that were preequilibrated on a water bath at 37 °C. At intervals of 0.25, 0.5, 1, 2, 3, 4, 5, and 10 min, the vials were removed from the water bath, and 3 volumes (0.6 mL) of cold methanol was added to deactivate the enzyme. The vials were agitated on a Vortex shaker for 20 s and then centrifuged for 4 min at 1000 rpm. Aliquots (50 μL) of the clear supernatant were analyzed by HPLC as described above. In addition to the parent compounds, a progressively increasing peak with a retention time identical with that of acrolein (2.6 min) was present in all of the chromatograms. The half-lives of the parent acetals were determined by linear least-square regression analysis of the pseudo-first-order reactions.

Cytotoxicity Studies. The toxicities of the compounds to L1210 murine leukemia cells were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described by Mossman.[43] The method is dependent on the reduction of MTT by the mitochondrial dehydrogenase of viable cells to a blue formazan product that can be measured spectrometrically.

The acetals were dissolved in sterile H_2O to provide stock solutions of 1 mg/mL concentration; these were further diluted with sterile H_2O to afford solutions suitable for the drug concentration range studied. All of the solutions were passed through a 0.22-μm filter (Millipore Corp.) immediately before use.

The L1210 cells were maintained in vitro by serial culture in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, L-glutamine (2 μmol/mL), 2-mercaptoethanol (10 μM), penicillin (50 U/mL), and streptomycin (50 μg/mL) at 37 °C in a humified atmosphere of 5% CO_2 and 95% air; the pH of the medium was 7.4. For the cytotoxicity studies, exponentially growing cells were incubated in the same medium at a concentration of 5×10^5/mL in 75 × 10 mm culture tubes with increasing drug concentrations for 1 h at 37 °C. The cells were washed twice with serum-free medium (2 mL) and harvested by centrifugation for 5 min at 1500 rpm. The cells were resuspended in drug-free medium at a concentration of 1.3×10^5/mL, and 150 μL aliquots (containing approximately 2×10^4 cells) were placed into a 96-well plate and incubated for 72 h at 37 °C. A solution of 75 μg of MTT in 15 μL of sterile H_2O was added to each well, and the plates were incubated for 4 h at 37 °C. Acid–2-propanol (180 μL of 0.04 N HCl in 2-propanol) was then added, and the mixtures were agitated with a pipet to dissolve the crystallized dye. The plates were read on a multiwell scanning spectrophotometer (ELISA reader) at a wavelength of 570 nm. The IC_{50} values were determined by plotting the drug concentration versus the cell viability as described by Mossman.[44] The values in Table II are the average of duplicate determinations.

Acknowledgment. This work was supported by Grant CA 42708 from the National Cancer Institute, National Institutes of Health.

Registry No. **1a**, 113341-60-9; **1b**, 123824-17-9; **1c**, 123824-18-0; **1d**, 123824-19-1; **1e**, 123824-20-4; **1f**, 123824-21-5; **1g**, 123824-22-6; **10**, 19790-60-4; 11, 123824-11-3; **12**, 123824-12-4; 14, 130197-72-7; $POCl_3$, 10025-87-3; NH_3, 7664-41-7; $MeNH_2$, 74-89-5; $EtNH_2$, 75-04-7; Me_2NH·HCl, 506-59-2; Et_2NH, 109-89-7; MeOH, 67-56-1; EtOH, 64-17-5; cyclohexylammonium hydrogen, 130197-71-6; *N,N*-bis(2-chloroethyl)phosphoramidate carboxylate esterase, 9016-18-6; benzyl alcohol, 100-51-6; acrolein, 107-02-8; bis(2-chloroethyl)amine hydrochloride, 821-48-7.

(44) Mossman, T. *J. Immunol. Methods* **1983**, *65*, 55.

Analogues of Methotrexate and Aminopterin with γ-Methylene and γ-Cyano Substitution of the Glutamate Side Chain: Synthesis and in Vitro Biological Activity[1]

Andre Rosowsky,*,† Henry Bader,† and James H. Freisheim‡

Dana-Farber Cancer Institute and Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115, and Department of Biochemistry, Medical College of Ohio, Toledo, Ohio 43699.
Received April 11, 1990

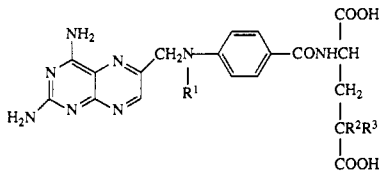
Analogues of methotrexate (MTX) and aminopterin (AMT) modified at the γ-position of the glutamate side chain were synthesized and evaluated as dihydrofolate reductase (DHFR) inhibitors and tumor cell growth inhibitors. Condensations of 4-amino-4-deoxy-N^{10}-methylpteroic acid (mAPA) with dimethyl DL-4-methyleneglutamate in the presence of diethyl phosphorocyanidate (DEPC) followed by alkaline hydrolysis yielded *N*-(4-amino-4-deoxy-N^{10}-methylpteroyl)-DL-4-methyleneglutamic acid (γ-methyleneMTX). Condensation of 4-amino-4-deoxy-N^{10}-formylpteroic acid (fAPA) with dimethyl-DL-4-methyleneglutamate by the mixed carboxylic–carbonic anhydride method yielded *N*-(4-amino-4-deoxypteroyl)-DL-4-methyleneglutamic acid (γ-methyleneAMT). Also prepared via DEPC coupling was a mixture of the four possible diastereomers of *N*-(4-amino-4-deoxy-N^{10}-methylpteroyl)-4-cyanoglutamic acid (γ-cyanoMTX). The requisite intermediate γ-*tert*-butyl α-methyl 4-cyanoglutamate, as a DL-threo/DL-erythro mixture, was prepared from methyl N^{α}-Boc-*O*-tosyl-L-serinate by reaction with sodium *tert*-butyl cyanoacetate followed by mild trifluoroacetic treatment to selectively remove the Boc group. The γ-methylene derivatives of MTX and AMT are attractive because of their potential to act as Michael acceptors within the DHFR active site. γ-CyanoMTX may be viewed as a congener of the nonpolyglutamated MTX analogue γ-fluoroMTX. In vitro bioassay data for the γ-methylene and γ-cyano compounds support the idea that the active site of DHFR, already known for its ability to tolerate modification of the γ-carboxyl group of MTX and AMT, can likewise accommodate substitution on the γ-carbon itself.

Analogues of the classical folic acid antagonists methotrexate (MTX, 1) and aminopterin (AMT, 2) with altered amino acid side chains have been the subject of intensive investigation for over 40 years.[2] Our own studies in this

† Dana-Farber Cancer Institute.
‡ Medical College of Ohio.

(1) Paper 40 in this series; for previous paper, see: Rosowsky, A.; Yu, C.-S. *Pteridines* **1989**, *1*, 143.

0022-2623/91/1834-0203$02.50/0



	R^1	R^2	R^3
1, MTX	CH_3	H	H
2, AMT	H	H	H
3	CH_3	$=CH_2$	
4	H	$=CH_2$	
5	CH_3	C≡N	H

area have included, for example, esters[3] and amides[4] of the glutamate moiety, compounds in which the γ-COOH is replaced by γ-SO_2OH[5] or γ-$PO(OH)_2$[6] groups, and compounds in which the side chain has been lengthened by insertion of as many as five extra CH_2 groups.[7] In the present paper we describe the synthesis and in vitro biological activity of analogues in which small lipophilic groups are introduced on the γ-carbon of an otherwise intact glutamate side chain; specifically, we describe the olefins **3** (γ-methyleneMTX) and **4** (γ-methyleneAMT) and the γ-cyano derivative **5** (γ-cyanoMTX), all of which were obtained as diastereomer mixtures.[8,9] Also presented are the results of bioassays measuring the ability of these compounds to inhibit dihydrofolate reductase (DHFR) in vitro and to inhibit the growth of WI-L2 cells in culture. These results add to our knowledge concerning the relationship between side-chain structure and biological activity in classical antifolates. Relevant work in this area has also been done recently with γ-fluoroMTX[10] as well as with γ-methylMTX, γ-(methylthio)MTX, and γ-hydroxyMTX.[11] Additionally, **3** and **4** are of interest because of the potential ability of the α,β-unsaturated ester group to alkylate nucleophiles by Michael addition and because of the chemical relationship of these olefins to *N*-(4-amino-4-deoxy-N^{10}-methylpteroyl)-DL-2-amino-4-hexenedioic acid and *N*-(4-amino-4-deoxypteroyl)-DL-2-amino-4-hexenedioic acid, which we have observed to have antifolate activity approximately equal to that of MTX.[7b]

Chemistry

The synthesis of the MTX and AMT analogues **3** and **4** with a γ-methylene group in the side chain is presented in Scheme I, and that of the γ-cyano analogue **5** is shown in Scheme II. The preparation of the key intermediates 4-amino-4-deoxy-N^{10}-methylpteroic acid (**8**) and 4-amino-4-deoxy-N^{10}-formylpteroic acid (**9**), as well as their conversion into a variety of MTX and AMT analogues, have been described by us previously.[3e,4d,5-7]

As shown in Scheme I, γ-methyleneMTX (**3**) and γ-methyleneAMT (**4**), as D,L mixtures, were obtained from the previously undescribed compound dimethyl DL-γ-methyleneglutamate hydrochloride (**11**·HCl), which we prepared in quantitative yield from the corresponding diacid by reaction with MeOH and $SOCl_2$. The known DL-diacid[12] was prepared by condensation of diethyl acetamidomalonate with ethyl 2-(bromoethyl)acrylate,[13] which gave diethyl 2-acetamido-2-carbethoxy-4-methyleneglutaric acid (**10**, 90%), followed by hydrolysis and decarboxylation in refluxing HCl (77% yield). Coupling of **8** and **11** by the DEPC method afforded diester **12** (54%), which on alkaline hydrolysis gave **3** (76%). Similarly, coupling of **9** and **11** followed by hydrolysis led to **13** (19%) and **4** (63%), isolated as a disodium salt. The condensation of **9** and **11** gave a low yield even though multiple additions of activating agent and amine were performed. Reactions of **9** with protected amino acids appear to be more capricious, in general, than those of **8**. Moreover, our experience has been that the purification of AMT analogues often entails greater loss due to oxidative decomposition than does the purification of the corresponding MTX analogues. This is unfortunate, since the AMT analogues typically have higher biological activity, and suggests that improvements in synthetic methodology in the AMT series would be worthwhile to pursue.

As shown in Scheme II, the Na salt of *tert*-butyl cyanoacetate was condensed with *N*-(*tert*-butyloxycarbonyl)-*O*-tosyl-L-serine methyl ester[14] to obtain **14** (69% yield). Selective hydrolysis of the *tert*-butyloxycarbonyl group without cleavage of the *tert*-butyl ester was achieved successfully with trifluoroacetic acid at 0 °C, giving the crystalline trifluoroacetate salt of **15** in 60% yield. Condensation of **15** with **8** by the DEPC method afforded cyano diester **16** (58%), which on saponification was converted to the desired cyano diacid **5** (57%). Although **16** and **5** were both TLC-homogeneous and gave satisfactory microchemical analyses, analysis by HPLC on a C_{18} reversed-phase column revealed two peaks. Moreover, the NMR spectrum of **16** contained two closely spaced γ-*tert*-butyl ester singlets at δ 1.38 and 1.42, with a total area corresponding to nine protons and two closely spaced α-methyl ester singlets at δ 3.60 and 3.70, with a total area

(2) For a comprehensive review of work in this and related areas of antifolate research, see: Rosowsky, A. *Prog. Med. Chem.* **1989**, *26*, 1.

(3) (a) Rosowsky, A. *J. Med. Chem.* **1973**, *16*, 1190. (b) Rosowsky, A.; Beardsley, G. P.; Ensminger, W. D.; Lazarus, H.; Yu, C. S. *J. Med. Chem.* **1978**, *21*, 380. (c) Rosowsky, A.; Yu, C. S. *J. Med. Chem.* **1983**, *26*, 1448. (d) Rosowsky, A.; Forsch, R. A.; Yu, C. S.; Lazarus, H.; Beardsley, G. P. *J. Med. Chem.* **1984**, *27*, 605. (e) Rosowsky, A.; Freisheim, J. H.; Bader, H.; Forsch, R. A.; Susten, S. S.; Cucchi, C. A.; Frei, E., III. *J. Med. Chem.* **1985**, *28*, 660.

(4) (a) Rosowsky, A.; Ensminger, W. D.; Lazarus, H.; Yu, C. S. *J. Med. Chem.* **1978**, *21*, 170. (b) Rosowsky, A.; Yu, C. S.; Uren, J.; Lazarus, H.; Wick, M. *J. Med. Chem.* **1981**, *24*, 559. (c) Rosowsky, A.; Forsch, R.; Uren, J.; Wick, M. *J. Med. Chem.* **1981**, *24*, 1450. (d) Rosowsky, A.; Bader, H.; Radike-Smith, M.; Cucchi, C. A.; Wick, M. M.; Freisheim, J. H. *J. Med. Chem.* **1986**, *29*, 1703.

(5) Rosowsky, A.; Forsch, R. A.; Freisheim, J. H.; Moran, R. G.; Wick, M. *J. Med. Chem.* **1984**, *27*, 600.

(6) Rosowsky, A.; Forsch, R. A.; Moran, R. G.; Kohler, W.; Freisheim, J. H. *J. Med. Chem.* **1988**, *31*, 1326.

(7) (a) Rosowsky, A.; Forsch, R.; Uren, J.; Wick, M.; Kumar, A. A.; Freisheim, J. H. *J. Med. Chem.* **1983**, *26*, 1448. (b) Rosowsky, A.; Bader, H.; Forsch, R. A.; Moran, R. G.; Freisheim, J. H. *J. Med. Chem.* **1988**, *31*, 763. (c) Rosowsky, A.; Bader, H.; Kohler, W.; Freisheim, J. H.; Moran, R. G. *J. Med. Chem.* **1988**, *31*, 1338.

(8) Compound **5**, with two chiral centers, consists of four diastereomers which can be named as 2*S*,4*S*, 2*R*,4*R*, 2*S*,4*R*, and 2*R*,4*S*. The racemic mixtures (2*S*,4*S*)/(2*R*,4*R*)-**5** and (2*S*,4*R*)/(2*R*,4*S*)-**5** can also be named as DL-*threo*-**5** and DL-*erythro*-**5**, respectively. Since **5** was obtained as a nonresolved mixture, we have chosen to omit stereochemical designations in the text and Experimental Section for the sake of brevity. Compounds **3** and **4** are chiral only at the α-carbon and are simple DL pairs.

(9) Our synthesis of compounds **3** and **5** has been cited briefly in an abstract: Rosowsky, A.; Bader, H.; Forsch, R. A.; Freisheim, J. H.; Moran, R. G. *Abstracts of the Ninth International Symposium on Pteridines and Folic Acid Derivatives*, Zurich, Switzerland, September 3–8, 1989; p 166 (Abstr. R-03).

(10) (a) Galivan, J.; Inglese, J.; McGuire, J. J.; Nimec, Z.; Coward, J. K. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 2598. (b) McGuire, J. J.; Graber, M.; Licato, N.; Vincenz, C.; Coward, J. K.; Nimec, Z.; Galivan, J. *Cancer Res.* **1989**, *49*, 4517. (c) Although the purified L-erythro isomer of γ-fluoroMTX did undergo some polyglutamylation in H35 cells, this was negligible in comparison with that of MTX.

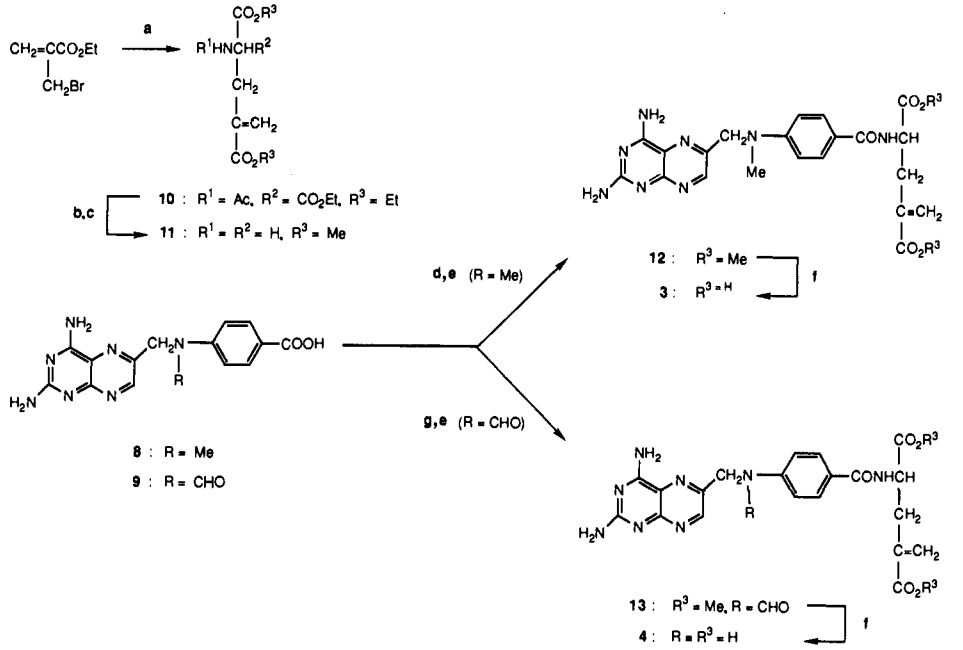
(11) Tsushima, T.; Kawada, K.; Ishihara, S.; Uchida, N.; Shiratori, O.; Higaki, J.; Hirata, M. *Tetrahedron* **1988**, *44*, 5375.

(12) Hellmann, H.; Lingens, F. *Chem. Ber.* **1956**, *89*, 77.

(13) Ferris, A. F. *J. Org. Chem.* **1955**, *20*, 780.

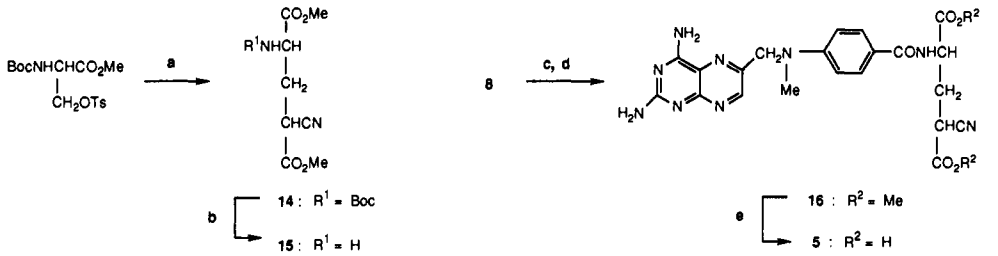
(14) Boggs, N. T., III; Goldsmith, B.; Gawley, R. E.; Koehler, K. A.; Hiskey, R. G. *J. Org. Chem.* **1979**, *44*, 2262.

Scheme I[a]



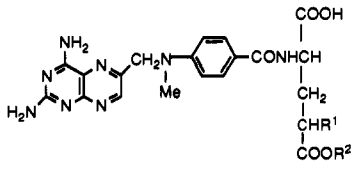
[a] (a) $AcNHCH(CO_2Et)_2$; (b) HCl; (c) $MeOH/SOCl_2$; (d) $DEPC/Et_3N$; (e) compound 11; (f) NaOH; (g) *i*-BuOCOCl/Et_3N.

Scheme II[a]



[a] (a) $NCCH_2CO_2Bu$-*t*/NaH; (b) TFA, 0 °C, 1 h; (c) $DEPC/Et_3N$; (d) compound 15; (e) NaOH.

corresponding to three protons. These results suggested that **16** and **5** (and by inference **15**) might be mixtures of threo and erythro isomers. Compound **16** gave two well-separated HPLC peaks (5.6 and 13.1 min; 30% MeCN in 0.1 M NH_4OAc, pH 7.8) with an approximate area ratio of 2:1, whereas **5** gave peaks (14.5 and 18.3 min; 5% MeCN in 0.1 M NH_4OAc, pH 7.5) with an approximate area ratio of 1:1. It thus appeared that some enrichment of the minor isomer might be occurring during purification of **16**. However, the stereochemical configuration of the two isomers is not known at present. Because the C≡N band at 2260 cm^{-1} in the IR spectrum of **5** was weak, we were concerned that the alkaline hydrolysis step had perhaps converted the nitrile group to an amide (**17**). That **16** was,



17: $R^1 = CONH_2$, $R^2 = H$
18: $R^1 = CH_2NH_2$, $R^2 = H$

in fact, a nitrile ester and not an amide ester was apparent from the mass spectrum, which contained a peak (M + 1) at 550 rather than 568. Moreover, an authentic specimen of **17** was obtained in 25% yield by hydrolysis of **16** with HBr in glacial acetic acid and was readily distinguishable from the nitrile. A major byproduct of the acid-catalyzed reaction of **16** was mAPA (**8**), which was isolated in 45% yield.

Because we were interested in also preparing the amine **18**, whose side chain may be viewed as being a hybrid of ornithine and glutamic acid, we attempted to selectively reduce the nitrile group in **5**. Treatment of the disodium salt of **5** with either BH_3/THF or sodium mono(trifluoroacetoxy)borohydride[15] unfortunately led only to recovered starting material. Hydrogenation over PtO_2 in $MeOH/NH_3$ likewise resulted in no reduction, while hydrogenation using Raney nickel in the same medium yielded multiple products, none of which gave a positive ninhydrin test for a primary amino group. Attempts were also made to reduce the CN group in **14** by hydrogenation over PtO_2, but these were largely unsuccessful due to the formation of multiple products. Because it was felt that failure to isolate an amine might be the result of spontaneous ring closure to an amide, attempts were made to hydrolyze the methyl ester in **14** with NaOH or $Ba(OH)_2$. However, this was likewise unsuccessful. Efforts to prepare **18** from **14** (or from **9** and a protected derivative of its still unknown precursor γ-carboxy-L-ornithine) were not pursued further.

(15) Umino, N.; Iwakuma, T.; Hoh, N. *Tetrahedron Lett.* **1976**, 2875.

Table I. Biological Activity of MTX and AMT Analogues with γ-Substitution on the Glutamate Side Chain

compd	DHFR activity, IC_{50}, μM	cell growth, IC_{50}, μM	
		WI-L2	L1210
1	0.025[a]	0.013	0.0046[a]
2	0.025[a]	0.0071	0.002[a]
3	0.044	0.048	0.87
4	0.088	0.020	0.029
5	0.057	ND[b]	0.018[c]
19	0.094	ND[b]	0.070

[a] Data from ref 7c. [b] ND = not determined. [c] The IC_{50} of 5 and 19 against MTX-resistant L1210/R81 cells was 80 and 154 μM, respectively, as compared with 200 μM for MTX.

Biological Activity

The ability of compounds 1–5 to inhibit purified DHFR from human leukemic lymphoblasts (WI-L2 cells) was determined spectrophotometrically at 340 nm as previously described.[16] The results are shown in Table I, along with the activity of amide 19. Also presented are the activities of these compounds as inhibitors of the growth of WI-L2 cells and L1210 murine leukemia cells in culture after 48 h of drug exposure. Compounds 3–5 were somewhat less potent than MTX or AMT as DHFR inhibitors, with IC_{50} values of 0.044, 0.088, and 0.057 μM, respectively. Cell growth inhibition also required somewhat higher concentrations of 3–5 than of MTX or AMT. However, it must be kept in mind that 3 and 4 are DL mixtures and that the L enantiomers probably account for most of the anti-DHFR activity as well as cell-growth inhibition. In the case of nitrile 5, which is a mixture of four diastereomers, it is again likely that only the isomers with the α-carbon in the L configuration possess substantial activity. Amide 17 was less potent than nitrile 5 in both assays. However, this does not necessarily indicate a difference between the two types of γ-substituents, since it was possible that the proportions of the four possible diastereomers in the test samples 5 and 19 were not the same.

The results of these assays may be compared with published values for other MTX analogues with γ-substituents. γ-FluoroMTX has been reported to bind to DHFR from human spleen cells with an IC_{50} of 0.95 nM, as compared with 0.75 nM for MTX, and to inhibit cultured H35 rat hepatoma cells with an IC_{50} ranging from 700 μM for 2-h exposure to 0.12 μM for 72-h exposure.[10a] This time dependence was consistent with the fact that both the DL-erythro and the DL-threo forms of γ-fluoroMTX are very poor substrates for FPGS and were very inefficiently polyglutamylated in cells.[10b,c] γ-FluoroMTX differed fundamentally in this respect from MTX, whose IC_{50} for 2-h treatment of H35 cells was 0.3 μM.[10a] DHFR inhibition data for γ-substituted MTX analogues were also reported recently by Tsushima and co-workers,[11] who found that the γ-Me, γ-SMe, and γ-OH compounds were comparable to MTX and γ-fluoroMTX in their ability to inhibit DHFR from bovine liver and chicken liver (30–50% inhibition at 0.03 μM as compared with 50% inhibition by MTX). These results demonstrated that the active site of DHFR is able to accommodate to changes on the γ-carbon itself just as easily as it can to changes on the γ-carboxyl. It is of interest to note that the maximally tolerated doses of γ-fluoroMTX (640 mg/kg, qd×5) and MTX (20 mg/kg, qd×5) produced similar increases in survival (221 and 241%, respectively) in mice with L1210 leukemia[11] but that the maximally tolerated dose as well as therapeutic effect decreased on replacement of F by Me, SMe, or OH, apparently as a manifestation of stereoelectronic substituent effects at the γ-position.

The findings of the present paper support the view that DHFR tolerates substitution on the γ-carbon of the glutamate moiety in MTX and AMT. Tumor cell growth inhibition on the other hand is likely to be affected in such analogues as a result of decreased transport and polyglutamylation. However, given the important role of hydrophobic interactions in the binding of classical antifolates to DHFR and the apparent tolerance of DHFR for some degree of γ-substitution on the side chain of the inhibitor, we believe it is worthwhile to examine a broader range of MTX and AMT analogues in which the hydrophobicity of the side chain is increased while the α- and γ-COOH groups are kept intact.

Experimental Section

IR spectra were obtained on a Perkin-Elmer Model 781 double-beam recording spectrophotometer, and UV spectra were recorded on a Varian Model 210 instrument. ^1H NMR spectra were obtained on a Varian EM360L spectrometer with Me_4Si or $Me_2Si(CH_2)_5SO_3Na$ as the reference. Optical rotations were measured on a Perkin-Elmer Model 241MC polarimeter. TLC analyses were done on fluorescent Baker Si250F silica gel plates, Eastman 13181 silica gel sheets, or Eastman 13254 cellulose sheets. Spots were visualized under 254-nm UV illumination or with the aid of ninhydrin. Column chromatography was on Baker 3405 (60–200 mesh), Baker 7024-1 "flash" silica gel, or Whatman DE-52 preswollen [*N*,*N*-(diethylamino)ethyl]cellulose (DEAE-cellulose). 4-Amino-4-deoxy-N^{10}-methylpteroic acid (mAPA, 8) and 4-amino-4-deoxy-N^{10}-formylpteroic acid (fAPA, 9) were prepared as reported;[3e,4d,5-7] other chemicals were purchased from Aldrich (Milwaukee, WI) or Bachem (Torrance, CA). Solvents used in moisture-sensitive reactions were dried over Linde 4A molecular sieves (Fisher, Boston, MA). HPLC was done on a Waters C_{18} radial compression cartridge column (5 μm particle size, 0.5 × 10 cm) connected to a Waters Model 400 instrument equipped with a Model 490 multiwavelength detector and a Model 660 programmable solvent gradient system. Melting points were taken on a Fisher-Johns hot stage apparatus (Fisher, Boston, MA) or in Pyrex capillary tubes in a Mel-Temp apparatus (Cambridge Laboratory Devices, Cambridge, MA) and are not corrected. Microanalyses were by Galbraith Laboratories (Knoxville, TN), MultiChem Laboratories (Lowell, MA), or Robertson Laboratory (Madison, NJ).

Dimethyl 4-Methylene-DL-glutamate Hydrochloride (11·HCl). Hydrolysis of 2-acetamido-2-carbethoxy-4-methyleneglutaric acid (10) in hot 12 N HCl as described[15] afforded 4-methylene-D,L-glutamic acid hydrochloride, mp 188–189 °C, in 77% yield. A solution of this salt (3.20 g, 0.015 mol) in MeOH (150 mL) was cooled to −30 °C and treated with $SOCl_2$ (13.5 mL) in small portions while the internal temperature was maintained between −30 and −20 °C. When addition was complete, the reaction mixture was allowed to come to room temperature and was left for 22 h. Solvent evaporation gave a thick oil (3.35 g, ca. 100% yield) which solidified on prolonged storage at room temperature. For analysis, a sample was kept at 60 °C over P_2O_5 for 18 h: IR (film) ν 3460 (NH_2), 3100 (NH_2^+), 1750, 1720 (ester C=O), 1630 (C=C), 820 (C=CH_2) cm^{-1}. Anal. ($C_8H_{13}NO_4$·HCl) C, H, Cl, N.

Dimethyl *N*-(4-Amino-4-deoxy-N^{10}-methylpteroyl)-4-methylene-DL-glutamate (12). A suspension of 8 (0.328 g, 0.93 mmol, assumed to contain 1.5 mmol of H_2O) in a solution of DEPC (0.455 g, 2.79 mmol) and Et_3N (0.47 g, 4.65 mmol) in dry DMF (20 mL) was stirred at room temperature for 4 h. To this mixture was then added a solution of 11·HCl (0.174 g, 0.93 mmol) in dry DMF (5 mL), and after 20 h the solvent was evaporated in vacuo. The residue was redissolved in $CHCl_3$ (300 mL), and the solution was washed with H_2O (250 mL) containing 28% NH_4OH (3 mL). The organic layer was evaporated to dryness, and the residue was dissolved in 95:5 $CHCl_3$–MeOH (3 mL) and applied onto a silica gel column (30 g, 2.0 × 27 cm). The column was eluted with the same solvent mixture, and fractions were monitored by TLC (silica gel, 9:1 $CHCl_3$–MeOH). Fractions showing a spot with R_f 0.31

(16) Susten, S. S.; Kempton, R. J.; Black, A. M.; Freisheim, J. H. *Biochem. Pharmacol.* 1984, *33*, 1957.

were pooled and evaporated. The residue was taken up in a small volume of MeOH, and the solution was added to Et_2O to precipitate the product. Drying in vacuo over P_2O_5 at 50 °C afforded a yellow powder (242 mg, 54% yield): mp 114–116 °C (softening above 106 °C). Anal. ($C_{23}H_{26}N_8O_5 \cdot 0.5H_2O$) C, H, N.

N-(4-Amino-4-deoxy-N^{10}-methylpteroyl)-4-methylene-DL-glutamic Acid (γ-MethyleneMTX, 3). A solution of diester **12** (42 mg) in a mixture of MeOH (2 mL) and 0.25 N NaOH (2 mL) was kept at 20 °C for 5 h while being monitored by TLC (silica gel, 5:4:1, $CHCl_3$–MeOH–28% NH_4OH) for the disappearance of diester (R_f 0.94) and the formation of diacid (R_f 0.51). Most of the solvent was removed under reduced pressure, a small amount of H_2O was added, and the pH was adjusted to 4.5 with 10% AcOH. After 18 h at 0 °C, the precipitated solid was filtered, washed with H_2O, and dried with the aid of a lyophilizer. Further drying in vacuo over P_2O_5 at 100 °C gave a yellow powder (31 mg, 76% yield): mp >300 °C dec; IR (KBr) ν 3410 (NH_2), 1640 (COOH), 1605 (amide C=O), 830 (C=CH_2) cm^{-1}. Anal. ($C_{21}H_{22}N_8O_5 \cdot 1.5H_2O$) C, H, N.

N-(4-Amino-4-deoxypteroyl)-4-methylene-DL-glutamic Acid (γ-MethyleneAMT, 4). Isobutyl chloroformate (68 mg, 0.5 mmol) was added at room temperature to a stirred solution of **9**·1.5H_2O (183 mg, 0.5 mmol) and Et_3N (303 mg, 3 mmol) in dry DMF (15 mL). After 20 min, diester **11**·HCl (112 mg, 0.5 mmol) was added, followed after 15 min by a second portion of isobutyl chloroformate (34 mg, 0.25 mmol). Alternating further additions of isobutyl chloroformate (34 mg, 0.25 mmol; 2 × 17 mg, 0.125 mmol) and **11**·HCl (56 mg, 0.25 mmol; 2 × 28 mg, 0.125 mmol) were then made at 15-min intervals to bring the total of these reactants to 1.0 mmol each. The progress of the reaction was monitored by TLC (silica gel, 28:12:1 $CHCl_3$–MeOH–28% NH_4OH) for the formation of product (R_f 0.85, blue–fluorescent) and the disappearance of starting material (R_f 0.15, blue–fluorescent). After evaporation to dryness under reduced pressure, the residue was taken up in 95:5 $CHCl_3$–MeOH and applied onto a silica gel column (12 g, 1.5 × 20 cm), which was eluted successively with 95:5 and 9:1 $CHCl_3$–MeOH. Fractions containing the desired product according to TLC were pooled, concentrated to a small volume, and added to excess Et_2O. The precipitate was filtered and redissolved in $CHCl_3$, and the solution was washed twice with an equal volume of H_2O, dried ($MgSO_4$), concentrated, and poured into excess Et_2O to obtain a yellow solid, which was collected and dried: yield 51 mg (19%); mp 131–133 °C; IR (KBr) ν 3420 (NH_2), 1725 (ester C=O), 1650 (amide C=O), 1505 (amide C=O, aromatic) cm^{-1}.

To complete the synthesis, a solution of **13** (47 mg, 0.085 mmol) in MeOH (2 mL) and 1 N NaOH (0.85 mL) was kept at room temperature for 22 h and then treated with a second portion of 1 N NaOH (0.85 mL). After another 26 h, the mixture was filtered, most of the solvent was evaporated, a small volume of water was added, and 10% AcOH was added until, at pH 6–7, a gelatinous yellow precipitate began to form. After storage at 0 °C for 2 days, the solid became more granular. The solid was collected by centrifugation, washed with H_2O, recentrifuged, and dried, first in a lyophilizer and then in vacuo at 80 °C over P_2O_5, to obtain the Na salt of **4** as a yellow powder (32 mg, 63% yield): mp 235–255 °C dec; TLC R_f 0.10 (silica gel, 5:4:1 $CHCl_3$–MeOH–28% NH_4OH); IR (KBr) ν 3430 (NH_2), 1645 (acid C=O), 1610 (amide C=O), 1520, 820, 850 (C=CH_2); MS (FAB) m/z found 451, calcd 451 (M − 1); found 473, calcd 473 (M − 1+ Na). Anal. ($C_{20}H_{18}N_8O_5Na_2 \cdot 0.66CH_3CO_2Na \cdot 2.5H_2O$) C, H, N.

γ-tert-Butyl α-Methyl N-(tert-Butyloxycarbonyl)-4-cyanoglutamate (14). To a stirred solution of *tert*-butyl cyanoacetate (7.34 g, 0.052 mol) in dry DMF (100 mL) were added NaH (1.37 g, 0.052 mol, 60% suspension in mineral oil) and, 20 min later, a solution of *N*-(*tert*-butyloxycarbonyl)-*O*-tosyl-L-serine methyl ester[14] (10 g, 0.026 mol) in DMF (50 mL) dropwise over 5 min. The initially formed precipitate redissolved, but after 23 h a thick gel was observed. Glacial AcOH (3.5 mL) was added, the solvent was distilled off under reduced pressure, and the residue was taken up in 10% AcOH. The solution was extracted with EtOAc (4 × 100 mL), the combined extracts were washed with saturated sodium bicarbonate, rinsed with saturated NaCl, and dried ($MgSO_4$). Evaporation of the EtOAc left a red oil, which was extracted repeatedly with cold petroleum ether (bp 40–60 °C) to remove the mineral oil and excess *tert*-butyl cyanoacetate. The oily residue (10.6 g) was dissolved in $CHCl_3$ and the solution was applied onto a silica gel column (300 g, 5.0 × 54 cm), which was eluted with $CHCl_3$. Fractions with R_f 0.29 (silica gel, $CHCl_3$) were pooled, evaporated, redissolved in toluene, and applied onto a second silica gel column (330 g, 5.0 × 60 cm), which was eluted successively with toluene and 97.5:2.5 $CHCl_3$–EtOAc. Fractions with R_f 0.30 (silica gel, 95:5 $CHCl_3$–EtOAc) were obtained with the latter eluent and were pooled and evaporated to a colorless oil (6.17 g, 69% yield): IR (film) ν 3390, 2260 (CN), 1710–1750 cm^{-1}; NMR ($CDCl_3$) δ 1.47 (s, 9 H, *t*-Bu), 1.50 (s, 9 H, *t*-Bu), 2.0–2.5 (m, 2 H, CH_2), 3.61 (s, 3 H, OMe), 4.40 (m, 1 H, α-CH), 5.0 (m, 1 H, α-CH). Anal. ($C_{16}H_{26}N_2O_6$) C, H, N.

γ-tert-Butyl α-Methyl 4-Cyanoglutamate (15). Compound **14** (1.02 g, 2.97 mmol) was shaken manually with trifluoroacetic acid (3 mL) for 15 min, and the resulting solution was kept at 0 °C for 1 h and poured into dry Et_2O (75 mL). The mixture was kept at 0 °C overnight, and the crystallized solid was filtered and dried in vacuo over P_2O_5 at 35 °C to obtain colorless needles (0.632 g, 60% yield): mp 108.5–109 °C; TLC R_f 0.95, ninhydrin-positive (silica gel, 5:4:1 $CHCl_3$–MeOH–28% NH_4OH); IR (KBr) ν 2250 (weak CN), 1750 (ester C=O), 1665 (CF_3COO^-) cm^{-1}; NMR (CD_3OD) δ 1.56 (s, 9 H, *t*-Bu), 2.45 (d, 2 H, CH_2), 3.3 (m, 1 H, α-CHCN), 3.86 (s, 3 H, OMe), 4.2 (m, 1 H, α-$CHNH_3^+$). Anal. ($C_{11}H_{18}N_2O_4 \cdot CF_3CO_2H$) C, H, F, N.

N-(4-Amino-4-deoxy-N^{10}-methylpteroyl)-4-cyanoglutamic Acid (γ-CyanoMTX, 5). To a stirred solution of DEPC (1.37 g, 8.4 mmol) and Et_3N (0.85 g, 8.4 mmol) in dry DMF (90 mL) was added **8**·1.5H_2O (0.986 g, 2.8 mmol). After 4 h at 25 °C, **15**·CF_3CO_2H (0.833 g, 2.33 mmol) was added, followed by another portion of Et_3N (0.475 g, 4.7 mmol). The solution was kept at 25 °C for 24 h, the DMF was evaporated under reduced pressure, and the residue was extracted twice with Et_2O and then taken up in $CHCl_3$ (150 mL). The $CHCl_3$ solution was washed with a mixture of 28% NH_4OH (4 mL) in H_2O (100 mL) and evaporated. The residue was taken up in warm 5:5:2 $CHCl_3$–MeCN–MeOH (12 mL) and applied onto a silica gel column (80 g, 3.0 × 37 cm), which was prepared and eluted with 5:5:1 $CHCl_3$–MeCN–MeOH. Fractions with R_f 0.50 (silica gel, 5:5:2 $CHCl_3$–MeCN–MeOH) were combined and evaporated, and the residue was recrystallized from a mixture of *i*-PrOH and MeOH. Drying in vacuo over P_2O_5 at 80 °C afforded γ-*tert*-butyl α-methyl *N*-(4-amino-4-deoxy-N^{10}-methylpteroyl)-4-cyanoglutamate (**16**) as a yellow solid (0.864 g, 58% yield): mp 184 °C (softening above 162 °C); IR (KBr) ν 3440, 2260 (very weak CN), 1740 (ester C=O), 1680–1610 cm^{-1}; NMR (DMSO-d_6) δ 1.38 and 1.42 (2 s, 9 H, *t*-Bu), 2.32 (m, 2 H, CH_2), 3.18 (s, 3 H, NCH_3), 3.60 and 3.70 (2 s, 3 H, OCH_3), 4.10 (m, 1 H, α-CH), 4.55 (m, 1 H, α-CH), 4.79 (s, 2 H, CH_2N), 6.54 (br s, 2 H, 4-NH_2), 6.84 (d, J = 4 Hz, $C_{3'}$- and $C_{5'}$-H), 7.70 (d, J = 4 Hz, $C_{2'}$- and $C_{6'}$-H, overlapping a br 2-NH_2 s), 8.58 (s 1 H, C_7); MS m/z found 550, calcd (M + 1) 550. HPLC analysis (C_{18} column, 30% MeCN in 0.1 M NH_4OAc, pH 7.8) gave two peaks in approximately 1:2 ratio with elution times of 5.6 and 13.1 min, respectively.

To complete the synthesis, a solution of diester **16** (402 mg, 0.75 mmol) in a mixture of DMSO (4 mL), MeOH (4 mL), and 1 N NaOH (3 mL) was kept at 25 °C for 24 h and acidified to pH 3 with 1 N HCl. After 1 h at 0 °C, the yellow solid was filtered and dried on a lyophilizer to obtain a yellow powder (351 mg) whose TLC (silica gel, 5:4:1 $CHCl_3$–MeOH–28% NH_4OH) showed mainly one spot with R_f 0.15. The solid was redissolved in 3% NH_4HCO_3 with enough NH_4OH added to bring the pH to 9.0, and the solution was applied onto a DEAE-cellulose column (50 g, 3.0 × 15.5 cm, HCO_3–form), which was eluted first with a large volume of H_2O to remove salts and then with 1% and 3% NH_4HCO_3. Fractions with R_f 0.15 were pooled, evaporated to a small volume, acidified to pH 4.0 with glacial AcOH, and left at 0 °C. The precipitated solid was collected, washed with H_2O, and dried on a lyophilizer and in vacuo over P_2O_5 at 60 °C to obtain a yellow powder (229 mg, 57% yield): mp 183 °C (prior softening); IR (KBr) ν 3430, 3200, 2255 (CN), 1650, 1610 cm^{-1}; MS m/z calcd 480 (M + 1), found 480. HPLC analysis (C_{18} column, 5% MeCN in 0.1 M NH_4OH, pH 7.5) gave two peaks of equal area with retention times of 14.5 and 18.3 min. Anal. ($C_{21}H_{21}N_9O_5 \cdot 2H_2O$) C, H, N.

N-(4-Amino-4-deoxy-N^{10}-methylpteroyl)-4-carbamoylglutamic Acid (γ-CarbamoylMTX, 17). A mixture of **16** (54

mg, 0.1 mmol) and 30% HBr–AcOH (1 mL) was stirred at 25 °C for 24 h and then warmed until all the solid was dissolved and left to stand at room temperature for 7 days. The solution was poured into excess Et_2O, and the solid was collected, redissolved in dilute NH_4OH, and purified by gradient chromatography on a C_{18} reversed-phase silica gel column, using 0.1 M NH_4OAc, pH 7.5, in reservoir A and 1:9 EtOH–0.1 M NH_4OAc, pH 7.5, in reservoir B. The fractions containing 17 (elution time 143 min) and mAPA (8) (elution time 190 min) were collected separately and freeze-dried. Each product was redissolved in dilute NH_4OH (pH 10), a small amount of undissolved gelatinous material was removed by filtration through a bed of silica gel, and the filtrate was freeze-dried. This process was repeated twice to obtain 16 mg (26%) of 17 as a yellow powder: mp 215 °C dec; TLC R_f 0.09 (silica gel, 5:4:1 $CHCl_3$–MeOH–NH_4OH); IR (KBr) ν 3450, 1670–1630, 1610 cm^{-1}; MS m/z calcd 498 (M + 1), found 498. HPLC analysis (C_{18} column, 5% EtOH in 0.1 M NH_4OAc, pH 7.5) revealed a single peak with a retention time of 11.7 min. The weight of mAPA (8) was 16 mg (45% yield); TLC R_f 0.57 (silica gel, 5:4:1 $CHCl_3$–MeOH–NH_4OH).

Acknowledgment. This work was supported in part by Grants CA25394 (A.R.) and CA41461 (J.H.F.) from the National Cancer Institute, DHHS.

Synthesis of Two Cyclopentenyl-3-deazapyrimidine Carbocyclic Nucleosides Related to Cytidine and Uridine

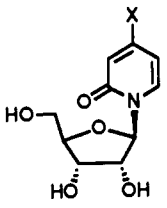
Richard R. Copp and Victor E. Marquez*

Laboratory of Medicinal Chemistry, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892. *Received May 10, 1990*

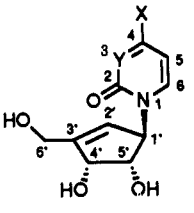
The cytosine analogue of neplanocin A, cyclopentenylcytosine (CPE-C, **3**), has significant antitumor and antiviral activity commensurate with the drug's ability to produce a significant depletion of cytidine triphosphate (CTP) levels that result from the potent inhibition of cytidine triphosphate synthetase. Another important antitumor agent, previously identified as a potent inhibitor of the same enzyme, is 3-deazauridine (**2**). The synthesis of the cyclopentenyl nucleosides 3-deaza-CPE-C (**5**) and 3-deaza-CPE-U (**6**) was undertaken in order to investigate the effects of a modified 3-deaza pyrimidine aglycon moiety on the biological activity of the parent CPE-C. These compounds were synthesized via an S_N2 displacement reaction on cyclopenten-1-ol methanesulfonate (**10**) by the sodium salt of the corresponding aglycon. In each case, separation and characterization of the corresponding N- and O-alkylated products was necessary before final removal of the blocking groups. The target compounds were devoid of in vitro antiviral activity against the HSV-1 and human influenza viruses. Although 3-deaza-CPE-C was nontoxic to L1210 cells in culture, 3-deaza-CPE-U displayed significant cytotoxicity against murine L1210 leukemia in vitro.

Introduction

The bioisosteric replacement of nitrogen by carbon in the pyrimidine heterocycle of the naturally occurring pyrimidine nucleosides cytidine and uridine has generated effective inhibitors of cell growth.[1-4] 3-Deazauridine (**2**),



1: X = NH_2
2: X = OH



3: X = NH_2, Y = N
4: X = OH, Y = N
5: X = NH_2, Y = CH
6: X = OH, Y = CH

and to a lesser extent 3-deazacytidine (**1**), produced significant growth inhibition against L-1210 leukemia cells in vitro.[4] The more effective of the two analogues, 3-deazauridine, also increased the survival time of mice bearing either this tumor or the *ara*-C-resistant variety (L-1210/*ara*-C), against which it displayed even better activity.[5] Other important biochemical properties resulting from this change were the increased stability of the glycosylic bond of both **1** and **2**, and the inertness of **1** toward deamination by cytidine deaminase (CDA).[6] The primary metabolic effect responsible for the biological activity of 3-deazauridine (**2**) was determined to be the strong depletion of CTP pools resulting from the inhibition of cytidine triphosphate synthetase by the metabolite 3-deazauridine 5′-triphosphate (3-deaza-UTP).[5,7,8]

Recently, we have discovered that a more radical change in the structure of cytidine, which consisted of replacing the tetrahydrofuran ring by a cyclopentene moiety,[9,10] produced an analogue (CPE-C, **3**) which was 3 orders of magnitude more potent than 3-deazauridine (**2**) as an antitumor agent and whose activity appeared to be commensurate with a sustained reduction of CTP pools.[10-13] The compound also displayed very potent antiviral activity and this was similarly correlated with the inhibition of cytidine triphosphate synthetase.[10,14,15] In support of this

(1) Robins, M. J.; Currie, B. L. *Chem. Commun.* **1968**, 1547.
(2) Currie, B. L.; Robins, R. K.; Robins, M. J. *J. Heterocycl. Chem.* **1970**, *7*, 323.
(3) Cook, P. D.; Day, R. T.; Robins, R. K. *J. Heterocycl. Chem.* **1977**, *14*, 1295.
(4) Bloch, A.; Dutschman, G.; Currie, B. L.; Robins, R. K.; Robins, M. J. *J. Med. Chem.* **1973**, *16*, 294.
(5) Brockman, R. W.; Shaddix, S. C.; Williams, M.; Nelson, J. A.; Rose, L. M.; Schabel, F. M., Jr. *Ann. N.Y. Acad. Sci.* **1975**, *255*, 501.
(6) Wang, M. C.; Bloch, A. *Biochem. Pharmacol.* **1972**, *21*, 1063.
(7) McPartland, R. P.; Wang, M. C.; Bloch, A.; Weinfeld, H. *Cancer Res.* **1974**, *34*, 3107.
(8) For a more recent review on 3-deazauridine, see: Moroconi, W. J.; Slavik, M.; Taylor, S. *Invest. New Drugs* **1986**, *4*, 67.
(9) Lim, M.-I.; Moyer, J. D.; Cysyk, R. L.; Marquez, V. E. *J. Med. Chem.* **1984**, *27*, 1536.
(10) Marquez, V. E.; Lim, M.-I.; Treanor, S. P.; Plowman, J.; Priest, M. A.; Markovac, A.; Khan, M. S.; Kaskar, B.; Driscoll, J. S. *J. Med. Chem.* **1988**, *31*, 1687.
(11) Glazer, R. I.; Knode, M. C.; Lim, M.-I.; Marquez, V. E. *Biochem. Pharmacol.* **1985**, *34*, 2535.
(12) Glazer, R. I.; Cohen, M. B.; Hartman, K. D.; Knode, M. C.; Lim, M.-I.; Marquez, V. E. *Biochem. Pharmacol.* **1986**, *35*, 1841.
(13) Moyer, J. D.; Malinowski, N. M.; Treanor, S. P.; Marquez, V. E. *Cancer Res.* **1986**, *46*, 3325.