Effects on the Firing Rate of Substantia Nigra DA Neurons.[23] The action potential of zona compacta DA cells was recorded in chloral-anesthetized rats by using standard extracellular recording techniques. DA cells were identified by waveform and firing pattern, and recording sites were verified histologically. Drugs were administered intraperitoneally via an indwelling catheter. Baseline firing rate was calculated by averaging the rate over the 2 min prior to drug injection. Drug effects were determined by averaging the response during the 1-min period of maximal inhibition. Drug-induced inhibition of firing was reversed with the DA antagonist haloperidol to confirm a DA agonist mechanism.

Inhibition of GBL-Stimulated DA Synthesis.[24] Compounds were administered to male Long-Evans rats (Blue Spruce Farms, Altamont, NY) 1 h before sacrifice, and GBL (750 mg/kg ip) and NSD 1015 (100 mg/kg ip) were administered 30 and 25 min, respectively, before sacrifice. Brain striatal levels of dihydroxyphenylalanine (DOPA) were analyzed by HPLC with electrochemical detection.[39]

Acknowledgment. We thank C. L. Christoffersen, A. E. Corbin, K. A. Serpa, Y. H. Shih, S. Z. Whetzel, and J. N. Wiley for biological data.

Supplementary Material Available: Tables listing fractional atomic coordinates and temperature factor parameters, bond lengths and angles, hydrogen bonding parameters, general displacement parameter expressions (13 pages); a table of observed and calculated structure amplitudes (23 pages). Ordering information is given on any current masthead page.

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Folate Analogues. 35. Synthesis and Biological Evaluation of 1-Deaza, 3-Deaza, and Bridge-Elongated Analogues of N^{10}-Propargyl-5,8-dideazafolic Acid[1]

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Structural modifications at the pyrimidine ring and at the C^9,N^{10}-bridge region of the thymidylate synthase (TS) inhibitors N^{10}-propargyl-5,8-dideazafolate (1; PDDF; CB 3717), 2-desamino-N^{10}-propargyl-5,8-dideazafolate (2, DPDDF), and 2-desamino-2-methyl-N^{10}-propargyl-5,8-dideazafolate (3, DMPDDF) have been carried out. Methods for the synthesis of 2-desamino-N^{10}-propargyl-1,5,8-trideazafolate (4), 2-desamino-2-methyl-N^{10}-propargyl-3,5,8-trideazafolate (5a), and 2-desamino-2-methyl-N^{10}-propargyl-5,8-dideaza-1,2-dihydrofolate (6) have been developed. The bridge-extended analogues isohomo-PDDF (7) and isohomo-DMPDDF (8) contain an additional methylene group interposed between N^{10} and the phenyl ring of 1 and 3, respectively. All new compounds were evaluated as inhibitors of TS and the growth of tumor cells in culture. Selected analogues were tested as substrates of folylpolyglutamate synthetase (FPGS) and striking differences in substrate activity were observed among these compounds, indicating that structural modifications at the pyrimidine ring of classical antifolates profoundly influence their polyglutamylation. Enzyme inhibition data established that both N^1 and N^3-H of the pyrimidine ring are essential for efficient binding of quinazoline-type antifolates to human TS.

The recent discovery of a number of very specific and powerful quinazoline antifolates that inhibit thymidylate synthase[2-4] (TS, EC 2.1.1.45) has stimulated the search for related compounds possessing more desirable therapeutic indices.[4,5] Striking biological results were obtained when the 2-amino group of the potent TS inhibitor N^{10}-propargyl-5,8-dideazafolate (PDDF; CB3717) was replaced with a methyl group.[3,4] The 2-desamino-2-methyl analogue 3 exhibited approximately the same substrate activity as 1 toward folylpolyglutamate synthetase (FPGS), indicating that polyglutamylation was not significantly affected by this structural change (Chart I). But the transport characteristics of 3 in H35 hepatoma cells were dramatically different from those of 1.[4] While 1 had no effect on MTX transport in H35 hepatoma cells, 3 inhibited MTX and (6*R*,6*S*)-5-formyltetrahydrofolate (folinic acid) influx into these cells efficiently. H35 R cells that were resistant to MTX by virtue of a transport defect were cross resistant to 3 but not to 1. Although 3 was a weaker inhibitor of TS compared to 1, it was 40-60 times more active as a growth inhibitor of Manca human lymphoma and H35 hepatoma cells. These results taken together indicated that further structural changes of 1 at the pyrimidine portion of the quinazoline ring might lead to the devel-

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[§] Burroughs Wellcome Co.

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(2) Jones, T. R.; Calvert, A. H.; Jackman, A. L.; Brown, S. J.; Jones, M.; Harrap, K. R. *Eur. J. Cancer* 1981, *17*, 11.
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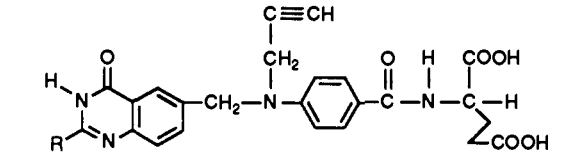
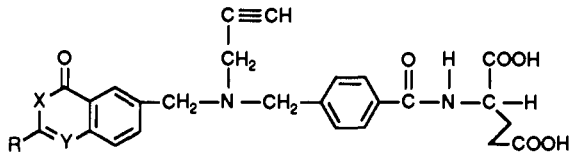
0022-2623/91/1834-2746$02.50/0

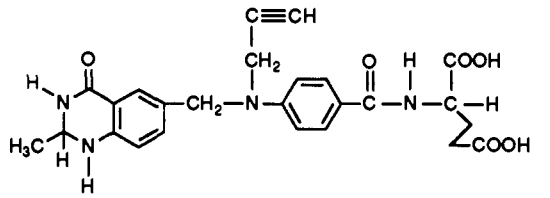
Chart I



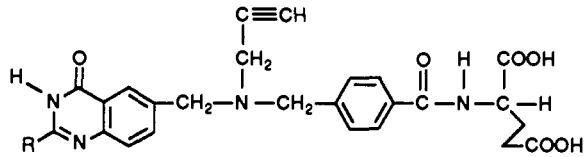
1: R = NH_2 (PDDF; CB 3717)
2: R = H (DPDDF)
3: R = CH_3 (DMPDDF)



4: R = H, X = NH, Y = CH
5a: R = CH_3, X = CH_2, Y = N



6



7: R = NH_2
8: R = CH_3

opment of antifolates with altered transport characteristics and tissue accumulation. Results with **3** suggested that its enhanced transport and tissue accumulation relative to **1** may compensate for its reduced inhibition of TS.

Previous results from this laboratory showed that substitution of N^3-H of **3** with a methyl group reduced TS inhibition by more than 1000-fold and dramatically lowered its cytotoxicity to tumor cells in culture.[4] Although we indicated from these results that the presence of N^3-H in **3** was necessary for binding to TS, alternate possibilities existed. For example, **3** can be enolized by a nucleophile present at the binding site of the enzyme and the inhibitor could bind to TS via the enol form as shown in Figure 1A. Such binding is not possible when N^3-H is substituted with a methyl group. On the other hand, if the N^3-H group in **3** is substituted with a methylene group as in B, it could undergo enolization and bind to the enzyme via the hydroxyl group (Figure 1B). However, if binding via N^3-H is necessary, the 3-methylene-substituted compound (**5a**) would be expected to be substantially less active as an inhibitor of TS relative to **3**. The importance of N^1 of the quinazoline ring in TS binding might be investigated by substituting it with a methenyl group that does not have the lone electrons necessary to interact with a substituent at the cofactor binding site of the enzyme through a hydrogen bond. Since 2-desamino-N^{10}-propargyl-5,8-dideazafolate (**2**) was previously shown to be a potent inhibitor of TS,[3] it was interesting to replace N^1 of **2** with a methenyl group and evaluate the resulting **4** as a TS inhibitor. The 1,2-dihydro derivative **6** was designed to evaluate the importance of the double bond between C^2 and N^1 of **3** in binding to TS. Reduction of this double bond would result in sp^3 hybridization of carbon 2, with the resultant steric

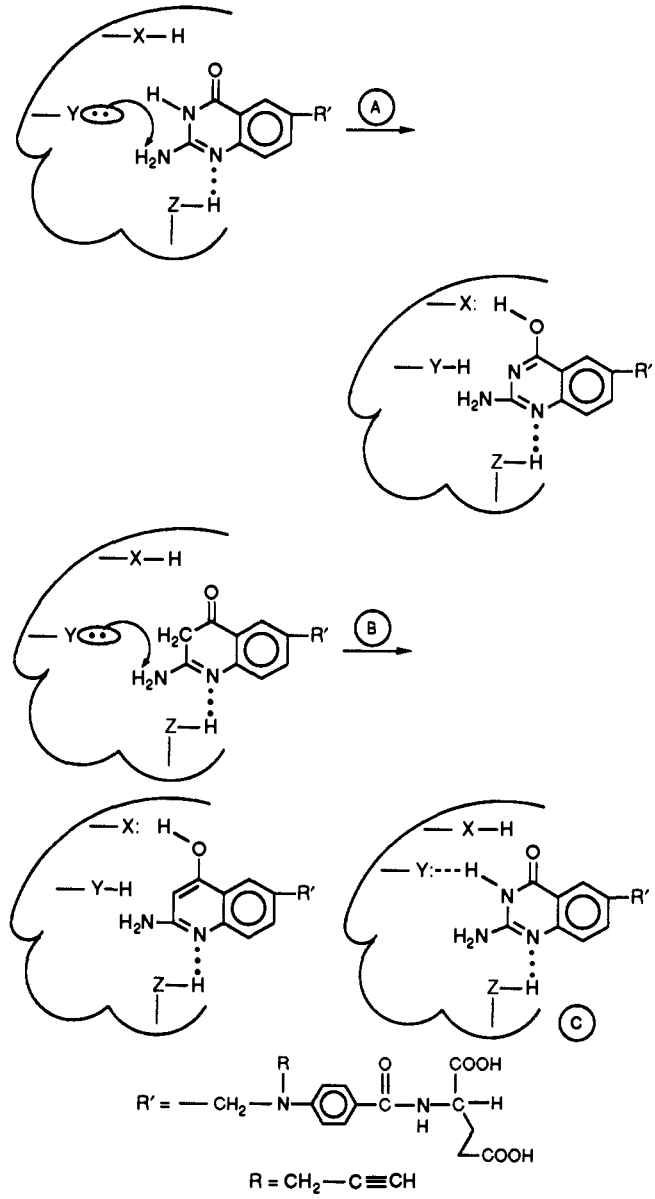


Figure 1.

alterations at N^1 and C^2. Although N^1 would still have the lone electrons, the extra hydrogens at N^1 and C^2 might be unfavorable for efficient binding interactions with the enzyme. The effect of this structural change of **3** on polyglutamylation and interaction with the reduced folate/MTX transport system in mammalian cells was of considerable interest to us.

Although the atomic structures of *E. coli* TS, CB3717, dUMP complex and TS, PDDF, FdUMP have been elucidated recently by X-ray crystallography,[6,7] it was of interest to study the importance of various substituents at the quinazoline ring of **1** in binding to the human enzyme. Such a study is relevant in view of the differences in species specificity of TS in response to inhibitors and pseudosubstrates.[8] For example (6*R*,6*S*)-tetrahydrohomofolate was shown to be a powerful inhibitor of *E. coli* TS,[9]

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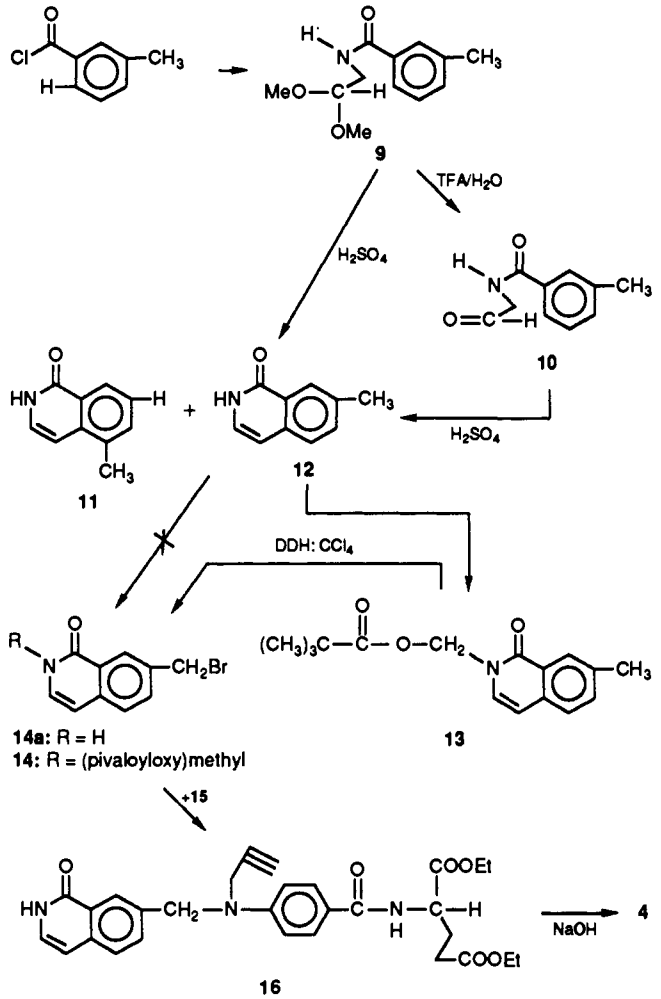
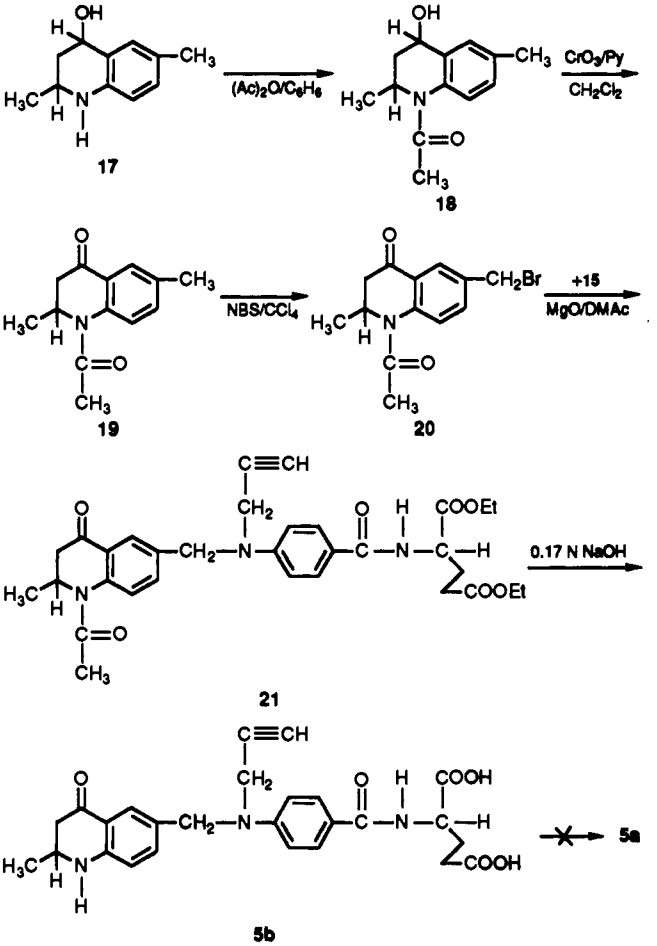
Scheme I



Scheme II



whereas it was a pseudocofactor of TS in *S. faecium* and *L. casei*.[10] Likewise, significant differences in enzyme inhibitory potencies by 1 and 3 exist among bacterial versus mammalian enzymes.[4,8,11] In addition, the use of carefully designed inhibitors is appropriate in studying the structural characteristics of the binding site of the human enzyme because the data are obtained from solutions rather than from crystals.

It was also appealing to test the interesting possibility of whether increasing the basicity at N^{10} of 1 and 3 might result in more water-soluble analogues without compromising their biological activities. The most desirable structural change to accomplish this objective was judged to be the interposition of a methylene group between N^{10} and the phenyl ring of 1 and 3 as shown in structures 7 and 8. However, there existed the possibility that the resulting steric effects might influence enzyme binding. Nevertheless, the data were expected to be useful in structure–activity relationship (SAR) studies of quinazoline antifolates that inhibit TS.

Chemistry. Our first attempt was directed toward the synthesis of the 1-deaza analogue 4. *m*-Toluic acid was converted to its acid chloride by reaction with $SOCl_2$ and coupled with aminoacetaldehyde dimethyl acetal to obtain the protected aldehyde 9. Treatment of 9 with aqueous TFA gave the aldehyde 10, which was cyclized to a mixture of isocarbostyryls 11 and 12 (Scheme I). The desired isomer 12 was the major product, the structure of which was established by NMR spectroscopy. Alternately, the protected aldehyde 9 could be cyclized directly with concentrated H_2SO_4 to a mixture of 11 and 12. Benzylic bromination of 12 with *N*-bromosuccinimide (NBS) or 1,3-dibromo-5,5-dimethylhydantoin (DDH) was unsuccessful due to the formation of products of unknown structures. However, protection of the NH via the (pivaloyloxy)methyl derivative 13 permitted benzylic bromination with NBS or DDH under standard conditions in CCl_4 to give the desired benzyl bromide 14. Reaction of 14 with diethyl *N*-[4-(propargylamino)benzoyl]-L-glutamate (15) in DMAc gave the diester 16, which was hydrolyzed to the target compound 4.

The strategy for the synthesis of the target 3-deaza compound 5a was based on the reaction of the 6-(bromomethyl)quinolinone derivative 20 with propargylamine 15 and the subsequent deblocking, oxidation, and hydrolysis of the reaction product 21 (Scheme II). The dimethylhydroxytetrahydroquinoline 17 was prepared by the acid-catalyzed condensation of *p*-toluidine and crotonaldehyde.[12] It was converted to the quinoline 19 by selective acetylation followed by Sarett oxidation. Benzylic bromination of 19 to 20 followed by reaction with the propargylamine 15 gave the diester 21. Hydrolysis of 21

(9) Goodman, L.; DeGraw, J. I.; Kisliuk, R. L.; Friedkin, M.; Pastore, J.; Crawford, E. J.; Plante, L. T.; Nahas, A.; Morningstar, J. F., Jr.; Kwok, G.; Wilson, L.; Donovan, E. F.; Ratzan, J. *J. Am. Chem. Soc.* **1964**, *86*, 308.

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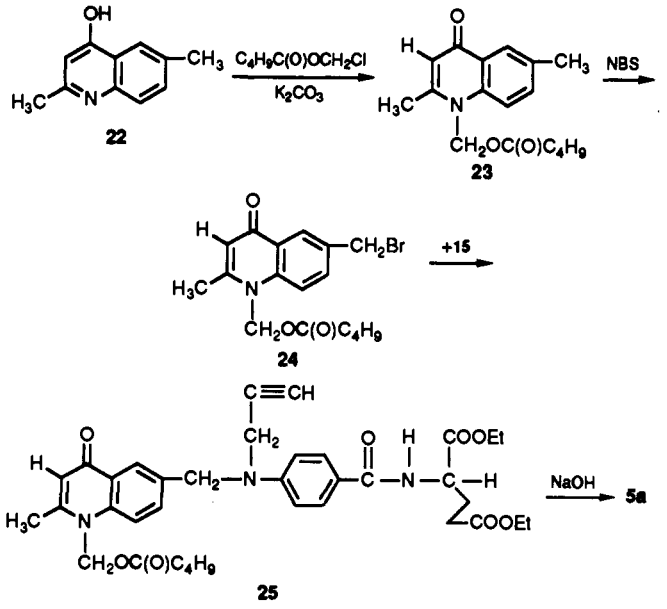
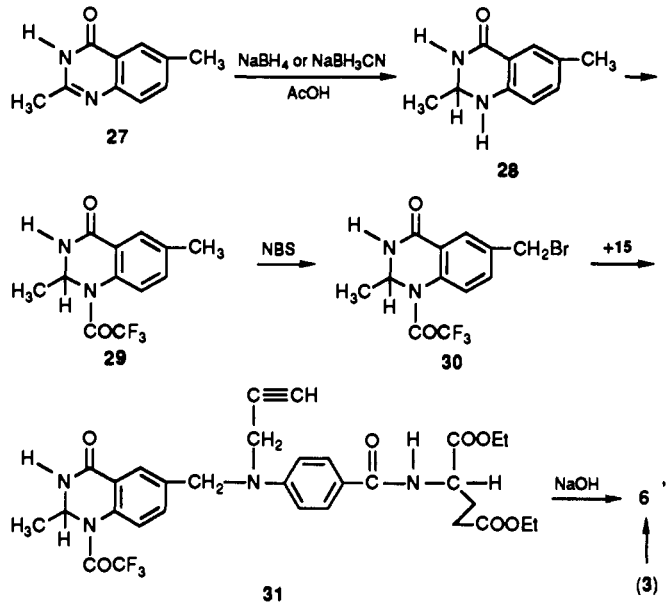
(12) Tamura, S.; Takiquehi, C.; Sakai, K. *Yakugaku Zasshi* **1956**, *76*, 912.

Scheme III



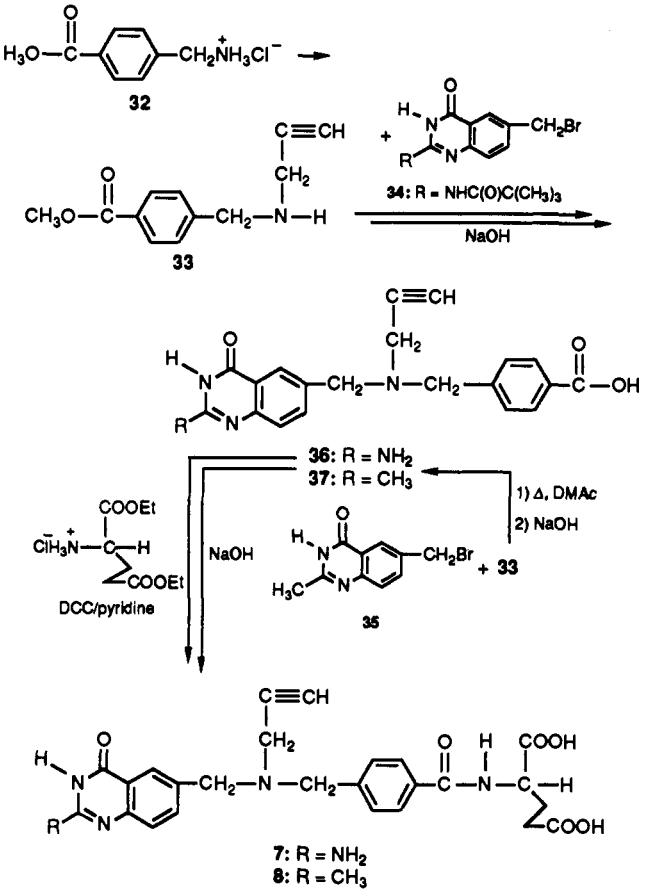
Scheme IV



gave **5b**. Quite unexpectedly, **5b** was found to be remarkably stable, and all attempts to convert it to the target compound **5a** by air, MnO_2, and several mild oxidative reagents were not successful. These results clearly indicated that an alternate method for the synthesis of **5a** that does not involve a tetrahydroquinoline as an intermediate was required.

2,6-Dimethyl-4-hydroxyquinoline (**22**) was prepared according to a literature procedure.[13] Because of its poor solubility in organic solvents, direct benzylic bromination was unsuccessful. Reaction of **22** with chloromethyl pivalate gave the *N*-[(pivaloyloxy)methyl]quinolinone **23**, having improved solubility relative to **23** (Scheme III). The infrared spectrum of **23** exhibited absorption bands at 1755 and 1600 cm^{-1}, indicating the presence of a conjugated ketone. The NMR and mass spectra of **23** (FAB; m/z = 288, MH^+) were also consistent with the proposed structure. The (pivaloyloxy)methyl derivative **23** reacted smoothly with NBS to give the corresponding bromo-

Scheme V



methyl derivative **24**, which on reaction with **15** followed by hydrolysis gave 3-deaza-DMPDDF (**5a**), which was purified by reverse phase chromatography on a C_{18} silica gel column.

The chemistry we developed for the synthesis of the 1,2-dihydro compound **6** is depicted in Scheme IV. 2,6-Dimethylquinazolin-4(3*H*)-one was reduced with $NaBH_4$ in HOAc to the corresponding dihydro derivative **28**, which on treatment with trifluoroacetic anhydride gave **29**. Benzylic bromination of **29** in the usual manner with NBS gave the bromomethyl derivative **30**, which was allowed to react with **15** in DMAc to obtain the diethyl ester **31**. Base hydrolysis of **31** gave the desired target compound **6**. Compound **6** was also prepared by the direct reduction of **3** with $NaBH_3CN$/HOAc in THF. However, under conditions required for the complete reduction of **3**, the product **6** was converted to a number of degradation products. Compound **6** was purified from the crude reduction product containing unreacted **3** by reverse phase chromatography on a C_{18} silica gel column.

Isohomo-PDDF (**7**) and isohomo-DMPDDF (**8**) were conveniently prepared according to the method in Scheme V. Methyl *p*-(aminomethyl)benzoate hydrochloride (**32**) was prepared from α-amino-*p*-toluic acid.[14] Alkylation of **32** with propargyl bromide gave methyl 4-(propargylamino)methylbenzoate (**33**), which on reaction with the (bromomethyl)quinazoline **34** in DMAc followed by hydrolysis of the resultant product gave the dideazapteroic acid analogue **36**. Likewise, reaction of **33** with **35** followed by hydrolysis gave the 2-desamino-2-methyl analogue **37**. Both **36** and **37** were converted to the target compounds **7** and **8**, respectively, by coupling with diethyl glutamate

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(14) Nair, M. G.; Baugh, C. M. *J. Med. Chem.* **1974**, *17*, 223.

Table I. Inhibition of Thymidylate Synthase by Analogues of 1

compound	I_{50} (μM) human TS[a]	I_{50} (μM) *E. coli* TS[a]
4 (1-deaza-PDDF)	0.74	
5a (3-deaza-DMPDDF)	1.4	
5b (3-deaza-1,2-dihydro-DMPDDF)	24.0	1.88
6 (1,2-dihydro-DMPDDF)	0.43	1.5
7 (isohomo-DMPDDF)	31.0	201.0
8 (isohomo-DMPDDF)	83.0	515.0
1 (PDDF)	0.010	0.030
3 (DMPDDF)	0.010	0.045

[a] TS from *E. coli* strains harboring the plasmids with *thy A* gene from SV40 transformed human fibroblasts from wild-type and *E. coli* were isolated and purified to near homogeneity according to procedures outlined by Dev et al.[21] Initial velocities for the enzyme were determined at pH 8.0 in the presence of 80 μM (6-*R*,*S*) H_4folate and 20 μM deoxyuridylate at 37 °C by the tritium release assay of Roberts (Roberts, D. *Biochemistry* 1966, 5, 3546–3548).

in pyridine, followed by hydrolysis and chromatographic purification. DCC was used as the coupling reagent in the above reaction.

Biological Evaluation and Discussion

(a) Inhibition of TS. All target compounds were evaluated as inhibitors of human TS. The data are presented in Table I. The 1-deaza analogue **4**, the 3-deaza analogue **5a**, and the 1,2-dihydro analogue **6** all were significantly weaker inhibitors of TS compared to **1**, **2**, or **3**. These results confirmed our earlier hypothesis[4] that N^3-H of the quinazoline ring is essential for binding to TS, presumably via a hydrogen bond to a suitable substituent at the cofactor binding site of the enzyme. In the *E. coli* enzyme this substituent at the binding site of the protein has been identified as Asp-221 by X-ray crystallography.[6] Results with **5a** indicate that 4-oxoquinazolines do not bind to TS after enolization, and the bound inhibitor is in the keto form, which is stabilized by hydrogen bonding through N^3-H. Replacement of N^1 of the quinazoline ring was detrimental for binding to the enzyme. It appears that the NH at position 3 of the quinazoline ring is necessary for direct interaction with a proton, on an enzyme substituent, which might stabilize binding as a result of a hydrogen bond (Figure 1C) or by indirect interaction via hydrogen bonding to Arg 21 through a fixed solvent molecule as shown by Matthews and co-workers for *E. coli* TS containing bound FdUMP and **1**.[7] The presence of a double bond at N^1 was found to be essential since the reduction of this bond of **3** resulted in loss of enzyme inhibition of several magnitudes relative to **3**, indicating that inhibitor binding to TS via N^1 is sensitive to changes of steric factors in this region. Results with bridge-elongated analogues **7** and **8** were disappointing. The enhanced basicity of N^{10} in **7** and **8** resulted in loss of enzyme inhibition several magnitudes greater relative to those of **1** and **3**. Whether this loss of activity was due to the displacement of the benzoylglutamate moiety by one carbon–carbon bond distance (~1.48 Å) from the unaltered 6-(propargylamino)methylquinazolines or was solely due to electronic factors (increase in basicity) could not be deduced from the available data.

The reduced TS inhibition by analogues **4**–**8** was reflected in their poor ability to inhibit the growth of tumor cells in culture (Table II). The only compound in this series that showed significant activity was **6**, which had an I_{50} of 0.6 μM against SW/480 cells. Under identical conditions the corresponding I_{50} for **3** was 0.05 μM. None of the compounds exhibited sufficient activity against these cells to warrant further studies.

Table II. Inhibition of Tumor Cell Growth by Analogues of 1

compound	I_{50} (μM) SW/480	MCF7	Manca human lymphoma
4 (1-deaza PDDF)	>100	22.0	9.0
5a (3-deaza-DMPDDF)	>50		>600
5b (3-deaza-1,2-dihydro-DMPDDF)	>100	>100	
6 (1,2-dihydro-DMPDDF)	0.6		
7 (isohomo-PDDF)	>100	>100	>80.0
8 (isohomo-DMPDDF)	>100	>100	
1 (PDDF)	0.8	0.8	0.56[4]
3 (DMPDDF)	0.05	0.007	0.013[4]

Table III. Inhibition of MTX Influx into MOLT-4 Cells by 1 and Analogues

compound	I_{50} (μM)
4 (1-deaza-PDDF)	4.5
5b (3-deaza-1,2-dihydro-DMPDDF)	4.5
1 (PDDF)	28.0
3 (DMPDDF)	1.5
7 (isohomo-PDDF)	(58% at 100 μM)
8 (isohomo-DMPDDF)	7.0
MTX	1.4
(6*R*,6*S*)-5-formyltetrahydrofolate	3.5
folic acid	>100

Table IV. Substrate Activity of 1 and Analogues for Hog Liver Folyl-polyglutamate Synthetase (FPGS)[a]

compound	app K_m (μM)	V_{max}, rel %[b]	rel $\frac{V_{max}}{\text{app } K_m}$
4 (1-deaza-PDDF)	43.7	16.5	0.38
5a (3-deaza-DMPDDF)	>200[c]		
5b (3-deaza-1,2-dihydro-DMPDDF)	>200[c]		
7 (isohomo-PDDF)	56.0	108	1.9
8 (isohomo-DMPDDF)	68.6	138	2.01
6 (1,2-dihydro-DMPDDF)	>200[d]		
1 (PDDF)	49.2	87.6	1.8
3 (DMPDDF)	66.7	88.7	1.33
(6*R*,6*S*)-H_4-folate	4.2	135	32.1
folic acid	104.6	80	0.77

[a] Assayed with partially purified hog liver FPGS as previously described.[23] [b] V_{max} relative to a control of 50 μM aminopterin included in each experiment. [c] 1% rel velocity at 200 μM. [d] 9% rel velocity at 200 μM. [e] 1.8% rel velocity at 200 μM.

The data in Table I show that both **1** and **3** were equipotent as inhibitors of human TS. However, **3** was 20–60 times better as an inhibitor of several human tumor cell lines in culture. The increased potency of **3** was previously attributed[4] to its enhanced transport to mammalian cells by the reduced folate transport system. Therefore, it was of interest to examine the relative efficiency of transport influx of various **1** analogues. The results of these studies are shown in Tables III and IV. The data presented in Table III clearly indicate that deletion of N^1 or N^3-H of the quinazoline ring enhanced the affinity relative to **1** of these deaza analogues for the reduced folate pathway of MOLT-4 cells. The most dramatic effect was seen with **3**, in which the 2-amino group of **1** was replaced with a methyl group. MTX and **3** showed approximately equal affinity for the reduced folate transporter in this cell line. However, modification of **3** by deletion of the N^3-H and reduction of the 1,2 double bond resulted in decreased affinity for this transport system. The assay employed in this study measures binding of the analogue to the reduced folate transporter since 5-formyltetrahydrofolate and MTX bind well to this transporter, while folate does not.[15,16]

Selected analogues were examined for their ability to function as substrates of partially purified hog liver FPGS. Their relative kinetic parameters are summarized in Table IV. It is noteworthy that the substrate activities of these modified quinazoline analogues were highly sensitive to structural changes at the pyrimidine portion of the quinazoline ring. For example, compounds 4 and 6 exhibited only very weak activity as a substrate to FPGS relative to tetrahydrofolate. These results confirm our earlier suggestion[4] that the enhanced cytotoxicity of 3 is most likely due to its efficient transport into tumor cells via the reduced folate transporter. The results clearly establish that the presence of both N^3-H and N^1 in the quinazoline ring is essential for efficient binding to human TS. However, these substituents are not critical for interaction with the reduced folate transporter of MOLT-4 cells. Although variations of the C^2 substituents are well tolerated by the enzyme, the exact role of the 4-oxo functionality in enzyme binding remains to be elucidated.

Experimental Section

Melting points were determined on a Fisher Model 355 digital melting point analyzer. NMR spectra were run in $CDCl_3$ or CF_3COOH on a 90-MHz Perkin-Elmer spectrometer with Me_4Si as an internal standard unless otherwise mentioned. Field strength of the various proton resonances is expressed in parts per million, and peak multiplicitly is depicted as follows: s, singlet; br, broad singlet; d, doublet; t, triplet, q, quartet; m, multiplet; c, unresolved multiplet, the center of which is given. The high-resolution NMR spectra of selected compounds were obtained from Burroughs Wellcome Co. The IR spectra were recorded on a Perkin-Elmer 1320 spectrophotometer. Ultraviolet spectra were recorded on a Bausch and Lomb Spectronic 2000 spectrometer interfaced with a Commodore Superpet computer or on a Gilford Response spectrometer. All HPLC analyses were done on a Waters 600 multisolvent delivery system equipped with a Model 481 UV detector and a Waters 740 data module. Mass spectra were determined by Dr. Susan Weintraub, University of Texas, San Antonio, TX, on a Finnigan-MAT Model 212 spectrometer in combination with an INCOS data system, or by Dr. F. A. Bencsath, University of South Alabama, Mobile, AL, on a VG 70-250 SEQ hybrid-tandem spectrometer. Column chromatography was performed on silica gel 60 (70–230 mesh ASTM) and TLC analysis on silica gel 60 F254 precoated plates. The following TLC solvent systems were used: (A) 5% CH_3OH in CH_2Cl_2; (B) 10% CH_3OH in CH_2Cl_2. For compounds for which only mass spectral data are given, their purity has been assessed to be >98% by TLC or HPLC. Where analyses are indicated only by symbols of the elements, analytical results obtained for these elements were within ±0.4% of the theoretical values.

***N*-(3-Toluoyl)aminoacetaldehyde Dimethyl Acetal (9).** To a stirring solution of 8 prepared from 3.4 g (25 mmol) of *m*-toluic acid in 100 mL of benzene were added 2.72 mL (25 mmol) of aminoacetaldehyde dimethyl acetal and 4.15 g (30 mmol) of K_2CO_3. The reaction mixture was stirred at room temperature for 18 h and evaporated. The residue was poured into 100 mL of ice water and extracted with CH_2Cl_2. The extract was washed with water and dried over Na_2SO_4. Removal of the solvent gave a gummy material: yield 3.9 g (70%); NMR ($CDCl_3$) δ 7.40 (c, 4 H, aromatic), 4.53 (t, 1 H, CH), 3.56 (t, 2 H, CH_2), 3.31 (s, 6 H, CH_3O), 2.26 (s, 3 H, CH_3); MS (FAB) *m/z* 224 (MH^+).

7-Methylisoquinolin-1(2*H*)-one (12). A solution of 8.0 g (35.9 mmol) of 9 in 75 mL of H_2SO_4 was stirred at 25 °C for 3 days and then poured into a mixture of 200 mL of EtOAc and 300 g of crushed ice. The EtOAc layer was washed with water, dried over Na_2SO_4, and concentrated. The residue thus obtained was recrystallized from EtOAc to yield 2.62 g of the desired compound 12 (39%): mp 189–191 °C (lit.[17] mp 184–185 °C); NMR ($CDCl_3$) δ 8.15 (d, 1 H, H^8, *J* = 7 Hz), 2.47 (s, 3 H, CH_3); IR (Nujol) 1660 (lactam), 870, 825 (2 adjacent H) cm^{-1}; MS(EI) *m/z* 160 (MH^+).

From the mother liquor of crystallization, compound 11 was separated: mp 168–170 °C (lit.[18] mp 162 °C); IR (Nujol) 1660 (lactam) 780 (3 adjacent H) cm^{-1}.

7-Methyl-2-[(pivaloyloxy)methyl]isoquinolin-1(2*H*)-one (13). To a solution of 954 mg (6 mmol) of 12 in 60 mL of THF were added 1.7 g (12 mmol) of K_2CO_3 and 1.7 mL (12 mmol) of chloromethyl pivalate. The reaction mixture was refluxed with stirring for 4 days. The progress of the reaction was monitored intermittently by TLC. The reaction mixture was evaporated, and the residue was dissolved in CH_2Cl_2 and applied on a silica gel column. The column was eluted with CH_2Cl_2 to obtain the desired 13: yield 1.32 g (80%); mp 84 °C; IR (Nujol) 1725 (ester) 1670 (lactam) 820 (2 adjacent H) cm^{-1}; NMR ($CDCl_3$) δ 8.34 (br, 1 H, H^5), 7.53 (d, 1 H, H^6, *J* = 2 Hz), 7.29 (d, 1 H, H^1, *J* = 8 Hz), 6.5 (d, 1 H, H^2, *J* = 8 Hz), 6.02 (δ, 2 H, CH_2), 2.58 (s, 3 H, CH_3), 1.29 (s, 9 H, *t*-Bu); MS *m/z* 273 (M^+).

7-(Bromomethyl)-2-[(pivaloyloxy)methyl]isoquinolin-1-(2*H*)-one (14). A mixture of 1.32 g (4.8 mmol) of 13, 690 mg (2.4 mmol) of 1,3-dibromo-5,5-dimethylhydantoin (DDH), and 200 mL of CCl_4 was heated to reflux under stirring. The clear solution was illuminated with a 250-W light bulb and 50-mg batches of benzoyl peroxide were added to it at 1-h intervals for 6 h. The reaction mixture was refluxed for 24 h, filtered, and concentrated. The residue was purified by column chromatography over silica gel with CH_2Cl_2 as the eluting solvent: yield 750 mg (44%); mp 103 °C; NMR ($CDCl_3$) δ 8.52 (d, 1 H, H^5, *J* = 2 Hz), 7.8 (dd, 1 H, H^7, *J* = 2 Hz), 7.58 (d, 1 H, H^8, *J* = 8 Hz), 7.39 (d, 1 H, H^1, *J* = 7 Hz), 6.53 (d, 1 H, H^2, *J* = 2 Hz), 6.04 (s, 2 H, CH_2), 4.70 (s, 2 H, CH_2Br), 1.29 (s, 9 H, *t*-Bu); MS (FAB) *m/z* 351, 353 (MH^+).

2-Desamino-N^{10}-propargyl-1,5,8-trideazafolic Acid (4). (a) Preparation of 16. A mixture of 750 mg (2.1 mmol) of 14, 767 mg (2.1 mmol) of 15 and 85 mg (2.1 mmol) of MgO in 2 mL of DMAc was stirred at 90–100 °C for 18 h and poured over 50 g of crushed ice. The precipitate of 16 thus formed was filtered, washed with water, and dried. The compound was judged to be pure by TLC and used for the next step.

(b) Hydrolysis of 16 to 4. The above solid was dissolved in 355 mL of CH_3CN and stirred with 105 mL of 0.1 N NaOH for 18 h at room temperature. The pH of the solution was adjusted to 7.5 with 1 N HCl and concentrated to ~40 mL. The concentrate was acidified with HOAc. The compound 4 separated as a precipitate was filtered. It was further purified by conversion to a sodium salt in 0.1 N NaOH and acidification with 1 N HCl. The solid thus separated was filtered, washed with distilled water, and dried over P_2O_5 under vacuum: yield 710 mg (72.3%); mp >300 °C; UV (0.1 N NaOH) λ_{max} 298.3 nm (ε = 26746); NMR (TFA) δ 8.71 (br, 1 H, H^5), 7.96 (C, 7 H, aromatic), 7.39 (d, 1 H, H^2, *J* = 12 Hz), 5.37, 4.77 (2s, 4 H, bridge CH_2, propargyl), 5.11 (br, 1 H, α-H of glutamate), 2.65 (c, 4 H, glutamate); MS (FAB) *m/z* 462 (MH^+). Anal. ($C_{25}H_{23}N_3O_6$) C, H, N.

4-Hydroxy-2,6-dimethyl-1,2,3,4-tetrahydroquinoline (17). To a solution of 6.43 g (60 mmol) of *p*-toluidine in 600 mL of 5% HCl was added 5.8 mL (60 mmol) of 85% crotonaldehyde. The reaction mixture was stirred for 2 days at room temperature and then neutralized with 40% NaOH. The crude product was collected, washed with water, and crystallized from 95% ethanol: yield of α-form 2.6 g (24.5%); mp 168–170 °C (lit.[12] mp 167–169 °C). From the EtOH mother liquor, 2.94 g (28%) of the β-form was obtained: mp 108–109 °C (lit.[12] mp 108–110 °C); IR (Nujol) 3210 (OH) cm^{-1}; NMR ($CDCl_3$) δ 7.3 (c, 3 H, aromatic), 4.0 (br, 1 H, NH), 2.6 (c, 1 H, H^2), 2.4 (s, 3 H, 6-CH_3), 2.1–1.7 (c, 2 H, H^3), 1.6 (d, 3 H, *J* = 7 Hz, 2-CH_3); MS *m/z* 177 (M^+).

1-Acetyl-4-hydroxy-2,6-dimethyl-1,2,3,4-tetrahydroquinoline (18). A mixture of 2.4 g (13.6 mmol) of 17, 1.4 mL

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(14.8 mmol) of acetic anhydride, and 40 mL of benzene was refluxed for 4.5 h, washed with 1% $NaHCO_3$, and dried over anhydrous Na_2SO_4. After removal of benzene, the residue was purified by chromatography on silica gel, eluting with 5% CH_3OH in CH_2Cl_2. A gum was obtained: yield 2.62 g (88%); IR (Nujol) 3200, 1740, 1650 cm^{-1}; NMR ($CDCl_3$) δ 7.2–7.6 (c, 3 H, aromatic), 2.5 (c, 1 H, H^2), 2.35 (s, 3 H, CH_3), 2.08 (s, 3 H, CH_3), 1.6–1.4 (c, 2 H, H^3), 1.1 (d, 3 H, CH_3, J = 7 Hz); MS m/z 219 (M^+).

1-Acetyl-4-oxo-2,6-dimethyl-1,2,3,4-tetrahydroquinoline (19). To a solution of 19.77 g (76.6 mmol) of chromium trioxide–pyridine complex in 400 mL of CH_2Cl_2 was added a solution of 2.78 (12.3 mmol) of 18 in 50 mL of CH_2Cl_2 at 10–15 °C. The reaction mixture was stirred at room temperature for 6 h and filtered, and the solution was washed with 1% HCl, 5% $NaHCO_3$, and water. After removal of the solvent, the residue was crystallized from CH_2Cl_2/ether to afford 1.8 g (68%) of white solid: mp 125–126 °C; IR (Nujol) 1690, 1650 cm^{-1}; NMR ($CDCl_3$) δ 7.5–7.1 (c, 3 H, aromatic), 2.7 (d, 2 H, H^3, J = 7 Hz), 2.6 (c, 1 H, H^2), 2.4 (s, 3 H, CH_3), 2.1 (s, 3 H, CH_3), 1.14 (d, 3 H, CH_3, J = 7 Hz); MS m/z 217 (M^+).

1-Acetyl-6-(bromomethyl)-2-methyl-2,3-dihydroquinolin-4(1*H*)-one (20). A mixture of 1.07 g (4.93 mmol) of 19, 0.9 g (5.06 mmol) of *N*-bromosuccinimide, 45 mg of benzoyl peroxide, and 25 mL of CCl_4 was refluxed with stirring and illuminated with a 250-W light bulb for 4 h. After filtration, the solution was concentrated and the residue was purified by chromatography on silica gel 60 with 3% MeOH in CH_2Cl_2 as the eluent: yield 1.14 g (78%) of gummy product; NMR ($CDCl_3$) δ 8.05–7.5 (c, aromatic H), 4.46 (s, 2 H, CH_2Br), 2.8 (d, 2 H, H^3, J = 6 Hz), 2.1 (s, 3 H, CH_3 CO), 1.1 (d, 3 H, CH_3); MS (FAB) m/z 296, 298 (1:1) (MH^+).

Diethyl 1-Acetyl-2-desamino-2-methyl-N^{10}-propargyl-3,5,8-trideaza-1,2-dihydrofolate (21). A mixture of 701 mg (2.4 mmol) of **20**, 792 mg (2.2 mmol) of propargylamine 15, and 96 mg (2.4 mmol) of MgO in 2.6 mL of DMAc was stirred at 86–95 °C for 18 h and then poured into ice water. The gummy product was dissolved in 50 mL of ethyl acetate. The solution was washed with water, dried over anhydrous Na_2SO_4, and concentrated. The residue was purified by chromatography on silica gel 60 with 4% CH_3OH in CH_2Cl_2 as the eluent: yield 900 mg (78%); TLC (4% CH_3OH in CH_2Cl_2), R_f 0.14; MS (FAB) m/z 576 (MH^+).

2-Desamino-2-methyl-N^{10}-propargyl-3,5,8-trideaza-1,2-dihydrofolic Acid (5b). To a solution of 580 mg (1 mmol) of 21 in 17 mL of ethanol were added 17 mL of water and 7 mL of 1 N NaOH. The reaction mixture was stirred at room temperature for 26 h, neutralized to pH 7 with 1 N HCl, and concentrated to about 10 mL under vacuum. The pH of the concentrate was adjusted to 4.5 with HOAc. After overnight refrigeration, the precipitate was collected, washed with water, and dried: yield 308 mg (64%); mp 199–201 °C dec. The crude product was purified by conversion to its ammonion salt and chromatography over a preparative C_{18} column using 8% acetonitrile in water as the eluting solvent. All the fractions corresponding to the product were pooled, concentrated, and acidified with 1 N HCl to pH 3.5. The precipitate was filtered, washed with water, and dried. **5b**: NMR (Me_2SO-d_6, 300 MHz) δ 7.82 (d, 1 H, J = 7.59 Hz, amide NH), 7.67 (d, 2 H, J = 9.11 Hz, $H^{2'}$ and $H^{6'}$), 7.51 (d, 1 H, $J_{5,7}$ = 2.2 Hz, H^5), 7.22 (dd, 1 H, $J_{7,8}$ = 8.68 Hz, $J_{7,5}$ = 2.2 Hz, H^7), 6.84 (d, 2 H, J = 8.68 Hz, $H^{3'}$ and $H^{5'}$), 6.75 (d, 1 H, $J_{8,7}$ = 8.68 Hz, H^8), 4.45 (s, 2 H, bridge CH_2), 4.25 (m, 1 H, α-H of glutamate), 4.17 (d, 2 H, J = 2.0 Hz, $CH_2C{\equiv}$), 3.62 (m, 1 H, H^2), 3.16 (t, 1 H, J = 2.0 Hz, $HC{\equiv}$), 2.2–2.4 (m, 4 H, H^3 and γ-H of glutamate), 1.8–2.0 (m, 2 H, β-H of glutamate), 1.20 (d, 3 H, J = 7.15 Hz, 2-CH_3); UV (0.1 N NaOH) λ_{max} 290 nm (ϵ = 19308); MS (FAB) m/z 478 (MH^+). Anal. ($C_{26}H_{27}N_3O_6$) C, H, N.

2,6-Dimethyl-1-[(pivaloyloxy)methyl]quinolin-4(1*H*)-one (23). To a solution of 1.74 g (10 mmol) of 2,6-dimethylquinolin-4(1*H*)-one (**22**) in 70 mL of DMF were added 2.07 g of K_2CO_3 (15 mmol) and 1.44 mL (10 mmol) of chloromethyl pivalate. The reaction mixture was stirred at 90 °C for 48 h. The solid was removed by filtration and 1.28 g of starting material (**22**) was recovered. The filtrate was concentrated under vacuum and the residue was extracted with CH_2Cl_2. The extract was washed with water and dried over anhydrous Na_2SO_4. After removal of the CH_2Cl_2, the residue was purified by chromatography on silica gel, using 2% CH_3OH in CH_2Cl_2 as the eluting solvent: yield 0.50 g (66% based on the expended starting material); mp 76–77 °C; NMR ($CDCl_3$) δ 7.9 (c, 1 H, aromatic), 7.55 (c, 1 H, aromatic), 6.9 (c, 1 H, aromatic), 6.02 (s, 2 H, CH_2), 2.50 (s, 3 H, CH_3), 2.70 (s, 3 H, CH_3), 1.20 (s, 9 H, *t*-Bu); IR (Nujol) 1755 (ester), 1600 (conjugated C=C) cm^{-1}; MS (FAB) m/z 288 (MH^+).

6-(Bromomethyl)-2-methyl-1-[(pivaloyloxy)methyl]-4-(1*H*)-one (24). To a solution of 0.38 g (1.32 mmol) of **23** in 20 mL of CCl_4 was added 0.24 g of NBS and 40 mg of benzoyl peroxide. The reaction mixture was refluxed and illuminated with a 250-W light bulb for 5 h. After cooling, the succinimide was filtered off and washed with CCl_4. The combined filtrate was concentrated and the residue was purified by chromatography on silica gel 60. Elution with 2% CH_3OH in CH_2Cl_2 gave a gummy material: yield 290 mg (60%); NMR ($CDCl_3$) δ 7.6 (c, 3 H, aromatic), 6.05 (s, 2 H, CH_2), 4.64 (s, 2 H, CH_2Br), 2.70 (s, 3 H, CH_3), 1.25 (s, 9 H, *t*-Bu); MS (FAB) m/z 366, 368 (MH^+).

Diethyl 2-Desamino-2-methyl-N^1-[(pivaloyloxy)-methyl]-N^{10}-propargyl-3,5,8-trideazafolate (25). A mixture of 240 mg (0.66 mmol) of compound **24**, 237 mg (0.66 mmol) of propargylamine 15, and 30 mg of MgO in 1.3 mL of DMAc was stirred at 90 °C for 19 h and then poured into ice water. The product was extracted with EtOAc and the extract was washed with water and dried over anhydrous Na_2SO_4. After removal of the solvent, the residue was purified by chromatography on silica gel 60 eluting with 2% CH_3OH in CH_2Cl_2: yield 220 mg (52%) of gummy material; TLC (5% CH_3OH in CH_2Cl_2) R_f 0.50; MS (FAB) m/z 646 (MH^+).

2-Desamino-2-methyl-3,5,8-trideazafolic Acid (5a). To a solution of 120 mg (0.19 mmol) of **25** in 4 mL of ethanol was added 1.2 mL of 1 N NaOH and 2.8 mL of water. The reaction mixture was stirred at 25 °C for 24 h and neutralized with 1 N aqueous HCl. After removal of the ethanol, the pH of the solution was adjusted to 3–4 with HOAc. The solid separated was collected, washed with water and dried: yield 60 mg (67%); mp 234–236 °C dec. It was further purified by conversion to the ammonium salt and subjected to preparative LPLC over a preparative C_{18} column with 8% CH_3CN in water as the eluent. All the fraction corresponding to the product was pooled, concentrated, and acidified with 1 N HCl to pH 3.5. The precipitate was filtered, washed with water, and dried. **5a**: UV (0.1 N NaOH) λ_{max} 300.5 nm (ϵ 20000); NMR (500 MHz, DMSO-d_6) δ 7.96 (d, 1 H, H^5, J = 2 Hz), 7.82 (d, 1 H, amide NH, J = 6.4 Hz), 7.68 (d, 2 H, $H^{2'}$, $H^{6'}$, J = 8.6 Hz), 7.52 (dd, 1 H, H^7, $J_{7,8}$ = 8.5 Hz, $J_{7,5}$ = 1.2 Hz), 7.46 (d, 1 H, H^8, J = 8.4 Hz), 6.86 (d, 2 H, $H^{3'}$, $H^{5'}$, J = 8.8 Hz), 5.89 (s, 1 H, H^3), 4.72 (s, 2 H, CH_2^9), 4.27 (m, 3 H, $CH_2C{\equiv}$, α-CH of glutamate), 3.19 (t, 1 H, $\equiv CH$, J = 2 Hz), 2.51 (s, 3 H, CH_3), 2.32 (m, γ-CH_2 of glutamate), 1.87 (m, 2 H, β-CH_2 of glutamate); MS (FAB) m/z 476 (MH^+). Anal. ($C_{26}H_{25}N_3O_6$) C, H, N.

2,6-Dimethyl-1,2,3,4-tetrahydroquinazolin-4-one (28). To a suspension of 2.95 (17 mmol) of 2,6-dimethylquinazolin-4-(3*H*)-one (**27**)[4] in 58 mL of HOAc was added 2.4 g (63 mmol) of $NaBH_4$ at 22–25 °C during 2 h. After that, the reaction mixture was stirred overnight at room temperature and neutralized with 20% NaOH. The solid was collected and washed with water. The crude product was crystallized from EtOH: yield 2.08 g (70%); mp 215–218 °C; NMR (TFA) δ 8.15–7.55 (c, 3 H, aromatic), 3.05 (c, 1 H, H^2), 2.55 (s, 3 H, CH_3), 1.95 (d, 3 H, CH_3, J = 7.8 Hz); IR (Nujol) 3280 (NH) 1645 (CO) cm^{-1}; MS m/z 176 (M^+).

2,6-Dimethyl-1-(trifluoroacetyl)-1,2,3,4-tetrahydroquinazolin-4-one (29). To a suspension of 2.24 g (12.7 mmol) of tetrahydroquinazolinone (**28**) in 20 mL of CH_2Cl_2 was added 2.15 mL (15.2 mmol) of trifluoroacetic anhydride at 10–15 °C. The reaction mixture was stirred overnight at room temperature, poured into ice water, and extracted with CH_2Cl_2. The CH_2Cl_2 layer was washed repeatedly with water, dried over Na_2SO_4, and evaporated. The residue was crystallized from EtOH: yield 2.25 g (65%); mp 129–130 °C; NMR ($CDCl_3$) δ 7.9–7.3 (c, 3 H, aromatic), 2.45 (s, 3 H, CH_3), 1.50 (d, 3 H, CH_3, J = 7.8 Hz); IR (Nujol) 3230 (NH), 1730 (CO), 1645 (CO) cm^{-1}; MS m/z 272 (M^+).

6-(Bromomethyl)-2-methyl-1-(trifluoroacetyl)-1,2,3,4-tetrahydroquinazolin-4-one (30). To a solution of 1.27 g (4.67 mmol) of **29** in 30 mL of CCl_4 were added 0.9 g (5.06 mmol) of NBS and 45 mg of benzoyl peroxide. The reaction mixture was refluxed and illuminated with a 250-W light bulb for 5 h. After removal of the solid succinimide, the solution was concentrated. The residue was purified by chromatography on silica gel 60,

eluting with 2% CH_3OH in CH_2Cl_2: yield 1.2 g (72%); mp 75–77 °C; NMR ($CDCl_3$) δ 8.0–7.6 (c, 3 H, aromatic), 4.50 (s, 2 H, CH_2Br), 1.51 (d, 3 H, CH_3, J = 7.5 Hz); MS (CI) m/z 351, 352 (MH^+).

Diethyl 2-Desamino-2-methyl-N^1-(trifluoroacetyl)-5,8-dideaza-1,2-dihydrofolate (31). A mixture of 405 mg (1.15 mmol) of bromide **30**, 414 mg of propargylamine **15**, and 48 mg (1.20 mmol) of MgO in 1 mL of DMAc was stirred at 80–90 °C for 18 h, poured into ice water, and extracted with EtOAc. Removal of the EtOAc gave the crude product, which was purified by chromatography on silica gel 60 with 4% CH_3OH in CH_2Cl_2 as the eluant. The appropriate fractions were concentrated under vacuum to afford 420 mg (58%): TLC (10% CH_3OH in CH_2Cl_2) R_f 0.69; NMR ($CDCl_3$) δ 7.9–7.3 (c, 7 H, aromatic), 4.15 (m, 4 H, CH_2, OCH_2CH_3), 1.10–1.50 (m, 9 H, CH_3, OCH_2CH_3); MS (FAB) m/z 631 (MH^+).

2-Desamino-2-methyl-N^{10}-propargyl-5,8-dideaza-1,2-dihydrofolic Acid (6). Method A. A mixture of 510 mg (0.81 mmol) of **31**, 18.8 mL of EtOH, 3.8 mL of water, and 5.67 mL of 1 N NaOH was stirred under nitrogen at room temperature overnight. The pH of the solution was adjusted to 7.5 with 1 N HCl. The solution was concentrated to ~10 mL and reneutralized to pH 4.5 with glacial HOAc. The precipitate was filtered, washed with water, and dried: yield 340 mg (87%); mp 198–200 °C dec; HPLC analysis indicated that the purity of the compound was 90%. It was purified by chromatography on a preparative C_{18} column with 5% CH_3CN in water as the eluent. All the fraction corresponding to the product was pooled, concentrated, and acidified with 1 N HCl to pH 3.5. The precipitate was filtered, washed with water, and dried. **6**: UV (0.1 NaOH) λ_{max} 297.4 nm (ε = 18011); NMR (DMSO-d_6, 300 MHz) δ 7.90 (d, 1 H, H^5, $J_{5,7}$ = 1.8 Hz), 7.66 (d, 2 H, $H^{2'}$, $H^{6'}$, J = 8.74 Hz), 7.54 (d, 1 H, H^1, $J_{1,2}$ = 1.9 Hz), 7.18 (dd, 1 H, H^7, $J_{7,8}$ = 7.9 Hz, $J_{7,5}$ = 2.1 Hz), 6.85 (d, 2 H, $H^{3'}$, $H^{5'}$, J = 8.90 Hz), 6.66 (d, 1 H, H^8, $J_{7,8}$ = 8.41 Hz), 4.45 (s, 2 H, CH_2^9), 4.09 (d, 2 H, J = 2.0 Hz, $CH_2C{\equiv}$), 4.04 (m, 1 H, α-CH of glutamate), 3.08 (t, 1 H, ≡CH, J = 2.0 Hz), 2.3 (m, 2 H, γ-CH_2 of glutamate), 1.82 (m, 2 H, β-CH_2 of glutamate); 1.14 (d, 3 H, 2-CH_3, J = 6.67 Hz); MS (FAB) m/z 479 (MH^+). Anal. ($C_{25}H_{26}N_4O_6$) C, H, N.

Method B. To a suspension of 100 mg (0.21 mmol) of **3** in 2.5 mL of THF and 0.4 mL of HOAc was added 120 mg (2 mmol) of sodium cyanoborohydride during 1 h at 20–25 °C. The reaction was monitored by HPLC until the peak of **3** disappeared. The total reaction time was ca. ~4 h. The reaction mixture was adjusted to pH 5–6 with NH_4OH and the THF was removed by evaporation under vacuum. The solution was then adjusted to pH 7–8 and applied to a C_{18} column, using 5% CH_3CN in water as the eluting solvent. The desired compound (**6**) thus obtained in 50% yield was identical in all respects (MS, NMR, HPLC) with an authentic sample of **6** obtained by the procedure outlined in Scheme IV.

Methyl 4-[(Propargylamino)methyl]benzoate (33). To a mixture of 3 g (15 mmol) of **32** and 4.14 g (30 mmol) of K_2CO_3 is 100 mL of CH_3OH was added dropwise 2.7 mL (30 mmol) of propargyl bromide. The reaction mixture was heated under reflux with stirring for 3 h and concentrated. The residue was purified by column chromatography over silica gel, using CH_2Cl_2 as eluting solvent: yield 530 mg (17%); NMR ($CDCl_3$) δ 8.13 (d, 2 H, H^2, H^6, J = 9 Hz), 7.53 (d, 2 H, H^3, H^5, J = 9 Hz), 3.99 (s, 3 H, CH_3O), 3.49 (d, 2 H, $CH_2C{\equiv}$, J = 2.2 Hz); MS (FAB) m/z 204 (MH^+).

N^{10}-Propargyl-5,8-dideazaisohomopteroic Acid (36). A mixture of 508 mg (2 mmol) of **34**,[22] 406 mg (2 mmol) of **33**, and 80 mg (2 mmol) of MgO in 5 mL of DMAc was stirred at 80 °C for 24 h and poured over 50 g of crushed ice. After the ice had melted, the solid that separated was washed with distilled water and dissolved in 25 mL of CH_3CN. A mixture of the solution and 75 mL of 0.1 N NaOH was stirred at room temperature for 18 h. The solution was neutralized with 1 N HCl and concentrated to ~20 mL. The pH of the concentrate was adjusted to 4–5 with HOAc. The solid separated was filtered, washed with distilled water, and dried over P_2O_5 under vacuum: yield 255 mg (35%); MS (FAB) m/z 363 (MH^+). The compound was judged to be pure by HPLC and used for the next step.

N^{10}-Propargyl-5,8-dideazaisohomofolic Acid (7). A mixture of 280 mg (0.77 mmol) of **36**, 206 mg (1 mmol) of DCC, and 240 mg (1 mmol) of diethyl L-glutamate and 20 mL of pyridine was stirred at 25 °C for 42 h. The pyridine was evaporated under reduced pressure and the residue was triturated with ice water and washed with distilled water. The crude product thus obtained was dissolved in 20 mL of 0.1 N NaOH was stirred at room temperature for 18 h. The pH of the mixture was adjusted to 7.5 with 1 N HCl and concentrated. The concentrate was acidified with HOAc and the solid was filtered, washed with distilled water, and dried: yield 150 mg (40%). It was further purified by conversion to ammonium salt and chromatographed over a preparative C_{18} column using 10% CH_3CN in water as the eluting solvent. **7**: mp >300 °C; UV (0.1 N NaOH) λ_{max} 250 nm (ε = 16438); NMR (TFA) δ 8.0–7.4 (c, 7 H, aromatic), 4.8 (s, 2 H, CH_2), 4.3 (m, 3 H, $CH_2C{\equiv}$, glu α-H), 3.8 (s, 2 H, CH_2), 3.1 (b, 1 H, ≡CH), 2.7–2.4 (c, 4 H, glutamate); MS (FAB) m/z 492 (MH^+). Anal. ($C_{25}H_{25}N_5O_6$) C, H, N.

2-Desamino-2-methyl-N^{10}-propargyl-5,8-dideazaisohomopteroic Acid (37). A mixture of 950 mg (2.6 mmol) of **35**,[4] 530 mg (2.6 mmol) of **33**, and 0.04 mg (2.6 mmol) of MgO in 5 mL of DMAc was stirred at 90–100 °C for 18 h and poured over 100 g of crushed ice. After the ice had melted, the water layer was decanted, and the gummy product was triturated with distilled water and decanted again. The solid thus obtained was dissolved in 65 mL of CH_3CN and stirred with 130 mL of 0.1 N NaOH at room temperature for 18 h. The solution was neutralized with 1 N HCl and concentrated to ~40 mL. The pH of the concentrate was adjusted to 4.5 with HOAc. The solid thus separated was filtered, washed with distilled water, and dried over P_2O_5: yield of **37**, 440 mg (47%); mp >300 °C; NMR (TFA) δ 7.9–7.6 (c, 7 H, aromatic), 4.78 (s, 2 H, CH_2), 3.82 (c, 4 H, CH_2, $CH_2C{\equiv}$), 2.94 (s, 3 H, CH_3); MS (FAB) m/z 362 (MH^+).

2-Desamino-2-methyl-N^{10}-propargyl-5,8-dideazaisohomofolic Acid (8). A mixture of 361 mg (1 mmol) of **37**, 240 mg (1 mmol) of diethyl L-glutamate, and 206 mg (1 mmol) of DCC in 20 mL of pyridine was stirred at 25 °C for 65 h. The pyridine was evaporated under reduced pressure and the residue triturated with 50 g of crushed ice and washed with distlled water. Diester of **8**: mp 137–140 °C; MS (FAB) m/z 547 (MH^+). The gummy product thus obtained was stirred with a mixture of 50 mL of 0.1 N NaOH and 17 mL of CH_3CN at room temperature for 18 h. The pH of the solution was adjusted to 7.5 with 1 N HCl and concentrate to ~20 mL. The concentrate was acidified to pH 4–5 with HOAc and the separated solid was filtered and washed with distilled water, yield 240 mg (49% based on **37**). It was further purified by conversion to the ammonium salt and chromatography over a preparative C_{18} column, using 7% CH_3CN in water as the eluting solvent. All the fractions corresponding to the product was pooled, concentrated, and acidified with HOAc to pH 4. The precipitate was filtered, washed with water, and dried. **8**: mp >300 °C; UV (0.1 N NaOH) λ_{max} 272 nm (ε = 10816); NMR (TFA) δ 7.9–7.3 (c, 7 H, aromatic), 4.8 (s, 2 H, CH_2), 3.8 (c, 4 H, CH_2, $CH_2C{\equiv}$); 3.2 (b, 1 H, ≡CH), 2.9 (s, 3 H, 2-CH_3), 2.7–2.3 (c, 4 H, glutamate); MS (FAB) m/z 491 (MH^+). Anal. ($C_{26}H_{26}N_4O_6$) C, H, N.

Biological Methods. Growth Inhibition. Cells were seeded into 96 well plates in 150 μL of medium (folate-free RPMI 1640, Gibco, 10% dialyzed FCS, Hazelton, 20 nM calcium leucovorin) by using a Perkin-Elmer propette. Seeding density was dependent upon the cell line used; SW480 cells were seeded at 8000 cells per well and MCF-7 cells were seeded at 10000 cells per well. Cultures were incubated for 24 h, 37 °C, 5% CO_2. Drug dilutions were prepared in medium, without leucovorin, at twice the concentration and were added to triplicate wells in 150 μL. Cultures were incubated for 96 h at 37 °C, 5% CO_2, and growth was assessed by using the MTT dye reduction assay. This assay was performed on a Perkin-Elmer propette by removing 200 μL of medium from each well and 100 μL of MTT [[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromode], Sigma, 5 mg/mL in phosphate-buffered saline] was added. Cultures were incubated for 1 h at 37 °C with 5% CO_2 on a rotating shaker platform. Following incubation, 150 μL of medium was removed and 100 μL of dimethyl sulfoxide (HPLC grade, EM Science) was added

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to each well. Plates were sonicated for approximately 10 s and the absorbance of each well was determined on a Titertek plate reader (Multiscan, MCC, Flow). The culture conditions for Manca human lymphoma cells were similar to those described for SW480 and MCF-7 cells.[11]

Transport Inhibition. Materials. ^{3}H-MTX was purchased from Amersham (specific activity 20 Ci/mmol) and purified by thin layer chromatography on Brinkmann Instruments, Inc., precoated cellulose TLC glass plates COL 300–25 a with 3% ammonium chloride mobile phase; R_f = 0.75. The purified MTX was extracted from the plate with water, dried under reduced pressure, and stored dry at −70 °C until use. Maximum storage time was 1 month. The procedure removed contaminants accounting for approximately 5% of the total radioactivity. Unlabeled MTX was a product of Sigma and was used without further purification.

Assay for Binding to the Reduced Folate Transporter. MOLT-4 human T-cell leukemia cells were grown in folate-free RPMI-1640 medium containing folate with 10% dialyzed fetal calf serum and 20 nM leucovorin to a density of ≤1 million cells per mL. The cells were harvested and 20 million cells were resuspended in 2.5 mL of assay buffer [107 mM NaCl, 20 mM Tris-HCl, 26.2 mM $NaHCO_3$, 5.3 mM KCl, 1.9 mM $CaCl_2$, 1 mM $MgCl_2$, and 7 mM glucose, pH 7.4 (Sirotnak et al.[19]) plus 10 mM dithiothreitol] at 37 °C.[16] To this was added 100 μL of MTX containing 1 μCi of purified ^{3}H-MTX. Aliquots of 0.45 mL were removed at specified times, layered on top of 0.5 mL of 1-bromododecane[19,20] in a 1.5-mL snap top disposable centrifuge tube, and centrifuged 30 s at 10000 rpm in a microcentrifuge to separate cells from medium and stop the reaction. The aqueous layer and most of the organic layer were then carefully removed, and tube bottom was cut off into a scintillation vial, and the pellet was extracted with 2 mL of 0.5 M NaOH at 37 °C overnight; 2 mL of 1 M HCl was then added, and the samples were counted in a liquid scintillation cocktail. Rates of MTX accumulation were linear for 8 to 10 min; all rates were calculated from uptake plots with four or five linear time points at ≤10 min. For determination of inhibition, test compounds at specified concentrations were added to the MTX solution at t = 0. Since the MTX concentration in the assay was well below K_m (K_m = 1 μM; G. K. Smith, unpublished), the calculated IC_{50} approximately equals K_i for a competitive inhibitor.

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Structure–Activity Relationships of Estrogenic Ligands: Synthesis and Evaluation of (17α,20*E*)- and (17α,20*Z*)-21-Halo-19-norpregna-1,3,5(10),20-tetraene-3,17β-diols

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As part our program to probe the molecular requirement for estrogen-receptor binding we undertook the synthesis and evaluation of the 17α,*E* and 17α,*Z* halovinyl estradiols. By use of an improved variation of the existing synthetic strategy, the targeted compounds were prepared stereospecifically and in 92–98% yields from the corresponding 17α,*E* or 17α,*Z* [(tri-*n*-butylstannyl)vinyl]estradiol 3-acetates. The novel estradiol derivatives were evaluated for their relative binding affinity (RBA) for the estrogen receptor with use of a rat uterine preparation. The results demonstrated a marked difference between the *E* and *Z* isomers and among the halogen employed. The *Z* isomers possessed significantly higher RBA values and the larger halogens (I, Br) were more effective than the smaller Cl substituent. These results modify the previous interpretations of estrogen-receptor binding for steroidal ligands. As a result, our design of (radio)halogenated ligands will incorporate this concern for *Z* vs *E* stereochemistry.

Our research laboratories have had a long standing interest in the development of a radiolabeled estrogen derivatives as potential in vitro/in vivo radiodiagnostic agents.[1-6] Among the criteria for such compounds are the need for high specific activity, appropriate radioemission properties, rapid labeling conditions, and high receptor affinity.[7-12] While our group, as well as others,[13-20] has made substantial progress on the first three criteria, the systematic evaluation of the receptor-binding characteristics of the ligands, as a prospective method for the development of the 19-norpregna-1,3,5(10),20-tetraene-3,17β-diol derivatives, has been generally deficient. From the beginning of our program we had an interest in the effect of stereoisomerism at the 21-position; however, the recent report of the synthesis and tissue distribution of the (17α,20*Z*)-21-iodo-19-norpregna-1,3,5(10),20-tetraene-3,17β-diol ((*Z*)-(iodovinyl)estradiol) (**21**) in which a com-

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