Folate Analogues. 32. Synthesis and Biological Evaluation of 2-Desamino-2-methyl-N^{10}-propargyl-5,8-dideazafolic Acid and Related Compounds[1]

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The chemical synthesis of three close analogues (2–4) of N^{10}-propargyl-5,8-dideazafolate (PDDF) is described. The quinazoline ring of 2 and 4 was constructed from the pivotal intermediate 9 in a novel and unambiguous manner during the final step of the synthesis under very mild conditions. 2-Desamino-2-methyl-N^{10}-propargyl-5,8-dideazafolate (DMPDDF) (2) was a strong inhibitor of human and *Lactobacillus casei* thymidylate synthases, whereas 2-desamino-2-(trifluoromethyl)-N^{10}-propargyl-5,8-dideazafolate (3) and 2-desamino-2,3-dimethyl-N^{10}-propargyl-5,8-dideazafolate (4) were only weak inhibitors of this enzyme. DMPDDF exhibited excellent growth inhibition of Manca human lymphoid leukemia and H35 hepatoma cells in culture. The inhibitor activities of 2 were 43- and 65-fold greater than that of PDDF, respectively, in these cell lines. H35R cells that are resistant to methotrexate by virtue of a transport defect were cross resistant to DMPDDF but not to PDDF. H35FF cells which have 70-fold greater amounts of thymidylate synthase compared to H35N cells were 130-fold resistant to DMPDDF. Furthermore, the toxicity of DMPDDF to H35 hepatoma cells could be completely reversed by thymidine, establishing its locus of action as thymidylate synthase. Trasnport studies in vitro established that DMPDDF effectively inhibits MTX influx into H35 hepatoma cells, whereas PDDF has no effect on MTX transport in this cell line. These data suggest that the greater activity of DMPDDF relative to PDDF is partly due to the ability of the former compound to enter cells via the MTX/reduced folate transport system. Enzyme inhibition data of 4 suggest that the presence of N^3H in DMPDDF is essential for binding to thymidylate synthase.

The remarkable potency of N^{10}-propargyl-5,8-dideazafolate (PDDF) as a specific inhibitor of the enzyme thymidylate synthase (TS, EC 2.1.1.45) coupled with its excellent in vivo activity as an antileukemic agent in murine tumor models[2] has prompted many investigators to synthesize several analogues of this lead compound as potential anticancer agents.[3-6] However, the clinical utility of PDDF has suffered due to its insolubility and subsequent nephrotoxicity.[7] Consequently PDDF has been withdrawn from further clinical trials. Like folate, and many antifolates, PDDF has been shown to be a substrate of folylpolyglutamate synthetase (FPGS) both in vitro[8] and in vivo.[9] Polyglutamyl metabolites of PDDF have been identified in animals[9] and tumor cells in culture,[10,11] the predominant form being the tetraglutamate or higher.[9,10] We have demonstrated previously that the synthetic polyglutamyl derivatives of PDDF were more inhibitory to human, *Lactobacillus casei*, and L1210 thymidylate synthases than PDDF.[12,13] From these data, it was reasonable to assume that like methotrexate, polyglutamylation of PDDF is a determinant of PDDF cytotoxicity. Therefore, analogues of PDDF that are capable of enhanced influx and polyglutamylation in tumor cells are potentially capable of exhibiting more favorable therapeutic indices compared to the parent compound. The replacement of the amino group at the 2-position of PDDF with a methyl or trifluoromethyl group was considered a logical approach to increase the lipophilicity of PDDF. After completion of our synthetic work of 2 and 3 and collection of their preliminary biological data, we became aware of a U.K. patent by Imperial Chemical Industries, covering a series of 2-desamino PDDF analogues in which compound 2 was also included as a potential antitumor agent.[14] However, the unambiguous chemistry we report in this paper for the synthesis of 2 and related compounds is different from that of the patent procedure. The excellent in vitro activity exhibited by 2 in cell culture, despite its relatively low inhibition of TS compared to PDDF, prompted us to examine whether the hydrogen at the 3-position of 2 was necessary for efficient binding to TS, presumably via hydrogen bonding. Therefore, compound 4 was prepared by replacing the 3-H of 2 with a methyl group to evaluate this possibility.

Chemistry

Commercially available 5-methylanthranilic acid (5) was

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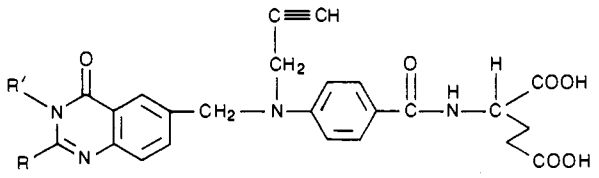


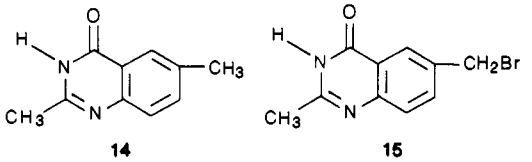
1: R = NH_2, R' = H
2: R = CH_3, R' = H
3: R = CF_3, R' = H
4: R = CH_3, R' = CH_3

acetylated according to a standard procedure with acetic anhydride to **6**. Benzylic bromination of **6** with dibromodimethylhydantoin (DDH) using benzoyl peroxide as a catalyst gave the desired benzyl bromide **7** in good yield. The preparation of diethyl *N*-[(propargylamino)-benzoyl]-L-glutamate (**8**) has been described previously.[24] Alkylation of **8** with **7** in the presence of MgO in DMAc gave the pivotal intermediate **9**.

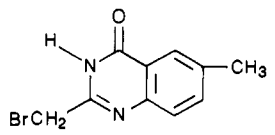
The carboxyl group of **9** was activated as the mixed anhydride with isobutyl chloroformate and reacted with gaseous ammonia. The intermediate thus obtained was subjected to base hydrolysis to obtain crude **2** that was purified by chromatography over DEAE-cellulose. Likewise, the mixed anhydride derived from **9** was treated with a neutralized solution of methylamine hydrochloride in DMF, and the resulting product was hydrolyzed with NaOH and purified by reverse-phase chromatography on a C_{18} column to obtain **4**. Both products **2** and **4** exhibited relevant NMR signals characteristic of the expected structures.

The synthesis of compound **3** was carried out by a different procedure. Conversion of **5** to **10** was accomplished by treatment of **5** with trifluoroacetic anhydride in CH_2Cl_2 (Scheme II). Reaction of **10** with ammonia followed by base treatment resulted in the formation of the desired quinazoline intermediate **11**. Bromination of **11** with DDH in the usual manner gave the benzylic bromide **12**. Alkylation of **8** with **12** was followed by base hydrolysis to obtain crude **3**, which was converted to the ammonium salt and purified by reverse-phase chromatography. Compound **3** exhibited spectral properties that were consistent with the expected structure.

While examining the synthetic route used by ICI for the preparation of **2**, it occurred to us that the structural integrity of the benzylic bromination product **15** is not un-



14 **15**



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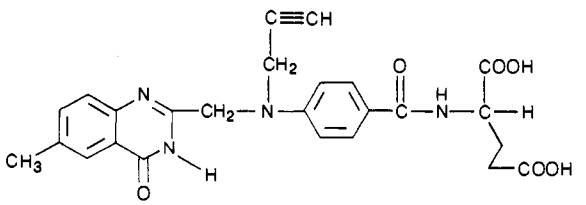

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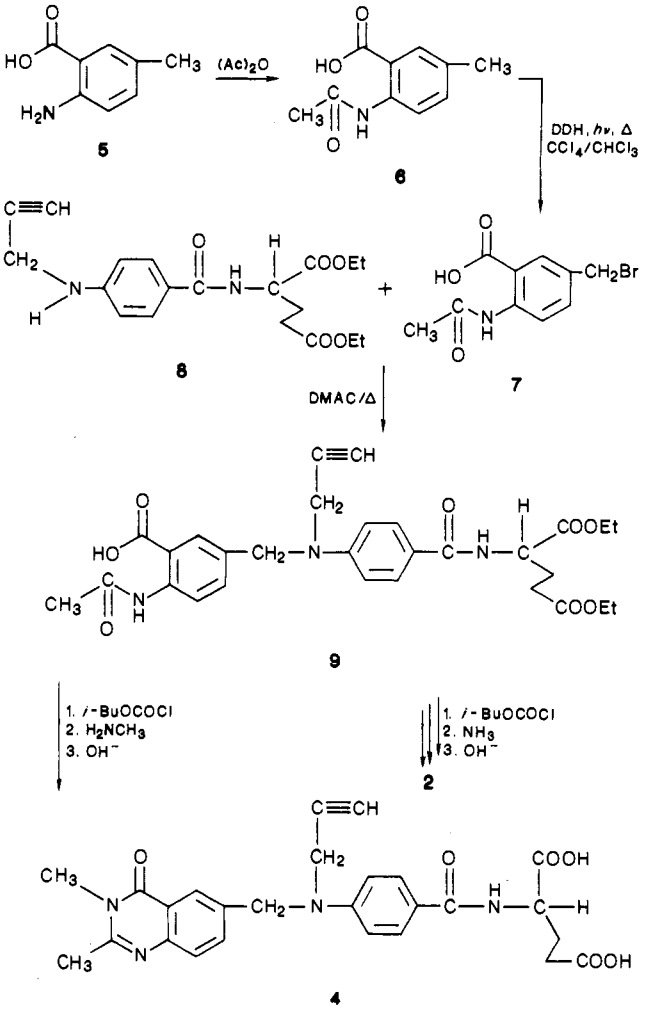
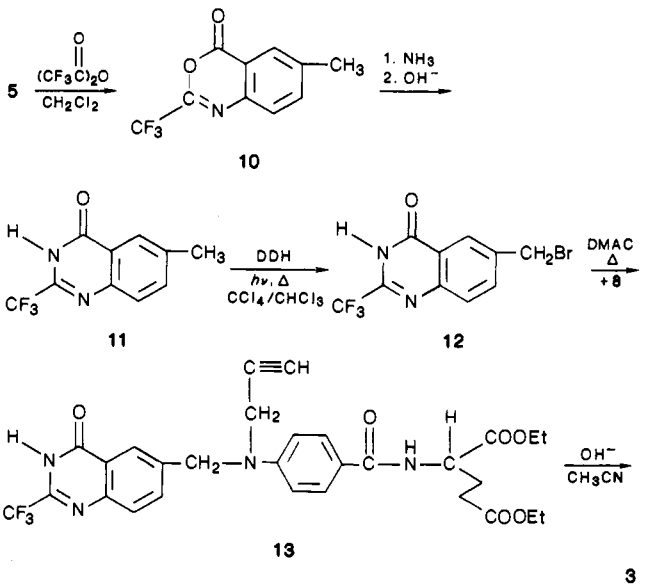
ambiguous, and an alternate structure **16** for this product was a theoretical possibility. Although bromination of the

Scheme I



Scheme II



2-methyl group could be prevented by the presence of a bulky N-protective group at N3 due to steric factors, the alternate possibility existed. Therefore, a direct comparison of our sample of **2**, prepared by the procedure of Scheme I with the sample of **2** obtained via bromination of **14**, appeared necessary to rule out an alternate structure **17** for the latter compound. This was accomplished as follows: The carboxyl group of **6** was activated as the mixed anhydride with isobutyl chloroformate. Reaction

of this mixed anhydride with ammonia followed by base hydrolysis gave 3,4-dihydro-2,6-dimethylquinazolin-4-one (14).[15] Bromination of 14 with DDH in the usual manner gave a single product 15. Reaction of 15 with 8 as described previously followed by base hydrolysis and chromatographic purification gave a product that was identical in all respects with the product prepared by the unambiguous procedure, thus confirming the structure of the compound prepared by ICI as 2.

Biological Evaluation and Discussion

All target compounds were evaluated as inhibitors of human thymidylate synthase derived from two sources. None of the compounds were better inhibitors of human TS compared to PDDF. The most inhibitory compound, 2, was at least 6 times less potent than PDDF as an inhibitor of human Manca lymphoma cell TS. DMPDDF (2) showed an I_{50} value of 9.8×10^{-9} M against purified TS derived from SV40-transformed human fibroblast cell lines. A similar pattern was obtained with *L. casei* TS, compound 2 being 5 times less potent than PDDF. The 2-trifluoromethyl analogue 3 weakly inhibited MCF-7 human breast cancer cells in culture (IC_{50} = 27 μM). Compound 3 was also remarkably less potent as a TS inhibitor ($I_{50} = 5.7 \times 10^{-6}$ M) in spite of its structural analogy with 2, indicating that electronic properties of substituents at the 2-position have a profound influence on enzyme inhibition. the 2,3-dimethyl analogue 4 was 1000 times weaker than PDDF as an inhibitor of TS with an I_{50} value of 11.3×10^{-6} M. Because of the low inhibitory activity of 3 and 4 toward TS and weak cytotoxicity against tumor cells in culture, they were not investigated further.

We also examined the inhibitory activity of DMPDDF against *L. casei* and purified human dihydrofolate reductase (DHFR). It was a very weak inhibitor of DHFR with an IC_{50} value of $>3 \times 10^{-5}$ M. Under the same conditions PDDF was a better inhibitor of this enzyme ($IC_{50} = 4.9 \times 10^{-6}$ M). The most promising compound, DMPDDF, was evaluated as an inhibitor of the growth of *Streptococcus faecium*, *L. casei*, and Manca human lymphoma cells. DMPDDF was 40 times more inhibitory than PDDF against the growth of Manca cells, even though it is only one-sixth as inhibitory as PDDF against Manca cell TS. Against *S. faecium* and *L. casei*, however, DMPDDF was less effective than PDDF as a growth inhibitor.

The antitumor potential of DMPDDF was evaluated in three strains of hepatoma cells in culture. The results are summarized in Tables II and III. DMPDDF was highly toxic to the wild type (H35N) cells with an IC_{50} of 5.5×10^{-8} M. Under the same conditions, the I_{50} of PDDF against the growth of H35N cell growth was 3.6×10^{-6} M. The activity of DMPDDF was in fact very close to that of methotrexate, which showed an IC_{50} value of 1×10^{-8} M against this cell line. As expected DMPDDF was approximately 130-fold less effective against TS amplified H35FF cell line.

Since the remarkable toxicity of DMPDDF against the wild type hepatoma cell line and human Manca lymphoma cell lines (64 and 40 times compared to PDDF) could not be explained in terms of its TS inhibition, it was evaluated as a inhibitor of other folate-dependent enzymes such as human and *L. casei* GAR formyltransferase and AICAR formyltransferase. The values in Table I show that DMPDDF has IC_{50} values greater than 6×10^{-5} M for these enzymes. These observations suggested that the excellent growth inhibitory activity exhibited by DMPDDF could be due to its metabolism to poly-γ-glutamates, which would be expected to be better inhibitors of TS.[13] The toxicity of DMPDDF in wild type H35 hepatoma cells could be completely reversed by thymidine, adducing independent evidence that its locus of action is thymidylate synthase.

It was quite surprising to note that DMPDDF was 40-fold less effective as an inhibitor of MTX transport resistant (H35R) cells. No such differential exist with PDDF as an inhibitor of H35N versus H35R cell growth. It is interesting to note that PDDF and DMPDDF are equitoxic to MTX-resistant (H35R) hepatoma cell lines. These striking results prompted us to investigate whether DMPDDF is taken up by H35N cells by the methotrexate transport system. Indeed, as shown in Table IV, DMPDDF effectively inhibited the transport of methotrexate, aminopterin, and (6*RS*)-5-formyltetrahydrofolate. Since MTX, (6*RS*)-5-formyltetrahydrofolate, and aminopterin share the same transport system in H35 cells for influx,[17-19] it was concluded that DMPDDF enters the cells

Table I. Inhibition of Folate-Dependent Enzymes by 2-Desamino-2-methyl-10-propargyl-5,8-dideazafolate (DMPDDF)

	IC_{50}, M			
	dihydrofolate reductase		thymidylate synthase	
	L. casei	human[a]	*L. casei*	human[b]
DMPDDF	3.5×10^{-5}	$>3.0 \times 10^{-5}$	1.0×10^{-7}	6.0×10^{-8}
PDDF	4.9×10^{-6} [c]		2.1×10^{-8} [c]	1.0×10^{-8}

	IC_{50}, M			
	GAR formyltransferase		AICAR formyltransferase	
	L. casei	human[b]	*L. casei*	human[b]
DMPDDF	$>1.7 \times 10^{-5}$	$>1.7 \times 10^{-5}$	$>6.0 \times 10^{-5}$	$>6.0 \times 10^{-5}$

[a] Pure human DHFR from lymphoblastoid cells (Prendergast, N.; Delcamp, T.; Smith, P.; Freisheim, J. H. *Biochemistry* **1988**, *27*, 3664). [b] Extracts of Manca human lymphoma cells. [c] Nair et al.[13]

Table II. Inhibition of the Growth of Microorganisms and Tumor Cells by 2-Desamino-2-methyl-N^{10}-propargyl-5,8-dideazafolate

	IC_{50}, nM		
	Manca[d]	*S. faecium*[e]	*L. casei*
DMPDDF	13.0	6.0	22.0
PDDF	560.0	0.13	0.4

	IC_{50}, nM		
	H35N	H35R[a]	H35FF[b]
DMPDDF	55.0	2240	7280
PDDF	3600	2000	*c*
MTX	10.0	906	10.0

[a] 100-fold resistant to methotrexate (MTX) via transport defect; also resistant to aminopterin. [b] Amplified for thymidylate synthase via (6*RS*)-5-formyltetrahydrofolate/FdUR selection. Equisensitive to trimetrexate, metoprin, and MTX. Contains $\simeq$70 times as much TS compared to H35N cells. H35 cells were grown as usual in 25 μM (6*RS*)-5-formyltetrahydrofolate with FdUR starting at 0.3 nM and increasing sequentially by 3-fold increments until 100 nM is reached. (6*RS*)-5-Formyltetrahydrofolate was held constant. The rationale for this procedure was according to Berger and coworkers (Berger, S. H.; Jenk, C. H.; Johnson, L. F.; Berger, F. G. *Mol. Pharmacol.* **1985**, *28*, 461). When TS inhibition was measured by ^{3}H release from [5-^{3}H]dUMP, values of 0.33 and 27.0 nmol/min per mg were observed for the wild type and FF amplified H35 cells, respectively. With the [^{3}H]FdUMP binding assay the respective values were 2.4 and 140. For methods, see: Tarantino; Galivan, J. *In Vitro* **1980**, *60*, 833. [c] Could not be measured; beyond the solubility of PDDF. [d] Manca cell growth assay described in: Thorndike, J.; Gaumont, Y.; Kisliuk, R. L.; Sirotnak, F. M.; Murthy, B. R.; Nair, M. G.; Piper, J. R. *Cancer. Res.* **1989**, *49*, 158. [e] Microbiological assay methods described in: Kisliuk, R. L.; Strumpf, D.; Gaumont, Y.; Leary, R. P.; Plante, L. J. *J. Med. Chem.* **1977**, *20*, 1531.

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Table III. Inhibition of Purified Human Thymidylate Synthase and the Growth of Colon Adenocarcinoma Cells by DMPDDF and Analogues

	IC_{50}, μM	
	human thymidylate synthase[a]	colon adenocarcinoma SW 480[b]
DMPDDF	0.0098	0.05
3	5.7	>100
4	11.3	>100

[a] Thymidylate synthase from a SV40-transformed human fibroblast cell line was cloned in *Escherichia coli* and the protein was purified to homogeneity by affinity chromatography (I. Dev and W. Dallas, personal communication). (Rode, W.; Scanlon, K. J.; Hynes, J. B.; Bertino, J. R. *J. Biol. Chem.* **1979**, *254*, 11538). It was assayed by the tritium-release method as described (Ferone, R.; Roland, S. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 5802), with 100 μM (6*RS*)-tetrahydrofolate as substrate. Standard errors of the IC_{50} values were less than ±10%. [b] SW-480 cells (15000/well) were seeded into 96-well plates in 150 μL of RPMI 1640 medium containing 10% dialyzed fetal calf serum and 20 nM (6*RS*)-5-formyltetrahydrofolate instead of folic acid as the folate source. Drug dilutions were prepared in culture medium at twice the final concentration and were added to triplicate wells in 150-μL aliquots. Cultures were incubated for 72 h at 37 °C in 5% CO_2. Effects on cell growth were determined by using the tetrazolium dye reduction assay described by Mossman (Mossman, T. *J. Immunol. Methods*, **1983**, *65*, 55–63). The 2-CF_3 analogue weakly inhibited MCF-7 human breast cancer cells in culture (IC_{50} = 27 μM).

Table IV. Inhibition of (6*RS*)-5-Formyltetrahydrofolate and Methotrexate Transport by DMPDDF in H35 Hepatoma Cells[a]

	percent uptake	
inhibitor	(6*RS*)-5-formyltetrahydrofolate (2 μM)	methotrexate (5 μM)
none	100[b]	100[b]
aminopterin (50 μM)	11.0	25.0
DMPDDF (50 μM)	8.5	25.0
methotrexate (50 μM)	22.0	25.0
(6*RS*)-5-formyltetrahydrofolate	22.0	25.0
PDDF (50 μM)	90.0	100
folic acid (50 μM)	83.0	85.0

[a] H35 hepatoma cells were cultured to confluence in 60-mm plates. They were then washed twice with Hanks BSS (4 mL) and incubated at 37 °C. The substances to be transported, [^{3}H]-(6*RS*)-5-formyltetrahydrofolate (2 μM), 3.8 × 10^5 dpm/nmol, and [^{3}H]methotrexate (5 μM), 5.5 × 10^5 dpm/nmol, were added in the presence or absence of inhibitor. (6*RS*)-5-Formyltetrahydrofolate uptake was linear for 10 min and MTX 4 min. These time points were used in this study. Sampling is done as described (Galivan, J. *Cancer Res.* **1979**, *39*, 735). The results are the average of duplicate samples. Error analysis of six identical duplicate sample was ±5%. [b] Actual control rates under these conditions were 0.25 nmol/g per min for (6*RS*)-5-formyltetrahydrofolate and 1 nmol/g per min for methotrexate.

by the MTX transport mechanism. Data presented in Table IV show that among the inhibitors tested, DMPDDF appears to be as effective as (6*RS*)-5-formyltetrahydrofolate, MTX, and aminopterin. Although the target enzyme is the same for both PDDF and DMPDDF, the mechanism of influx of these two compounds to H35N cells is not identical, and this difference in transport and accumulation appears to have a profound influence on the cytotoxicity of these compounds. The differential cytotoxicity of DMPDDF and PDDF in the cell lines examined suggest that in spite of the less potent inhibition of the target enzyme by the former, it could still exhibit better cytotoxicity than the latter by altered transport and accumulation. Therefore, development of PDDF analogues that are capable of altered transport and metabolism as antitumor agents is appealing.

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. Field strength of the various proton resonances is expressed in parts per million, and peak multiplicity is depicted as follows: s, singlet; br, broad singlet; d, doublet; t, triplet; q, quartet; c, unresolved multiplet, the center of which is given. Ultraviolet spectra were recorded on a Bausch and Lomb spectronic 2000 spectrometer interfaced with a Commodore superpet computer or a Gilford Response spectrometer. All HPLC analyses were done on a Waters 600 multisolvent delivery system equipped with a model 481 UV detector and Waters 740 data module. All mass spectra were determined by Dr. Susan Weintraub, University of Texas, San Antonio, TX. Elemental analysis were done by Galbraith Laboratories, Inc., Knoxville, TN. Where analyses are indicated only by symbols of the elements, analytical results obtained for these elements or functions were within ±0.4% of the theoretical values.

2-Acetamido-5-methylbenzoic Acid (6). To a stirring solution of 20 g (0.13 mol) of 2-amino-5-methylbenzoic acid (**5**) in 500 mL of water and 18 mL of concentrated HCl was added 25.6 mL of acetic anhydride during a period of 10 min and the stirring was allowed to continue for 15 more min. This solution was added to a stirring solution of 100 mL of 33% NaOAc maintained at 0 °C and the resulting precipitate of **6** was filtered, washed with water, and dried over P_2O_5 in vacuum: yield 19.6 (76.8%); mp 187 °C; NMR (TFA) δ 7.53 (dd, 1 H, J = 8 Hz, aromatic), 8.07 (d, 1 H, J = 2 Hz, aromatic), 8.12 (d, 1 H, J = 8 Hz, aromatic), 2.5 (s, 3 H, CH_3), 2.45 (s, 3 H, CH_3); MS (FAB) m/z 194 (MH^+).

2-Acetamido-5-(bromomethyl)benzoic Acid (7). A mixture of 7.72 g (40 mmol) of **6** and 5.72 g (20 mmol) of 1,3-dibromo-5,5-dimethylhydantoin (DDH) in 1 L of 1:1 CCl_4 and $CHCl_3$ was heated to reflux with stirring. The clear solution was illuminated with a 250-W light bulb, and 50-mg batches of benzoyl peroxide was added to it at 1-h intervals for 6 h. The reaction mixture was allowed to reflux for 18 h, concentrated to ~500 mL under reduced pressure, and cooled. The first crystals were filtered, washed with cold $CHCl_3$ and dried to obtain 3.8 g (35%) of pure **7**. An additional 6 g of crude **7**, contaminated with the degradation products of DDH, was recovered from the filtrate: mp 192–95 °C; NMR (TFA) δ 8.4 (d, 1 H, J = 7 Hz, aromatic), 8.23 (d, 1 H, J = 2 Hz, aromatic), 7.66 (dd, 1 H, J = 7 Hz, 2 Hz, aromatic), 4.45 (s, 2 H, CH_2Br), 2.45 (s, 3 H, CH_3); MS (FAB) m/a, 272, 274 (MH^+). Anal. ($C_{10}H_{10}BrNO_3$) C, H, Br.

2-Desamino-2-methyl-N^{10}-propargyl-5,8-dideazafolic Acid (2) and Its 3-Methyl Analogue (4). A mixture of 2-acetamido-5-(bromomethyl)benzoic acid (273 mg, 1 mmol), 360 mg (1 mmol) of **8**, and 50 mg of MgO in 2 mL of DMAc was stirred in a test tube at 90–100 °C for 18 h and poured over 50 g of crushed ice. After the ice had melted, the water layer was decanted and the gummy product triturated with distilled water and decanted again. The amorphous product **9** was dried over P_2O_5 in vacuum: yield 480 mg (87%); MS (FAB) m/z 552 (MH^+). The compound was judged to be pure by TLC and used for the next step.

(a) Preparation of 2. To a stirring solution of 551 mg (1 mmol) of **9** in 25 mL of dry DMF was added 0.15 mL (1.3 mmol) of *N*-methylmorpholine. The solution was cooled to 0 °C, and 0.15 mL (1.15 mmol) of isobutyl chloroformate was added. After 15 min at 0 °C, the solution was removed from the ice bath and allowed to warm to room temperature (~25 °C) and kept for 30 more min. The reaction mixture was again cooled to 0 °C and ammonia was bubbled through till the solution became distinctly basic. The reaction flask was stoppered and stirring continued at 25 °C for 18 h. On evaporation of DMF at 70 °C under reduced pressure and addition of 50 g of crushed ice, a yellow gum was obtained. The gummy product was separated by decantation and

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stirred with 70 mL of a 5:2 mixture of 0.1 N NaOH and acetonitrile for 16 h. The pH of the hydrolysate was adjusted to 7.5 with 1 N HCl and evaporated to ~20 mL. On addition of glacial HOAc, a thick yellow precipitate was formed, which was filtered, washed with water, and dried. Examination of this product by HPLC revealed the presence of several impurities that were removed by ion-exchange chromatography on DEAE-cellulose using a linear NACl gradient. **2**: yield 220 mg (46%); mp 158–60 °C; UV (0.1 N NaOH) λ_{max} 299.5 nm (ϵ = 26 199); NMR (TFA) δ 8.54 (d, 1 H, J = 2 Hz, aromatic), 8.15, 7.85 (c, 6 H, aromatic), 5.35, 4.77 (s, s, 2 H, 2 H, benzyl, propargyl), 5.11 (br, 1 H, α-H, glutamate), 3.0 (s, 3 H, 2-CH_3), 2.56–2.86 (c, 4 H, glutamate); MS (FAB) m/z 477 (MH^+). Anal. ($C_{25}H_{24}N_4O_6 \cdot 0.75H_2O$) C, H, N, O.

(b) Preparation of 4. Compound **9** (1 mmol) was activated to the mixed anhydride as described above, and stirred with 120 mg (3 mmol) of methylamine hydrochloride and 1 mL of triethylamine. After 18 h, DMF was removed under reduced pressure, and the residue triturated with 50 g of ice cold water. The gummy product thus obtained was stirred with a mixture of 20 mL of acetonitrile and 50 mL of 0.1 N NaOH for 16 h. The hydrolysate was worked up as described for **2** to obtain the crude product that was converted to the ammonium salt and purified by reverse phase chromatography on a C_{18} column using 10% acetonitrile in water as the eluting solvent. Yield, 120 mg (24%); mp, 138–40 °C; UV (0.1 N NaOH) λ_{max} 298 nm (ϵ = 27,283). NMR (TFA) δ 8.47, 8.07, 7.8 (c, 7 H, aromatic), 5.3, 4.72 (s, s, 2 H, 2 H, benzyl, propargyl), 5.07 (br, 1 H, H, glutamate) 3.85 (s, 3 H, N-Me) 3.05 (s, 3 H, 2-CH_3) 2.85-2.52 (C, 4 H, glutamate). MS (FAB) m/z 491 (MH^+). Anal. ($C_{26}H_{26}N_4O_6 \cdot 0.5H_2O$) C, H, N.

6-Methyl-2-(trifluoromethyl)-4*H*-benzoxazin-4-one (10). To a solution of 20 g of 2-amino-5-methylbenzoic acid (**5**) in 250 mL of CH_2Cl_2 was added slowly with stirring 35 mL of trifluoroacetic anhydride. The dark brown solution thus obtained was stirred overnight at 25 °C and evaporated under reduced pressure and the residue triturated with 500 g of crushed ice. The dark solid was filtered and washed repeatedly with small amounts of MeOH, whereupon white crystals of **10** were obtained. This compound (**10**) is unstable in air and quickly converted to 6-methyl-2-(trifluoroacetamido)benzoic acid, unless stored under strictly anhydrous conditions. **10**: yield 17.5 g (53%); mp 113 °C; NMR ($CDCl_3$) δ 8.1 (d, 1 H, J = 2 Hz, aromatic), 7.74 (c, 2 H, aromatic), 2.54 (s, 3 H, methyl); MS (FAB) m/z 230 (MH^+). Anal. ($C_{10}H_6F_3NO_2$) C, H, N.

6-Methyl-2-(trifluoromethyl)-4(3*H*)-quinazolinone (11). A solution of 9.16 g (40 mmol) of **10** in 50 mL of dry DMF was cooled to 0 °C in an ice bath, and a stream of ammonia was passed through with stirring for 30 min, when the solution became distinctly basic. This reaction mixture was stirred for 18 h at 25 °C and then evaporated to dryness at 60–70 °C under reduced pressure. The resulting gum was treated with 200 mL of 0.5 M NaOH for 16 h and acidified with 1 N HCl to pH 7.5. The white thick precipitate thus obtained was washed with water and dried: yield 8.5 g (92%); mp >300 °C; NMR (TFA) δ 8.3 (br, 1 H, aromatic), 7.95 (br, 2 H, aromatic), 2.6 (s, 3 H, CH_3); MS (FAB) m/z 229 (MH^+). Anal. ($C_{10}H_7F_3N_2O$) C, H, N.

6-(Bromomethyl)-2-(trifluoromethyl)-4(3*H*)-quinazolinone (12). A suspension of 2.28 g (10 mmol) of **11** in 400 mL of CCl_4 in a 1-L three-necked round-bottomed flask was heated to reflux when a clear solution was obtained. To this solution was added portionwise 1.43 g (10 mmol) of 2,4-dibromo-5,5-dimethylhydantoin during 10 min, and the reflux was allowed to continue for 18 h under illumination from a 250-W light bulb. During the first 6 h 50 mg each of benzoyl peroxide was added to the reaction vessel at 1-h intervals. After completion of the reaction, the turbid solution was filtered hot, and the filtrate was concentrated to ~150 mL and chilled to obtain the first crops of crystals (~400 mg) that was a mixture of dimethylhydantoin and the desired bromination product in ~1:1 ratio (NMR). Concentration of the filtrate to ~75 mL followed by overnight refrigeration gave crystals of pure **12**: yield 800 mg; mp >300 °C dec; NMR (TFA) δ 8.52 (d, 1 H, aromatic), 8.95 (c, 2 H, aromatic), 4.7 (s, 2 H, bromomethyl); MS (FAB) m/z 306, 308 (1:1) (MH^+). Anal. ($C_{10}H_6BrF_3N_2O$) C, H, Br.

2-Desamino-2-(trifluoromethyl)-N^{10}-propargyl-5,8-dideazafolate (3). (a) Preparation of 13. A mixture of 645 mg (~2 mmol) of bromide **12**, 720 mg (2 mmol) of diethyl *N*-[(propargylamino)benzoyl]-L-glutamate (**8**) and 800 mg (2 mmol) of MgO in 2 mL of DMAC was stirred at 80–100 °C for 16 h, poured over 100 g of crushed ice, and triturated to obtain a creamy white solid. The solid was filtered, washed twice with cold water, and dried over P_2O_5 in vacuum. TLC of the product showed the presence of a trace amount of **8**, which could be removed by trituration with 100 mL of ether and subsequent decantation. The soft white solid appeared to be hygroscopic at this stage: yield 950 mg (74%); TLC (5% MeOH in CH_2Cl_2), silica gel plates, R_f 0.35; MS (FAB) m/z 587 (MH^+).

(b) Hydrolysis of 13 to 3. The above solid was dissolved in 20 mL of THF and stirred with 200 mL of 0.1 N NaOH for 18 h and evaporated under reduced pressure at 30–35 °C to 150 mL. The pH of the solution was adjusted to 7.5 with 1 N HCl, reevaporated under reduced pressure to ~50 mL, and cooled to ~10 °C, and the pH of the concentrate was readjusted to 4.5 with glacial HOAc, whereupon a thick white precipitate was obtained. After overnight refrigeration, the precipitate was filtered, washed with water, and dried: yield 435 mg (51%); mp 176–180 °C; HPLC analysis indicated that the compound was contaminated with ~7% of impurities. It was further purified by conversion to the ammonium salt and chromatography over a preparative C_{18} column using 10% acetonitrile in water as the eluting solvent. **3**: NMR (TFA) δ 8.15 (s, 1 H aromatic), 7.7–7.4 (c, 6 H, aromatic), 4.9 (s, 2 H, bridge CH_2), 4.7 (t, 1 H, α proton of glutamate), 4.35 (s, 2 H, propargyl), 2.55–2.0 (c, 4 H, glutamate); UV (0.1 N NaOH) λ_{max} 298.6 nm (ϵ = 33 814); MS (FAB) m/z 531 (MH^+). Anal. ($C_{25}H_{21}F_3N_4O_6$) C, H, N.

4(3*H*)-2,6-Dimethylquinazolinone (14). To a solution of 1.92 g (10 mmol) of **6** in 50 mL of dry DMF, maintained at 0 °C, was added 1.63 mL (12.5 mmol) of *N*-methylmorpholine followed by 1.13 mL (10 mmol) of isobutyl chloroformate. After the solution was stirred for 15 min at this temperature, ammonia was bubbled through the solution for 20 min, when the solution became distinctly basic. The reaction mixture was stoppered and allowed to stir at 25 °C for 18 h. The solvent was removed by evaporation under reduced pressure at 65–70 °C, and the resulting viscous material was stirred with a mixture of 300 mL of 0.1 N NaOH and 100 mL of acetonitrile for 18 h. Most of the acetonitrile was removed by rotary evaporation, the pH was adjusted to 7.5 with 1 N HCl, and the solution thus obtained was concentrated under reduced pressure to 50 mL. The pH of this solution was adjusted to 5.0 with glacial HOAc whereupon crystals of **14** separated as white needles. These crystals were filtered, washed with water, and dried: yield 1.5 g (86%); mp 255 °C; NMR (TFA) δ 7.95 (d, 1 H, aromatic, J = 2 Hz), 6.5, 7.4 (d, d, 2 H, aromatic, J = 9 Hz), 2.65 (s, 3 H, CH_3), 2.24 (s, 3 H, CH_3); MS (FAB) m/z 175 (MH^+).

4(3*H*)-6-(Bromomethyl)-2-methylquinazolinone (15). To a refluxing solution of 3.5 g (20 mmol) of **14** in 300 mL of a 1:1 mixture of CCl_4 and $CHCl_3$ was added 2.86 g (10 mmol) of DDH and 50 mg of benzoyl peroxide. The solution was illuminated with a 250-W light bulb, and the refluxing was continued for 18 h, with the portionwise addition of 50 mg of benzoyl peroxide every hour for the first 6 h. The reaction mixture was concentrated to ~150 mL and cooled, and the crystals formed were filtered, washed with CCl_4, and dried: yield 2.5 g (50%); mp >300 °C; NMR (TFA) δ 8.35 (s, 1 H, aromatic), 8.0, 7.8 (d, d, 2 H, 2 H, J = 9 Hz, aromatic), 4.55 (s, 2 H, CH_2Br), 3.0 (s, 3 H, CH_3); MS (FAB) m/z 253, 255 (MH^+).

Preparation of 2 from 15. A mixture of 253 mg (1 mmol) of **15**, 360 mg (1 mmol) of **8**, and 40 mg (1 mmol) of MgO was stirred at 90–100 °C in 2 mL of DMAc for 16 h and poured over 50 g of ice and triturated. The solid thus obtained was separated by decantation and stirred with a mixture of 50 mL of 0.1 N NaOH and 20 mL of acetonitrile for 18 h. The pH of the solution was adjusted to 7.5 with 1 N HCl and chromatographed on a DEAE-cellulose column. The major product thus obtained in 67% yield was identical in all respects (MS, NMR, HPLC) with an authentic sample of **2** obtained by the procedure outlined in Scheme I.

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Synthesis and Tumor Uptake of 5-^{82}Br- and 5-^{131}I-Labeled 5-Halo-1-(2-fluoro-2-deoxy-β-D-ribofuranosyl)uracils

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A synthesis of 5-bromo- and 5-iodo-1-(2-fluoro-2-deoxy-β-D-ribofuranosyl)uracil (3 and 4) and their 5-^{82}Br and 5-^{131}I analogues has been developed. The tissue distribution of the radiolabeled compounds in BDF_1 mice bearing Lewis lung tumors has been investigated. After injection of the radiolabeled analogues of compounds 3 and 4 there was a rapid initial excretion of activity. Compound 3 was excreted unchanged in the urine. Residual activity in mice after 4 h showed a distribution characteristic of bromide (Br^-). Compound 4 was excreted mainly as unchanged starting material with increasing amounts of iodide (I^-) detected at later time periods, in addition to 5-iodouridine and unidentified metabolites at shorter time periods. Both 3 and 4 demonstrated a remarkable in vivo stability relative to related 5-substituted nucleosides that do not contain the 2′-fluoro group. The tumor uptake was minimal, with only the 5-bromo analogue demonstrating a slight elevation in tumor to blood ratios relative to other tissues. Compounds 3 and 4 were shown to compete with thymidine for the same binding site in the transport of nucleosides across the cell membrane in mouse erythrocytes. The inhibition constants (K_i) show that the compounds were weak competitors of thymidine binding to pyrimidine nucleoside transporter compared to physiological nucleosides. Other evidence indicates that compounds 3 and 4 are not substrates for mammalian kinase enzymes.

Pyrimidine nucleosides satisfy many of the requirements of a good tumor diagnostic agent. For example, naturally occurring pyrimidine nucleosides are used as building blocks for DNA and RNA, and many pyrimidine nucleoside analogues become trapped in neoplastic tissue by mimicking this behavior. Compounds such as 5-bromo-2′-deoxyuridine and 5-iodo-2′-deoxyuridine are incorporated into the DNA of animal and human tumor cells.[1,2] The radiolabeled deoxythymidine analogue [^{131}I]-5-iodo-2′-deoxyuridine has been shown to accumulate in a variety of animal tumors[3-6] and has recently been labeled with ^{123}I to scintigraphically image experimental tumors.[7] 5-Fluoro analogues such as 5-fluorouracil and 5-fluoro-2′-deoxyuridine have demonstrated selective accumulation in animal[8-10] and human tumors.[11,12] A number of ^{18}F-labeled pyrimidine nucleosides have also shown promise as imaging agents in studies with experimental in vivo tumor models.[13] The related arabino configuration nucleosides, 5-iodo- and 5-bromo-1-(2-deoxy-β-D-arabinofuranosyl)uracil, have also shown biological activity. These compounds, originally synthesized by Fox and co-workers,[14] were shown to have both antiherpetic activity against herpes simplex type 1 and type 2 infected cells and cytotoxicity toward normal lymphocytic cells in culture.

A series of radiolabeled pyrimidine nucleoside analogues with the structure 5-halo-1-(2-fluoro-2-deoxy-β-D-ribofuranosyl)uracil have been synthesized and investigated for their potential as scintigraphic radiotracers in diagnostic oncology. The presence of the 2′-fluoro ribo substituent has been shown to confer biochemical stability to other pyrimidine nucleosides by making them less susceptible to phosphorylytic cleavage catalyzed by the enzyme pyrimidine phosphorylase.[15,16] Two of the 5-halo compounds in this series, 5-chloro and 5-fluoro-1-(2-fluoro-2-deoxy-β-D-ribofuranosyl)uracil, were recently prepared as ^{14}C-labeled analogues and were shown to have a high tumor to blood ratio in Lewis lung tumor bearing mice.[16] We now describe an original synthesis of the compounds 3 and 4 and report the biological distribution of their radiohalogenated derivatives in BDF_1 mice bearing a Lewis lung carcinoma. A structural diagram equivalent to 3 and 4 was presented in an earlier paper on a series of 5-substituted nucleosides.[17] However, this structure appears to be in

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