Synthesis of 5-Methyl-5-deaza Nonclassical Antifolates as Inhibitors of Dihydrofolate Reductases and as Potential Antipneumocystis, Antitoxoplasma, and Antitumor Agents[1]

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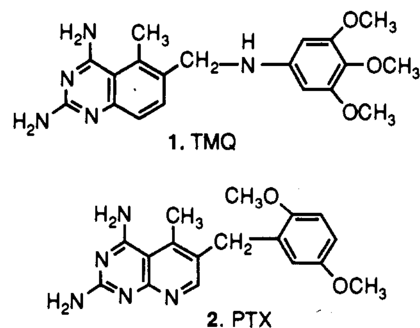
A series of 2,4-diamino-5-methyl-6-(anilinomethyl)pyrido[2,3-*d*]pyrimidines **4–9** were synthesized as 5-deaza nonclassical antifolates containing trimethoxy, dichloro-, or trichlorophenyl substitutions and a *N*-H, *N*-CH_3, or *N*-CHO at the 10-position. The compounds were evaluated as inhibitors of dihydrofolate reductases (DHFR) from *Pneumocystis carinii* (*P. carinii*), *Toxoplasma gondii* (*T. gondii*), rat liver (RL), and *Lactobacillus casei* (*L. casei*); as inhibitors of *T. gondii* and *P. carinii* cell growth in culture; and as antitumor agents. The compounds were prepared by modifications of procedures for classical 5-deaza folates. 2,4-Diamino-5-methyl-6-[(3′,4′,5′-trimethoxy-*N*-methylanilino)methyl]pyrido[2,3-*d*]pyrimidine (**5a**) exhibited high potency as well as selectivity (compared to RL DHFR) for *P. carinii* and *T. gondii* DHFR. Compound **5a** is one of the most potent and selective nonclassical folate inhibitors of *T. gondii* DHFR known. The N-10 formyl analogue 2,4-diamino-5-methyl-6-[(*N*-formyl-3′,4′,5′-trimethoxyanilino)methyl]pyrido-[2,3-*d*]pyrimidine (**6a**) had decreased potency, but it maintained high selectivity for *T. gondii* DHFR. The corresponding chloro-substituted analogues maintained potency or had decreased potency; N-10 substitution did not increase potency or selectivity to the extent observed in the 3′,4′,5′-trimethoxy series. Partial reduction of the B ring to afford the dihydro analogue 2,4-diamino-5-methyl-6-[(*N*-formyl-3′,4′,5′-trimethoxyanilino)methyl]-5,8-dihydropyrido[2,3-*d*]pyrimidine (**7**), its 5,6,7,8-tetrahydropyrido[2,3-*d*]pyrimidine analogue **8**, and 2,4-diamino-5-methyl-6-[(3′,4′,5′-trimethoxyanilino)methyl]-5,6,7,8-tetrahydropyrido[2,3-*d*]pyrimidine (**9**) resulted in a significant decrease in potency. In *T. gondii* cell culture inhibitory studies, 2,4-diamino-5-methyl-6-[(3′,4′,5′-trimethoxyanilino)methyl]pyrido[2,3-*d*]pyrimidine (**4a**), **5a**, and **6a** were less potent compared to their DHFR inhibitory potencies. Against *P. carinii* cells in culture, **4a** and **5a** at 10 μg/mL were as effective as the clinically used combination of trimethoprim/sulfamethoxazole (50/250 μg/mL). With the exception of the B ring reduced analogues **7–9**, all of the compounds were significantly cytotoxic to leukemia CCRF-CEM cells in culture. The chloro-substituted analogues, in general, were more potent against a variety of other tumor cells in culture than the trimethoxy analogues. These results were corroborated by the preclinical tumor screening program at the National Cancer Institute where the most potent compound 2,4-diamino-5-methyl-6-[(3′,4′-dichloroanilino)methyl]pyrido[2,3-*d*]pyrimidine (**4b**) was found to inhibit the growth of 26 tumor cell lines at an $IG_{50} < 1.00 \times 10^{-8}$ M.

Infection with *Pneumocystis carinii* (*P. carinii*) and *Toxoplasma gondii* (*T. gondii*) remain the principal cause of death in patients with Acquired Immunodeficiency Syndrome (AIDS)[2] in the United States. Currently used therapeutic regimens are beset with high cost, toxicity (nonselective), and possibly resistance.[3] Thus there is an urgent need for more selective treatments of these infections.

Allegra *et al.*[4,5] reported trimethoprim (TMP), a first line drug, and pyrimethamine, which are currently used agents, as comparatively weak inhibitors of dihydrofolate reductase (DHFR) from *P. carinii* and *T. gondii*; consequently, they must be used with sulfonamides to provide synergistic effects. However the nonclassical antifolate trimetrexate (TMQ) 1 was 100–10 000 times more potent than TMP or pyrimethamine against DHFR from *P. carinii* and *T. gondii*. In addition the pyrido[2,3-*d*]-pyrimidine piritrexim (PTX, 2) was found to have potency similar to that of 1.[5] These results for *P. carinii* DHFR have been confirmed by Broughton and Queener.[6] Both



1. TMQ

2. PTX

1 and **2** are much more lipid soluble than classical antifolates such as methotrexate (MTX) and do not require the cell-transport mechanism that MTX and other classical antifolates utilize and are thus able to penetrate these

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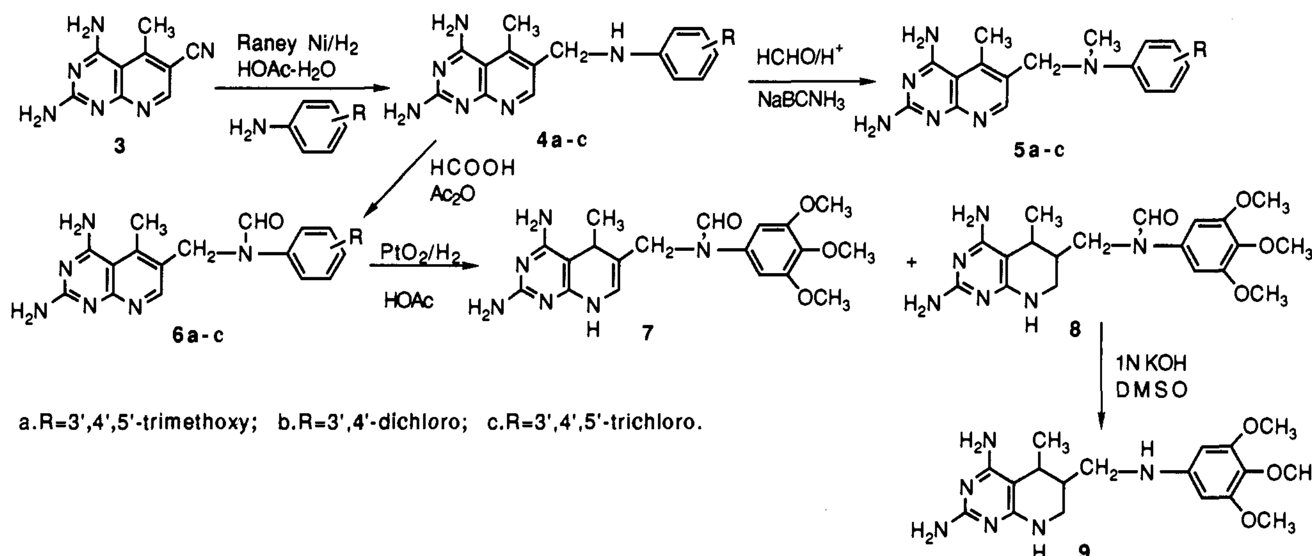
Scheme I



organisms by passive diffusion. This lipid solubility also allows penetration into the CNS where *T. gondii* infections can occur. In clinical trials,[7] TMQ along with host rescue with leucovorin (which circumvents DHFR inhibition in host cells but not in *P. carinii* or *T. gondii* because it is not taken up efficiently by these organisms) proved to be a viable alternate therapy and has been approved by the FDA. However, 1 and 2 are not selective and inhibit mammalian DHFR with equal or greater potency than that displayed toward nonmammalian forms of DHFR.

To investigate the possibility of selective inhibition of DHFR from *P. carinii* and *T. gondii*, as well as antitumor activity and in view of our continued interest in classical and nonclassical 5-deaza folates,[8-11] we synthesized a number of nonclassical, lipophilic 2,4-diamino-5-methyl-6-substituted-pyrido[2,3-*d*]pyrimidines **4-6** as hybrid molecules based on the structures of **1** and **2**. These pyrido-[2,3-*d*]pyrimidines are similar to **2** in that they incorporate the N-8 nitrogen instead of the C-8 of **1** and contain the 6-substitution pattern of **1** rather than that of **2**. The N-8 nitrogen was expected to provide additional binding to DHFR compared to **1** on the basis of the report that the N-8 nitrogen of MTX plays a role in the binding of MTX to various DHFR's.[12] The N-10 nitrogen, which is absent in **2**, allows for a variety of N-10 substitutions. Further, conformational studies[13] of **1** predicted that analogues with N-10 CH_3 substituents were more conformationally restricted in their side chains compared to **1** yet were capable of attaining minimum-energy conformations similar to **1**. We initially chose the 3,4,5-trimethoxy, electron-donating substitution pattern on the phenyl ring similar to **1** and the 3,4,5-trichloro and 3,4-dichloro electron-withdrawing groups. In addition, we synthesized the N-10 CH_3- and formyl-substituted analogues. Molecular modeling studies using SYBYL 5.5[14] and its SEARCH and MAXIMIN2 options showed that partial reduction of the B ring of **4a** caused the side chain, a trimethoxyanilino moiety, to adopt alternate orientations compared to that of the unreduced precursors. We reasoned that such alternate orientations of the side chain may allow for selectivity against DHFR from *P. carinii* and/or *T. gondii* as has been proposed for TMP[15] with respect to bacterial DHFR compared to mammalian DHFR. In order to study the effect of partial reduction of the B ring on the inhibitory activity against *P. carinii* DHFR and *T. gondii* DHFR and antitumor activity, we prepared the partially reduced analgoues **7-9**.

Chemistry

The syntheses of the target compounds **4-9** are shown in Scheme I. The key intermediate, 2,4-diamino-6-cyano-5-methylpyrido[2,3-*d*]pyrimidine **3** was prepared in 27% yield by the procedure of Piper *et al.*[16] This consisted of the condensation of triethyl ortho acetate with malononitrile to afford 2-amino-4-methyl-6-chloropyridine-3,5-dicarbonitrile, which upon reductive dechlorination with H_2/5% Pd/$BaCO_3$ and subsequent condensation with guanidine gave **3**. Reductive condensation of **3** with the appropriate aniline in 70% acetic acid and Raney Ni at atmospheric pressure afforded **4a-c** in 18-23% yield. A mixture of three products was obtained in each of these reductive condensations: unreacted aniline, the desired product (**4a-c**), and a tailing fluorescent spot as observed on TLC. For the synthesis of **4a**, the mixture of the three products obtained was isolated as a solid. The trimethoxyaniline was removed by repeated washing with acetone, and separation of the desired product **4a** from the tailing fluorescent spot was carried out by crystallization from AcOH/MeOH. Separation of the mixture obtained in the synthesis of **4b** and **4c** was accomplished by column chromatography and gradient elution with EtOAc/MeOH. A 30-45% yield of the fluorescent spot was isolated in all three condensation reactions. The mass spectra and ^1H NMR of this fluorescent product indicated it to be the 6-hydroxymethyl analogue of **3**, suggesting that the low yield of the reductive condensation was, in part, due to the fact that a considerable amount of nitrile was overreduced, via the aldehyde to the alcohol, prior to condensation with the substituted anilines.

Methylation of compounds **4a-c** at N-10 was carried out by reductive alkylation using formaldehyde (37%) and sodium cyanoborohydride in a modification of a method reported by Borch and Hassid[17] and applied to classical antifolates by Temple *et al.*[18] and later by Taylor *et al.*[19] and Piper *et al.*[20] We found that the methylation of **4a** as a suspension in acetonitrile afforded the desired N-10 monomethylated product **5a**. In addition, during the reductive methylation a pH between 2 and 3 (1 N HCl)

Table I. Inhibitory Concentrations (IC_{50}, nM) against DHFR's and Selectivity Ratios[6,21]

compd	*P. carinii*	RL	selectivity ratio RL/*P. carinii*	*T. gondii*	selectivity ratio RL/*T. gondii*	*L. casei*[22]
4a	86	2.1	0.02	7.4	0.28	22
5a	13.2	7.6	0.58	0.58	8.94	200
6a	550	110	0.20	13.0	8.46	
4b	320	53	0.17	28.0	1.89	860
5b	100	42	0.42	27.0	1.56	960
6b	510	140	0.27	83.0	1.69	
4c	63	33	0.52	12.0	2.80	
5c	104.5	36.3	0.35	38.1	1.05	36
6c	520	250	0.48	94.0	2.7	
7	>2600	>2600				
8	>1200	>1200				
9	>3700	≈3700				
TMQ	42	3	0.07	10	0.3	27
PTX	38	1.5	0.04	11	0.14	
TMP	40000	400000	10	100000	4	103

not only prevented multiple methylation but also increased the rate and yield of the reaction compared to neutral pH conditions. The methylation reaction of the dichloro congener **4b** and that of the trichloro **4c** did not go to completion at neutral pH, even after prolonged reaction (>48 h) and the use of a large excess of reagents (8–10 equiv of HCHO and $NaCNBH_3$), and afforded low yields of 25–30% for **5b** and less than 10% for **5c**. However, at a pH 2–3, no multiple methylated products were detected by TLC or mass spectra, and significantly improved yields of pure products were obtained. The reaction essentially went to completion within 1 h for **4a** and 3–4 h for the dichloro analogue **4b** with only trace amounts of starting materials still present. In the case of the trichloro analogue **4c**, the reaction required 15 h as indicated by TLC. Separation of **5c** from a small amount of the starting material **4c** was carried out by column chromatography, affording a 43% yield of **5c**. The position of methylation was confirmed to be exclusively at N-10 for **5a–c** by ^1H NMR and NOE studies. For example, the newly introduced CH_3 of **5c** showed a strong NOE with the 2′- and 6′-aromatic protons and with the H-7 in the 2D NOESY spectra indicating that methylation had occurred exclusively at the N-10 position. The lack of NOE between the newly introduced CH_3 and the exchangeable amino protons indicated that the methyl group had not been introduced at either the 2- or the 4-amino groups.

The N-10 formyl compounds **6a–c** were prepared by reaction of **4a–c** with 98% formic acid and a catalytic amount of acetic anhydride. The reaction went smoothly in good to high yields with no side products. The ^1H NMR of the formylated analogues **6a–c** showed the 9-CH_2 as a singlet in contrast to the doublet observed in **4a–c** arising from the coupling with the adjacent NH.

Catalytic hydrogenation of both **4a** and **5a** to afford the B ring reduced target compounds resulted in benzylic cleavage, and the corresponding 5,6-dimethyl-5,6,7,8-tetrahydropyrido[2,3-*d*]pyrimidine was isolated from each reaction mixture. Similar benzylic cleavage has been reported for classical bicyclic 5-deaza folates.[19] This cleavage occurred both under pressure as well as atmospheric hydrogenation over PtO_2, as indicated by TLC. The strategy of N-10 formylation to prevent benzylic type cleavage for classical folates reported by Taylor *et al.*[19] was adapted in our nonclassical 5-methyl series. Thus, the *N*-CHO compound **6a**, upon hydrogenation over PtO_2 at 50 psi, afforded a mixture of the corresponding 5,8-dihydro analogue **7** and 5,6,7,8-tetrahydro analogue **8** with no benzylic cleavage side product detectable by mass spectra. The ratio of the two products **7**:**8** depended upon the reaction time. A 4-h reaction afforded compound **7** and **8** in a 1:3.5 ratio based on the purified yields obtained after silica gel column chromatography. Reduction of **6a** for 10 h afforded compound **8** exclusively. The structure of **7** was established by mass spectra and the proton coupling pattern of its ^1H NMR. The purified dihydro product **7** was stable to air oxidation at room temperature. Compound **9** was obtained by hydrolysis of the *N*-formyl group of **8** with 1 N KOH in DMSO. The structures of **8** and **9** were confirmed by 2D-COSY and by irradiation decoupling studies. The 2D-COSY spectra of compounds **8** and **9** did not show any discernable H5–H6 coupling, which suggested a dihedral angle near 90° and consequently a cis orientation of H5–H6. However, no NOE could be detected between H5 and H6 of compound **8**.

Biological Results and Discussion

Compounds **4a–c**, **5a–c**, **6a–c**, **7**, **8**, and **9** were evaluated as inhibitors of *P. carinii* DHFR, *T. gondii* DHFR, and rat liver (RL) DHFR.[6,21] Selectivity ratios were determined compared to RL DHFR. These results are listed in Table I. In the electron-donating trimethoxy series, N-10 methylation increased potency against both *P. carinii* and *T. gondii* DHFR, with IC_{50} values reduced 6.5-fold for *P. carinii* DHFR and 8.7-fold for *T. gondii* DHFR. Selectivity for *P. carinii* DHFR and *T. gondii* DHFR also inceased significnatly for both enzymes (29-fold and 32-fold, respectively). N-10 formylation decreased inhibitory potency against all three DHFR's, but with concomitant changes in potency toward RL DHFR, N-10 formylation produced nearly as much selectivity for *T. gondii* DHFR as does N-10 methylation. This pattern indicated that N-10 substitution could be important for selectivity against *T. gondii* DHFR. Since the methyl and formyl moieties are electronically quite different, these results with *T. gondii* DHFR suggests steric restriction to side-chain flexibility as a possible reason for the effects of these substitutions.[13] In the electron-withdrawing series, N-10 formylation decreased potency for both the dichloro and trichloro analogues, but selectivity was not changed. All of the chlorinated analogues were equally potent (**6a–c** against *P. carinii* DHFR) or less potent compared to their corresponding trimethoxy analogues against all three DHFR's (except **4c** against *P. carinii* DHFR). The potencies for *P. carinii* and *T. gondii* DHFR's for the N-10 unsubstituted analogues were marginally better than N-10 methylated or N-10 formylated analogues (**4c** *vs* **5c**, **4c** *vs*

Table II. Inhibitory Concentrations (IC_{50}, μM) against *T. gondii* Cell Growth in Culture[23,a]

compd	IC_{50}	compd	IC_{50}
4a	23.0	**6a**	inactive
5a	4.7		

[a] Uracil is incorporated into nucleic acid by *T. gondii* grown in culture. Because mammalian cells do not avidly incorporate uracil, the incorporation of uracil can be used as an index of growth of *T. gondii* on host cells. For these experiments *T. gondii* was grown on HEL (human embryonic lung) cells with Minimum Essential Medium (MEM) supplemented with glutamine (2 mM), penicillin/streptomycin (100 units/mL and 100 μg/mL, respectively), and fetal bovine serum (10%). HEL cells were grown to confluency in 24-well tissue culture plates using MEM. To inoculate, old medium was replaced with 0.5 mL of fresh medium containing 5×10^4 *T. gondii* tachyzoites obtained from mouse peritoneal fluid or from tissue culture. Four hours after addition of the inoculum, an additional 0.5 mL of medium containing drug or control diluent was added to the wells. Twenty-four hours after inoculation, 1 μCi of tritiated uracil (New England Nuclear) was added to each well and incubation continued for additional 24 h. At the end of this period, the medium was sampled to determine remaining counts in the supernatant. The cells were dislodged, suspended by agitation, and filtered through glass fiber filters (Whatman) using a well-washing apparatus with a 24-place filter manifold. Each well is washed with cold isotonic saline and added to the filter. The filters are washed with approxiately 20 mL of cold isotonic saline, dried overnight, removed to scintillation vials, and counted with Ultima Gold Scintillant (Packard). This method is similar to the micromethod of Mack and Mcleod.[23]

6c); thus the dramatic increase in potency produced by N-10 methylation in the trimethoxy series for *T. gondii* DHFR was clearly absent. These results suggest that the electronic nature of the substituent on the phenyl ring was also important for selectivity and potency. Partial saturation of the B ring was detrimental to activity against *P. carinii* and RL DHFR as shown by the significantly lower IC_{50} values of **7**, **8**, and **9** compared to their unsaturated analogues. In summary, for inhibition of *T. gondii* DHFR as well as *P. carinii* DHFR, the electron-donating trimethoxy substitution pattern on the phenyl ring along with N-10 methylation increased both potency and selectivity in the analogues studied. It was interesting to note that all of the compounds in this study, with the exception of the B ring reduced analogues, were more selective than TMQ for *P. carinii* DHFR and *T. gondii* DHFR (**4a** being equiselective). Further the most inhibitory compound **5a** was 3 times as potent and 8 times as selective as TMQ against *P. carinii* DHFR. Against *T. gondii* DHFR, **5a** was 12 times as potent and 31 times as selective as TMQ. To our knowledge compound **5a** is one of the most selective and potent nonclassical inhibitors known with respect to *T. gondii* DHFR. Inhibition of *Lactobacillus casei* (*L. casei*) DHFR (Table I) by compounds **4a** and **5a** more closely resembled the pattern obtained with RL DHFR than that obtained with *P. carinii* DHFR and *T. gondii* DHFR in that **4a** was more inhibitory than its methylated derivative **5a**. Compound **5c** was about as inhibitory as TMP for the *L. casei* enzyme but lacked the selectivity seen with TMP. The cell culture inhibition results against *T. gondii* for **4a**, **5a**, and **6a** are reported in Table II.[23] These IC_{50} values are higher than the DHFR inhibitory potencies and suggest a lower level of transport into *T. gondii* cells in culture. The glucuronic acid salt of **4a** was prepared and tested against *P. carinii* cells in culture.[24,25] The ELISA results are shown in Figure 1. At 10 μg/mL the glucuronic acid salt of **4a** was as effective as the clinically used combination of trimethoprim/sulfamethoxazole TMP/SMX (50/250 μg/mL). The inhibition of *P. carinii* growth in culture by the Giemsa

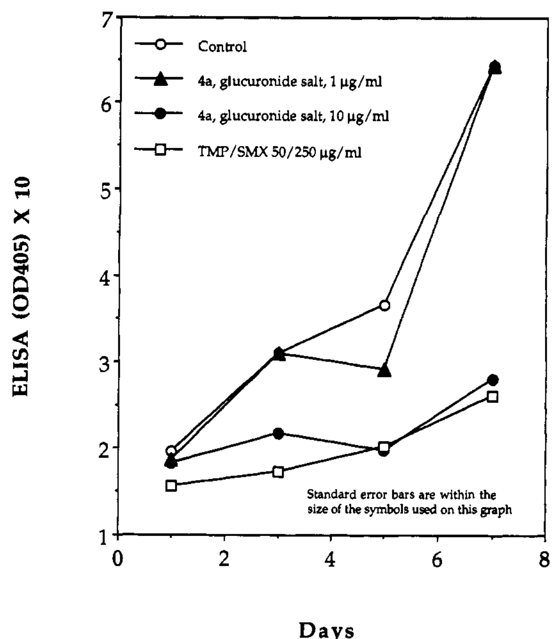


Figure 1. Time course of inhibition of *P. carinii* growth in culture. The glucuronide salt of **4a** was added to culture media at day 0 to yield a final concentration of 1 or 10 μg/mL. An 8-day-old monolayer of HEL cells was inoculated to yield 1.55 ± 0.25 organisms per 1-mL well. Each experimental condition was run in quadruplicate. Samples were taken at days 0, 1, 3, 5, and 7 for ELISA. The samples from four wells were pooled and handled as one sample through preparation for the ELISA; triplicate determinations were made from the same pooled sample. A polyclonal antiserum derived from convalescent rats that had been immunosuppressed and transtracheally inoculated with *P. carinii* was used for ELISA. The technique as applied to both culture and animal samples is fully described elsewhere.[25]

Table III. Inhibitory Concentrations (μg/mL) against *P. carinii* Cell Growth in Culture[24,a]

compd	IC_{50}	% inhibition
4a	1	56 ± 6
4a	10	77 ± 1
4a glucuronide salt	1	2 ± 8
4a glucuronide acid	10	57 ± 7
5a	1	65 ± 5
5a	10	74 ± 5
TMP/SMX	50/250	84 ± 3

[a] Human embryonic lung fibroblastic (HEL) were cultured in 24-well tissue culture plates with minimum essential medium containing 10% fetal bovine serum. On days 8 or 9 when the monolayers were confluent, they were inoculated with homogenates of rat lungs infected with *P. carinii* to yield 6×10^5 viable trophozoites in each well. Compounds were diluted in culture medium and added to appropriate wells. The final volume of each well was 1 mL. Plates were incubated at 35 °C in 5% oxygen and 5–10% carbon dioxide, with the balance being nitrogen. Separate plates were harvested for analysis at 1, 3, 5, and 7 days after inoculation. At these times 10 μL of the culture supernate was removed from each well, air-dried onto a 1-cm^2 area of a slide, fixed with 100% methanol, and stained with Giemsa. Both trophozoites and cysts are counted in ten randomly selected 100× fields. Two counters evaluate the slides independently as blinded samples. The means of both sets of counts are averaged for the final value. Control wells to which no drug was added served as reference for unhibited growth. This table contains data from two independent experiments.

method for compounds **4a**, its glucuronide salt, compound **5a**, and TMP/SMX is shown in Table III. In this study at 10 μg/mL both **4a** and **5a** were equiactive with TMP/SMX (50/250 μg/mL), but the glucuronide salt of **4a** was somewhat less active.

Compounds **4a**, the glucuronide salt of **4a**, and **5a** were tested in the *P. carinii* mouse model.[26] At 2 mg/kg/day

Table IV. IC_{50}[a] (μM) against Tumor Cell Growth in Culture[27-29]

compd	colon HCT116	melanoma SK5 Mel	kidney ACHN	breast MCF7	leukemia CCRF-CEM
4a	71.7	>100	>100	>100	0.13
5a	37.9	>100	>100	>100	0.02
6a	>112	>112	>112	>100	0.61
4b	28.6	7.73	22.9	94.5	0.10
5b	5.30	21.2	70.2	9.6	0.01
6b	16.0	25.1	91.4	106.3	0.21
4c				>100	0.20
5c				18.2	0.20
5c				18.2	0.03

[a] IC_{50} = concentation of drug required to decrease cell viability as measured by MTA (MTT assay) by 50% after 3 days of treatment.

none of the compounds showed any inhibitory activity, and no toxicity was observed. These compounds are currently being evaluated at higher doses, and the results will be reported elsewhere.

Antitumor activity was evaluated as a measure of growth inhibition of colon HCT116, melanoma SK5Mel, breast MCF7, kidney ACHN, and leukemia CCRF-CEM cells in culture and are reported in Table IV.[27-29] In the leukemia CCRF-CEM cytotoxicity study the compounds were very potent, and N-10 methylation in all three phenyl substitution patterns increased potency by almost 1 order of magnitude. The 3,4-dichloro-*N*-CH_3 analogue **5b** was the most active. Good cytotoxicity was demonstrated by the dichlorophenyl and trichlorophenyl analogues in the other tumor cells in Table IV, and with the exception of the colon cell line the trimethoxy compounds were essentially inactive. In the National Cancer Institute preclinical *in vitro* tumor screening program[30] the dichloro and trichloro analogues were significantly more inhibitory than the trimethoxy analogues. Compound **4b** was the most active and demonstrated an IG_{50} of $<1.0 \times 10^{-8}$ M aginst 26 of the tumors tested and is currently undergoing further evaluation. The selectivity of **5a** for *P. carinii* and *T. gondii* DHFR compared to RL DHFR and the comparative lack of selectivity of the chloro analogues was corroborated by the tumor inhibitory results (with the exception of the CCRF-CEM leukemia cell line). On the basis of this report we have undertaken an extensive structure–activity/selectivity study of bicyclic and tricyclic compounds which include conformationally restricted analogues related to the pyrido[2,3-*d*]pyrimidines reported in this study as selective inhibitors of *P. carinii* and *T. gondii* DHFR and as antitumor agents, the results of which will be the topic of future publications.

Experimental Section

Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. Nuclear magnetic resonance spectra for proton (^1H NMR) were recorded on a Bruker WH-300 (300 MHz) spectrometer. The data was accumulated by 16k size with a 0.5-s delay time and 70° tip angle with internal standard TMS; s = singlet, d = doublet, t = triplet, m = multiplet. Chemical shifts (δ) listed for multiplets were measured from the approximate centers, and relative integrals of peak areas agreed with those expected for the assigned structures. Mass spectra were recorded on a Varian MATCH-311A mass spectrometer in the fast-atom-bombardment (FAB) mode. Thin-layer chromatography (TLC) was performed on silica gel plates with fluorescent indicator and were visualized with light at 254 and 366 nm. Ratios of solvents are volume/volume. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, TN, or Atlantic Microlabs, Norcross, GA. Analytical results indicated by element symbols were within ±0.4% of the theoretical values.

2,4-Diamino-5-methyl-6-[(3′,4′,5′-trimethoxyanilino)methyl]pyrido[2,3-*d*]pyrimidine (4a). A stirred solution of the nitrile **3** (3.50 g, 17.50 mmol) and trimethoxyaniline (4.60 g, 25.1 mmol) in 70% AcOH (600 mL) containing damp Raney Ni (10 g) was hydrogenated at atmospheric pressure for 24 h. The mixture was treated with Norit and filtered (Celite mat). The solvent was evaporated (water aspirator, bath to 40 °C) with the aid of an added portion of EtOH. A solution of the residue in warm EtOH (20 mL) was added in a thin stream to a stirred saturated Na_2CO_3 solution (200 mL) at room temperature. The mixture was stirred for an additional 20 min, and a yellow precipitate formed which was collected, washed with H_2O, and dried. This crude product contained trimethoxyaniline, which was removed by repeated treatment with acetone and then CH_3OH (50 mL). The insoluble material was suspended in 200 mL of EtOH and stirred for 1 h. The solid was collected by filtration and dissolved in a minimum amount of glacial AcOH, and the cloudy solution was clarified (Celite), concentrated, diluted with MeOH (80 mL), and cooled in a freezer to afford a yellow solid (2.20 g). TLC analysis of this product showed a red spot (R_f 0.56, MeOH/$CHCl_3$, 1:8, with three drops of NH_4OH) with a tailing baseline fluorescent spot. The crude product was dissolved in a minimum amount of AcOH and evaporated under reduced pressure to an oily residue which was diluted with MeOH and cooled in a freezer overnight to afford 1.0 g (19%) of **4a** as a yellow solid: mp 295–299 °C; ^1H NMR (Me_2SO-d_6) δ 2.66 (s, 3 H, 5-CH_3), 3.51 (s, 3 H, 4′-OCH_3), 3.67 (2 s, 6 H, 3′,5′-OCH_3), 4.17 (d, 2 H, CH_2N, J = 4.9 Hz), 5.66 (t, 1 H, NH, J = 4.9 Hz), 5.93 (s, 2 H, Ar-H), 6.21 (br s, 2 H, NH_2), 6.99 (br s, 2 H, NH_2), 8.48 (s, 1 H, H_7); MS *m/e* 371 (MH^+) for $C_{18}H_{22}N_6O_3$. Anal. ($C_{18}H_{22}N_6O_3 \cdot 1.2CH_3COOH \cdot H_2O$) C, H, N.

The glucuronide salt of **4a** was prepared by dropwise addition of a solution of glucuronic acid (0.08 g, 0.41 mmol) in 30 mL of water to a suspension of **4a** (0.08g, 0.22 mmol) in 30 mL of MeOH. The resulting solution was stirred for 5 min and the concentrated to dryness under vacuum. The residue was stirred in CH_3CN, filtered, and washed with MeOH and Et_2O to give 0.14 g (86%) of a solid: MS *m/e* 371 (MH^+) for $C_{18}H_{22}N_6O_3$. Anal. ($C_{18}H_{22}N_6O_3 \cdot 1.5C_6H_9O_7 \cdot 3H_2O$) C, H, N.

2,4-Diamino-5-methyl-6-[(3′,4′-dichloroanilino)methyl]pyrido[2,3-*d*]pyrimidine (4b). A stirred solution of **3** (3.0 g, 15 mmol) and 3,4-dichloroaniline (2.64 g, 16.6 mmol) in 80% AcOH (550 mL) containing damp Raney Ni (19 g) was hydrogenated at atmospheric pressure for 24 h. The mixture was then treated with Norit and filtered through Celite. The solvent was removed from the filtrate by evaporation under reduced pressure (H_2O aspirator, bath to 40 °C) with the aid of an added portion of MeOH. To a solution of the residue in warm EtOH (25 mL) was added silica gel (15 g). The resulting suspension was evaporated under vacuum to dryness and loaded on a funnel (3$^1/_2$-in. in diameter) containing 85 g of silica gel. The funnel was eluted under slightly reduced pressure (water aspirator) with EtOAc (500 mL) to remove the unreacted dichloroaniline and then eluted sequentially with EtOAc/MeOH (8:1 1 L, 6:1 1 L, 4:1 2 L), and fractions of 70 mL each were collected. The fractions containing the desired product (R_f 0.55 in MeOH/$CHCl_3$, 4:1, with 3 drops of NH_4OH) were combined and left overnight at room temperature. The yellow solid which separated was collected to afford 1.2 g (23%) of **4b**: mp >300 °C; ^1H NMR (DMSO-d_6) δ 2.60 (s, 3 H, CH_3), 4.20 (d, 2 H, CH_2NH, J = 4.6 Hz), 6.25 (br s, 2 H, NH_2), 6.38 (t, 1 H, NH, J = 4.6 Hz), 6.62 (dd, 1 H, ArH-6′), 6.82 (d, 1 H, ArH-2′), 7.01 (br s, 2 H, NH_2), 7.27 (d, 1 H, ArH-5′), 8.47 (s, 1 H, H_7); MS *m/e* 349 (MH^+), for 351 (MH + 2) for $C_{15}H_{14}N_6Cl_2$. Anal. ($C_{15}H_{14}N_6Cl_2$) C, H, N, Cl.

2,4-Diamino-5-methyl-6-[(3′,4′,5′-trichloroanilino)methyl]pyrido[2,3-*d*]primidine (4c). A stirred solution of nitrile **3** (3.0 g, 15 mmol) and trichloroaniline (3.0 g, 15.4 mmol) in 70% AcOH (400 mL) containing damp Raney Ni (20 g) was hydrogenated at atmospheric pressure for 20 h. The mixture was then treated with Norit and filtered through Celite. The solvent was removed from the filtrate by evaporation under reduced pressure (H_2O aspirator, bath to 40 °C) with the aid of an added portion of EtOH. To a solution of the residue in warm EtOH (20 mL) was added 10 g of silica gel. The resulting mixture was evaporated to dryness and loaded on a funnel (3$^1/_2$ in. in diameter) containing 90 g of silica gel. The funnel was eluted with EtOAc/MeOH (4:1)

under slightly reduced pressure (H_2O aspirator). The fractions containing the desired product (R_f 0.37 MeOH/$CHCl_3$ (4:1) with 3 drops of NH_4OH) were combined and left at room temperature overnight. The desired compound separated to afford 1.1 g (18%) of **4c**: mp >300 °C; ^1H NMR (DMSO-d_6) δ 2.58 (s, 3 H, CH_3), 4.21 (d, 2 H, CH_2NH, J = 4.6 Hz), 6.31 (br s, 2 H, NH_2) 6.60 (t, 1 H, $NHCH_2$, J = 4.6 Hz), 6.85 (s, 2 H, ArH), 7.06 (br s, 2 H, NH_2), 8.46 (s, 1 H, H_7); ^1H NMR (TFA-d) δ 3.09 (s, 3 H, CH_3), 5.13 (s, 2 H, CH_2), 7.65 (s, 2 H, ArH), 9.06 (s, 1 H, H_7); MS m/e 383 (MH^+), 385 (MH + 2), 387 (MH + 4) for $C_{15}H_{13}N_6Cl_3$. Anal. ($C_{15}H_{13}N_6Cl_3$) C, H, N, Cl.

2,4-Diamino-5-methyl-6-[(3',4',5'-trimethoxy-*N*-methylanilino)methyl]pyrido[2,3-*d*]pyrimidine (5a). Method A. To a stirred suspension of **4a** (0.50 g, 1.35 mmol) and 37% formaldehyde (0.5 mL) in CH_3CN (10 mL) at 25 °C under N_2 was added sodium cyanoborohydride (0.25 g, 4.39 mmol). Glacial AcOH (0.10 mL) was then added to the reaction mixture dropwise via a syringe over a period of 5 min. After the mixture was stirred for 2 h, additional glacial AcOH (0.2 mL) was added and the reaction mixture was stirred at room temperature until no starting material could be detected on TLC (R_f for **4a** was 0.56, R_f for **5a** was 0.67 in MeOH/$CHCl_3$ (1:8) with 3 drops of NH_4OH). The reaction mixture was then diluted with MeOH and refrigerated overnight. The solid formed was filtered and washed with water, MeOH, and Et_2O to give 0.22 g (42%) of **5a**: mp >300 °C (decomposition ≈ 265 °C); ^1H NMR (Me_2SO-d_6) δ 2.62 (s, 3 H, 5-CH_3), 2.85 (s, 3 H, N-CH_3), 3.55 (s, 3 H, 4'-OCH_3), 3.71 (s, 6 H, 3',5'-OCH_3), 4.47 (s, 2 H, CH_2), 6.05 (s, 2 H, Ar-H), 6.22 (s, 2 H, NH_2), 6.99 (br, s, 2 H, NH_2), 8.27 (s, 1 H, H_7); MS m/e 385 (MH^+) for $C_{19}H_{24}N_6O_3$. Anal. ($C_{19}H_{24}N_6O_3$·0.6CH_3COOH·H_2O) C, H, N.

Method B. The procedure was the same as in the method A except 1 N HCl was used instead of glacial AcOH and the pH was maintained between 2 and 3. The reaction was completed in 1 h. The reaction was taken to dryness under vacuum (bath temperature <40 °C). To the residue was added CH_3CN (10 mL), and the mixture was refrigerated overnight. The product which separated was obtained as the HCl salt 0.56 g (75%). On the basis of TLC the product was identical to that obtained in method A: MS m/e 385 (MH^+) for $C_{19}H_{24}N_6O_3$. Anal. ($C_{19}H_{24}N_6O_3$·1.50HCl·0.75CH_3CN) C, H, N, Cl.

2,4-Diamino-5-methyl-6-[(*N*-methyl-3',4'-dichloroanilino)methyl]pyrido[2,3-*d*]pyrimidine (5b). To a stirred suspension of **4b** (0.50 g, 1.43 mmol) and 37% formaldehyde (0.5 mL) in acetonitrile (15 mL) at 25 °C under N_2 was added $NaCNBH_3$ (0.25 g, 4.39 mmol). A 1 N HCl solution was then added to the reaction mixture dropwise and the pH adjusted to between 2 and 3. After the mixture was stirred for 2 h, additional 1 N HCl solution was added and the pH adjusted to 2. At this stage only trace amounts of **4b** remained unreacted. The reaction mixture was stirred overnight and the pH was maintained between 2 and 3, after which no starting material could be detected on TLC (**4b** R_f = 0.55, **5b** R_f = 0.66 in MeOH/CHCl, 1:8, with 3 drops of NH_4OH). The reaction mixture was then taken to dryness, diluted with MeOH, and kept in the freezer overnight. The solid which remained was filtered and washed (MeOH and Et_2O) to afford 0.32 g (56% yield) of **5b**: mp >300 °C; ^1H NMR (DMSO-d_6) δ 2.60 (s, 3 H, 5-CH_3), 2.95 (s, 3 H, N-10-CH_3), 4.62 (s, 2 H, CH_2N), 6.71 (dd, 1 H, ArH-6', J = 1.9 and 9.0 Hz), 6.91 (d, 1 H, ArH-2', J = 1.9 Hz), 7.34 (d, 1 H, ArH-5', J = 9 Hz), 7.03 (br s, 2 H, NH_2), 7.83 (br s, 2 H, NH_2), 8.13 (s, 1 H, H_7); MS m/e 363 (MH^+), 365 (MH + 2) for $C_{16}H_{16}N_6OCl_2$. Anal. ($C_{16}H_{16}N_6Cl_2$·1.55H_2O·0.2CH_3CN) C, H, N, Cl.

2,4-Diamino-5-methyl-6-[(3',4',5'-trichloro-*N*-methylanilino)methyl]pyrido[2,3-*d*]pyrimidine (5c). To a stirred suspension of **4c** (0.1 g, 26 mmol) and 37% formaldehyde (0.2 mL) in acetonitrile (6 mL) at room temperature under N_2 was added $NaCNBH_3$ (0.05 g, 0.88 mmol) from a freshly opened can. A 1 N HCl solution (bubbled with N_2 for 1 min) was then added slowly to the reaction mixture to maintain the pH between 2 and 3. Additional amounts of formaldehyde (0.2 mL), $NaCNBH_3$ (0.05 g), and 1 N HCl solution were added after 4, 6, and 8 h. The reaction was stopped after 15 h. The starting material **4c** could still be detected on TLC (R_f 0.37 MeOH/$CHCl_3$ (1:4) with 3 drops of NH_4Cl). To the reaction mixture was added 4 g of silica gel, and the residue obtained, after removal of solvents under vacuum, was applied as a plug on a column containing 100 g of silica gel. The column was sequentially eluted with $CH_3OH/CHCl_3$ (1 L, 1:10 and 1 L, 2:9). The fractions containing the desired product (R_f 0.40; $CH_3OH/CHCl_3$ (1:4) with 3 drops of NH_4OH) were combined and evaporated to dryness. The residue was dissolved in a minimum amount of AcOH and filtered. The filtrate was evaporated to dryness and diluted with acetone and left in the freezer overnight to afford a yellow solid which was collected to give 0.045 g (43% yield) of **5c**: mp >300 °C; ^1H NMR (DMSO-d_6) δ 2.55 (s, 3 H, 5-CH_3), 2.92 (s, 3 H, N-10-CH_3), 4.60 (s, 2H, 9-CH_2), 6.28 (br s, 2 H, NH_2), 6.96 (s, 2 H, 2',6'-H), 7.05 (br s, 2 H, NH_2), 8.10 (s, 1 H, H_7); MS m/e 399 (MH^+), 401 (MH + 2), 403 (MH + 4) for $C_{16}H_{15}N_6Cl_3$. Anal. ($C_{16}H_{15}N_6Cl_3$·2.1CH_3COOH·0.7HCl) C, H, N, Cl.

2,4-Diamino-5-methyl-6-[(*N*-formyl-3',4',5'-trimethoxyanilino)methyl]pyrido[2,3-*d*]pyrimidine (6a). A solution of **4a** (0.30 g, 0.81 mmol) in 98% formic acid (3 mL) was stirred at room temperature for 5 h, after which the solvent was removed under reduced pressure. The residue was diluted with MeOH and refrigerated overnight. The mixture was filtered to afford 0.30 g (93%) of **6a** as an off-white solid: mp 270–272 °C; ^1H NMR (Me_2SO-d_6) δ 2.59 (s, 3 H, 5-CH_3), 3.65 (s, 3 H, 4'-OCH_3), 3.74 (s, 6 H, 3',5'-OCH_3), 5.08 (s, 2 H, CH_2N), 6.64 (s, 2 H, Ar-H, overlapped with peak at 6.50 (br s, 2 H, NH_2), 7.30 (br, s, 2 H, NH_2) 8.15 (s, 1 H, H_7), 8.40 (s, 1 H, N-CHO), 8.50 (s, 1.1 H, *H*COOH); MS m/e 399 (MH^+) for $C_{19}H_{22}N_6O_4$. Anal. ($C_{19}H_{22}N_6O_4$·1.1HCOOH) C, H, N.

2,4-Diamino-5-methyl-6-[(*N*-formyl-3',4'-dichloroanilino)methyl]pyrido[2,3-*d*]pyrimidine (6b). A solution of **4b** (0.30 g, 0.86 mmol) in 98% formic acid (2 mL) and acetic anhydride (0.16 mL) was stirred at room temperature for 5 h, and the solvent was removed under reduced pressure. The residue was diluted with MeOH and refrigerated overnight. The precipitate obtained was filtered to afford 0.30 g (92% yield) of **6b** as an off-white solid: mp 270–275 °C; ^1H NMR (DMSO-d_6) δ 2.60 (s, 3 H, CH_3), 5.10 (s, 2 H, CH_2N), 6.63 (br s, 2 H, NH_2), 7.27 (br s, 2 H, NH_2), 7.37 (d, 1 H, ArH-6'), 7.59 (d, 1 H, ArH-5'), 7.78 (d, 1 H, ArH-2'), 8.23 (s, 1 H, H_7), 8.49 (s, 1 H, N-CHO), 8.66 (s, 0.9 H, HCOOH); MS m/e 377 (MH^+), 379 (MH + 2) for $C_{16}H_{14}N_6OCl_2$. Anal. ($C_{16}H_{14}N_6OCl_2$·0.9HCOOH·1.1H_2O) C, H, N, Cl.

2,4-Diamino-5-methyl-6-[(*N*-formyl-3',4',5'-trichloroanilino)methyl]pyrido[2,3-*d*]pyrimidine (6c). A solution of **4c** (0.10 g, 0.26 mmol) in a mixture of 98% formic acid (3 mL) and Ac_2O (0.15 mL) was stirred at room temperature for 5 h, after which the solvents were removed under reduced pressure. The residue was diluted with MeOH and refrigerated overnight. The solid which separated was filtered to afford 0.07 g (65% yield) of **6c** as a white solid: mp 269–272 °C; ^1H NMR (DMSO-d_6) δ 2.54 (s, 3 H, CH_3), 5.10 (s, 2 H, CH_2), 6.55 (br s, 2 H, NH_2), 7.20 (br s, 2 H, NH_2), 7.83 (s, 2 H, ArH), 8.17 (s, 1 H, H_7), 8.48 (s, 1 H, N-CHO), 8.65 (s, 0.9 H, HCOOH); ^1H NMR (TFA-d) δ 3.08 (s, 3 H, CH_3), 5.36 (s, 2 H, CH_2N), 8.18 (s, 1 H, HCON), 8.60 (s, 1 H, HCOOH), 8.94 (s, 1 H, H_7); MS m/e 411 (MH^+), 413 (MH + 2), 415 (MH + 4) for $C_{16}H_{13}N_6OCl_3$. Anal. ($C_{16}H_{13}N_6OCl_3$·0.9HCOOH) C, H, N, Cl.

2,4-Diamino-5-methyl-6-[(*N*-formyl-3',4',5'-trimethoxyanilino)methyl]-5,8-dihydro- and -5,6,7,8-tetrahydropyrido-[2,3-*d*]pyrimidine 8. To a solution of **6a** (0.50 g, 1.25 mmol) in glacial AcOH (20 mL) was added platinum oxide (0.1 g), and the suspension was hydrogenated in a Parr apparatus (50 psi) for 4 h at room temperature. The reaction mixture was filtered through Celite and the filtrate evaporated under reduced pressure. The residue was dissolved in 4 mL of MeOH, loaded on a flash silica gel (150 g) chromatographic column, and eluted under air pressure sequentially with MeOH/EtOAc (1:10, 1 L, 2:10, 1 L, and 3:10, 1 L), collecting 100-mL fractions. Fractions 10–20 (R_f 0.42 EtOAc/MeOH (4:1) with one drop of NH_4OH) were combined and concentrated to 10 mL under reduced pressure. The cloudy solution was clarified by filtration and the filtrate refrigerated overnight. The yellow solid which separated, on filtration afforded 0.05 g (10% yield) of the dihydro compound **7**: mp 146–149 °C; ^1H NMR (DMSO-d_6) δ 1.00 (d, 3 H, 5-CH_3, J = 6.3 Hz), 3.26 (q, 1 H, 5-H, J = 6.3 Hz), 3.62 (s, 3 H, 4'-OCH_3), 3.72 (s, 6 H, 3',5'-OCH_3), 3.95 (d, 1 H, H_9, J = 15.2 Hz), 4.90 (d, 1 H, H_9, J = 15.2 Hz), 5.42 (br s, 2 H, NH_2), 5.82 (br s, 2 H, NH_2), 5.87 (d, 1 H, H_7, J = 4.9 Hz), 6.69 (s, 2 H, Ar-H), 7.70 (d, 1 H, NH,

J = 4.9 Hz), 8.48 (s, 1 H, N-CHO); MS m/e 401 (MH^+) for $C_{19}H_{24}N_6O_4$. Anal. ($C_{18}H_{24}N_6O_4 \cdot 2.1CH_3COOH$) C, H, N.

The tetrahydro compound 8 (0.17 g, yield 34%) was obtained from fractions 26–30 (R_f 0.22 MeOH/$CHCl_3$ (1:4) with 1 drop of NH_4OH): mp 166–170 °C; ^1H NMR (DMSO-d_6) δ 0.89 (d, 3 H, CH_3), 1.89 (m, 1 H, H_6), 2.68 (m, 1 H, H_5), 3.01 (m, 2 H, C_7H_2), 3.66 (s, 3 H, 4′-OCH_3), 3.76 (s, 6 H, 3′,5′-OCH_3), 3.96 (m, 2 H, C_9H_2), 4.90 (br s, 2 H, NH_2), 5.79 (br s, 2 H, NH_2), 6.35 (br s, 1 H, NH), 6.79 (s, 2 H, Ar-H), 8.38 (s, 1 H, CHO); MS m/e 403 (MH^+) for $C_{18}H_{27}N_6O_4$. Anal. ($C_{18}H_{26}N_6O_4 \cdot 1.5CH_3COOH \cdot 2.2H_2O$) C, N, H.

2,4-Diamino-5-methyl-6-[(3′,4′,5′-trimethoxyanilino)methyl]-5,6,7,8-tetrahydropyrido[2,3-*d*]pyrimidine (9). To a solution of 8 (0.5 g, 12.4 mmol) in DMSO (7 mL) was added 1 N KOH solution until the solution just became turbid. N_2 was bubbled through the reaction mixture for 2 min and the flask stoppered tightly and stirred for 3 days at room temperature, and for 4 h at 35 °C, and again at room temperature for an additional 12 h. The white solid was collected by filtration to afford 0.15 g (32% yield) of 9: mp 250–252 °C; ^1H NMR (DMSO-d_6) δ 0.86 (d, 3 H, 5-CH_3), 1.96 (m, 1, H, H_6), 2.83 (m, 1 H, H_5), 2.96 (m, 3 H, 9-CH_2 and H_7), 3.19 (m, 1 H, H_7), 3.52 (s, 3 H, 4′-OCH_3), 3.69 (s, 6 H, 3′,5′-OCH_3), 5.10 (s, 2 H, NH_2), 5.40 (s, 3 H, NH_2 and N_{10}-H), 5.86 (s, 2 H, Ar-H), 6.11 (d, 1 H, N_8-H); MS m/e 375 (MH^+) for $C_{18}H_{26}N_6O_3$. Anal. ($C_{18}H_{26}N_6O_3 \cdot 0.2H_2O$) C, H, N.

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