5,10-Methylenetetrahydro-5-deazafolic Acid and Analogues: Synthesis and Biological Activities[1a,b]

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The synthesis of 5,10-methylene-5-deazatetrahydrofolic acid (2), a stable, rigid analogue of 5,10-methylenetetrahydrofolate (1), is reported as a potential inhibitor of thymidylate synthase. The target compound was obtained by a Fisher-indole type cyclization of the hydrazone 16 from 2-amino-6-hydrazino-4-oxopyrimidine (10) and diethyl *N*-[4-(3-formyl-1-pyrrolyl)benzoyl]-L-glutamate (15) followed by catalytic reduction of the product 17. Similarly, modification of the Fisher-indole type cyclization of the appropriate hydrazone precursors 11 and 12 afforded the nonclassical analogues 3-amino-7,8,9-trimethyl-2*H*-pyrrolo[3',4':4,5]pyrido[2,3-*d*]pyrimidin-1-one (4) and 3-amino-8-benzyl-7,9-dimethyl-2*H*-pyrrolo[3',4':4,5]pyrido[2,3-*d*]pyrimidin-1-one (5), respectively. The target compound 2, its aromatic precursor 18, and the nonclassical analogue 4 were evaluated as inhibitors of the growth of Manca human lymphoma cells and also as inhibitors of human dihydrofolate reductase, human thymidylate synthase, glycinamide ribonucleotide formyltransferase, and aminoimidazole carboxamide ribonucleotide formyltransferase. Compound 18 showed weak inhibition of lymphoma cell growth (IC_{50} = 42 μM) and of AICAR formylTF (IC_{50} = 17 μM). Compounds 2 and 4 did not inhibit lymphoma cell growth or thymidylate synthase. The inactivity of 2 was attributed to its lack of flexibility leading to its inability to bind to thymidylate synthase.

5,10-Methylenetetrahydrofolate (5,10-CH_2THF) (1) carries a labile methylene group, clamped between its N-5 and N-10 atoms, which it donates to deoxyuridine monophosphate (dUMP) in the thymidylate synthase (TS) catalyzed formation of deoxythymidine monophosphate (dTMP).[2] This reaction is the final step in the sole de novo pathway for the synthesis of dTMP. Thus inhibition of TS is an important goal in cancer chemotherapy.[3]

The conformational aspects of the cofactor 1 as it pertains to the mechanism of action of TS suggest that tricyclic 1 is initially recognized by TS and upon binding causes a significant conformational change in the enzyme.[4a,b] That the N-5,N-10 methylene bridge must open to a bicyclic system prior to the transfer of the methylene unit is supported by chemical studies.[5a,b]

The solution conformation of the cofactor 1 has been reported by Poe et al.,[6] and the stereochemistry defined by Slieker and Benkovic.[7] On the basis of the reported solution conformation, we considered that a rational approach to the design of a tricyclic cofactor analogue as an inhibitor of TS would be to introduce stability into the tricyclic system. The replacement of the N-5 with a carbon was expected to afford a stable semirigid 5-deaza analogue of 1. Thus the energy-minimized conformation (SYBYL Maximin) of 5,10-methylenetetrahydro-5-deazafolic acid (2) could be superimposed atom for atom on the solution conformation of 1. In order to function as a cofactor a labile methylene group is essential. In the cofactor 1 this is attained by the fact that the methylene moiety is flanked by two nitrogens. Compound 2, a 5-deaza analogue of 1, was expected to be stable under catalytic conditions, bind to TS, and prevent the binding of 1 and hence the transfer of the methylene unit to dUMP. Thus compound 2 was expected to function as an inhibitor of TS and as an antitumor agent.

Chemistry

Cyclocondensation of various biselectrophiles with substituted 6-aminopyrimidines provides a one-step approach to the synthesis of 5,6- and 6,7-disubstituted pyrido[2,3-*d*]pyrimidines. We have reported the synthesis of similar tricyclic systems using keto esters,[8] chlorovinyl aldehydes,[9] and vinyl aldehydes[10] as biselectrophiles with 6-aminopyrimidines. Initial attempts at the synthesis of the tricyclic ring system of 2 involved using biselectrophiles derived from N-substituted pyrroles. Though the methods

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(1) (a) Taken in part from the thesis submitted by J.P. to the Graduate School of Duquesne University in partial fulfillment of the requirements for the degree of Doctor of Philosophy, March 1990. (b) Presented in part at the *9th International Symposium on Pteridines and Folic Acid Derivatives Chemical, Biological and Clinical Aspects*; Zurich, Switzerland, September 3-8, 1989. *Chemistry and Biology of Pteridines 1989*; Curtis, H.-Ch., Ghisla, S., Blau, N., Eds.; Walter deGruyter: Berlin, 1990; pp 1060-1063.

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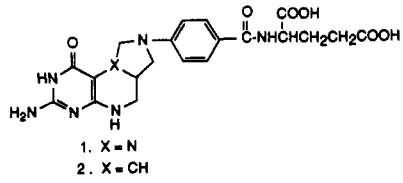
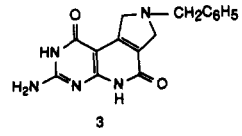
were fraught with difficulties we were able to synthesize the tricyclic pyridone **3** using ethyl *N*-benzyl-3-carbethoxypyrrolidin-4-one.[11] However, removal of the lactam carbonyl was not possible via reduction or chlorination followed by reduction as had been successful in previous reports of similar systems.[12]



1. X = N
2. X = CH



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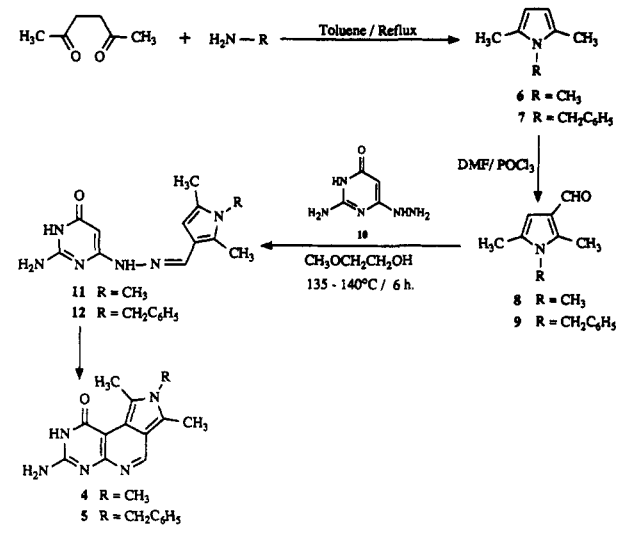
Synthetic problems in the preparation of appropriate biselectrophiles and their cyclocondensations or in their subsequent manipulations to the desired heterocyclic nucleus necessitated a reevaluation of the synthetic approach. The most obvious alternative was to adopt the multistep approach that has been utilized in the literature.[13] This involves a systematic construction of one ring followed by the other, in a stepwise fashion, in order to generate the desired tricyclic ring system. This procedure was utilized by Taylor et al.[14] in the synthesis of a number of tricyclic analogues of folic acid. While our work was in progress, Su and Watanabe[15] reported the synthesis of 2,4-diamino-6-(4-methoxyphenyl)-6,7-dihydropyrrolo[3,4-*c*]pyrido[2,3-*d*]pyrimidine via such an eight-step synthetic sequence. However, this method was reported to be unsuccessful in furnishing key intermediates required for the synthesis of 5,10-methylene-5-deazafolic acid.[15]

Instead of attempting a stepwise ring building approach, we chose to pursue an alternate strategy. A brief consideration of the mechanism of biselectrophile (ketoaldehydes) cyclocondensation with substituted 6-aminopyrimidines indicated that cyclocondensation commenced via attack by the most nucleophilic 5-position of the pyrimidine on the more reactive moiety of the biselectrophile (e.g. the aldehyde carbonyl in the keto aldehydes). Subsequent attack by the 6-amino group of the pyrimidine on the less reactive moiety of the biselectrophile (the ketone carbonyl in keto aldehydes) then serves to complete the cyclocondensation to afford the linear 6,7-disubstituted pyrido[2,3-*d*]pyrimidine. In order to synthesize the angular i.e. 5,6-disubstituted pyridopyrimidines, a reversal in this direction of cyclocondensation was required. That is, if the aldehyde were forced to react at the 6-amino

Scheme I. Synthesis of **4** and **5**



position of the pyrimidine, then ring closure at C-5 would afford the desired angular pyridopyrimidine skeleton. It was envisioned that hydrazone formation at the 6-position of the pyrimidine could be used as the first step in this strategy toward the synthesis of the desired tricyclic system.

A search of the literature for compounds possessing a similar pyrrolo[3,4-*c*]pyridine type substructure revealed supporting evidence for the feasibility of the proposed approach. Fritz and Schenk[16,17] have reported the reaction of phenylhydrazine with N-substituted pyrrole-3-aldehydes to afford the corresponding hydrazones. Subsequent cyclization of the hydrazones under acidic conditions proceeded via cyclization being forced at the carbon atom β to the aldehyde to afford the 2*H*-pyrrolo[3,4-*c*]quinolines. An independent and unambiguous synthesis of the 2-benzyl-1,3-dimethyl-2*H*-pyrrolo[3,4-*c*]quinoline proved that a Fischer-indole type cyclization does indeed provide a route to tricyclic pyrrolo[3,4-*c*]pyridine derivatives.[16,17] Extension of this synthetic procedure to the use of a 6-hydrazinopyrimidine, instead of phenylhydrazine, was thus anticipated to afford the desired pyrrolo[3,4-*c*]pyrido-[2,3-*d*]pyrimidine ring system.

Two potential targets, **4** and **5**, were identified for synthesis via this novel route. These model compounds were selected for the following reasons: (a) to investigate the feasibility of this cyclization strategy so as to adapt it for the synthesis of 5,10-methylene-5-deazafolate; (b) two methyl groups were introduced on the pyrrole atoms α to the nitrogen atom, for synthetic simplicity and to counter any possible acid-catalyzed polymerization of pyrroles;[18] and (c) these model compounds were anticipated to possess biological activity and were expected to be potential inhibitors of folate metabolizing enzymes.

The appropriate pyrroles **6** and **7** were first synthesized as shown in Scheme I. Acetonylacetone and benzylamine were reacted to afford 1-benzyl-2,5-dimethylpyrrole (**7**) as a yellow crystalline solid. Formylation of commercially available 1,2,5-trimethylpyrrole (**6**) with dimethylforma-

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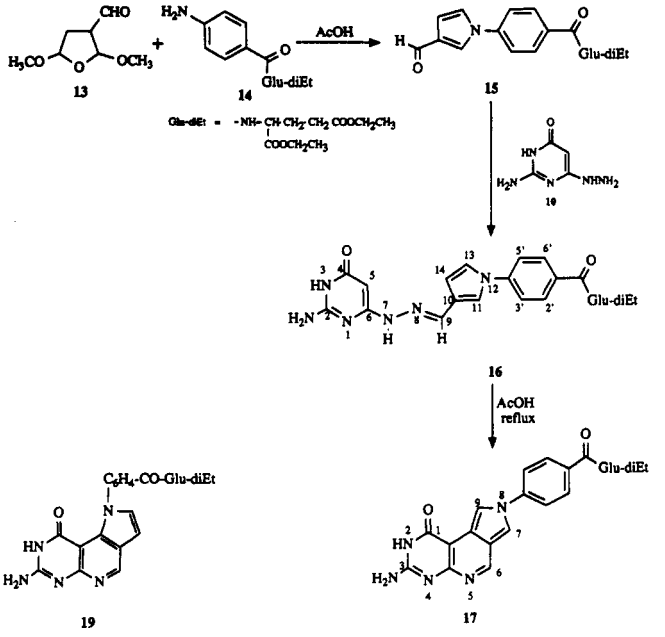
(18) Smith, G. F. The Acid Catalyzed Polymerization of Pyrroles and Indoles. *Adv. Heterocycl. Chem.* **1963**, *2*, 287–309.

mide and phosphorus oxychloride afforded the 3-formylpyrrole 8 in 91% yield. Similarly, 7 was formylated to afford 1-benzyl-2,5-dimethyl-3-formylpyrrole (9) as a tan solid in 96% yield. Commercially available 2-amino-6-chloro-4-oxopyrimidine was treated with excess hydrazine and refluxed to afford the desired hydrazinopyrimidine 10 in 90% yield. 3-Formyl-1,2,5-trimethylpyrrole (8) was refluxed with an equimolar amount of 10 in methoxyethanol to afford 3-formyl-1,2,5-trimethylpyrrole *N*-(2-amino-4-oxo-6-pyrimidinyl)hydrazone (11) in 83% yield. The ^{1}H NMR of 11 in DMSO-d_6 indicated the pyrimidine H-5 proton signal at 5.04 ppm which exchanged upon addition of D_2O. The pyrrole H-4 signal was observed at 5.98 ppm and the vinylic proton of the synthesized hydrazone occurred at 7.94 ppm. This together with the absence of a formyl proton signal at 9.70 ppm and the appropriate elemental analysis established the structure of the hydrazone 11. Reaction of 9 and hydrazinopyrimidine 10 in refluxing methoxyethanol similarly afforded 1-benzyl-2,5-dimethyl-3-formylpyrrole *N*-(2-amino-4-oxo-6-pyrimidinyl)hydrazone (12) as a tan powder in 82% yield.

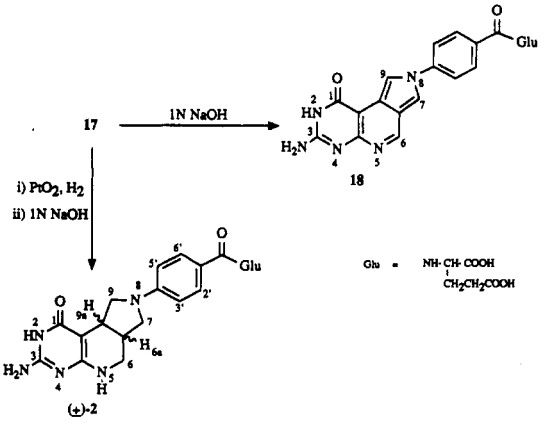
Cyclization of the hydrazone 11 in 30% HBr in CH_3COOH afforded the desired tricyclic 2-amino-4-oxo-5,6,7-trimethylpyrrolo[3,4-*c*]pyrido[2,3-*d*]pyrimidine (4) (90% yield). The ^{1}H NMR of 4 in DMSO-d_6 indicated the absence of the pyrimidine H-5 and pyrrole H-4 proton signals observed in the hydrazone 11, which indicated that the reaction proceeded via ring fusion at the pyrimidine C-5 and pyrrole C-4 to afford the product 4. In addition, a new aromatic signal was observed at 8.24 ppm. The ^{13}C NMR of 4 corroborated the tricyclic structure of 4 as assigned. Similarly, 12 on cyclization afforded after workup a 40% yield of 2-amino-6-benzyl-5,7-dimethyl-4-oxopyrrolo[3,4-*c*]pyrido[2,3-*d*]pyrimidine (5) as a yellow hygroscopic solid. The ^{1}H NMR spectrum in DMSO-d_6 once again indicated the absence of the pyrimidine H-5 and pyrrole H-4 signals of the hydrazone 12 and the appearance of a new aromatic signal integrating for one proton at 8.37 ppm.

Extrapolation of this strategy to the synthesis of target compound 2 first involved the synthesis of the appropriate pyrrole-3-aldehyde. While formylations using the Vilsmeier-Haack reaction of 1,2,5-trisubstituted pyrroles 6 and 7 readily afforded the corresponding pyrrole-3-aldehydes 8 and 9, this was not expected in the formylation of 1-substituted pyrroles without substitutions in the 2- and 5-positions, where isomeric mixtures were possible. Thus, an alternate method was devised as shown in Scheme II. 3-Formylpyrrole and its 1-substituted derivatives have been prepared from 2,5-dimethoxytetrahydrofuran-3-carboxaldehyde (13).[19,20] Reaction of 13 with commercially available diethyl (4-aminobenzoyl)-L-glutamate (14) in CH_3COOH afforded the diethyl *N*-[4-(3-formyl-1-pyrrolyl)benzoyl]-L-glutamate (15) as a viscous red oil. An attempt at distillation of this oil under high vacuum and temperature proved unsuccessful. The ^{1}H NMR of 15 in deuterated chloroform indicated the absence of the methoxy signals and the presence of three new aromatic signals at 6.87, 7.20, and 7.77 ppm, which were assigned to the pyrrole ring protons. The signal at 9.90 ppm was assigned to the aldehydic proton. These chemical shift

Scheme II. Synthesis of the Tricyclic 17



Scheme III. Synthesis of 18 and 2



positions correlated with those reported in the literature[21] for 1-substituted pyrrole-3-aldehydes.

Reaction of the 6-hydrazinopyrimidine 10 with the pyrrole aldehyde 15 afforded the desired hydrazone 16 as a yellow powder in 80% yield. Proof of the structure of the product 16 was obtained from its IR, ^{1}H NMR, and ^{13}C NMR spectra. Assignment of the pyrrole protons was accomplished via nuclear Overhauser experiments (NOE) on the ^{1}H NMR spectrum of 16 in DMSO-d_6. The proton-decoupled ^{13}C NMR spectrum of 16 in CF_3COOD indicated the presence of 25 distinct carbon atoms as expected for 16. Analysis of the proton-coupled ^{13}C NMR spectrum also indicated the correct number of methyl, methylene, methine, and quaternary C atoms as predicted for 16. These data together with elemental analysis of 16 established its structure.

Cyclization of hydrazones 11 and 12 to afford pyrrolo fused pyridopyrimidine hydrobromides 4 and 5, respectively, had been particularly facile in HBr/CH_3COOH. However, cyclization of the hydrazone 16 in HBr/CH_3-COOH led to the formation of intractable tars. Acid-catalyzed polymerizations are known to occur for pyrroles

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that are not substituted at the 2- or 5-positions.[18] In order to decrease the acidity of the reaction mixture, the hydrazone 16 was refluxed in glacial acetic acid under nitrogen, for a period of 24 h. Workup of the resultant precipitate afforded a brown powder. TLC analysis of the product indicated it to be a mixture of two major spots. Purification via column chromatography afforded the desired tricyclic compound 17 in an overall yield of 45%, as a brown solid. While the other product was isolated, it was not identified.

The aliphatic region of the ^{1}H NMR of 17 indicated signals for all the protons in the diethyl glutamate moiety, as expected. The absence of the pyrimidine H-5 proton in DMSO-d_6 at ca. 5.2 ppm and that of the hydrazone NH signal at 10.5 ppm substantiated that a Fischer-indole type cyclization via concomitant loss of ammonia had occurred. While the newly formed H-6 proton signal was anticipated to be downfield at 8.5–9.0 ppm, it could not be unequivocally identified apart from the other aromatic pyrrole protons which were tentatively assigned at 7.53 and 8.02 ppm. The proton-decoupled ^{13}C NMR of 17 in both DMSO-d_6 and CF_3COOD revealed the expected number (25) of carbon atoms and was in accord with the expected structure of 17.

Saponification of the tricyclic diester 17 in 1 N NaOH for 10 h, followed by acidification and chromatography on a cellulose column, afforded the pyrrolo fused pyrido[2,3-*d*]pyrimidine glutamic acid derivative 18 in 95% yield. The ^{1}H NMR of 18 in DMSO-d_6 indicated three aromatic signals at 6.93, 7.53, and 8.53 ppm, as was observed with the ester 17. The NMR spectrum of 18 in CF_3COOD exhibited these aromatic signals at 7.05, 7.40, and 8.65 ppm, which were exchanged about 90% on standing for 72 h. Similar proton exchange has been observed in related heterocyclic systems.[22] The ^{13}C NMR was in accord with the structure of 18 as designated. NOE experiments were performed on 18 in a mixture of DMSO-d_6 and D_2O and were restricted to the aromatic region of the spectrum so as to unequivocally assign the protons (H-6, H-7, and H-9) corresponding to the signals observed at 6.93, 7.53, 8.53. Irradiation of the signal at 6.93 ppm effected only one of the three aromatic signals at 7.53 ppm. This established that the proton corresponding to the signal at 6.93 ppm was in the immediate vicinity of the proton which occurred at 7.53 ppm. Irradiation of the signal at 7.53 ppm exhibited effects on the protons occurring at 6.93 ppm and the AB q signal at 7.71 ppm. This established that the H-7 proton occurs at 7.53 ppm and was in close proximity to the H-6 proton at 6.93 ppm as well as to the ortho protons of the phenyl ring which occur at 7.71 ppm (AB q). Thus H-9 must be assigned to the signal at 8.53 ppm and it should exhibit NOE effect only on the ortho protons at 7.71 ppm. Irradiation at 8.5 ppm did indeed lead to an effect on only the 7.71 ppm AB q signal. This confirmed the proton assignments for 18 as follows: H-6 = 6.93 ppm, H-7 = 7.53 ppm, and H-9 = 8.53 ppm. The downfield shift (1.00 ppm) of H-9 as compared to chemically similar H-7 could be attributed to the anisotropy of the carbonyl on the 1-position resulting in a deshielding effect.[23] The upfield position (6.93 ppm) of the H-6 signal was unexpected.[24]

Selected decoupling experiments in the ^{13}C NMR of 18 in DMSO-d_6 were performed for all five aromatic proton signals which led to the identification of their chemical shifts in the ^{13}C NMR spectrum. It was thus established that C-6 occurred as a doublet centered at 110.11 ppm (J_{C-H} = 177 Hz). C-7 and C-9 exhibited signals in close proximity to each other, as expected, at 120.72 and 120.45 ppm, respectively. Both exhibited the same $^1J_{C-H}$ = 189 Hz. This ^{13}C NMR data also served to establish the fact that the product isolated from the cyclization of the hydrazone 16 was indeed the pyrrolo[3,4-*c*]pyridine type compound as in 17 and 18, and not the isomeric pyrrolo-[2,3-*d*]pyridine type system as in 19. This was important to establish because cyclization of the hydrazone 16 could also occur at the pyrrole 2-position to afford 19 and/or at the pyrrole 4-position to afford 17. The equivalent nature of both pyrrole methine carbon atoms (C-7 and C-9) as indicated by their close chemical shifts and identical coupling constants in the ^{13}C NMR further supported the pyrrolo[3,4-*c*]pyridine type structure as in 18 and not the pyrrolo[2,3-*d*]pyridine as in 19. In addition, it is known that for 3-arylpyrroles the α-carbon generally occurs about 10 ppm downfield as compared to the β-carbon atom in the ^{13}C NMR.[25-28] Thus, C-α occurs between 114.5 and 118.8 ppm ($^1J_{C\alpha-H}$ = 182–184 Hz), whereas C-β occurs in 105.6–106.1 ppm ($^1J_{C\beta-H}$ = 169–171 Hz). Both C-7 and C-9 of 19 were found to occur near 120 ppm ($^1J_{CH}$ = 189 Hz), indicating their C-α nature and hence the pyrrolo-[3,4-*c*]pyridine structure of 18 as shown.

Rings B and C of the tricyclic compound 17 were reduced using platinum oxide in 90% aqueous trifluoroacetic acid at 50 psi H_2 pressure. Reduction was allowed to proceed only until 90% of the calculated amount of H_2 was consumed (48 h) to afford the reduced product contaminated with some starting 17. Longer hydrogenation times reduced yields of the desired product 2 and afforded additional unidentified products. The crude product was directly saponified to afford 2 contaminated with about 12% (^{1}H NMR) of 18. Purification, to remove 18, was carried out by column chromatography using cellulose and eluting with 15% aqueous acetic acid. Fractions containing the reduced product were pooled and freeze-dried to afford 2 as a tan powder in 80% yield. TLC analysis of the product in three different solvent systems indicated a single diffused spot which suggested that only one geometric isomer had been formed rather than a mixture of both cis and trans isomers at the B/C ring junction. While double-bond migration is commonly observed with palladium catalysts, this is generally not the case when platinum catalysts are used in hydrogenation reactions.[29,30] Since hydrogenation of 17 afforded only one product, it must

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Table I. Cell Growth and Enzyme Inhibition Data (IC_{50}, μM)

compd[a]	Manca cell growth	DHFR human[c]	DHFR *L. casei*	TS human[d]	TS *L. casei*	GAR formyl TF[e]	AICAR formyl TF[e]
2	>20[f]	>40[g]	5	>40		>40	>40
4	>10	>95[h]			95	>95	>95
18	42	>80[i]			>100	>95	17

[a] Compounds were dissolved in DMSO. Controls indicated that DMSO did not interfere with the assays at concentrations used. [b] Manca human lymphoma cells[33] were obtained from Dr. F. M. Sirotnak, Memorial Sloan-Kettering Institute for Cancer Research, and grown in RPMI medium with 10% fetal calf serum. Growth was estimated by tetrazolium reduction. [c] Recombinant human DHFR supplied by Dr. J. H. Freisheim.[44] [d] Recombinant human TS supplied by Dr. D. V. Santi.[37] [e] (6*R*)-10-Formyltetrahydrofolate used as substrate. [f] No inhibition. [g] 34% inhibition. [h] No inhibition. [i] 23% inhibition.

have been the cis isomer. Booth et al.[31] have demonstrated that chemical shift positions of ring junction carbon atoms of fused ring systems are significantly different in the cis and trans isomers. The ^{13}C NMR spectrum of **2** exhibited the expected number of carbon atom signals. This further suggested that only the cis isomer was formed since a ^{13}C NMR spectrum of a cis/trans mixture would exhibit more than the required number of carbon atom signals. It should be noted that reduction of the B and C rings of **17** results in the generation of two chiral centers at C-6a and C-9a. Thus *cis*-5,10-methylenetetrahydro-5-deazafolate **2** was synthesized and isolated as a diastereomeric mixture, without any of the trans isomer. In this cis mixture one diastereomer has the C ring above the plane of the pyrimidine A ring similar to the proposed conformation of the cofactor **1**. The other diastereomer of **2** has its C ring below the plane of the pyrimidine A ring.

The ^1H NMR spectrum of **2** confirmed the reduction of the pyridopyrimidine system. In the case of the aromatic tricyclic precursor **18**, the AB q system for the phenyl moiety was found to occur at 7.69 and 8.02 ppm (J = 8.32 Hz) in DMSO-d_6. Hydrogenation of the pyrrolo system to the corresponding alicyclic pyrrolidino system should alter the availability of the lone pair of electrons on N-8, thereby altering the chemical shifts of the AB q system. For the hydrogenated product **2** the AB q system was observed at 6.55 and 7.75 ppm (J = 8.7 Hz). These values correlate well with those observed for other tetrahydro, tricyclic, classical folates such as **1** reported in the literature. In addition the ^1H NMR spectrum of **2** also revealed the presence of the NH-5 proton signal at 6.49 ppm. The absence of aromatic signals at 6.93, 7.53 and 8.53 ppm of purified **2** coupled with the appearance of the requisite new signals in the aliphatic methylene and methine region (2.0–4.0 ppm) that integrated for eight protons confirmed that reduction of **17** was complete to afford **2** in the purified sample. An analytical sample of **2** was obtained by ion-exchange chromatography using a DEAE-cellulose column and eluting with aqueous ammonium bicarbonate solution.

The structure of **2** was further confirmed by elemental analysis and also by the ^{13}C NMR spectra. To assign the protons of **2**, especially in the aliphatic region, a number of additional NMR experiments were performed. Homonuclear shift correlated two-dimensional NMR spectroscopy (COSY 2D) of a sample of **2** in CF_3COOD revealed the relationships between some of these proton signals and assisted in their assignment. The C-9 axial proton was expected to be shielded by the anisotropy of the C-1 carbonyl group as has been reported for 5,10-methylenetetrahydrofolate[6] and occurred at 4.47 ppm, about 0.3 ppm upfield from the C-9 equatorial proton. The resonance signal of the bridghead proton at C-6a overlaps with the multiplet from one of the H_β protons of the glutamate residue at 2.33–2.38 ppm. The other bridgehead proton at C-9a resonated as a multiplet (or a doublet of a doublet) at 4.25 ppm. This downfield shift of the C-9a methine proton relative to H-6a was due to its location α to an aromatic ring.[32] These assignments were further supported by selected decoupling experiments performed on this sample in its ^{13}C NMR. The proton assignments in the NMR of **2** in DMSO-d_6 were also similarly identified using COSY 2D and NOE experiments.

Biological Results and Discussion

The target compound **2** and compounds **4** and **18** were evaluated as inhibitors of the growth of Manca human lymphoma cells[33,34] (Table I). Both **2** and **4** were found to be inactive. However, the aromatic precursor of **2**, compound **18**, had marginal inhibitory activity. The compounds were also evaluated as inhibitors of folate metabolizing enzymes: human dihydrofolate reductase,[35] *Lactobacillus casei* dihydrofolate reductase,[36] human TS,[37] *Lactobacillus casei* TS and glycinamide ribonucleotide formyltransferase, and aminoimidazole carboxamide ribonucleotide formyltransferase (AICAR formyl TF) from *Lactobacillus casei*.[38] Compounds **2**, **4**, and **18** were inactive against the enzymes tested (Table I) except for **18**, which had an IC_{50} of 17 μM against *L. casei* AICAR formyl TF, and compound **2**, which had an IC_{50} = 5μM against *L. casei* dihydrofolate reductase. The lack of activity of the target compound **2** against human TS appears to be due to its lack of flexibility which was recently reported to be required for the substrate activity of 5,-10-CH_2THF. In crystal studies of TS Hardy et al.[39] were

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unable to dock the solution conformation of the tricyclic 5,10-CH_2THF on the folate binding site of TS. Further Mathews et al.[40] were also unable to accommodate the tricyclic solution conformation of the cofactor on the TS active site in their crystal studies. It was suggested that 5,10-CH_2THF binds to TS in a conformation, different from its solution conformation, which is conducive to its collapse from a tricyclic to the bicyclic 5-imminium ion in keeping with the proposed mechanism of TS. Thus reports of crystal structures of TS, which have only recently become available, strongly suggest that the enzyme does not contain a stable binding site for the cofactor 1 in its tricyclic form. Transient binding of the cofactor is known to cause a large conformational change in TS,[4a,b] possibly due, in part, to the collapse of the tricyclic cofactor 1 to the bicyclic imminium ion which is the bound folate species prior to the ternary (TS–dUMP–CH_2THF) complex formation. This has recently been modeled using the crystal structure of TS.[41] The previous report[12] of the inactivity of the homologous compound 5-deaza-5,10-methylenetetrahydrohomofolate from this laboratory further corroborates the inability of rigid tricyclic mimics of 1 to bind to TS.

Our studies with rigid and semirigid 5-deaza tricyclic analogues of the cofactor 1 attest to the problem of designing compounds by analogy to unbound solution conformations of substrates rather than their bound conformations.[42,43]

The significant activity of compound 18 as an inhibitor of AICAR formylTF has initiated a study of analogues that is currently underway.

Experimental Section

Starting materials used in synthetic procedures were obtained from Aldrich Chemical Co., Milwaukee, WI. Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. Catalytic hydrogenation reactions were performed in a Parr pressure reaction apparatus. Infrared (IR) spectra were determined neat or as Nujol mulls on a Perkin-Elmer 1430 ratio recording infrared spectrophotometer and are reported in reciprocal centimeters. Nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Varian EM-360 (60 MHz) or Bruker WH-300 (300 MHz and 500 MHz) spectrometers, and ^{13}C NMR spectra were obtained on a Bruker WH-300 instrument at 75.46 MHz, 90 pulse, and 14 μs. The data was accumulated by 16K size with 0.5 s delay time and 70° tip angle. The chemical shift values are expressed in δ values (parts per million) relative to tetramethylsilane as an internal standard: s = singlet, d = doublet, t = triplet, m = multiplet. Thin-layer chromatography (TLC) was performed on Eastman Kodak chromatogram sheets (silica gel and cellulose) with fluorescent indicator. Proportions of solvents used for TLC are by volume. Spots on TLC were detected by UV light (254 and 350 nm). Elemental analyses were performed by Atlantic Microlab, Inc., Atlanta, GA, and Galbraith Laboratories, Knoxville, TN. Element compositions are within ±0.4% of the calculated values. Compounds were dried in an Abderhalden drying apparatus, over phosphorus pentoxide for a period of 24–48 h, at ≤0.20 mmHg (with heating over ethanol or toluene) prior to submission for elemental analysis. Molecular modeling and energy minimization were carried out using SYBYL from Tripos Associates, St. Louis, MO.

1-Benzyl-2,5-dimethylpyrrole (7). Acetonylacetone (11.7 mL, 100 mmol) and benzylamine (10.9 mL 100 mmol) were dissolved in toluene (25 mL) and heated under reflux, in a flask fitted with a Dean–Stark trap until separation of water ceased (4 h). The reaction was cooled to room temperature, allowed to stand overnite, and concentrated in vacuo to yield an amber oil which solidified to a yellow solid upon cooling. This was distilled under reduced pressure (78–80 °C at 2–3 μHg) to yield 16.69 g (90%) of 7 as a pale yellow crystalline solid: mp 40–41 °C (lit.[17] mp 40–41 °C); TLC (a) ethyl acetate/silica gel, R_f = 0.72; (b) ethyl acetate/methanol (1:1)/silica gel, R_f = 0.86; (c) ethyl acetate/methanol (1:1) + 2 drops AcOH, silica gel, R_f = 0.84; IR 2930, 2860, 1455, 1410, 1300, 748, 725 cm^{-1}; ^{1}H NMR (60 MHz) ($CDCl_3$) δ 2.10 (s, 6 H, CH_3), 4.95 (s, 2 H, NCH_2), 5.83 (s, 2 H, 3- and 4-CH), 6.87 (m, 2 H, C_6H_5), 7.20 (m, 3 H, C_6H_5).

3-Formyl-1,2,5-trimethylpyrrole (8). $POCl_3$ (10.4 mL, 110 mmol) was added dropwise with stirring to 10.6 mL (140 mmol) of DMF in a three-neck flask, under nitrogen, at 20–25 °C over a period of 30 min. To this mixture was added trichloroethylene (20 mL) followed by the addition of 10.92 g (100 mmol) of 1,2,5-trimethylpyrrole in 5 mL of trichloroethylene over 30 min at a temperature maintained below 25 °C. The mixture was stirred for 15 min at 55 °C and then at 85 °C for 2–3 min. The reaction was cooled to room temperature and treated with 200 mL of ice/water, the mixture was made alkaline (pH 10) with a solution of NaOH (30%) and heated at 85 °C for 10 min, and the phases were separated. The aqueous phase was extracted with 2 × 25 mL of CH_2Cl_2, and the combined organic phase was dried ($MgSO_4$) and concentrated to yield a dark brown residue. This was first treated with 50 mL of ether, decanted, and then treated with 500 mL of acetone/ether (1:1). The combined supernatents were dried ($MgSO_4$) and concentrated, and the residue was washed with 200 mL of ether to yield 7.2 g of a yellowish brown solid. The ether washings were allowed to evaporate to yield an additional 5.3 g of 8 as a yellowish-brown crystalline solid (91%): mp 95–96 °C (lit.[45] mp 96.5–97 °C); TLC (a) ethyl acetate/silica gel, R_f = 0.49; (b) ethyl acetate/methanol (1:1)/silica gel, R_f = 0.76; (c) ethyl acetate/methanol (1:1) + 2 drops AcOH/silica gel, R_f = 0.75; IR 2725, 1637 (C=O), 1530, 1469, 1405, 1377, 1335, 1181, 840, 815, 662 cm^{-1}; ^{1}H NMR (60 MHz) ($CDCl_3$) δ 2.17 (s, 3 H, CH_3), 2.40 (s, 3 H, CH_3), 3.37 (s, 3 H, NCH_3), 6.18 (s, 1 H, CH), 9.73 (s, 1 H, CHO).

1-Benzyl-2,5-dimethyl-3-formylpyrrole (9). To 14.8 mL (192 mmol) of DMF in a three-necked flask under nitrogen at 10 °C was added dropwise with stirring 4.5 mL (48 mmol) $POCl_3$ over a period of 30 min. To this mixture was added a solution of 8.43 g (45.6 mmol) of 7 in 4 mL of DMF over a period of 30 min at 10 °C. The reaction mixture was heated at 100 °C for 2 h, cooled to room temperature, and poured into ice. The mixture was brought to pH 11 with NaOH (30%). The precipitate that formed was filtered and air-dried to yield 9.0 g of a tan solid. The filtrate upon storage at 10 °C yielded an additional 0.33 g of 9 as a fine crystalline, white solid (96% yield): mp 108–109 °C (lit.[17] mp 108–109 °C); TLC (a) ethyl acetate/silica gel, R_f = 0.62; (b) ethyl acetate/methanol (1:1)/silica gel, R_f = 0.81; (c) ethyl acetate/methanol (1:1) + 2 drops AcOH/silica gel, R_f = 0.81; IR 2730, 1649 (C=O), 1565, 1440, 1375, 1350, 1150, 1035, 838, 820, 730, 725, 690, 663 cm^{-1}; ^{1}H NMR (60 MHz) (DMSO-d_6) δ 2.07 (s, 3 H, CH_3), 2.37 (s, 3 H, CH_3), 5.13 (s, 2 H, CH_2), 6.20 (s, 1 H, CH), 6.93–7.25 (m, 5 H, C_6H_5), 9.73 (s, 1 H, CHO).

2-Amino-6-hydrazino-4-oxopyrimidine (10). 2-Amino-6-chloropyrimidin-4-ol (1.46 g, 10 mmol) and 1.17 g (20 mmol) hydrazine hydrate (55% aqueous solution) were mixed with 75 mL of water and heated at reflux for 1.5 h at 110–120 °C. The reaction mixture was cooled to room temperature and filtered. The residue was washed with 800 mL of water and air-dried to

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yield 1.28 g (90%) of a white powder: mp 292–293 °C (lit.[46] mp 297 °C); TLC ethyl acetate/methanol (1:1) + 2 drops acetic acid/silica gel, R_f = 0.56; IR 3430, 3300, 3090 (NH_2), 1675 (C=O), 975, 780 cm^{-1}. Compound **10** was insoluble in DMSO. Its ^1H NMR in CF_3COOD did not reveal any signals since all proton signals were found to exchange.

3-[[2-(2-Amino-4-oxopyrimidin-6-yl)hydrazono]methyl]-1,2,5-trimethylpyrrole (11). A mixture of 3-formyl-1,2,5-trimethylpyrrole (**8**) (2.74 g, 20 mmol) and **10** (2.82 g, 20 mmol) in methoxyethanol (125 mL) was refluxed at 135 °C for 6 h. The mixture was cooled to room temperature and filtered. The filtrate was treated with 100 mL of ethanol/ether (1:1), allowed to stand for 3 h, and filtered to yield 2.7 g of a tan, finely crystalline product. The filtrate was concentrated in vacuo and the residue triturated with ethanol overnight, filtered, washed with ether, and air-dried to afford an additional 1.64 g (total yield 83%) of a tan powder. An analytical sample was prepared by recrystallization from a mixture of water/ethanol/DMSO (8:1:1) to yield **11** as fine tan crystals: mp 258 °C; TLC (a) ethyl acetate/methanol (1:1) + 2 drops AcOH/silica gel, R_f = 0.63; (b) 1-butanol/acetic acid/water (3:1:3)/cellulose, R_f = 0.93; IR 3430, 3310, 3120 (N–H), 1680 (C=O), 1610, 1465, 1375, 1230, 1042, 965, 790 cm^{-1}; ^1H NMR (500 MHz) (DMSO-d_6) δ 2.14 (s, 3 H, CH_3), 2.25 (s, 3 H, CH_3). 3.35 (s, 3 H, NCH_3), 5.04 (s, 1 H, H_5) (exchanges with D_2O), 5.98 (s, 1 H, CH), 6.20 (s, 2 H, NH_2) (exchanges with D_2O), 7.94 (s, 1 H, CH), 9.75 (s, 1 H, NH) (exchanges with D_2O), 9.79 (s, 1 H, NH) (exchanges with D_2O); (CF_3COOD) δ 2.30 (s, 3 H, CH_3), 2.58 (s, 3 H, CH_3), 3.62 (s, 3 H, NCH_3), 8.32 (s, 1 H, CH). Anal. ($C_{12}H_{16}N_6O\cdot0.4H_2O$) C, H, N.

1-Benzyl-3-[[2-(2-amino-4-oxopyrimidin-6-yl)hydrazono]methyl]-2,5-dimethylpyrrole (12). A mixture of **10** (2.82 g, 20 mmol) and **9** (4.26 g, 20 mmol) in methoxyethanol (150 mL) was refluxed at 125–130 °C for a period of 6 h. The reaction mixture was allowed to cool to room temperature and filtered. The filtrate was treated with an equal amount of ethanol and concentrated in vacuo. The residue obtained was treated with excess ethanol and further concentrated to yield a viscous brownish residue that was triturated with anhydrous ether overnight. Filtration afforded 5.50 g (82%) of **12** as a semicrystalline tan-brown powder: mp 150 °C dec; TLC (a) ethyl acetate/methanol (1:1) + 2 drops AcOH/silica gel, R_f = 0.69; (b) 1-butanol/acetic acid/water, (3:1:3)/cellulose, R_f = 0.95; IR 3430, 3310, 3120 (N–H), 1610 (C=O), 1225, 1040, 975, 800, 725 cm^{-1}; ^1H NMR (500 MHz) (DMSO-d_6) δ 2.08 (s, 3 H, CH_3), 2.18 (s, 3 H, CH_3), 5.07 (s, 1 H, H_5) (exchanges with D_2O), 5.09 (s, 2 H, NCH_2), 6.10 (s, 1 H, CH), 6.20 (s, 2 H, NH_2) (exchanges with D_2O), 6.91 (m, 2 H, C_6H_5), 7.23–7.34 (m, 3 H, C_6H_5), 7.99 (s, 1 H, CH=N), 9.75 (br, 2 H, NH's) (exchanges with D_2O). An analytical sample was prepared by recrystallization from a mixture of water and DMF (9:1). Anal. ($C_{18}H_{20}N_6O$) C, H, N.

3-Amino-7,8,9-trimethyl-2*H*-pyrrolo[3′,4′:4,5]pyrido[2,3-*d*]pyrimidin-1-one Hydrobromide (4). The hydrazone **11** (1.56 g, 6 mmol) was heated in 30 mL of 30% HBr in AcOH at 70–80 °C for 2 h. The resulting yellow suspension was allowed to cool to room temperature and filtered. The residue was washed with cold AcOH and then with ether and dried to yield 1.81 g (90%) of the tricyclic hydrobromide **4** as a yellow solid. Recrystallization from acetic acid/water (14:1) afforded an analytical sample of **4**: mp 158–160 °C; TLC (a) ethyl acetate/methanol (1:1) + 2 drops AcOH/silica gel, R_f = 0.61; (b) 1-butanol/acetic acid/water, (3:1:3)/cellulose, R_f = 0.91; IR 3280, 3100 (N–H), 1690, 1660 cm^{-1}; ^1H NMR (500 MHz) (DMSO-d_6) δ 2.24 (s, 3 H, CH_3) 2.49 (s, 3 H, CH_3), 3.54 (s, 3 H, NCH_3), 7.39 (s, 1 H, NH), 8.16 (br s, 2 H, NH_2) (exchanges with D_2O), 8.24 (s, 1 H, CH); (CF_3COOD) (500 MHz) δ 2.51 (s, 3 H, CH_3), 2.69 (s, 3 H, CH_3), 3.71 (s, 3 H, CH_3), 8.83 (s, 1 H, CH); ^{13}C NMR (CF_3COOD) (125 MHz) C_6 = 155.38 ppm; $^1J_{C-H}$ = 157 Hz. Anal. ($C_{12}H_{13}N_5O\cdot1.2HBr\cdot1.3CH_3COOH\cdot0.2H_2O$) C, H, N, Br.

3-Amino-8-benzyl-7,9-dimethyl-2*H*-pyrrolo[3′,4′:4,5]pyrido[2,3-*d*]pyrimidin-1-one Hydrobromide (5). The hydrazone **12** (2.52 g, 7.5 mmol) was heated in 40 mL of 30% HBr in AcOH at 70–80 °C for 4 h. The resulting reddish-yellow suspension was allowed to cool to room temperature and filtered. The residue was washed with cold AcOH and then with ether and dried to yield 1.30 g (40%) of the tricyclic hydrobromide as a yellow solid. Recrystallization from acetic acid/water (12:1) afforded an analytical sample of **5**: mp 215–217 °C; TLC (a) $CHCl_3$/methanol (9:1)/silica gel R_f = 0.10; (b) $CHCl_3$/methanol (3:1)/silica gel, R_f = 0.49; ^1H NMR (500 MHz) (DMSO-d_6) δ 2.16 (s, 3 H, CH_3), 2.48 (s, 3 H, CH_3), 5.38 (s, 2 H, CH_2), 6.99 (m, 2 H, C_6H_5), 7.30–7.36 (m, 3 H, C_6H_5), 7.47 (s, 1 H, NH), 8.30 (br, 2 H, NH_2) (exchanges with D_2O), 8.37 (s, 1 H, CH); (CF_3COOD) (500 MHz) δ 2.43 (s, 3 H, CH_3), 2.56 (s, 3 H, CH_3), 5.30 (s, 2 H, CH_2), 6.93 (m, 2 H, C_6H_5), 7.35 (m, 3 H, C_6H_5), 8.82 (s, 1 H, CH); ^{13}C NMR (CF_3COOD) (75 MHz) C_6 = 155.60 ppm ($^1J_{C-H}$ = 158 Hz). Anal. ($C_{18}H_{17}N_5O\cdot1.0HBr\cdot1.0H_2O$) C, H, N, Br.

Diethyl *N*-[4-(3-Formyl-1-pyrrolyl)benzoyl]-L-glutamate (15). To a mixture of 2,5-dimethoxy-3-formyltetrahydrofuran (**13**) (6.44 g, 20 mmol) and diethyl (4-aminobenzoyl)glutamate (**14**) (3.20 g, 20 mmol) was added 25 mL of AcOH. The reaction was exothermic and turned a deep red color. The reaction mixture was heated at reflux (bath temperature 110 °C) for 2 h. The mixture was cooled to room temperature and poured into 100 mL of ice/water, stirred, and treated with 50 mL of ether. The layers were separated, and the aqueous phase was treated with 5 mL of 30% NaOH and extracted with 5 × 50 mL of $CHCl_3$. The combined $CHCl_3$ extracts were dried ($MgSO_4$) and concentrated in vacuo to give 8.00 g (100%) of the formylpyrrole **15** as a viscous, dark red oil: ^1H NMR (300 MHz) ($CDCl_3$) δ 1.22 (t, 3 H, CH_3), 1.32 (t, 3 H, CH_3), 2.17 (m, 1 H, H_β), 2.32 (m, 1 H, H_β), 2.52 (m, 2 H, H_γ), 4.12 (q, 2 H, $COOCH_2$), 4.25 (q, 2 H, $COOCH_2$), 4.80 (m, 1 H, H_α), 6.83 (m, 1 H, CH), 7.16 (m, 1 H, CH), 7.41 (d, 1 H, CONH), 7.47 (AB q, 2 H, 3′,5′-CH), 7.75 (m, 1 H, CH), 7.97 (AB q, 2 H, 2′,6′-CH), 9.85 (s, 1 H, CHO). This was used without further purification.

Diethyl *N*-[4-[3-[[(2-Amino-4-oxopyrimidin-6-yl)hydrazono]methyl]pyrrol-1-yl]benzoyl]-L-glutamate (16). A mixture of **15** (1.40 g 3.5 mmol) and **10** (0.49 g 3.5 mmol) in methoxyethanol (20 mL) was heated at 135 °C for 6 h. The reaction mixture was allowed to cool to room temperature and filtered. The yellow filtrate was treated with 20 mL of ethanol/ether (1:1) and then concentrated in vacuo at 45 °C. The residue was treated with ether (100 mL) and further concentrated to yield a crystalline residue that was triturated with ether, filtered, and air-dried to afford 1.44 g (80%) of the hydrazone **16** as a yellow powder: mp 140–145 °C; TLC (a) ethyl acetate/MeOH (1:1)/silica gel, R_f = 0.68; (b) $CHCl_3$/MeOH/NH_4OH (5:4:1)/silica gel, R_f = 0.75; (c) 1-butanol/acetic acid/water (3:1:3)/cellulose, R_f = 0.94; IR 3430, 3310, 3150 (N–H), 1730, 1710, 1660, 1600, 1460, 1375, 1220, 800 cm^{-1}; ^1H NMR (500 MHz) (DMSO-d_6) δ 1.16 (t, 3 H, CH_3), 1.20 (t, 3 H, CH_3), 2.03 (m, 1 H, H_β), 2.10–2.13 (m, 1 H, H_β), 2.46 (m, 2 H, H_γ), 4.05 (m, 2 H, $COOCH_2$), 4.13 (m, 2 H, $COOCH_2$), 4.45 (m, 1 H, H_α), 5.20 (s, 1 H, H_5) (exchanges with D_2O), 6.31 (br s, 2 H, NH_2) (exchanges with D_2O), 6.65 (s, 1 H, H_{14}), 7.59 (s, 1 H, H_{13}), 7.78 (AB q, 2 H, 3′,5′-CH), 7.86 (s, 1 H, H_{11}), 7.99 (AB q, 2 H, 2′,6′-CH), 8.01 (s, 1 H, H_9), 8.78 (d, 1 H, CONH) (exchanges with D_2O), 10.06 (br s, 1 H, N_3-N) (exchanges with D_2O), 10.30 (br s, 1 H, N_7-H) (exchanges with D_2O). Anal. ($C_{25}H_{29}N_7O_6\cdot0.3C_3H_8O_2$) C, H, N.

***N*-[4-(3-Amino-1-oxopyrrolo[3′,4′:4,5]pyrido[2,3-*d*]pyrimidin-8-yl)benzoyl]-L-glutamic Acid (18).** The hydrazone **16** (1.57 g, 3.0 mmol) in AcOH (20 mL) was refluxed at 120–130 °C, under nitrogen, for a period of 24 h. The reaction mixture was cooled to room temperature and poured into 100 mL of ice/water. The precipitate formed was filtered, washed with excess water until the washings were neutral, and air-dried overnight to yield 1.35 g of a brownish tan powder. A 200-mg sample was purified by column chromatography on silica gel (5 × 25 cm) using $CHCl_3$/methanol (9:1) as eluant. Thirty 10-mL fractions were collected and fractions 21–30 were pooled and concentrated to afford 105 mg of **17** as a tan powder. The remainder of the crude product was similarly purified by column chromatography to afford a total yield of 0.63 g (45%) of the tricyclic compound **17** as a tan powder: mp 254 °C dec; TLC (a) $CHCl_3$/methanol/NH_4OH (5:4:1)/silica gel, R_f = 0.81; (b) ethyl acetate/MeOH (1:1)/silica gel, R_f = 0.70; (c) CH_3CN/water (9:1)/silica gel, R_f = 0.66; (d) 1-butanol/acetic acid/water (3:1:3)/cellulose, R_f = 0.94; IR 3340, 3200 (N–H), 1730, 1650, 1605, 1505, 1455, 1375, 1200, 850, 765 cm^{-1}; ^1H NMR (500 MHz) (DMSO-d_6) δ 1.17 (t, 3 H, CH_3), 1.20 (t, 3 H,

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CH_3), 2.03–2.06 (m, 1 H, H_β), 2.11–2.15 (m, 1 H, H_β), 2.47 (m, 2 H, H_γ), 4.06 (q, 2 H, $COOCH_2$), 4.12 (q, 2 H, $COOCH_2$), 4.46 (m, 1 H, H_α), 6.52 (br s, 2 H, NH_2) (exchanges with D_2O), 6.92 (s, 1 H, H_6), 7.53 (s, 1 H, H_7), 7.69 (AB q, 2 H, 3′,5′-CH), 8.02 (AB q, 2 H, 2′,6′-CH), 8.53 (s, 1 H, H_9), 8.79 (d, 1 H, CONH) (exchanges with D_2O), 10.45 (br s, 1 H, NH) (exchanges with D_2O); (CF_3COOD) (300 MHz) δ 1.32 (t, 3 H, CH_3), 1.39 (t, 3 H, CH_3), 2.35 (m, 1 H, H_β), 2.51 (m, 1 H, H_β), 2.74 (m, 2 H, H_γ), 4.29 (q, 2 H, $COOCH_2$), 4.41 (q, 2 H, $COOCH_2$), 4.99 (m, 1 H, H_α), 6.97 (s, 0.07 H, H_6), 7.33 (s, 0.07 H, H_7), 7.65 (AB q, 2 H, 3′,5′-CH), 8.03 (AB q, 2 H, 2′,6′-CH), 8.58 (s, 0.06 H, H_9). Compound **17** (1.10 g, 2.2 mmol) was dissolved in methoxyethanol (25 mL) and treated with 1 N NaOH (12 mL). The solution was stirred at room temperature for 10 h. The reaction mixture was then treated with water (10 mL), cooled in an ice bath, and acidified to pH 4 with AcOH. The mixture was freeze-dried to afford 1.07 g of the crude free acid **18** as a brown powder. The crude acid dissolved in water (6 mL) and loaded onto a cellulose column (2.3 × 20 cm). The column was first eluted with 100 mL of 0.1 M NaH_2PO_4 (pH 6.8) to remove impurities and salts. The column was then eluted with 50% AcOH, collecting fifty 10-mL fractions. The fractions containing product (25–50) as seen on TLC were pooled (250 mL) and lyophilized. The residue was then treated with 15 mL of ice cold water, filtered, and dried over P_2O_5 (0.005 mmHg) for 24 h to yield 0.89 g (95%) of **18** as a yellowish brown solid: mp >260 °C dec; TLC (a) $CHCl_3$/MeOH/NH_4OH (5:4:1)/silica gel, R_f = 0.50; (b) 1-butanol/AcOH/water (3:1:3)/cellulose, R_f = 0.46; (c) acetic acid/water (1:1)/cellulose, R_f = 0.50; ^1H NMR (500 MHz) (DMSO-d_6) δ 1.98–2.01 (m, 1 H, H_β), 2.10–2.12 (m, 1 H, H_β), 2.38 (t, 2 H, H_γ), 4.42 (m, 1 H, H_α), 6.49 (br s, 2 H, NH_2) (exchanges with D_2O), 6.93 (s, 1 H, H_6), 7.53 (s, 1 H, H_7), 7.69 (AB q, 2 H, 3′,5′-CH), 8.02 (AB q, 2 H, 2′,6′-CH), 8.53 (s, 1 H, H_9), 8.66 (d, 1 H, CONH) (exchanges with D_2O), 10.43 (br s, 1 H, NH) (exchanges with D_2O), 12.55 (br s, 2 H, COOH) (exchanges with D_2O); (CF_3COOD) (300 MHz) δ 2.47 (m, 1 H, H_β), 2.67 (m, 1 H, H_β), 2.88 (m, 2 H, H_γ), 5.15 (m, 1 H, H_α), 7.05 (s, 0.08 H, H_6), 7.39 (s, 0.08 H, H_7), 7.72 (AB q, 2 H, 3′,5′-CH), 8.08 (AB q, 2 H, 2′,6′-CH), 8.65 (s, 0.06 H, H_9). Anal. ($C_{21}H_{18}N_6O_6 \cdot 3.0H_2O$) C, H, N.

***cis*-5,10-Methylenetetrahydro-5-deazafolic Acid (2).** The tricyclic compound **17** (0.25 g, 0.5 mmol) was dissolved in 25 mL of aqueous CF_3COOH (90%). This solution was a mixed with PtO_2 (0.04 g) and the mixture hydrogenated in a Parr apparatus under 50 psi of hydrogen pressure at room temperature until approximately 90% of the calculated amount of hydrogen was consumed (48 h). The catalyst was filtered and treated with 15 mL of ethanol, and the filtrate was concentrated under reduced pressure to a brown, syrupy residue. This was dissolved in methoxyethanol (10 mL), treated with 1 N NaOH (5 mL), and stirred at room temperature for 10 h. The reaction mixture was then cooled in an ice bath, acidified with AcOH to pH 4, and stirred at <10 °C for 1 h. The reaction mixture was then lyophilized to yield a brown residue. This residue was treated with ice-cold water (20 mL), allowed to stand for 1 h, and then filtered, and the residue was washed with (20 mL) of ice-cold water and ether and air-dried to yield 0.22 g (crude yield 97%) of a tan powder. This was purified by column chromatography on cellulose (2.5 × 34 cm, packed wet in 15% AcOH). The sample (0.11 g) was loaded and eluted with 15% AcOH, collecting 5-mL fractions. Fractions (3–15) containing the product (TLC) were pooled and freeze-dried to a brown residue that was washed with ice-cold water, filtered, and air-dried to yield 0.09 g of **2** as a tan powder (overall yield 80%). A 15-mg sample of this product was finally purified by ion-exchange chromatography on a DEAE-cellulose column using 3% NH_4HCO_3 as eluant. Four-milliliter fractions were collected and those containing the product (80–180) as seen by TLC were pooled and freeze-dried to afford 13 mg of **2** as a light tan powder: mp >300 °C; TLC (a) acetic acid/water (1:3)/cellulose, R_f = 0.53; (b) acetic acid/water (1:1)/cellulose, R_f = 0.83; (c) 1-butanol/acetic acid/water (3:1:3)/cellulose, R_f = 0.79; (d) 0.1 M NaH_2PO_4 (pH 6.8)/cellulose, R_f = 0.20; IR 3330, 3180 (N–H), 1780, 1680, 1605, 1510, 1455, 1375, 1195, 765 cm^{-1}; ^1H NMR (500 MHz) (DMSO-d_6) δ 1.91–2.01 (m, 1 H, H_β), 2.05–2.09 (m, 2 H, H_β and H_6), 2.33–2.38 (m, 4 H, H_γ, H_6, and H_{6a}), 3.44 (m, 1 H, H_{9a}), 3.48 (m, 1 H, $H_{9equitorial}$), 3.57 (m, 1 H, H_{9axial}), 3.67–3.70 (m, 2 H, H_7), 4.37 (m, 1 H, H_α), 6.48 (br s, 2 H, NH_2) (exchanges with D_2O), 6.49 (br s, 1 H, NH) (exchanges with D_2O), 6.55 (AB q, 2 H, 3′,5′-CH), 7.76 (AB q, 2 H, 2′,6′-CH), 8.20 (d, 1 H, CONH) (exchanges with D_2O), 10.46 (br s, 1 H, NH) (exchanges with D_2O), 12.32 (br, 2 H, COOH) (exchanges with D_2O); (CF_3COOD) (300 MHz) δ 2.46 (m, 1 H, H_β), 2.69 (m, 2 H, H_β and H_{6a}), 2.90 (m, 3 H, H_γ and H_6), 3.23 (m, 1 H, H_6), 4.25 (m, 1 H, H_{9a}), 4.47 (m, 3 H, H_7 + $H_{9equitorial}$), 4.80 (m, 1 H, H_{9axial}), 5.18 (m, 1 H, H_α), 7.96 (AB q, 2 H, 3′,5′-CH), 8.25 (AB q, 2 H, 2′,6′-CH). Anal. ($C_{21}H_{24}N_6O_6 \cdot H_2O$) C, H, N.

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