Synthesis and Biological Evaluation of 5-Deazaisofolic Acid, 5-Deaza-5,6,7,8-tetrahydroisofolic Acid, and Their N^9-Substituted Analogues

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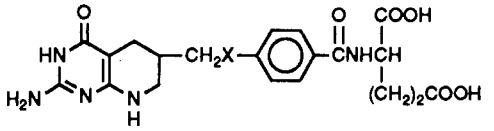
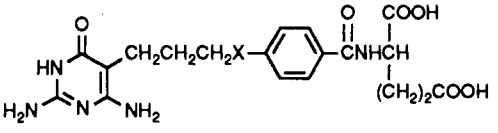
Prompted by recent disclosures concerning the potent antitumor activities of 5-deaza-5,6,7,8-tetrahydrofolic acid and 5,10-dideaza-5,6,7,8-tetrahydrofolic acid (DDATHF), we have prepared 5-deazaisofolic acid (**3a**) and 5-deaza-5,6,7,8-tetrahydroisofolic acid (**4a**). Reductive condensation of 2,6-diamino-3,4-dihydro-4-oxopyrido[2,3-*d*]pyrimidine with di-*tert*-butyl *N*-(4-formylbenzoyl)-L-glutamate and subsequent deprotection with trifluoroacetic acid yielded 5-deazaisofolic acid in good yield. Catalytic hydrogenation of this analogue then gave **4a**. The 9-CH_3 and 9-CHO modifications of **3a** and the 9-CH_3 derivative of **4a** were also synthesized. Each of the new analogues was evaluated with a variety of folate-requiring enzymes as well as MCF-7 cells in culture. Compound **4a** had an IC_{50} of ca. 1 μM against MCF-7 cells and was nearly 100-fold less potent than DDATHF in this regard. The three oxidized isofolate analogues were all poor inhibitors of tumor cell growth.

Numerous deaza analogues of folic acid have been synthesized and found to display promising levels of antitumor activity.[1,2] Of particular recent interest have been analogues of 5,6,7,8-tetrahydrofolic acid devoid of a nitrogen atom at position 5. For example, the compound 5,10-dideaza-5,6,7,8-tetrahydrofolic acid (DDATHF, **1a**) was shown to be a potent inhibitor of glycinamide ribonucleotide transformylase (GAR Tfase), the first of the two folate-requiring enzymes in the de novo biosynthetic pathway of purines.[3-5] Reversal studies using normal metabolites further implicated GAR Tfase as the primary metabolic target of **1a** in cell culture.[3,5] The structurally related compound 5-deaza-5,6,7,8-tetrahydrofolic acid (5-DATHF, **1b**) was also found to be an effective inhibitor of GAR Tfase and the growth of various tumors, having similar levels of activity as **1a**.[6,7]



1a: X = CH_2
b: X = NH

A second class of inhibitors of GAR Tfase has recently been identified. Two novel open-chain versions of **1a** and **1b** in which the C_7 carbon unit is deleted have recently been elaborated. The 5,10-dideaza derivative of this type has been designated as 7-DM-DDATHF (**2a**) since it is the



2a: X = CH_2
b: X = NH

7-desmethylene analogue of **1a**.[8] It was found to be somewhat less inhibitory toward the growth of CCRF-CEM cells than **1a** or **1b**.[9] The acyclic counterpart of 5,6,7,8-tetrahydrofolic acid and **1b** has been designated 5-DACTHF (**2b**).[10] This latter compound and its polyglutamylated homolgues inhibited GAR Tfase as well as 5-aminoimidazole-4-carboxamide ribonucleotide transformylase (AICAR Tfase) and was also effective against P388 leukemia in mice.[10]

Encouraged by these results demonstrating the chemotherapeutic potential of inhibitors of de novo purine biosynthesis, we embarked upon the synthesis of 5-deaza analogues of isofolic acid. This paper reports the preparation and biological properties of 5-deazaisofolic acid (**3a**), its 9-methyl (**3b**) and 9-formyl (**3c**) modifications, as well as 5-deaza-5,6,7,8-tetrahydroisofolic acid (**4a**) and its 9-methyl derivative (**4b**). The structures of these new analogues are presented in Table I. It will be noted that **3a** is also the 8-aza analogue of 5,8-dideazaisofolic acid (IAHQ), a compound developed earlier in this laboratory, which has demonstrated activity in several different animal models of cancer.[11-13]

Chemistry

The routes used to synthesize the new 5-deaza analogues of isofolic acid are depicted in Scheme I. Our initial approach was to prepare the known compound 2-amino-3,4-dihydro-4-oxo-6-nitropyrido[2,3-*d*]pyrimidine (**5**) and then convert it to the corresponding acetylated derivative

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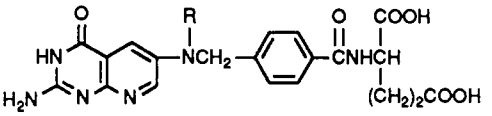
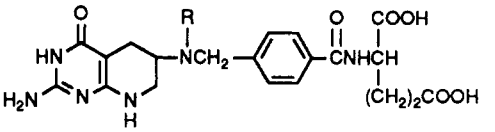
Table I. Biochemical and Antitumor Effects of 5-Deazaisofolic Acid, 5-Deaza-5,6,7,8-tetrahydroisofolic Acid, and Their 9-Substituted Derivatives



3a: R = H
b: R = CH_3
c: R = CHO

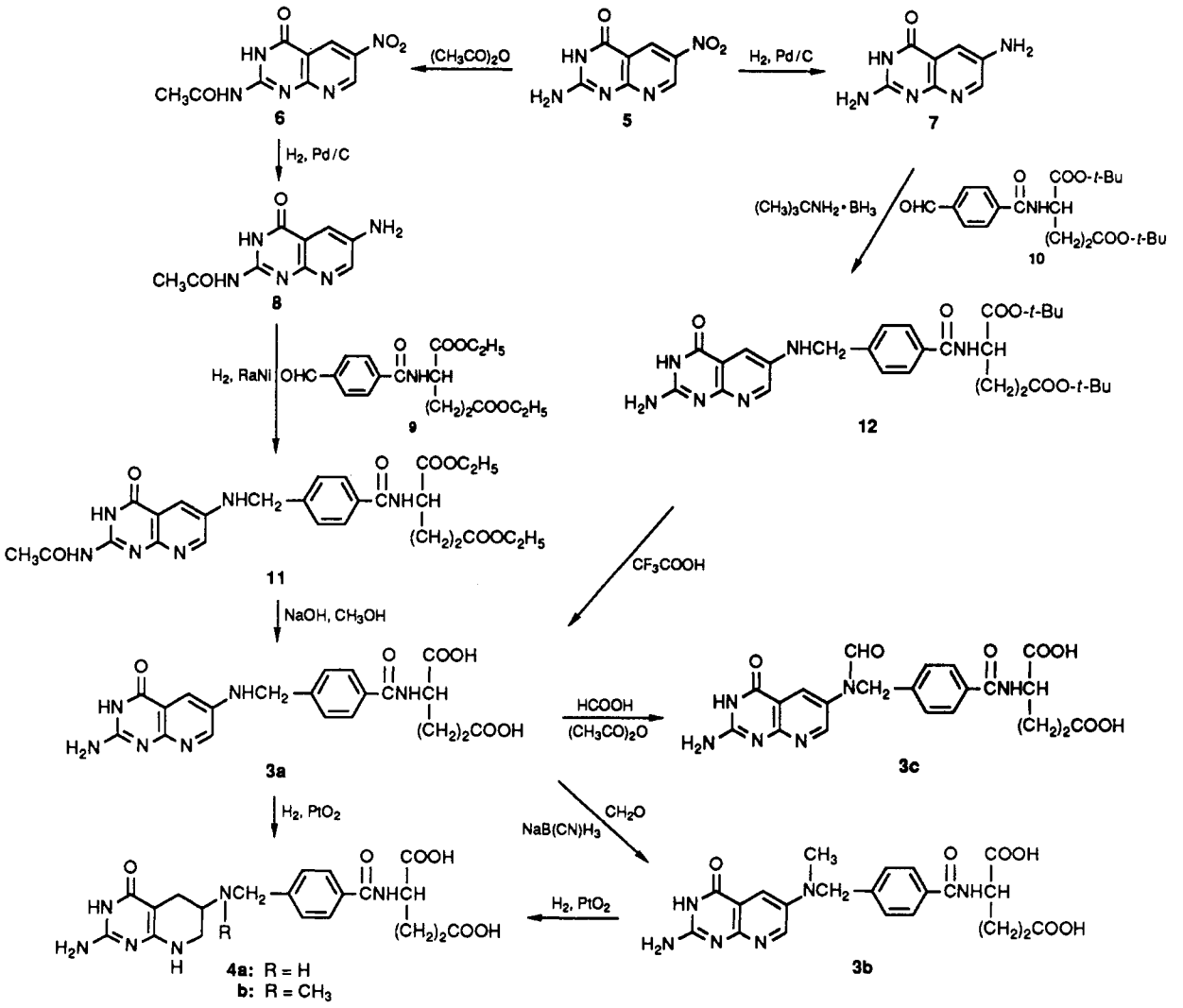


4a: R = H
b: R = CH_3

compd	I_{50},[a] μM: GAR TFase hog liver	AICAR TFase MOLT-4	TS human	DHFR WIL2	ED_{50},[a] μM: MCF-7 cell growth	K_i,[a] μM: [^{3}H]MTX influx MOLT-4
3a	85.9	62.8	7.1	0.21[a]	>100	>30
3b	≫100	141	0.48	0.25	35	
3c	≫100	49.9	28.8	1.95	>100	
4a	5.3[b]	≫100	≫100	>1000	1.2[b]	17.5[b]
4b	26	>100	≫100	>1000	4.5	22
5-DACTHF	3.0[c]	94[c]	≫100[c]	≫100[c]	0.037	1.0
DDATHF	0.22		>1000[d]	>100[d]	0.018	1.5

[a] Limits of variability < ±15%. [b] Average of two separate determinations. [c] Reported previously; cf. ref 10. [d] Reported previously; cf. ref 3.

Scheme I. Synthetic Routes to 5-Deazaisofolic Acid, 5-Deaza-5,6,7,8-tetrahydroisofolic Acid, and Their 9-Substituted Modifications



6 in order to improve solubility in organic solvents.[14] The 6-amino modification (8) was then obtained by catalytic hydrogenation. Compound 8 was then condensed reductively with diethyl *N*-(4-formylbenzoyl)-L-glutamate (9)[15] in the presence of Raney nickel. The fully protected in-

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Table II. Comparison of the Substrate Activities of Newly Synthesized Analogues for Hog Liver Folylpolyglutamate Synthetase[a]

compd	rel V_{max}[b]	app K_m, μM	rel V_{max}/ app K_m
3a	31.3	133 ± 18.5	0.23
3b	47.1	55.3 ± 11.4	0.85
3c		≫100	
4a	69.8	67.4 ± 9.2	1.0
4b	65.9	9.4 ± 3.4	7.0
5-DACTHF	100	6.7 ± 1.2	14.9
DDATHF	97.1	17.5 ± 2.6	5.6

[a] For purification and methods, cf. ref 10. [b] V_{max} relative to a control of 50 μM aminopterin included in each experiment.

termediate (**11**), which was obtained in very low yield, was then deblocked in base to afford 5-deazaisofolic acid (**3a**). Since large quantities of **3a** were required for the preparation of derivatives, a more satisfactory approach was developed. Compound **5** as the hydrochloride salt was subjected to catalytic hydrogenation to afford 2,6-diamino-3,4-dihydro-4-oxopyrido[2,3-*d*]pyrimidine (**7**) in good yield. This represents a considerable improvement over the earlier procedure, which involved the use of stannous chloride followed by the addition of gaseous hydrogen sulfide.[14] Reductive amination of di-*tert*-butyl *N*-(4-formylbenzoyl)-L-glutamate (**10**)[16] to **7** in the presence of *tert*-butylamine–borane proceeded smoothly to give di-*tert*-butyl 5-deazaisofolate (**12**). Treatment of compound **12** with anhydrous trifluoroacetic acid then gave the target compound **3a** in respectable overall yield.

The 9-CH_3 modification (**3b**) was obtained by treating **3a** with aqueous formaldehyde in the presence of sodium cyanoborohydride. Treatment of **3a** with a mixture of formic acid and acetic anhydride yielded the 9-CHO derivative **3c**. Reduction of **3a** and **3b** using hydrogen with platinum oxide as the catalyst gave the target compounds 5-deaza-5,6,7,8-tetrahydroisofolic acid (**4a**) and 9-methyl-5-deaza-5,6,7,8-tetrahydroisofolic acid (**4b**). These compounds were obtained as diastereomeric mixtures which were not resolved.

Biological Evaluation

As shown in Table I, each of the newly synthesized folate analogues was evaluated as an inhibitor of hog liver GAR Tfase and human AICAR Tfase, thymidylate synthase (TS), and dihydrofolate reductase (DHFR). The compounds 5-DACTHF and DDATHF were also included in each of the biological evaluations in order to permit the direct comparison of results obtained under identical experimental conditions. Target compounds were also tested as inhibitors of the growth of MCF-7 cells and **4a** and **4b** were studied with regard to their affinity for the reduced folate transporter protein. Finally, each analogue was evaluated as a substrate for hog liver folylpolyglutamate synthetase (FPGS). The bioassay results are presented in Table II.

Discussion

Each of the target compounds was evaluated against human DHFR. Compounds **3a** and **3b** are modest inhibitors of this enzyme and the I_{50} values are within 2-fold of the values of the corresponding 5,8-dideazafolates.[17] The 9-CHO modification (**3c**), however, is 7-fold less inhibitory than 9-formyl-5,8-dideazaisofolic acid.[17] As expected the 5,6,7,8-tetrahydro derivatives **4a** and **4b** as well as 5-DACTHF and DDATHF are not inhibitory toward DHFR, since they are analogues of the product of this reaction, 5,6,7,8-tetrahydrofolic acid.[3,10] Against human TS **3a** is a weak inhibitor, while its 9-CH_3 derivative is significantly more effective and its 9-CHO modification is approximately 4-fold less potent. A similar pattern was reported for the corresponding 5,8-dideazaisofolates using L1210 leukemia TS.[17] As is the case for 5-DACTHF and DDATHF, the reduced 5-deazaisofolates **4a** and **4b** are not inhibitory toward TS.[3,10] Interestingly, compounds **3a–c** do not effectively inhibit either GAR Tfase or AICAR Tfase. In addition, these analogues are poor inhibitors of the growth of MCF-7 cells in culture. It is remarkable that 5,8-dideazaisofolic acid is greater than 100-fold more effective as an inhibitor of MCF-7 cell growth than its counterpart, 5-deazaisofolic acid (**3a**).[18] This can be attributed at least in part to the fact that **3a** is considerably less effective as a substrate for FPGS (Table II) than its dideaza counterpart.[19]

The reduced analogue **4a** is a reasonably effective inhibitor of GAR Tfase, being less than 2-fold as potent as 5-DACTHF. However, it is more than 30-fold less potent than 5-DACTHF as an inhibitor of the growth of MCF-7 cells. The comparatively low level of cell growth inhibition by **4a** can be attributed to its reduced rate of influx into cells implied by its poor affinity to the reduced folate transporter protein as well as its lower substrate activity for FPGS (cf. Table II). The addition of a 9-CH_3 group to **4a** yielding **4b** gives rise to a 5-fold reduction in GAR Tfase inhibiton. As shown in Table II, **4b** is an effective substrate for FPGS, being 7-fold superior to **4a** and comparable to DDATHF. However, like **4a** it does not have good affinity for the reduced folate transporter protein as indicated by its large K_i value for the inhibition of the uptake of [^{3}H]MTX into MOLT-4 cells. The net result is that **4b** is nearly 4-fold less active as an inhibitor of cell growth than **4a**. Neither **4a** nor **4b** binds effectively to AICAR Tfase.

From the results presented in Table I, it can be seen that both DDATHF and 5-DACTHF should be readily taken up by MOLT-4 cells. Furthermore, while 5-DACTHF is approximately 14-fold less effective as an inhibitor of GAR Tfase than DDATHF, it is only 2-fold less active toward MCF-7 cells. However, as shown in Table II, 5-DACTHF is considerably better as a substrate for FPGS than DDATHF. It appears, therefore, that even a modest inhibitor of GAR Tfase can possess potent cell-killing activity provided that it can effectively gain entry into target cells and then be readily converted into polyglutamate metabolites. The key determinant of growth-inhibitory potency of these analogues appears to be their rapid conversion to high molecular weight polyglutamates, which are selectively retained by cells and which are more potent inhibitors of GAR Tfase. For example, the hexaglutamate of 5-DACTHF is approximately 38-fold more effective as an inhibitor of GAR Tfase from L cells than the parent compound.[10]

Experimental Section

Melting points were determined on a Mel-temp apparatus and are uncorrected. Elemental analyses were performed by Atlantic Microlabs, Inc., Atlanta, GA. Analytical samples gave combustion

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values for C, H, and N within ±0.4% of the theoretical values. Solvation due to H_2O was confirmed by the presence of a broad peak centered at approximately 3.4 ppm in the ^1H NMR spectrum which was transformed into a sharp singlet (DOH) by the addition of D_2O. The presence of CH_3COOH was confirmed by a peak at 2.0 ppm in the ^1H NMR spectrum of compound **12**. The presence of CF_3COOH was confirmed by ^{19}F NMR for compound **4b**, which contains CF_3COOH in the empirical formula. All intermediates were free of significant impurities on TLC on silica gel (Eastman 13181). Free acids **3a–c** and **4a,b** were checked for purity by TLC on cellulose (Eastman 13254). Column chromatographic separations for **11** were performed on Baker silica gel (60–200 mesh) and for compounds **3a–c** on Cellulose Mikro-Kristallin, Avicel from EM Science. The UV spectra were determined on a Hewlett-Packard 8451A spectrophotometer in 0.1 N phosphate buffer, pH 7.0. High-resolution ^1H NMR spectra were acquired on a Varian VXR-400. NMR values for chemical shifts are presented in parts per million downfield from Me_4Si as the internal standard, and the relative peak areas are given to the nearest whole number. Positive (M + 1) and negative (M − 1) ion FAB spectra were obtained on a VG 70SQ mass spectrometer at the Chemistry Department, University of South Carolina, Columbia, SC, by Dr. Michael Walla.

2,6-Diamino-3,4-dihydro-4-oxopyrido[2,3-*d*]pyrimidine (7). A suspension of 2-amino-3,4-dihydo-4-oxo-6-nitropyrido[2,3-*d*]-pyrimidine (**5**) as the hydrochloride salt[14] (2 g, 8.2 mmol) in DMF (900 mL) and 2-methoxyethanol (100 mL) was warmed to improve solubility and then cooled to room temperature. To this was added 10% Pd/C (1 g) and the mixture was hydrogenated at 42 psi until the theoretical uptake of H_2 had occurred (1 h). The fluorescent greenish solution was filtered through Celite and the filtrate evaporated to dryness. The resultant yellowish green solid (1.65 g) was suspended in H_2O (50 mL) and the pH of the suspension brought to 9.5 by dropwise addition of NH_4OH. This suspension was stirred at room temperature for 1 h and the solid separated by filtration and washed with H_2O and Me_2CO. The solid was dried under vacuum at 90 °C overnight to afford 1.13 g (78%) of yellowish product: mp >300 °C dec; TLC, no satisfactory system found: ^1H NMR (Me_2SO-d_6) δ 5.14 (s, 2, 6-NH_2), 6.70 (s, 2, 2-NH_2), 7.40 (d, 1, H_5, J_m = 3.03 Hz), 8.05 (d, 1, H_7, J_m = 3.04 Hz); FAB/MS *m/z* 178 (M + 1). Anal. ($C_7H_7N_5O$·0.25H_2O) C, H, N.

2-Acetamido-6-amino-3,4-dihydro-4-oxopyrido[2,3-*d*]pyrimidine (8). A solution of 2-acetyl derivative **6**[14] (3.01 g, 12.1 mmol) in DMF (175 mL) and 2-methoxyethanol (75 mL) was charged with 10% Pd/C (3 g) and hydrogenated at ~56 psi until the theoretical uptake of H_2 had occurred (30 min). The fluorescent solution was filtered through Celite to remove the catalyst and the filtrate evaporated to dryness to give a yellow solid, which upon recrystallization from DMF–H_2O gave 1.98 g (76%) of pure yellow product: mp >250 °C dec; TLC R_f 0.60 ($CHCl_3$–MeOH 8:2); ^1H NMR (Me_2SO-d_6) δ 2.15 (s, 3, CH_3CO), 5.70 (s, 2, 6-NH_2), 7.48 (d, 1, H_5, J_m = 2.5 Hz), 8.31 (d, 1,H_7, J_m = 2.5 Hz); FAB/MS *m/z* 220 (M + 1). Anal. ($C_9H_9N_5O_2$·H_2O) C, H, N.

Diethyl 2-Acetyl-5-deazaisofolate (11). A mixture of **8** (4.5 g, 20.54 mmol), diethyl *N*-(4-formylbenzoyl)-L-glutamate (**9**)[15] (6.7 g, 20 mmol), and Raney Ni (about 1.5 g damp) in 70% AcOH–H_2O (225 mL) was hydrogenated at ambient temperature with the pressure maintained at ~55 psi for 18 h. The mixture was treated with Norit and filtered through Celite. The filtrate was basified to pH 8 with NH_4OH. The resulting yellow solid was isolated by filtration, washed with H_2O and hexane, and then dried under vacuum. Next, it was treated with 100 mL of $CHCl_3$ and filtered to remove some insoluble pink solid. The solution was washed successively with 2 × 100 mL of 10% $NaHSO_3$, 1 × 100 mL of H_2O, 2 × 100 mL of 10% citric acid, and 2 × 100 mL of H_2O and then dried over $MgSO_4$. The solvent was removed under vacuum and the resultant red oily material was triturated several times with Et_2O to afford a pink-yellowish solid, which was dried, dissolved in $CHCl_3$–DMF, coated on silica gel, and then applied to a silica gel column (24 × 2.4 cm). After flushing the column with $CHCl_3$, the product was eluted with successive gradients of $CHCl_3$–MeOH: 99:1, 98:2, 97:3, and 95:5. Appropriate fractions were pooled and evaporated to dryness to afford 1.4 g (13%) of shining yellowish product: mp 220–222 °C (with preliminary softening and darkening); TLC R_f 0.65 ($CHCl_3$–MeOH 8:2); ^1H NMR (Me_2SO-d_6) δ 1.16 (m, 6, CH_2CH_3), 1.95–2.19 (m, 2, Glu β-CH_2 superimposed on 2.14, s, 3, CH_3CO), 2.42 (t, 2, Glu γ-CH_2, J = 15 Hz), 4.0–4.16 (m, 4, CH_2CH_3), 4.40–4.50 (m, 3, Glu α-CH and $NHCH_2$), 7.04 (t, 1, $NHCH_2$, J = 12 Hz), 7.24 (app d, 1, H_5, $J_{5,7}$ = 2.4 Hz), 7.48 (d, 2, 3′, 5′, J_o = 8 Hz), 7.83 (d, 2, 2′, 6′, J_o = 8 Hz), 8.44 (app d, 1, H_7, $J_{7,5}$ = 2.4 Hz), 8.67 (d, 1, CONH, J = 7.2 Hz), 11.57 (br s, 1, lactam NH or CH_3CONH), 11.94 (br s, 1, lactam NH or CH_3CONH); FAB/MS *m/z* 539 (M + 1). Anal. ($C_{26}H_{30}N_6O_7$·1.25H_2O) C, H, N.

Di-*tert*-butyl 5-Deazaisofolate (12). A suspension of **7** (0.64 g, 3.61 mmol) in glacial AcOH (250 mL) was warmed to improve solubility and cooled to ambient temperature. To this suspension was added di-*tert*-butyl *N*-(4-formylbenzoyl)-L-glutamate (**10**)[16] (1.18 g, 3.0 mmol), dissolved in AcOH (25 mL), with stirring. This mixture was charged with 3-Å molecular sieves and stirred under N_2 with exclusion of moisture for 18 h. Next, $(CH_3)_3CNH_2$·BH_3 (0.097 g, 1.12 mmol) was added and the solution was stirred at room temperature for 2 h and then warmed to 80 °C for an additional 2 h. The reaction mixture was filtered and the filtrate evaporated to dryness with the addition of EtOH under vacuum to afford a dark yellow solid, which upon crystallization from MeOH–DMF gave 1.17 g (71%) of the yellow product: mp >300 °C dec; TLC R_f 0.50 ($CHCl_3$–MeOH 8:2); ^1H NMR (Me_2SO-d_6) δ 1.37 [s, 9, $C(CH_3)_3$], 1.40 [s, 9, $C(CH_3)_3$], 1.85–2.06 (m, 2, Glu β-CH_2), 2.32 (t, 2, Glu γ-CH_2, J = 15.2 Hz), 4.29–4.35 (m, 1, Glu α-CH), 4.39 (d, 2, $NHCH_2$, J = 5.6 Hz), 6.30–6.40 (br s, 2, 2-NH_2), 6.57 (t, 1 $NHCH_2$, J = 12 Hz), 7.19 (d, 1, H_5, $J_{5,7}$ = 2.8 Hz), 7.46 (d, 2, 3′, 5′, J_o = 8 Hz), 7.82 (d, 2, 2′, 6′, J_o = 8.2 Hz), 8.22 (app d, 1, H_7), 8.53 (d, 1, CONH, J = 8 Hz) 11.56 (br s, 1, lactam NH); FAB/MS *m/z* 553 (M + 1). Anal. ($C_{28}H_{36}N_6O_6$·CH_3COOH) C, H, N.

5-Deazaisofolic Acid (3a). Method A. A solution of **11** (0.30 g, 0.53 mmol) in MeOH (50 mL) containing 1 N NaOH (3 mL) was stirred under N_2 at room temperature for 48 h and then at 45–50 °C for 2 h. The reaction mixture was cooled to room temperature and its pH adjusted to 3.5 by the dropwise addition of glacial AcOH. The cloudy solution was refrigerated overnight, the resultant precipitate was collected by centrifugation and washed with H_2O, Me_2CO, and Et_2O, and the product was dried under vacuum at 80 °C overnight to afford 0.16 g (68.6%) of yellow solid: mp >250 °C dec; TLC R_f 0.42 (5% NH_4HCO_3); UV λ_{max} 204 (ϵ 29.9 × 10^3), 240 (26 × 10^3), 280 (17.1 × 10^3), 372 sh (4.2 × 10^3); ^1H NMR (Me_2SO-d_6) δ 1.88–2.12 (m, 2, Glu β-CH_2), 2.34 (t, 2, Glu γ-CH_2, J = 14.8 Hz), 4.36–4.41 (m, 3, Glu α-CH and $NHCH_2$), 6.26–6.38 (br s, 2, 2-NH_2), 6.58 (t, 1, $NHCH_2$, J = 12.4 Hz), 7.19 (d, 1, H_5, $J_{5,7}$ = 2.8 Hz), 7.46 (d, 2, 3′, 5′, J_o = 8 Hz), 7.83 (d, 2, 2′, 6′, J_o = 8.2 Hz), 8.22 (app d, 1, H_7), 8.53 (d, 1, CONH, J = 7.6 Hz); FAB/MS *m/z* 441 (M + 1), 439 (M − 1). Anal. ($C_{20}H_{20}N_6O_6$·3H_2O) C, H, N.

Method B. A 0.30 g (0.49 mmol) sample of **12** was dissolved in 20 mL of CF_3COOH and stirred at ambient temperature for 2 h. The CF_3COOH was removed under vacuum and the residue triturated three times with Et_2O and dried under vacuum. The solid was dissolved in 10 mL of 1 N NaOH and reprecipitated by adjusting the pH to 3.5 by the dropwise addition of 1 N HCl. The solid was collected by filtration, washed with H_2O and Et_2O, and dried under vacuum. This impure compound was purified by dissolving it in 0.5 N NaOH (5 mL) and applying the solution to a cellulose column (14 × 1.5 cm) packed in 5% NH_4HCO_3. The product was eluted with 5% NH_4HCO_3. Appropriate fractions were pooled and the pH was ajusted to 3.5 by the dropwise addition of 1 N HCl. The cloudy suspension was left to stand at 4 °C to afford a yellow precipitate, which was collected by filtration, washed with H_2O and Et_2O, and dried under vacuum at 90 °C to yield 0.142 g (66%) of **3a**, identical by melting point, TLC, and NMR with the sample described in method A. Anal. ($C_{20}H_{20}N_6O_6$·0.5H_2O) C, H, N.

9-Methyl-5-deazaisofolic Acid (3b). A 0.30 g (0.66 mmol) sample of **3a** was dissolved in 18 mL of 0.2 N NaOH and then neutralized to pH 6.4 with 1 N HCl. To this was added 0.31 g (3.94 mmol) of 38% HCHO followed by 0.062 g (0.987 mmol) of $NaB(CN)H_3$. The pH of the reaction mixture was maintained at 6.5 for 90 min by occasional addition of 1 N HCl, and reaction was allowed to continue for 20 h. The solution was then adjusted to pH 11 with 1 N NaOH with stirring for 15 min to allow HCN gas to escape. Next it was acidified to pH 4 with 1 N HCl to

precipitate the product, which was collected by filtration, washed with H_2O and then Et_2O, and dried at 100 °C under vacuum to obtain 0.19 g of product. This material was purified by cellulose column chromatography using a 1.5 × 20 cm column. A solution of 0.25% NH_4HCO_3 was used to elute the product. Appropriate fractions were pooled and acidified to pH 4 with 1 N HCl to afford a yellow solid, which was collected by filtration washed with H_2O and then Me_2CO, and dried at 80 °C under vacuum to obtain 0.175 g (56%): mp >230 °C dec; TLC R_f 0.70 (5% NH_4HCO_3); UV λ_{max} 204 (ϵ 30.4 × 10^3), 237 (23.4 × 10^3), 288 (16.2 × 10^3), 394 sh (3.3 × 10^3); ^{1}H NMR (Me_2SO-d_6) δ 1.88–2.11 (m, 2, Glu β-CH_2), 2.34 (t, 2, Glu γ-CH_2, J = 14.5 Hz), 3.08 (s, 3, NCH_3), 4.34–4.41 (m, 1, Glu α-CH), 4.68 (s, 2, NCH_2), 6.26–6.44 (br s, 2, 2-NH_2), 7.32 (d, 2, 3′, 5′, J_o = 8.4 Hz), 7.38 (d, 1, H_5, $J_{5,7}$ = 3.2 Hz), 7.82 (d, 2, 2′, 6′, J_o = 8.4 Hz), 8.36 (br s, 1, H_7), 8.52 (d, 1, CONH, J = 7.6 Hz); FAB/MS m/z 455 (M + 1), 453 (M − 1). Anal. ($C_{21}H_{22}N_6O_6$·H_2O) C, H, N.

9-Formyl-5-deazaisofolic Acid (3c). A 0.15 g (0.33 mmol) sample of **3a** was dissolved in 10 mL of 98% HCOOH and to this solution was added 5 mL of Ac_2O with stirring at room temperature. After 90 min, the cream product was precipitated by the addition of Et_2O, followed by chilling in an ice bath for 30 min. The precipitate was collected by filtration, washed with H_2O, Me_2CO, and Et_2O, and then dried under vacuum at 80 °C to afford 0.10 g of solid. This was purified by dissolving it in 0.5 mL of 1 N NaOH and applying the solution to a cellulose column (1.5 × 20 cm) packed in 0.5% NH_4HCO_3. A solution of 0.25% NH_4HCO_3 was used to elute the product. Appropriate fractions were pooled and acidified to pH 4 with 1 N HCl and the resultant cloudy solution was left to stand at 4 °C overnight. The resulting dark yellowish precipitate was isolated by filtration, washed with H_2O, Me_2CO, and Et_2O, and dried under vacuum at 80 °C to obtain 0.08 g (50.4%) of **3c**: mp >235 °C dec; TLC R_f 0.91 (5% NH_4CO_3); UV λ_{max} 204 (ϵ 32.4 × 10^3), 274 (14.5 × 10^3), 326 sh (4.3 × 10^3); ^{1}H NMR (Me_2SO-d_6) δ 1.88–2.10 (m, 2, Glu β-CH_2), 2.34 (t, 2, Glu γ-CH_2, J = 14.4 Hz), 4.32–4.38 (m, 1, Glu α-CH), 5.12 (s, 2, NCH_2), 6.66–6.88 (br s, 2, 2-NH_2), 7.33 (d, 2, 3′, 5′, J_o = 8.4 Hz), 7.78 (d, 2, 2′, 6′, J_o = 8.4 Hz), 8.09 (d, 1, H_5, $J_{5,7}$ = 2.8 Hz), 8.52 (d, 1, CONH, J = 7.2 Hz), 8.60 (s, 1, CHO), 8.63 (d, 1, H_7, $J_{7,5}$ = 2.8 Hz); FAB/MS m/z 469 (M + 1), 467 (M − 1). Anal. ($C_{21}H_{20}N_6O_7$·1.25H_2O) C, H, N.

5-Deaza-5,6,7,8-tetrahydroisofolic Acid (4a). To a solution of **3a** (0.110 g, 0.222 mmol) in CF_3COOH (40 mL) was added PtO_2 (0.110 g), and the suspension was hydrogenated (45–55 psi) in a Parr apparatus for 18 h at 25 °C. The solution was filtered through Celite and the filtrate evaporated under reduced pressure with the help of added portions of Et_2O. The residue was treated with 10% NH_4OH (15 mL) and clarified by filtration. The pH of the light-yellow filtrate was adjusted to 4 by the dorpwise addition of AcOH. The solution was then cooled and diluted with EtOH (75 mL) to effect precipitation. The white solid was collected by filtration, washed with EtOH, and dried under vacuum at 60 °C to obtain 0.073 g (68%) of **4a**: mp >260 °C dec; TLC R_f 0.64 (5%, NH_4HCO_3); UV λ_{max} 204 (ϵ 24.7 × 10^3), 220 (27.5 × 10^3), 276 (11.9 × 10^3); ^{1}H NMR (D_2O) δ 2.0–2.28 (m, 2, Glu β-CH_2), 2.32–2.38 (m, 2, Glu γ-CH_2), 2.70–2.87 (m, 2, C_5-CH_2), 3.55–3.64 (m, 2, C_7-CH_2), 3.79–3.91 (m, 1, C_6-CH), 4.33–4.38 (m, 1, Glu α-CH), 4.44 (s, 2, NHCH_2), 7.63 (d, 2, 3′, 5′, J_o = 8.36 Hz), 7.88 (d, 2, 2′, 6′, J_o = 8.32 Hz) (The confident interpretation of this NMR spectrum was accomplished with the help of the two dimensional COSY spectrum which contained the appropriate cross correlation peaks.); FAB/MS m/z 445 (M + 1), 443 (M − 1). Anal. ($C_{20}H_{24}N_6O_6$·2H_2O) C, H, N.

9-Methyl-5-deaza-5,6,7,8-tetrahydroisofolic Acid (4b). A 0.108 g (0.228 mmol) sample of **3b** was dissolved in CF_3COOH (50 mL), and PtO_2 (0.075 g) was added. The suspension was hydrogenated (50 psi) for 18 h at 25 °C. The solution was filtered through Celite and the filtrate was evaporated to dryness under reduced pressure at 40 °C with the help of added portions of Et_2O. The residue was treated with 10% NH_4OH (10 mL) and filtered. The pH of the clear, colorless filtrate was adjusted to 4 with AcOH and the solution was evaporated to near dryness (2–5 mL) under reduced pressure and diluted with EtOH (50 mL) to precipitate a white solid. The product was isolated by filtration, washed with EtOH, and dried under vacuum at 65 °C to afford 0.082 g (75%) of **4b**: mp >300 °C dec; TLC R_f 0.66 (5% NH_4HCO_3); UV λ_{max} 204 (ϵ 26.3 × 10^3), 222 (28.8 × 10^3), 276 (12 × 10^3); ^{1}H NMR (D_2O) δ 2.0–2.25 (m, 2, Glu β-CH_2), 2.32–2.37 (m, 2, Glu γ-CH_2), δ 2.8 (app d, 3, N-CH_3), 2.84–2.89 (m, 2, C_5-CH_2), 3.66–3.71 (m, 2, C_7-CH_2), 3.73–3.79 (m, 1, C_6-CH), 4.33–4.38 (m, 1, Glu α-CH), 4.44 (s, 2, NCH_2), 7.60 (d, 2, 3′, 5′, J_o = 8.32 Hz), 7.88 (app d, 2, 2′, 6′, J_o = 8.4 Hz); FAB/MS m/z 459 (M + 1), 457 (M − 1). Anal. ($C_{21}H_{26}N_6O_6$·0.75CF_3COOH·H_2O) C, H, N.

Biological Evaluations. Homogeneous DHFR was obtained from human WIL2 cells as described earlier.[20] It was assayed spectrophotometrically at 340 nm using 9 μM dihydrofolate, 30 μM NADPH, 0.15 M KCl in 0.05 M Tris buffer (pH 7.4); [DHFR] = 0.0086 μM by MTX titration. Thymidylate synthase was obtained by cloning the gene from an SV 40-transformed human fibroblast cell line in *Escherichia coli*.[21] The protein was purified to homogeneity by affinity chromatography.[22] It was assayed by the ^{3}H-release method described by Ferone and Roland.[23] Compounds were evaluated as inhibitors of purified GAR Tfase according to the literature method.[10,24] Partially purified AICAR Tfase was obtained from MOLT-4 cells by a modification of the procedure of Mueller and Benkovic.[10,25] The continuous spectrophotometric assay was used to determine the I_{50} values.[26] The inhibition of the influx of [^{3}H]MTX into MOLT-4 cells was measured as described earlier.[27] The growth inhibitory activities of the newly synthesized compounds toward MCF-7 cells were evaluated according to the literature method.[28] Hog liver FPGS was purified by a modification of the method of Cichowicz and Shane.[10,29] The enzyme was assayed by the charcoal-absorption method,[30] with the KCl concentration being reduced to 20 mM. Aminopterin was employed as the standard substrate.

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Registry No. **3a**, 130327-67-2; **3b**, 130327-68-3; **3c**, 130327-69-4; **4a** (diastereomer 1), 130327-70-7; **4a** (diastereomer 2), 130350-21-9; **4b** (diastereomer 1), 130327-78-5; **4b** (diastereomer 2), 130327-77-4; **4b**·TFA (diastereomer 1), 130327-79-6; **4b**·TFA (diastereomer 2), 130327-80-9; **5**·HCl, 130327-71-8; **6**, 130327-72-9; **7**, 91996-79-1; **8**, 130327-73-0; **9**, 56277-36-2; **10**, 87597-84-0; **11**, 130327-74-1; **12**·AcOH, 130327-76-3.

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