6-Substituted 2,4-Diamino-5-(4-carbethoxyphenylazo)pyrimidines as Potential Precursors of Tetrahydropteridine Antimetabolites[1]

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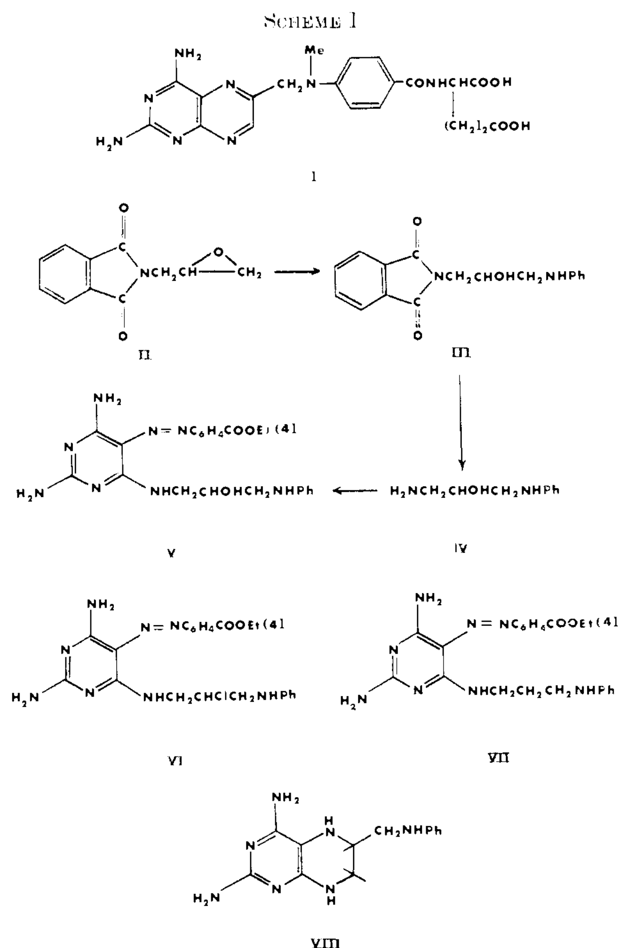
2,4-Diamino-5-(4-carbethoxyphenylazo)pyrimidines bearing the 3-anilino-2-hydroxypropylamino or 3-anilino-2-chloropropylamino substituents in the 6 position have been synthesized and compared in biological systems with 2,4,6-triamino-5-(4-carbethoxyphenylazo)pyrimidine to test the hypothesis that the former compounds might undergo an *in vivo* conversion to a tetrahydropteridine. No evidence to support this hypothesis could be found, and it was demonstrated that azopyrimidines are inhibitors, rather than substrates, of rat azo reductases.

As part of a continuing program[3-5] of synthesis and evaluation of substituted pyrimidines as folic acid antagonists, we have considered the possibility of synthesizing compounds that are potential precursors of folic acid antagonists which may be converted *in vivo* to active antagonists. The advantages of this type of approach to the design of chemotherapeutically effective compounds have been discussed several times.[6-9] The conversion of an inactive to an active compound by enzymatic activation occurring preferentially in the tumor cell offers to cancer chemotherapy possibilities of increased selectivity of action, reduced systemic toxicity, and possible reduction in the development of resistance to the drug.

Our preliminary approach to this problem has been to design compounds that may be converted *in vivo* to folic acid antagonists of the 2,4-diaminopteridine class of which the best known member is amethopterin (I) (see Scheme I). The compounds selected were appropriately substituted 5-arylazopyrimidines (V and VI) which, conceivably, could cyclize to a tetrahydropteridine (VIII) after reductive fission of the azo linkage.[10] Compound VII was included as a control in which this reductive cyclization could not occur.

SCHEME I



Results and Discussion

The results presented in Table I show that compounds V–VII are inhibitors of folic acid reductase, binding somewhat less efficiently than folic acid. The effect of introducing the 6-X substituents of compounds V–VII into 2,4,6-triamino-6-(4-carbethoxyphenylazo)-pyrimidine ($[I]/[S]_{50} = 0.011$)[4] is to produce decreases in binding capacity of approximately 100-fold. In the work of Baker and Shapiro[11] it is also evident that introduction of two hydrophobic substituents into 2,4-diaminopyrimidines does not produce an additive increase in binding capacity toward dihydrofolic reductase. Thus, replacement of the 6-methyl group of 2,4-diamino-5-(4-phenylbutyl)-6-methylpyrimidine by phenyl leads to an approximately 50-fold loss in effectiveness of binding, although 2,4-diamino-6-phenylpyrimidine binds to dihydrofolic reductase seven times more effectively than 2,4-diamino-6-methylpyrimidine. One explanation of these findings is that

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(10) G. R. Ramage and G. Trappe [*J. Chem. Soc.*, 4410 (1952)] have, however, noted that reduction of 4-(2-chloroethyl)amino-5-nitropyrimidines and subsequent refluxing in ethanolic solution yielded glyoxalinopyrimidines, formed by cyclization onto a ring nitrogen, rather than pteridines or tetrahydropteridines.

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TABLE I

TOXICITY AND ANTITUMOR ACTIVITY OF 6-SUBSTITUTED 2,4-DIAMINO-5-(4-CARBETHOXYPHENYLAZO)PYRIMIDINES

No.	$([I]/[S])_{50}$[a]	Mouse toxicity,[b] mg/kg	Rat toxicity,[c] mg/kg qd 5-9	Dose, mg/kg qd 5-9[d]	% body wt change[e]	T/C[f]
V	1.5	>1600	>400	400	+6	0.5
VI	3.75	300	140	100	+3	0.67
				50	+19	0.9
VII	1.2	1100	550	400	−9	0.46

[a] Folic acid reductase inhibition. Ratio of concentration of inhibitor required for 50% inhibition of enzyme activity to concentration of substrate, [folate] = 8×10^{-5} M. [b] Approximate acute LD_{50} (see Experimental Section). [c] Approximate LD_{50}, five daily doses on days 5-9 (see Experimental Section). [d] Against established Murphy-Sturm lymphosarcoma. [e] Weight of rats on day 5 taken as 100%. [f] Ratio of volume of treated and control tumors estimated on day 12.

the binding site of folic and dihydrofolic reductase contains only one hydrophobic bonding area. Introduction of two hydrophobic groups into the 2,4-diaminopyrimidine structure may cause one of these groups to interact at a nonhydrophobic binding area with resulting desorption of the molecule from the enzyme.

Compounds V-VII are relatively nontoxic and exert little or no antitumor action even at dose levels approaching the toxic range (Table I). The broad similarity in toxicities and antitumor activities of compounds V-VII does not lend any support to the idea that V and VI may be converted *in vivo* to the pteridine, VIII, which might be regarded as a potentially more cytotoxic agent.

Confirmatory evidence for this view was obtained by *in vitro* studies carried out to determine the ease of reduction of the azo linkage in azopyrimidines and azobenzenes. Rat liver homogenate fortified with an NADPH-generating system as recommended by Mueller and Miller[12] was employed as the reducing medium. As anticipated,[13] substituted azobenzenes (2-N,N-diethylaminoazobenzene, 2-carboxy-2′-N,N-diethylaminoazobenzene, and 2-carbomethoxy-2′-N,N-diethylaminoazobenzene) were reduced relatively rapidly (100-400 mμmoles/min per g of tissue wet weight), but the 5-arylazopyrimidines V-VII, 2,4,6-triamino-5-phenylazopyrimidine, 2,4,6-triamino-5-(4-carbethoxyphenylazo)pyrimidine, and 2,4,6-triamino-5-(4-carboxy-L-glutamylphenylazo)pyrimidine were completely resistant to reduction even after a 2-hr incubation period. Furthermore, it was established that reduction of 2-carboxy-2′-N,N-diethylaminoazobenzene was inhibited by 2,4,6-triamino-5-(4-carboxy-L-glutamylphenylazo)pyrimidine with the latter exhibiting an $[I]/[S]_{50}$ ratio of 1.5. It is thus probable that 5-arylazopyrimidines are inhibitors, rather than substrates, of liver azo reductases.

Experimental Section[14]

N-(2-Hydroxy-3-anilino)propylphthalimide (III).—N-(2,3-epoxypropyl)phthalimide[15] (II, 10 g, 0.05 mole) and aniline (4.6 g, 0.05 mole) in methanol (100 ml) were refluxed for 12 hr. The solution was cooled, and the precipitate was filtered, washed with cold methanol (20 ml), and recrystallized from methanol to give III, mp 151-152°, in >90% yield.

Anal. Calcd for $C_{17}H_{16}N_2O_3$: C, 68.95; H, 5.44; N, 9.46. Found: C, 69.06; H, 5.46; N, 9.51.

N-Phenyl-2-hydroxy-1,3-propanediamine (IV).—III (10 g, 0.035 mole) and HCl (100 ml, *d* 1.2) were refluxed for 6 hr. The mixture was diluted with water (50 ml) and left overnight at 5°. The calculated quantity of phthalic acid was filtered and the filtrate was spin evaporated *in vacuo* to give a residue which was recrystallized from methanol (charcoal) to give the dihydrochloride of IV, mp 177-179° (yield 6.2 g, 79%).

Anal. Calcd for $C_9H_{16}Cl_2N_2O$: C, 45.20; H, 6.74; Cl, 29.63; N, 11.7. Found: C, 45.04; H, 6.70; Cl, 29.75; N, 11.4.

2,4,6-Triamino-5-(4-carbethoxyphenylazo)-6-N-(2-hydroxy-3-anilino)propylpyrimidine (V).—Compound IV (3.32 g, 0.02 mole as free base from IV·2HCl and NaOEt) and 2,4-diamino-5-(4-carbethoxyphenylazo)-6-chloropyrimidine[4] (2.85 g, 0.01 mole) suspended in ethanol (100 ml) were heated at 100° to complete solution. On cooling, V crystallized as orange needles with mp 201-203° (4.0 g, 90%). An analytical sample (from methanol) had mp 202-204°.

Anal. Calcd for $C_{22}H_{26}N_8O_3$: C, 58.25; H, 5.78; N, 24.7. Found: C, 57.95; H, 5.84; N, 24.59.

2,4,6-Triamino-5-(4-carbethoxyphenylazo)-6-N-(2-chloro-3-anilino)propylpyrimidine (VI).—Compound V (2.25 g, 0.005 mole) was finely powdered and suspended in dry ether (50 ml) at 0°. Thionyl chloride (0.9 g, 0.0075 mole) was added with vigorous stirring, and the mixture was maintained at 35° for 1.5 hr. Excess thionyl chloride was destroyed with ethanol (2 ml), and the product was filtered, washed with ether, and recrystallized (ethanol) to give VI as the **hydrochloride** in yellow plates, mp 146-150°, in near quantitative yield. Despite repeated recrystallization, it was impossible to obtain a completely satisfactory elemental analysis for this material.

Anal. Calcd for $C_{22}H_{26}Cl_2N_8O_2$: C, 52.25; H, 5.2; Cl, 14.02; N, 22.17. Calcd for $C_{22}H_{26}Cl_2N_8O_2 \cdot 2H_2O$: C, 48.8; H, 4.83; Cl, 13.1; N, 20.7. Found: C, 48.75; H, 4.50; Cl, 12.34; N, 19.84.

2,4,6-Triamino-5-(4-carbethoxyphenylazo)-6-N-(3-anilino)-propylpyrimidine (VII).—N-Phenyl-1,3-propanediamine[16] (2.92 g, 0.02 mole) and 2,4-diamino-5-(4-carbethoxyphenylazo)-6-chloropyrimidine (2.85 g, 0.01 mole) suspended in ethanol (100 ml) were heated at 100° to complete solution. On cooling, VII crystallized as orange needles, mp 178-181° (3.8 g, 88%). An analytical sample (from methanol) had mp 182-184°.

Anal. Calcd for $C_{22}H_{26}N_8O_2$: C, 60.85; H, 6.04; N, 25.8. Found: C, 60.54; H, 5.91; N, 25.91.

Inhibition of Folic Acid Reductase.[4]—A high-speed, supernatant fraction of rat liver homogenate was used as the source of folic acid reductase.[17] The assay procedure for folic acid reductase activity was similar to that described by Werkheiser.[18]

Reduction of Azo Compounds.[12]—A 10% suspension of rat liver homogenate was prepared in ice-cold, 0.25 M sucrose containing 0.01 M phosphate buffer, pH 7.4. The incubation mixture contained 15.2 μmoles of fructose 1,6-diphosphate, 200 μg of NAD, 200 μg of NADP, 120 μmoles of nicotinamide, 10 μmoles of $MgCl_2$, 200 μmoles of KCl, 50 μmoles of phosphate buffer, pH 7.4, and 0.5 ml of rat liver homogenate in a total volume of 3.0 ml. Azo compound, dissolved in dimethyl sulfoxide or 0.02 M NaOH was added to a final concentration of 75 mμmoles. Blank samples contained all constituents except the azo compound. Duplicate mixtures were incubated at 37° with shaking and,

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(14) Melting points were recorded on a Thomas-Kofler hot stage and are corrected. Analyses are by Galbraith Laboratories, Inc., Knoxville, Tenn., and by Dr. A. E. Bernhardt, Mulheim, Ruhr, Germany.

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after various time intervals, the reaction was stopped by the addition of 3.0 ml of 20% (w/v) aqueous trichloroacetic acid. Azo compound remaining was extracted with *n*-butyl alcohol and estimated spectrophotometrically.

Biological Test Methods.—Toxicity determinations were performed using male Swiss mice (22-26 g). The compound, dissolved in saline or suspended in 10% gum acacia, was administered by intraperitoneal injection to groups of 3-6 mice/dose level. Deaths within a 21-day period were recorded and approximate LD_{50} values were estimated graphically from per cent mortality/log dose plots.

Antitumor activities of the compounds against the Murphy-Sturm lymphosarcoma were assessed as follows. The tumor was implanted subcutaneously into male Holtzman rats using a trochar and cannula. Five days later when the tumor had reached a size of about 5 g, the compound was injected intraperitoneally daily for 5 days. Control animals received the vehicle only. On day 12, the volumes of the tumors were calculated from measurements taken by a caliper,[8] and the mean tumor volume of treated rats was compared with the mean tumor volume of control rats (T/C in Table I). Rats were subsequently observed to determine whether complete regression of the tumors occurred.

Synthesis of Fluorinated Pyrimidines and Triazines[1,2]

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The synthesis of fluorinated pyrimidines and triazines as potential thymidylate synthetase inhibitors was accomplished by treating the appropriate carbonyl compound with sulfur tetrafluoride in the presence of hydrofluoric acid. The anomers of 5-trifluoromethyl-6-aza-2'-deoxyuridine were prepared by conventional procedures from 5-trifluoromethyl-6-azauracil. Some preliminary biological results are presented.

One possible approach to the selective control of DNA synthesis and mitosis is by inhibition of the synthesis of thymidine 5'-phosphate (TMP). Thymidylate synthetase[3] catalyzes the conversion of 2'-deoxyuridine 5'-monophosphate (dUMP) to TMP in the presence of the carbon donor, N^5,N^{10}-methylenetetrahydrofolic acid (CH_2THFA). The reaction has been studied in several laboratories[3] and the requirements suggest a sequential reaction of the enzyme with the cofactor (CH_2THFA) followed by reaction of this complex with the substrate dUMP.

Since 5-fluoro-2'-deoxyuridine 5'-monophosphate[3e] (FdURP) is known to be a strong inhibitor of the enzyme, substitution of fluorine for hydrogen in the methyl group of thymine might also confer inhibitory properties. The increase of electronegativity associated with the trifluoromethyl group might be a desirable feature, since Baker in 1960,[4] had postulated that an increased acidity of the N-3 hydrogen could improve the properties of the analogs and allow them to be more strongly bound to the enzyme receptor site.

Thus, 5-trifluoromethyluracil (**2**), 5-difluoromethyluracil (**4a**), and 5-trifluoromethyl-6-azauracil (**6**) and its α- and β-2'-deoxyribosides (**7a** and **b**) were synthesized in an effort to study the requirements of an effective antimetabolite of the thymidylate synthetase sequence of reactions. During the course of this work Heidelberger and co-workers[5] reported the synthesis of 5-trifluoromethyluracil (**2**) and 5-trifluoromethyl-2'-deoxyuridine by another route. The synthesis of 5-trifluoromethyl-6-azauracil (**6**) and the 2'-deoxyriboside anomers (**7**) was communicated[2c,d] jointly with Shen and co-workers.[6]

The initial approaches to the synthesis of **2** *via* primary ring synthesis utilizing ethyl 3,3,3-trifluoropropionate[7] in analogy to Whitehead's[8] synthesis of 5-carbethoxyuracil were unsuccessful. The second approach was based on the aromatic character of C_5 in uracil.[9] Since the trifluoromethyl radical has been reported to add readily to various aromatic systems[10] this procedure was applied to uracil. Photochemical attempts were unsuccessful; thermal decomposition of trifluoromethyl iodide in the presence of mercuric chloride[11] gave uracil-5-carboxylic acid (**1**) indicating that reaction occurred followed by hydrolysis.

Since sulfur tetrafluoride was introduced as a reagent for the conversion of the carboxyl group to the trifluoromethyl group, reports have appeared describing the selective nature of this reagent.[12] Raasch noted the protective effect and Martin, *et al.*,[13] the enhance-

(1) This work was generously supported by grant CA-5639 from the National Cancer Institute, National Institutes of Health, U. S. Public Health Service.

(2) Preliminary communications of portions of this work have appeared: (a) M. P. Mertes and S. E. Saheb, *J. Pharm. Sci.*, **52**, 508 (1963); (b) *J. Med. Chem.*, **6**, 619 (1963); (c) *J. Heterocyclic Chem.*, **2**, 491 (1965); (d) M. P. Mertes, S. E. Saheb, and D. Miller, *ibid.*, 493 (1965). Portions of this work were presented at the Symposium on Newer Concepts of Structure Activity Relationships, 112th Meeting of the American Pharmaceutical Association, Detroit, Mich., March 1965, Abstract A-III. While this paper was in press a similar publication appeared: A. Dipple and C. Heidelberger, *J. Med. Chem.*, **9**, 715 (1966).

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