Found: C, 34.1; H, 3.6; N, 16.4. ^{1}H NMR (Figure 1) δ 3.30 (dd, $J_{5eq,5ax}$ = 17.7, $J_{5eq,4}$ = 5.6 Hz, H5eq), 3.36 (dd, $J_{5ax,5eq}$ = 17.7, $J_{5ax,4}$ = 6.4 Hz, H5ax), 4.23 (dd, $J_{4,5ax}$ = 6.4, $J_{4,5eq}$ = 5.6 Hz, H4); ν_{max} 3200, 1730, 1680, 1180 cm^{-1}; *m/z* 174 (M, 100%), 129 (48), 86 (25), 59 (40), 44 (27); $[\alpha]^{25}_D$ = +83.1° (*c* 0.2, methanol). TDHO analyzed by HPLC as described above had a retention time of 11 min 19 s with λ_{max} 204, 278, and 376 nm (absorbance ratios A_{278}/A_{204} = 6.9, A_{376}/A_{204} = 0.079). A trace amount of L-dihydroorotic acid eluted with a retention time of 9 min 21 s.

2-Oxo-1,2,3,6-tetrahydropyrimidine-4,6-dicarboxylic Acid. 2-Hydroxypyrimidine-4,6-dicarboxylic acid and 2-oxo-1,2,3,6-tetrahydropyrimidine-4,6-dicarboxylic acid (HDDP) were synthesized as described by Christopherson et al. (1987). HDDP was obtained from water as colorless crystals, mp >225 °C dec. Anal. Calcd for $C_5H_5N_2O_3$: 141.0300. Found: 141.0299. ^{1}H NMR (Figure 1) δ 4.63 (d, $J_{6,5}$ = 4.4 Hz, H6), 5.75 (d, $J_{5,6}$ = 4.4 Hz, H5); ν_{max} 3320, 1710, 1680, 1640 cm^{-1}; *m/z* 141 (M − 45, 100%), 95 (78), 44 (35). HDDP analyzed by HPLC as described above had a retention time of 29 min 1 s with λ_{max} 208 and 275 nm (absorbance ratio A_{275}/A_{208} = 0.33).

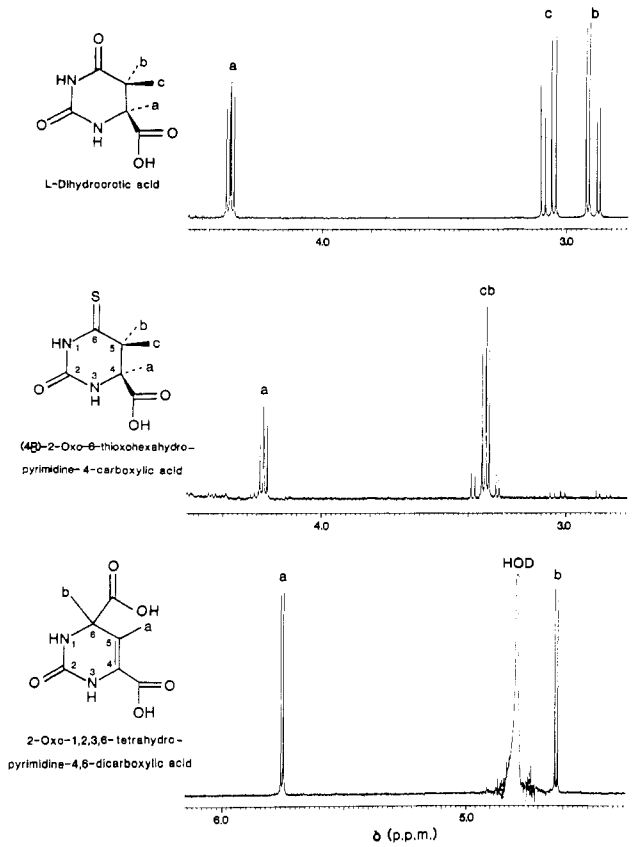


FIGURE 1: Proton nuclear magnetic resonance spectra for L-dihydroorotic acid, (4*R*)-2-oxo-6-thioxohexahydropyrimidine-4-carboxylic acid (TDHO), and 2-oxo-1,2,3,6-tetrahydropyrimidine-4,6-dicarboxylic acid (HDDP). Nonexchangeable hydrogen atoms are labeled alphabetically, and peak assignments are made on the spectra. Details are given under Experimental Procedures.

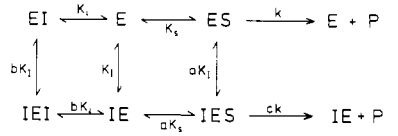
cis-2-Oxohexahydropyrimidine-4,6-dicarboxylic Acid. A suspension of 2-hydroxypyrimidine-4,6-dicarboxylic acid (6.66 g, 36.2 mmol) in methanol (350 mL) was saturated with dry HCl gas and then refluxed for 1.5 h, filtered hot, and cooled and the methanol removed. The residue was recrystallized from methanol to give dimethyl 2-hydroxypyrimidine-4,6-dicarboxylate as colorless crystals (4.75 g, 62%), mp 186–188 °C. Anal. Calcd for $C_8H_8N_2O_5$: C, 45.3; H, 3.8; N, 13.2. Found: C, 45.0; H, 3.5; N, 13.0. ^{1}H NMR (DMSO-d_6) δ 3.99 (s, 2 × CO_2CH_3), 4.10 (br s, OH), 7.84 (s, H5); ν_{max} 3460, 3433, 3329, 1747, 1672, 1653, 1611, 1457, 1442, 1267, 1234 cm^{-1}; *m/z* 212 (M, 8%), 182 (24), 154 (100), 139 (11), 121 (25), 93 (31), 81 (14), 66 (20).

Palladium on charcoal (10%, 40 mg) was added to a stirred solution of dimethyl 2-hydroxypyrimidine-4,6-dicarboxylate (1.0 g, 4.7 mmol) in methanol (150 mL). This mixture was then stirred under a hydrogen atmosphere for 16 h and filtered and the solvent removed to give a white solid. The product was recrystallized from methanol/ether to give colorless needles (750 mg, 74%), mp 178–179 °C. ^{1}H NMR (DMSO-d_6)[2] δ 2.28 (ddd, $J_{5ax,5eq}$ = 14.0, $J_{5ax,4(6)}$ = 6.0 Hz, H5ax), 2.36 (ddd, $J_{5eq,5ax}$ = 14.0, $J_{5eq,4(6)}$ = 4.0 Hz, H5eq), 4.12 (dd, $J_{4(6),5ax}$ = 6.0, $J_{4(6),5eq}$ = 4.0 Hz, H4 and H6); ν_{max} 3249, 1751, 1695, 1533, 1450, 1253, 1202, 1042 cm^{-1}; *m/z* 216 (M, 10%), 157 (100), 114 (73), 97 (34), 82 (23).

The hexahydropyrimidine dimethyl ester (200 mg, 0.93 mmol) was dissolved in a solution of sodium hydroxide (1 M, 7 mL) and refluxed for 30 min. The reaction mixture was cooled to 0 °C and acidified with Dowex 50W X8 (H^+) to pH 3–4, and the water was removed by freeze-drying to give the product as colorless crystals (94 mg, 54%). ^{1}H NMR δ 2.38 (ddd, $J_{5ax,5eq}$ = 13.5, $J_{5ax,4(6)}$ = 5.6 Hz, H5ax), 2.50 (ddd, $J_{5eq,5ax}$ = 13.5, $J_{5eq,4(6)}$ = 5.6 Hz, H5eq), 4.25 (dd, $J_{4(6),5ax}$ = 5.6, $J_{(4,6),5eq}$ = 5.6 Hz, H4 and H6); *m/z* 144 (M − 44, 1%), 100 (6), 71 (4), 56 (5), 44 (100).

[2] Couplings measured from D_2O exchange spectrum.

Scheme I



cis- and trans-2-Oxohexahydropyrimidine-4,6-dicarboxylic Acid. Triethylamine (0.1 mL, 0.71 mmol) was added to a solution of the *cis*-hexahydropyrimidine dimethyl ester (100 mg, 0.46 mmol) in methanol (20 mL) and stirred at room temperature for 14 days. The solvent was removed to give a colorless crystalline solid (100 mg, 100%) which was a mixture of cis:trans diastereoisomers in the ratio 55:45 as analyzed by ^{1}H NMR. ^{1}H NMR (DMSO-d_6) (trans diastereoisomer) δ 2.11 (dd, $J_{5ax(5eq),4}$ = 6.1, $J_{5ax(5eq),6}$ = 6.1 Hz, H5ax and H5eq), 3.99 (ddd, $J_{4(6),5ax}$ = 6.1, $J_{4(6),5eq}$ = 6.1, $J_{4(6),NH}$ = 2.3 Hz, H4 and H6). The diastereoisomeric mixture of the hexahydropyrimidine dimethyl esters (100 mg, 0.71 mmol) was hydrolyzed, as above, to give the diastereomeric mixture of the dicarboxylic acids as colorless crystals (64 mg, 74%). ^{1}H NMR (trans diastereoisomer) δ 2.34 (dd, $J_{5ax(5eq),4}$ = 6.3, $J_{5ax(5eq),6}$ = 6.3 Hz, H5ax and H5eq), 4.13 (dd, $J_{4(6),5ax}$ = 6.3 Hz, H4 and H6).

Analysis of Kinetic Data. Data were fitted to the appropriate velocity equation by using the DNRP53 program for nonlinear regression analysis (Duggleby, 1984). This program is written in BASIC and was run on an IBM XT microcomputer. It was assumed that all data points were equally accurate and the (C) option was selected for constant weighting of all experimental points. Data obtained for inhibition of dihydroorotase by various analogues were consistent with the model of Scheme I, where all species are assumed to be in rapid equilibrium and conversion of dihydroorotate to carbamyl aspartate is the rate-limiting step. S is the substrate dihydroorotate, P is the product carbamylaspartate, I is inhibitor, *a* and *b* are interaction factors, and *c* is a constant. The complexes EI and IE are not identical. For EI, I competes with S for binding to the active site of E while for IE, I binds at a site distinct from the active site and may influence the subsequent binding of S or the rate of catalysis. With the assumptions described above, a velocity equation can be derived from Scheme I:

$$\frac{v}{V} = \frac{1 + \left(\frac{c}{aK_I}\right)I}{\left(1 + \frac{K_s}{S}\right) + \left(\frac{K_s}{K_iS} + \frac{K_s}{K_IS} + \frac{1}{aK_I}\right)I + \left(\frac{K_s}{bK_iK_IS}\right)I^2} \quad (2)$$

When $a \rightarrow \infty$ and $b \rightarrow \infty$, the complexes IES and IEI are present at negligible concentrations, and when $K_i \ll K_I$ (Scheme I), eq 2 simplifies to the velocity equation describing competitive inhibition:

$$\frac{v}{V} = \frac{S}{S + \left(1 + \frac{I}{K_i}\right)K_s} \quad (3)$$

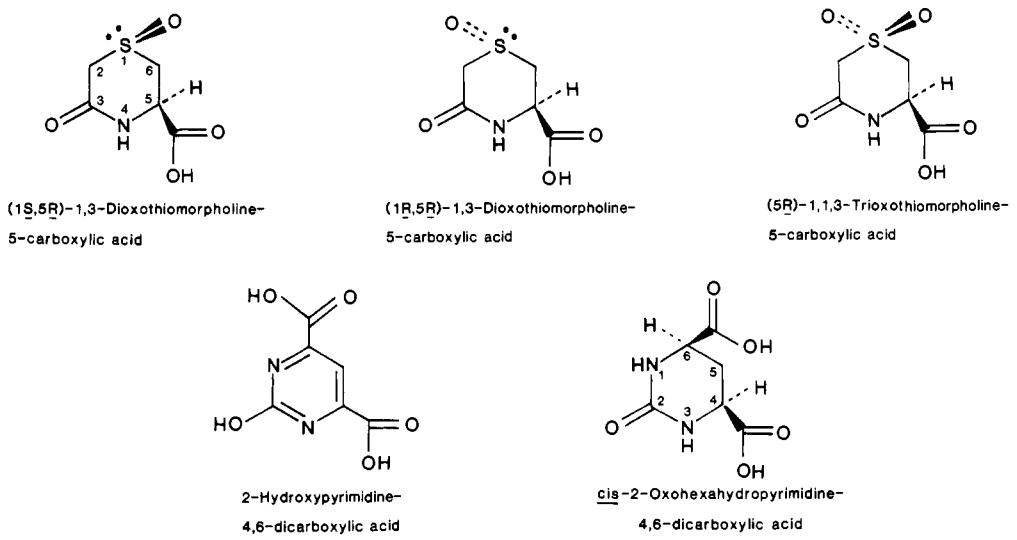


FIGURE 2: Pyrimidine analogues synthesized as potential inhibitors of dihydroorotase.

When $b \rightarrow \infty$, $c = 0$ (IES is a nonproductive enzyme–substrate complex), and $K_I \ll K_i$, eq 2 reduces to an equation describing noncompetitive inhibition:

$$\frac{v}{V} = \frac{S}{\left(1 + \frac{I}{aK_I}\right)S + \left(1 + \frac{I}{K_I}\right)K_s} \quad (4)$$

When S is constant, eq 2 can be rewritten in the general form:

$$v = \frac{v_0(1 + \delta I)}{1 + \beta I + \gamma I^2} \quad (5)$$

where v_0 is the uninhibited reaction velocity when $I = 0$ and β, γ, and δ are empirical constants; eq 5 is a 2/1 velocity equation when written in reciprocal form. From eq 2, when $c = 0$ or $aK_I \gg c$, $\delta = 0$ and eq 5 reduces to a 2/0 velocity equation in reciprocal form.

Results

A variety of pyrimidine analogues have been synthesized as potential inhibitors of dihydroorotase (Figures 1 and 2), but only (4*R*)-2-oxo-6-thioxohexahydropyrimidine-4-carboxylate (TDHO) and 2-oxo-1,2,3,6-tetrahydropyrimidine-4,6-dicarboxylate (HDDP) were potent inhibitors of the enzyme. Levenson and Meyer (1984) synthesized a cyclic thiomorpholine sulfoxide of undefined stereochemistry at position 1 and found it to be a moderately effective inhibitor of dihydroorotase. We have also synthesized this analogue and have separated the two diastereoisomers (1*S*,5*R*)- and (1*R*,5*R*)-1,3-dioxothiomorpholine-5-carboxylic acid (Figure 2) by fractional crystallization. Neither isomer in pure form significantly inhibited dihydroorotase. Levenson and Meyer (1984) also synthesized a cyclic thiadiazine sulfone and found moderate inhibition of dihydroorotase. We have synthesized a similar sulfone, (5*R*)-1,1,3-trioxothiomorpholine-5-carboxylic acid (Figure 2), but found no inhibition of dihydroorotase. The sulfone analogue of dihydroorotate (Figure 2) has two oxygen atoms as substituents of the sulfur in similar positions to the oxygens at position 6 of the dihydropyrimidine ring of the proposed transition state for the reaction catalyzed (eq 1).

It was proposed in earlier studies that 5-fluoroorotate, like orotate, is a competitive inhibitor of dihydroorotase (Christopherson & Jones, 1980a). However, the inhibition patterns of Figure 3a,b show that while orotate is competitive with respect to dihydroorotate, 5-fluoroorotate is a noncompetitive inhibitor. 5-Fluoroorotate has a dissociation constant for interaction with the free enzyme of $K_I = 15$ μM compared with orotate which has a dissociation constant of $K_i = 81$ μM (Scheme I). 6-Thioorotate synthesized by the method of Daves et al. (1961) was an effective inhibitor of dihydroorotase with an apparent inhibition constant of 82 μM, and in the presence of 100 μM $ZnCl_2$, a much lower value of 3.8 μM was obtained. 6-L-Thiodihydroorotate (TDHO) was synthesized as a potential chelating inhibitor of dihydroorotase (cf. eq 1) and is a potent competitive inhibitor with $K_i = 0.85$ μM. In the presence of 100 μM $ZnCl_2$, the K_i value for TDHO decreases to 0.17 μM and the inhibition pattern remains competitive (Figure 3c).

2-Hydroxypyrimidine-4,6-dicarboxylic acid (Figure 2) was synthesized as a second type of transition-state analogue but was also found to be inactive. Reduction of this analogue with 2 equiv of hydrogen gave 2-oxo-1,2,3,6-tetrahydropyrimidine-4,6-dicarboxylic acid (HDDP), a potent competitive inhibitor of dihydroorotase with a K_i value of 0.74 μM (Figure 3d). Reduction of the aromatic precursor with 4 equiv of hydrogen gave *cis*-2-oxohexahydropyrimidine-4,6-dicarboxylic acid or a mixture of the cis and trans isomers (55:45) as described under Experimental Procedures. The cis isomer was inactive and the cis–trans mixture gave an apparent K_i value of 26 μM, from which an approximate K_i value for the trans isomer alone of 12 μM was calculated. Data from the inhibition patterns of Figure 3 were fitted to the appropriate velocity equation, and the parameter values obtained are listed in Table I along with dissociation constants for the substrates (K_s values, Scheme I). The presence of Zn^{2+} increases the apparent K_s value for dihydroorotate from 20 μM to 65 μM, consistent with moderate competitive inhibition. Excess zinc ions are a competitive inhibitor for carboxypeptidase A (Hirose et al., 1987).

The presence of Zn^{2+} (100 μM) enhances the binding of TDHO and 6-thioorotate to dihydroorotase but does not significantly affect the interaction of HDDP or other inhibitors with the enzyme. The ^1H NMR spectrum (Figure 1) and the ultraviolet absorption spectrum for TDHO in the pressure of excess Zn^{2+} were unchanged,[3] suggesting that there is no direct interaction between TDHO and Zn^{2+} in aqueous solution. Data for inhibition of dihydroorotase by TDHO in the presence

[3] R. J. Goodridge and R. I. Christopherson, unpublished experiments.

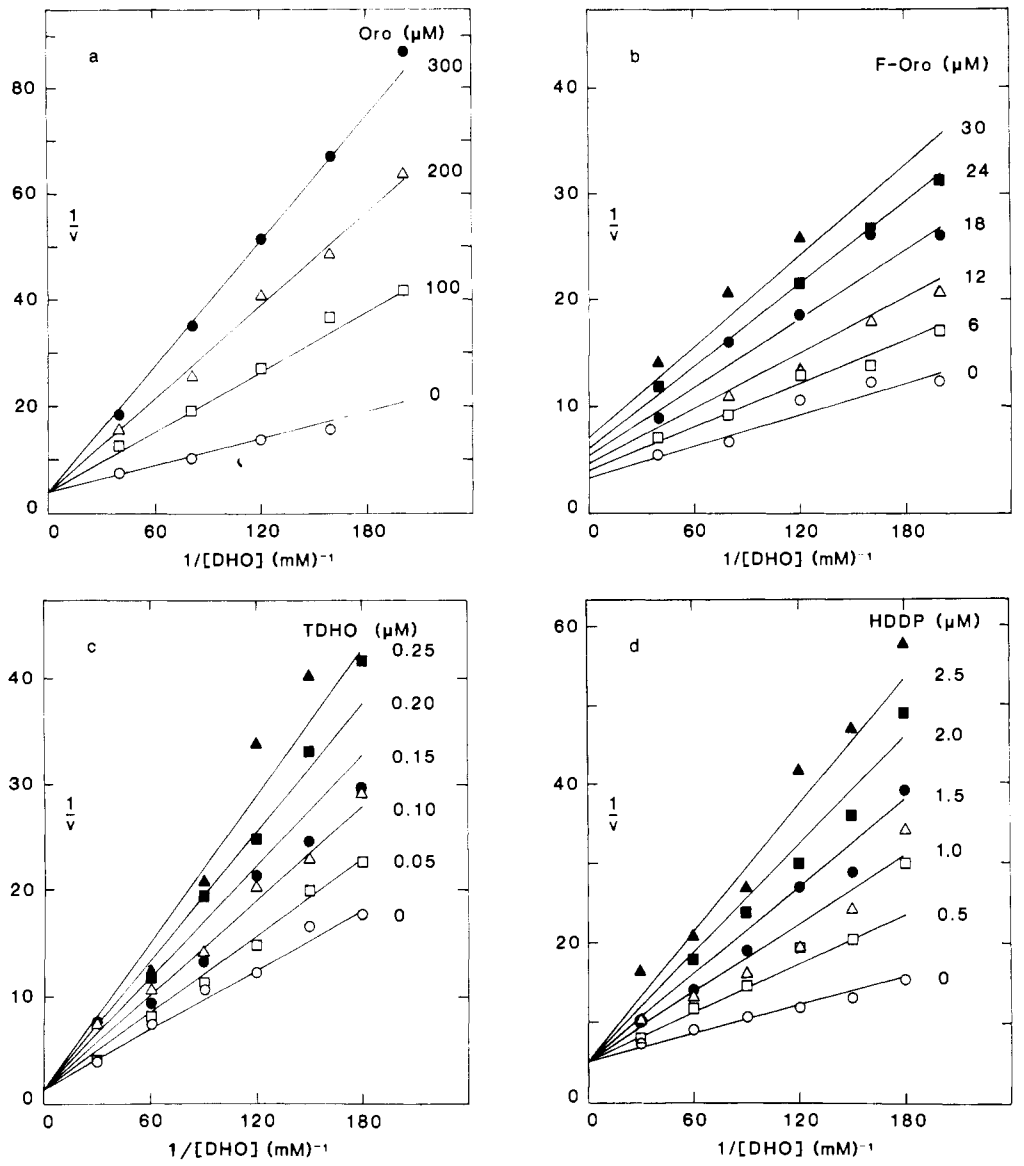


FIGURE 3: Inhibition patterns for orotate (Oro), 5-fluoroorotate (F-Oro), TDHO, and HDDP with respect to dihydroorotate (DHO). Data for competitive inhibition by Oro, TDHO, and HDDP were fitted to eq 3; data for noncompetitive inhibition by F-Oro were fitted to eq 4. Assays were initiated with 4.1 ng of dihydroorotate synthetase as described under Experimental Procedures. Reaction velocity units are μM/min.

of 100 μM $ZnCl_2$ were fitted to eq 3 for classical competitive inhibition (Figure 3c), but the inhibition pattern becomes nonlinear with respect to substrate and inhibitor at high concentrations (0.25 μM) of TDHO. Attempts to fit such data to the overall velocity equation describing the model of Scheme I (eq 2) were unsuccessful. The effect of Zn^{2+} at higher concentrations of TDHO is illustrated in Figure 4. A plot of $1/v$ versus TDHO concentration shows upward curvature in the presence of Zn^{2+} but yields a classical linear Dixon plot in the absence of Zn^{2+}. The data showing upward curvature were fitted to eq 5 by using the program DNRP53; the very small value for δ obtained reduced eq 5 from a 2/1 velocity equation to a 2/0 equation in reciprocal form. The parameter values obtained were not well-defined, probably due to errors inherent in measuring very low reaction velocities; the curve through the experimental data should be regarded as a simulation.

A variety of divalent metal chlorides would not substitute for $ZnCl_2$ in inducing the greatly enhanced inhibition of Figure 4. At a concentration of 0.5 μM TDHO, the ratio of the inhibited reaction velocity (v_i) to the activity in the absence of inhibitor (v_0) was determined in the presence of 100 μM

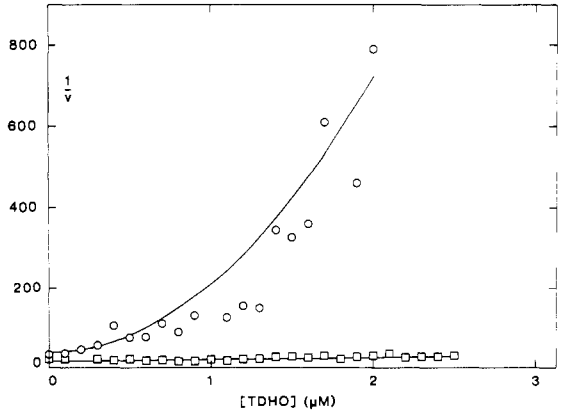


FIGURE 4: Dixon plots for inhibition of dihydroorotase by TDHO alone (□) and in the presence of 100 μM $ZnCl_2$ (○). Initial reaction velocities (μM/min) were determined with 12.5 μM [^{14}C]dihydroorotate and 1.5 ng of dihydroorotate synthetase. Data obtained in the presence of 100 μM $ZnCl_2$ (○) were fitted to eq 5 to yield parameter values of $\nu_0 = 0.027 \pm 0.009$ μM/min, $\delta = 7.7 \times 10^{-9} \approx 0$ μM^{-1}, $\beta = 0.17 \pm 1.6$ μM^{-1}, and $\gamma = 4.5 \pm 1.3$ μM^{-2}.

concentrations of various divalent metal ions. Values for v_i/v_0 obtained were Zn^{2+} (~0), Ca^{2+} (0.74), Co^{2+} (0.84), Mn^{2+}

Table I: Dissociation Constants for the Interaction of Substrates and Inhibitors with Dihydroorotase[a]

compound	dissociation constant (μM)
N-carbamyl-L-aspartate	420 ± 20[b]
L-dihydroorotate	20 ± 2[b]
	65 ± 20[d]
orotate	81 ± 9
5-fluoroorotate	K_i = 15 ± 4, a = 480 ± 130[e]
6-thioorotate	82 ± 8[c]
	5.2 ± 1.9[c,d]
L-6-thiodihydroorotate (TDHO)	0.85 ± 0.14
	0.17 ± 0.02[d]
2-oxo-1,2,3,6-tetrahydropyrimidine-4,6-dicarboxylate (HDDP)	0.74 ± 0.10
cis- and *trans*-2-oxohexahydropyrimidine-4,6-dicarboxylate	26 ± 3[c]

[a] Data were obtained at pH 7.4 by using 19 different substrate concentrations to obtain K_s values for carbamylaspartate and dihydroorotate. Dissociation constants for substrate analogues were obtained for the degradative reaction from inhibition patterns of the type shown in Figure 3. Within the concentration ranges of substrates and inhibitors used in these experiments, all substrate analogues except for 5-fluoroorotate acted as competitive inhibitors. [b] Interaction as a substrate. [c] Apparent K_i values were obtained by fitting data from linear Dixon plots to the equation describing simple competitive inhibition (eq 3), with S = 12.5 μM and K_s = 19.6 μM or 65.2 μM in the presence of 100 μM $ZnCl_2$. [d] Dissociation constant measured in the presence of 100 μM $ZnCl_2$. [e] Data were fitted to eq 4 describing noncompetitive inhibition where $K_I \ll K_i$, $b \rightarrow \infty$, and $c = 0$ (Scheme I).

(0.90), Ni^{2+} (0.91), Mg^{2+} (~1.0), and no divalent metal ion (~1.0).

Dihydroorotase is inactivated in a time-dependent manner by L-cysteine at high concentrations (50 mM; Christopherson & Jones, 1980a). This inhibitory effect is preceded by an activation at lower concentrations (0.1 μM) which is also induced by oxidized forms of L-cysteine (L-cysteic acid and L-cysteinesulfinic acid) and by iminodiacetate. At higher concentrations, L-cysteine and iminodiacetate are effective inhibitors of dihydroorotase; L-cysteic acid and L-cysteinesulfinic acid have little subsequent inhibitory effect. The data were fitted to eq 5, and values for the empirical constants β, γ, and δ are listed in Table II. Some of the parameter values obtained are not well-defined; apparent inhibition constants have been calculated for these four compounds from values for β, γ, and δ by using eq 6 as defined in footnote *b* to Table II.

Discussion

Interactions of all inhibitors with dihydroorotase can be accounted for by the kinetic model of Scheme I where all enzyme species are in rapid equilibrium and the conversion of ES and IES to product (P) is rate-limiting. Orotate (Figure 3a) and 6-thioorotate are simple competitive inhibitors of dihydroorotase; of the four possible enzyme–inhibitor complexes (EI, IE, IES, and IEI; Scheme I) only EI is formed at significant concentrations. In the presence of 100 μM $ZnCl_2$, 6-thioorotate is the most potent of these competitive inhibitors (K_i = 5.2 μM, Table I). The noncompetitive inhibition pattern of Figure 3b indicates that 5-fluoroorotate interacts with dihydroorotase in a different way, forming an IE complex and a nonproductive IES complex (Scheme I). The interaction factor of 479 (a, Table I) is consistent with S or I binding less strongly to the EI or ES complexes, respectively (Scheme I). It is not clear why substitution of a fluorine atom at position 5 of orotate should change the type of inhibition from competitive (EI) to noncompetitive (IE, IES), but the electronegativity of fluorine would withdraw electron density from the pyrimidine ring and there is also the possibility of fluorine interacting directly with the enzyme. L-6-Thiodihydroorotate (TDHO) is a potent competitive inhibitor of dihydroorotase (K_i = 0.85 μM, Figure 3c, Table I) which interacts more strongly with the enzyme than the substrate/product dihydroorotate (K_s = 19.6 μM, Table I). The substitution of sulfur for oxygen at position 6 has significantly enhanced the binding of TDHO, which may be attributed to interaction with a zinc atom at the active site of dihydroorotase as originally proposed by Christopherson and Jones (1980a); the presence of zinc was subsequently demonstrated by Kelly et al. (1986). Such a sulfur–zinc coordination bond has been demonstrated by X-ray crystallographic studies for the interaction between (3-mercapto-2-benzylpropanoyl)-L-alanylglycinamide and the zinc protease, thermolysin (Monzingo & Matthews, 1982). The observation that low concentrations of Zn^{2+} enhance the binding of 6-thioorotate (K_i = 5.2 μM) and TDHO (K_i = 0.17 μM) but no other inhibitor suggests that these two mercaptan inhibitors form a ternary complex with Zn^{2+} and the enzyme; Zn^{2+} does not interact significantly with TDHO alone in aqueous solution. The inhibition pattern for TDHO in the presence of 100 μM Zn^{2+} intersects on the $1/v$ ordinate (Figure 3c), suggesting that an IES complex (Scheme I) is not formed. The upward curvature of the inhibition pattern (Figure 3c) at higher concentrations of inhibitor and lower concentrations of substrate as shown in Figure 4 indicates positive cooperativity for the binding of TDHO and perhaps formation of an IEI complex (Scheme I) in the presence of Zn^{2+}.

2-Oxo-1,2,3,6-tetrahydropyrimidine-4,6-dicarboxylic acid (Figure 1, HDDP) is also a potent competitive inhibitor (K_i = 0.74 μM) that binds more tightly to dihydroorotase than carbamylaspartate or dihydroorotate and may be regarded as a transition-state analogue (Wolfenden, 1969). The cyclic sulfone (Figure 2) may differ from the transition state (eq 1) because sulfur is larger than carbon and a negative charge is not delocalized between the two oxygens of the sulfone (cf. eq 1). The binding of HDDP at the active site of dihydroorotase may resemble that of the specific thermolysin inhibitor *N*-(1-carboxy-3-phenylpropyl)-L-leucyl-L-tryptophan (CLT) where the two oxygens of the *N*-(carboxymethyl) group form a bidentate complex with zinc at the active site, in a manner analogous to the presumed transition state formed during peptide hydrolysis (Monzingo & Matthews, 1982; cf. eq 1). Construction of Dreiding models of HDDP and the cis and trans isomers of 2-oxohexahydropyrimidine-4,6-dicarboxylate indicated that the structure of the trans isomer could be superimposed on that of HDDP (Figure 1) in various conformations whereas the two carboxyl groups of the cis isomer (Figure 2) could not be superimposed on those of HDDP. The

Table II: Values of Kinetic Parameters for Inhibition of Dihydroorotase by Analogues Giving Nonlinear Dixon Plots[a]

compound	β (μM^{-1})	γ (μM^{-2})	δ (μM^{-1})	K_i^{app} (μM)[b]
L-cysteine[c]	9.9 ± 11	0.022 ± 0.025	15 ± 17	980
L-cysteic acid[c]	64 ± 10	0.0022 ± 0.0058	74 ± 11	47000
L-cysteine-sulfinic acid[c]	52 ± 13	0.013 ± 0.009	69 ± 15	11000
iminodiacetate[c]	5.4 ± 2.1	0.044 ± 0.018	11 ± 4	360

[a] Data were obtained at pH 7.4 with 25 μM [^{14}C]dihydroorotate as substrate, and the reaction was initiated by addition of 2.4 ng of dihydroorotate synthetase to 25-μL assay mixtures. Dihydroorotase activities in the absence of inhibitor (v_0) were normalized to a value of 0.064 μM/min. [b] Equating the terms of eq 5 to those of eq 2, it can be shown that $\delta/\gamma = bcK_i(S + K_s)/aK_s$, which can be rewritten as $\delta K_s/\gamma(S + K_s) = bcK_i/a = K_i^{app}$ (eq 6). Values of 19.6 μM and 25 μM have been assigned to K_s and S, respectively, to calculate K_i^{app}. [c] To allow for time-dependent inhibition, samples for determination of reaction velocities were taken at 7.5, 15, and 22.5 min after mixing dihydroorotate synthetase into the reaction mixture.

cis isomer did not bind to dihydroorotase and probably cannot adopt the conformation of the transition state (eq 1). The D isomer of dihydroorotate is a poor substrate when compared with the L isomer (Christopherson & Jones, 1980a), and it is evident therefore that the two carboxyl groups of HDDP and the trans isomer provide important attachment points to the enzyme. The position of the carboxyl group on carbon 6 probably mimicks the positions of the two oxygen atoms of the transition state (eq 1) while the carboxyl group on carbon 4 would be in the same position for inhibitors and substrate. HDDP (K_i = 0.74 μM) binds more tightly to dihydroorotase than carbamylaspartate (K_s = 420 μM, Table I), which is also a dicarboxylic acid, because the latter is not constrained in aqueous solution to the near-planar configuration that must precede ring closure.

L-Cysteine (Christopherson & Jones, 1980a) and iminodiacetate show a time-dependent inhibition of dihydroorotase, perhaps due to formation of coordination complexes with a zinc atom at the active site of the enzyme. Coombs et al. (1962) have demonstrated a similar effect for L-cysteine upon bovine carboxypeptidase A. Plots of $1/v$ versus inhibitor concentration for L-cysteine and iminodiacetate showed an initial activation at low inhibitor concentrations (0.1 μM), followed by subsequent inhibition at higher concentrations, where these plots approach linear asymptotes. These data were fitted to a 2/1 velocity equation (eq 5) consistent with formation of an IE complex (Scheme I) at lower concentrations of I with greater affinity for S than free E and, at higher concentrations of I, formation of an IEI complex where the second molecule of I competes directly with S for binding to E. The two oxidized forms of L-cysteine tested (L-cysteic acid and L-cysteinesulfinic acid) may form the IE complex but do not form significant concentrations of IEI because they activate dihydroorotase at low concentrations but do not significantly inhibit the enzyme at higher concentrations. Christopherson and Jones (1980a) proposed that the amino and thiolate groups of L-cysteine form a bidentate complex with zinc at the active site of dihydroorotase while the carboxylate may form an electrostatic interaction with a positive charge on the active site which normally interacts with the carboxyl group of dihydroorotate.

As mentioned in the introduction, dihydroorotase shares a number of unrelated properties with a family of proteolytic enzymes collectively called the "zinc proteases". The success of the mercaptan analogue TDHO and the transition-state analogue HDDP as potent inhibitors of dihydroorotase provides a further parallel with the zinc proteases although, to our knowledge, Zn^{2+} does not enhance the inhibition by mercaptan analogues of these proteases. Reactions catalyzed by zinc proteases involve one substrate and two products whereas dihydroorotase has a single substrate and product. Some inhibitors of the zinc proteases that incorporate structural features of the two products may therefore bind more tightly to their target enzymes than the inhibitors of dihydroorotase described here.

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