dissolved in physiological salt. LD50 values were determined on five groups of 10 animals each and calculated according to the method of Litchfield and Wilcoxon.[56]

The doses of the oximes **1b** and **1e** producing no observable symptoms, i.e., the so-called sign free dose, were determined under similar experimental conditions.

The protective effects of **1b** and **1c** were evaluated against paraoxon in male albino rats (Wistar), with use of sign free dose conditions. Paraoxon dissolved in physiological salt was administrated subcutaneously while the oxime and atropine sulfate (17.4 mg/kg) were administered intramuscularly 1 min after intoxication. The protective index was calculated as the ratio LD50 (paraoxon + oxime)/LD50 (paraoxon). The protective index of atropine sulfate (17.4 mg/kg) was similarly calculated.

Acknowledgment. We are indebted to Dr. M. P. Simonnin for assistance in the analysis of NMR spectra and to Isabelle Callebat for therapeutic determinations.

Registry No. **1a**, 306-44-5; **1b**, 112740-61-1; **1c**, 112740-56-4; **1c**·Na, 112740-55-3; **1d**, 112740-58-6; **1d**·Na, 112740-57-5; **1e**, 112740-60-0; **2**, 112740-54-2; AChE, 9000-81-1; Me_2S, 75-18-3; PNPA, 830-03-5; VX, 50782-69-9; $(CH_3)_3SiCl$, 75-77-4; (*E*)-$HON{=}CHCO_2Et$, 31767-15-4; $MeSOCH_2^-$, 13810-16-7; $MeSO_2CH_2^-$, 29119-74-2; (*E*)-$BrCH_2COCH{=}NOH$, 112740-59-7; *p*-$MeOC_6H_4OH$, 150-76-5; *p*-MeC_6H_4OH, 106-44-5; PhOH, 108-95-2; *p*-AcC_6H_4OH, 99-93-4; *m*-AcC_6H_4OH, 121-71-1; *p*-ClC_6H_4OH, 106-48-9; 3,4-Cl_2-1-OHC_6H_3, 95-77-2; 3,5-Cl_2-1-OHC_6H_3, 591-35-5; 3,4,5-Cl_3-1-OHC_6H_3, 609-19-8; sarin, 107-44-8; paraoxon, 311-45-5.

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Methotrexate Analogues. 31. Meta and Ortho Isomers of Aminopterin, Compounds with a Double Bond in the Side Chain, and a Novel Analogue Modified at the α-Carbon: Chemical and in Vitro Biological Studies[1]

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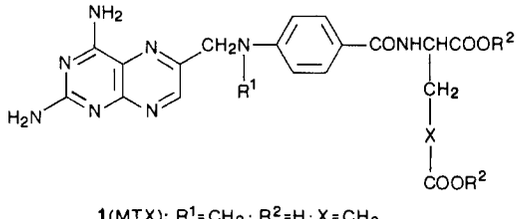
Five heretofore undescribed analogues of methotrexate (MTX) and aminopterin (AMT) were synthesized and tested as dihydrofolate reductase (DHFR) inhibitors and tumor cell growth inhibitors. The meta isomer of AMT was obtained from 2,4-diamino-6-(bromomethyl)pteridine and *m*-(aminobenzoyl)-L-glutamic acid, while the ortho isomer was obtained via the same route by using α-methyl γ-*tert*-butyl *o*-(aminobenzoyl)-L-glutamate instead of the free acid. Analogues of MTX and AMT containing a double bond in the side chain were prepared from dimethyl D,L-2-amino-4-hexenedioate and 4-amino-4-deoxy-N^{10}-methylpteroic acid and 4-amino-4-deoxy-N^{10}-formylpteroic acid, respectively. Finally, a positional isomer of MTX with the CH_2CH_2COOH moiety moved from the α-carbon to the adjacent carboxamide nitrogen was synthesized from 3-[*N*-(carboxymethyl)amino]propanoic acid diethyl ester and 4-amino-4-deoxy-N^{10}-methylpteroic acid. The positional isomers of AMT were weak DHFR inhibitors and showed very little growth-inhibitory activity against L1210 murine leukemia cells or the MTX-resistant L1210/R81 mutant line in culture. The MTX and AMT analogues with the CH_2CH_2COOH moiety replaced by a $CH_2CH{=}CHCOOH$ side chain showed anti-DHFR activity similar to that of the previously described saturated compound *N*-(4-amino-4-deoxy-N^{10}-methylpteroyl)-L-2-aminoadipic acid, but were less potent than the parent drugs. The MTX analogue with the CH_2CH_2COOH side chain displaced from C to N was weakly bound to DHFR, confirming the importance of an intact CONH moiety, and showed greatly diminished cell growth inhibitory potency relative to MTX. None of the compounds was a substrate for folylpolyglutamate synthetase (FPGS) from mouse liver. Furthermore, inhibition of folic acid polyglutamylation in vitro at equimolar 500 μM concentrations of drug and substrate was negligible. The structural changes embodied in these five novel compounds are therefore too great for binding to the FPGS active site.

Structural analogues of methotrexate (MTX, **1**) and aminopterin (AMT, **2**) have been studied extensively as means of gaining a better understanding of the molecular features that are optimal for enzyme binding, cell membrane penetration, and in vitro/in vivo antitumor activity.[2]



1 (MTX): $R^1 = CH_3$; $R^2 = H$; $X = CH_2$
2 (AMT): $R^1 = R^2 = H$; $X = CH_2$
5: $R^1 = CH_3$; $R^2 = H$; $X = CH{=}CH$
6: $R^1 = R^2 = H$; $X = CH{=}CH$
16: $R^1 = CH_3$; $R^2 = Me$; $X = CH{=}CH$

Work in this laboratory has focused mainly on the amino acid side chain,[1,3-13] though this is clearly not the only

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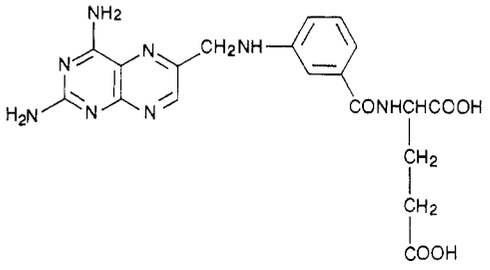
region of the molecule where systematic structural variations are possible. Five unusual new compounds are described in this paper: (a) **3** and **4**, the meta and ortho isomers of AMT; (b) D,L-**5** and D,L-**6**, which are MTX and



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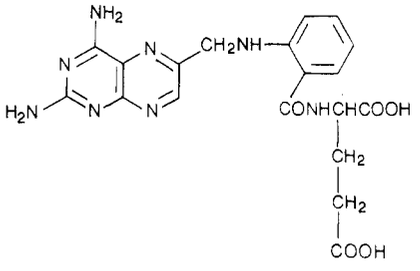

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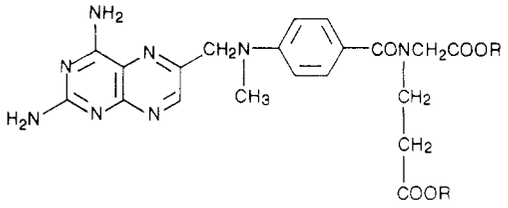
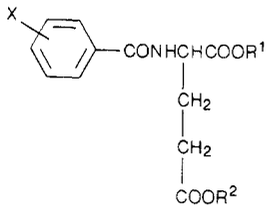
AMT analogues with a double bond in the side chain; and (c) **7**, a novel structure with the CH_2CH_2COOH side chain moved from the α-carbon to the adjacent amide nitrogen. In addition to the synthesis of these compounds, the results of enzyme activity and cell growth assays are briefly presented.



7: R=H
21: R=Et

Chemistry. Compound **3** was obtained in modest yield (18%) by condensing 2,4-diamino-6-(bromomethyl)pteridine[14] with *N*-(*m*-aminobenzoyl)-L-glutamic acid (**8**) in *N*,*N*-dimethylacetamide at 60 °C in the presence of diisopropylethylamine. The amine **8** was obtained by catalytic reduction of the *m*-nitrobenzoyl derivative **9** (79% yield), which was prepared from L-glutamic acid by silylation of the carboxyl groups with Me_3SiCl/Et_3N, followed directly by N-acylation with *m*-nitrobenzoyl chloride and desilylation with MeOH (98% yield). An unsuccessful attempt was also made to condense the (bromomethyl)pteridine with γ-ethyl *N*-(*m*-aminobenzoyl)-L-glutamate (**10**), which was obtained by hydrogenation of nitro ester

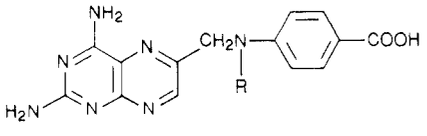


8: X = 3-NH_2; $R^1 = R^2 = H$
9: X = 3-NO_2; $R^1 = R^2 = H$
10: X = 3-NH_2; R^1 = H; R^2 = Et
11: X = 3-NO_2; R^1 = H; R^2 = Et
12: X = 2-NO_2; R^1 = Me; R^2 = *t*-Bu
13: X = 2-NH_2; R^1 = Me; R^2 = *t*-Bu

11. The inability of **10** to react in the desired manner with the (bromomethyl)pteridine was ascribed to extensive amino ester self-condensation. It should be noted that the NH_2 group in **10** is meta to the carboxamide moiety whereas para derivatives have generally been used until now.

For the preparation of **4**, we elected to use a method we have previously followed to obtain AMT analogues from 2,4-diamino-6-(hydroxymethyl)pteridine[15] without intermediate isolation of the 6-bromomethyl compound. In addition, we chose to use a glutamate diester in the hope that this would improve the yield and facilitate purification. α-Methyl γ-*tert*-butyl glutamate was N-acylated with *o*-nitrobenzoyl chloride to form **12** (94%), and the latter was sequentially reduced and treated with HCl to obtain **13**·HCl (96%). The diester formed in the coupling reaction was hydrolyzed directly with base to allow purification by anion-exchange chromatography. It is of interest to note that strong base treatment in this instance removed both the methyl and *tert*-butyl ester groups. Facile alkaline hydrolysis of a *tert*-butyl ester has been observed previously from the γ-position of AMT.[15] The final purified yield of **4** by this method was unfortunately only 1.5%, probably as a result of steric hindrance since the NH_2 group was now ortho to the carboxamide moiety. Because enough material was isolated for preliminary bioassay, no attempt to optimize the reaction conditions was made. However, our results suggest that coupling of 2,4-diamino-6-(bromomethyl)pteridine gives low yields of *o*- and *m*-(aminobenzoyl)amino acid derivatives irrespective of whether the bromo compound is isolated first or is used in situ.

Compounds D,L-**5** and D,L-**6**, which are to our knowledge the first examples of MTX and AMT analogues containing a double bond in the amino acid side chain, were obtained from 4-amino-4-deoxy-N^{10}-methylpteroic acid (**14**) and 4-amino-4-deoxy-N^{10}-formylpteroic acid (**15**) by methods previously described.[15] The yield of the ester **16** from **14**



14: R=CH_3
15: R=CHO

via diethyl phosphorocyanidate (DEPC) coupling was 47%; the overall yield of D,L-**5** after hydrolysis with $Ba(OH)_2$ was 38%. The yield of the crude N^{10}-formyl diethyl ester of D,L-**6** from **15** via mixed carboxylic–carbonic anhydride coupling was 65%; the overall yield of D,L-**6** itself after alkaline hydrolysis of the blocking groups was 34%. The

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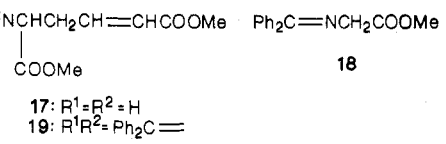
starting material, dimethyl D,L-2-amino-4-hexenedioate (17·HCl), was prepared by an adaptation of the procedure



$R^1R^2NCHCH_2CH{=}CHCOOMe$ (COOMe) $Ph_2C{=}NCH_2COOMe$

18

17: $R^1{=}R^2{=}H$
19: $R^1R^2{=}Ph_2C{=}$

of Joucla and co-workers.[16] The benzophenone imine derivative **18** of methyl glycinate was treated with lithium diisopropylamide in THF, and the resulting anion was allowed to react with methyl 4-bromocrotonate in the presence of HMPA to obtain the protected amino diacid **19**. The imino group was found to be hydrolyzed in 76% yield, with no loss of the ester groups, by overnight two-phase treatment with 1 N HCl and Et_2O at room temperature.[17] The overall yield of **17**·HCl starting from ethyl glycinate hydrochloride was 61%.

Finally, for the synthesis of **7**, ethyl glycinate was condensed with ethyl acrylate as described by Hering[18] to obtain the amino diester **20** (65%), which was condensed with **14** by the DEPC method to obtain **21** (53%). Hydrolysis of the ester groups with $Ba(OH)_2$ then gave **7** (76%; 40% from **14**).

$EtOOCCH_2CH_2NHCH_2COOEt$

20

Biological Activity. Compounds **3–7** were assayed as inhibitors of dihydrofolate reductase (DHFR) isolated from L1210 murine leukemia cells and purified by affinity chromatography on MTX-Sepharose as previously described.[19] The binding affinity of the AMT positional isomers **3** and **4** was found to be enormously reduced in comparison with AMT. The meta isomer **3** had an IC_{50} of 200 μM as compared with 0.002 μM for AMT, and the ortho isomer **4** was bound even more weakly (20% inhibition at 50 μM). It is clear from these data that DHFR inhibition requires substitution on the phenyl ring to be para, probably because only this arrangement allows the molecule to adopt the optimal three-dimensional conformation for tight binding to the enzyme active site. Compounds **3** and **4** were also tested as substrates for mouse liver folylpolyglutamate synthetase (FPGS) and as competitive inhibitors of the enzyme as previously described[20] but were found to have neither substrate nor inhibitor activity. Not surprisingly in view of the low binding affinity of **3** and **4** for DHFR, these compounds were weak growth inhibitors of tumor cell growth in culture. The IC_{50} of **3** against L1210 mouse leukemia cells was 1.5 μM while that of **4** was 64 μM. Even lower activity was observed against CEM human lymphoblasts. Thus, activity relative to AMT was diminished 10^3–10^4-fold by moving N^{10} from the para to the meta or ortho positions. It is of interest to note that a compound claimed to be the meta isomer of folic acid reportedly shows antifolate activity in culture.[21]

Compounds D,L-**5** and D,L-**6** were found to be potent DHFR inhibitors, with IC_{50}'s of 32 and 46 nM, respectively. These values were close to those of MTX and AMT, both of which are L-enantiomers. It has been shown that L-MTX is ca. 10-fold more potent than D-MTX as a DHFR inhibitor.[22] Thus, it is reasonable to assume that the IC_{50} values obtained with D,L-**5** and D,L-**6** are approximately twice as high as they would be with the L-enantiomers, i.e., that the IC_{50}'s of L-**5** and L-**6** would be in the 15–25 μM range. The IC_{50} of *N*-(4-amino-4-deoxy-N^{10}-methylpteroyl)-L-2-aminoadipic acid, the saturated analogue of **5**, is in the 30–40 μM range.[5] Introduction of a C=C bond in the aminoadipate side chain therefore did not diminish DHFR binding and may have even enhanced it. The presence of the C=C bond would be expected to shorten the side chain and introduce conformational rigidity relative to aminoadipate.

In growth inhibition assays against cultured L1210 cells, D,L-**5** and D,L-**6** gave IC_{50}'s of 0.35 and 0.031 μM, respectively, as compared with our earlier value of 0.030 μM for the L-2-aminoadipate analogue of MTX.[5] Therefore, the introduction of a double bond had about the same favorable effect in this assay as it did on DHFR binding. The IC_{50}'s of MTX and AMT against L1210 cells, on the other hand, were 0.005 and 0.002 μM, respectively. Thus, notwithstanding their potent anti-DHFR activity, which was similar to that of MTX and AMT, compounds D,L-**5** and D,L-**6** were 10–100-fold less effective than MTX and AMT as inhibitors of cell proliferation. The most likely explanation of these results is that D,L-**5** and D,L-**6** are less efficiently transported into cells than MTX and AMT and are not converted to polyglutamates. The inability of the saturated L-2-aminoadipate analogue of MTX to form polyglutamates in the presence of folylpolyglutamate synthetase (FPGS) from mouse liver was reported earlier.[20] In the present work, we have found that D,L-**5** and D,L-**6** similarly lack substrate activity when incubated with FPGS in the presence of an equimolar concentration (500 μM) of folic acid. We conclude that replacement of the CH_2CH_2COOH moiety in MTX or AMT by $CH_2CH{=}CHCOOH$ is not tolerated by FPGS even though it is well tolerated by DHFR.

Assays of D,L-**5** and D,L-**6** as inhibitors of the growth of the MTX-resistant L1210/R81 cell line were also performed in order to determine whether resistance could be overcome. The IC_{50}'s for both compounds were >300 μM, demonstrating essentially complete cross-resistance with MTX.

Compound **7** was found to have an IC_{50} of 0.038 μM in the DHFR inhibition assay, a value in the general range of other active MTX analogues. However, the data were unusual in that the titration curve for enzyme activity became horizontal at approximately 67% inhibition and remained horizontal for all inhibitor concentrations up to 0.23 μM. This extreme example of curvilinearity in a DHFR titration curve is not often seen and cannot be interpreted without more detailed kinetic analysis. Assays of FPGS substrate activity with **7** were similarly negative. The weak inhibitory effect of **7** against DHFR as well as its lack of activity as an FPGS substrate were supported by low growth inhibitory activity against cultured cells. The IC_{50} against L1210 cells was 12 μM, while the IC_{50} against L1210/R81 cells was 180 μM. Overall, therefore, we conclude from these results that moving the CH_2CH_2COOH moiety of MTX from the α-carbon to the amide nitrogen leads to a marked loss of biological activity. We reported earlier that modification of the CONH region in

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MTX by reduction to CH_2NH is highly detrimental to DHFR binding, FPGS substrate activity, and cell growth inhibition.[4] One explanation proposed for these results was that the NHCO group can serve as a hydrogen bond donor, whereas in the reduced analogue the nitrogen is an acceptor. The same explanation may apply here, except that in 7 the amide nitrogen is retained but is alkyl substituted.

Experimental Section

Infrared spectra were obtained on a Perkin-Elmer Model 781 double-beam spectrophotometer, and UV spectra were obtained on a Varian Model 215 UV/vis instrument. NMR spectra were recorded on a Varian T60 spectrometer using Me_4Si as the internal reference standard. Melting points were determined on a Fisher-Johns apparatus (corrected) or in Pyrex capillary tubes in a Mel-Temp apparatus (Laboratory Devices, Inc., Cambridge, MA) (not corrected). TLC was carried out on plastic sheets (Eastman 13181 silica gel, Eastman 13254 cellulose) or glass plates (Baker 250F, Whatman MK6F) containing a fluorescent indicator. Spots were visualized in a viewing chamber under 254-nm illumination or, where appropriate, with the aid of ninhydrin spray. Column chromatography was on Baker 3405 silica gel (60–200 mesh), Whatman DE-52 preswollen (diethylamino)cellulose (DEAE-cellulose), or Merck LiChroprep C_{18} silica gel (40–60 μm particle size) for LPLC. Waters C_{18} reversed-phase radial compression cartridges (5 μm particle size, 0.5 × 10 cm) were used for HPLC on a Waters Model 400 instrument equipped with a Model 490 multiwavelength detector and Model 660 solvent gradient system. 4-Amino-4-deoxy-N^{10}-methylpteroic acid (**14**), 4-amino-4-deoxy-N^{10}-formylpteroic acid (**15**), and 2,4-diamino-6-(hydroxymethyl)pteridine were obtained as previously described.[15] Methyl *N*-(diphenylmethylene)glycinate (**18**) was prepared from methyl glycinate hydrochloride and benzophenone imine by the method of O'Donnell and Polt.[17] 2,4-Diamino-6-(bromomethyl)pteridine hydrobromide was a generous gift from Dr. J. R. Piper, Southern Research Institute, Birmingham, AL. Other chemicals were from Aldrich, Milwaukee, WI, Chemical Dynamics, South Plainfield, NJ, Sigma, St. Louis, MO, and Fisher, Boston, MA. Et_3N and solvents used for moisture-sensitive reactions were dried over Davison 4A molecular sieves. Microchemical analyses were by Galbraith Laboratories, Knoxville, TN, and Multi Chem, Lowell, MA, and were within ±0.4% of theory unless otherwise specified.

***N*-(*m*-Nitrobenzoyl)-L-glutamic Acid (9).** Et_3N (3.03 g, 0.03 mmol) and Me_3SiCl (3.25 g, 0.03 mol) were added to a stirred suspension of L-glutamic acid (1.47 g, 0.01 mol) in CH_2Cl_2 (50 mL), and after the mixture was stirred for 23 h at 25 °C, a solution of *m*-nitrobenzoyl chloride (1.86 g, 0.01 mol) was added, followed by another portion of Et_3N (1.11 g, 0.011 mol). After addition, stirring at 25 °C was continued for 4 h, MeOH (2.5 mL) was added, and the solution was extracted with H_2O (2 × 100 mL). The aqueous layer was applied onto an anion-exchange column (Amberlite CG-400, 100–200 mesh, 40 g). The column was eluted first with H_2O and then with 20% AcOH. The AcOH eluate was evaporated, the residue taken up in benzene–hexane, and the solution reevaporated to obtain a thick oil (3.19 g, 98%): IR (film) 1700–1750 (acid C=O), 1650 (amide C=O), 1530, 1350 (NO_2) cm^{-1}. Anal. ($C_{12}H_{12}N_2O_7 \cdot 0.33C_6H_{14}$) C, H, N.

***N*-(*m*-Aminobenzoyl)-L-glutamic Acid (8).** The nitrobenzoyl derivative **9** (2.71 g, 9.15 mmol) was dissolved in EtOH (25 mL) and hydrogented in the presence of 5% platinum on carbon (0.3 g), and the product was crystallized from 1:1 MeOH–*i*-PrOH: yield 1.92 g (79%); mp 164–165 °C (lit.[21] mp 166–167 °C).

γ-Ethyl *N*-(*m*-Nitrobenzoyl)-L-glutamate (11). A suspension of γ-ethyl L-glutamate (1.75 g, 0.01 mmol), Et_3N (2.02 g, 0.02 mol), and Me_3SiCl (2.17 g, 0.02 mol) in CH_2Cl_2 (50 mL) was stirred at 25 °C for 23 h. The mixture was then cooled to 0 °C, and a solution of *m*-nitrobenzoyl chloride (1.86 g, 0.01 mol) in CH_2Cl_2 (10 mL) was added, followed by another portion of Et_3N (1.11 g, 0.011 mol). After 24 h, EtOH (3.5 mL) was added, and the solution was extracted with H_2O (2 × 125 mL). The organic layer was separated, H_2O was added, and the pH of the aqueous layer was adjusted to 8 with 0.1 N NaOH. The aqueous layer was separated, acidified to pH 2, and extracted with CH_2Cl_2. The CH_2Cl_2 extract was dried ($MgSO_4$) and evaporated, and the residue was crystallized from hot 2:1 benzene–hexane to obtain colorless needles (2.12 g, 65%): mp 125.5 °C; TLC (cellulose, pH 7.4 phosphate buffer) R_f 0.82; IR (KBr) 1720 (acid C=O), 1740 (acid and ester C=O), 1645 (amide C=O), 1540, 1350 (NO_2) cm^{-1}. Anal. ($C_{14}H_{16}N_2O_7$) C, H, N.

γ-Ethyl *N*-(*m*-Aminobenzoyl)-L-glutamate (10). A solution of the nitrobenzoyl derivative **11** (2.09 g, 6.44 mmol) in a mixture of EtOH (15 mL) and glacial AcOH (5 mL) was hydrogenated for 3 h in the presence of 5% platinum on carbon (0.5 g) in a Parr apparatus at an initial pressure of 50 psi. After removal of the catalyst, the solution was evaporated in vacuo to obtain a viscuous oil (1.9 g, 100%): TLC, blue fluorescent spot, R_f 0.92 (cellulose, 7.4 phosphate buffer), R_f 0.84 (silica gel, 4:1 EtOH–NH_4OH); IR (film) 3350, 2500, 1720 (acid and ester C=O) cm^{-1}. Anal. ($C_{14}H_{18}N_2O_5$) C, H, N.

α-Methyl γ-*tert*-Butyl *N*-(*o*-Nitrobenzoyl)-L-glutamate (12). Et_3N (2.22 g, 0.022 mol) was added at 0 °C to a solution of α-methyl γ-*tert*-butyl L-glutamate hydrochloride (2.54 g, 0.01 mol) and *o*-nitrobenzoyl chloride (1.85 g, 0.01 mol) in CH_2Cl_2 (35 mL). The mixture was stirred for 3 h at 25 h and then extracted successively with 30-mL portions of 0.2 N HCl, H_2O, saturated NH_4HCO_3, and H_2O, dried ($MgSO_4$), and evaporated. Crystallization of the residue from 1:1 benzene–hexane gave colorless needles (3.44 g, 94%): mp 96–97 °C; TLC R_f 0.72 (silica gel, 9:1 $CHCl_3$–MeOH); IR (KBr) 3310, 1760 (ester C=O), 1720 (ester C=O), 1650 (amide C=O), 1540, 1360 (NO_2) cm^{-1}; NMR ($CDCl_3$) δ 1.43 (s, 9 H, *t*-Bu), 1.6–2.5 (m, 4 H, CH_2CH_2), 3.78 (s, 3 H, OCH_3), 4.83 (m, 1 H, α-CH), 7.5–7.8 (m, 4 H, aromatic protons), 8.0 (m, 1 H, NH). Anal. ($C_{17}H_{22}N_2O_7 \cdot 0.75H_2O$) C, H, N.

Treatment of this material with 0.3 N NaOH at 25 °C for 4 h resulted in partial hyrolysis of the amide bond.

α-Methyl γ-*tert*-Butyl *N*-(*o*-Aminobenzoyl)-L-glutamate Hydrochloride (13·HCl). Compound **12** (1.18 g, 3.1 mmol) was dissolved in a mixture of EtOH (15 mL) and AcOH (5 mL) and hydrogenated for 3 h in the presence of 10% platinum on carbon (0.12 g). The residue after solvent evaporation was redissolved in anhydrous Et_2O, ethereal HCl was added, the mixture was stirred at 0 °C, and the precipitate was collected and dried over P_2O_5 at 80 °C to obtain a hygroscopic HCl salt (1.12 g, 96%): mp 73–77 °C; TLC, blue fluorescent spot, R_f 0.79 (silica gel, 9:1 MeOH–$CHCl_3$). Anal. ($C_{17}H_{24}N_2O_5 \cdot HCl \cdot 0.25H_2O$) C, H, N.

***N*-[3-[*N*-[(2,4-Diaminopteridin-6-yl)methyl]amino]benzoyl]-L-glutamic Acid (3, "meta-Aminopterin").** Compound **8** (165 mg, 0.55 mmol) and *i*-Pr_2EtN (207 mg, 1.6 mmol) were added to a stirred solution of 2,4-diamino-6-(bromomethyl)pteridine hydrobromide (88% purity, 191 mg, 0.5 mmol) in *N*,*N*-dimethylacetamide (1.5 mL). The mixture was kept at 60 °C (bath temperature) for 4 h, the solvent was evaporated in vacuo, 0.1 N NaOH was added to the residue, some insoluble material was removed by filtration, and the filtrate was acidified to 4.2 with AcOH to obtain a flocculent yellow solid. The solid was taken up in 3% NH_4NCO_3 (2 mL), and the solution was applied onto a DEAE-cellulose column (22 × 1.0 cm), the column was eluted with 3% NH_4HCO_3, and fractions containing a TLC spot with R_f 0.23 (silica gel, 5:4:1 $CHCl_3$–MeOH–NH_4OH) were pooled and freeze-dried to obtain a yellow solid (45 mg, 18%): IR (KBr) 3430, 1645, 1400 cm^{-1}; UV (0.1 N HCl) λ_{max} 243 nm (ε 21 200), 290 infl (6100), 335 (10 600), 350 infl (8800); UV (0.1 N NaOH) λ_{max} 258, 370 nm. Anal. ($C_{19}H_{20}N_8O_5 \cdot 4H_2O$) C, H, N.

***N*-[2-[*N*-[(2,4-Diaminopteridin-6-yl)methyl]amino]benzoyl]-L-glutamic Acid (4, "ortho-Aminopterin").** Br_2 (0.435 mL, 8.7 mmol) was added slowly at 5 °C to a stirred solution of Ph_3P (2.28 g, 8.7 mmol) in dry *N*,*N*-dimethylacetamide (10 mL). Solid 2,4-diamino-6-(hydroxymethyl)pteridine (1.09 g, 2.9 mmol) was added, the mixture was stirred at 25 °C for 18 h, and to the resultant solution were then added compound **13**·HCl (1.09 g, 2.9 mmol) and 98% BaO (0.91 g, 5.8 mmol). The mixture was stirred at 55 °C for 24 h, cooled to room temperature, and poured into 1 N NaOH (45 mL) and H_2O (100 mL). After 4 h of stirring, a solid (A) was removed by filtration. The filtrate was acidified to pH 4.4 with AcOH, and a second solid (B) was collected and dried in a freeze-drying apparatus. Solid A was extracted with CH_2Cl_2 under reflux, the insoluble Ph_3PO was removed by filtration, and the filtrate was evaporated. The residue was combined with solid B and sonicated in 1.5% NH_4HCO_3 (45 mL). The

pH was adjusted to 11, some insoluble material was removed by filtration, the pH was readjusted to 8.5 with NH_4OH, and the solution was applied onto a DEAE-cellulose column (24 × 1.5 cm). The column was eluted with 3% NH_4HCO_3, and fractions showing a TLC spot with R_f 0.28 (silica gel, 5:4:1 $CHCl_3$–MeOH–NH_4OH) or R_f 0.19 (silica gel, 15:5:1 $CHCl_3$–MeOH–AcOH) were combined, evaporated, and dried in a lyophilizer to obtain a bright-yellow powder (23 mg, 1.5%); IR (KBr) 3420, 1635, 1645 cm^{-1}; UV (0.1 N HCl) λ_{max} 243 nm (ϵ 22 000), 335 (10 300), 350 infl (8800) 290 infl (7900); UV (0.1 N NaOH) λ_{max} 256, 372 nm. Anal. ($C_{19}H_{20}N_8O_5 \cdot 3.5H_2O \cdot 0.33CH_3COOH$) C, H, N.

Dimethyl D,L-2-Amino-4-hexenedioate Hydrochloride (17·HCl). Methyl *N*-(diphenylmethylene)glycinate (**18**)[17] (2.53 g, 0.01 mol) was added to a solution of $LiN(Pr\text{-}i)_2$, which had been freshly prepared by adding *n*-BuLi in THF (4 mL of 2.5 M solution, 0.01 mol) to a solution of *i*-Pr_2NH (1.01 g, 0.01 mol) in dry THF (20 mL, distilled from Na) under N_2 at −78 °C. The solution was stirred at −78 °C for 5 min, and HMPA (5 mL, dried over molecular sieves) was added, followed after another 5 min by methyl 4-bromocrotonate (1.79 g, 0.01 mol). The reaction mixture was kept at −78 °C for 30 min and then allowed to warm to room temperature overnight. After quenching with glacial AcOH (0.3 mL), the mixture was partitioned between Et_2O and 5% $NaHCO_3$. The Et_2O layer was washed with H_2O, dried ($MgSO_4$), and evaporated. The residual oil was redissolved in a small volume of Et_2O, light petroleum ether (bp 30–60 °C) (60 mL) was added, and the mixture was chilled until an oil separated. The solvent was decanted, and the oil was triturated again with light petroleum ether. The decantates were combined and evaporated to give **19** as an oil (2.93 g, 83%): NMR ($CDCl_3$) δ 2.75 (t, J = 7 Hz, 2 H, CH_2), 3.65 and 3.69 (2 overlapping s, 6 H, CH_3O), 4.17 (t, J = 6 Hz, 1 H, α-CH), 5.75 (d, J = 16 Hz, 1 H, CH=), 6.7 (m, 1 H, CH=), 7.3 (br m, 10 H, aromatic protons). The product (2.93 g) was dissolved in Et_2O (40 mL), and the solution was chilled in an ice bath and stirred vigorously while 1 N HCl (10 mL, 10 mmol) was added dropwise over 30 min. The two-phase mixture was allowed to come to room temperature and stirred overnight. The aqueous layer was separated, concentrated, and freeze-dried, and the light-yellow solid (**17**·HCl) was dried in vacuo at 75 °C over P_2O_5: yield 1.42 g (76%, overall 61%); mp 97–108 °C; TLC, ninhydrin positive spot, R_f 0.7 (cellulose, 2:1 EtOH–3% NH_4Cl); NMR (D_2O) δ 2.92 (t, J = 7 Hz, 2 H, CH_2), 3.73 (s, 3 H, OCH_3), 3.83 (s, 3 H, OCH_3), 4.35 (t, J = 7 Hz, α-CH), 6.03 (d, J = 16 Hz, 1 H, CH=), 6.9 (m, 1 H, CH=). This material was used in the next reaction without further purification.

Dimethyl *N*-(4-Amino-4-deoxy-N^{10}-methylpteroyl)-D,L-2-amino-4-hexenedioate (16). Compound **14** (180 mg, 0.5 mmol) was added in portions over 3 min to a solution of Et_3N (303 mg, 3 mmol) and diethyl phosphorocyanidate (245 mg, 1.5 mmol) in dry DMF (20 mL). After 5 h at room temperature, **17**·HCl (112 mg, 0.5 mmol) was added, followed by a second portion of Et_3N (50 mg, 0.5 mmol). The reaction mixture was left to stir at room temperature for 40 h and evaporated to dryness under reduced pressure. The residue was partitioned between $CHCl_3$ and dilute NH_4OH, and the residue, after evaporation of the $CHCl_3$ layer, was applied onto a column of silica gel (15 g). Elution with 19:1 $CHCl_3$–MeOH yielded a product showing a major TLC spot with R_f 0.4 and a second spot with R_f 0.5 (silica gel, 19:1 $CHCl_3$–MeOH). The impure product was rechromatographed on a similar column, the fractions showing only a single TLC with R_f 0.4 were pooled and evaporated, and the resulting glassy solid was triturated with Et_2O to obtain a light-yellow powder (117 mg, 47%): mp 131–138 °C; IR (KBr) 3400, 3210, 2960 (br), 1725, 1640, 1610 cm^{-1}. Anal. ($C_{23}H_{26}N_8O_5 \cdot 0.5H_2O$) C, H, N.

***N*-(4-Amino-4-deoxy-N^{10}-methylpteroyl)-D,L-2-amino-4-hexenedioic Acid (5).** $Ba(OH)_2 \cdot 8H_2O$ (158 mg, 0.5 mmol) was added to solution of diester **16** (107 mg, 0.217 mmol) in 50% EtOH (15 mL), and the mixture was stirred at room temperature overnight. A small amount of water containing NH_4HCO_3 (80 mg) was then added, the mixture was stirred vigorously for 5 min, the $BaCO_3$ was removed by filtration, and the filtrate was concentrated by rotary evaporation to remove most of the EtOH. Acidification with 10% AcOH and chilling gave a precipitate, which was filtered and dried in a lyophilizer to a light-yellow powder (88 mg, 81%): IR (KBr) 3430, 1640, 1605 cm^{-1}; HPLC on C_{18}-bonded silica gel [reservoir A, 0.025 M NH_4OAc, pH 6.0, containing 2.5% MeCN; reservoir B, 0.1 M NH_4OAc, pH 6.0, containing 10% MeCN; 0.7 mL/min over 20 min] showed a single peak eluting at 14.8 min. Anal. ($C_{21}H_{22}N_8O_5 \cdot 1.75H_2O$) C, H, N.

***N*-(4-Amino-4-deoxypteroyl)-D,L-2-amino-4-hexenedioic Acid (6).** A suspension of compound **15** (188 mg, 0.5 mmol) in dry DMF (15 mL) was treated with Et_3N (101 mg, 1 mmol) and *i*-BuOCOCl (68 mg, 0.5 mmol) and the resulting clear solution was stirred for 10 min. Compound **17**·HCl (112 mg, 0.5 mmol) was then added, followed by Et_3N (40 mg, 0.4 mmol) and *i*-BuOCOCl (27 mg, 0.2 mmol) after a brief period of stirring. After 10 min, a second portion of **17**·HCl (45 mg, 0.2 mmol) was added. Finally a third cycle of addition of Et_3N (20 mg, 0.2 mmol), *i*-BuOCOCl (14 mg, 0.1 mmol), and **17**·HCl (22 mg, 0.1 mmol) was carried out. The reaction mixture was concentrated to dryness in vacuo, and the residue was partitioned between $CHCl_3$ and $NaHCO_3$. The $CHCl_3$ layer was evaporated, and the residue was chromatographed on a column of silica gel (13 g), which was eluted with 19:1 $CHCl_3$–MeOH. Pooled TLC-homogeneous fractions were evaporated, the glassy residue was triturated with Et_2O, and the resulting solid was filtered and dried in vacuo at 60 °C over P_2O_5 to a light-yellow powder (165 mg, 65%): mp 194–197 °C; IR (KBr) 3450, 3200 (sh), 2960 (br), 1725 (ester C=O), 1660, 1635, 1605 cm^{-1}. This material was suspended directly in MeOH (3 mL) and treated with 1 N NaOH (1 mL). When the solid dissolved, the MeOH was partly evaporated, and more 1 N NaOH was added occasionally until TLC showed hydrolysis to be complete. Hydrolysis was followed by the gradual disappearance of fluorescent spots corresponding first to unchanged starting material and then, transiently, to the N^{10}-formyl derivatives of the monoester(s) and diacid. The pH was adjusted to 8 with HCl, the solution was applied onto a DEAE-cellulose column (22 × 1.5 cm, HCO_3^- form), and the column was eluted first with a large volume of H_2O to remove salts and then successively with 0.2 M and 0.4 M NH_4HCO_3. Pooled TLC-pure fractions were freeze-dried to obtain a still impure light-yellow solid (126 mg, 77% crude yield). Further purification of this material was carried out by gradient elution from a C_{18}-bonded silica gel LPLC column [400 mL of 0.1 M NH_4OAc, pH 7.0, containing increasing ammounts of EtOH from 5% to 10%]. Fractions that were TLC-pure were pooled and freeze-dried, and the resulting solid was suspended in dilute NH_4OH. A small amount of insoluble material (apparently some silica gel that had been stripped from the column during chromatography) was removed by filtration, the filtrate was chilled and acidified with 10% AcOH, and the precipitate was collected and dried in vacuo over P_2O_5 at 60 °C to obtain a light-yellow solid (55 mg, 34%). HPLC on C_{18}-bonded silica gel [reservoir A, 0.1 M NH_4OAc, pH 7.0, containing 1% MeCN; reservoir B, same buffer containing 8% MeCN; 0.9 mL/min over 20 min] showed a strong peak at 16.7 min along with a trace of a UV-absorbing impurity (<1% by peak height). Anal. ($C_{20}H_{20}N_8O_5 \cdot 0.75CH_3COOH \cdot 1.5H_2O$) C, H, N.

3-[*N*-(Carboxymethyl)amino]propanoic Acid Bis(ethyl ester) (20). A mixture of ethyl glycinate hydrochloride (17.9 g, 0.2 mol) in absolute EtOH (15 mL) containing ethyl acrylate (15 g, 0.15 mol) and Et_3N (20.2 g, 0.2 mol) was stirred in the dark for 6 days. The thick solid was filtered with the aid of Et_2O, the filter cake was washed generously with Et_2O, and the combined filtrates were evaporated. Distillation of the residue yielded a colorless liquid (19.8 g, 65%): bp 137–140 °C (11 Torr) [lit.[18] bp 108–111 °C (1.5 Torr)]; IR (NaCl) 3340, 2990, 1740 (ester C=O) cm^{-1}; NMR ($CDCl_3$) δ 1.27 (t, J = 7 Hz, 6 H, CH_3), 1.85 (s, 1 H, NH, exchangeable in D_2O), 2.48 (t, J = 5 Hz, 2 H, CH_2CO), 2.93 (t, J = 5 Hz, 2 H, NCH_2CH_2), 3.38 (s, 2 H, NCH_2CO), 4.13 (2 q, J = 7 Hz, 4 H, CH_2O).

3-[*N*-(4-Amino-4-deoxy-N^{10}-methylpteroyl)-*N*-(carboxymethyl)amino]propanoic Acid Bis(ethyl ester) (21). Compound **14** (720 mg, 2 mmol) was added in small portions over 5 min to a solution of Et_3N (606 mg, 6 mmol) and diethyl phosphorocyanidate (978 mg, 6 mmol) in dry DMF (75 mL). After overnight stirring, **20** (812 mg, 4 mmol) was added, and the solution was left at room temperature for 4 days. The residue after rotary evaporation was swirled with Et_2O, and the Et_2O was decanted. The Et_2O-insoluble material was partitioned between $CHCl_3$ and dilute NH_4OH, the $CHCl_3$ layer was evaporated, and the crude product was chromatographed on a silica gel column (50 g, 45 × 2.5 cm), which was eluted successively with 19:1 and

9:1 $CHCl_3$–MeOH. Pooled TLC-pure fractions were evaporated to a glassy solid, which was triturated with Et_2O, filtered, and dried in vacuo at 60 °C over P_2O_5 to obtain a yellow powder (544 mg, 53%): TLC R_f 0.7 (silica gel, 9:1 $CHCl_3$–MeOH); mp 94–105 °C; IR (KBr) 3440 (br), 3200 (br), 2990, 1740 (ester C=O), 1630 (sh), 1615 cm^{-1}. Anal. ($C_{24}H_{30}N_8O_5 \cdot 0.5H_2O$) C, H, N.

3-[*N*-(4-Amino-4-deoxy-N^{10}-methylpteroyl)-*N*-(carboxymethyl)amino]propanoic Acid (7). A solution of **21** (510 mg, 1 mmol) in 50% EtOH (50 mL) was treated with $Ba(OH)_2 \cdot 8H_2O$ (630 mg, 1 mmol) and stirred at room temperature overnight. A solution of NH_4HCO_3 (300 mg, 4 mmol) in a small volume of H_2O was then added, and after 5 min of vigorous stirring, the $BaCO_3$ was removed by filtration. The filtrate was concentrated to remove EtOH and acidified with 10% AcOH. The precipitated solid was collected and dried by lyophilization to obtain a light-yellow powder (362 mg, 76%): TLC R_f 0.9 (cellulose, pH 7.4 phosphate buffer); IR (KBr) 3400 (br), 3200 (sh), 1720 (sh), 1645, 1615 cm^{-1}. Anal. ($C_{20}H_{22}N_8O_5 \cdot 1.75H_2O$) C, H, N.

Biological Assays. The ability of compounds **3**–**7** to inhibit DHFR from murine leukemic cells, to act as substrates and/or inhibitors of partially purified FPGS from mouse liver, and to inhibit the growth of L1210, L1210/R81, and CEM cells was evaluated by methods described earlier.[15,19,20]

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Registry No. L-**3**, 112113-76-5; L-**4**, 112113-77-6; DL-**5**, 112113-78-7; DL-**6**, 112113-79-8; DL-**6** (R^1 = CHO, R^2 = Me), 112113-89-0; **7**, 112113-80-1; L-**8**, 112138-39-3; L-**9**, 5420-67-7; L-**10**, 112113-81-2; L-**11**, 112113-82-3; L-**12**, 112113-83-4; L-**13**·HCl, 112113-84-5; **14**, 19741-14-1; **15**, 89043-75-4; DL-**16**, 112113-85-6; DL-**17**ηdtHCl, 112113-86-7; **18**, 81167-39-7; DL-**19**, 112113-87-8; **20**, 3783-61-7; **21**, 112113-88-9; DHFR, 9002-03-3; H-L-Glu-OH, 56-86-0; $O_2NC_6H_4$-*m*-COCl, 121-90-4; H-L-Glu(OEt)-OH, 1119-33-1; H-L-Glu(*t*-BuO)-OMe·HCl, 6234-01-1; $O_2NC_6H_4$-*o*-COCl, 610-14-0; H-Gly-OEt·HCl, 623-33-6; 2,4-diamino-6-(bromomethyl)pteridine hydrobromide, 52853-40-4; methyl 4-bromocrotonate, 1117-71-1; ethyl acrylate, 140-88-5.

New Actinomycin D Analogues as Superior Chemotherapeutic Agents against Primary and Advanced Colon Tumors and Colon Xenografts in Nude Mice

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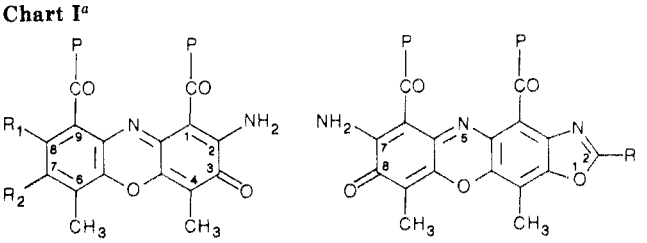
"Reverse" analogues (RAD's) of actinomycin D (AMD) and their antitumor activity against mouse and human colon tumor cells are reported. RAD's are tetracyclic, and they have an oxazole ring fused on the tricyclic phenoxazine chromophore of AMD. The oxazole ring in RAD is substituted at the C-2 carbon with either a CH_3 (in RAD I), a C_6H_5 (in RAD II), or a $CH_2CONH(CH_2)_4NH_2$ (in RAD III) group. In tumor cells and rat hepatic microsomes, RAD's are metabolized to a tricyclic "symmetrical" analogue of AMD (SAD) with the loss of the oxazole ring and its substituents. RAD and SAD are very active in priming superoxides in the presence of microsomal enzymes as well as in inhibiting the synthesis of DNA and the growth of human colon tumor HT-29 cells in vitro. RAD III and SAD efficiently cleave closed circular plasmid pBR322 DNA like the antitumor agent bleomycin. In addition to their strong inhibitory activity against P388 and B_{16} tumors in vitro and in vivo, RAD III and SAD demonstrate high levels of activity against primary C26 and advanced C38 colon tumors in mice and against a xenograft of human colon adenocarcinoma CX-1 in athymic mice. In all these biological activities, the analogues demonstrate superiority to AMD in several experimental tumors. Also, the analogues, in contrast to AMD, show reduced toxicity in tumor-free mice, which is possibly due to the metabolic deactivation of SAD in host organs.

In search for actinomycin D (AMD, 1, Chart I) analogues with a broader antitumor activity and reduced host toxicity, considerable work has been done in our laboratories.[1-6] Recently, we reported the synthesis of two new classes of actinomycin D analogues; one class has a tetracyclic chromophoric structure, which features an oxazole ring attached to the phenoxazinone tricyclic ring of actinomycin D. We termed this class "reverse" analogues (RAD).[7] The other class has the same tricyclic phenoxazinone ring of AMD, but carries two extra groups, an amino and a hydroxyl group. These moieties are substituted at the C-7 and C-8 positions. In this process, the unsymmetrical molecule of AMD is made symmetrical, and

Chart I[a]



[a] P = Thr-D-Val-Pro-Sar-MeVal

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