Methotrexate Analogues. 18. Enhancement of the Antitumor Effect of Methotrexate and 3',5'-Dichloromethotrexate by the Use of Lipid-Soluble Diesters

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Lipophilic methotrexate (MTX) and 3',5'-dichloromethotrexate (DCM) diesters were prepared by HCl-catalyzed esterification or by neutral esterification using cesium carbonate and an alkyl or aralkyl halide in Me_2SO. The products were tested for in vivo antitumor activity against L1210 leukemia in mice to test whether all MTX and DCM diesters are therapeutically equivalent in this species. Contrary to what has been found with simple primary dialkyl esters, ortho-substituted dibenzyl esters of MTX produce longer survival on a q3d×3 schedule than does MTX itself and show a dose-sparing effect comparable to that of MTX at shorter treatment intervals. Thus, MTX bis(6-chloropiperonyl) ester at an MTX-equivalent dose of 5.5 mg/kg gave a +88% increase in median life span (ILS), whereas for MTX a +88% ILS required 30 mg/kg. When the MTX-equivalent dose of MTX bis(6-chloropiperonyl) ester was increased to 40 mg/kg, a +167% ILS was observed, as compared with a +100% ILS with 60 mg/kg of the parent drug. High activity (>100% ILS) was likewise shown by the bis(2,5-dimethylbenzyl), bis(2,6-dichlorobenzyl), and di-3-picolyl esters of MTX and by the bis(1-methylbutyl) ester of DCM. The results of this study indicate that MTX and DCM esters are *not* therapeutically equivalent in mice, despite the high serum esterase activity in this species, and that an up to 10-fold reduction in total administered dose on the q3d×3 schedule is feasible by this approach.

A number of straight-chain alkyl diesters of methotrexate (MTX) amd 3',5'-dichloromethotrexate (DCM) were synthesized by Loo et al.[1-3] and independently in this laboratory[4] as an extension of earlier work of Eisenfeld, Mautner, and Welch,[5] in which the dimethyl ester of MTX was studied in an attempt to overcome lack of penetration of the blood–brain barrier by MTX. The replacement of negatively charged carboxylate groups by lipid-solubilizing hydrocarbon moieties made esters attractive not only as potential means of drug delivery to the central nervous system but also as candidates for the percutaneous treatment of psoriasis.[6,7] More recently we reported that MTX di-*n*-butyl ester (DBMTX), in addition to having unexpected effects on thymidine uptake and thymidine incorporation into DNA,[8,9] is active in vitro against MTX-resistant human leukemic lymphoblasts (CCRF-CEM cells) with normal dihydrofolate reductase levels but an impaired active transport mechanism for MTX.[10] These cells were, in fact, collaterally sensitive to DBMTX, and we postulated that this was due to a decreased ability to transport not only MTX but also reduced folates. Similar collateral sensivity to conventional lipid-soluble antifolates was also noted in our study[10] and was confirmed subsequently by other investigators using several different MTX-resistant cell lines with a defect in MTX transport.[11-13]

The metabolic fate of DBMTX has been shown to be species dependent.[14,15] In the human and the rhesus monkey, serum esterase activity is relatively low, and conversion of DBMTX to MTX by cleavage of the α- and γ-ester groups is a gradual process, with the γ-*n*-butyl ester (γ-MBMTX) being the predominant metabolite for a significant period of time.[15] However, in mice, a species with high serum esterase activity, both esters are cleaved rapidly, so that within a few minutes only MTX is detectable.[16] Since straight-chain alkyl diesters containing up to eight carbon atoms per alkyl group seem to have essentially the same antitumor activity as MTX itself against L1210 leukemia in mice,[2,4] the diesters have come to be regarded as being therapeutically equivalent, in the mouse at least, to MTX itself. We now present evidence that the nature of the ester group can, in fact, favorably influence the antitumor activity of MTX esters even in the mouse and that when the same ester group is compared, this effect is more pronounced in DCM than in MTX.

Chemistry. Diesters reported in this paper were prepared by HCl-catalyzed esterification[3] or by neutral esterification using cesium carbonate and the appropriate alkyl or aralkyl halide. The latter procedure, adapted by us[17] from a method developed for use with simpler *N*-acyl amino acids by Wang et al.,[18] is preferred when the esterifying alcohol is not a liquid, is uneconomical to use in excess, or is acid labile. The reacion may be performed equally well in Me_2SO or DMF, and aralkyl chlorides are sufficiently reactive in these polar solvents to be used instead of the less readily available bromides or iodides. Yields of primary and secondary diesters obtained by neutral esterification (Table I) ranged from 31 to 76% but were not optimized in every instance. Several variations

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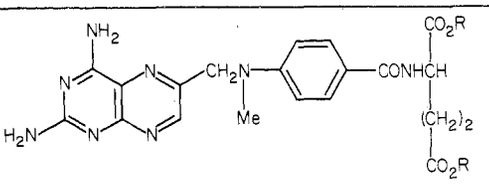
Table I. Physical Constants of Methotrexate Diesters 1-11



no.	R	mp, °C	yield, %	method[a]	R_f[b]	formula	anal.[c]
1	$CH_2(CH_2)_{10}CH_3$	128-130	74	D	0.84	$C_{44}H_{70}N_8O_5$	C, H, N
2	$CH_2(CH_2)_{14}CH_3$	130-138	58	D	0.91	$C_{52}H_{86}N_8O_5$	C, H, N
3	$CH_2(CH_2)_{16}CH_3$	160-168	76	D	0.83	$C_{56}H_{94}N_8O_5$	C, H, N
4	$CH_2C_6H_5$	146-147.5	70	A	0.31[d]	$C_{34}H_{34}N_8O_5 \cdot 0.3H_2O$[e]	C, H, N
			68	B			
			50	E			
			29	F			
5	$C(CH_3)_3$	134-136	15	F	0.42[f]	$C_{28}H_{38}N_8O_5 \cdot H_2O$	C, H, N
6	$CH_2C_6H_3(2,5\text{-}Me_2)$	135-137	70	D	0.80	$C_{38}H_{42}N_8O_5$	C, H, N
7	$CH_2C_6H_2(2,4,6\text{-}Me_3)$	115-123	49	B	0.72	$C_{40}H_{46}N_8O_5$	C, H, N
8	$CH_2C_6H_3(2,6\text{-}Cl_2)$	176-180	64	C	0.60[d]	$C_{34}H_{30}N_8Cl_4O_5$	C, H, N, Cl
9	$CH_2C_6H_2[6\text{-}Cl\text{-}3,4\text{-}(OCH_2O)]$	130-138	47	D	0.84	$C_{36}H_{32}N_8Cl_2O_5 \cdot 0.1CHCl_3$	C, H, N, Cl
10	$CH_2(3\text{-}C_5H_4N)$	115-125	31	A	0.59	$C_{32}H_{32}N_{10}O_5 \cdot 0.5H_2O$	C, H, N
11	$\alpha\text{-}CH_2(3\text{-}C_5H_4N)$, $\gamma\text{-}CH_2(CH_2)_2CH_3$	151-153	66	G	0.71	$C_{30}H_{35}N_9O_5 \cdot 0.2H_2O$	C, H, N

[a] See Experimental Section. [b] Unless otherwise noted, R_f values are for silica gel plates developed with 3:1 $CHCl_3$-MeOH. [c] Analyses were within ± 0.4% of theoretical values for the indicated elements. [d] $CHCl_3$-MeOH, 6:1. [e] The microanalytical data are for a sample prepared via method F by Nick Papathanasopoulos. [f] Toluene-MeOH, 4:1.

of the basic method were investigated, but only with the dibenzyl ester **4** was a comparison of yields made. As indicated in Table I, the highest yield of **4** (70%) was achieved by adding benzyl chloride to the preformed dicesium salt of MTX. Essentially the same yield was obtained, however, by simply adding cesium carbonate to a suspension of MTX disodium salt. Thus when starting with MTX disodium salt it is not necessary to prepare the free acid in a separate step. As an alternative to the cesium salt method, the use of a crown ether to augment the nucleophilicity of the carboxylate ion was also examined, but the yield was somewhat decreased. A notable observation, on the other hand, was that when the alkylating reagent was 2,6-dichlorobenzyl bromide, neutral esterification of MTX could be achieved successfull *even with the disodium salt*. In contrast to the reaction of ordinary alkyl bromides, which usually required 24–48 h and an excess of reagent, esterification with 2,6-dichlorobenzyl bromide was complete after just 3 h and required no more than stoichiometric proportions of reactants, with no cesium carbonate. The most likely explanation for the higher reactivity in this instance is that (a) electron-withdrawing *o*-chloro substituents activate the neighboring benzylic carbon for nucleophilic attack, and (b) displacement of a bulky bromine atom by carboxylate oxygen provides relief of steric strain. 2,6-Dichlorobenzyl bromide has likewise been found to react efficiently with the disodium salts of folic acid and 5-formyltetrahydrofolic acid (folinic acid).[4] In only one instance was direct esterification of MTX by the cesium carbonate method unsuccessful. This was in the attempted synthesis of MTX di-*tert*-butyl ester (**11**), which had to be made instead from 4-amino-4-deoxy-N^{10}-methylpteroic acid and di-*tert*-butyl glutamate via mixed anhydride coupling. The yield by the latter method was only 15%, but probably would have been higher if our newer diethyl phosphorocyanidate procedure[19,20] had been used. It thus appears that while neutral esterification may be used to obtain a wide variety of primary and secondary diesters of MTX, tertiary diesters are not accessible by this route.

Antitumor Activity. In vivo assays of the effect of MTX diesters were carried out in L1210 leukemic mice according to a standard NCI protocol.[21] The tumor (10^5 cells in the ascites form) was implanted ip on day 0, and treatment was given on days 1, 4, and 7 by ip injection. Nonesterified MTX and DCM were administered in solution at pH 7.5–8.5. However, because of their very low water solubility, the diesters were administered with 10% Tween 80 in sterile water or 0.9% NaCl as the vehicle. Even under these conditions, complete solubilization was not achieved with the more lipophilic compounds, so that in all cases the highest doses were administered as partially dissolved suspensions. Since there was some concern thast the use of 10% Tween 80 might alter the membrane permeability of the tumor cells in the peritoneal cavity, control experiments in which MTX was given ip in 10% Tween 80 rather than in water showed no significant difference in either optimal dose or the therapeutic response. Comparison of the various primary dialkyl ester of MTX (Table II, entries 1–8) did not disclose any greater prolongation in survival than with MTX itself, in agreement with earlier reports.[2,4] The highly hindered di-*tert*-butyl ester **5** (entry 9) was much less active than MTX, suggesting that cleavage of the α-ester is probably essential for activity.[14] On the other hand, several of the substituted dibenzyl esters (entries 10–13) gave ILS values that were markedly higher than those of MTX. The best examples were the bis(2,6-dichlorobenzyl) ester **8** and bis(6-chloropiperonyl) ester **9** which gave a +167% ILS at 20 and 40 mg/kg, respectively, with no weight loss indicative of toxicity. In pooled control experiments using MTX, the optimally tolerated q3d×3 dose of 30 mg/kg gave only a +88% ILS, and doubling of the dose to 60 mg/kg improved survival only marginally, with some weight loss. More interesting than just the increased ILS with these substituted dibenzyl esters was the finding that the equiactive molar dose was much lower than with MTX

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Table II. Antitumor Activity of Methotrexate and 3′,5′-Dichloromethotrexate Diesters against L1210 Leukemia in Mice

entry[a]	parent drug	ester group	dose,[b] mg/kg	no. of mice	av wt on day 1, g/ mouse	7-day wt change g/mouse	7-day wt change %	median survival, days range	median survival, days T/C	% ILS
1	MTX	*n*-propyl	20 (17)	5	21.4	+2.1	+10	11-17	13/9	+44
			40 (34)	5	21.1	−0.3	−1	16-19	19/9	+100
2	MTX	*n*-butyl	15 (12)	10	25.3[c]	−4.2	−16	15-20	16/9	+77
			30 (24)	10	26.1	−4.1	−16	11-17	15/9	+66
			60 (48)	10	24.9	−5.1	−20	7-24	17/9	+88
3	MTX	*n*-pentyl	10 (8)	5	21.5	−0.6	−3	10-15	12/9	+33
			20 (16)	5	20.8	+0.4	+2	14-18	14/9	+55
4	MTX	3-methylbutyl	10 (8)	5	21.9	+3.0	+10	11-13	12/9	+33
			20 (16)	5	23.7	+1.9	+9	12-16	14/9	+55
			40 (32)	5	23.2	−1.0	−5	11-21	17/9	+88
5	MTX	*n*-octyl	7.5 (5)	5	26.4	−0.4	−2	10-14	13/9	+44
			15 (10)	5	25.2	−0.8	−3	14-18	15/9	+66
6	MTX	2-ethoxyethyl	20 (14)	5	22.6	+1.5	+7	11-15	13/9	+44
			40 (28)	5	23.1	+0.5	+2	12-20	14/9	+55
7 (1)	MTX	*n*-dodecyl	30 (17)	9	23.2	+3.0	+13	10-14	12/9	+33
			60 (34)	9	24.2	0.0	0	8-21	18/9	+100
8 (4)	MTX	benzyl	15 (7)	5	25.8	+0.4	+2	12-14	12/9	+33
			30 (14)	5	25.6	−1.6	−6	17-22	18/9	+100
9 (5)	MTX	*tert*-butyl	400 (320)	5	27.6	−2.6	−9	14-20	15/11	+36
10 (6)	MTX	2,5-dimethylbenzyl	10 (6.5)	5	23.0	+1.6	+7	13-18	17/9	+88
			20 (13)	5	22.0	+0.7	+3	14-23	19/9	+111
			40 (26)	5	20.7	+0.7	+3	15-23	23/9	+155
11 (7)	MTX	2,4,6-trimethylbenzyl	20 (13)	5	20.4	+0.8	+4	11-19	13/9	+44
			40 (26)	5	22.0	0.0	0	11-21	17/9	+88
12 (8)	MTX	2,6-dichlorobenzyl	10 (6)	5	21.6	+1.4	+6	15-21	15/9	+66
			20 (12)	5	22.4	+0.8	+4	17-25[d]	24/9	+167
13 (9)	MTX	6-chloropiperonyl	10 (5.5)	5	22.5	+2.1	+9	13-20	17/9	+88
			20 (11)	5	22.2	+1.0	+4	13-22	18/9	+100
			40 (22)	5	21.4	+0.2	+1	22-27[d]	24/9	+167
14 (10)	MTX	3-picolyl	10 (7)	5	21.3	+1.1	+5	14-22	17/9	+88
			20 (14)	5	22.0	+1.0	+5	15-17	16/9	+77
			40 (28)	5	26.0	−2.0	−8	21-31	22/9	+144
15 (11)	MTX	α-*n*-butyl, γ-3-picolyl	20 (15)	5	21.1	+2.4	+11	12-14	12/8	+50
			40 (30)	5	21.2	+1.7	+8	14-17	18/8	+75
			80 (60)	5	22.0	+0.3	+1	13-20	19/8	+137
16	DCM	*n*-butyl	45 (30)	5	25.0	+1.0	+4	14-19	18/11	+64
			90 (60)	5	27.8	−3.8	−13	15-24	20/11	+82
17	DCM	2-methylpropyl	40 (28)	5	21.4	+0.1	+1	12-18	13/9	+44
			80 (56)	5	19.8	+1.3	+7	12-23	19/9	+111
18	DCM	3-methylbutyl	20 (16)	5	22.1	+2.7	+12	12-13	13/9	+44
			40 (32)	5	21.2	+0.1	+1	13-19	15/9	+67
			80 (64)	5	20.8	−1.0	−5	10-23	18/9	+100
19	DCM	1-methylbutyl	20 (16)	5	22.1	+2.6	+17	11-14	14/9	+55
			40 (32)	5	20.6	+0.1	+7	13-19	15/9	+66
			80 (64)	5	21.2	−1.5	−7	13-26	21/9	+133
			120 (96)	10	21.5	+1.4	+6	13-36[e]	22/9	+145
			140 (112)	5	21.6	−3.2	−24	8-27	23/9	+155
20	DCM	*n*-octyl	45 (32)	5	26.2	−1.4	−5	11-13	13/9	+44
			90 (64)	5	28.2	−3.6	−13	13-19	15/9	+66
			180 (128)	5	28.2	−5.8	−21	14-23	20/9	+122
MTX			15	19	23.1	+2.1	+9	13-22	16/9	+77
			30	23	24.6	−0.6	−2	14-23	17/9	+88
			60	19	23.9	−1.3	−5	9-28	18/9	+100
DCM			120	10	22.4	+2.4	+11	9-15	11/9	+22
			160	10	22.5	+2.9	+13	10-16	13/9	+44
			200	5	24.2	+1.8	+8	13-18	16/9	+78
			240	5	22.4	+2.1	+10	13-18	15/9	+67
			280	5	21.4	+1.4	+7	14-20	17/9	+89

[a] Entries 1-6 and 15-19 are previously characterized compounds for this laboratory.[4] [b] Numbers in parentheses are MTX- or DCM-equivalent doses in milligrams per kilogram. [c] Weight change in this experiment was recorded on day 11 instead of day 7. [d] One animal surviving to day 30 was included in the T/C. [e] Two animals surviving to day 60 were included in the T/C.

itself. With the bis(6-chloropiperonyl) ester **9**, for example, an ILS of +88% was observed at 10 mg/kg, a molar dose corresponding to only 5.5 mg/kg of the parent acid. When this is compared with the 30 mg/kg of MTX to obtain the same +88% ILS, it is evident that the introduction of two aralkyl ester groups leads to a nearly 6-fold decrease in the total amount of administered drug. Since the host toxicity of MTX would be expected to reflect inter alia the total drug load, it appears that substituted dibenzyl esters may have a sparing effect relative to the parent acid. From the limited number of diesters tested to date, it is not possible to draw firm conclusions regarding optimal structure–activity requirements for this sparing effect. We initially speculated that the presence of one or two *o*-chloro substituents was a critical factor, but this seems not to be true, since the bis(2,5-dimethylbenzyl) ester **6** (entry 10) also produced about twice as great an ILS as MTX at the same molar dose. That ortho substitution as such is responsible

can be ruled out as well, since the very active di-3-picoyl ester **10** (entry 14) obviously lacks this structural feature. It must therefore be concluded that a larger number of analogues will have to be tested before a definitive structure–activity pattern begins to emerge.

Antitumor assays of the dialkyl esters of DCM (Table II, entries 16–20) showed all of them to be superior to DCM free acid in terms of molar potency, even though this had not been true with dialkyl esters of MTX. The best example was DCM bis(1-methylbutyl) ester, a secondary alkyl derivative. This compound (entry 19) gave a +145% ILS at 120 mg/kg (q3d×3) with 2 out of 10 long-term survivors (60 days). Further dose escalation to 140 mg/kg gave a +155% ILS, but there was marked weight loss. We observed survival increases of 60–90% with DCM on the q3d×3 schedule at 200–280 mg/kg. This may be compared with similar 60–70% increases in survival with the di-*n*-butyl, bis(3-methylbutyl), and bis(1-methylbutyl) esters at DCM-equivalent doses of only ~30 mg/kg. We have given DCM at doses of up to 400 mg/kg (q3d×3) without obtaining more than a +90% ILS. It thus appears that simple C_4 and C_5 dialkyl esters of DCM are capable of decreasing the total drug burden by as much as 10-fold relative to the parent acid and that esters of secondary alcohols (cf. entry 19) are as effective as those of primary alcohols in terms of both molar potency and increased survival.

It is reasonable to expect that secondary alkyl esters, and perhaps certain substituted benzyl esters with bulkyl ortho substituents, would be intermediate in chemical stability between nonhindered primary alkyl esters and highly hindered compounds such as **5**, which shows minimal activity. Such esters of intermediate stability might therefore be predicted to show improved prodrug action, and the antitumor effect of these compounds on the q3d×3 schedule might be more appropriately compared with that of the parent drug when the latter is given at more closely spaced intervals. In our experience the optimal MTX dose on the q3d×3 and qd×9 schedules is 30 and 4 mg/kg, respectively, i.e., a ~2.5-fold reduction in total administered dose on daily treatment. When MTX is given b.i.d.×10, we have observed the optimal dose to be just 0.5 mg/kg, i.e., a total of 10 mg/kg corresponding to a 9-fold decrease relative to the q3d×3 regimen. It can thus be seen that several of the MTX diesters have a potency on the q3d×3 schedule that falls between that of MTX given qd×9 and that of MTX b.i.d.×10. A similar interpretation of the data for DCM diesters can be made.

Although our results are consistent with the idea that diester derivatives of MTX act solely as slow-release agents of the parent acid, we cannot at this time exclude the possibility that some of their antitumor activity partially reflects the effect of cytotoxic γ-ester metabolites with the ability to bind almost as tightly as MTX to the target enzyme dihydrofolate reductase. It is also reasonable to expect that the favorable dose effect observed with MTX and DCM diesters is partly due to the fact that these compounds form sparingly soluble depots in the peritoneal cavity and thus are only slowly absorbed into the circulation. This would simulate, and be pharmacokinetically equivalent to, slow intravenous infusion. Additional chemical and pharmacological studies on these and related MTX and DCM diesters would assist the further development of this special class of lipid-soluble antifols[10] as therapeutic agents.

Experimental Section

Melting points (uncorrected) were determined in Pyrex capillary tubes in a Mel-Temp apparatus (Laboratory Devices, Cambridge, MA). Rotary evaporation of aqueous, Me_2SO, and DMF solutions was performed at 30–40 °C (bath temperature) with the aid of a vacuum pump and dry ice/acetone to cool the receiver. The Me_2SO and DMF used as reaction solvents were dried over Linde 4Å molecular sieves (Fisher Scientific, Boston, MA). Alkyl and aralkyl halides were purchased from Aldrich Chemical Co., Milwaukee, WI, and were used as received. Cesium carbonate was obtained from Alfa Products, Danvers, MA. TLC was carried out on Anasil OF silica gel plates (250-μm thickness, Analabs, North Haven, CT), with spots being visualized at 254 nm in a viewing chamber. Column chromatography was performed on Baker 5-3405 silica gel (60–200 mesh). Infrared spectra were recorded on a Perkin-Elmer Model 137B double-beam recording spectrophotometer, and NMR spectra were obtained on a Varian T-60A instrument with tetramethylsilane as the reference. Analytical samples were dried overnight at 75 °C (0.01 mmHg) over P_2O_5. Microanalyses were performed by Galbraith Laboratories, Knoxville, TN.

MTX disodium salt was provided by the National Cancer Institute, and DCM free acid was generously donated by Lederle Laboratories, Pearl River, NY. For the preparation of MTX free acid, the disodium salt was dissolved in water, the pH was adjusted to 6.0 with glacial acetic acid, and the precipitate was collected, washed with ice-cold water, followed by ethanol and ether, and, finally, dried. For stoichiometry calculations, the free acid was assumed to be a dihydrate. To prepare MTX dicesium salt, the free acid was suspended in water, the pH was adjusted to 7.5–8.0 with 20% aqueous cesium carbonate, and the solution was concentrated to dryness. As much water as possible was removed by repeated entrainment with benzene on a rotary evaporator, and the residual solid was washed with ether, filtered, pulverized until yellow in a mortar, and dried in vacuo at 75 °C. Stoichiometry calculations were based on the amount of free acid used.

Synthesis from MTX Disodium Salt (Method A). MTX Dibenzyl Ester (4). The dicesium salt corresponding to 0.1 g (0.2 mmol) of MTX free acid was suspended in Me_2SO (4 mL), and to this suspension was added with stirring a solution of benzyl chloride (0.1 g, 0.8 mmol) in Me_2SO (1 mL). After being stirred at room temperature for 20 h, the solution was poured into water. The emulsion was broken up by addition of solid NaCl, and the product was filtered, washed with water, and dried in vacuo over P_2O_5. The crude diester was dissolved in a minimum of $CHCl_3$ and applied onto a silica gel column (20 g), which was eluted consecutively with 99:1 (200 mL), and 96:4 (200 mL) $CHCl_3$–MeOH. Individual 8-mL fractions were monitored by TLC, and appropriately pooled fractions (tubes 54–68) were combined and evaporated to obtain a yellow solid (0.1 g): IR (KCl) ν_{max} 3450, 1750 (ester C=O), 1615–1640 (amide C=O), 1565, 1540, 1515, 1450, 1385, 1355, 1315, 1250, 1205, 1100, 915, 830, 740–770, 700 cm^{-1}; NMR (Me_2SO-d_6) δ 2.2 (m, CH_2CH_2), 3.2 (s, NCH_3), 3.3 (s, NCH_2), 4.5 (m, α-CH), 5.09 (s, γ-CO_2CH_2Ar), 5.12 (s, α-CO_2CH_2Ar), 6.4–8.6 (complex m, 2- and 4-NH_2, CONH, pteridine C-7, aromatic protons).

Synthesis from MTX Disodium Salt and Cesium Carbonate (Method B). MTX Bis(2,4,6-trimethylbenzyl) Ester (7). 2,4,6-Trimethylbenzyl chloride (0.28 g, 1.6 mmol) was added to a stirred suspension of MTX disodium salt (0.2 g, 0.4 mmol) and cesium carbonate (0.26 g, 0.8 mmol) in Me_2SO (10 mL). After being stirred at room temperature for 24 h, the mixture was concentrated to dryness, and the residue was triturated with water (25 mL), filtered, and dried in vacuo over P_2O_5. The crude diester (0.33 g) was dissolved in a minimal volume of 98:2 $CHCl_3$–MeOH and applied onto a silica gel column (12 g), which was eluted with the same mixture. Individual 9-mL fractions were monitored by TLC, pooled appropriately (tubes 16–55), and evaporated to a yellow solid (0.15 g): IR (KCl) ν 3450, 2970, 1740 (ester C=O), 1640 (amide C=O), 1565, 1505, 1450, 1380, 1250, 1180, 1100, 1030, 890, 830, 770 cm^{-1}.

Synthesis from MTX Disodium Salt Without Cesium Carbonate (Method C). MTX Bis(2,6-dichlorobenzyl) Ester (8). 2,6-Dichlorobenzyl bromide (0.1 g, 0.4 mmol) was added to a suspension of MTX disodium salt (0.1 g, 0.2 mmol) in Me_2SO (5 mL), and the mixture was stirred at room temperature for 3 h. The clear solution was concentrated to an amber semisolid on the rotary evaporator. Trituration with water (20 mL) produced a solid, which was collected, dried in vacuo over P_2O_5,

redissolved in a minimal volume of 98:2 $CHCl_3$–MeOH, and applied onto a silica gel column (6 g). The column was eluted with the same solvent mixture, and individual 8-mL fractions were appropriately pooled (tubes 13–37) and evaporated: yield 0.1 g; IR (KCl) ν_{max} 3450, 1740 (ester C=O), 1615–1640 (amide C=O), 1565, 1535, 1505, 1450, 1370, 1350, 1250, 1205, 1100, 975, 830, 780, 770 cm^{-1}.

Synthesis from MTX Free Acid and Cesium Carbonate (Method D). MTX Bis(6-chloropiperonyl) Ester (9). 6-Chloropiperonyl chloride (0.17 g, 0.8 mmol) was added to a suspension of MTX free acid (0.1 g, 0.2 mmol) and cesium carbonate (0.13 g, 0.4 mmol) in Me_2SO (5 mL). After being stirred at room temperature for 45 h, the reaction mixture was concentrated to dryness, and the residue was triturated with water, suction filtered, and dried in vacuo over P_2O_5. The crude diester (0.12 g) was dissolved in a minimal volume of 98:2 $CHCl_3$–MeOH and applied onto a silica gel column (5 g), which was eluted with the same solvent mixture. Individual 8-mL fractions were monitored by TLC, appropriately pooled (tubes 17–25), and evaporated to obtain a yellow solid (0.08 g): IR (KCl) ν_{max} 3450, 1740 (ester C=O), 1615–1640 (amide C=O), 1565, 1535, 1505, 1480, 1450, 1420, 1330–1370, 1240, 1200, 1125, 1100, 1040, 1000, 860, 830, 780, 725, 695 cm^{-1}; NMR ($CDCl_3$) δ 2.3 (m, CH_2CH_2), 3.1 (s, NCH_3), 4.5 (m, CH_2N and α-CH), 5.0 (s, γ-CO_2CH_2Ar), 5.1 (s, α-CO_2CH_2Ar), 5.9 (s, OCH_2O), 6.2–8.6 (complex m, 2- and 4-NH_2, CONH, pteridine C-7, and aromatic protons).

Synthesis from MTX Disodium Salt and 18-Crown-6 (Method E). MTX Dibenzyl Ester (4). Methanol (30 mL) was added to a mixture of MTX disodium salt (0.2 g, 0.4 mmol) and 18-crown-6 (0.264 g, 0.1 mmol; Aldrich Chemical Co., Milwaukee, WI). The resulting solution was evaporated to dryness on the rotary evaporator, and the last traces of alcohol were removed from the orange residue by entrainment with benzene (50 mL). The residue was then suspended in Me_2SO (10 mL), a solution of benzyl chloride (0.2 g, 1.6 mmol) in Me_2SO (2 mL) was added, and the mixture was stirred at room temperature for 20 h. The Me_2SO was removed by rotary evaporation, and the product was triturated with water, filtered, dried in vacuo over P_2O_5, and purified by chromatography on a silica gel column (6 g): yield 0.13 g.

Synthesis from 4-Amino-4-deoxy-N^{10}-methylpteroic Acid (Method F). MTX Dibenzyl Ester (4). The pteroic acid (1.1 g, 3.0 mmol)[19] was dissolved in hot DMF (100 mL). The stirred solution was allowed to cool at room temperature, and to it were added successively trimethylamine (1 mL) and isopropyl chloroformate (0.55 g, 4.5 mmol). After 15 min to complete the formation of the mixed anhydride, a solution of dibenzyl L-glutamate hydrochloride salt (2.2 g, 6 mmol) in DMF (20 mL) containing triethylamine (1 mL) was added. The reaction was heated to 80 °C for 30 min, the DMF was evaporated under reduced pressure, and the semisolid residue was triturated with 4% NH_4OH until solidification occurred. The product was collected, washed with water, dried over P_2O_5, and chromatographed on a silica gel column (70 g): yield 0.55 g.

Synthesis from an MTX Ester and Cesium Carbonate in DMF Solvent (Method G). MTX γ-*n*-Butyl α-3-Picolyl Ester (11). 3-Picolyl chloride hydrochloride salt (0.2 g, 1.15 mmol) was added to a mixture of MTX γ-*n*-butyl ester (0.39 g, 0.76 mmol) and cesium carbonate (0.62 g, 1.9 mmol) in DMF (30 mL). After being stirred at room temperature for 22 h, the reaction mixture was filtered, and the filtrate was evaporated to dryness under reduced pressure. The solid was triturated with ether, dried in vacuo over P_2O_5, dissolved in a minimum of 95:5 $CHCl_3$–MeOH, and applied onto a silica gel column (25 g), which was eluted with the same mixed solvent (900 mL). Individual 9-mL fractions were monitored by TLC, appropriately pooled (tubes 27–80), and evaporated to obtain a yellow solid (0.3 g): IR (KCl) ν_{max} 3330, 2950, 1740 (ester C=O), 1615–1640 (amide C=O), 1560, 1515, 1450, 1300–1375, 1250, 1200, 1100, 830, 770, 715 cm^{-1}; NMR ($CDCl_3$ + Me_2SO-d_6) δ 0.9 (t, $CH_3CH_2CH_2$), 1.4 (m, $CH_2CH_2CH_3$), 2.3 (m, glutamyl CH_2CH_2), 4.0 (t, γ-CO_2CH_2), 4.6 (m, CH), 4.7 (s, NCH_2), 5.1 (s, α-$CH_2C_5H_4N$), 5.8–8.6 (complex m, 2- and 4-NH_2, CONH, pteridine C-7, aromatic protons).

Antitumor Assay. In vivo testing of the MTX esters were performed in B6D2F_1J male mice (Jackson Laboratories, Bar Harbor, ME) according to a standard National Cancer Institute Protocol.[21] Animals weighing 20–25 g were inoculated ip with 10^5 L1210 murine leukemia cells on day 0, randomized into test and control groups, and injected ip on days 1, 4, and 7 with solutions (or suspensions) of the various compounds in 10% Tween 80 made up with sterile H_2O or 0.9% NaCl. A minumum of four dose levels covering a 6- to 8-fold range of concentrations was used. Animals were watered and fed a standard laboratory diet ad libitum. Individual weights were recorded on days 1 and 7, and the mean weight change was calculated for each group and expressed as a percentage. The day of death was recorded for each animal, and the median day of death was used to calculate survival relative to untreated controls. Increased life span (ILS) was calculated from the following formula:

$$\% \text{ ILS} = 100[(T/C) - 1]$$

where T and C are the median day of death for the treated and control groups, respectively. For the sake of brevity, the data in Table II include only nontoxic doses at which the percent ILS was equal to or greater than +33%.

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Registry No. **1**, 86669-30-9; **2**, 86669-31-0; **3**, 86669-32-1; **4**, 60219-06-9; **5**, 86669-33-2; **6**, 86669-34-3; **7**, 86669-35-4; **8**, 86669-36-5; **9**, 86688-51-9; **10**, 86669-37-6; **11**, 86669-38-7; di-*n*-propyl methotrexate, 50714-20-0; di-*n*-butyl methotrexate, 50602-77-2; di-*n*-pentyl methotrexate, 50602-78-3; bis(3-methylbutyl) methotrexate, 50714-23-3; di-*n*-octyl methotrexate, 50602-80-7; bis(2-ethoxyethyl) methotrexate, 50714-27-7; di-*n*-butyl 3′,5′-dichloromethotrexate, 86669-39-8; bis(2-methylpropyl) 3′,5′-dichloromethotrexate, 86669-40-1; bis(3-methylbutyl) 3′,5′-dichloromethotrexate, 86669-41-2; bis(1-methylbutyl) 3′,5′-dichloromethotrexate, 86669-42-3; di-*n*-octyl 3′,5′-dichloromethotrexate, 86669-43-4; dicesium methotrexate, 86669-44-5; 2,4,6-trimethylbenzyl chloride, 1585-16-6; disodium methotrexate, 7413-34-5; 2,6-dichlorobenzyl bromide, 20443-98-5; 6-chloropiperonyl chloride, 23468-31-7; methotrexate, 59-05-2; 4-amino-4-deoxy-N^{10}-methylpteroic acid, 19741-14-1; dibenzyl L-glutamate hydrochloride, 4561-10-8; 3-picolyl chloride hydrochloride, 6959-48-4.