of the heamtocrit at a given compound concentration. The intracellular Hb concentration was determined by a Coulter hemoglobinometer and Diluter 2. Red cell lysis and cyanomet-Hb conversion was done with Zapolgobin (Coulter). The average MCHC values (nine measurements at a given compound concentration) are given in Table III.

Filterability Assay. The apparatus was previously described,[16] except an effective membrane diameter of 2.3 cm was used with a 5-μm pore size polycarbonate membrane (Bio-Rad). A sickle cell suspension (Hct = 1%) was deoxygenated at 37 °C with the same gas mixture used in the morphological assay above. The equilibrium P_{O_2} of each sample suspension was checked just prior to filtration by an anaerobic withdrawal and placement of a 100-μL portion into an O_2 analyzer (Radiometer BMS MK2). This same gas was used to push 2.5 mL of treated or untreated cell suspension through the 5-μm pore size membrane at a pressure of 3.1 cmHg as measured on Croft pressure gauge. The cells were always exposed to this gaseous environment during filtration. After 5–6 min, SS cells that had passed through the membrane into the lower chamber of the filter assembly were harvested and weighed, and a correction for their weight due to intersitial solvent was made with ^{3}H-labeled methoxyinulin (NEN).

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Registry No. 1, 13734-34-4; 1 (anhydride), 33294-54-1; 2, 88932-67-6; 3, 88932-68-7; 3 (free base), 88932-73-4; 4, 88932-69-8; 5, 88932-70-1; 5 (free base), 88932-75-6; 6, 88932-71-2; 7, 88932-72-3; 7 (free base), 88932-74-5; 2-aminopyridine, 504-29-0; 3-aminopyridine, 462-08-8; 4-aminopyridine, 504-24-5.

Methotrexate Analogues. 19. Replacement of the Glutamate Side Chain in Classical Antifolates by L-Homocysteic Acid and L-Cysteic Acid: Effect on Enzyme Inhibition and Antitumor Activity

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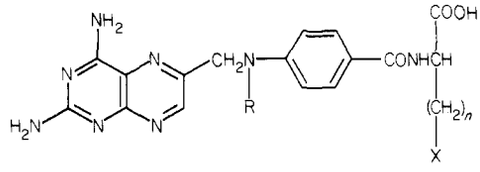
Methotrexate (MTX) and aminopterin (AMT) analogues containing L-homocysteic acid or L-cysteic acid in place of L-glutamic acid were synthesized and tested as inhibitors of dihydrofolate reductase from L1210 cells and folyl polyglutamate synthetase from mouse liver. The ID_{50} against dihydrofolate reductase was comparable for the MTX and AMT analogues (0.04–0.07 μM), whereas the ID_{50} against folyl polyglutamate synthetase was 3- to 4-fold lower for the AMT analogues (40–60 μM) than for the MTX analogues (100–200 μM). Thus, N^{10}-substitution has a greater effect on binding to folyl polyglutamate synthetase than dihydrofolate reductase. The cytotoxicity of these compounds was assayed in vitro against L1210 cells, and the AMT analogues again proved more potent (ID_{50} = 0.03–0.05 μM) than the MTX analogues (ID_{50} = 0.1–0.4 μM). A similarly increased potency was observed for the AMT analogues against L1210 leukemia in vivo. Though differential cell uptake cannot be ruled out as the basis of increased potency, it is possible that part of the activity of the AMT analogues involves interference with the intracellular polyglutamation of reduced folate cofactors, i.e., that they are "self-potentiating antifolates". Of the four compounds reported, the most active was *N*-(4-amino-4-deoxypteroyl)-L-homocysteic acid, which produced a 138% increase in life span (ILS) in L1210 leukemic mice when given on a modified bid × 10 schedule at a dose of 2 mg/kg. A comparable ILS was obtained with AMT itself at 0.24 mg/kg. Thus, replacement of γ-CO_2H by γ-SO_3H in the side chain does not decrease therapeutic effect. However, a higher dose is required, presumably to offset pharmacological differences reflecting the inability of the sulfonate group to be polyglutamated.

As part of a larger program on side-chain modified antifolates related to methotrexate (MTX) and aminopterin (AMT),[1-4] we were interested in preparing and evaluating the biological activity of compounds in which the γ-carboxyl of the glutamate moiety is replaced by another acidic group. Replacement of CO_2H by SO_3H seemed attractive because of the ready availability of L-homocysteic acid. Our rationale for this structural modification was that it would yield analogues that are incapable of forming polyglutamate conjugates inside the cell, while retaining a good affinity for dihydrofolate reductase. Polyglutamation of MTX and other classical antifolates has become widely recognized as an important determinant of toxicity and selectivity.[5-9] In this context we postulated that non-



	R	n	X
MTX	Me	2	COOH
AMT	H	2	COOH
1	Me	2	SO_2OH
2	Me	1	SO_2OH
3	H	2	SO_2OH
4	H	1	SO_2OH

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[⊥] Dana-Farber Cancer Institute and the Department of Dermatology Harvard Medical School.

(1) Rosowsky, A.; Yu, C.-S. *J. Med. Chem.* **1983**, *26*, 1448 (paper 18 in this series).
(2) Rosowsky, A.; Forsch, R.; Uren, J.; Wick, M.; Kumar, A. A.; Freisheim, J. H. *J. Med. Chem.* **1983**, *26*, 1719 (paper 20 in this series).
(3) Rosowsky, A.; Moran, R. G.; Forsch, R.; Colman, R.; Wick, M. *Biochem. Pharmacol.*, in press (paper 17 in this series).
(4) Rosowsky, A.; Forsch, R. *J. Med. Chem.* **1982**, *25*, 1454 (paper 16 in this series).
(5) Poser, R. G.; Sirotnak, F. M.; Chello, P. L. *Cancer Res.* **1981**, *41*, 1757.

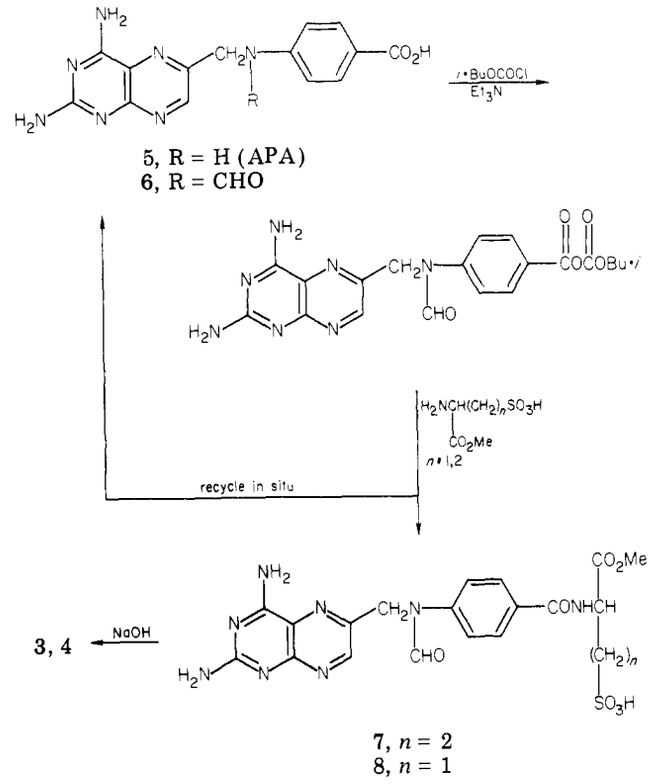
0022-2623/84/1827-0600$01.50/0

polyglutamated MTX and AMT analogues might be therapeutically advantageous in a setting where polyglutamation is more destructive to normal host proliferative tissues than to tumor, as was likely in a recently described patient with small-cell lung carcinoma who relapsed on MTX treatment and whose tumor formed abnormally low amounts of polyglutamates even though MTX active transport appeared to be intact and dihydrofolate reductase content was normal.[10] In this paper, we report the synthesis of the L-homocysteic acid analogues **1** and **3** of MTX and AMT, respectively. In addition, we report the preparation of the corresponding L-cysteic acid analogues **2** and **4**. A preliminary account of work on the DL form of **1** appears separately.[3]

Chemistry. 4-Amino-4-deoxy-N^{10}-methylpteroic acid (mAPA) was condensed by the phosphorocyanidate method[11,12] with the methyl ester of either L-homocysteic acid or L-cysteic acid. The amino esters were prepared, as HCl salts, by treatment of the acids with $SOCl_2$ and MeOH. The yields of crude methyl ester HCl salts were almost quantitative. The salts were deliquescent solids that gave the expected spectral properties and were therefore used without further purification. The crude product of each coupling reaction was purified by DEAE-cellulose column chromatography, with 3% NH_4HCO_3 as the eluent. Freeze–drying of TLC-homogeneous fractions afforded the MTX analogues **1** and **2** in 77 and 79% yield, respectively. Elemental analyses were consistent with hydrated ammonium salts, and it was assumed that the sulfonic acid group is the one to which ammonia remains bound.

For the preparation of the AMT analogues **3** and **4**, a formyl group was selected for N^{10} protection.[13] Reaction of 4-amino-4-deoxypteroic acid (APA, **5**) with Ac_2O and 98% HCOOH yielded the N^{10}-formyl derivative **6** in 82% yield (Scheme I). This heretofore unknown compound could be distinguished readily from **5** on the basis of its higher R_f value on cellulose TLC and by the fact that the TLC spot was blue-fluorescent whereas that of **5** was absorbent under 254-nm illumination. In addition, the UV spectrum in 0.1 N HCl showed a bathochromic shift in the low-wavelength maximum from 243 to 247 nm, and there was disappearance of the strong peak at 297 nm. Attempted coupling of **6** with L-homocysteic acid or the methyl ester of L-cysteic acid via the phosphorocyanidate method did not proceed well, in contrast to numerous reactions in the N^{10}-methyl series that have been carried out successfully in this laboratory. We ascribed this to a deactivating effect by the N^{10}-formyl on the ability of the *p*-carboxyl to form a mixed carboxylic–phosphoric anhydride, the putative intermediate in the phosphorocyanidate coupling reaction. Condensation was achieved satisfactorily, on the other hand, via a modified mixed carboxylic–carbonic anhydride technique that we believe may be of general use in situations involving deactivated carboxylic acids. The N^{10}-formyl derivative **6** was first activated with just over 1 equiv of *i*-BuOCOCl and 3 equiv of Et_3N. When the amino ester HCl salt was added and TLC was performed, it was observed that, in addition to the expected coupling products **7** or **8**, some unchanged **6** was regenerated. This indicated probable reaction of the amino esters at the wrong carbonyl group of the mixed anhydride, and it appeared that the extent of this unfavorable reaction was ca. 50%. Accordingly, 0.5 equiv of *i*-BuOCOCl and 1.5 equiv of Et_3N were added directly to reactivate the starting compound in situ, and another portion of the amino ester HCl salt was added. The process was carried out with three cycles of activation and coupling in the case of compound **3** and twice with compound **4**. Increasing production of the N^{10}-formylated adducts **7** and **8** at each cycle was monitored by TLC, which showed the increasing presence of a blue-fluorescent spot at an R_f higher than that of **6**, in parallel with the eventual disappearance of almost all the fluorescence corresponding to the starting material. The coupling products **7** and **8** were not isolated but instead were treated directly with NaOH at room temperature[14] until TLC showed complete deprotection as evidenced by the conversion of the blue-fluorescent spot to a slower-moving UV-absorbing spot. Since the N^{10}-formyl group is cleaved rapidly under saponification conditions, separate steps for ester and amide cleavage were avoided. The yields of the AMT analogues **3** and **4** obtained in this manner were 72 and 83%, respectively, after purification by DEAE-cellulose column chromatography. As with the MTX analogues, compounds **3** and **4** gave elemental analyses consistent with hydrated monoammonium salts. Quantitative UV absorption spectra were likewise in accord with the formulation of the products as N^{10}-unsubstituted compounds.

Scheme I



(6) Jolivet, J.; Schilsky, R. L.; Bailey, B. D.; Drake, J. C.; Chabner, B. A. *J. Clin. Invest.* **1982**, *70*, 351.
(7) Rosenblatt, D. S.; Whitehead, V. M.; Vera Matiaszuk, N.; Potter, A.; Vuchich, M.-J.; Beaulieu, D. *Mol. Pharmacol.* **1982**, *21*, 718.
(8) Galivan, J.; Nemec, Z. *Cancer Res.* **1983**, *43*, 551.
(9) Fry, D. W.; Anderson, L. A.; Borst, M.; Goldman, I. D. *Cancer Res.* **1983**, *43*, 1087.
(10) Curt, G.; Carney, D.; Jolivet, J.; Bailey, B. D.; Schepartz, S.; Chabner, B. A. *Proc. Am. Assoc. Cancer Res.* **1983**, *24*, 283.
(11) Rosowsky, A. Forsch, R.; Uren, J.; Wick, J. *J. Med. Chem.* **1981**, *24*, 1450.
(12) Rosowsky, A.; Wright, J. E.; Ginty, C.; Uren, J. *J. Med. Chem.* **1982**, *25*, 960.
(13) Erickson, J.; Mathews, C. *Biochem. Biophys. Res. Commun.* **1971**, *43*, 1164.
(14) Erickson, J.; Mathews, C. *J. Biol. Chem.* **1972**, *14*, 105.

Table I. Inhibition of L1210 Murine Leukemia Dihydrofolate Reductase by L-Homocysteic Acid and L-Cysteic Acid Analogues of Methotrexate and Aminopterin

compd	ID_{50},[a] μM
1 (mAPA-L-HCysA)	0.041
2 (mAPA-L-CysA)	0.045
3 (APA-L-HCysA)	0.063
4 (APA-L-CysA)	0.065
methotrexate (mAPA-L-Glu, MTX)	0.050
aminopterin (APA-L-Glu, AMT)	0.040

[a] Micromolar concentrations to achieve 50% inhibition of enzyme activity by spectrophotometric assay at 340 nm.[19] Purified dihydrofolate reductase was used. The assay was in 0.05 M potassium phosphate buffer, pH 7.0, at 22 °C. The enzyme (0.08 μM) was incubated with the inhibitor and NADPH (100 μM) for 2 min prior to the addition of dihydrofolate (80 μM) to initiate the reaction.

Table II. Cytotoxicity of L-Homocysteic Acid and L-Cysteic Acid Analogues of Methotrexate and Aminopterin against L1210 Murine Leukemia Cells in Culture

compd	ID_{50},[a] μM
1 (mAPA-L-HcysA)	0.18
2 (mAPA-L-CysA)	0.34
3 (APA-L-HCysA)	0.031
4 (APA-L-CysA)	0.044
methotrexate (mAPA-L-Glu, MTX)	0.012
aminopterin (APA-L-Glu, AMT)	0.0031

[a] Micromolar concentration to inhibit cell proliferation by 50% relative to untreated controls after 48 h of continuous drug exposure.[20]

Biological Activity. Compounds 1–4 were tested as inhibitors of purified dihydrofolate reductase from L1210 murine leukemia, and their activity was compared to that of MTX and AMT. As may be seen from Table I, the ID_{50} values for all four compounds were within 50% of those of their respective parent molecules. Interestingly, the MTX analogues 1 and 2 were slightly more potent than MTX, whereas with the AMT analogues 3 and 4 activity was somewhat lower than that of AMT. It thus appears that the effect of modifying the side chain is not the same in the MTX and AMT series. Inhibition by the L-homocysteic acid analogues 1 and 3 was linear up to a concentration of 0.012 μM, whereas the L-cysteic acid analogues 2 and 4 exhibited curvilinear inhibition (data not shown). This difference suggests that the rate of dissociation of the enzyme–drug complex may be faster for the shorter-chain compounds.

The cytotoxicity of 1–4 against L1210 leukemia cells in culture was likewise assayed (Table II). The AMT analogues 3 and 4 were more potent than the MTX analogues 1 and 2, and the extent of the difference was roughly the same as that between the parent drugs, i.e., 10- to 20-fold. For each pair of analogues, the L-homocysteic acid derivative was somewhat more potent than the L-cysteic acid derivative. There was greater variation in potency among the four compounds than would be predicted on the basis of the enzyme inhibition data alone, suggesting that they may not enter cells with the same ease. The most potent member of the series was compound 3, which had an ID_{50} of 0.031 μM.

Because of our recent observation that the DL form of 1 inhibits partly purified mouse liver folyl polyglutamate synthetase in vitro, we tested compounds 1–4 in this system. As shown in Table III, the K_i for the MTX analogues 1 and 2 was in the 100–200 μM range, whereas the K_i for the AMT analogues 3 and 4 was in the 40–60 μM range. Thus, in contrast to its small effects on dihydrofolate reductase inhibition, N^{10}-substitution caused a roughly 3- to 4-fold decrease in binding to folyl polyglutamate synthetase. On the other hand, in both the MTX and AMT series, a decrease in chain length by one CH_2 group appears to have minimal effect. A similar small difference in dihydrofolate reductase inhibition has been observed between MTX and its L-aspartate analogue.[15] However, it should be noted that we have recently observed that the deletion of a single CH_2 group from the MTX side chain markedly impedes polyglutamation.[16] These findings thus illustrate the important concept that optimal substrate and inhibitor activity toward a given enzyme need not necessarily have the same stringent structural requirements. Inhibition of folyl polyglutamate synthetase also does not appear to depend on the stereochemistry of the amino acid, as the L enantiomers of 1 and 3 showed K_i values very close to those of the corresponding DL forms (Table III).

Table III. Inhibition of Mouse Liver Folyl Polyglutamate Synthetase by L-Homocysteic Acid and L-Cysteic Acid Analogues of Methotrexate and Aminopterin

compd	K_i,[a] μM
1 (mAPA-L-HCysA)[b]	198 ± 31 (2)
2 (mAPA-L-CysA)	136 ± 31 (2)
3 (APA-LHCysA)[b]	59 ± 28 (2)
4 (APA-L-CysA)	43 ± 8 (2)

[a] Data were obtained from a minimum of two saturation curves and are the means plus or minus standard deviation for the number of experiments given in parentheses. [b] The K_i for the DL enantiomers of 1 (see ref 3) and 3 were 59 ± 28 (4) and 45 ± 6 (3) μM, respectively.

It was of interest to correlate the cytotoxicities of compounds 1–4 with their ability to inhibit dihydrofolate reductase and folyl polyglutamate synthetase. As the data in Tables I–III indicate, while the difference in the ID_{50} between the L-homocysteic acid analogues 1 and 3 against dihydrofolate reductase was only 1.4-fold, there was a 3-fold difference in the ID_{50} against folyl polyglutamate synthetase. Similarly for the L-cysteic acid analogues 2 and 4, the ID_{50} difference against dihydrofolate reductase was 1.4-fold, whereas against folyl polyglutamate synthetase this difference was 3-fold. Since the difference in cytotoxicity between 1 and 3 and between 2 and 4 was 6- and 8-fold, respectively, the cell-killing effect of these compounds appears to correlate better with folyl polyglutamate synthetase inhibition than with dihydrofolate reductase inhibition. These results, which are supported by the in vivo data (see below), suggest that folyl polyglutamate synthetase inhibition may play a role in the growth-inhibitory action of these compounds. However, we cannot at this time discount the possibility that the greater cytotoxicity of the AMT analogues reflects more efficient uptake into cells.

The in vivo antitumor activity of 1–4 was evaluated against L1210 leukemia in mice, on a twice-daily schedule. This mode of administration was chosen on the basis that, since polyglutamation of these compounds is precluded, frequent doses should be superior to intermittent doses in maintaining effective drug concentrations in the tumor. As indicated in Table IV, the L-homocysteic acid analogues 1 and 3 were clearly superior to the L-cysteic acid analogues 2 and 4. Whereas 32 mg/kg of 1 produced a 144% increase in life span (ILS), the same dose of 2, representing es-

(15) Mead, J. A. R.; Greenberg, N. H.; Schrecker, A. W.; Seeger, D. R.; Tomcufcik, A. S. *Biochem. Pharmacol.* **1965**, *14*, 105.
(16) Moran, R. G.; Rosowsky, A.; Colman, P.; Forsch, R.; Chan, K. *Proc. Am. Assoc. Cancer Res.* **1983**, *24*, 278.

Table IV. Antitumor Activity of L-Homocysteic Acid and L-Cysteic Acid Analogues of Methotrexate and Aminopterin against L1210 Leukemia in Mice

compd	dose,[a] mg/kg	no. of mice	7-day wt change, %	survival, days range	survival, days T/C, median	ILS, %
1 (mAPA-L-HCysA)	16	5	−9	19-21	21/9	+133
	32	5	−12	21-24	22/9	+144
2 (mAPA-L-CysA)	16	5	+2	11-14	12/9	+33
	32	5	−1	12-16	13/9	+44
3 (APA-L-HCysA)	0.5	5	−1	13-16	15/8	+88
	1.0	5	−8	13-17	16/8	+100
	2.0	5	−8	16-20	19/8	+138
4 (APA-L-CysA)	8	5	+2	12-15	13/8	+62
	12	5	+2	13-15	13/8	+62
	16	5	0	15-17	15/8	+88
methotrexate (MTX)	0.5	5	−1	15-18	16/9	+78
	0.75	5	−5	16-21	18/9	+100
	1.0	5	−8	3-22[b]	21/9	+133
aminopterin (AMT)	0.06	5	+1	11-13	12/8	+50
	0.12	5	+3	12-15	14/8	+75
	0.24	5	−12	12-20	19/8	+138

[a] Groups of five B6D2F$_1$J male mice were injected ip with 10^5 L1210 cells on day 0, and treatment was started on day 1. Drugs were administered ip in sterile water on a bid(1-4), qd(5,6), bid(7-10) schedule, with a single double-dose injection on days 5 and 6. [b] One injection death occurred on day 3 in this group.

sentially the same molar amount, gave only a 44% ILS. Similarly, the L-homocysteic acid **3** gave a 122% ILS at 2 mg/kg, whereas the L-cysteic acid **4** gave only a 33% ILS at the same dose. As in the in vitro assays against L1210 cells, the AMT analogues were roughly 10-fold more potent than the corresponding MTX analogues.

We recently proposed[3] that classical antifolates structurally modified to cause dual inhibition of dihydrofolate reductase and folyl polyglutamate synthetase may be of therapeutic interest, in that the prevention of the polyglutamation of reduced folates may decrease the dose of antifolate needed to cause arrest of DNA synthesis and, therefore, cell death. The compounds described in this paper possess the desired property of dual enzyme inhibition, and at least one of them (**3**) shows significant in vivo antitumor activity (>120% ILS) at doses in the 1 to 2 mg/kg range. These compounds could, in principle, be regarded as "self-potentiating" antifolates. However, it must be noted that unless the concentration of compounds such as **3** in the tumor at the therapeutically active in vivo dose reaches at least 50 μM, their effect on polyglutamation would be negligible. Future efforts addressing this problem will include the synthesis of other analogues that we hope will retain dihydrofolate reductase affinity while showing higher activity against folyl polyglutamate synthetase.

Experimental Section

Infrared spectra were obtained on a Perkin-Elmer Model 137B double-beam recording spectrophotometer, UV spectra were determined on a Cary 210 instrument, and NMR spectra were recorded on a Varian T60A spectrometer with Me_4Si as the internal reference. TLC was carried out on Eastman 13181 silica gel or Eastman 13254 cellulose sheets containing a fluorescent indicator. Spots were visualized under 254-nm illumination. Ion-exchange chromatography was performed on Whatman DE-52 preswollen [2-(diethylamino)ethyl]cellulose (DEAE-cellulose). Rotary evaporation of DMF solutions were performed with the aid of a vacuum pump at a bath temperature of 35–40 °C, with dry ice/acetone used to cool the receiver. The DMF used in coupling reactions was dried over Linde 4A molecular sieves. Microanalyses were performed by Galbraith Laboratories, Knoxville, TN, and were within ±0.4% of theoretical values unless noted otherwise.

L-Cysteic acid, L-homocysteic acid, and DL-homocysteic acid were obtained from Sigma Chemical Co., St. Louis, MO. 4-Amino-4-deoxy-N^{10}-methylpteroic acid was prepared as previously described[11,12] and was assumed to be the dihydrate for purposes of molar calculations. Aminopterin was obtained through the auspices of the National Cancer Institute, Bethesda, MD. Diethyl phosphorocyanidate was synthesized as reported.[11,17] Other reagents were of standard grade and were used without purification.

***N*-(4-Amino-4-deoxy-N^{10}-methylpteroyl)-L-homocysteic Acid (1).** A stirred suspension of L-homocysteic acid (0.55 g, 0.003 mol) in MeOH (25 mL) cooled in an ice bath was treated dropwise with $SOCl_2$ (5 mL) over 20 min so that the internal temperature did not exceed 12 °C. After being stirred overnight at room temperature, the mixture, which was now homogeneous, was evaporated to dryness. The methyl ester HCl salt was dried in vacuo at 60 °C over P_2O_5: yield 0.56 g (80%); IR (KBr) ν 3410, 2940, 1740 (ester C=O) cm^{-1}; NMR (D_2O) δ 2.43 (m, 2 H, CH_2), 3.0 (m, 3 H, $CH_2SO_3^-$ and α-CH), 3.87 (s, 3 H, OCH_3). A portion of this material was used in the next step, with no further purification.

Small portions of mAPA (1.08 g, 0.003 mol) were added to a stirred solution of diethyl phosphorocyanidate (1.47 g, 0.009 mmol) and Et_3N (0.91 g, 0.009 mol) in dry DMF (100 mL) at a rate such that the mAPA dissolved as it was being added. After the solution was stirred for 4 h, an additional 0.001 mol of each reagent was added to complete the activation of the CO_2H group. To the solution was then added another portion of Et_3N (0.91 mg, 0.009 mol), followed by the amino ester HCl salt (0.7 g, 0.003 mol). The solution was left at room temperature for 3 days and concentrated to dryness by rotary evaporation. The residue was taken up in 0.1 N NaOH; after 30 min, excess NH_4HCO_3 was added, and the solution was freeze-dried. The residue was redissolved in a minimum of H_2O, and the solution was applied onto a DEAE-cellulose column that had been first equilibrated with 3% NH_4HCO_3 and then washed to neutrality with H_2O. The column was eluted with a large volume of H_2O until the eluate was free of salts, and the product (**1**) was taken off with 3% NH_3HCO_3. Individual 5–10-mL volumes of the eluate were monitored by TLC, and homogeneous fractions were pooled and freeze-dried to obtain analytically pure **1** as a bright-yellow powder: yield 1.33 g (77%); R_f 0.8 (cellulose, pH 7.4 phosphate); IR (KBr) ν 3400, 1640, 1615 cm^{-1}; UV λ_{max} (pH 7.4) 259 nm (ϵ 24400), 302 (25300), 373 (8200); UV λ_{max} (0.1 N HCl) 242 nm (ϵ 18600), 305 (23000). Anal. ($C_{19}H_{22}N_8O_6S \cdot NH_3 \cdot 4H_2O$) C, H, N, S.

***N*-(4-Amino-4-deoxy-N^{10}-methylpteroyl)-L-cysteic Acid (2).** The same procedure as in the preparation of **1** was followed. L-Cysteic acid hydrate (935 mg, 5.0 mmol) was converted to its methyl ester HCl salt in ca. 100% yield: IR (KBr) ν 3410, 2940, 1740 (ester C=O) cm^{-1}; NMR (D_2O) δ 3.5 (m, 3 H, $CH_2SO_3^-$ and α-CH), 3.88 (s, 3 H, OCH_3). The yield of **2** from 110 mg (0.5 mmol) of the amino ester HCl salt was 215 mg (79%) after desalting and

(17) Yamada, S.; Kasai, Y.; Shioiri, T. *Tetrahedron Lett.* **1973**, 1595.

chromatography on DEAE-cellulose: R_f 0.8 (cellulose, pH 7.4 phosphate); IR (KBr) 3290, 1615 (sh), 1590 cm^{-1}; UV λ_{max} (pH 7.4) 258 nm (ϵ 23500), 300 (24100), 372 (7900); UV λ_{max} (0.1 N HCl) 242 nm (ϵ 17900), 306 (22200). Anal. ($C_{18}H_{20}N_8O_6S \cdot NH_3 \cdot 2.75H_2O$) C, H, N, S.

***N*-(4-Amino-4-deoxypteroyl)-L-homocysteic Acid (3).** To a suspension of the N^{10}-formyl derivative **6** (187 mg, 0.5 mmol) in dry DMF (20 mL) at room temperature was added Et_3N (152 mg, 1.5 mmol), followed by *i*-BuOCOCl (65 μL, 68 mg, 0.5 mmol). After 10 min, another 10 μL of *i*-BuOCOCl was added to remove a trace of remaining cloudiness, and 5 min later the amino acid ester HCl salt (117 mg, 0.5 mmol) was added in a single portion. Stirring was continued for 10 min, and another 0.75 mmol of Et_3N and 0.25 mmol of *i*-BuOCOCl were added, followed 15 min later by 0.25 mmol of the amino ester salt. The cycle was repeated once more with 0.15 mmol of Et_3N and 0.05 mmol of *i*-BuOCOCl (15 min), followed by 0.05 mmol of amino ester salt (10 min). Thus, the approximate total of each reactant was as follows: **6**, 0.5 mmol; Et_3N, 2.4 mmol; *i*-BuOCOCl, 0.88 mmol; amino ester salt, 0.8 mmol. TLC (cellulose, pH 7.4 phosphate) was carried out during the reaction to monitor the disappearance of **6** (R_f 0.5, blue fluorescence) and concomitant formation of the blocked coupling product **7** (R_f 0.7, blue fluorescence). After the final addition of the amino ester salt, the reaction mixture was stirred for 10 min and concentrated to dryness by rotary evaporation, and the residue was taken up in a minimum of H_2O. Dropwise addition of 2 N NaOH was carried out until the TLC (R_f 0.5, dark absorbing spot) showed loss of the N^{10}-formyl group. Solid NH_4HCO_3 was added to bring the pH to 8, and the solution was freeze-dried. The residue was taken up in a minimum of H_2O and applied onto a DEAE-cellulose column that had been initially equilibrated with 3% NH_4HCO_3 and then washed to neutrality with H_2O. The column was eluted with a large volume of H_2O to remove salts and then with 3% NH_4HCO_3 to elute the product (**3**). Freeze-drying of pooled TLC-homogeneous fractions of the 3% NH_4HCO_3 eluate gave **3** as a bright-yellow powder: yield 223 mg (83%); IR (KBr) ν 2940–3230, 1625 (sh), 1585 cm^{-1}; UV λ_{max} (pH 7.4) 260 nm (ϵ 27400), 282 (26100), 370 (8900); UV λ_{max} (0.1 N HCl) 243 nm (ϵ 18300), 290 (20100). Anal. ($C_{18}H_{20}N_8O_6S \cdot NH_3 \cdot 2.5H_2O$) C, H, N, S.

The DL form of **3** (see Table III, footnote *b*) was prepared in the same manner and was characterized spectrally as well as by microanalysis. Anal. ($C_{18}H_{20}N_8O_6S \cdot 0.5NH_3 \cdot 2H_2O$).

***N*-(4-Amino-4-deoxypteroyl)-L-cysteic Acid (4).** The same procedure as in the preparation of **3** was followed. The mixed anhydride coupling reaction, with TLC monitoring of the disappearance of **6**, was performed according to the following sequence: (1) **6** (0.5 mmol), Et_3N (1.5 mmol), and *i*-BuOCOCl (0.5 mmol), 10 min; (2) *i*-BuOCOCl (0.08 mmol), 5 min; (3) amino ester salt (0.6 mmol), 15 min; (4) Et_3N (0.75 mmol) and *i*-BuOCOCl (0.25 mmol), 10 min; (5) amino ester salt (0.25 mmol), 15 min; (6) Et_3N (0.38 mmol) and *i*-BuOCOCl (0.13 mmol), 10 min; (7) amino ester salt (0.13 mmol), 10 min. The yield of **4** after N^{10}-formyl group cleavage and DEAE-cellulose desalting and chromatography as described above was 189 mg (72%): IR (KBr) ν 3030–3230, 1590–1615 cm^{-1}; UV λ_{max} (pH 7.4) 260 nm (ϵ 26900), 282 (25900), 370 (8700). Anal. ($C_{17}H_{18}N_6O_6S \cdot 0.9NH_3 \cdot 2.5H_2O$) C, H, N, S.

4-Amino-4-deoxypteroic Acid (5).[18] Aminopterin (2.4 g, 0.005 mol) was suspended in 1 M NaOAc (500 mL) containing $ZnCl_2$ (0.1 g), and 2 N NaOH was added dropwise with stirring until a clear solution formed. Glacial AcOH was then added dropwise to bring the pH to 7.5, and 5 μL of carboxypeptidase G_1 (4000 units/mL) was added. The mixture was shaken at 37 °C for 1 day, cooled to 5 °C, and suction filtered. The solid was washed thoroughly with H_2O and dried in vacuo on a freeze-drying apparatus to obtain **5** (1.43 g, 86%) as an orange-yellow solid. The analytical sample was prepared by passing a small amount of this material through a microcrystalline cellulose column, with 0.1 M glycine, pH 10, as the eluent. Appropriate fractions were pooled and acidified with AcOH, and the precipitate was collected, washed with H_2O, and dried in vacuo over P_2O_5: R_f 0.1 (cellulose, pH 7.4 phosphate), dark absorbing spot; UV λ_{max} (0.1 N HCl) 243 nm (ϵ 18500), 297 (23900), 337 (infl) (13700); UV λ_{max} (0.1 N NaOH) 261 nm (ϵ 30400), 371 (9100). Anal. ($C_{14}H_{13}N_7O_2 \cdot 1.1CH_3CO_2H \cdot H_2O$) C, H, N.

4-Amino-4-deoxy-N^{10}-formylpteroic Acid (6). A portion of the material obtained in the preceding experiment (1.3 g, 0.004 mol) was added, without purification, to the formylation reagent obtained by combining Ac_2O (25 mL) and 98% HCO_2H (100 mL) and allowing the heat of reaction to dissipate. When addition of **5** to this mixture was complete, the temperature was raised to 100 °C for 1 h. Rotary evaporation and trituration of the residue with H_2O gave a solid, which was filtered and dried in vacuo on a lyophilizer to obtain **6** as an off-white powder: yield 1.16 g (82%); R_f 0.4 (cellulose, pH 7.4 phosphate), blue fluorescent spot; UV λ_{max} (0.1 N HCl) 247 nm (ϵ 23100), 337 (9800); UV λ_{max} (pH 7) 258 nm (ϵ 28000), 370 (7200). Anal. ($C_{15}H_{13}N_7O_3 \cdot 1.75H_2O$) C, H, N.

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Registry No. 1, 89043-71-0; **2**, 89043-72-1; **3**, 89043-73-2; **4**, 89043-74-3; **5**, 36093-85-3; **6**, 89043-75-4; **7**, 89043-76-5; **8**, 89043-77-6; L-homocysteic acid, 14857-77-3; L-homocysteic acid methyl ester hydrochloride salt, 89043-78-7; 4-amino-4-deoxy-N^{10}-methylpteroic acid, 19741-14-1; L-cysteic acid, 498-40-8; L-cysteic acid methyl ester hydrochloride salt, 89043-79-8; aminopterin, 54-62-6; dihydrofolate reductase, 9002-03-3; folyl polyglutamate synthetase, 63363-84-8.

(18) The preparation of this compound by total chemical synthesis has been described: Piper, J. R.; Montgomery, J. A. *J. Org. Chem.* **1977**, *42*, 208. Nair, M. G.; Adapa, S. R.; Bridges, T. W. *Ibid.* **1981**, *46*, 3152. The properties of our sample were in accord with those given in the literature.

(19) Gunderson, L. E.; Dunlap, R. B.; Harding, N. G.; Freisheim, H. J.; Otting, F.; Huennekens, F. M. *Biochemistry* **1972**, *11*, 1018.

(20) Rosowsky, A.; Kim, S.-H.; Ross, J.; Wick, M. *J. Med. Chem.* **1982**, *25*, 171.