Design and Synthesis of Tetrahedral Intermediate Analogues as Potential Dihydroorotase Inhibitors

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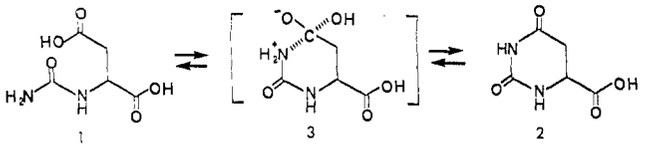
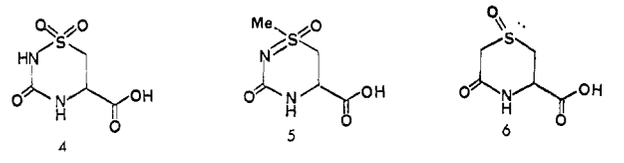
Three new heterocyclic analogues (4–6) of dihydroorotic acid were designed, synthesized, and tested as inhibitors of dihydroorotase. Each compound possessed a tetrahedral sulfur atom at the position equivalent to carbon 4 in the dihydroorotate ring in an attempt to mimic the presumed tetrahedral transition state in the course of the enzymatic reaction. Additionally, *N*-carbamyl-3-phosphonoalanine was prepared and evaluated as a dihydroorotase inhibitor. Compounds 4 and 6 were modest inhibitors (I_{50}'s of 0.52 and 0.18 mM, respectively), but the other candidate inhibitors showed little inhibition at 1 mM.

Dihydroorotase (EC 3.5.2.3) catalyzes the cyclization of *N*-carbamylaspartate (1) to dihydroorotate (2), the third

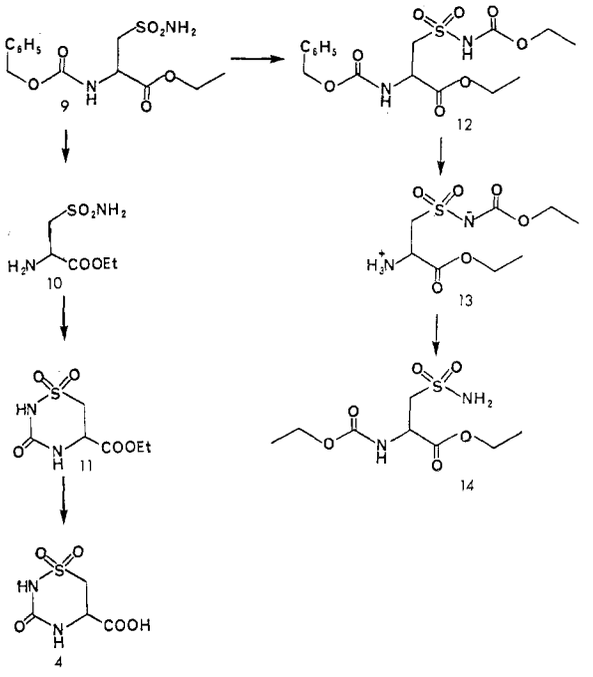




step in de novo pyrimidine biosynthesis. In higher organisms, dihydroorotase is part of a multienzyme complex also containing the first two enzymes in that pathway, carbamyl phosphate synthetase and aspartate transcarbamylase.[2] That this enzyme might present a likely target for potential anticancer drugs is indicated by the fact that it has been found to have elevated levels in certain hepatomas[3] and that inhibition of the pathway at the prior step (aspartate transcarbamylase) by *N*-(phosphonoacetyl)-L-aspartic acid has been shown to have clinical potential.[4]

Only a few inhibition studies have been carried out with dihydroorotase;[5-8] no really tight-binding inhibitors have been found. We have sought to apply recent concepts regarding the design of analogues of high-energy intermediates and/or transition states in enzyme-catalyzed reactions as potential very tight-binding inhibitors to this key enzyme in pyrimidine biosynthesis. In order to do this, the enzyme's catalytic mechanism must first be considered. Dihydroorotase does not appear to belong to the class of serine or cysteine proteases that from an acylenzyme intermediate. Other proteases, such as carboxypeptidase A and angiotensin converting enzyme, possess zinc in the active site, and interesting inhibitors of the former con-

Scheme I



taining tetrahedral phosphorus[10] and of the latter containing a sulfhydryl[11] have been successfully designed. Dihydroorotase from bacteria may have zinc,[8] and indirect evidence has been obtained by Christopherson and Jones[7] that the mammalian enzyme has a catalytically significant metal ion. This report describes the preparation and evaluation of several candidate inhibitors (4–6) of dihydroorotase with a tetrahedral atom at the reaction center that are intended to mimic postulated catalytic intermediate 3. Additionally, at neutral pH, compound 4 possesses a negative charge in the sulfonamide group, which could conceivably mimic a change present in the transition state.

Chemistry. *N*-(Benzyloxycarbonyl)-L-cysteamide ethyl ester (9) was prepared in four steps from L-cystine by the method of Baganz and Dransch.[12] Hydrogenation of the blocked sulfonamide gave the syrupy free base of the sulfonamide ester (10), which, upon treatment with 1,1'-carbonyldiimidazole gave the ethyl ester of the 1,2,4-thiadiazine dioxide (11). Saponification of the ester afforded the final product, 3-oxo-3,4,5,6-tetrahydro-2*H*-1,2,4-thiadiazine-5-carboxylic acid 1,1-dioxide (4), as shown

(1) Current address: Cetus Corp., Berkeley, CA 94710.
(2) (a) Hoogenrad, N. J.; Levine, R. L.; Cretchmer, N. *Biochem. Biophys. Res. Commun.* **1971**, *44*, 981. (b) Shoaf, W. T.; Jones, M. E. *Ibid.* **1971**, *45*, 796.
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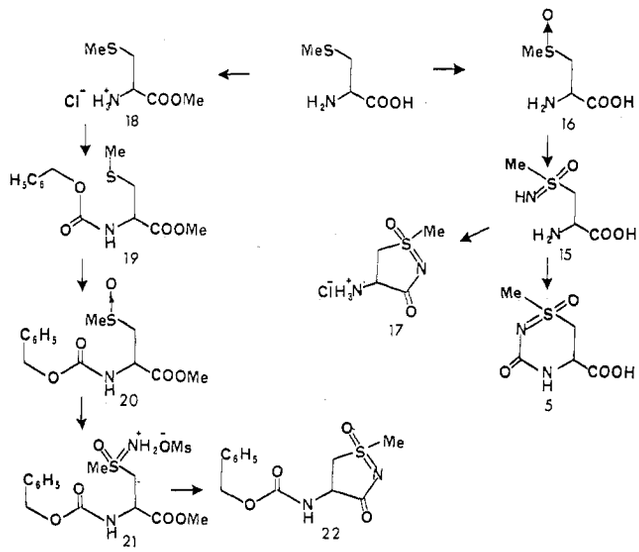
Scheme II



in Scheme I. In addition to 1,1′-carbonyldiimidazole, both phosgene and *p*-nitrophenyl chloroformate were found to be effective synthons for the introduction of a carbonyl group and consequent formation of the heterocyclic ring. We found the use of 1,1′-carbonyldiimidazole to proceed more cleanly and in higher yield, however.

An alternate route to the product was explored wherein sulfonamide **9** was treated with ethyl chloroformate to yield the dicarbamate **12**. Subsequent removal of the benzyl carbamate by hydrogenolysis gave a good yield of the zwitterion **13**, which was characterized by NMR (α-H at 4.18 ppm, NH_3^+ at 6.10 ppm). In an attempted ring-closure experiment in an NMR tube, **13** was heated at 100 °C in Me_2SO-d_6. Apparently, the sulfonamide was a better leaving group than ethoxide from the cyclic transition state, and the reaction led to an overall intramolecular transcarbonylation, with the only product observed being *N*,3-bis(ethoxycarbonyl)cysteamide (**14**) (α-H at 4.42 ppm, coupled to NH at 7.52 ppm). In retrospect, this result could be predicted on the basis of the relative pK_as of ethanol (ca. 16) and the sulfonamide (ca. 10).

The synthesis of analogue **5** was suggested by analogy to the potent transition-state analogue inhibitor of glutamine synthetase, methionine sulfoximine.[13] Sulfoximines, which are imino analogues of sulfones, are tetrahedral at sulfur and, unlike sulfones, constitute centers of asymmetry.[14] The structurally analogous inhibitor for dihydroorotase was seen as being most easily accessible (as shown in Scheme II) from *S*-methylcysteine sulfoximine (**15**), which was obtained from the corresponding sulfoxide (**16**) by treatment with sodium azide under conditions similar to those used for the Schmidt reaction, i.e., NaN_3 and H_2SO_4 at elevated temperatures for extended times.[15] We found that the overall yield of product was improved somewhat by the use of sodium *m*-periodate[16] instead of hydrogen peroxide[15] for the production of the *S*-methylcysteine sulfoxide (**16**) used as starting material. Furthermore, we found that the reaction of **16** with hydrazoic acid did not require the elevated temperature reported earlier, although yields in this step were quite low. Treatment of **15** with 1,1′-carbonyldiimidazole and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in dimethylformamide gave the desired analogue, 3-oxo-1-methyl-3,4,5,6-tetrahydro-1*H*-1,2,4-thiadiazine-5-carboxylic acid 1-oxide (**5**).

Scheme III

The inherent nucleophilicity of the sulfoximine nitrogen was demonstrated during an attempt to synthesize the methyl ester hydrochloride of the amino acid sulfoximine **15**. Treatment of **15** with HCl in methanol resulted in the formation of two products; one was the expected methyl ester hydrochloride of **15**, and the second product was generated by intramolecular ring closure of the sulfoximine nitrogen to the ester group. When the reaction was conducted without a cooling bath, only the 4-amino-4,5-dihydro-1-methyl-1*H*,3*H*-isothiazol-3-one 1-oxide hydrochloride salt (**17**) was observed by NMR analysis of the product mixture.

An alternate route to the sulfoximine was sought with the hope of circumventing the somewhat harsh conditions required for the hydrazoic acid amination reaction. Treatment of *S*-methyl-L-cysteine with HCl in anhydrous methanol gave the methyl ester hydrochloride (**18**). Protection of the amino group as the benzyl carbamate gave the fully protected amino acid (**19**). Periodate oxidation of the sulfide to the corresponding sulfoxide (**20**) gave a mixture of diastereomers, which were separated by fractional cystallization. The two isomers differed in melting point by 60 °C and exhibited marked differences in solubility in benzene and chloroform; the lower melting isomer was very soluble, but the higher melting isomer was only sparingly so. The two isomers also differed greatly in their reactivity toward the powerful aminating reagent, *O*-(mesitylenesulfonyl)hydroxylamine (MSH),[17] which has been found to be the reagent of choice for the synthesis of N-unsubstituted sulfoximines from the corresponding sulfoxides.[18,19] With the lower melting isomer a very facile reaction with MSH occured in benzene at room temperature, giving the pure mesitylenesulfonate of **21** directly. Once again, the proclivity of the sulfoximine nitrogen to act as a nucleophile was evidenced when, in an attempt to isolate the free base of the sulfoximine, treatment of the salt of **21** with aqueous bicarbonate led only to the ring-closed product, 4-[(benzyloxycarbonyl)amino]-4,5-dihydro-1-methyl-1*H*,3*H*-isothiazol-3-one 1-oxide (**22**). Attempts to react the higher melting isomer with MSH under similar conditions were not fruitful, and forcing conditions (i.e., higher temperatures and/or concentrations of MSH) lead only to extensive decomposition.

Although compound **6** lacks certain binding points (particularly the N-3 of the dihydropyrimidine) of intermediate **3**, it does possess tetrahedral geometry at the appropriate position. Furthermore, the sulfoxide oxygen has greater hydrogen bond forming capability than either sulfonamide or sulfone oxygen.[20] For the synthesis of **6**,

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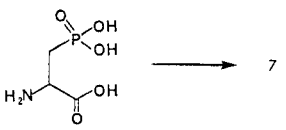
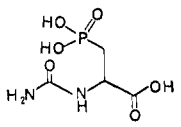
Scheme IV



24

cysteine was converted to *S*-(carboxymethyl)cysteine, which was cyclized to 3-oxo-3,4,5,6-tetrahydro-2*H*-1,4-thiadiazine-5-carboxylic acid (**23**), according to the methods of Ozawa.[21] The thiazinone was then oxidized with sodium metaperiodate to give the desired 3-oxo-3,4,5,6-tetrahydro-2*H*-1,4-thiazine-5-carboxylic acid 1-oxide (**6**), presumably as a mixture of diastereomers, as shown in Scheme III.

The tight binding of phosphoramidon [*N*-[*N*-[[(6-deoxy-α-L-mannopyranosyl)oxy]hydroxyphosphinyl]-L-leucyl]tryptophan] to the zinc-containing peptidase thermolysin[22] and of certain *N*-phosphoryl amino acids to carboxypeptidase A[10a] led us to consider *N*-carbamyl-3-phosphonoalanine (7) as a likely candidate transition-state



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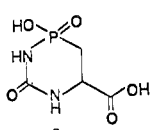

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analogue inhibitor of dihydooratase. Additionally, phosphadiazine **8**, the phosphorus isoster of the transition-state **3** was considered because of the success of Bartlett and co-workers in the design of Z-NHCH$_2$PO$_2$(H)-Phe, a potent inhibitor of carboxypeptidase A.[10a]

3-Phosphonoalanine (**24**) was prepared by the methods of Chambers and Isbell.[23] Treatment with KOCN, using the method of Nyc and Mitchell,[24] gave the desired *N*-carbamyl-3-phosphonoalanine (**7**), as shown in Scheme IV. Unfortunately, numerous methods to convert 3-phosphonoalanine to phosphadiazine **8** all lead to failure, due mainly to the presence of both a carboxylic and phosphonic acid group in the same molecule.

Enzymatic Evaluation of Inhibitors. The isolation of mammalian dihydroorotase was greatly facilitated by the use of hampster cells that had been made resistant to *N*-(phosphonoacetyl)-L-aspartic acid. These cells overaccumulate the multienzyme complex containing dihydroorotase[25] and provide an excellent source for this enzyme in quantity.[26]

The enzyme activity was assayed in the retrosynthetic direction by incubation of a mixture of the enzyme, labeled substrate (dihydroorotate), and the potential inhibitor. Aliquots were removed and purified by thin-layer chromatography, and the extent of reaction progress was determined by scintillation counting, following the technique of Christopherson and Jones.[7] Assays were conducted at pH 7.4 with 10 μM dihydroorotate (the K_m for dihydroorotase from mouse Ehrlich ascites is about 4 μM at pH 7.37). Compounds **4**–**7** and **23** and *d*-3-phosphonoalanine were tested as possible inhibitors of dihydroorotase. Of these compounds, cyclic sulfonamide **4** showed an I_{50} of 0.52 mM, and cyclic sulfoxide **6** showed an I_{50} of 0.18 mM. The I_{50}'s of the other compounds were above 1 mM. Since inhibitors should have inhibition constants substantially less than the K_m of substrate to be considered successful transition-state analogues, we must conclude that either these compounds do not adequately mimic the presumed tetrahedral intermediate or that our assumptions regarding the enzyme's mechanism are incorrect.

Experimental Section

Melting points were determined in a Thomas-Hoover Uni-Melt apparatus using capillary tubes and were uncorrected. Proton magnetic resonance spectra were run on either a Varian FT-80 at 80 MHz or on a Varian T-60 at 60 MHz. Chemical shifts are reported downfield from internal Me$_4$Si. Evaporations were conducted under high vacuum at room temperature on a rotary evaporator, unless otherwise specified. Chemicals (reagents and starting materials) that were commercially available were obtained either from Aldrich Chemical Co. or Sigma Chemical Co. Thin-layer chromatography was performed on Bakerflex (J. T. Baker Co.) silca gel 1B2-F plates (2.5 × 7.5 cm) containing fluorescent indicator and an inert binder. Spots were visualized either by UV light or by spraying the plates with a 1% *tert*-butyl hypochlorite in cyclohexane solution, followed by a 1% sodium iodide in 1% aqueous soluble starch solution.[27] Compounds having an active NH show up as dark purple spots on a gray background. Column chromatography was performed with Merck silca gel 60 (230–400 mesh). Columns were dry packed. Elemental analyses were performed either by the microanalytical lab in the Department of Chemistry at the University of California at Berkeley or by MWH Laboratories in Phoenix, AZ.

Ethyl 3-Oxo-3,4,5,6-tetrahydro-2*H*-1,2,4-thiadiazine-5-carboxylate 1,1-Dioxide (11). To a solution of the ester **10** (2.97 g, 11.5 mmol) in 80 mL of dry chloroform was added dropwise a solution of 1,1′-carbonyldiimidazole (20 g, 123 mmol) in 100 mL of chloroform. Two hours after all additions had been made, 75–100 mL of chloroform was removed under reduced pressure, and stirring was continued for another 1.5 h, at which time all the remaining chloroform was removed on the rotary evaporator. The residue was taken up in 50 mL of water to decompose the excess 1,1′-carbonyldiimidazole. The pH of the aqueous solution was adjusted to 3 by the batchwise addition of Dowex 50-X8 (H$^+$, 100–200 mesh), and the resin was removed by filtration and washed with ethanol and water. The filtrate was concentrated on the rotary evaporator to dryness, and the residue was fractionated on a silica gel column (3 × 30 cm) with chloroform/acetic acid (1:1) as eluant. A total of 2.19 (86%) of product was obtained from the column fractions after recrystallization from ethanol: mp 200–201 °C dec; NMR (Me$_2$SO-d_6) δ 1.19 (t, 3 H, J = 7.1 Hz), 3.68 (d, 2 H, J = 5.4 Hz), 4.14 (q, 2 H, J = 7.1 Hz), 4.49 (dt, 1 H, J_t = 5.4 Hz, J_d = 3.1 Hz), 7.86 (d, 1 H, J = 3.1 Hz). Anal. (C$_6$H$_{10}$N$_2$O$_5$S) C, H, N.

3-Oxo-3,4,5,6-tetrahydro-2*H*-1,2,4-thiadiazine-5-carboxylic Acid 1,1-Dioxide (4). The ethyl ester **11** (192 mg, 0.864 mmol) was dissolved in 25 mL of 95% ethanol, and to the solution was added 2.5 mL of 4 N NaOH. The solution became cloudy and was stirred at room temperature. After 30 min, the solution was adjusted to pH 3 by the batchwise addition of Dowex 50-X8 (H$^+$). The resin was removed by filtration and washed well with ethanol and water. The filtrate and combined washings were concentrated under reduced pressure, and the residue was crystallized from water to yield 99 mg (59%); mp 194-197 °C dec; NMR (Me$_2$SO-d_6) δ 3.69 (d, 2 H, J = 5.7 Hz), 4.38 (dt, 1 H, J_t = 5.7 Hz, J_d = 3.2 Hz), 7.85 (d, 1 H, J = 3.2 Hz). Anal. (C$_4$H$_6$N$_2$O$_5$S) H, N; C: calcd, 24.75; found, 25.24.

3-Oxo-1-methyl-3,4,5,6-tetrahydro-1*H*-1,2,4-thiadiazine-5-carboxylate 1-Oxide (5). To a solution of 1,8-diazabicyclo-[5.4.0]undec-7-ene (DBU; 38 mg, 0.25 mmol) in 1 mL of Me$_2$SO was added *S*-methylcysteine sulfoximine[15] (20 mg, 0.12 mmol).

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The mixture was heated gently to dissolve the amino acid. To this was added 1,1'-carbonyldiimidazole (35 mg, 0.216 mmol). After 1 h, the solution was diluted with 5 mL of water, and to this was added batchwise Dowex 50-X8 (H^+) until the pH of the solution was ~3. The resin was removed by filtration and washed with water. The filtrate and washings were concentrated under reduced pressure. Residual Me_2SO was removed by repeated washing of the residue with toluene. The semicrystalline residue was dissolved in a minimum of hot water and filtered. Upon cooling, crystals formed and were removed by filtration and found to be pure by TLC and NMR: yield 20 mg (87%); mp 181–182.5 °C; NMR (Me_2SO-d_6) δ 3.27 (s, 3 H), 3.63 (m, 2 H), 4.43 (dd, 1 H, J_d = 5.0 and 9.0 Hz). Anal. ($C_5H_8N_2O_4S$) C, H, N.

Methyl *S*-Methylcysteinate Hydrochloride (18). Into a stirred suspension of *S*-methylcysteine (10.15 g, 75.07 mmol) in cold anhydrous methanol (130 mL) was bubbled dry HCl gas at room temperature. When all the solid had dissolved, the solution was cooled in an ice bath, and the solution was saturated with HCl at 0–5 °C and allowed to remain overnight at 4 °C. After evaporation under reduced pressure, the residue was taken up in methanol, and ether was added until cloudy. The flask was chilled overnight, and the next day the prismatic crystals that had formed were removed by filtration: yield 13.12 g (94%); mp 154–156 °C; NMR (D_2O) δ 2.17 (s, 3 H), 3.05 (dd, 1 H, J_{vic} = 3.0 and J_{gem} = 8.0 Hz), 3.26 (d, 1 H, J = 8.0 Hz), 3.88 (s, 3 H), 4.42 (dd, 1 H, J_d = 6.0 and 8.0 Hz. Anal. ($C_5H_{11}NO_2S$·HCl) C, H, N.

Methyl *N*-(Benzyloxycarbonyl)-*S*-methylcysteinate (19). To a stirred, ice-cold mixture of potassium bicarbonate (8.51 g, 85 mmol) and **18** (3.16 g, 17.02 mmol) in 85 mL each of water and ethyl acetate was added dropwise, over 15 min, benzyl chloroformate (3.2 g, 18.82 mmol). After 3 h, the ice bath was removed, and the mixture was allowed to attain room temperature. The organic phase was separated, washed with 75 mL of 0.1 N HCl and 75 mL of water, dried over magnesium sulfate, and filtered, and the filtrate was taken to dryness under reduced pressure on the rotary evaporator to yield 3.71 g of a syrup. The material crystallized as fine, fibrous needles from ether/hexanes to give 4.27 g (88.5%) of pure product: m.p. 62–64 °C; NMR ($CDCl_3$) δ 2.03 (s, 3 H), 2.88 (d, 2 H, J = 6.0 Hz), 3.70 (s, 3 H), 4.53 (dt, 1 H, J_d = 8.0 Hz, J_t = 6.0 Hz), 5.07 (s, 2 H), 5.58 (br d, 1 H, J = 8.0 Hz), 7.28 (s, 5 H). Anal. ($C_{13}H_{17}NO_4S$) C, H, N.

Methyl *N*-(Benzyloxycarbonyl)-*S*-methylcysteinate Sulfoxide (20). To an ice-cold solution of the sulfide **19** (4.0 g, 14.2 mmol) in 45 mL of methanol was added dropwise, with stirring, a solution of sodium metaperiodate (3.17 g, 14.82 mmol) in 20 mL of water. Much material precipitated from the reaction mixture. The ice bath was removed, and stirring was continued. After 45 min, the mixture was filtered to remove the sodium iodate. The salt was washed with methanol, and the combined washings and filtrate were concentrated under reduced pressure to ~25 mL and extracted with dichloromethane (4 × 25 mL). The combined organic extracts were washed with 25 mL of water, dried over magnesium sulfate, and filtered, and the filtrate was evaporated to dryness. The residue was crystallized from chloroform/ether, The first crop of crystals obtained weighed 2.15 g (50%) and melted at 148–150 °C. The second crop weighed 1.67 g (40%) and melted at 90–92 °C (overall yield of 90%). These were apparently the two epimeric sulfoxides, i.e., the (*R*)- and (*S*)-sulfoxides of the L-amino acid: NMR ($CDCl_3$) δ 2.62 (s, 3 H), 3.25 (m, 2 H, high melting isomer), 3.31 (m, 2 H, low melting isomer), 3.77 (s, 3 H), 4.81 (m, 1 H, high melting isomer), 4.73 (m, 1 H, low melting isomer), 5.13 (s, 2 H), 6.09 (br d, 1 H, J = 7.0 Hz), 7.33 (s, 5 H). Anal. ($C_{13}H_{17}NO_5S$) C, H, N correct for each of the isomers.

Methyl *N*-(Benzyloxycarbonyl)-*S*-methylcysteinate Sulfoximine Mesitylenesulfonate (21). To a solution of the low-melting isomer of **20** (500 mg, 1.67 mmol) in 5 mL of benzene was added *O*-(mesitylenesulfonyl)hydroxylamine[17] (450 mg, 209 mmol). The solution was stirred at room temperature. The mesitylenesulfonate salt of the sulfoximine crystallized from the reaction mixture and was removed by filtration to yield 760 mg (88%) of product: mp 138–140 °C; NMR (D_2O) δ 2.22 (s, 3 H), 2.60 (s, 6 H), 3.78 (d, 3 H, J = 6.0 Hz), 3.97 (s, 3 H), 4.60 (m, 2 H), 4.93 (dd, 1 H, J_d = 4.8 and 8.0 Hz), 5.15 (s, 2 H), 6.85 (s, 2 H), 7.35 (s, 5 H). Anal. ($C_{22}H_{30}N_2O_8S_2$) H, N; C: calcd, 51.35; found, 50.89.

4-[(Benzyloxycarbonyl)amino]-4,5-dihydro-1-methyl-3*H*-isothiazol-3-one 1-Oxide (22). Compound **20** (900 mg, 3.01 mmol) was stirred overnight with 800 mg (3.72 mmol) of *O*-(mesitylenesulfonyl)hydroxylamine in 15 mL of $CHCl_3$. This solution was then shaken with 50 mL of 5% $NaHCO_3$, and the aqueous phase was extracted with 5 × 15 mL of $CHCl_3$. The combined organic phases were extracted with 5 mL of H_2O, dried ($MgSO_4$), and evaporated. The residue was dissolved in 25 mL of CH_2Cl_2 and extracted with 3 × 25 mL of 0.5 N HCl. These aqueous extracts were neutralized by the addition of Na_2CO_3, and then extracted with 4 × 25 mL of CH_2Cl_2. The organic extract was dried and evaporated, and the residue was dissolved in $CHCl_3$. Addition of hexane induced crystallization: yield 50 mg; mp 197–199 °C; NMR (Me_2SO-d_6) δ 3.51 (s, 3 H), 3.93 (m, 2 H), 4.61 (m, 1 H), 5.08 (s, 2 H), 7.33 (s, 5 H). Anal. ($C_{12}H_{14}N_2O_4S$) C, H, N.

3-Oxo-3,4,5,6-tetrahydro-2*H*-1,4-thiazine-5-carboxylic Acid 1-Oxide (6). 3-Oxo-3,4,5,6-tetrahydro-2*H*-1,4-thiazine-5-carboxylic acid (**23**) was prepared according to the procedure in the literature from *S*-(carboxymethyl)cysteine.[21] To a solution of **23** (50 mg, 0.31 mmol) in 2 mL of 50% aqueous ethanol was added at room temperature a solution of sodium metaperiodate (70 mg, 0.327 mmol) in 2 mL of water. After 1 h, the solution was concentrated under reduced pressure, and the precipitated sodium iodate was removed by filtration. Addition of ethanol to the filtrate induced the product to crystallize: yield 45 mg (82%); mp 160–163 °C dec. Anal. ($C_5H_7NO_4S$) C, H, N.

***N*-Carbamyl-3-phosphono-*dl*-alanine (7).** 3-Phosphonoalanine[23] (**24**; 1 g, 5.91 mmol) and potassium cyanate (1.2 g, 14.8 mmol) were dissolved in 25 mL of water, and the pH was adjusted to 7 with 4 N NaOH. The mixture was heated at 60 °C for 2 h, concentrated under vacuum to 5 mL, and loaded onto a column (2.6 × 20 cm) of Dowex 50-X8 (H^+ form). The column was washed with water, and the acidic, ninhydrin-negative fractions were pooled and adjusted to neutrality with 1 N NaOH. The solution was concentrated to a foam under high vacuum (1.1 g). Anal. ($C_4H_7N_2O_6P$·2Na·2H_2O) C, H; N: calcd, 9.59; found, 8.60.

Enzyme Assay Materials. Dihydroorotase was isolated from *N*-(phosphonoacetyl)aspartate-resistant hamster cells (supplied by Dr. G. R. Stark) by the method of Coleman et al.[26] The dihydroorotate used as substrate was prepared from [2-^{14}C]orotate (New England Nuclear) by enzymatic reduction using the method of Kensler et al.[28]

Enzyme Assays. The compounds were tested as inhibitors of dihydroorotase in the degradative direction, with dihydroorotate as substrate, by the radiometric method of Christopherson and Jones.[7] Each reaction mixture contained 10 μM dihydro[^{14}C]-orotate (specific activity 25 Ci/mol); 50 mM Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], pH 7.4; 7.5 μL of enzyme stock solution; and 0.1–1 mM of the compound being tested in a total volume of 25 μL. After 15 min at 29 °C (which we found to be still in the linear portion of the reaction), 5-μL samples were spotted on PEI-cellulose plated (J. T. Baker Co.), which were developed with 0.19 mM LiCl. Each chromatogram was cut into four strips (0–16, 16–42, 42–72, and 72–92% of the length of the chromatogram). The second strip contained *N*-carbamylaspartate. The strips were placed in 5-mL scintillation cocktail containing toluene, Triton X-100 (2:1 v/v), PPO (2,5-diphenyloxazole; 5.5 g/L), and POPOP [1,4-bis[2-(5-phenyloxazolyl)]benzene; 0.1 g/L], and radioactivity was determined with a Beckman LS 7000 scintillation counter. Relative counts in *N*-carbamylaspartate (counts in CAA/total counts on plate) in the inhibited mixture were compared to relative counts in CAA for uninhibited controls to give percent inhibition. All points were the average of duplicate incubations, which rarely differed by more than 10% and never by more than 20%. Further inhibitor levels were chosen to bracket the 50% inhibition level, and I_{50}'s were determined graphically. The values given are representative; repeat determinations (at least two) never varied by more than 20%.

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Registry No. 4, 87862-93-9; 5, 87862-94-0; 6, 87862-95-1; 7, 87862-96-2; 10, 34234-57-6; 11, 87862-97-3; 15, 87862-98-4; 18, 34017-27-1; 19, 76646-28-1; 20 [(*R*)-sulfoxide], 87862-99-5; 20 [(*S*)-sulfoxide], 87863-00-1; 21, 87863-02-3; 22, 87863-03-4; 23, 62305-89-9; 24, 20263-06-3; 1,1′-carbonyldiimidazole, 530-62-1; DBU, 6674-22-2; *S*-methylcysteine, 1187-84-4; benzyl chloroformate, 501-53-1; *O*-(mesitylenesulfonyl)hydroxylamine, 36016-40-7; dihydroorotase, 9024-93-5.

Improved Synthesis and Antitumor Evaluation of 5,8-Dideazaisofolic Acid and Closely Related Analogues[1]

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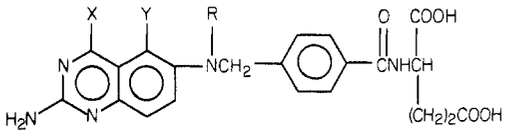
A new synthetic route to 5,8-dideazaisofolic acid (IAHQ) is described which precludes the possibility of contamination due to its 4-amino counterpart 5,8-dideazaisoaminopterin. Substitution of D-glutamic acid in this synthetic scheme gave D-IAHQ. The 9-formyl, 9-methyl, 5-methyl, and 5,9-dimethyl modifications of IAHQ were also prepared. These compounds, together with several structurally related or isomeric analogues, were studied for inhibitory effects upon the growth of four human gastrointestinal adenocarcinoma cell lines in vitro. In general, the compounds having a normal folate configuration at positions 9 and 10 are more active than their reversed bridge isomers. The lack of antitumor activity of D-IAHQ provides indirect evidence concerning the mechanism of action of IAHQ.

5,8-Dideazaisofolic acid (IAHQ, **1a**) was first described in 1975 as part of an ongoing synthetic program concerned with quinazoline analogues of folic acid.[2] Earlier studies had shown that its isomer 5,8-dideazafolic acid (AHQ), which has a normal folate configuration at positions 9 and 10, and the 10-CH_3 analogue (10-CH_3-AHQ) were effective inhibitors of thymidylate synthase from several different sources.[3–5] In vitro, 10-CH_3-AHQ inhibited the growth of mouse neuroblastoma cells, although not nearly as effectively as certain structurally related 2,4-diaminoquinazolines, which were shown to be potent inhibitors of dihydrofolate reductase (DHFR).[5]

Preliminary studies with IAHQ showed that at low doses using a single dose regimen on day 1 after tumor inoculation, the compound was ineffective against L1210 leukemia in mice.[2] IAHQ was a moderately effective inhibitor of rat liver DHFR, being some 10-fold less inhibitory than AHQ.[6] Subsequently, a large series of quinazoline analogues of folic acid was evaluated as inhibitors of thymidylate synthase from *Lactobacillus casei* and from L1210 leukemia cells.[7] IAHQ was found to be an effective inhibitor of the L1210 enzyme; however, AHQ and 10-CH_3-AHQ were significantly more inhibitory toward this enzyme. More recent studies revealed that IAHQ was an effective inhibitor of the growth of human colon adenocarcinoma cells (HCT-8) in vitro, thus generating interest in this compound for potential use in the treatment of methotrexate (MTX) unresponsive tumors.[8] Significant activity against colon tumor 38 in mice was also demonstrated, and when a regimen of 85 mg/kg on days 2 and 10 following tumor inoculation was used, there were 6 of 20 tumor-free animals after 90 days.[8] MTX was not effective in this model.[9] It was also found that IAHQ protected newborn hamsters from mortality due to transplantable human osteosarcoma cells, whereas MTX had no effect against this xenograph at the maximally tolerated dose.[10]

Chemistry. This paper describes a new unequivocal synthetic route to **1a**, which precludes the possibility of



1a, X = OH; Y = R = H
b, X = NH_2; Y = R = H
c, X = OH; Y = CH_3; R = H
1d, X = OH; Y = H; R = CHO
e, X = OH; Y = H; R = CH_3
f, X = OH; Y = CH_3; R = CH_3

trace contamination due to its 4-amino counterpart, 5,8-dideazaisoaminopterin (**1b**). The latter compound was shown to be a reasonably potent inhibitor of DHFR from rat liver.[6] Numerous earlier synthetic efforts employed diethyl esters of glutamate, which were removed in the final step under hydrolysis conditions using sodium hydroxide. During this study, the carboxyl groups of glutamic acid were protected by the use of *tert*-butyl esters, which are removed readily at ambient temperature under mildly acidic conditions. This modification yields final

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