Notes

Synthesis and Biological Activity of 4-Amino-7-oxo-Substituted Analogues of 5-Deaza-5,6,7,8-tetrahydrofolic Acid and 5,10-Dideaza-5,6,7,8-tetrahydrofolic Acid

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The 4-amino-7-oxo-substituted analogues of 5-deaza-5,6,7,8-tetrahydrofolic acid (5-DATHF) and 5,10-dideaza-5,6,7,8-tetrahydrofolic acid (DDATHF) were synthesized as potential antifolates. Treatment of the α,*β*-unsaturated esters **11a**−**c**, obtained in one synthetic step from commercially available para-substituted methyl benzoates (**9a**−**c**) and methyl 2-(bromomethyl)-acrylate (**10**), with malononitrile in NaOMe/MeOH afforded the corresponding pyridones **12a**−**c**. Formation of the pyrido[2,3-*d*]pyrimidines **13a**−**c** was accomplished upon treatment of **12a**−**c** with guanidine in methanol. After the hydrolysis of the ester group present in **13a**−**c**, the resulting carboxylic acids **14a**−**c** were treated with diethyl cyanophosphonate in Et_3N/DMF and coupled with L-glutamic acid dimethyl ester to give **15a**−**c**. Finally, the basic hydrolysis of **15a**−**c** yielded the desired 4-amino-7-oxo-substituted analogues **16a**−**c** in 20−27% overall yield. Compounds **16a**−**c** were tested in vitro against CCRF-CEM leukemia cells. The results obtained indicated that our 4-amino-7-oxo analogues are completely devoid of any activity, the IC_{50} being higher than 20 *μ*g/mL for all cases except **14c** for which a value of 6.7 *μ*g/mL was obtained. These results seem to indicate that **16a**−**c** are inactive precisely due to the presence of the carbonyl group in position C7, the distinctive feature of our synthetic methodology.

Introduction

Chemotherapeutic agents that act on DNA synthesis have been widely used in the treatment of cancer. Nearly all types of cells can synthesize the necessary nucleotides de novo or from the degradation products of nucleic acids. While the de novo synthetic route is almost the same in all kinds of cells, recovery pathways are quite different in characteristics and distribution. The fact that cancer cells are dependent on the de novo synthesis of purines more than normal cells allows an anticancer agent which acts on this pathway, while leaving the recovery pathway unaltered, to have a certain degree of selectivity and, consequently, lower toxicity.[1]

Folic acid (FA, **1**) and derivatives, especially 5,6,7,8-tetrahydrofolic acid (H_4FA), participate as coenzymes in the transference, oxidation, and reduction of carbon units in numerous biochemical routes (Figure 1).[2] Thus, acting on folic acid metabolism provides a way to intervene in the de novo biosynthesis of nucleotides. This objective has been sought by several different approaches.

A traditional approach is the inhibition of dihydrofolate reductase (DHFR), the enzyme which catalyzes the transformation of 7,8-dihydrofolic acid (H_2FA) to H_4-FA. The synthesis of potent DHFR inhibitors (Figure 2) such as aminopterin (AMT, **2**) and methotrexate

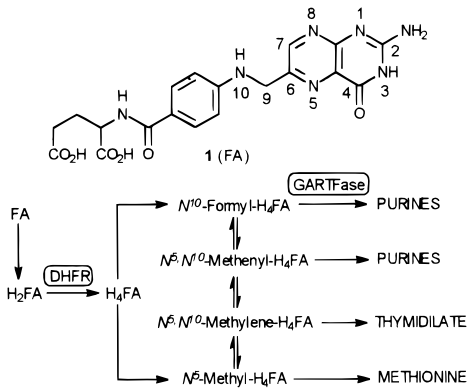

Figure 1.

(MTX, **3**),[3] currently widely used for cancer chemotherapy, has brought about the development of a large series of analogues.[4,5]

More recently, a new mechanism of action was found in the case of 5-deaza-5,6,7,8-tetrahydrofolic acid (5-DATHF, **4**) and 5,10-dideaza-5,6,7,8-tetrahydrofolic acid (DDATHF, **5**) synthesized by E. C. Taylor et al. (Figure 2).[6,7] These compounds inhibit glycinamide ribonucleotide transformylase (GARTFase), responsible for the transformation of glycinamide ribonucleotide (GAR) into α-*N*-formylglycinamide ribonucleotide (FGAR), therefore inhibiting the biosynthesis of purines. The 6*R*-diaste-

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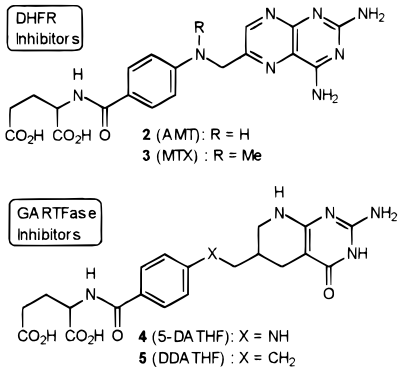


Figure 2.

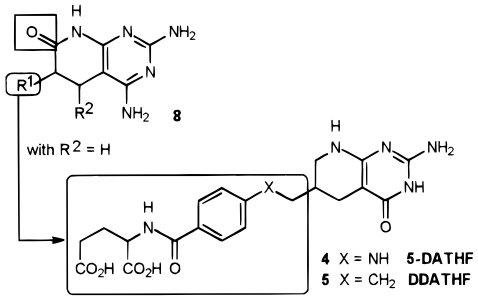
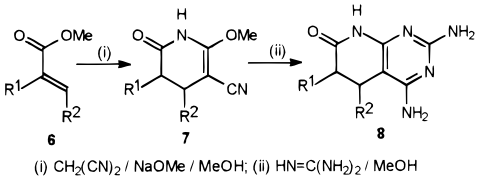


Figure 3.

Scheme 1



reomer of DDATHF (lometrexol), in which the H−C6 atom has the same spatial orientation as in H_4FA, has recently finished phase II clinical trials.[8,9]

Usually these folic acid analogues are synthesized through a strategy employing the reaction of a conveniently substituted benzoate and a preformed bicyclic heterocyclic compound, normally obtained by cyclization of adequate substituents present in a pyrimidine ring.

During the past few years, our group has developed an alternative strategy for the formation of 5,6,7,8-tetrahydropyrido[2,3-*d*]pyrimidin-7-ones of general structure **8** (Scheme 1) by cyclization with guanidine of 2-methoxy-6-oxo-1,4,5,6-tetrahydropyridine-3-carbonitriles (**7**), obtained by reaction of an α,*β*-unsaturated ester **6** and malononitrile in NaOMe/MeOH.[10−15]

A structural comparison (Figure 3) between **8** and the antifolates 5-DATHF (**4**) and DDATHF (**5**) showed that, if it were possible to introduce the long side chain present on C6 of the inhibitors, new analogues with potential activity could be obtained, the main difference being the presence of a carbonyl group at C7 and an amino group at C4.

Now, we report the use of our methodology for the synthesis of **16a**−**c** (Scheme 2), 4-amino-7-oxo-substituted analogues of 5-DATHF (**4**) and DDATHF (**5**), and the determination of their biological activity.

Results and Discussion

Chemistry. A retrosynthetic analysis of structures **16a**−**c**, according to the methodology depicted in Scheme 1, led to the α,*β*-unsaturated esters **11a**−**c**, which were further disconnected to a nucleophilic para-substituted methyl benzoate **9a**−**c** and methyl 2-(bromomethyl)-acrylate (**10**). Although commercially available, this was obtained in a 67% yield over two steps by following the method of J. Villieras and M. Rambaud[16] starting from trimethyl phosphonoacetate. Then, the treatment of **10** with methyl 4-methylaminobenzoate (**9a**) in methanol afforded methyl *p*-[*N*-(2-methoxycarbonylallyl)-*N*-methylamino]benzoate (**11a**) in 99% yield (Scheme 2). Similarly, methyl *p*-[*N*-(2-methoxycarbonylallyl)-amino]benzoate (**11b**) was obtained in 74% yield by reaction of methyl 2-(bromomethyl)acrylate (**10**) and methyl 4-aminobenzoate (**9b**) in the presence of Et_3N in toluene.

Finally, the synthesis of methyl *p*-(3-methoxycarbonyl-3-butenyl)benzoate (**11c**) was achieved by using a metal-catalyzed cross coupling reaction according to the methodology described by P. Knochel et al.[17,18] Thus, the organometallic derivative **9c** was formed by the slow addition of methyl *p*-bromomethylbenzoate, obtained by methylation of *p*-bromomethylbenzoic acid with diazomethane, to a suspension of activated zinc in anhydrous THF at 0 °C in an inert atmosphere. The resulting mixture was added to a solution of CuCN and LiCl in THF at −78 °C, the transmetalation being ensured by warming the solution to −20 °C for 5 min. After the solution was cooled to −78 °C, methyl 2-(bromomethyl)-acrylate (**10**) was added to the mixture of **9c** in THF to afford **11c**. The yield obtained was found to be dependent on the rate of addition of the methyl *p*-bromomethylbenzoate to the zinc suspension. Table 1 summarizes the yields of **11c** and the two byproducts obtained, **17** and **18**, as a function of the rate of addition of methyl *p*-bromomethylbenzoate.

Formation of **18** can be explained by a Wurtz-type coupling of the Zn organometallic intermediate. This kind of reaction is frequent in benzylic or allylic bromides, with **18** even being the only product isolated.[19] The amount of **18** could be minimized by a careful control of the rate of addition of the methyl *p*-bromomethylbenzoate, which must be approximately a drop every 5 s.[20]

As for the formation of **17**, it can only be explained by the presence of water in the medium that destroys either the Zn or the Cu organometallic. To minimize the amount of **17** formed (expt 4), it was necessary to dry the glassware in an oven, to use THF freshly distilled over $LiAlH_4$, and to dry the LiCl over P_2O_5 at 60 °C under reduced pressure for 24 h.

The quality and activation of the zinc employed was also critical for the progress of the reaction. In the present work, we used Zn from Fluka (art. 96453, puriss., grit, ≥99.5%), which was cleaned with diluted HCl and activated with 1,2-dibromoethane in boiling THF. A coupling reaction carried out with Zn dust did not afford the desired product.

In the optimal experimental conditions found (expt 4), methyl *p*-(3-methoxycarbonyl-3-butenyl)benzoate (**11c**) was obtained in a 77% yield.

The syntheses of the 2-methoxy-6-oxo-1,4,5,6-tetrahydropyridine-3-carbonitriles **12a**−**c** were carried out by following the general method developed by our group (Scheme 1) which has been tested with a wide range of

Scheme 2

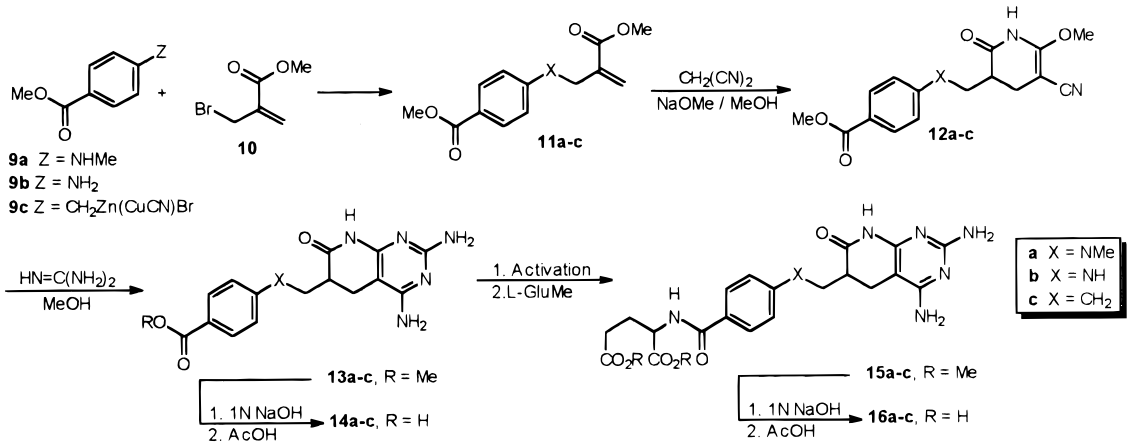
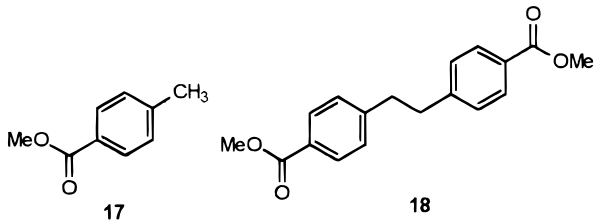


Table 1. Yields of **11c** and the Two Byproducts Obtained, **17** and **18**, as a Function of the Rate of Addition of Methyl *p*-Bromomethylbenzoate to the Zinc Suspension



entry	rate of add.[a] (g/h)	**11c** (%)	**17** (%)	**18** (%)
expt 1	3.7	16	24	14
expt 2	0.9	28	16	5
expt 3	2.9	55	21	3
expt 4[b]	2.5	77	2	0.5

[a] Rate of addition of methyl *p*-bromomethylbenzoate to the zinc suspension. [b] LiCl was dried over P_2O_5 at 60 °C under reduced pressure for 24 h.

α,β-unsaturated esters.[21] The yields obtained depend on the nature and position of the substituents present in the ester. In particular, yields are lower in 2-substituted acrylates due to formation of the Michael bisadduct as a byproduct. Thus, in the case of methyl methacrylate, it was necessary to apply a simplex optimization method to find the optimal reaction conditions.[22] Similarly, **11a**−**c** underwent Michael addition followed by cyclization when treated with malononitrile in NaOMe/MeOH to afford pyridones **12a**−**c** in 40−55% yield (Scheme 2). Compounds **12a**−**c** were obtained as racemic mixtures and used without separation. However, enantioresolution of **12a** and **12c** is possible by using reversed phase HPLC on a vancomycin chiral stationary phase (CHIROBIOTIC V) column from Advanced Separation Technologies (Whippany, NJ), employing a mixture of 90% buffer solution (1% triethylammonium acetate) and 10% acetonitrile as eluent.[23]

Next, the 2,4-diaminopyrido[2,3-*d*]pyrimidines **13a**−**c** were obtained in 80−95% yield by treatment of the corresponding compounds **12a**−**c** with 2 equiv of guanidine in MeOH at reflux for 20 h. The syntheses of **13a**−**c** prove again the general applicability of the methodology depicted in Scheme 1. Subsequently, compounds **13a**−**c** were hydrolyzed in 1 N aqueous NaOH to afford the corresponding carboxylic acids in almost quantitative yield.

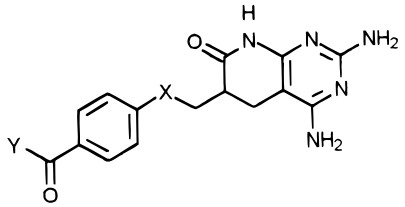
Our initial strategy for the syntheses of the folic acid analogues **15a**−**c** was to activate the carboxylic acids **14a**−**c** by using 2-chloro-4,6-dimethoxy-1,3,5-triazine (**19**) in the presence of *N*-methylmorpholine (NMM, **20**) and to react the resulting ester with L-glutamic acid dimethyl ester, following the method described by Z. Kaminski.[24] This reagent has been widely used in the formation of folic acid analogues and, in particular, was employed by E. C. Taylor in the synthesis of DDATHF.

However, the treatment of **14a** or **14b** in the aforementioned reaction conditions did not yield the corresponding amides **15a,b**, either at room temperature or at 70 °C, the starting acids being recovered and a byproduct being formed. This compound was identified as the 2,4-dimethoxy-6-(*N*-morpholin)-1,3,5-triazine (**22**). Formation of **22** could be rationalized as follows: the low solubility of the starting acids **14a,b** in DMF precludes the ionization of the carboxylic acid by the NMM, and then the 2-chloro-4,6-dimethoxy-1,3,5-triazine (**19**) undergoes the nucleophilic substitution of the chlorine atom by the NMM with formation of the quaternary ammonium salt **21**, which finally reverts to **22** during the evaporation of the DMF (80 °C at 2−4 mmHg) due to the demethylation of **21** caused by the chloride ion.

However, when we used triazine **19** for the coupling of the also poorly soluble **14c**, we obtained the desired **15c** in 58% yield. This result led us to think that the low solubility is not the only factor which favors the formation of **22**. So we decided to test the coupling of L-glutamic acid dimethyl ester with *p*-toluic acid and *p*-methylaminobenzoic acid using the 2-chloro-4,6-dimethoxy-1,3,5-triazine (**19**) and NMM. In the first case, we obtained the coupled product in 75% yield, but in the second one, **22** was obtained as the predominant product. Therefore, the use of **19** and NMM for the coupling of folic acid analogues which contain a nitrogen atom in the CH_2X bridge should not be recommended.

This unexpected problem led us to change the activating agent, and we shifted to diethyl cyanophosphonate which has successfully been used in the coupling of L-glutamic acid dimethyl ester with pyrrolo[2,3-*d*]pyrimidines[25] and other nitrogenated compounds.[26] Thus, the treatment of the acids **14a**−**c** with diethyl cyanophosphonate in DMF using Et_3N as the base, followed by addition of L-glutamic acid dimethyl ester, gave the corresponding coupled products **15a**−**c** in 70−85% yield.

Table 2. Structures and in Vitro Cytotoxicity Assay (IC_{50}) Conducted in CCRF-CEM Human Leukemia Cells



compd	X	Y	IC_{50} (μg/mL)
13a	NMe	OMe	>20
13b	NH	OMe	>20
13c	CH_2	OMe	>20
14a	NMe	OH	>20
14b	NH	OH	>20
14c	CH_2	OH	6.7
16a	NMe	Glu[a]	>20
16b	NH	Glu	>20
16c	CH_2	Glu	39.2

[a] Glu = $HO_2CCH_2CH_2CH(CO_2H)NH$-.

The last step for the syntheses of the folic acid analogues **16a**−**c** is the basic hydrolysis of the ester groups present in the glutamate unit. Hydrolyses carried out using 1 N NaOH at room temperature during 24 h afforded the corresponding compounds **16a**−**c** in 85−95% yield.

To sum up, compounds **16a**−**c**, which are the 4-amino-7-oxo-substituted analogues of 5-DATHF (**4**) and DDATHF (**5**), have been obtained in 6 steps, from commercially available compounds, in 25, 19, and 27% total yields, respectively.

Biological Evaluation. Compounds **13a**−**c**, **14a**−**c**, and **16a**−**c** were tested in vitro against CCRF-CEM leukemia cells (Table 2).[27] This is a very common T cell derived lymphoblastic leukemia that has been widely used as a discriminatory test. Both DDATHF (**5**) and methotrexate (**3**) demonstrated potent growth inhibitory activity against the CCRF-CEM cells, the IC_{50} of DDATHF and methotrexate being 0.007 μg/mL and 0.004 μg/mL, respectively.

The results obtained indicated that our 4-amino-7-oxo analogues are completely devoid of any activity, the IC_{50} being higher than 20 μg/mL for all cases except **14c** for which a value of 6.7 μg/mL was obtained. This lack of activity cannot be attributed to the low solubility of these compounds in aqueous system because, in the test performed, all the compounds are dissolved in DMSO and then diluted into aqueous buffer solution to the desired concentration. Both DDATHF and methotrexate are not very soluble even under these conditions, yet they are very potent and cytotoxic compounds.

Compounds **16a**−**c** present a 2,4-diaminopyrimidine ring and thus are primarily targeted at DHFR, and it is known that oxygenation at the 7 position is, in general, not good for DHFR binding. Thus, for instance, methotrexate inhibited rat liver DHFR with an IC_{50} of 23 nM, whereas the corresponding value for 7-hydroxymethotrexate was 4000 nM.[28]

Consequently, taking into account that the 4-amino-substituted analogue of DDATHF was able to inhibit bovine liver DHFR with an IC_{50} of 71 nM,[7] it has to be concluded that our 4-amino-7-oxo-substituted analogues of DDATHF are inactive precisely due to the presence of the carbonyl group in position C7, the distinctive feature of our synthetic methodology. The only remaining question is if such negative effect of the carbonyl group would be also present in 7-oxo-substituted analogues of DDATHF which would be targeted against glycinamide ribonucleotide transformylase (GARTFase). Experiments are being conducted to clarify this point.

Experimental Section

All melting points, determined with a Büchi 530 capillary apparatus, and boiling points, determined during distillation, are uncorrected. Infrared spectra were recorded in a BOMEM Michelson 100 and a Nicolet Magna 560 FTIR spectrophotometers. UV spectra were registered in a Hewlett-Packard 8450 instrument. ^1H and ^{13}C NMR spectra were determined in a Varian Gemini-300 operating at a field strength of 300 and 75.5 MHz, respectively. Chemical shifts are reported in parts per million (δ) and coupling constants (J) in Hz using, in the case of ^1H NMR, TMS or sodium 2,2,3,3-tetradeuteriotrimethylsilylpropionate as an internal standard and setting, in the case of ^{13}C NMR, the references at the signal of the solvent: 77.0 ppm ($CDCl_3$); 39.5 ppm (DMSO-d_6); 163.8 ppm (CF_3COOD, TFA-d). Standard and peak multiplicities are designated as follows: s, singlet; d, doublet; dd, doublet of doublets; t, triplet; brs, broad singlet; br, broad signal; m, multiplet. Mass spectra (m/z (%), EI, 70 eV) were obtained on a Hewlett-Packard 5995 A spectrometer. FAB(+)-HRMS were registered at the Servicio de Espectrometría de Masas (Universidad de Córdoba) using a VG Autospec spectrometer (resolution 8000, 3-nitrobenzyl alcohol as matrix). Elemental microanalyses were obtained on a Carlo-Erba CHNS-O/EA 1108 analyzer and gave results for the elements stated with ±0.4% of the theoretical values. *N*,*N*-Dimethylformamide (DMF) was dried over activated (250 °C) 4-Å molecular sieves. Tetrahydrofuran (THF) was distilled from $LiAlH_4$ and kept over 4-Å molecular sieves. MeOH refers to methanol, Et_2O refers to diethyl ether, AcOEt refers to ethyl acetate, and Carbitol refers to 2-(2-ethoxyethoxy)ethanol. Thin layer chromatographies (TLC) were performed on precoated sheets of silica 60 Polygram SIL N-HR/UV_{254} (Macherey Nagel art. 804023). Dry-column chromatography was performed using silica gel 70−230 mesh (ASTM) (Merck art. 7734 or Macherey Nagel art. 81533). Flash chromatography was performed using silica gel 230−400 mesh (ASTM) (Macherey Nagel art. 81538). Diazomethane in Et_2O was prepared starting from diazald (*N*-methyl-*N*-nitroso-*p*-toluenesulfonamide) by using the method and apparatus described by M. Hudlicky.[29]

Methyl 2-(Bromomethyl)acrylate (10).[16] A saturated aqueous solution of 33.81 g (0.24 mol) potassium carbonate was slowly added (30 min) to a mixture of 25.86 g (0.15 mol) of trimethyl phosphonoacetate and 50.50 g (0.59 mol) of a 35% aqueous solution of formaldehyde stirred at room temperature. At the end of the addition, the temperature rose to 30−35 °C, and stirring was maintained for 1 h. Then, a saturated solution of ammonium chloride (150 mL) was added, and the resulting mixture was extracted with CH_2Cl_2 (3 × 50 mL). The organic extracts were dried ($MgSO_4$) and concentrated to give 16.24 g (0.14 mol, 89%) of methyl 2-(hydroxymethyl)acrylate as a colorless liquid which was used without any further purification. To a stirred solution of 13.60 g (0.12 mol) of methyl 2-(hydroxymethyl)acrylate in 250 mL of Et_2O was added 14.60 g (0.054 mol) of phosphorus tribromide at −20 °C. The resulting mixture was heated to room temperature and maintained with stirring for 3 h. Then it was cooled to −20 °C, and 150 mL of water were added. The mixture was extracted with hexane (3 × 50 mL), dried ($MgSO_4$), and concentrated in vacuo to give 13.36 g (0.091 mol, 78%) of **10** which was used without any further purification.

Methyl *p*-[*N*-(2-Methoxycarbonylallyl)-*N*-methylamino]benzoate (11a). A solution of 10.80 g (0.06 mol) of methyl 2-(bromomethyl)acrylate (**10**) in 20 mL of MeOH was added to a suspension of 10.00 g (0.06 mol) of methyl 4-methylaminobenzoate (**9a**) in 30 mL of MeOH. The resulting mixture was stirred for 12 h at room temperature. Then the solvent

was removed at reduced pressure, and the residue was suspended in water. The mixture was neutralized (pH = 7−8) with a saturated solution of $NaHCO_3$ and extracted with CH_2Cl_2 (5 × 50 mL). The combined extracts were dried ($MgSO_4$) and concentrated in vacuo to give 16.90 g (0.06 mol, 99%) of **11a** as a yellow solid: mp 40−42 °C; IR ($CHCl_3$), 3000, 1715 (C=O), 1610, 1525, 770 cm^{-1}; UV (MeOH) λ_{max} (log ϵ) 202 (2.600), 306 (2.678) nm; MS *m/z* 263 (60) [M^+], 232 (40), 204 (40), 178 (100). Anal. ($C_{14}H_{17}NO_4$) C, H, N.

Methyl *p*-[*N*-(2-Methoxycarbonylallyl)amino]benzoate (11b). A solution of 3.00 g (0.017 mol) of methyl 2-(bromomethyl)acrylate (**10**) in 10 mL of toluene was added to a suspension of 2.53 g (0.017 mol) of methyl 4-aminobenzoate (**9b**) and 1.69 g (0.017 mol) of Et_3N in 20 mL of toluene. The resulting mixture was stirred for 96 h at room temperature. Then the mixture was filtered to separate the triethylammonium bromide formed and was concentrated in vacuo. The solid obtained was column chromatographed using AcOEt/hexane (1:2) as eluent to give 2.61 g (0.01 mol, 74%) of **11b** as a yellow solid: mp 63−65 °C; IR (film), 3330 (N−H), 1700 (C=O), 1610, 1525, 770 cm^{-1}; UV (MeOH) λ_{max} (log ϵ) 202 (2.334), 301 (2.381) nm; MS *m/z* 249 (99) [M^+], 218 (59), 164 (100), 158 (42), 130 (65). Anal. ($C_{13}H_{15}NO_4$) C, H, N.

Methyl *p*-(3-Methoxycarbonyl-3-butenyl)benzoate (11c). A suspension of 5.00 g (23 mmol) of *p*-bromomethylbenzoic acid (Fluka art. 18520) in 100 mL of Et_2O was treated with an ethereal solution of diazomethane[29] (5.35 g (25 mmol) of diazald in 250 mL of Et_2O and 1.50 g (27 mmol) of KOH in 25 mL of EtOH). The resulting solution was stirred for 12 h, extracted with a 10% $NaHCO_3$ solution, dried ($MgSO_4$), and concentrated in vacuo to give 5.22 g (23 mmol, 98%) of **9c** as a colorless solid: mp 52−55 °C. A mixture of 3.63 g (56 mmol) of Zn (obtained by washing 4.00 g of Zn, Fluka art. 96453, puriss., grit, ≥ 99.5%, with 10% aqueous HCl for 2 min and then rinsing with water and acetone), 10 mL of anhydrous THF, and a few drops of 1,2-dibromoethane was heated at reflux for 5 min in an inert atmosphere. After the solution was cooled, a solution of 10.51 g (46 mmol) of **9c** in 20 mL of anhydrous THF was added dropwise at 0 °C during 4 h by using a peristaltic pump (Pharmacia Biotech peristaltic pump P-1). After being stirred for 2−3 h at 0 °C, the resulting solution was added by using the aforementioned peristaltic pump to a suspension of 4.11 g (46 mmol) of CuCN and 3.89 g (92 mmol) of LiCl (dried over P_2O_5 at 60 °C under reduced pressure for 24 h) in 100 mL of anhydrous THF cooled to −78 °C in an inert atmosphere. The resulting mixture was warmed to −20 °C for 5 min and cooled again to −78 °C. Then, a solution of 9.15 g (50 mmol) of methyl 2-(bromomethyl)acrylate (**10**) in 50 mL of anhydrous THF was added dropwise. The mixture was warmed to 0 °C and stirred for 12 h. Then it was poured into a mixture of CH_2Cl_2 and a saturated solution of ammonium chloride, stirred, and filtered to separate the inorganic salts formed. The two layers were separated, and the aqueous layer was extracted with CH_2Cl_2 (3 × 50 mL). The combined organic extracts were washed with water and with brine, dried ($MgSO_4$), and concentrated in vacuo. The crude material was purified by flash chromatography using AcOEt/hexane (1:10) as eluent to give 8.76 g (35 mmol, 77%) of **11c** as a colorless oil which crystallizes on standing: mp 39−41 °C; IR (film) 3000, 1720 (C=O), 1630, 1610, 770 cm^{-1}; MS *m/z* 248 (0.6) [M^+], 217 (1.7), 149 (100). Anal. ($C_{14}H_{16}O_4$) C, H.

General Method for the Syntheses of Pyridones 12. Methyl *p*-[*N*-(5-Cyano-6-methoxy-3,4-dihydro-2-pyridon-3-ylmethyl)-*N*-methylamino]benzoate (12a). A solution of 1.16 g (15.5 mmol) of malononitrile in 7 mL of MeOH was added all at once to a solution of 0.49 g (21.2 mmol) of Na in 13 mL of MeOH in an inert atmosphere. After this solution was stirred for a few minutes, a solution of 3.85 g (14.6 mmol) of methyl *p*-[*N*-(2-methoxycarbonylallyl)-*N*-methylamino]benzoate (**11a**) in 7 mL of MeOH was added dropwise. The mixture was heated at reflux for 3 h and, after being cooled, was concentrated in vacuo. The residue obtained was dissolved in water, cooled in an ice bath, and carefully neutralized (pH = 8) with a 3% HCl solution. The solid formed was filtered, washed with cool water, and dissolved in CH_2Cl_2. The aqueous layer formed was extracted with CH_2Cl_2 (2 × 50 mL). The combined organic extracts were washed with water, dried ($MgSO_4$), and concentrated to give 2.62 g (8.0 mmol, 55%) of **12a** as a colorless solid: mp 177−178 °C; IR (KBr) 3210, 3110 (N−H), 2200 (CN), 1715 (COOMe), 1690 (CONH), 1640, 1615 (C=C), 770 cm^{-1}; UV (MeOH) λ_{max} (log ϵ) 309.0 (3.158); MS *m/z* 329 (29) [M^+], 298 (12), 178 (100). Anal. ($C_{17}H_{19}N_3O_4$) C, H, N.

Methyl *p*-[*N*-(5-Cyano-6-methoxy-3,4-dihydro-2-pyridon-3-ylmethyl)amino]benzoate (12b). The procedure was the same as that stated above for **12a** but using 3.00 g (12 mmol) of methyl *p*-[*N*-(2-methoxycarbonylallyl)amino]benzoate (**11b**) in 6 mL of MeOH, 0.41 g (18 mmol) of Na in 12 mL of MeOH, 0.95 g (14 mmol) of malononitrile in 6 mL of MeOH, and refluxed for 2 h. Neutralization was accomplished with 25% acetic acid. The yield was 1.62 g (5 mmol, 43%) of **12b** as a colorless solid: mp 181−183 °C; IR (KBr) 3400 (N−H), 3200, 3100 (CON−H), 2200 (CN), 1700 (COOMe), 1690 (CONH), 1640, 1605 (C=C), 770 cm^{-1}; UV (MeOH) λ_{max} (log ϵ) 313.3 (2.413); MS *m/z* 315 (10) [M^+], 284 (4), 164 (100), 151 (47). Anal. ($C_{16}H_{17}N_3O_4$) C, H, N.

Methyl *p*-[2-(5-Cyano-6-methoxy-3,4-dihydro-2-pyridon-3-yl)ethyl]benzoate (12c). The procedure was the same as that stated above for **12a** but using 6.71 g (27 mmol) of methyl *p*-(3-methoxycarbonyl-3-butenyl)benzoate (**11c**) in 15 mL of MeOH, 0.93 g (41 mmol) of Na in 20 mL of MeOH, 2.14 g (32 mmol) of malononitrile in 15 mL of MeOH, and refluxed for 2.5 h. Neutralization was accomplished with 3% HCl. The yield was 4.37 g (14 mmol, 51%) of **12c** as a colorless solid: mp 146−147 °C; IR (KBr) 3205, 3105 (N−H), 2200 (CN), 1720 (COOMe), 1695 (CONH), 1640, 1610 (C=C), 770 cm^{-1}; MS *m/z* 314 (20) [M^+], 283 (17), 152 (100). Anal. ($C_{17}H_{18}N_2O_4$) C, H, N.

General Method for the Syntheses of 2,4-Diamino-7-oxo-5,6,7,8-tetrahydropyrido[2,3-*d*]pyrimidines 13. Methyl *p*-[*N*-(2,4-Diamino-7-oxo-5,6,7,8-tetrahydropyrido[2,3-*d*]pyrimidin-6-ylmethyl)-*N*-methylamino]benzoate (13a). A 0.79-g (4-mmol) portion of guanidine carbonate was added to a solution of 0.20 g (9 mmol) of Na in 25 mL of MeOH, and the mixture was refluxed for 15 min. After the solution was cooled, the sodium carbonate formed was filtered, and 1.35 g (4 mmol) of methyl *p*-[*N*-(5-cyano-6-methoxy-3,4-dihydro-2-pyridon-3-ylmethyl)-*N*-methylamino]benzoate (**12a**) was added. The mixture was heated at reflux for 20 h and cooled to room temperature. The solid was filtered, washed with MeOH and Et_2O, and dried over P_2O_5 to give 1.18 g (3 mmol, 83%) of **13a** as a colorless solid: mp 282 °C; IR (KBr) 3600, 3440, 3340, 3210, 3060 (N−H), 1685 (COOMe), 1660 (CONH), 1610, 1570 (C=C, C=N), 770 cm^{-1}; MS *m/z* 356 (15) [M^+], 325 (8), 178 (100). Anal. ($C_{17}H_{20}N_6O_3 \cdot H_2O$) C, H, N.

Methyl *p*-[*N*-(2,4-Diamino-7-oxo-5,6,7,8-tetrahydropyrido[2,3-*d*]pyrimidin-6-ylmethyl)amino]benzoate (13b). The procedure was the same as that stated above for **13a** but using 0.074 g (3.2 mmol) of Na in 6 mL of MeOH, 1.03 g (1.76 mmol) of guanidine carbonate, and 0.5 g (1.6 mmol) of methyl *p*-[*N*-(5-cyano-6-methoxy-3,4-dihydro-2-pyridon-3-ylmethyl)-amino]benzoate (**12b**). The yield was 0.48 g (1.4 mmol, 88%) of **13b** as a colorless solid: mp 275−278 °C; IR (KBr) 3470, 3380, 3220, 3100 (N−H), 1690 (C=O), 1630, 1610, 1570 (C=C, C=N), 765 cm^{-1}; MS *m/z* 342 (15) [M^+], 191 (74), 178 (33), 120 (100). Anal. ($C_{16}H_{18}N_6O_3$) C, H, N.

Methyl *p*-[2-(2,4-Diamino-7-oxo-5,6,7,8-tetrahydropyrido[2,3-*d*]pyrimidin-6-yl)ethyl]benzoate (13c). The procedure was the same as that stated above for **13a** but using 0.17 g (7.4 mmol) of Na in 20 mL of MeOH, 0.66 g (3.7 mmol) of guanidine carbonate, and 1.16 g (3.7 mmol) of methyl *p*-[2-(5-cyano-6-methoxy-3,4-dihydro-2-pyridon-3-yl)ethyl]-benzoate (**12c**). Reflux time was 38 h. The yield was 1.22 g (3.6 mmol, 97%) of **13c** as a colorless solid: mp 320 °C dec; IR (KBr) 3500, 3380, 3330, 3200, 3160 (N−H), 1700 (COOMe),

1680 (CONH), 1630, 1570 (C═C, C═N) cm^{-1}; MS FAB(+) *m/z* 364 $[M + Na]^+$, 342 $[M + H]^+$, 341 $[M^+]$. Anal. ($C_{17}H_{19}N_5O_3$) C, H, N.

General Method for the Hydrolysis of the Ester Group present in Pyrido[2,3-*d*]pyrimidines 13. *p*-[*N*-(2,4-Diamino-7-oxo-5,6,7,8-tetrahydropyrido[2,3-*d*]-pyrimidin-6-ylmethyl)-*N*-methylamino]benzoic Acid (14a). A suspension of 500 mg (1.4 mmol) of methyl *p*-[*N*-(2,4-diamino-7-oxo-5,6,7,8-tetrahydropyrido[2,3-*d*]-pyrimidin-6-ylmethyl)-*N*-methylamino]benzoate (**13a**) in a 0.5 N aqueous solution of NaOH was heated at reflux until dissolution of the solid and then was stirred at room temperature for 12 h. The resulting solution was filtered through a Lida filter (47-mm filter membrane, 0.45 μm nylon, art. NY504700, Lida Manufacturing Corp., 9115 26th Avenue, Kenosha, WI), and the filtrate was acidified with concentrated acetic acid. The resulting precipitate was filtered (sometimes a centrifugation was required), washed with water, and dried over P_2O_5 to give 532 mg (1.35 mmol, 96%) of **14a·3H$_2$O** as a colorless solid: mp 202−209 °C dec; IR (KBr) 3500−2500 (COO−H and N−H), 1660 (C═O), 1600, 1560 (C═C, C═N), 775 cm^{-1}; MS *m/z* 191 (35) $[C_8H_9N_5O^+]$, 151 (100), 134 (53). Anal. ($C_{16}H_{18}N_6O_3·3H_2O$) C, H, N.

***p*-[*N*-(2,4-Diamino-7-oxo-5,6,7,8-tetrahydropyrido[2,3-*d*]pyrimidin-6-ylmethyl)amino]benzoic Acid (14b).** The procedure was the same as that stated above for **14a** but using 1.000 g (3 mmol) of methyl *p*-[*N*-(2,4-diamino-7-oxo-5,6,7,8-tetrahydropyrido[2,3-*d*]pyrimidin-6-ylmethyl)amino]benzoate (**13b**) in 15 mL of a 1 N aqueous solution of NaOH. Yield was 1.030 g (3 mmol, 99%) of **14b·1.5H$_2$O** as a colorless solid: mp 285−287 °C; IR (KBr) 3500−2500 (COO−H and N−H), 1700 (C═O), 1650, 1600 (C═C, C═N), 780 cm^{-1}; MS *m/z* 191 (100) $[C_8H_9N_5O^+]$, 137 (31), 120 (49). Anal. ($C_{15}H_{16}N_6O_3·1.5H_2O$) C, H, N.

***p*-[2-(2,4-Diamino-7-oxo-5,6,7,8-tetrahydropyrido[2,3-*d*]pyrimidin-6-yl)ethyl]benzoic Acid (14c).** The procedure was the same as that stated above for **14a** but using 1.70 g (5 mmol) of methyl *p*-[2-(2,4-diamino-7-oxo-5,6,7,8-tetrahydropyrido[2,3-*d*]pyrimidin-6-yl)ethyl]benzoate (**13c**) in 100 mL of a 1 N aqueous solution of NaOH. A digestion in acetic acid of the crude material obtained gave 1.79 g (4.6 mmol, 92%) of **14c·CH$_3$CO$_2$H** as a colorless solid: mp >275 °C; IR (KBr) 3500−2500 (COO−H and N−H), 1710 (C═O), 1680−1620 (C═O, C═C, C═N), 1560 (C═C, C═N), 770 cm^{-1}. Anal. ($C_{16}H_{17}N_6O_3·CH_3CO_2H$) C, H, N.

General Method for the Syntheses of 16a−c. *N*-{*p*-[*N*-(2,4-Diamino-7-oxo-5,6,7,8-tetrahydropyrido[2,3-*d*]pyrimidin-6-ylmethyl)-*N*-methylamino]benzoyl}glutamic Acid (16a). To a solution of 1.00 g (2.9 mmol) of *p*-[*N*-(2,4-diamino-7-oxo-5,6,7,8-tetrahydropyrido[2,3-*d*]pyrimidin-6-ylmethyl)-*N*-methylamino]benzoic acid (**14a**) in 50 mL of anhydrous DMF in an inert atmosphere was added 0.74 g (7.3 mmol) of Et_3N. The resulting mixture was stirred at room temperature for 10 min. Then 1.19 g (7.3 mmol) of diethyl cyanophosphonate was added, and the resulting mixture was stirred for 4 h. Next, 0.74 g (7.3 mmol) of Et_3N and 1.54 g (7.3 mmol) of L-glutamic acid dimethyl ester hydrochloride were added. The mixture was stirred for 24 h at room temperature in an inert atmosphere. The solution was concentrated in vacuo, and the resulting solid was suspended in water, basified with an aqueous solution of $NaHCO_3$, sonicated, filtered (or centrifugated), washed with water and with MeOH, and dried over P_2O_5 to give 1.02 g (2.0 mmol, 70%) of dimethyl *N*-{*p*-[*N*-(2,4-diamino-7-oxo-5,6,7,8-tetrahydropyrido[2,3-*d*]pyrimidin-6-ylmethyl)-*N*-methylamino]benzoyl}glutamate (**15a**) which was used without further purification: mp 172−175 °C; IR (KBr) 3450, 3330, 3205 (N−H), 1740 (COOMe), 1685, 1635 (CONH), 1605, 1570, 1510 (C═C, C═N), 775 cm^{-1}; HRMS FAB(+) calcd for $C_{23}H_{30}N_7O_6$ [M + H], 500.2258; found, 500.2248. A suspension of 0.500 g (10 mmol) of **15a** in 8 mL of a 1 N aqueous solution of NaOH was stirred at room temperature for 24 h. The solution obtained was filtered through a Lida filter (47 mm, 0.45 μm nylon) and cooled in an ice bath. The resulting solution was carefully acidified (pH = 6−7) with 25% aqueous acetic acid and stirred in the cold for 2 h. The resulting solid was filtered (or centrifugated), washed with water, and dried over P_2O_5 to give 0.420 g (0.9 mmol, 89%) of **16a** as a colorless solid: mp 230 °C; IR (KBr) 3500−2500 (COO−H), 3420, 3360, 3210 (N−H), 1640 (C═O), 1610, 1560, 1500 (C═C, C═N), 765 cm^{-1}; ^{1}H NMR (TFA-*d*), δ 2.34 (br, 1H, C3−H), 2.54 (br, 1H, C3−H), 2.68−2.86 (m, 3H, C4−H, C5″-H), 3.05−3.08 (m, 1H, C5″-H, C6″-H), 3.50 (s, 3H, N−CH_3), 3.98−4.08 (m, 1H, C6′-H), 4.18−4.21 (m, 1H, C6′-H), 5.02 (br, 1H, C2−H), 7.77 (AA′BB′, $^3J_{HH}$ = 8 Hz, 2H, C3′-H), 8.06 (AA′BB′, $^3J_{HH}$ = 8 Hz, 2H, C2′-H); ^{13}C NMR (TFA-*d*), δ 21.4 (C5″), 27.8 (C3), 31.7 (C4), 36.1 (C6″), 49.0 (N−CH_3), 55.0 (C2), 62.1 (C6′), 82.3 (C4″a), 124.0 (C3′), 132.6 (C2′), 137.9 (C1′), 144.0 (C4′), 155.0 (C8′a), 156.2 (C4′, C2′), 171.1 (C1′-*C*ONH), 176.0 (C7″), 178.4 (C5), 182.0 (C1); HRMS FAB(+) calcd for $C_{21}H_{26}N_7O_6$ [M + H], 472.1945; found, 472.1939. Anal. ($C_{21}H_{25}N_7O_6·2.5H_2O$) C, H, N.

***N*-{*p*-[*N*-(2,4-Diamino-7-oxo-5,6,7,8-tetrahydropyrido-[2,3-*d*]pyrimidin-6-ylmethyl)amino]benzoyl}glutamic Acid (16b).** The procedure was the same as that stated above for **16a** but using 0.468 g (1.4 mmol) of *p*-[*N*-(2,4-diamino-7-oxo-5,6,7,8-tetrahydropyrido[2,3-*d*]pyrimidin-6-ylmethyl)amino]-benzoic acid (**14b**) in 20 mL of anhydrous DMF, 0.345 g (3.4 mmol) of Et_3N, 0.558 g (3.4 mmol) of diethyl cyanophosphonate, 0.345 g (3.4 mmol) of Et_3N, and 0.723 g (3.4 mmol) of L-glutamic acid dimethyl ester hydrochloride to afford 0.583 g (1.2 mmol, 84%) of dimethyl *N*-{*p*-[*N*-(2,4-diamino-7-oxo-5,6,7,8-tetrahydropyrido[2,3-*d*]pyrimidin-6-ylmethyl)amino]benzoyl}-glutamate (**15b**) as a colorless solid: mp 172−174 °C; IR (KBr) 3330, 3200 (N−H), 1730 (COOMe), 1675, 1635 (CONH), 1600, 1560, 1500 (C═C, C═N), 770 cm^{-1}; MS *m/z* 191 (26) $[C_8H_9N_5O^+]$, 120 (100). Then, using 0.492 (1.0 mmol) of **15b** in 8.5 mL of 1 N aqueous NaOH gave 0.380 g (0.8 mmol, 83%) of **16b** as a colorless solid: mp >200 °C dec; IR (KBr) 3500−2500 (COOH), 3350, 3210 (N−H), 1650 (C═O), 1610, 1560, 1510 (C═C, C═N), 770 cm^{-1}; ^{1}H NMR (TFA-*d*), δ 2.31−2.38 (m, 1H, C3−H), 2.55−2.77 (m, 4H, C3−H, C4−H, C5″-H), 3.17−3.14 (m, 1H, C5″-H), 3.58 (br, 1H, C6″-H), 3.86−3.90 (m, 1H, C6′-H), 4.00−4.07 (m, 1H, C6′-H), 4.95 (dd, $^3J_{HH}$ = 5 Hz, $^3J_{HH}$ = 8 Hz, 1H, C2−H), 7.77 (AA′BB′, $^3J_{HH}$ = 8 Hz, 2H, C3′-H), 8.09 (AA′BB′, $^3J_{HH}$ = 8 Hz, 2H, C2′-H); ^{13}C NMR (TFA-*d*), δ 21.5 (C5″), 27.6 (C3), 31.5 (C4), 36.8 (C6″), 54.8 (C2), 55.0 (C6′), 86.6 (C4″a), 125.1 (C3′), 132.1 (C2′), 137.0 (C1′), 139.1 (C4′), 154.6 (C8′a), 155.6 (C4′), 157.0 (C2′), 171.4 (C1′-*C*ONH), 176.3 (C7″), 178.5 (C5), 181.9 (C1); HRMS FAB(+) calcd for $C_{20}H_{24}N_7O_6$ [M + H], 458.1788; found, 458.1781. Anal. ($C_{20}H_{23}N_7O_6·0.5CH_3CO_2H·1.5H_2O$) C, H, N.

***N*-{*p*-[2-(2,4-Diamino-7-oxo-5,6,7,8-tetrahydropyrido-[2,3-*d*]pyrimidin-6-yl)ethyl]benzoyl}glutamic Acid (16c).** The procedure was the same as that stated above for **16a** but using 0.458 g (1.4 mmol) of *p*-[2-(2,4-diamino-7-oxo-5,6,7,8-tetrahydropyrido[2,3-*d*]pyrimidin-6-yl)ethyl]benzoic acid (**14c**) in 20 mL of anhydrous DMF, 0.345 g (3.4 mmol) of Et_3N, 0.558 g (3.4 mmol) of diethyl cyanophosphonate, 0.345 g (3.4 mmol) of Et_3N, and 0.723 g (3.4 mmol) of L-glutamic acid dimethyl ester hydrochloride to afford 0.549 g (1.1 mmol, 81%) of dimethyl *N*-{*p*-[2-(2,4-diamino-7-oxo-5,6,7,8-tetrahydropyrido-[2,3-*d*]pyrimidin-6-yl)ethyl]benzoyl}glutamate (**15c**) as a colorless solid: mp >300 °C; IR (KBr) 3320, 3210 (N−H), 1740 (COOMe), 1650 (CONH), 1570, 1500 (C═C, C═N), 790 cm^{-1}. Then, using 1.000 g (2.1 mmol) of **15c** in 16 mL of 1 N aqueous NaOH afforded 1.008 g (2.0 mmol, 94%) of **16c·3.5H$_2$O** as a colorless solid: mp 246−247 °C; IR (KBr) 3600−2500 (COO−H), 3330, 3200 (N−H), 1630−1670 (C═O), 1540, 1490 (C═C, C═N), 760 cm^{-1}; ^{1}H NMR (TFA-*d*), δ 2.12−2.20 (m, 2H, C6′-H), 2.29−2.42 (m, 1H, C3−H), 2.53−2.62 (m, 1H, C3−H), 2.66−2.97 (m, 7H, C5″-H, C6″-H, C5′-H, C4−H), 5.03 (dd, $^3J_{HH}$ = 5 Hz, $^3J_{HH}$ = 9 Hz, 1H, C2−H), 7.34 (AA′BB′, $^3J_{HH}$ = 8 Hz, 2H, C3′-H), 7.75 (AA′BB′, $^3J_{HH}$ = 8 Hz, 2H, C2′-H); ^{13}C NMR (TFA-*d*), δ 26.2 (C5″), 27.8 (C3), 31.6 (C4), 34.5 (C6′), 35.3 (C5′), 44.5 (C6″), 55.0 (C2), 84.2 (C4″a), 129.6 (C3′), 131.0 (C2′), 131.1 (C1′), 149.0 (C4′), 151.4 (C8′a), 155.8 (C4′, C2′), 174.5 (C1′-*C*ONH), 178.6 (C7″), 182.2 (C5), 183.3 (C1); HRMS FAB(+)

calcd for $C_{21}H_{25}N_6O_6$ [M + H], 457.1836; found, 457.1826. Anal. ($C_{21}H_{24}N_6O_6 \cdot 3.5H_2O$) C, H, N.

In Vitro Cell Culture Studies. Dose response curves were generated to determine the concentration required for 50% inhibition of growth (IC_{50}) of CCRF-CEM human leukemia cells.[27] Test antifolate compounds were dissolved initially in pure DMSO at a concentration of 4 mg/mL and further diluted with cell culture medium (Roswell Park Memorial Institute, RPMI-1640 media) to the desired concentration. CCRF-CEM leukemia cells in complete medium were added to 24-well cluster plates at a final concentration of 4.8×10^4 cells/well in a total volume of 2.0 mL. Test compounds at various concentrations were added to duplicate wells so that the final volume of DMSO was 0.5%. The plates were incubated for 72 h at 37 °C in a 5% CO_2-in-air atmosphere. At the end of the incubation, cell numbers were determined on a ZBI Coulter counter. Control wells usually contained $(4-6) \times 10^5$ cells at the end of the incubation.

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Supporting Information Available: ^{1}H NMR and ^{13}C NMR spectral data for compounds **11a**−**c**, **12a**−**c**, **13a**−**c**, **14a**−**c**, and **15a**−**c** (4 pages). Ordering information is given on any current masthead page.

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