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Inhibition of Murine Thymidylate Synthase and Human Dihydrofolate Reductase by 5,8-Dideaza Analogues of Folic Acid and Aminopterin[1]

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A series of 5,8-dideaza analogues of folic acid, isofolic acid, aminopterin, and isoaminopterin were evaluated for inhibition of thymidylate synthase, TS, from mouse L1210 leukemia cells with 10-propargyl-5,8-dideazafolic acid, CB3717, **4a**, as the reference inhibitor. These compounds were also tested as inhibitors of human dihydrofolate reductase, DHFR, obtained from WIL2 cells. None of the analogues studied were as potent as **4a** toward TS; however, 9-methyl-5,8-dideazaisoaminopterin, **6d**, was only 2.5-fold less effective. Compound **4a** was prepared by direct alkylation of the di-*tert*-butyl ester of 5,8-dideazafolic acid followed by hydrolysis of the resulting diethyl ester, which resulted from concomitant transesterification. It was found to be identical with a sample of **4a** prepared by earlier methodology by using a variety of spectroscopic techniques. Its isomer, 9-propargyl-5,8-dideazaisofolic acid, **4b**, which was synthesized by an analogous approach, was found to be dramatically less inhibitory toward TS than **4a**. Each of the 2,4-diamino derivatives, including those possessing an allyl or propargyl group at N^9, was an excellent inhibitor of DHFR, having a level of potency similar to that of methotrexate, MTX. However, many of these 5,8-dideazaaminopterin analogues were far more inhibitory toward TS than MTX.

From a historical perspective, interest in 5,8-dideaza analogues of folic acid as potential antitumor agents has waxed and waned during the past two decades. For example, 10-methyl-5,8-dideazafolic acid, **5b**, was reported to be a potent inhibitor of thymidylate synthase, TS, from C 1300 mouse neuroblastoma cells.[2] It also inhibited the growth of C 1300 cells in vitro, although not as effectively as related compounds having a 2,4-diamino configuration.[2] Protection studies using thymidine and leucovorin indicated that the cytotoxic action of **5b** was attributable at least in part to the inhibition of TS.[2] It should also be noted that compounds such as **5b** have been shown to be surprisingly effective inhibitors of dihydrofolate reductase, DHFR, from rat liver.[3] However, for an inhibitor of TS to be useful chemotherapeutically, it should not have too high an affinity for DHFR, since DHFR levels are normally much greater than those of TS in mammalian cells. Subsequently, a large series of 5,8-dideazafolates prepared in this laboratory were evaluated for inhibitory effects against TS from *Lactobacillus casei* and from L1210 leukemia cells.[4] Large differences in inhibitory potency between the two enzymes were observed for several analogues, with some compounds displaying selectivity for murine TS.[4] Such observations emphasize that bacterial TS is not suitable for evaluating folate analogues targeted for human neoplastic disease.

Interest in 5,8-dideazafolates was rekindled by studies conducted in the United Kingdom, where synthetic efforts were focused upon the introduction of new substituents located at position 10.[5] The best inhibitor of L1210 leukemia TS was found to be 10-propargyl-5,8-dideazafolic acid, **4a**, which became referred to as CB3717.[5] On the basis of its high degree of efficacy against L1210 leukemia in mice, CB3717 was selected for phase I and II clinical trials.[6] Unfortunately, renal toxicity and hepatotoxicity have caused a cessation of these studies.[7]

Concurrent to the development of CB3717, 5,8-dideazaisofolic acid, **1a**, IAHQ, was identified as a compound having potential for colorectal adenocarcinoma in studies using the human cell line HCT-8 in vitro.[8] Ironically, IAHQ was not a particularly effective inhibitor of mammalian TS, although its cytotoxicity was almost completely reversed by thymidine.[4,8] It was proposed that the mechanism of action of IAHQ involves its intracellular conversion to poly-γ-L-glutamyl metabolites, which are significantly more potent as inhibitors of TS, particularly in the presence of high concentrations of 2′-deoxyuridine monophosphate, dUMP, which accumulate due to the inhibition of TS.[8] Although numerous analogues of IAHQ were prepared, such as **1b–f**, none has demonstrated superior cytotoxicity toward human gastrointestinal tumor cells in vitro.[9]

It was of interest, therefore, to prepare the bridge-reversed isomer of CB3717, 9-propargyl-5,8-dideazaisofolic acid, **4b**, as well as related 9-substituted analogues. Each of the new compounds synthesized as well as members of a large series of related analogues was evaluated for inhibition of L1210 leukemia TS as well as human tumor DHFR in an effort to develop new structure–activity patterns to facilitate the design of more selective inhibitors of TS.

Chemistry. Of the potential synthetic routes to the bridge-reversed isomer **4b** of CB3717, direct alkylation of the di-*tert*-butyl ester **2b** of IAHQ[9] appeared to be the most straightforward approach. Recent studies concerning the alkylation of the diethyl esters of 8-deazafolate and 8-deazaaminopterin showed that selective alkylation at N^{10} could be achieved by using ethanol rather than a polar aprotic solvent.[10] As shown in Scheme I, the desired

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Scheme I

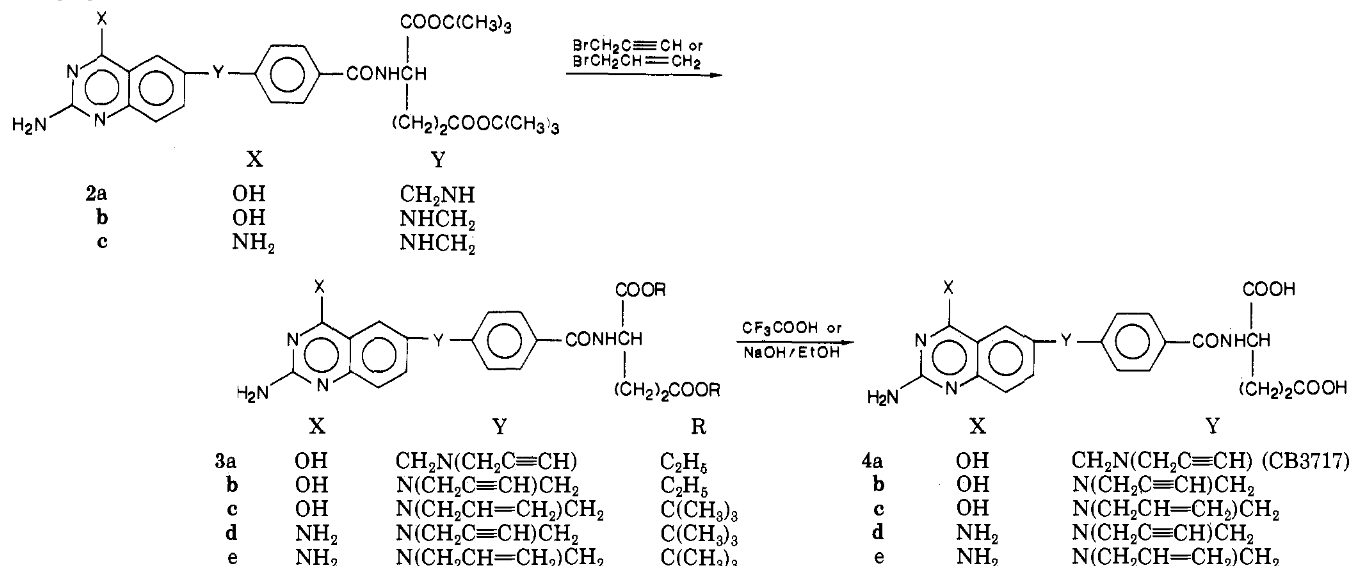

Table I. Properties of N-Alkylated Compounds Synthesized

no.	X	Y	R	meth	reactn time, h	yield, %	mp, °C	formula	anal.
3a	OH	$CH_2N(CH_2C{\equiv}CH)$	C_2H_5	A	24	41	134–140[a]	$C_{28}H_{31}N_5O_6{\cdot}0.1H_2O$	C,H,N
3b	OH	$N(CH_2C{\equiv}CH)CH_2$	C_2H_5	A	34	41	155–165	$C_{28}H_{31}N_5O_6{\cdot}1.2H_2O$[b]	C,H,N
3c	OH	$N(CH_2CH{=}CH_2)CH_2$	$C(CH_3)_3$	B	24	26	126–139	$C_{32}H_{41}N_5O_6{\cdot}1.0H_2O$	C,H,N
3d	NH_2	$N(CH_2C{\equiv}CH)CH_2$	$C(CH_3)_3$	B	19	55	120–130	$C_{32}H_{40}N_6O_5{\cdot}1.5H_2O$[c]	C,H,N
3e	NH_2	$N(CH_2CH{=}CH_2)CH_2$	$C(CH_3)_3$	B	24	55	180–200 dec	$C_{32}H_{42}N_6O_5{\cdot}1.25H_2O$[d]	C,H,N
4a	OH	$CH_2N(CH_2C{\equiv}CH)$	H	C	1.5	87	220–221[e]	$C_{24}H_{23}N_5O_6{\cdot}0.8H_2O$	C,H,N
4b	OH	$N(CH_2C{\equiv}CH)CH_2$	H	C	1.5	67	235 dec	$C_{24}H_{23}N_5O_6{\cdot}0.7H_2O$[f]	C,H,N
4c	OH	$N(CH_2CH{=}CH_2)CH_2$	H	D	1.5	48	171–182 dec	$C_{24}H_{25}N_5O_6{\cdot}0.5H_2O$	C,H,N
4d	NH_2	$N(CH_2C{\equiv}CH)CH_2$	H	D	12	58	219–221 dec	$C_{24}H_{24}N_6O_5{\cdot}1.7H_2O$[g]	C,H,N
4e	NH_2	$N(CH_2CH{=}CH_2)CH_2$	H	D	1.25	58	222–225 dec	$C_{24}H_{26}N_6O_5{\cdot}0.75H_2O$	C,H,N

[a] Jones et al.[5] reported mp 143–147 °C. [b] Anal. C, H; N: calcd, 12.62; found, 11.98. [c] Anal. C, H; N: calcd, 13.65; found, 13.14. [d] Anal. C, H; N: calcd, 13.70; found, 13.18. [e] Jones et al.[5] reported mp 232–235 °C. [f] Also contained 0.1 Me_2CO, which was confirmed by NMR. [g] Anal. C, H; N: calcd, 16.57; found 16.14.

alkylation product **3b** was obtained by the reaction of **2b** with propargyl bromide in refluxing ethanol, although transesterification occurred, catalyzed by hydrogen bromide liberated during the alkylation step. Careful saponification of **3b** yielded 9-propargyl-5,8-dideazaisofolic acid, **4b**, in respectable yield. The FAB/MS spectrum of **4b** showed the expected MH^+ ion at m/e 478, together with peaks of m/e 331 (amide bond cleavage) and m/e 213 (due to scission of the N^9–C^{10} bond). This spectrum did not establish unequivocally the location of the propargyl group. However, the 400-MHz ^1H NMR spectrum showed a peak at 6.16 ppm integrating to two protons, which is characteristic of the 2-NH_2 group in compounds possessing the 2-NH_2, 4-OH configuration. In addition, the signal due to the N^9 proton had disappeared in proceeding from **2b** to **4b**. The utility of this direct alkylation procedure was further demonstrated by the preparation of CB3717 from di-*tert*-butyl 5,8-dideazafolate, **2a**,[11] which is also depicted in Scheme I. The resulting **4a** was shown to be identical with a sample of the compound prepared by the original synthetic methodology[5] by 400-MHz NMR as well as ultraviolet and FT-IR spectroscopy.

The reaction of the di-*tert*-butyl ester **2b** with allyl bromide at ambient temperature in dimethylacetamide gave the 9-allyl derivative **3c**, which was deprotected in trifluoroacetic acid to yield 9-allyl-5,8-dideazaisofolic acid, **4c**, as shown in Scheme I. In this instance, deesterification did not occur due to the ability of dimethylacetamide to act as a hydrogen bromide scavenger. The reaction of 5,8-dideazaisoaminopterin di-*tert*-butyl ester, **2c**,[11] with either propargyl bromide or allyl bromide in dimethylacetamide gave the 9-propargyl and 9-allyl esters, **3d** and **3e** respectively, which were subsequently converted to the free acids **4d** and **4e** by using trifluoroacetic acid. The FAB mass spectrum for 9-propargyl-5,8-dideazaisoaminopterin, **4d**, showed MH^+ at m/e 477 as well as peaks at m/e 264 and 212 due to fragmentation of the N^9–C^{10} bond. The high-resolution NMR spectra of the di-*tert*-butyl esters **3d** and **3e** demonstrated that alkylation had occurred exclusively at N^9 due to the disappearance of the signal due to the N^9 proton and the presence of broad peaks between 6.10 and 6.50 ppm (2-NH_2) and at 7.40–7.80 ppm (4-NH_2) integrating to approximately two protons each (data available as supplementary material). The physical properties of these propargyl and allyl derivatives as well as the reaction conditions employed for preparing them are summarized in Table I. Complete ultraviolet spectral

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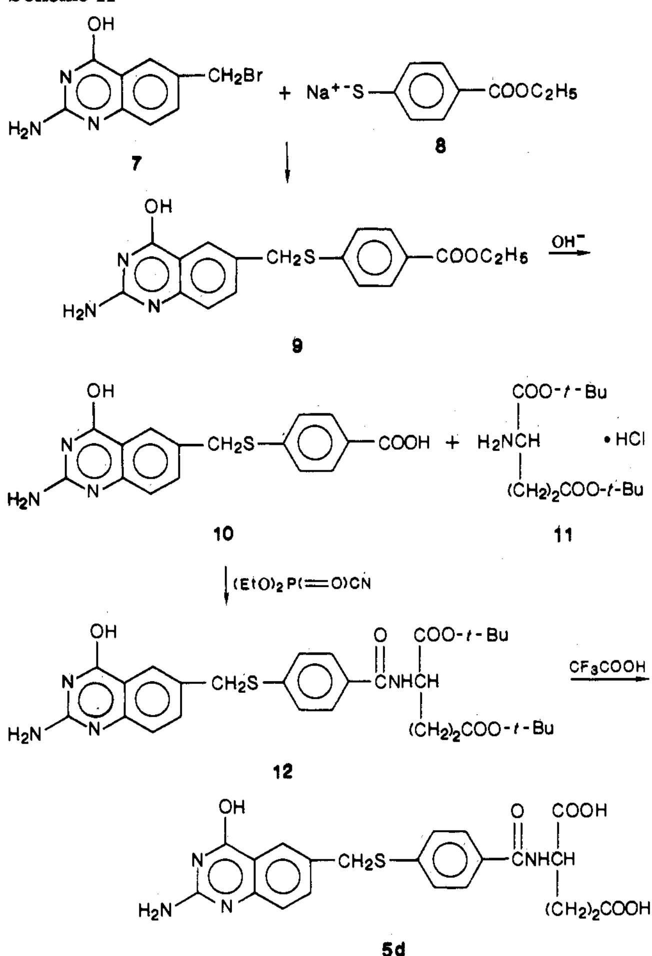
Table II. Ultraviolet Spectral Data of Target Compounds[a]

no.	max, nm	ε	min, nm	ε
4a	302	26 277	284	23 170
	278	23 441	251.5	10 709
	226	51 218		
4b	348	3884	316	3045
	278	17 855	263	15 658
	233	45 419		
4c	370	3283	319	1896
	284	17 956	266	13 404
	235	41 617		
4d	368	3562	317	1654
	282	18 067	266	16 032
	238	45 550		
4e	388	3492	326	1579
	287	18 481	269	14 754
	239	43 025		
5d	279	26 809	254	13 404
	228	49 362		

[a] Spectra determined in 0.1 N NaOH.

Scheme II



data for the compounds prepared for this study are presented in Table II.

The folate analogue 10-thia-5,8-dideazafolic acid, **5d**, was first elaborated in low yield by the reaction of 2-amino-6-(bromomethyl)-4-hydroxyquinazoline, **7**,[12] with the sodium salt of diethyl *N*-(4-mercaptobenzoyl)-L-glutamate, followed by saponification of the resulting product in dilute sodium hydroxide.[13] Since supplies of **5d** had been exhausted, a new stepwise procedure for its preparation was developed as depicted in Scheme II. Compound **7** reacted with the sodium salt of ethyl 4-mercaptobenzoate to yield the ethyl ester **9**, which upon saponification gave 10-thia-5,8-dideazapteroic acid, **10**, in good yield. Coupling of this key intermediate to di-*tert*-butyl glutamate,[11] by using diethyl phosphorocyanidate afforded the di-*tert*-butyl ester **12**. Upon treatment with trifluoroacetic acid, **12** was converted into **5d** in respectable overall yield. This synthetic approach to **5d** precludes possible racemization of the L-glutamyl moiety and avoids decomposition due to prolonged exposure to basic conditions, which has been observed with compounds of this type.

Table III. Inhibition of Thymidylate Synthase (L1210) and Dihydrofolate Reductase (WIL2) by 5,8-Dideaza Analogues of Folic Acid

no.	X	Y	TS: I_{50},[a,b] μM	DHFR: I_{50},[b] μM
1a	H	$NHCH_2$	1.3	0.11
1b	CH_3	$NHCH_2$	2.2	0.46
1c	Cl	$NHCH_2$	4.0	0.10
1d	H	$N(CH_3)CH_2$	0.2	0.30
1e	CH_3	$N(CH_3)CH_2$	0.7	3.2
1f	H	$N(CHO)CH_2$	11.0	0.28
4b	H	$N(CH_2C{\equiv}CH)CH_2$	8.6	3.0
4c	H	$N(CH_2CH{=}CH_2)CH_2$	6.1	1.4
5a	H	CH_2NH	3.4	0.034
5b	H	$CH_2N(CH_3)$	0.13	0.083
5c	H	$CH_2N(CHO)$	9.8	0.011
5d	H	CH_2S	0.22	0.33
5e	H	CH_2O	12.0	0.17
4a (CB3717)	H	$CH_2N(CH_2C{\equiv}CH)$	0.014	0.91

[a] Average of three determinations. [b] Limits of variability <±15%.

Table IV. Inhibition of Thymidylate Synthase (L1210) and Dihydrofolate Reductase (WIL2) by 5,8-Dideaza Analogues of Aminopterin

no.	X	Y	TS: I_{50},[a,b] μM	DHFR: I_{50},[b] μM
6a	H	$NHCH_2$	0.55	0.0086
6b	CH_3	$NHCH_2$	0.15	0.0041
6c	Cl	$NHCH_2$	0.098	ND
6d	H	$N(CH_3)CH_2$	0.033	0.0046
6e	H	$N(CHO)CH_2$	>10.0	0.0057
4d	H	$N(CH_2C{\equiv}CH)CH_2$	4.4	0.0053
4e	H	$N(CH_2CH{=}CH_2)CH_2$	3.2	0.0054
6f	H	CH_2NH	5.0	0.0045
6g	H	$CH_2N(CH_3)$	0.6	0.0039
6h	Cl	CH_2NH	0.19	0.0038
6i	H	$CH_2N(CHO)$	>10.0	0.0058
MTX			20.0	0.0038

[a,b] See footnotes, Table III.

Biological Evaluation. Each of the target compounds together with a wide variety of structurally related compounds was evaluated as an inhibitor of L1210 leukemia TS[14] and human WIL2 DHFR.[15] For compounds having the 2-NH_2, 4-OH configuration, the results obtained are

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presented in Table III, while data obtained for analogues of aminopterin and methotrexate, MTX, are summarized in Table IV. Values obtained for MTX and **4a** are included for reference purposes.

Discussion

In order to obtain meaningful structure–activity patterns, we evaluated a wide variety of classical 5,8-dideazafolate analogues as inhibitors of TS obtained from an L1210 leukemia cell line resistant to **4a** by virtue of overproduction of TS.[14] The enzyme concentration was carefully monitored by titration with 5-fluoro-2′-deoxyuridine monophosphate, FdUMP, in order that each inhibitor was evaluated under identical conditions. The results presented in Table III for compounds having the 2-NH_2, 4-OH configuration show that the normal C^9–N^{10}-bridged analogue **5a** is a modest inhibitor and that methylation at N^{10} (**5b**) enhances potency by greater than 20-fold. The presence of a propargyl group at N^{10} (**4a**) increases inhibition by an additional order of magnitude, making **4a** the most potent of the compounds studied. Similar results were reported earlier, using L1210 TS, with **4a** having an I_{50} of 0.02 μM.[16] The current data are also in accord with the I_{50} values for **5a**, **5b**, and **4a**, using TS obtained from human leukemia K562 cells.[10] In addition, a K_i of 0.02 μM was obtained for **4a** against TS from human HeLa S3 cells.[17] These results suggest that the L1210 TS is an adequate model for human TS at least as far as folate analogues are concerned. The N^{10}–CHO modification **5c** is 3-fold less inhibitory than the unsubstituted analogue **5a**. The I_{50} value for **5c** is much higher than that reported earlier against L1210 TS.[4] A possible explanation for this discrepancy is that the sample evaluated in the earlier study had undergone hydrolysis to **5a** and that a lower enzyme concentration was employed in the assay. However, compound **5c** continues to be a valuable ligand for the affinity chromatographic purification of TS.[18] This demonstrates that potential ligands for affinity chromatography need not be potent inhibitors of the target enzyme. The replacement of the N^{10} by sulfur (**5d**) results in a 15-fold decrease in I_{50}. The analogous replacement by oxygen (**5e**), however, results in a compound with reduced inhibitory potency as compared to **5a**.

For compounds having an isofolate structure, the unsubstituted analogue **1a** is a somewhat better inhibitor than its isomer **5a**. The inclusion of CH_3 or Cl at position 5 (**1b** and **1c**) causes a slight reduction in affinity for TS. The 9-CH_3 analogue **1d** is a significantly superior inhibitor than **1a**, but the presence of a second CH_3 at position 5 reduces potency by about 3-fold. The presence of a formyl group at N^9 (**1f**) causes a substantial reduction in inhibition, as is the case for its isomer **5c**. Finally, the 9-propargyl (**4b**) and 9-allyl (**4c**) derivatives are less inhibitory than the parent compound **1a**. In fact, **4b** is ca. 600-fold less inhibitory than its isomer having a normal folate configuration, **4a**.

The results obtained against TS with compounds having a 2,4-$(NH_2)_2$ configuration are summarized in Table IV. As has been reported from numerous other studies, MTX is a poor inhibitor of this enzyme. Removal of the two pyrazine ring nitrogens as in 5,8-dideazaaminopterin, **6f**, results in a 4-fold augmentation in inhibitory potency. The N^{10}–CH_3 modification, **6g**, is 8-fold and the 5-Cl modification, **6h**, 26-fold more potent than **6f**. The inclusion of a formyl group at N^{10} (**6i**) again results in a poor inhibitor of TS. Turning to compounds that may be considered as analogues of isoaminopterin, it will be seen that 5,8-dideazaisoaminopterin, **6a**, is nearly 10-fold more inhibitory than its normal-bridged isomer **6f**. The presence of a CH_3 or Cl group at position 5 (**6b** and **6c**) is modestly beneficial for inhibitory potency. However, the presence of a CH_3 group at N^9 (**6d**) results in a compound that is ca. 17-fold more inhibitory than the parent compound **6a** and that is only 2.5-fold less inhibitory than CB3717 (**4a**). In this series of compounds, formylation is also highly deleterious to inhibitory activity (**6e**). It was disappointing, therefore, to ascertain that the 9-propargyl (**4d**) and 9-allyl (**4e**) derivatives in this series were approximately 100-fold poorer inhibitors than **6d**.

It may be concluded that the region on TS that is juxtaposed to the 9–10 region of 5,8-dideaza analogues of the 4-OH or 4-NH_2 types is highly hydrophobic. There is also considerably greater bulk tolerance near N^{10} as opposed to N^9. However, since nonpolar substituents located at position 5 are favorable to binding for 2,4-$(NH_2)_2$ derivatives, but slightly unfavorable in the case of analogues of **1a**, suggests that 2-NH_2, 4-OH analogues bind to TS in a somewhat different conformation than analogues 2,4-$(NH_2)_2$ analogues.

Turning next to the inhibitory effects against human DHFR, it will be seen in Table IV that each of the 2,4-$(NH_2)_2$ derivatives is a potent inhibitor, with some compounds being indistinguishable from MTX. The higher I_{50} value obtained for 5,8-dideazaisoaminopterin, **6a**, is significant, however, since similar results were also obtained by using rat liver DHFR.[19] The potent inhibitory effects of the 9-propargyl (**4d**) and 9-allyl (**4e**) modifications help to support the contention that alkylation did occur at N^9 during their syntheses, since substitution at either N^2 or N^4 would be expected to result in dramatically poorer inhibitors of DHFR. It will be seen from results presented in Table III that folate analogues having a normal bridge are good inhibitors of DHFR, with the N^{10}–CHO modification (**5c**) having an I_{50} value of 0.011 μM and 5,8-dideazafolic acid (**5a**) being only 3-fold less inhibitory. Similar results were reported for these compounds when the enzyme isolated from rat liver was used.[3] Substitution of the N^{10} by sulfur or oxygen (**5d** and **5e**) leads to reduced affinity for DHFR, emphasizing the importance of N^{10} for effective inhibition of DHFR for 5,8-dideazafolates. The presence of a propargyl at N^{10} is highly detrimental to binding, with **4a** being 26-fold less inhibitory than **5a**. Almost identical results were reported earlier for the relative inhibitory activities of **4a** and **5a** against DHFR from L1210 leukemia cells.[16]

For the 5,8-dideaza analogues of isofolic acid, the parent compound **1a** is 3-fold less inhibitory than its isomer **5a**. The 5-Cl modification **1c** is equipotent, while the 5-CH_3 (**1b**) and 9-CH_3 (**1d**) modifications are from 3- to 4-fold less inhibitory than **1a**. The 5,9-$(CH_3)_2$ (**1e**), 9-propargyl (**4b**), and 9-allyl (**4c**) derivatives are significantly less inhibitory than **1a**, indicative of steric intolerance between DHFR and inhibitors of this type in the C^5–N^9 region of the molecule.

It is believed that the results presented herein indicate that new 5,8-dideaza analogues of folic acid can be pre-

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pared that have greater selectivity for TS and that may also have properties that will render them superior to those compounds current under evaluation as antitumor agents. Efforts along these lines are currently in progress and will be the subject of forthcoming papers.

Experimental Section

Melting points were determined on a Mel-Temp apparatus and are uncorrected. Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN. All analytical samples were dried under vacuum at 100 °C unless stated otherwise and gave combustion values of C, H, and N within ±0.4% of the theoretical values. Solvation due to water was confirmed by the presence of a broad peak centered at approximately 3.4 ppm in the ^{1}H NMR spectrum, which was transformed into a sharp singlet (DOH) by the addition of D_2O. All intermediates were free of significant impurities on TLC using silica gel media (Baker 1B2-F). Free acids were assayed on Eastman 13254 cellulose (0.1 M phosphate buffer, pH 7.5). Column chromatographic separations were performed on Kieselgel 60 (70–230 mesh) obtained from E. Merck and Co. Compounds 12 and **5d** were analyzed by using a Waters Associates HPLC system consisting of a solvent programmer model 441 and a UV (254 nm) detector. A reverse-phase Bondapak C-18 (3.9 mm × 30 cm) column and two solvent systems A and B were used: A, 0.1% CF_3COOH in H_2O; B, 0.1% CF_3COOH in 1-propanol.[20] The following gradient conditions were employed: 0–30% B, 40 min, flow rate 1 mL/min. Samples for HPLC were dissolved in Me_2SO just prior to injection. The UV spectra were determined by using a Cary 219 spectrophotometer. The ^{1}H NMR spectra were determined by using a Varian EM 390 spectrometer operating at 90 MHz. High-resolution ^{1}H NMR spectra were acquired on a Bruker AM-300 or Bruker WH-400 spectrometer at the Magnetic Resonance Laboratory, University of South Carolina, Columbia, SC. NMR values for chemical shifts are presented in parts per million downfield from Me_4Si as the internal standard. The relative peak areas are given to the nearest whole number. Structurally consistent FAB mass spectra for compounds **4b** and **4d** were obtained on a Finnigan MAT 212 spectrometer using argon bombardment. Infrared spectra were run on a Mattson Polaris FT-IR spectrometer.

Homogeneous DHFR was obtained from human WIL2 cells as described earlier.[15] It was assayed spectrophotometrically at 340 nm by using 9 μM dihydrofolate, 30 μM NADPH, 0.15 M KCl in 0.05 M Tris buffer (pH 7.4); [DHFR] = 0.0076 μM by MTX titration. MTX was a gift from Dr. Suresh Kerwar, Lederle Laboratories, Pearl River, NY. TS was purified from an L1210 cell line resistant to **4a** by virtue of overproducing this enzyme by ca. 50-fold.[14] This cell line as well as an authentic sample of **4a** were generous gifts from Drs. A. L. Jackman and H. A. Calvert, Institute of Cancer Research, Royal Cancer Hospital, Sutton, Surry, U.K. TS was purified to homogeneity by affinity chromatography using **5c** immobilized on aminoethyl-Sepharose as described previously.[18] The enzyme activity was estimated by a modification of the ^{3}H release method of Roberts.[21] The standard reaction mixture in a total volume of 80 μL contained 3.6 nmol of [5-^{3}H]dUMP (ca. 3 × 10^7 cpm/μmol), 25 nmol of CH_2FAH_4, 1.6 μmol of 2-mercaptoethanol, 2.9 μmol of NaF, 2.9 μmol of sodium phosphate buffer, pH 7.5, 0.085% Triton X-100, and 0.24 pmol of TS. Enzyme concentration was determined by titration with FdUMP. Enzyme was preincubated with [5-^{3}H]-dUMP plus or minus inhibitor for 10 min at 37 °C followed by the addition of *dl*-L-5,10-methylenetetrahydrofolic acid, CH_2FAH_4, to start the reaction. Blank counts per minute were determined in the absence of CH_2FAH_4. The reaction was terminated after 1 h of incubation at 37 °C by addition of 200 μL of a suspension of charcoal (Norit, 100 mg/mL) in 2% CCl_3COOH. The mixture was centrifuged at 16000*g* for 5 min. A 100-μL sample of the supernatant was added to 10 mL of Ecolume (ICN) and counted in a Beckman LS-100C liquid scintillation counter. All assays were performed in duplicate. Activity of the enzyme is expressed as a percent of control with no inhibitor present. Synthetic methods for the preparation of compounds other than those described below have been reported elsewhere: **1a**, **1b**, **1d**, **1e**, **1f**;[9] **1c**, **6c**, **6h**;[22] **5a**, **5b**;[12] **5c**;[3] **5e**;[13] **6a**, **6d**, **6e**, **6f**, **6g**, **6i**;[19] **6b**.[23]

Method A. N-Alkylated Diethyl Esters 3a,b. A mixture of the appropriate di-*tert*-butyl ester (0.885 mmol) and excess propargyl bromide (6 mL of an 80% solution in toluene) in absolute EtOH (35 mL) was heated at reflux for the time specified in Table I. The solvent was removed under vacuum and the residue partitioned between $CHCl_3$ and aqueous $NaHCO_3$ (5% w/v). The organic layer was washed twice with H_2O and dried ($MgSO_4$). The solvent was removed at reduced pressure and the residue purified on a silica gel column by elution of the product with $CHCl_3$–MeOH, 95:5 (**3a**), or $CHCl_3$–MeOH, 90:10 (**3b**). The compounds were dried under vacuum at 65 °C for 11 h.

Method B. N-Alkylated Di-*tert*-butyl Esters 3c–e. To a solution of the appropriate di-*tert*-butyl ester (2.68 mmol) in Me_2NAc (23 mL) was added propargyl bromide (19 mL of an 80% solution in toluene). The solution was stirred at ambient temperature for the time specified in Table I. The solvent was removed at reduced pressure and the residue dissolved in $CHCl_3$. This solution was washed with $NaHCO_3$ (5% w/v) and H_2O. After drying over $MgSO_4$, the solvent was removed under vacuum and the residue purified on a silica gel column ($CHCl_3$–MeOH, 90:10). Compounds **3c,d** were dried under vacuum at 65 °C for 24 h, while compound **3e** was dried similarly at 100 °C.

Method C. Compounds 4a,b. To a solution of the appropriate diethyl ester, **3a** or **3b** (0.19 mmol), in EtOH (2.2 mL) was added 1 N NaOH (1 mL). The solution was stirred at ambient temperature for 1.5 h. TLC indicated the reaction to be complete. The solution was brought to pH 3.5 with 0.5 N HCl. The precipitate was isolated by centrifugation, washed with H_2O, and dried. Compound **4b** was also washed once with Me_2CO. The products were dried under vacuum at 100 °C for 9 h.

Method D. Compounds 4c–e. The appropriate di-*tert*-butyl ester (0.163 mmol) was dissolved in CF_3COOH (1.5 mL) and the solution stirred at room temperature for the time specified in Table I. Completion of the reaction was determined by TLC. The reaction mixture was poured into cold Et_2O (25 mL). The precipitated solid was isolated by centrifugation, washed with Et_2O, and dried. Next, it was resuspended in H_2O (2.5 mL) and basified to pH 9 with concentrated NH_4OH. The solution was filtered, if necessary, and then acidified to pH 3.5 with 0.5 N HCl. The precipitated solid was collected by centrifugation, washed with H_2O, and dried. Compounds **4d,e** were also washed with Me_2CO. The products were dried under vacuum at 100 °C for 12 h.

Ethyl 10-Thia-5,8-dideazapteroate (9). A solution of 2.7 g (7.5 mmol) of diethyl 4,4′-dithiobisbenzoate[24,25] in 100 mL of EtOH was reduced with 0.95 g (10 mmol) of $NaBH_4$ at ambient temperature. This was added portionwise to a suspension of 3.8 g (15 mmol) of 2-amino-6-(bromomethyl)-4-hydroxyquinazoline, **7**,[12] in 20 mL of DMF, and the resulting mixture was stirred at ambient temperature for 18 h. A negative active halogen test[26] was obtained, indicating that **7** had been consumed, and the EtOH was removed under reduced pressure. The addition of 550 mL of H_2O gave a precipitate, which was separated by filtration and washed with H_2O. After recrystallization from DMF–H_2O, there was obtained 4.74 g (89%) of white crystalline solid: mp 268 °C dec; TLC, R_f 0.33 ($CHCl_3$–MeOH, 7:3); NMR (300 MHz, Me_2SO-d_6) δ 1.63 (t, 3, CH_3, J = 7.15 Hz), 4.50 (s, 2, CH_2S), 4.65 (q, 2, CH_2, J = 7.15 Hz), 7.53 (d, 2, 3′, 5′, J_o = 8.48 Hz), 7.63 (d, 1, H_8, $J_{7,8}$ = 8.51 Hz), 8.26 (d, 2, 2′, 6′, J_o = 8.48 Hz + d, 1, H_7, $J_{7,8}$ = 8.51 Hz), 8.42 (d, 1, H_5, $J_{5,7}$ = 1.80 Hz). Anal. ($C_{18}H_{17}N_3O_3S$) C, H, N.

10-Thia-5,8-dideazapteroic Acid (10). A suspension of compound **9** (1.82 g, 5 mmol) in 120 mL of 0.2 N NaOH was stirred at ambient temperature for 45 h. Traces of insoluble material were removed by filtration, and the pH of the filtrate was adjusted

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to 6 with concentrated HCl. The product was isolated by centrifugation and washed with 3 × 40 mL of H_2O. After drying, there was obtained 1.2 g (73%) of white crystalline powder: mp 339–341 °C dec; TLC, R_f 0.58 (Whatman KC18F-reverse phase, EtOH–MeCN, 8:2); NMR (300 MHz, CF_3COOD) δ 4.56 (br s, 2, CH_2S) 7.59 (br s, 2, 3′, 5′), 7.64 (br s, 1, H_8), 8.21 (br s, 2, 2′, 6′ + br s, 1, H_7), 8.48 (br s, 1, H_5). Anal. ($C_{16}H_{13}N_3O_3S{\cdot}0.5H_2O$) C, H, N.

Di-*tert*-butyl 10-Thia-5,8-dideazafolate (12). To a suspension of **10** (0.2 g, 0.6 mmol) in 10 mL of DMF were added di-*tert*-butyl L-glutamate hydrochloride, **11** (0.195 g, 0.66 mmol), and diethyl phosphorocyanidate (0.108 g, 0.66 mmol) in 1.0 mL of DMF. The suspension was treated with 0.134 g (1.32 mmol) of Et_3N in 2 mL of DMF, and the resulting mixture was stirred under N_2 at ambient temperature for 1.5 h. It was then poured into a mixture of EtOAc–C_6H_6 (3:1) and the organic layer washed successively with 50 mL of H_2O, 60 mL of saturated aqueous Na_2CO_3, 50 mL of H_2O, and 60 mL of saturated NaCl. After drying over $MgSO_4$, the solvent was removed under vacuum. The crude product was applied to a silica gel column and eluted with $CHCl_3$–MeOH, 9:1. Fractions homogeneous by TLC were pooled and evaporated to obtain an off-white powder, which was recrystallized from $CHCl_3$-*n*-hexane, 1:3. The precipitate was separated by centrifugation to yield 0.27 g (81%) of crystalline white powder: mp 190–192 °C; TLC, R_f 0.79 ($CHCl_3$–MeOH, 4:1); HPLC, 47.5 min; NMR (300 MHz, Me_2SO-d_6) δ 1.37 (s, 9, C-$(CH_3)_3$), 1.40 (s, 9, $C(CH_3)_3$), 1.70–2.05 (m, 2, glu β-CH_2), 2.32 (t, 2, glu γ-CH_2, J = 7.4 Hz), 4.26–4.33 (m, 1, glu α-CH), 4.39 (s, 2, CH_2S), 6.44 (br s, 2, NH_2), 7.14 (d, 1, H_8, $J_{7,8}$ = 8.45 Hz), 7.42 (d, 2, 3′, 5′, J_o = 8.46 Hz), 7.59 (dd, 1, H_7, $J_{7,8}$ = 8.45 Hz, $J_{5,7}$ = 2.03 Hz), 7.78 (d, 2, 2′, 6′, J_o = 8.46 Hz), 7.92 (d, 1, H_5, $J_{5,7}$ = 2.03 Hz), 8.54 (d, 1, CON*H*, J = 7.53 Hz). Anal. ($C_{29}H_{36}N_4O_6S{\cdot}H_2O$) C, H, N.

10-Thia-5,8-dideazafolic Acid (5d). Compound **12** (0.217 g, 0.40 mmol) was dissolved in CF_3COOH (10 mL). After the reaction mixture was stirred under N_2 at ambient temperature for 1 h, the solution was evaporatd under reduced pressure. The yellow oily residue was treated with 40 mL of Et_2O, and the off-white precipitate was separated by centrifugation and washed with 3 × 30 mL of Et_2O. The crude product was dissolved in 30 mL of H_2O, and the resulting white suspension was basified to pH 11 with 1 N NaOH. Traces of insoluble material were removed by filtration, and the filtrate was brought to pH 3.5 with 1 N HCl. The white precipitate was separated by centrifugation, washed three times with H_2O, and dried under vacuum at 80 °C for 6 h, yielding 0.138 g (79%) of white crystalline powder: mp 224–225 °C (lit.[13] mp > 220 °C dec); TLC, R_f 0.62; HPLC, 34.6 min; NMR (300 MHz, Me_2SO-d_6) δ 1.85–2.12 (m, 2, glu, β-CH_2), 2.49 (t, 2, glu γ-CH_2), 4.31–4.37 (m, 1, glu α-CH), 4.37 (s, 2, CH_2S), 6.39 (br s, 2, NH_2), 7.12 (d, 1, H_8, $J_{7,8}$ = 8.46 Hz), 7.42 (d, 2, 3′, 5′, J_o = 8.46 Hz), 7.58 (dd, 1, H_7, $J_{7,8}$ = 8.46 Hz, $J_{5,7}$ = 2.10 Hz), 7.78 (d, 2, 2′, 6′, J_o = 8.46 Hz), 7.90 (d, 1, H_5, $J_{5,7}$ = 2.10 Hz), 8.56 (d, 1, CON*H*, J = 7.63 Hz). Anal. ($C_{21}H_{20}N_4O_6S$) C, H, N.

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Registry No. **1a**, 56239-21-5; **1b**, 56277-35-1; **1c**, 102743-78-2; **1d**, 87614-69-5; **1e**, 87597-82-8; **1f**, 87539-56-8; **2a**, 88050-25-3; **2b**, 87597-85-1; **2c**, 88050-31-1; **3a**, 76858-74-7; **3b**, 111718-80-0; **3c**, 111718-81-1; **3d**, 111742-50-8; **3e**, 111742-51-9; **4a**, 76849-19-9; **4b**, 111718-82-2; **4c**, 111718-83-3; **4d**, 111742-52-0; **4e**, 111742-53-1; **5a**, 2854-11-5; **5b**, 5854-12-6; **5c**, 61038-31-1; **5d**, 64088-74-0; **5e**, 64088-76-2; **6a**, 56239-22-6; **6b**, 56277-34-0; **6c**, 97541-74-7; **6d**, 93502-95-5; **6e**, 93502-96-6; **6f**, 18921-68-1; **6g**, 27244-49-1; **6h**, 20242-62-0; **6i**, 61075-41-0; **7**, 58677-08-0; **9**, 111718-84-4; **10**, 111718-85-5; **11**, 32677-01-3; **12**, 111718-86-6; TS, 9031-61-2; DHFR, 9002-03-3; $BrCH_2C{\equiv}CH$, 106-96-7; $BrCH_2CH{=}CH_2$, 106-95-6; EtOCO-*p*-$C_6H_4SSC_6H_4$-*p*-COOEt, 20057-83-4.

Supplementary Material Available: Table V containing high-resolution ^1H NMR data for compounds presented in Table I (3 pages). Ordering information is given on any current masthead page.

Synthesis and Potential Antipsychotic Activity of 1*H*-Imidazo[1,2-*c*]pyrazolo[3,4-*e*]pyrimidines

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The synthesis of a series of 1*H*-imidazo[1,2-c]pyrazolo[3,4-*e*]pyrimidines is reported along with the effects of these compounds in preclinical tests for antipsychotic activity. Certain of these compounds displayed antipsychotic-like effects in conditioned avoidance tests, but unlike currently used antipsychotic drugs, they did not have affinity for brain dopamine receptors. These compounds also did not cause dystonias predictive of extrapyramidal side effects in monkeys at doses that produced behavioral effects. On the basis of this unique biological profile, a member of this series, 7,8-dihydro-8-ethyl-1,3,5-trimethyl-1*H*-imidao[1,2-c]pyrazolo[3,4-*e*]pyrimidine (**19**, CI-943), has been selected for clinical evaluation as an antipsychotic agent.

Although dopamine antagonist antipsychotic drugs continue to be the preferred method for the treatment of schizophrenia, the need exists for a new generation of agents with improved efficacy and reduced neurological side effects.[1] Available antipsychotics are effective in controlling the positive symptoms of schizophrenia such as delusions, hallucinations, and loose associations but are ineffective in treating the negative symptoms including social withdrawal and blunted affect. Moreover, these agents cause side effects such as extrapyramidal syndrome and tardive dyskinesia. In recent years research has been focused on identifying compounds with improved clinical profiles. However, despite the desire for novel antipsychotics, many of these so-called "atypical" agents are dopamine antagonists that have profiles similar to those of existing agents.[2]

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