dissolved in Me_2SO (1% final concentration). The growth rate inhibition of cells was determined with a ZBI Coulter Counter. Dose–effect relationship from the results obtained after 48 h of culture were calculated by a least-squares procedure. Linear regressions were determined as the percentage inhibition of the growth as a function of the logarithm of the dose. The concentration of drug that lowered control growth cell by 50% (ID50) was estimated from these equations.

Antitumor Studies. Experiments with **9** were performed on L1210 lymphoid leukemia, P388 lymphocytic leukemia, sarcoma 180, and B16 melanocarcinoma. The protocols were already described.[21] Antitumor activity was expressed as (T/C) 100, *T* being the median survival time of treated animals and *C* the median survival time of controls. A significant tumor activity is considered for T/C > 125%. Drug was dissolved in saline solution (0.15 M NaCl).

Mutagenic Test. The mutagenic property of **9** was determined by the selection of his^+ revertants of *Salmonella typhimurium* histidine auxotroph strains TA98 and TA100 according to the procedure described by Ames et al.,[18] with minor modifications as follows: overnight cultures growing in Oxoid nutrient broth (Oxoid Ltd., England) were subcultured into fresh media of the same composition. The subcultures were allowed to grow for 2 h, subdivided into 20-mL aliquots, and reincubated during 5 h in the absence (controls) or in the presence of various concentrations of drug. After the incubation was terminated, bacteria were removed by centrifugation and washed once with the Oxoid medium. Final pellets of bacteria were resuspended in Oxoid to obtain an absorbance of 2.50 at 650 nm. Aliquots of these bacteria suspensions were diluted in the top agar (0.6% Difco agar, 0.6% NaCl) and distributed on the petri plates. Histidine revertants were counted after 48 h incubation at 37 °C.

Acknowledgment. This investigation was supported by contrat de recherche libre 78.1160-3 from INSERM. The technical assistance of Georgette François for cytotoxicity and antitumoral tests is gratefully acknowledged.

Registry No. 2, 7556-92-5; 3, 87039-48-3; 4, 87039-49-4; 5, 87039-50-7; 6, 87039-51-8; 9, 87039-52-9; 10, 87039-53-0; 11, 87039-54-1; 13, 87039-55-2; 14, 87039-56-3; aziridine, 151-56-4.

(21) Labarde, J. F.; Sournies, F.; Cros, S.; Francois, G.; Van De Grampel, J. C.; Van Der Huizen, A. A. *Cancer Lett.* 1981, *12*, 245.

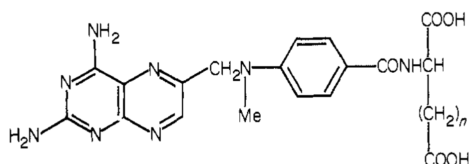
Methotrexate Analogues. 20. Replacement of Glutamate by Longer-Chain Amino Diacids: Effects on Dihydrofolate Reductase Inhibition, Cytotoxicity, and in Vivo Antitumor Activity

Andre Rosowsky,*,† Ronald Forsch,† Jack Uren,† Michael Wick,‡ A. Ashok Kumar,§ and James H. Freisheim§

Dana-Farber Cancer Institute and the Departments of Pharmacology and Dermatology, Harvard Medical School, Boston, Massachusetts 02115, and the Department of Biological Chemistry, University of Cincinnati Medical Center, Cincinnati, Ohio 45267. *Received May 2, 1983*

Chain-extended analogues of methotrexate were synthesized by condensation of 4-amino-4-deoxy-N^{10}-methylpteroic acid with esters of L-α-aminoadipic, L-α-aminopimelic, and L-α-aminosuberic acids, followed by ester hydrolysis with acid or base. Coupling was accomplished in up to 85% yield by the use of the peptide bond forming reagent diethyl phosphorocyanidate at room temperature. The products were found to bind bacterial (*Lactobacillus casei*) and mammalian (L1210 mouse leukemia) dihydrofolate reductase with an affinity comparable to methotrexate and were also equitoxic to L1210 cells in culture. Cytotoxicity increased up to 3-fold as the number of CH_2 groups in the amino acid side chain was extended from two to five. The α-aminoadipate and α-aminopimelate analogues were poor substrates for carboxypeptidase G_1, confirming that this enzyme has a strict requirement for a C-terminal L-glutamic acid residue. The in vivo antitumor activity of the chain-extended analogues against L1210 leukemia in mice was comparable to that of the parent drug on the qd×9 schedule, but higher doses were required to achieve the same increase in survival. The results were consistent with findings, reported separately, that these compounds are poor substrates for folate polyglutamate synthetase and therefore would not be expected to form γ-polyglutamates once they enter a cell. This distinctive property has potential therapeutic implications for the treatment of certain MTX-resistant tumors whose resistance may be associated with a lower than normal capacity to form γ-polyglutamates in comparison with proliferative tissues such as intestinal mucosa or marrow.

Previous work in this laboratory and others has led to the recognition that the terminal region of the glutamate side chain in classical antifols, such as methotrexate (MTX, 1), is amenable to structural modification with no signif-



1-4, *n* = 2–5

icant loss of binding to the target enzyme dihydrofolate reductase.[1-8] As part of a continuing program of systematic molecular change aimed at the development of new or improved folate antagonists as antitumor agents,[9,10] we

† Dana-Farber Cancer Institute and the Department of Pharmacology, Harvard Medical School.

‡ Dana-Farber Cancer Institute and the Department of Dermatology, Harvard Medical School.

§ University of Cincinnati Medical Center.

(1) Rosowsky, A.; Beardsley, G. P.; Ensminger, W. D.; Lazarus, H.; Yu, C.-S. *J. Med. Chem.* 1978, *21*, 380 (part 11 of this series).
(2) Szeto, D. W.; Cheng, Y. C.; Rosowsky, A.; Yu, C.-S.; Modest, E. J.; Piper, J. R.; Temple, C., Jr.; Eliott, R. D.; Rose, J. D.; Montgomery, J. A. *Biochem. Pharmacol.* 1979, *28*, 2633.
(3) Rosowsky, A.; Forsch, R.; Uren, J.; Wick, M. *J. Med. Chem.* 1981, *24*, 1450 (part 14 of this series).
(4) Piper, J. R.; Montgomery, J. A.; Sirotnak, F. M.; Chello, P. L. *J. Med. Chem.* 1982, *25*, 182.
(5) Rosowsky, A.; Wright, J. E.; Ginty, C.; Uren, J. *J. Med. Chem.* 1982, *25*, 960 (part 15 of this series).
(6) Rosowsky, A.; Wright, J. E.; Shapiro, H.; Beardsley, G. P.; Lazarus, H. *J. Biol. Chem.* 1982, *257*, 14162 (part 17 of this series).
(7) Kumar, A. A.; Freisheim, J. H.; Kempton, R. J.; Anstead, G. M.; Black, A. M.; Judge, L. *J. Med. Chem.* 1983, *26*, 111.
(8) Kumar, A. A.; Kempton, R. J.; Anstead, G. M.; Freisheim, J. H. *Biochemistry* 1983, *22*, 390.

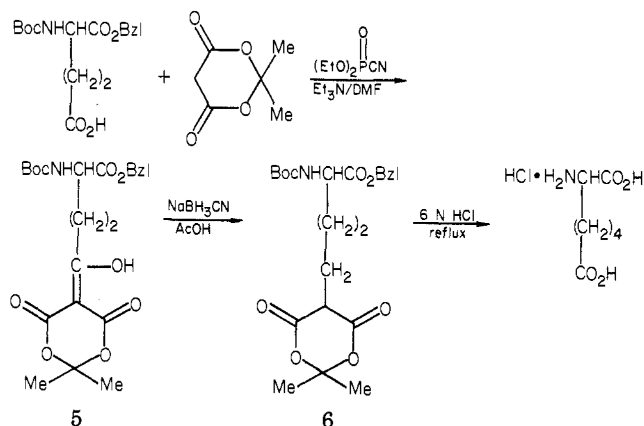
0022-2623/83/1826-1719$01.50/0 © 1983 American Chemical Society

were interested in synthesizing MTX analogues in which L-glutamate is replaced by other L-amino dicarboxylic acids of longer chain length. The shorter-chain L-aspartate analogue was reported a number of years ago to be less active than MTX in vitro and in vivo.[11] Low activity was ascribed to a combination of decreased dihydrofolate reductase affinity and inefficient cell uptake. We postulated that these effects could reflect increased ionization of the α-COOH group due to the proximity of the β-COOH group; we also reasoned that if the distance between the carboxyls were *extended*, the acidity and extent of dissociation of the α-COOH could be decreased. In addition, the introduction of extra CH_2 groups was expected to enhance lipid solubility and, thus, increase the amount of drug able to penetrate cells by passive diffusion as opposed to carrier-mediated active transport.

As a first step toward chain-extended MTX analogues with greater lipid solubility, we have synthesized the α-L-aminoadipic, α-L-aminopimelic, and α-L-aminosuberic acid derivatives **2–4**. All three compounds were found to bind to bacterial (*Lactobacillus casei*) and mammalian (L1210 mouse leukemia) dihydrofolate reductase with an affinity comparable to that of MTX. Cytotoxicity to L1210 cells in culture was likewise comparable to, or slightly greater than, that of MTX, indicating that the ability of these analogues to efficiently cross the cell membrane was preserved. Lastly, in vivo antitumor assay against L1210 leukemia in mice revealed that all three compounds prolonged survival to about the same extent as MTX. However, optimal therapeutic activity required higher doses and more frequent injection. Two novel properties of compounds **2–4** are their very low substrate activities for the enzymes carboxypeptidase G_1 and folate polyglutamate synthetase. The latter characteristic[12,13] probably accounts for the higher dosage and frequency requirements of these compounds relative to MTX. Lack of polyglutamation is potentially a therapeutic asset, because in the treatment of MTX-resistant tumors with a *capacity for polyglutamation that is low in comparison with normal proliferative tissue*, dose escalation should be tolerated better with non-polyglutamatable compounds than with MTX.

Chemistry. At the time this work began, synthetic methods for the synthesis of chain-lengthened homologues of L-glutamic acid were generally unsatisfactory.[14,15] Fortunately, a superior route to L-α-aminoadipic acid recently became available through the work of Scott and Wilkinson.[16] In this approach, N^{α}-(benzyloxycarbonyl)-L-lysine[17,18] is converted to L-α-aminoadipic acid by sequential *N,N*-dichlorination (NaOCl, 5 °C), dehydrochlorination to a nitrile, and vigorous acid hydrolysis (6 N HCl, reflux) to simultaneously hydrolyze the nitrile to a carboxylic acid and deprotect the amino group. Esterification of the product gave the heretofore not characterized diethyl ester as an HCl salt. Subsequent to this work, two other satisfactory routes to L-α-aminoadipic acid from L-lysine were reported.[19,20]

Scheme I



By following the sequence in Scheme I, we solved the problem of obtaining L-α-aminopimelic acid from L-glutamic acid without having to perform homologation in two stages.[15] 2,2-Dimethyl-1,3-dioxane-4,6-dione ("Meldrum's acid") was acylated with α-benzyl *N*-[*tert*-butyloxycarbonyl)-L-glutamate in the presence of diethyl phosphorocyanidate.[21] The resulting enolic tricarbonyl compound **5** was reduced directly with sodium cyanoborohydride in glacial acetic acid[22] to form **6** (75%). In the last step, hydrolysis in refluxing 6 N HCl led to ring opening, decarboxylation, and deprotection of the amino and α-carboxy groups. A 75% yield of L-α-aminopimelic acid was obtained. Retention of stereochemical integrity was established by measurement of the optical rotation and comparison with literature data.[15,23] Prior to being coupled to 4-amino-4-deoxy-N^{10}-methylpteroic acid, the amino acid was converted to its oily di-*tert*-butyl ester by reaction with excess *tert*-butyl acetate and 70% $HClO_4$.[24] To avoid polymerization during storage, we used the amino ester as soon as it was formed. It should be noted that the novel application of Meldrum's acid described here can, in principle, find use in the synthesis of other rare long-chain amino dicarboxylic acids.

Coupling of the amino diesters **7–9** to 4-amino-4-deoxy-N^{10}-methylpteroic acid (Scheme II) was performed with the aid of diethyl phosphorocyanidate, which we recently showed to be superior to other reagents for this purpose.[3,5] In the case of esters **7** and **8**, the activation and coupling steps were performed at 0 °C, and the total reaction time was 2 h. With ester **9**, both the activation (overnight) and the coupling (4 days) were conducted at room temperature. The yields of products (**10**, 51%; **11**, 71%; **12**, 82%) indicated that a lower temperature gives better results even though a longer time is required. All three products (**10–12**) were soluble in $CHCl_3$ and could be purified readily by silica gel column chromatography. Saponification of **10** and **12** was accomplished in 75–90%

(9) Rosowsky, A.; Moran, R. G.; Forsch, R.; Colman, P.; Wick, M. *Biochem. Pharmacol.*, in press (part 18 of this series).
(10) Rosowsky, A.; Yu, C.-S. *J. Med. Chem.*, in press (part 19 of this series).
(11) Mead, J. A. R.; Greenberg, N. H.; Schrecker, A. W.; Seeger, D. R.; Tomcufcik, A. S. *Biochem. Pharmacol.* **1965**, *14*, 105.
(12) Forsch, R.; Moran, R. G.; Rosowsky, A.; Wick, M. *Proc. Am. Assoc. Cancer Res.* **1983**, *24*, 274.
(13) Moran, R. G.; Rosowsky, A.; Colman, P. D.; Forsch, R.; Chan, K., submitted for publication in *Mol. Pharmacol.*
(14) Rudinger, J.; Farkasova, H. *Collect. Czech. Chem. Commun.* **1963**, *28*, 2941.
(15) Farkasova, H.; Rudinger, J. *Collect. Czech. Chem. Commun.* **1965**, *30*, 3117.
(16) Scott, A.; Wilkinson, T. *Synth. Commun.* **1980**, *10*, 127.
(17) Scott, J.; Parker, D.; Parrish, D. *Synth. Commun.* **1981**, *11*, 303.
(18) Benoiton, L. *Biochem. Prep.* **1966**, *11*, 80.
(19) Baldwin, J. E.; Harrison, P.; Murphy, J. A. *J. Chem. Soc., Chem. Commun.* **1982**, 818.
(20) Buckley, T. F.; Rapoport, H. *J. Am. Chem. Soc.* **1982**, *104*, 4446.
(21) Shioiri, T.; Hamada, Y. *J. Org. Chem.* **1978**, *43*, 3631.
(22) Nutaitis, C.; Schultz, R. A.; Obaza, J.; Smith, F. X. *J. Org. Chem.* **1980**, *45* 4606.
(23) Wade, R.; Birnbaum, S. M.; Winitz, M.; Koegel, R. J.; Greenstein, J. P. *J. Am. Chem. Soc.* **1957**, *79*, 648.
(24) Taschner, E.; Chimiak, A.; Bator, B.; Sokolowska, T. *Justus Liebigs Ann. Chem.* **1961**, *646*, 134.

Scheme II

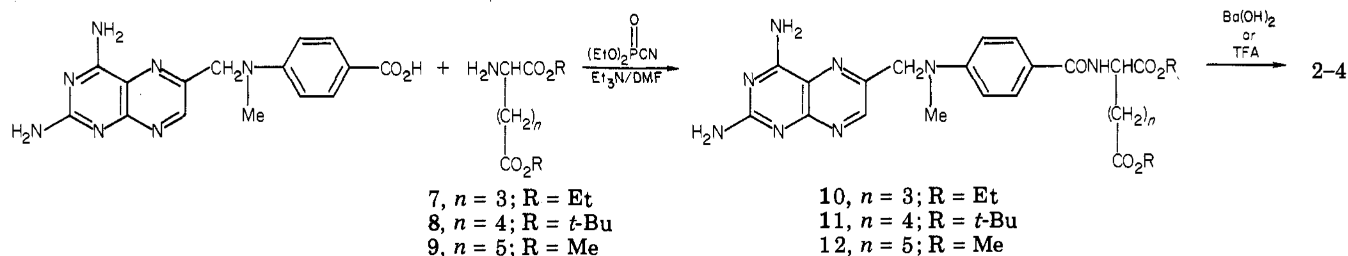


Table I. Dihydrofolate Reductase Inhibition and Cytotoxicity of Chain-Extended MTX Analogues

	DHFR: ID_{50}, nM					
	[^{3}H]MTX binding assay		spectrophotometric assay			L1210 cells:
compd	*L. casei*/MTX[a]	L1210	*L. casei*/MTX[a]	L1210	L1210/R71[b]	ID_{50},[a] nM
MTX (1)	11	2.0[a]	11	9.0	32	20
2	12	1.4	6.9	6.7	39	30
3	18	2.8	6.3	6.9	39	10
4					35	6.3

[a] Average of two separate experiments, each with a standard error of less than 10%. [b] Measurements performed at the University of Cincinnati.

yield by overnight treatment with a stoichiometric amount of $Ba(OH)_2 \cdot 8H_2O$ in 50% ethanol at room temperature.[3] With the di-*tert*-butyl ester **11**, hydrolysis was effected in 88% yield with trifluoroacetic acid (room temperature, 10 min). Final purification was by DEAE-cellulose ion-exchange chromatography, with 3% NH_4HCO_3 as the eluent. Freeze–drying of pooled eluates resulted in conversion of the ammonium salts to free acids, isolated as di- or trihydrates.[25]

Biological Activity. The ability of **2** and **3** to bind to dihydrofolate reductase was assayed with bacterial and mammalian enzyme as described.[3] In each experiment, MTX was included as a positive control to allow determination of relative binding affinity. The results are shown in Table I. Against the *Lactobacillus casei* enzyme, the ID_{50} for **2** and **3** was in the 10–20-nM range by competitive radioligand binding assay and in the 5–10-nM range by spectrophotometric assay. The dihydrofolate reductase binding affinity of **2** and **3** did not differ from that of MTX by more than a factor of two, regardless of what test system was employed. Compounds **2–4** were also tested against dihydrofolate reductase isolated from MTX-resistant L1210/R71 cells grown in culture.[26] The enzyme was purified to homogeneity by affinity column chromatography.[27] Once again the ID_{50} values for MTX and its homologues **2–4** were essentially the same (30–40 nM). An increase in distance between the side-chain COOH groups is therefore not detrimental to binding to any of the three enzymes tested.

To compare the biological activity of MTX and the chain-extended compounds in intact cells, we determined the cytotoxicity of **2–4** against L1210 cells in culture (Table I). Proliferation at 48 h was inhibited by 50% in the presence of 20 nM MTX, 30 nM **2**, 10 nM, **3**, and 6.3 nM **4**. By comparison, aminopterin had an ID_{50} of 3.9 nM. Thus, **4** appears to have a potency intermediate between MTX and aminopterin against these cells.

To determine whether extension of the side chain would affect cleavage by carboxypeptidase G_1, we tested the substrate activity of **2** and **3** in vitro using a spectrophotometric assay based on the change of UV absorbance at 320 nm when 4-amino-4-deoxy-N^{10}-methylpteroic acid is formed.[28] As shown in Table II, the K_m of MTX in this assay was 4.84 μM, whereas for **2** and **3** and K_m was 196 and 481 μM, respectively. The V_{max} for the three compounds did not vary greatly. When the assay was run under conditions of competitive kinetics, with a fixed MTX concentration and variable concentrations of **2** or **3**, K_i values of ca. 3 and 22 μM, respectively, were obtained. Thus, **2**, which differs from MTX by just one CH_2, was a 7-fold better competitive inhibitor than **3**. Carboxypeptidase G_1 evidently has more stringent structural requirements than dihydrofolate reductase in this regard. The relatively low substrate activity of **2** and **3** for carboxypeptidase G_1 is of interest in view of previous reports concerning the use of this enzyme to potentiate the antitumor activity of nonclassical antifols via depletion of circulating reduced folates.[29,30] Since MTX is rapidly cleaved by the enzyme,[31,32] its use in this context would be precluded. Analogues such as **2** or **3** would not be subject to this restriction.

Table II. Substrate Activity of Chain-Elongated MTX Analogues for Carboxypeptidase G_1

compd	K_m, μM	V_{max}, min^{-1}	K_i, μM
MTX (1)	4.84	0.134	
2	196	0.124	2.77, 3.22
3	481	0.440	22

To compare the in vivo effects of MTX and compounds **2–4**, we carried out treatment of L1210 leukemia in mice (Table III). On an intermittent dose schedule (q2d×5), **2** and **3** produced a 50–70% increase in life span (ILS) at 40 mg/kg, mice (Table III) compared with a 122% ILS for

(25) Piper, J. R.; Montgomery, J. A. *J. Org. Chem.* **1977**, *42*, 208.
(26) Jackson, R. C.; Niethammer, D.; Huennekens, F. M. *Cancer Biochem. Biophys.* **1975**, *1*, 151.
(27) Kaufman, B. T.; Kemerer, V. F. *Arch. Biochem. Biophys.* **1977**, *179*, 420.
(28) McCullough, J. L.; Chabner, B. A.; Bertino, J. R. *J. Biol. Chem.* **1971**, *246*, 7207.
(29) Chabner, B. A.; Chello, P. L.; Bertino, J. R. *Cancer Res.* **1972**, *32*, 2114.
(30) Kalghatgi, K.; Moroson, B.; Horvath, C.; Bertino, J. R. *Cancer Res.* **1979**, *39*, 3441.
(31) Chabner, B. A.; Johns, D. G.; Bertino, J. R. *Nature (London)* **1972**, *239*, 395.
(32) Abelson, H. T.; Ensminger, W. D.; Rosowsky, A.; Uren, J. *Cancer Treat. Rep.* **1978**, *62*, 1549.

Table III. Antitumor Activity of Chain-Extended MTX Analogues against L1210 Leukemia in Mice

compd	schedule	dose, mg/kg	no. of mice	7-day wt change, %	survival, days: range [a]	survival, days: median T/C	% ILS
MTX (1)	q2d×5	10	5	+11	14–18	17/9	+89
		15	5	+8	17–22	20/9	+122
	qd×9	2	5	+6	14–18	16/9	+78
		4	5	−2	12–19	17/9	+89
		6	5	−2	11–22+	21/9	+133
	b.i.d.×10 [b]	0.25	5	+1	12–15	13/8	+63
		0.5	5	+1	15–17	16/8	+100
		0.75	5	+3	15–17+	17/8	+112
2	q2d×5	20	5	+8	12–20	13/9	+44
		40	5	+7	12–20	14/9	+55
	qd×9 (expt 1)	2	5	+10	9–11	10/8.5	+18
		4	5	+7	8–17	11/8.5	+29
		8	5	+2	12–19	12/8.5	+41
	qd×9 (expt 2)	20	5	+7	12–16	14/9	+56
		40	5	+2	17–19	18/9	+100
		60	5	−6	12–22+	21/9	+133
	b.i.d.×10 [b]	9	5	−3	16–18	17/9	+89
		12	5	−3	9–24	18/9	+100
		15	5	−7	10–24	21/9	+133
3	q2d×5	20	5	+6	13–18	13/9	+44
		40	5	+7	10–22	15/9	+67
	1d×9 (expt 1)	2	5	+10	11–16	12/8.5	+41
		4	5	+4	12–20	13/8.5	+52
		8	5	+4	14–22	14/8.5	+65
	qd×9 (expt 2)	20	5	−2	10–24	18/9	+100
		40	5	−4	10–23	21/9	+133
4	qd×9	10	5	+11	12–15	13/9	+44
		20	5	+7	15–17	16/9	+78
		30	5	+9	17–18	17/9	+89
		40	5	+6	13–20+	19/9	+111

[a] A plus sign indicates one animal surviving beyond the last day shown. This mouse was included in the treated (T) group for the purpose of determining the median survival day. [b] On days 5 and 6 of the b.i.d. × 10 schedule, mice received a single double-dose injection because of weekend scheduling.

MTX at its maximally tolerated dose of 15 mg/kg. On the daily schedule (qd×9), 2 at 60 mg/kg gave a 133% ILS, 3 at 40 mg/kg also gave a 133% ILS, and 4 at 40 mg/kg gave a 111% ILS. At 60 mg/kg, 2 became toxic (data not shown). In comparison, MTX at 4 and 6 mg/kg prolonged survival by 89 and 133%, respectively, with 4 mg/kg being generally better tolerated than the higher dose. It thus appears that 2–4 are as active as MTX on the qd×9 schedule in terms of survival but are roughly one-tenth as potent on a molar basis. Because of the improved therapeutic effect of 2 and 3 on the daily schedule, MTX and 2 were also compared on a twice-daily regimen (b.i.d.×10). At 15 mg/kg, 2 gave a 133% ILS, as compared with 111% for MTX at 0.75 mg/kg. Thus, there was an identical 3.6-fold reduction in total administered dose in changing from qd×9 to b.i.d.×10 treatment, but the degree of improvement in therapeutic efficacy with more frequent dosing was not greater for 2 than for MTX.

The findings reported here are of mechanistic interest and may have clinical applications. A notable aspect of the cellular pharmacology of 2–4, recently cited in an abstract[12] and to be published in detail elsewhere,[13] is that these compounds are *poor substrates for folyl polyglutamate synthetase*. The role of polyglutamation has increasingly come to be recognized as a major determinant of the cytotoxicity and therapeutic selectivity of MTX in vivo.[33–47] If polyglutamation in marrow or other sensitive host tissues were to exceed that in a tumor, the amount of MTX needed to destroy the tumor would be likely to cause unacceptable host toxicity. In this case, an analogue of MTX whose cytotoxic action *does not involve polyglutamation* might offer a therapeutic advantage, since a tumor with low polyglutamation capacity would be no less sensitive than normal proliferative tissues. It may be noted that compounds such as 2–4 may find use in long-term, low-dose regimens, as in psoriasis treatment where progressive MTX polyglutamate accumulation in hepatocytes[48,49] may be responsible for the hepatotoxicity typically associated with this type of chronic therapy.[50]

(33) Schilsky, R. L.; Bailey, B. D.; Chabner, B. A. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 2919.
(34) Galivan, J. *Mol. Pharmacol.* **1980**, *17*, 105.
(35) Poser, R. G.; Sirotnak, F. M.; Chello, P. L. *Cancer Res.* **1981**, *41*, 4441.
(36) Fry, D. W.; Yalowich, J. C.; Goldman, I. D. *J. Biol. Chem.* **1982**, *257*, 1890.
(37) Fry, D. W.; Anderson, L. A.; Borst, M.; Goldman, I. D. *Cancer Res.* **1983**, *43*, 1087.
(38) White, J. C.; Goldman, I.D. *Mol. Pharmacol.* **1976**, *12*, 711.
(39) Jackson, R. C.; Niethammer, D.; Hart, L. I. *Arch. Biochem. Biophys.* **1977**, *182*, 646.
(40) White, J. C.; Goldman, I. D. *J. Biol. Chem.* **1981**, *256*, 5722.
(41) Balinska, M.; Galivan, J.; Coward, J. K. *Cancer Res.* **1981**, *41*, 2751.
(42) For a dissenting report on the effluxing ability of MTX polyglutamates, see: Poser, R. G.; Sirotnak, F. M.; Chello, P. L. *Biochem. Pharmacol.* **1980**, *29*, 2701.
(43) Jacobs, S.; Adamson, R. H.; Chabner, B. A.; Derr, C. I.; Johns, D. G. *Biochem. Biophys. Res. Commun.* **1975**, *63*, 692.
(44) Sirotnak, F. M.; Chello, P. L., Piper, J. R.; Montgomery, J. A. *Biochem. Pharmacol.* **1978**, *27*, 1821.
(45) Rosenblatt, D. S.; Whitehead, V. M.; Dupont, M. M.; Vuhich, M. J.; Vera, N. *Mol. Pharmacol.* **1978**, *14*, 210.
(46) Curt, G.; Jolivet, J.; Carney, D.; Bailey, B. D.; Chabner, B. A. *Proc. Am. Assoc. Cancer Res.* **1982**, *23*, 9.
(47) Jolivet, J.; Schilsky, R. L.; Bailey, B. D.; Drake, J. C.; Chabner, B. A. *J. Clin. Invest.* **1982**, *70*, 351.
(48) Gewirtz, D. A.; White, J. C.; Randolph, J. K.; Goldman, I. D. *Cancer Res.* **1979**, *39*, 2914.
(49) Balinska, M.; Nimec, Z.; Galivan, J. *Arch. Biochem. Biophys.* **1982**, *216*, 466.

Further studies on 2–4 and similar compounds are planned in our laboratory as part of a continuing program of development of side-chain-altered classical antifols.

Experimental Section

Infrared spectra were obtained on a Perkin-Elmer Model 137B double-beam recording spectrophotometer, optical rotations were determined on a Perkin-Elmer Model 241MC polarimeter, and NMR spectra were recorded on a Varian T60A instrument with Me_4Si as the internal reference. TLC was carried out on Eastman 13181 silica gel or Eastman 13254 cellulose sheets (fluorescent indicator), and spots visualized in a viewing chamber under 254-nm illumination or with the aid of ninhydrin spray as appropriate. Column chromatography was performed on Baker 3405 silica gel (60–200 mesh) or Whatman DE-52 preswollen diethylaminoethylcellulose (DEAE-cellulose). Melting points were measured in Pyrex capillary tubes in a Mel-Temp apparatus (Laboratory Devices, Inc., Cambridge, MA) and are not corrected. Microanalyses were performed by Galbraith Laboratories, Knoxville, TN, and were within ±0.4% of theoretical values unless otherwise noted.

L-α-Aminoadipic acid was synthesized from N^{α}-(benzyloxycarbonyl)-L-lysine[17] according to the method of Scott and Wilkinson.[16] α-Benzyl *N*-(*tert*-butyloxycarbonyl)-L-glutamate and *N*-(benzyloxycarbonyl)-L-α-aminosuberic acid were purchased from Chemical Dynamics Corp., South Plainfield, NJ. Cesium carbonate was obtained from Alfa Products, Danvers, MA. 4-Amino-4-deoxy-N^{10}-methylpteroic acid and diethyl phosphorocyanidate were prepared as previously described.[3] DMF used in the coupling reaction was dried over Linde 4A molecular sieves. Unless otherwise specified, solutions in extraction solvents were dried over Na_2SO_4 before being evaporated.

Diethyl L-α-Aminoadipate (7). Anhydrous HCl gas was bubbled through a suspension of L-α-aminoadipic acid (4.0 g, 0.025 mol) in absolute EtOH (25 mL). Another 50 mL of EtOH was added, the mixture was stirred under reflux overnight, and the solvent was evaporated. The residue was partitioned between Et_2O and aqueous K_2CO_3, and the organic layer was washed with brine, dried, and evaporated to a small volume. Addition of ethereal HCl gave the hydrochloride salt (2.7 g, 42%) as a waxy solid. The analytical sample was recrystallized from EtOAc and dried in vacuo at 60 °C over P_2O_5 overnight: mp 107–109 °C; IR (KBr) ν 3410, 2930, 1735 (ester C=O) cm^{-1}. Anal. ($C_{10}H_{19}NO_4$·HCl) C, H, N, Cl.

Diethyl *N*-(4-Amino-4-deoxy-N^{10}-methylpteroyl)-L-α-aminoadipate (10). 4-Amino-4-deoxy-N^{10}-methylpteroic acid (1.6 g, 0.005 mol) was added in small portions to a stirred solution of diethyl phosphorocyanidate (2.5 g, 0.015 mol) and Et_3N (1.5 g, 0.015 mol) in dry DMF (150 mL). The solution was heated in an oil bath at 80 °C for a few minutes, then cooled, and treated with another portion of Et_3N (1.0 g, 0.010 mol), followed by diethyl L-α-aminoadipate hydrochloride (1.3 g, 0.005 mol). The reaction mixture was then heated again at 80 °C for 2 h, cooled, and evaporated to dryness under reduced pressure. The residue was taken up in $CHCl_3$, and the solution was washed with H_2O and evaporated. Column chromatography on silica gel (95:5 $CHCl_3$–MeOH), pooling of appropriate column eluates, and trituration of the evaporated fractions with Et_2O afforded **10** as a bright-yellow powder (1.2 g, 51%): mp 113–123 °C; IR (KBr) ν 3390, 2920, 1720 (ester C=O), 1615-1640 (amide C=O) cm^{-1}. Anal. ($C_{25}H_{32}N_8O_5$·0.5H_2O) C, H, N.

***N*-(4-Amino-4-deoxy-N^{10}-methylpteroyl)-L-α-aminoadipic Acid (2).** A solution the diester **10** (1 g, 0.002 mol) in a mixture of 95% EtOH (30 mL) and H_2O (25 mL) was stirred at room temperature overnight with $Ba(OH)_2$·8H_2O (1.26 g, 0.004 mol). To the suspension was then added a solution of Na_2SO_4 (0.57 g, 0.004 mol) in a minimum of H_2O. After the solution was stirred for a few minutes, the $BaSO_4$ was filtered off, and the aqueous layer was extracted with $CHCl_3$ and acidified with 10% AcOH. The precipitate was filtered and dried under high vacuum in a lyophilization apparatus to obtain **2** as a yellow powder (0.8 g, 85%). This material was pooled with the product (1.2 g, 78%) from another larger run and purified by ion-exchange chromatography on DEAE-cellulose, using 3% ammonium bicarbonate as the eluent. Appropriate TLC-pure fractions were combined and reduced to a small volume by lyophilization, and the product was precipitated by addition of 10% AcOH: R_f 0.8 (cellulose, 0.1 M phosphate buffer, pH 7.4); IR (KBr) ν 3360, 1610–1460 cm^{-1}. Anal. ($C_{21}H_{24}N_8O_5$·2H_2O) C, H, N.

Benzyl 2-[(*tert*-Butyloxycarbonyl)amino]-5-[5-(2,2-dimethyl-4,6-dioxo-1,3-dioxanyl)]pentanoate (6). To a mixture of α-benzyl *N*-(*tert*-butyloxycarbonyl)-L-glutamate (10.1 g, 0.03 mol) and 2,2-dimethyl-1,3-dioxane-4,6-dione (3.60 g, 0.025 mol) in dry DMF (125 mL) in an ice bath were added successively diethyl phosphorocyanidate (4.90 g, 0.03 mol) and Et_3N (8.05 g, 0.08 mol). The mixture was stirred in the ice bath for 2 h and then left at room temperature overnight. Rotary evaporation left an oil, which was taken up in 1:1 benzene–EtOAc. After the solution was washed with H_2O, 5% $NaHCO_3$, and brine, the solvents were evaporated under reduced pressure to obtain **5** as a gum. A solution of this material in glacial AcOH (35 mL) was treated with $NaBH_3CN$ (3.14 g, 0.05 mol) over 1–2 min, and after being stirred for 1 h, the mixture was diluted with ice-cold 0.5 N HCl (125 mL) and extracted with benzene. The organic layer was washed successively with H_2O, 5% $NaHCO_3$, and brine and then dried and evaporated to obtain **6** as a white solid (8.46 g, 75%). The analytical sample was prepared by column chromatography on silica gel (95:5 $CHCl_3$–MeOH): mp 110–112 °C (slow gas evolution at 120 °C); IR (KBr) ν 3350, 2940, 1775 (sh), 1745, 1680, 1510 cm^{-1}; NMR ($CDCl_3$) δ 1.4–2.2 [m, 21 H, *t*-C_4H_9, $(CH_2)_3$, and $C(CH_3)_2$], 3.51 (m, 1 H, malonyl CH), 4.0–4.5 (m, 2 H, α-CH and NH), 5.19 (s, 2 H, benzylic CH_2), 7.35 (s, 5 H, aromatic protons). Anal. ($C_{23}H_{31}N_8O_5$·0.75H_2O) C, H; N: calcd, 3.03; found, 3.53.

L-α-Aminopimelic Acid. Compound **6** (2.3 g, 0.005 mol) was heated overnight in refluxing 6 N HCl (15 mL), and the cooled solution was extracted with Et_2O. The aqueous layer was evaporated under reduced pressure, and the crude HCl salt was redissolved in 0.1 N HCl (5 mL), which was then adjusted to pH 5 with pyridine. Absolute EtOH (15 mL) was added, and the mixture was left in the freezer overnight. The crystals were filtered, washed with 95% EtOH, and dried in vacuo over P_2O_5 at 95 °C to obtain analytically pure product (0.66 g, 75%): α_D^{25} +21.6° (*c* 1, 5 M HCl) [lit.[15] +21.6°; lit.[23] +21.5°]. Anal. ($C_7H_{13}NO_4$·0.3H_2O) C, H, N.

Di-*tert*-Butyl *N*-(4-Amino-4-deoxy-N^{10}-methylpteroyl)-L-α-aminopimelate (11). A mixture of L-α-aminopimelic acid (0.35 g, 0.002 mol) and 70% $HClO_4$ (0.32 g, 0.0022 mol) in *tert*-butyl acetate (30 mL) was stirred at room temperature overnight until it became homogeneous. After an additional day, the solution was extracted with cold 0.5 N HCl (2 × 10 mL), the aqueous layer was carefully neutralized with small portions of powdered $NaHCO_3$, and the product was extracted into Et_2O. The Et_2O layer was washed with brine, dried, and evaporated to obtain the diester **8** as an oil (0.31 g, 54%): IR (neat) ν 3400 (sh) 2980, 1730 (C=O) cm^{-1}. 4-Amino-4-deoxy-N^{10}-methylpteroic acid (0.37 g, 0.001 mol) was added in small portions to a solution of diethyl phosphorocyanidate (0.51 g, 0.0031 mol) and Et_3N (0.3 g, 0.003 mol) in dry DMF (35 mL). After 2 min in an oil bath at 80 °C, the solution was cooled, and to it was added another portion of Et_3N (0.1 g, 0.001 mol), followed by a solution of di-*tert*-butyl L-α-aminopimelate (0.31 g, 0.001 mol) in a small volume of DMF. The reaction mixture was heated at 80 °C for 2 h and then left overnight at room temperature. Rotary evaporation gave a semisolid that dissolved in $CHCl_3$. The $CHCl_3$ solution was washed with dilute ammonia and evaporated, and the residue was chromatographed on silica gel (95:5 $CHCl_3$–MeOH). Appropriate TLC-homogeneous fractions were pooled and evaporated, and the residue was triturated with Et_2O to obtain the product as a bright-yellow powder (0.44 g, 71%): mp 148–150 °C; IR (KBr) 3390, 2980, 1725 (ester C=O), 1610–1630 cm^{-1}. Anal. ($C_{30}H_{42}N_8O_5$·0.25H_2O) C, H, N.

***N*-(4-Amino-4-deoxy-N^{10}-methylpteroyl)-L-α-aminopimelic Acid (3).** The diester **11** (1.8 g, 0.003 mol) was dissolved in trifluoroacetic acid (10 mL), and the solution was left to stand at room temperature for 10 min, before being added dropwise to a stirred solution of K_2CO_3 (10 g) in H_2O (40 mL). Additional K_2CO_3 was added in small portions until a homogeneous solution was obtained, and the pH was adjusted to 5 with 10% AcOH.

(50) Tobias, H.; Auerbach, R. *Arch. Intern. Med.* **1973**, *132*, 391.

After overnight refrigeration, the precipitate was filtered and dried in a high-vacuum lyophilizer to obtain a yellow powder (1.4 g, 88% assuming $2.25H_2O$): IR (KBr) ν 3330, 2980 (sh), 1710 (sh), 1640, 1600 cm^{-1}. Anal. ($C_{22}H_{26}N_8O_5 \cdot 2.25H_2O$) C, H, N.

Dimethyl L-α-Aminosuberate (9). A solution of *N*-(benzyloxycarbonyl)-L-α-aminosuberic acid (0.32 g, 0.001 mol) in dry DMF (5 mL) was treated with cesium carbonate (0.65 g, 0.002 mol) and methyl iodide (0.57 g, 0.004 mol), and the mixture was stirred at room temperature overnight. Rotary evaporation under reduced pressure left an oil, which was partitioned between benzene and H_2O. After being washed thoroughly with H_2O, the benzene layer was evaporated to give the dimethyl ester as an oil (0.36 g, 100%): IR (neat) ν 3340 (NH), 2940, 1725 (broad, C=O) cm^{-1}; NMR ($CDCl_3$) δ 1.2–1.9 [m, 8 H, $(CH_2)_4$], 2.28 (m, 2 H, CH_2CO_2), 3.65 (s, 3 H, OCH_3), 3.72 (s, 3 H, OCH_3), 4.05 (1 H, α-CH), 5.13 (s, br m, 3 H, NH and benzylic CH_2), 7.37 (s, 5 H, aromatic protons). The crude N-protected diester (0.35 g, 0.001 mol) in MeOH (20 mL) containing glacial AcOH (0.5 mL) and 5% Pd/C (50 mg) was shaken under H_2 (3 atm) for 3 h. The catalyst was filtered off, the solvent was evaporated, the residue was partitioned between CH_2Cl_2 and 5% aqueous $NaHCO_3$, and the organic layer was dried and evaporated to obtain the deblocked diester as an oil: 0.20 g (93%); IR (neat) ν 3280 (NH), 2920, 1725 (ester C=O) cm^{-1}; NMR ($CDCl_3$) δ 1.3–1.9 [m, 10 H, $(CH_2)_4$ and NH_2], 2.29 (m, 2 H, CH_2CO_2), 3.50 (m, 1 H, α-CH), 3.64 (s, 3 H, OCH_3), 3.69 (s, 3 H, OCH_3).

The **hydrochloride salt** was prepared in 79% yield by treating the base with anhydrous HCl in Et_2O. The product crystallized from EtOAc–hexane in the form of colorless fibers: mp 103–104 °C. Anal. ($C_{10}H_{20}ClNO_4$) C, H, N, Cl.

Dimethyl *N*-(4-Amino-4-deoxy-N^{10}-methylpteroyl)-L-α-aminosuberate (12). 4-Amino-4-deoxy-N^{10}-methylpteroic acid (0.36 g, 0.001 mol) was added to a solution of diethyl phosphorocyanidate (0.41 g, 0.0025 mol) and Et_3N (0.25 g, 0.0025 mol) in dry DMF (40 mL). The mixture was stirred at room temperature overnight, and additional portions of diethyl phosphorocyanidate (0.21 g, 0.0013 mol) and Et_3N (0.13 g, 0.0013 mol) were added to complete the activation of the COOH group. After 2 h, a solution of dimethyl L-α-aminosuberate (0.20 g, 0.0009 mol) in a small volume of DMF was added, and the reaction mixture was left to stir at room temperature for 4 days. Rotary evaporation left a semisolid that dissolved readily in $CHCl_3$. The $CHCl_3$ solution was washed with dilute ammonia and evaporated, and the residue was chromatographed on silica gel with 95:5 $CHCl_3$–MeOH as the eluent. Appropriate TLC-homogeneous fractions were pooled and evaporated, and the residue was triturated with Et_2O to obtain the product as a yellow powder (0.46 g, 85%): mp 112–118 °C; IR (KBr) ν 3330, 1730 (ester C=O), 1615–1635 (amide C=O) cm^{-1}. Anal. ($C_{25}H_{32}N_8O_5 \cdot 0.75H_2O$) C, H, N.

***N*-(4-Amino-4-deoxy-N^{10}-methylpteroyl)-L-α-aminosuberic Acid (4).** A solution of the diester 12 (0.27 g, 0.0005 mol) in 1:1 EtOH–H_2O (20 mL) was treated with $Ba(OH)_2 \cdot 8H_2O$ (0.32 g, 0.001 mol) and left to stir at room temperature overnight. To the reaction mixture was then added a solution of Na_2SO_4 (0.14 g, 0.001 mol) in a small volume of H_2O. After 5 min of vigorous stirring, the $BaSO_4$ was filtered off, and the filtrate was acidified with 10% AcOH and placed in the refrigerator until precipitation was complete. The solid was collected, washed with H_2O, and dried under high vacuum in a lyophilization apparatus to obtain a yellow powder (0.24 g, 87%): R_f 0.8 (cellulose, 0.1 M phosphate buffer, pH 7.4); IR (KBr) ν 3330, 2890, 1705, 1590–1625 cm^{-1}. Anal. ($C_{23}H_{28}N_8O_5 \cdot H_2O$) C, H, N.

Enzyme Assays. Dihydrofolate reductase affinity measurements by competitive [^{3}H]MTX binding assay were carried out at 37 °C in 0.1 M potassium phosphate buffer, pH 6.8, as reported earlier.[51] *Lactobacillus casei*/MTX dihydrofolate reductase was kindly provided by Dr. Bing Wong, New England Enzyme Center, Boston, MA. For the assay using enzyme from L1210 leukemia, the ascites cells from tumor-bearing mice were washed and lysed according to Arons et al.[52] In the determination of dihydrofolate reductase inhibition by spectrophotometric assay (ΔA_{340}),[53] the reaction was carried out at 22 °C in 0.1 M Tris buffer, pH 7.0. The enzyme was isolated from L1210/R71 cells (MTX resistant)[26] and purified by affinity chromatography on MTX–agrose.[27] The concentration of enzyme in the assay mixture was 62 nM, and the enzyme was incubated with inhibitor and NADPH for 2 min prior to the addition of dihydrofolate to initiate the reaction.

Substrate activity for carboxypeptidase G_1 was measured according to the method of McCullough et al.,[28] using a sterile enzyme preparation obtained from Dr. Herbert Abelson.[32] The assay takes advantage of the change in absorbance at 320 nm that occurs when 4-amino-4-deoxy-N^{10}-pteroic acid is formed from MTX or its analogues. The enzyme solution had an activity of ca. 1100 units/mL. The assay was performed at 37 °C in 0.05 M potassium phosphate buffer, pH 6.7. A typical assay mixture contained 100 μM $ZnSO_4$, 60 μM sustrate (e.g., MTX), and 10 μL of enzyme in a 1-mL volume. The enzyme solution was added at various dilutions to give a ΔA_{320} of 0.02–0.2 absorbance unit/min. Dilutions of the stock solution of carboxypeptidase G_1 were made in 1% bovine serum albumin. Kinetic constants (K_m and V_{max}) were obtained by standard Lineweaver–Burke plots, with a linear correlation coefficient (r^2) of 0.94–0.99. Competitive inhibition of MTX cleavage by compounds 2 and 3 was assayed both by keeping the substrate concentration constant and varying the inhibitor concentration, or vice versa. Analysis of the data by standard methods[54] showed inhibition to be competitive.

Cytotoxicity and in Vivo Antitumor Assays. Growth inhibition of L1210 cells in culture was measured after 48 h of continuous exposure as described earlier.[55]

In vivo antitumor activity was determined against L1210 ascitic leukemia in mice according to a standard NCI protocol.[56] Male $B6D2F_1J$ mice weighing 20–25 g were randomized into groups after being inoculated intraperitoneally with 10^5 L1210 cells on day 0. Drugs were dissolved in water at pH 7.5–8.0 and were given daily for 9 days (qd×9) by intraperitoneal injection starting on day 1. Animals were weighed after 7 days, and the weight change was recorded. The increase in life span (%ILS) was calculated from the formula, % ILS = $[(T/C) - 1]100$, where T and C are the median survival in days of treated and control mice, respectively. ILS values of +25% or higher are considered significant in this assay.

Acknowledgment. The enzyme inhibition assays performed at the University of Cincinnati were supported by NCI Grant CA 11666 (to J.H.F.). The other work described in this paper was supported in part by NCI Grants CA 25394 and CA30897 (to A.R) and by Program Project Grant CA 19589 and Core Grant CA 06516. The technical assistance of Carol Ginty (enzyme assays), gerda Swedowsky (cytotoxicity assays), and Polly Currier (antitumor assays) is gratefully acknowledged.

Registry No. 2, 87206-08-4; 3, 87206-09-5; 4, 87174-83-2; 5, 87174-84-3; 6, 87174-85-4; 7·HCl, 87174-86-5; 8, 87174-87-6; 9, 87206-10-8; 10, 87206-11-9; 11, 87174-88-7; 12, 87174-89-8; L-α-aminoadipic acid, 1118-90-7; 4-amino-4-deoxy-N^{10}-methylpteroic acid, 19741-14-1; α-benzyl *N*-(*tert*-butyloxycarbonyl)-L-glutamate, 30924-93-7; 2,2-dimethyl-1,3-dioxane-4,6-dione, 2033-24-1; L-α-aminopimelic acid, 26630-55-7; *tert*-butyl acetate, 540-88-5; *N*-(benzyloxycarbonyl)-L-α-aminosuberic acid, 66713-87-9; dimethyl *N*-(benzyloxycarbonyl)-L-α-aminosuberate, 87174-90-1; dimethyl L-α-aminosuberate hydrochloride, 87206-12-0.

(51) Rosowsky, A.; Yu, C.-S.; Uren, J.; Lazarus, H.; Wick, M. *J. Med. Chem.* 1981, *24*, 559 (part 13 of this series).
(52) Arons, E.; Rothenberg, S.; da Costa, M.; Fischer, C.; Iqbal, M. *Cancer Res.* **1975**, *35*, 2033.
(53) Gunderson, L.; Dunlap, R. B.; Harding, N. G. L.; Freisheim, J. H.; Otting, F.; Huennekens, F. M. *Biochemistry* **1972**, *11*, 1018.
(54) Dixon, M.; Webb, E. C. "Enzymes"; Academic Press: New York, 1964, pp 318–331.
(55) Rosowsky, A.; Kim, S.-H.; Ross, J.; Wick, M. M. *J. Med. Chem.* **1982**, *25*, 171.
(56) Geran, R. I.; Greenberg, N. H.; Macdonald, M. M.; Schumacher, A. M.; Abbott, B. J. *Cancer Chemother. Rep., Part 3* **1972**, *3*(3), 1.