Synthesis and Biological Activity of 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)

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We recently described the syntheses of **12a**−**c**, 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), in six steps from commercially available *p*-substituted methyl benzoates in 20−27% overall yields. Such analogues were tested in vitro against CCRF-CEM leukemia cells and showed that they are completely devoid of any activity, the IC_{50} being higher than 20 μg/mL for all cases. To clarify if the presence of the carbonyl group in position C7, the distinctive feature of our synthetic methodology, is the reason for this lack of activity, we have now obtained the 7-oxo substituted analogues of 5-DATHF and DDATHF, **18a**−**c**, in 10−30% overall yield. Testing of **18a**−**c** in vitro against CCRF-CEM leukemia cells revealed that these compounds are totally inactive. A molecular modeling study of **18b** inside the active site of the complex *E. coli* GARTFase-5-DATHF-GAR pointed to an electronic repulsion between the atoms of the 7-oxo group and the carbonyl group of Arg90 as a possible explanation for the inactivity of **18a**−**c**.

Introduction

For over 40 years Methotrexate (MTX, **1**)[1] has been the antifolate most widely used, alone or in combination with other agents, in the treatment of cancer[2,3] (Figure 1). MTX acts as a competitive inhibitor of dihydrofolate reductase (DHFR) enzyme that takes part in the folic acid (FA, **2**) metabolism catalyzing the reduction of 7,8-dihydrofolic acid (H_2FA) to 5,6,7,8-tetrahydrofolic acid (H_4FA)[4,5] (Figure 1). Folic acid and its derivatives are responsible for the de novo biosynthesis of nucleotides, participating as coenzymes in the transfer, oxidation, and reduction of carbon units in numerous biochemical routes, such as the thymidylate cycle, de novo synthesis of purines, methionine regeneration from homocysteine, and the interconversion of serine and glycine.[4,5]

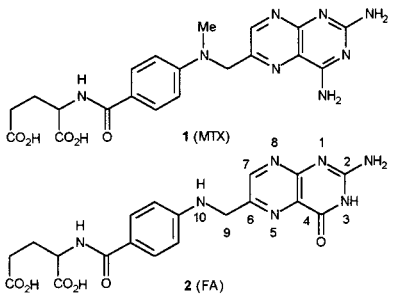

Figure 1.

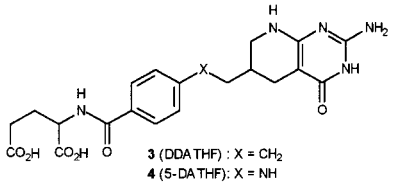

Figure 2.

Despite the good results, MTX presents, among others, a limited antitumor spectrum and acquisition of drug resistance through four main mechanisms:[3,6] membrane impaired transport, impaired polyglutamation, DHFR increase activity, and decrease in the enzyme affinity. Due to these deficiencies, great efforts have been made in searching for new compounds that overcome, entirely or partially, these drawbacks.[7−10]

In this context, E. C. Taylor[11] et al. obtained 5,10-dideaza-5,6,7,8-tetrahydrofolic acid (DDATHF, **3**)[12] and 5-deaza-5,6,7,8-tetrahydrofolic acid (5-DATHF, **4**)[13] (Figure 2). These compounds inhibit glycinamide ribonucleotide transformylase (GARTFase),[14] responsible for the transformation of glycinamide ribonucleotide (GAR) into α-*N*-formylglycinamide ribonucleotide (FGAR), therefore inhibiting the biosynthesis of purines. The 6*R*-diastereomer of DDATHF[15] (Lometrexol, LY264618), topological analogue of natural 6*S*-H_4FA,[16] has recently finished phase II clinical trials.[17−19]

More recently, our group has obtained compounds **12a**−**c**, 4-amino-7-oxo substituted analogues of DDATHF and 5-DATHF, employing an alternative shorter strategy for the formation of the 5,6,7,8-tetrahydropyrido-[2,3-*d*]pyrimidin-7-one moiety (Scheme 1).[20] Thus, cyclization with guanidine of 2-methoxy-6-oxo-1,4,5,6-tetrahydropyridine-3-carbonitriles (**8a**−**c**), obtained by reaction of an α,*β*-unsaturated ester **7a**−**c** and malononitrile in NaOMe/MeOH, afforded the corresponding compounds **9a**−**c**. After the hydrolysis of the ester group present in **9a**−**c**, the resulting carboxylic acids **10a**−**c** were treated with diethyl cyanophosphonate in Et_3N/DMF and coupled with L-glutamic acid dimethyl ester to give **11a**−**c**. Finally, the basic hydrolysis of **11a**−**c** yielded the desired 4-amino-7-oxo substituted analogues **12a**−**c** in 20−27% overall yield. Formation of key intermediates **7a**−**c** was achieved from commercially available benzoates **5a**−**c** and methyl 2-(bromomethyl)-acrylate (**6**). Unfortunately, biological evaluation of analogues **12a**−**c** showed no activity against CCRF-

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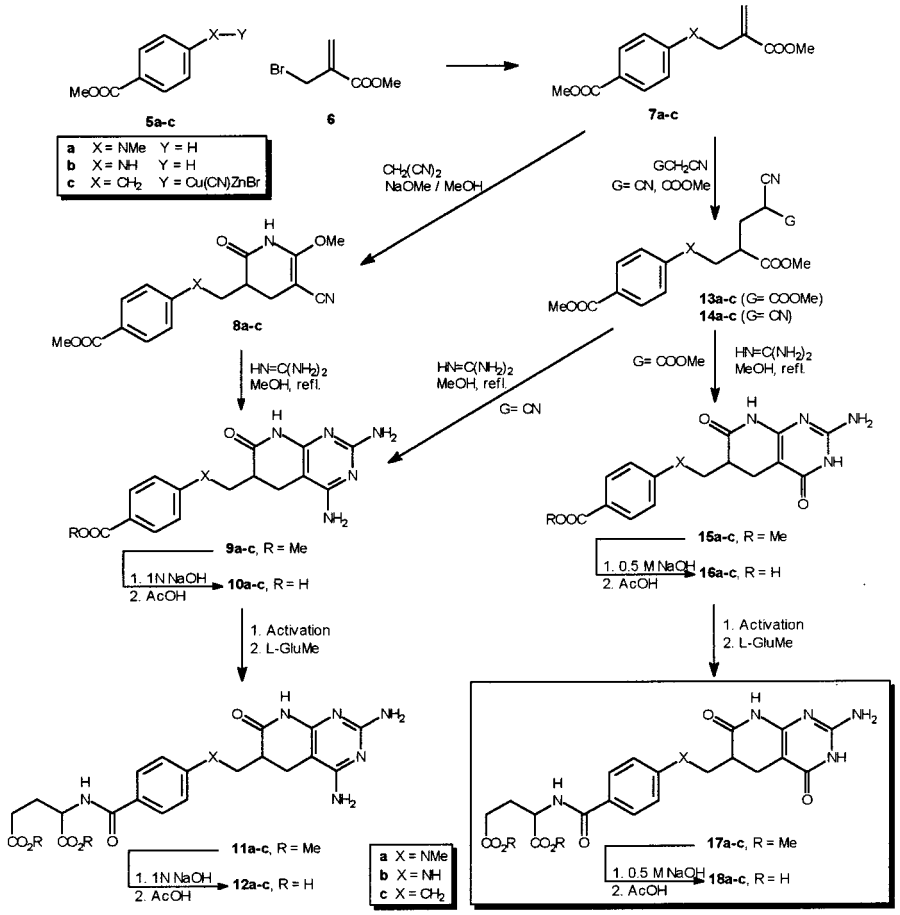
Scheme 1



CEM leukemia cells, the IC_{50} being higher than 20 μg/mL for all cases. Both DDATHF (**3**) and Methotrexate (**1**) 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.

Compounds **12** present a 2,4-diaminopyrimidine ring, so they are primarily targeted at DHFR, and it is known that oxygenation at the 7 position is in general not good for DHFR binding. Consequently, taking into account that the 4-amino substituted analogue of DDATHF was capable of inhibiting bovine liver DHFR with an IC_{50} of 71 nM,[12] 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 effects of the carbonyl group would be also present in 7-oxo substituted analogues of DDATHF, which would be targeted against glycinamide ribonucleotide transformylase (GARTFase).

Now, we report the synthesis of **18a**−**c**, 7-oxo substituted analogues of DDATHF (**3**) and 5-DATHF (**4**), and the determination of their biological activity.

Results and Discussion

Chemistry. Adducts **13a**−**c** (G = COOMe) were obtained in 41−80% yield, after column chromatography purification, by reaction of the previously described α,β-unsaturated esters **7a**−**c** with methyl cyanoacetate in NaOMe/MeOH (Scheme 1).[21] The use of other experimental conditions, such as phase transfer catalysis in toluene using potassium carbonate in the presence of benzyltriethylammonium chloride, gave lower yields and allowed the isolation and characterization of the corresponding Michael bisadducts (**19a**−**c**) (Figure 3). Compounds **13** (G = COOMe) were obtained as diastereomers and used without further separation. The subsequent treatment of **13a**−**c** with guanidine in MeOH at reflux afforded, in one step, the 2-amino-4-oxopyrido-[2,3-*d*]pyrimidines **15a**−**c** in 52−87% yield (Scheme 1). This methodology was also applicable to the cyclization with guanidine of the Michael adducts **14a**−**c** (G = CN), obtained by reaction of α,β-unsaturated esters **7a**−**c** with malononitrile, which led to the 2,4-diaminopyrido-[2,3-*d*]pyrimidines **9a**−**c**, common precursors of the previously described 4-amino-7-oxo substituted analogues **12a**−**c** (**7** → **8** → **9** → **10** → **11** → **12**) (Scheme 1).

Hydrolysis of the ester group present in **15a**−**c** was achieved in 0.5 N aqueous NaOH to yield the carboxylic

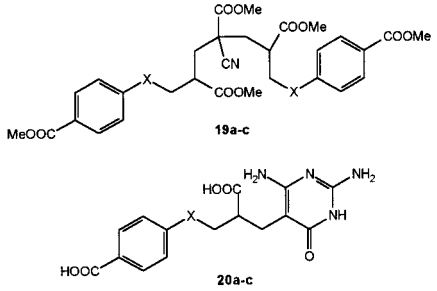


Figure 3.

Table 1. In Vitro Cytotoxicity Assay (IC_{50}) Conducted in CCRF-CEM Human Leukemia Cells

compd	X	IC_{50} (μg/mL)
MTX		0.004
DDATHF		0.007
18a	NMe	>50
18b	NH	20
18c	CH_2	>50

acids **16a**−**c** in almost quantitative yield. If such hydrolysis was carried out in 1 N aqueous NaOH, a mixture of two products was obtained: the expected acid **16** and a monocyclic compound **20** (Figure 3) resulting from the opening of the pyridone ring as the ^{13}C NMR data reveals. Further treatment of this mixture in acetic acid at reflux reverted to the corresponding acid **16**.

The coupling of **16a**−**c** with L-glutamic acid dimethyl ester was achieved by using diethyl cyanophosphonate as the activating agent and Et_3N in DMF at room temperature, the corresponding esters **17a**−**c** being obtained in 53−82% yield (Scheme 1).

The last step for the synthesis of the folic acid analogues is the basic hydrolysis of the ester groups present in the glutamate unit of **17a**−**c**. Hydrolyses carried out using 0.5 N NaOH at room temperature afforded the final compounds **18a**−**c** in 61−95% yield (Scheme 1).

To summarize, compounds **18a**−**c**, which are 7-oxo substituted analogues of 5-DATHF (**4**) and DDATHF (**3**), have been obtained in six steps from commercially available compounds in 22%, 9%, and 27% global yields, respectively. The methodology used also allows the synthesis of the 2,4-diamino-7-oxo analogues **12**.

Biological Evaluation. Compounds **18a**−**c** were tested in vitro against CCRF-CEM[22] leukemia cells (Table 1). This is a very common T-cell derived lymphoblastic leukemia that has been widely used as a discriminatory test. The results obtained indicated that our 7-oxo analogues are completely devoid of any activity, with IC_{50} values being higher than 20 μg/mL for all cases. This lack of activity can be attributed, at first sight, to electronic considerations due to the presence of the carbonyl group at the C-7 position. In fact, this is the only different feature with respect to DDATHF and 5-DATHF.

In this context, the X-ray structure of the complex *E. coli* GARTFase-5-DATHF-GAR[23] (Protein Data Bank, Brookhaven National Laboratory; http://pdb.pdb.bnl.gov) allowed us to determine (Weblab Viewer 2.01; Molecular Simulations Inc.; http://www.msi.com) the closest residues surrounding C7 in 5-DATHF. Among them, Arg90 forms an H-bond with N8, and Phe88 is placed near C7 at a distance (3.84 Å) that avoids steric interactions with the inhibitor.

Consequently, a first explanation for the lack of activity of **18a**−**c** would imply the existence of amide-iminol tautomerism in the pyridone ring. The presence of an iminol tautomer would disable the H-bond interaction between Arg90 and N8. Nevertheless, spectroscopic data for **18a**−**c** are consistent only with the existence of amide tautomers. This fact is in accordance with the rest of the 7-oxopyrido[2,3-*d*]pyrimidines previously obtained in our group.

On the other hand, we have proved, by modeling our 7-oxo substituted analogues, that **18a**−**c** could adopt a similar conformation to 5-DATHF inside the active site of the enzyme. Then we have replaced the structure of 5-DATHF by the structure of **18b** in the active site of the enzyme and determined the resulting interactions. The replacement shows that Phe88 would be face-to-face to the 7-oxo group of **18b** at 2.68 Å. This would produce electronic repulsion between the oxygen atoms of both carbonyl groups, and as a consequence, the conformation of amino acids surrounding the bicyclic unit would be distorted, disrupting any possible H-bridge interaction. This phenomenon would give a possible explanation for the total absence of activity of **18a**−**c**.

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 BOMEM Michelson 100 and 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. Mass spectra (*m*/*z* (%), EI, 70 eV) were obtained on a Hewlett-Packard 5995 A spectrometer. Elemental microanalyses were obtained on a Carlo-Erba CHNS-O/EA 1108 analyzer and gave results for the elements stated within ±0.4% of the theoretical values.

General Method for the Synthesis of Michael Adducts 13. Dimethyl 2-Cyano-4-{[(4-methoxycarbonylphenyl)-methylamino]methyl}pentanedioate (13a). To a solution of 2.20 g (9.5 mmol) of sodium in 100 mL of methanol was added 9.40 g (9.5 mmol) of methyl cyanoacetate. Next, 5.00 g (1.9 mmol) of methyl *p*-[*N*-(2-methoxycarbonylallyl)-*N*-methylamino]benzoate (**7a**) were added dropwise. The resulting mixture was heated to 35 °C for 90 min and, after being cooled, was neutralized with concentrated acetic acid. The solvent was removed in vacuo, and the residue was treated with 100 mL of CH_2Cl_2 and 100 mL of water. The two layers were separated, and the aqueous layer was extracted with (2 ×25 mL) of CH_2Cl_2. The combined organic extracts were washed with water, dried ($MgSO_4$), and concentrated in vacuo. The crude material was purified by column chromatography using AcOEt/hexane (2:1) as eluent to give 3.5 g (9.7 mmol, 51%) of **13a** as a colorless oil: Anal. ($C_{18}H_{22}N_2O_6$) C, H, N.

General Method for the Synthesis of 2-Amino-4,7-dioxo-3,4,5,6,7,8-hexahydropyrido[2,3-*d*]pyrimidines 15. Methyl 4-[*N*-(2-Amino-4,7-dioxo-3,4,5,6,7,8-hexahydropyrido[2,3-*d*]pyrimidin-6-ylmethyl)-*N*-methylamino]benzoate (15a). A 2.2 g (12.2 mmol) portion of guanidine carbonate was added to a solution of 0.56 g (24.3 mmol) of Na in 15 mL of methanol, and the mixture was refluxed for 15 min. After the solution was cooled, the sodium carbonate formed was filtered, and 0.88 g (2.4 mmol) of dimethyl 2-cyano-4-{[(4-methoxycarbonylphenyl)methylamino]methyl}pentanedioate (**13a**) in 15 mL of methanol were added. The mixture was heated at reflux for 24 h and cooled to room temperature. The solid was filtered, washed with MeOH, and dried in vacuo over P_2O_5 to give 0.75 g (2.1 mmol, 87%) of **15a** as a white solid: mp >250 °C; Anal. ($C_{17}H_{19}N_5O_4 \cdot H_2O$) C, H, N.

General Method for the Hydrolysis of the Ester Group present in Pyrido[2,3-*d*]pyrimidines 15. 4-[*N*-(2-Amino-4,7-dioxo-3,4,5,6,7,8-hexahydropyrido[2,3-*d*]pyrimidin-6-ylmethyl)-*N*-methylamino]benzoic Acid (16a). A suspension of 0.50 g (1.4 mmol) of methyl 4-[*N*-(2-amino-4,7-dioxo-3,4,5,6,7,8-hexahydropyrido[2,3-*d*]pyrimidin-6-ylmethyl)-*N*-methylamino]benzoate (**15a**) in 7 mL of a 0.5 M aqueous NaOH was stirred at room temperature until solution occurred. 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 (pH 5−6).

The resulting precipitate was filtered, washed with water, and dried at vacuum over P_2O_5 to give 0.47 g (1.37 mmol, 98%) of **16a** as a white solid: mp >250 °C; Anal. ($C_{16}H_{17}N_5O_4 \cdot 0.75H_2O$) C, H, N.

General Method for the Synthesis of 17a−c. Dimethyl *N*-{4-[*N*-(2-Amino-4,7-dioxo-3,4,5,6,7,8-hexahydropyrido-[2,3-*d*]pyrimidin-6-ylmethyl)-*N*-methylamino]benzoyl}-L-glutamate (17a). To a solution of 1.0 g (2.92 mmol) of 4-[*N*-(2-amino-4,7-dioxo-3,4,5,6,7,8-hexahydropyrido[2,3-*d*]pyrimidin-6-ylmethyl)-*N*-methylamino]benzoic acid (**16a**) in 15 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 1.2 g (7.3 mmol) of diethyl cyanophosphonate was added, and the resulting mixture was stirred for 90 min. Next, 0.74 g (7.3 mmol) of Et_3N and 1.54 g (7.3 mmol) of L-glutamic acid dimethyl ester hydrochloride in 15 mL of DMF were added. After stirring for 24 h at room temperature in an inert atmosphere, the solution was concentrated in vacuo. The resulting crude was suspended in water, basified (pH 8) with a saturated aqueous solution of $NaHCO_3$, filtered, washed with water, dried over P_2O_5, and digested with MeOH to give 0.77 g (1.54 mmol, 53%) of **17a** as a white-off solid: mp > 250 °C; Anal. ($C_{23}H_{28}N_6O_7$) C, H, N.

General Method for the Synthesis of 18a−c. *N*-{4-[*N*-(2-Amino-4,7-dioxo-3,4,5,6,7,8-hexahydropyrido[2,3-*d*]-pyrimidin-6-ylmethyl)-*N*-methylamino]benzoyl}-L-glutamic Acid (18a). A suspension of 0.15 g (0.30 mmol) of dimethyl *N*-{4-[*N*-(2-amino-4,7-dioxo-3,4,5,6,7,8-hexahydropyrido[2,3-*d*]pyrimidin-6-ylmethyl)-*N*-methylamino]benzoyl}-L-glutamate (**17a**) in 10 mL of 0.5 M NaOH was stirred at room temperature until solution occurred. The resulting solution was filtered through a Lida filter (47 mm filter membrane, 0.45μm Nylon, Art NY504700, Lida Manufacturing Corp., 9115 26th Avenue, Kenosha, WI), and the filtrate was acidified with concentrated acetic acid (pH 5−6). The resulting precipitate was filtered, washed with water, and dried at vacuum over P_2O_5 to give 0.135 g (0.286 mmol, 95%) of **18a** as a white solid: mp > 250 °C; Anal. ($C_{21}H_{24}N_6O_7 \cdot 3.9H_2O$) 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.[22] 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: Experimental details and spectral data for compounds **13a−c**; **15a−c**; **16a−c**; **17a−c**; **18a−c**; **14a−c**; **9a−c**; **19a−c**; and **20a**,**b**. This material is available free of charge via the Internet at http://pubs.acs.org.

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