Methotrexate Analogues. 21. Divergent Influence of Alkyl Chain Length on the Dihydrofolate Reductase Affinity and Cytotoxicity of Methotrexate Monoesters

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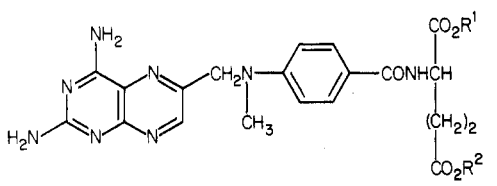
n-Octyl, *n*-dodecyl, and *n*-hexadecyl α- and γ-esters of methotrexate (MTX) were compared with the previously described α- and γ-*n*-butyl esters and with MTX as inhibitors of dihydrofolate reductase (DHFR) and human leukemic lymphoblasts (CEM cells) in culture. The overall order of activity in both test systems was MTX > MTX γ-esters > MTX α-esters. In the DHFR assay the activity of the α-esters followed the order $C_4 > C_8 \simeq C_{12} > C_{16}$, whereas for the γ-esters this order was $C_4 \simeq C_8 > C_{12} > C_{16}$. On the other hand, the order of cytotoxic activity in culture in both series was $C_{16} > C_{12} > C_8 > C_4$. Increasing the alkyl chain length in the ester moiety therefore decreases DHFR affinity but increases cytotoxicity. The most potent member of the compounds tested was the γ-*n*-hexadecyl ester, whose IC_{50} against CEM cells was 0.11 μM as compared with 0.025 μM for MTX. In a comparison of the effect of treatment with the γ-*n*-hexadecyl ester (10^{-5} M, 1 h) on DNA synthesis in CEM and CEM/MTX cells, the latter of which are 120-fold resistant to MTX by virtue of a transport defect, the ester produced only 4-fold less inhibition in the resistant line than in the parental line. These results suggest possible use of this compound or related derivatives in the treatment of MTX-resistant tumors with impaired transport.

Monoesters[1–4] and diesters[2,4–10] of the antitumor agent methotrexate (MTX) are of potential therapeutic interest



MTX, $R^1 = R^2 = H$
MTX α-esters, $R^1 = n\text{-}C_8H_{17}$ (1), $n\text{-}C_{12}H_{25}$ (3), $n\text{-}C_{16}H_{33}$ (5), $R^2 = H$
MTX γ-esters, $R^1 = H$; $R^2 = n\text{-}C_8H_{17}$ (2), $n\text{-}C_{12}H_{25}$ (4), $n\text{-}C_{16}H_{33}$ (6)

because of their enhanced lipophilicity relative to the parent acid. Replacement of the ionizable carboxyl groups in the side chain by hydrophobic esters offers an improved opportunity for uptake into cells by passive diffusion as opposed to active transport and, therefore, represents an approach to the treatment of tumors that are MTX resistant by virtue of a transport defect.[2,11,12]

In an earlier paper[1] we reported that, in a series of short-chain MTX monoesters ranging from one to four carbons in length, γ-esters were more toxic to cultured human leukemic lymphoblasts (CEM cells) than the corresponding α-esters. On the other hand, activity among the γ-esters varied in the order Me > Et > *n*-Bu, whereas for the α-isomers, the opposite seemed to be the case (*n*-Bu > Et). In order to further delineate the structure–activity relationships among these compounds, we have now examined th cytotoxicity and dihydrofolate reductase (DHFR) inhibitory activity of several pairs of monoesters of longer chain length. We describe here the chemical synthesis of the α- and γ-*n*-octyl, α- and γ-*n*-dodecyl, and α- and γ-hexadecyl esters (1–6) and compare their in vitro effects with those of MTX and its α- and γ-*n*-butyl esters. Increasing the length of the alkyl ester had a negative effect on DHFR binding in both series but produced an increase in cytotoxicity consistent with either enhanced uptake or more extensive cleavage to MTX intracellularly. The α- and γ-*n*-hexadecyl esters were roughly 10 times more potent than the α- and γ-*n*-butyl esters, with the γ-*n*-hexadecyl ester (6) showing activity only 4-fold lower than that of MTX itself. We also report that 6 qualitatively resembles MTX di-*n*-butyl ester in its ability to block not only the incorporation of deoxyuridine (dUrd) into DNA but also that of thymidine (dThd).[8,9] This effect, which does not occur with MTX, has been ascribed to interference with pyrimidine nucleoside uptake[9] and is of biochemical interest because the reutilization of exogenous dThd by cells represents a potential mechanism of MTX resistance.[13]

Chemistry. α-Esters and γ-esters of MTX were prepared previously by acid-catalyzed esterification in the presence of a limited amount of HCl (1.5 equiv/mol of MTX[1] or by partial saponification of a diester with NaOH (1.0 equiv/mol of MTX diester).[1,14] With both methods the monoesters were unavoidably accompanied by some of the corresponding diester, as well as by free MTX. The γ-isomer was the preponderant monoester under the acidic conditions and was the sole isolated product under the alkaline conditions. In the present work we employed neutral esterification with an alkyl halide and Cs_2CO_3,[15] using 1 molar equiv of alkyl halide instead of 2. While this method again gave mainly diesters, the desired monoesters

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were formed in amounts that were sufficient for our intended in vitro bioassays. For the three alkyl halides used (Table I), the yields of the diesters were 20–40% and those of the α- and γ-esters were 5–10 and 10–15%, respectively. Thus, the use of a single molar equivalent of alkyl halide did not completely prevent diester formation, suggesting that a second esterification of the glutamate side chain is facilitated when one of the carboxyls is already esterified. The slight preponderance of the γ-ester is consistent with either more rapid esterification of the γ- and α-COOH in MTX or more rapid formation of diester from α- than from γ-ester.

As in our previous work,[1] the monoesters were separable from one another, as well as from the diesters and MTX, by *dry-column* chromatography on silica gel. The compounds designated as γ-esters were consistently eluted more slowly than the α-esters, in agreement with the relative acidities of the free COOH groups in MTX.[16] The monoesters were also resolvable by TLC on silica gel. As shown in Table I, compounds formulated as α-esters (**1**, **3**, and **5**) had R_f values in the 0.2–0.3 range, whereas the putative γ-isomers (**2**, **4**, and **6**) had R_f values of 0.1–0.2. There was likewise a consistent pattern in melting temperatures, with the fast-moving isomers giving melting point (decomposition) values 20–30 °C higher than those of the slow-moving isomers. These trends were in agreement with our earlier observations with the methyl, ethyl, and *n*-butyl esters[1] and were further reinforced by the bioassay results discussed below.

Because of the importance of unequivocally establishing the location of the ester groups in compounds **1–6**, we felt that the characterization of these compounds by NMR spectrometry would be worthwhile. For this purpose we found it very convenient to convert each monoester to a mixed alkyl 2,6-dichlorobenzyl diester by reaction with 2,6-dichlorobenzyl bromide and Na_2CO_3 in DMF.[10,15] The reactivity of 2,6-dichlorobenzyl bromide is sufficiently high to obviate the need for Cs_2CO_3. As expected, the R_f's of the diesters were much higher than those of the monoesters and became progressively higher with longer chain length. On the other hand, the isomeric mixed diesters in each pair (e.g., **7** and **8**) could not be resolved. The benzylic CH_2 protons in the α-(2,6-dichlorobenzyl) esters **7**, **9**, and **11** gave rise to NMR singlets that were consistently at lower field than those of **8**, **10**, and **12**, respectively. Conversely, the aliphatic CH_2 protons adjacent to oxygen in **7**, **9**, and **11** gave triplets that were at higher field than those in **8**, **10**, and **12**. While these differences in chemical shift (ca. 0.08 ppm in the case of the benzylic protons and 0.10–0.15 ppm in the case of the simple *O*-alkyl protons) were small, they were reproducible and could be clearly discerned in mixtures of the isomeric diesters. The lower-field absorption of the benzylic protons in **7**, **9**, and **11** and of the *O*-alkyl protons in **8**, **10**, and **12** is in accord with a greater deshielding effect by the α-carboxyl group and leaves little doubt about the designation of compounds **1**, **3**, and **5** as α-esters and of compounds **2**, **4**, and **6** as γ-esters.

Final proof of the α- or γ-ester structure was achieved in the case of the γ-*n*-hexadecyl ester **6** by unequivocal chemical synthesis (Scheme I). Benzyl *N*-(*tert*-butyloxycarbonyl)-L-glutamate was esterified with *n*-hexadecyl bromide and Cs_2CO_3 in DMF to obtain the diester **13** (96%). Catalytic debenzylation afforded the monoeser **14** (92%), which was then subjected sequentially to (a) reesterification with 2-(trimethylsilyl)ethanol and DCC in the presence of pyridine, (b) acidic cleavage of the *N*-

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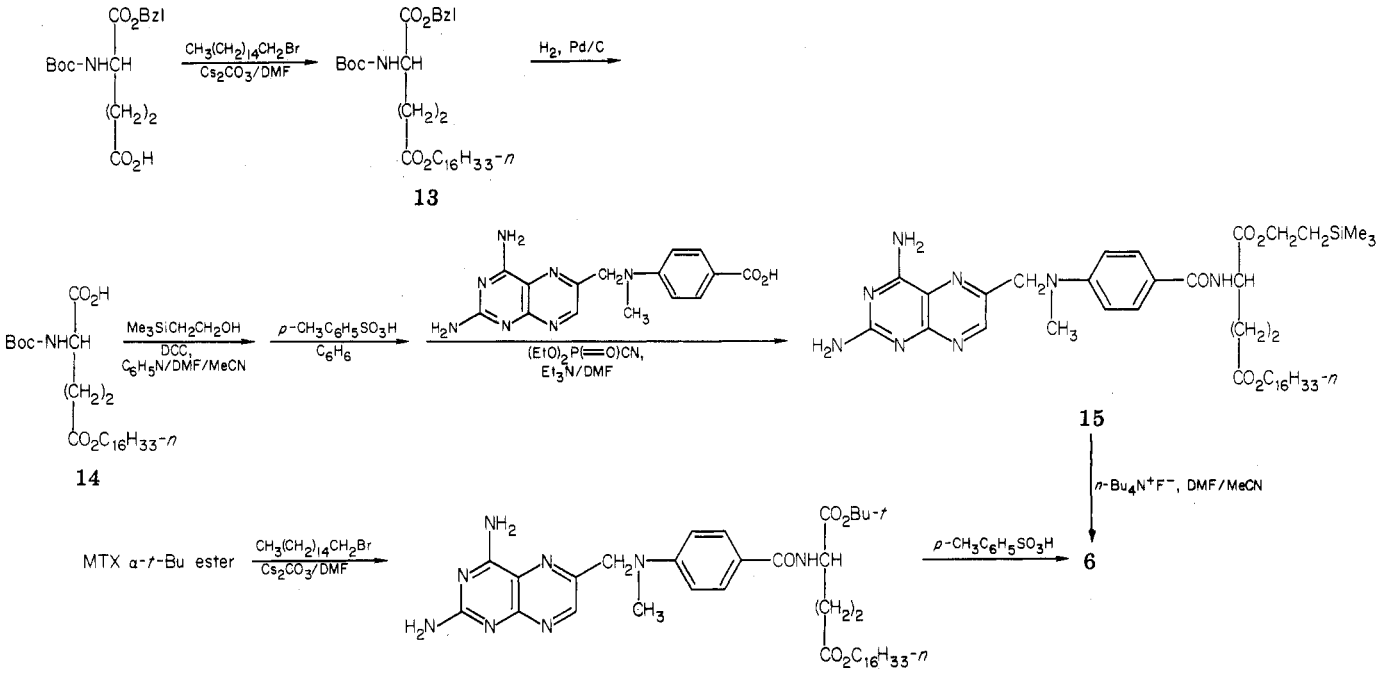
Table I. Physical Constants of Methotrexate Mono- and Diesters

compd	R^1	R^2	method[a]	yield,[b] %	R_f[c]	mp,[d] °C	^{1}H NMR shifts,[e] δ OCH_2-alkyl	^{1}H NMR shifts,[e] δ OCH_2-aryl	formula[f]
1	*n*-C_8H_{17}	H	A	11 (37)	0.23	148–155			$C_{28}H_{38}N_8O_5 \cdot 0.7CHCl_3$
2	H	*n*-C_8H_{17}	A	9	0.11	168–173			$C_{28}H_{38}N_8O_5 \cdot 0.5CHCl_3$
3	*n*-$C_{12}H_{25}$	H	A	6 (25)	0.24	150–158			$C_{32}H_{46}N_8O_5 \cdot 0.7CHCl_3$
4	H	*n*-$C_{12}H_{25}$	A	13	0.12	177–185			$C_{32}H_{46}N_8O_5 \cdot 0.4CHCl_3$
5	*n*-$C_{16}H_{33}$	H	A	11 (33)	0.27	156–165			$C_{36}H_{54}N_8O_5 \cdot 0.4CHCl_3$
6	H	*n*-$C_{16}H_{33}$	A, C, D	15, 53, 39	0.19	197–205			$C_{36}H_{54}N_8O_5 \cdot 0.3CHCl_3$
7	*n*-C_8H_{17}	$CH_2C_6H_3Cl_2$-2,6	B	50	0.65	100–106	4.07	5.27	$C_{35}H_{42}N_8Cl_2O_5 \cdot 0.1CHCl_3 \cdot 1.7CH_3OH$
8	$CH_2C_6H_3Cl_2$-2,6	*n*-C_8H_{17}	B	56	0.65	150–155	3.92	5.35	$C_{35}H_{42}N_8Cl_2O_5$
9	*n*-$C_{12}H_{25}$	$CH_2C_6H_3Cl_2$-2,6	B	41	0.72	151–155	3.98	5.15	$C_{39}H_{50}N_8Cl_2O_5 \cdot 0.05CHCl_3$
10	$CH_2C_6H_3Cl_2$-2,6	*n*-$C_{12}H_{25}$	B	51	0.72	156–161	3.84	5.24	$C_{39}H_{50}N_8Cl_2O_5 \cdot 0.05CHCl_3$
11	*n*-$C_{16}H_{33}$	$CH_2C_6H_3Cl_2$-2,6	B	60	0.75	150–154	3.97	5.15	$C_{43}H_{58}N_8Cl_2O_5$
12	$CH_2C_6H_3Cl_2$-2,6	*n*-$C_{16}H_{33}$	B	18	0.75	150–153	3.87	5.23	$C_{43}H_{58}N_8Cl_2O_5 \cdot 0.08CHCl_3$
15	$(CH_3)_3SiCH_2CH_2$	*n*-$C_{16}H_{33}$	E	47	nd	94–98			$C_{41}H_{66}N_8O_5Si \cdot 0.5H_2O$
16	*t*-C_4H_9	*n*-$C_{16}H_{33}$	F	71	nd	146–152			$C_{40}H_{62}N_8O_5$

[a] See Experimental Section for identification of methods A–F. [b] Numbers in parentheses are the yield of diester obtained along with the α- and γ-esters in method A. [c] Silica gel, 3:1 $CHCl_3$–MeOH. [d] These compounds characteristically melt with decomposition over a broad range. [e] Spectra run in $CHCl_3$ solutions containing 1% Me_4Si. [f] Correct C, H, and N analyses were obtained for all compounds, and correct Cl analyses were also obtained for compounds 7–12.

Scheme I



(*tert*-butyloxycarbonyl) group with *p*-toluenesulfonic acid, and (c) coupling to 4-amino-4-deoxy-N^{10}-methylpteroic acid with diethyl phosphorocyanidate.[17-19] Selective removal of the α-ester with tetrabutylammonium fluoride in DMF[20] then gave **6**. The overall six-step yield was 22%. Alternatively, MTX α-*tert*-butyl ester[3] was esterified with *n*-hexadecyl bromide and Cs_2CO_3 to give diester **16** (71%), and the α-ester was selectively removed by acidolysis with *p*-toluenesulfonic acid to form **6** (39%). The properties of **6** prepared by these two routes and of the product obtained directly from MTX by neutral esterification were indistinguishable. Therefore, the minor isomer isolated from the direct synthesis had to be the α-isomer.

Biological Activity. To assess the effect of increasing alkyl chain length on DHFR binding, we tested compounds **1–6** as inhibitors of the reduciton of dihydrofolate by a partially purified enzyme extract from beef liver.[21] A spectrophotometric assay was used, which measures absorbance change at 340 nm when the cofactor NADPH is converted to its oxidized form. The primary sequence of beef liver DHFR, its extensive homology to other mammalian enzymes, and the similar affinities of the bovine and human enzyme for MTX have been documented.[22-24] As shown in Table II, MTX α-*n*-butyl ester and the other α-esters (**1**, **3**, and **5**) were less potent than the corresponding γ-esters. DHFR binding also appeared to be influenced more by changes in alkyl chain length in the α-esters than in the γ-esters. Thus, a 6- to 8-fold IC_{50} increase was observed as the length of the α-ester increased from C_4 to C_8, whereas in the γ-series the same 6- to 8-fold increase did not occur until the alkyl group was extended from C_4 to C_{12}. Over the entire C_4–C_{16} range the IC_{50} increase was three times greater in the α series than in the γ series, and there was a similar difference relative to MTX. These results were in accord with the general view that the α-carboxyl in MTX contributes greatly to binding by interacting with a highly conserved arginine on the DHFR, whereas the γ-carboxyl lies in a region of relatively high bulk tolerance.[25,26] Since the introduction of the *n*-dodecyl and *n*-hexadecyl groups in **4** and **6**, respectively, caused a 6-fold decrease in IC_{50} relative to **2**, it appears that their may exist at some distance from the active site a domain not compatible with *long* hydrophobic sustituents at the γ-terminal position of the glutamate moiety. However, in the absence of detailed studies on these compounds, including K_i determinations and analysis of the kinetics of inhibition (competitive vs. noncompetitive), this interpretation is only tentative.

To establish whether there is a correlation between DHFR affinity and cytotoxicity among MTX α- and γ-esters, we compared their growth inhibitory activity against human leukemic lymphoblasts (CEM cells)[27] in culture. As shown in Table II, the IC_{50} values were consistently higher for the γ-esters, in agreement with the DHFR binding data. In both series there was an increase in the IC_{50} from C_4 to C_8 and a subsequent decline following the order $C_8 > C_{12} > C_{16}$. The *n*-hexadecyl esters were 7- to 10-fold more potent than the corresponding *n*-butyl esters,

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Table II. In Vitro Biological Activity of Methotrexate Long-Chain Monoesters

compd	DHFR inhibn: IC_{50},[a] μM	CEM cell inhibn: IC_{50},[b] μM
	α-Esters	
α-*n*-Bu[c]	0.056	2.0
1	0.36	3.0
3	0.44	2.1
5	1.2	0.25
	γ-Esters	
γ-*n*-Bu[c]	0.0054	0.78
2	0.0054	0.92
4	0.034	0.37
6	0.037	0.11
MTX	0.0033	0.025[d]

[a] IC_{50} values were determined spectrophotometrically at 340 nm with DHFR obtained from beef liver.[21] The assay mixture contained NADPH (100 μM), dihydrofolate (25 μM), 2-mercaptoethanol (10 mM), EDTA (1 mM), and KCl (0.15 M) in 1 mL of 0.1 M Tris, pH 7.5.[6] The enzyme was preincubated with TPNH for 3 min prior to dihydrofolate addition to initiate the reaction. [b] Cytotoxicity was determined as reported previously,[28] with cells being counted after 48 h of drug exposure. Compounds were dissolved in Me_2SO (1 mg/mL) and a 10-fold dilution with Me_2SO was made before addition to the growth medium. The final Me_2SO concentration was 0.1%. Control experiments showed no effect on cell growth at this concentration. Triplicate tubes were used at each dose, and results were averaged. [c] The α- and γ-*n*-butyl esters were prepared as previously described.[1] [d] Average of four separate determinations.

though they were still 5- to 10-fold less potent than MTX itself. It thus appears that, in contrast to the effect of the esters on DHFR binding in a cell-free system, an increase in ester chain length results in higher potency in intact cells. We believe this is consistent with the greater lipophilicity of the longer-chain derivatives, which would be expected to favor passive diffusion across the cell membrane. An additional factor that could play a role, particularly with the *n*-hexadecyl esters and derivatives of longer chain length, is the possible formation of micelles that can enter cells by a pinocytotic process bypassing the normal carrier-mediated active-transport pathway for MTX.

In view of our earlier finding of an unexpected effect by MTX di-*n*-butyl ester on dThd incorporation into DNA in L1210 leukemic cells in culture,[8] it was of interest to determine whether this is also observed with a monoester. We chose the *n*-hexadecyl analogue **6** because it seemed to be the most cytotoxic member of the series. CEM and CEM/MTX cells, which are resistant to MTX by virtue of a transport defect,[2] were treated with 10^{-7}–10^{-4} M **6** for 1 h at 37 °C and were then pulsed for 0.5 h with [^{3}H]dThd or [^{3}H]dUrd. The DNA was precipitated with acid, and the acid-insoluble fraction was counted. Results were expressed as a percentage of untreated controls (Table III). In both CEM and CEM/MTX cells, the concentration required in a 1-h exposure to achieve 50% inhibition of dThd and dUrd incorporation was 10^{-6}–10^{-5} M. Thus, **6** behaved qualitatively like the di-*n*-butyl ester[8] in that it blocked both dThd and dUrd incorporation into DNA, whereas MTX in this concentration range affects only the incorporation of dUrd. Since the transport-defective CEM/MTX cells are known to be 120-fold less sensitive to MTX than the parental CEM line,[2] our present finding that 1-h treatment with 10^{-5} M **6** inhibits DNA synthesis in the resistant cells only 4-fold less than in the sensitive cells suggests that this compound, like the di-*n*-butyl and γ-*n*-butyl esters we have previously described,[2,8] is a reasonable candidate for use against MTX-resistant human tumors with impaired transport.

Table III. Inhibitory Effect of Methotrexate γ-Hexadecyl Ester (**6**) on Deoxyuridine and Thymidine Incorporation into DNA of DEM and CEM/MTX Cells in Culture

concn, M	[^{3}H]dUrd, % of controls[a]		[^{3}H]dThd, % of controls[a]	
	CEM	CEM/MTX	CEM	CEM/MTX
10^{-7}	117	95	92	81
10^{-6}	64	103	67	108
10^{-5}	14	55	25	15
10^{-4}	7	2	17	8

[a] Control values (counts per minute/10^5 cells) in the acid-insoluble fraction were 4200 ± 900 and 11 500 ± 2500, respectively, in the [^{3}H]dUrd assay and 1200 ± 100 and 2600 ± 200, respectively, in the [^{3}H]dThd assay. Differences were significant ($p < 0.05$) at and above 10^{-6} M for the CEM cells and at or above 10^{-5} M for the CEM/MTX cells.

Experimental Section

Infrared spectra were obtained on a Perkin-Elmer Model 137B double-beam recording spectrophotometer, and NMR spectra were recorded on a Varian T60A instrument with Me_4Si as the internal reference. TLC was performed on Eastman 13181 silica gel sheets or Anasil OF silica gel plates (250-μm thickness), with spots being visualized at 254 nm in a viewing chamber or with ninhydrin spray where appropriate. Dry-column chromatography was carried out on Woelm activity grade III 30-mm silica gel (ICN Pharmaceuticals, Cleveland, OH) as previously described.[1] Ordinary column chromatography employed Baker 3405 silica gel (60–200 mesh). Melting points were measured in Pyrex capillary tubes in a Mel-Temp apparatus (Laboratory Devices, Cambridge, MA) and are not corrected. Elemental analyses were performed by Galbraith Laboratories Knoxville, TN, and were within ±0.4% of theoretical values unless otherwise specified.

α-Benzyl *N*-(*tert*-butyloxycarbonyl)-L-glutamate was purchased from Chemical Dynamics, South Plainfield, NJ. *n*-Hexadecyl bromide was obtained from Eastman, Rochester, NY, and Cs_2CO_3 was purchased from Alfa, Beverly, MA. 2,6-Dibromobenzyl chloride and 2-(trimethylsilyl)ethanol were from Aldrich Chemical Co., Milwaukee, WI. 4-Amino-4-deoxy-N^{10}-methylpteroic acid, MTX α-*tert*-butyl ester, and diethyl phosphorocyanidate were prepared as previously described.[18,19] DMF and Me_2SO were dried over Linde 4A molecular sieves prior to use.

MTX α- and γ-Esters. Reaction of MTX with *n*-Hexadecyl Bromide and Cesium Carbonate. Method A. A mixture of MTX free acid (4 g, 0.008 mol) and Cs_2CO_3 (2.6 g, 0.008 mol) in dry Me_2SO (150 mL) was stirred at room temperature for 1.5 h, and a solution of *n*-hexadecyl bromide (2.93 g, 0.0096 mol) in Me_2SO (50 mL) was added. After being stirred for 46 h, the mixture was concentrated to dryness by rotary evaporation (45 °C, vacuum pump), and the residue was triturated with H_2O until the product solidified. A few drops of 1 N HCl were added to bring the pH to 3, and after overnight storage in the refrigerator, the solid was collected, washed with H_2O, and dried in vacuo over P_2O_5; crude yield 6.3 g. Half the product was chromatographed on a silica gel *dry column*, which was eluted successively with 95:5 (2000 mL), 9:1 (2000 mL), 85:15 (2000 mL), 4:1 (2000 mL), and 3:1 (1000 mL) $CHCl_3$–MeOH. Individual 20-mL fractions were monitored by TLC (silica gel, 3:1 $CHCl_3$–MeOH), and appropriate volumes were pooled and evaporated. Tubes 30–45 yielded MTX di-*n*-hexadecyl ester, R_f 0.91, identical with an authentic specimen.[10] Tubes 65–270 yielded MTX α-*n*-hexadecyl ester (**5**), and tubes 285–430 yielded MTX γ-*n*-hexadecyl ester (**6**). The second half of the product was chromatographed in the same manner. Total recoveries of purified products were as follows: diester, 2.48 g; α-ester, 0.58 g; γ-ester, 0.83 g.

Esterification of MTX Monesters with 2,6-Dichlorobenzyl Bromide and Sodium Carbonate. Method B. 2,6-Dichlorobenzyl bromide (36 mg, 0.148 mmol) was added to a mixture of

MTX α-*n*-hexadecyl ester (50 mg, 0.074 mmol) and Na_2CO_3 (7.8 mg, 0.074 mmol) in dry DMF (3 mL). After the mixture was stirred for 2.5 h at room temperature, the solvent was removed by rotary evaporation (40 °C, vacuum pump), and the residue was triturated with 0.05 M potassium phosphate buffer to remove any unchanged **5**. The solid remaining was filtered, washed with H_2O, and dried in vacuo over P_2O_5: crude yield 62 mg. Chromatography on a silica gel column (7 g) with 99:1 (200 mL) and 98:2 (200 mL) $CHCl_3$–MeOH as the eluents gave pure **11** as a bright yellow solid (37 mg).

α-Benzyl γ-*n*-Hexadecyl *N*-(*tert*-Butyloxycarbonyl)-L-glutamate (13). A solution of α-benzyl *N*-(*tert*-butyloxycarbonyl)-L-glutamate (1.67 g, 0.05 mol) in dry DMF (20 mL) was stirred with Cs_2CO_2 (1.63 g, 0.05 mol) and *n*-hexadecyl bromide (1.52 g, 0.05 mol) at room temperature for 2.5 days. The DMF was evaporated, and the residue was partitioned between C_6H_6 and H_2O. The organic layer was washed thoroughly with H_2O and evaporated to give, after drying in vacuo (P_2O_5, 45 °C), 2.96 g (96% yield) of white solid: mp 65–66 °C (MeOH); IR (KBr) ν 3310, 2900, 1730 (ester C=O), 1710 (ester C=O), 1625, 1520, 760, 700 cm^1; NMR ($CDCl_3$) δ 0.6–2.5 [m, 44 H, $CH_3(CH_2)_{14}$, glutamyl CH_2CH_2, $(CH_3)_3CO$], 4.05 (t, J = 6 Hz, 2 H, CO_2CH_2), 4.95–5.30 (m, 3 H, $ArCH_2$ and α-CH), 7.37 (s, 5 H, aromatic protons). Anal. ($C_{33}H_{55}NO_6$) C, H, N.

γ-*n*-Hexadecyl *N*-(*tert*-Butoxycarbonyl)-L-glutamate (14). A solution of **13** (8 g, 0.0142 mol) in *i*-PrOH (200 mL) was shaken in a Parr low-pressure hydrogenation apparatus with H_2 (2–3 atm) and 5% Pd/C (200 mg) at room tempature overnight. Filtration of the catalyst and solvent removal gave a white solid, which was recrystallized from *n*-hexane: yield 6.19 g (92%); mp 68–69 °C; IR (KBr) ν 3330, 2900, 1735 (ester C=O), 1705, 1685, 1500 cm^{-1}; NMR ($CDCl_3$) δ 0.7–1.8 [m, 40 H, $CH_3(CH_2)_{14}$, $(CH_3)_3CO$], 1.9–2.6 (m, 4 H, glutamyl CH_2CH_2), 4.05 (t, J = 6 Hz, 2 H, CO_2CH_2), 5.20 (m, 1 H, α-CH), 8.45 (m, 1 H, NH). Anal. ($C_{26}H_{49}NO_6$) C, H, N.

MTX α-[2-(Trimethylsilyl)ethyl] γ-*n*-Hexadecyl Ester (15). Method E. A solution of the monoester **14** (5.7 g, 12 mmol) in a mixture of MeCN (30 mL) and DMF (5 mL) was treated with pyridine (1.8 mL) and 2-(trimethylsilyl)ethanol (1.6 g, 13.2 mmol) with cooling in an ice bath. DCC (2.7 g, 13 mmol) was then added, and the reaction mixture was left at 0 °C for 1 h and in the refrigerator overnight. The semisolid mass was triturated with EtOAc (20 mL) and filtered, and the filter cake was washed with EtOAc. The combined filtrates were evaporated, the residue was dissolved in C_6H_6, and a small amount of insoluble material was filtered off. The filtrate was then washed with 0.1 N HCl, rinsed with H_2O, and evaporated to an oil (6.8 g, 100%) whose NMR spectrum showed it to be pure enough for direct use in the next step: IR (neat) ν 3340, 2910, 1725 (ester C=O) cm^{-1}; NMR (CCl_4) δ 0.01 [s, 11 H, $(CH_3)_3SiCH_2$], 0.90–1.90 [m, 40 H, $CH_3(CH_2)_{14}$, $(CH_3)_3CO$], 2.10–2.50 (m, 4 H, glutamyl CH_2CH_2), 4.00–4.50 (m, 4 H, CO_2CH_2), 5.21 (d, J = 8 Hz, 1 H, α-CH).

A solution of the above diester (567 mg, 1 mmol) in dry C_6H_6 (20 mL) containing *p*-toluenesulfonic acid monohydrate (190 mg, 1 mmol) was refluxed for 1 h and evaporated to dryness to an oil, which gradually solidified on being scratched with a spatula (643 mg, 100% yield): NMR ($CDCl_3$) δ 0.7–1.7 [m, 31 H, $CH_3(CH_2)_{14}$], 2.1–2.6 (m, 7 H, $ArCH_3$ and glutamyl CH_2CH_2), 3.8–4.4 (m, 5 H, CO_2CH_2 and α-CH), 7.11 (d, J = 8 Hz, aromatic protons), 7.70 (d, J = 8 Hz, aromatic protons), 7.9–8.2 (broad, 3 H, NH_3^+) [the $(CH_3)_3SiCH_2$ protons were visible at ca. 0.0 but could not quantitated because Me_4Si was added as an internal standard].

4-Amino-4-deoxy-N^{10}-methylpteroic acid (360 mg, 1 mmol) was added in small portions to a solution of diethyl phosphorocyanidate (489 mg, 3 mmol) and Et_3N (303 mg, 3 mmol) in dry DMF (25 mL). The solution was heated for 2 min at 80 °C (oil bath temperature), cooled to room temperature, and treated with another portion of Et_3N (202 mg, 2 mmol), followed by the above tosylate salt of α-[2-(trimethylsilyl)ethyl] γ-*n*-hexadecyl L-glutamate (643 mg, 1 mmol) in a small volume of DMF. The mixture was stirred for 2 h in the 80 °C bath, and then the solvent was removed by rotary evaporation. The residue was dissolved in $CHCl_3$, and the solution was extracted with 5% $NaHCO_3$, rinsed with H_2O, and evaporated to a solid, which was purified by silica gel column chromatography with 95:5 $CHCl_3$–MeOH as the eluent: yield 360 mg; IR (KBr) ν 3330, 3900, 1736 (ester C=O), 1660, 1610 cm^{-1}.

MTX α-*tert*-Butyl γ-*n*-Hexadecyl Ester (16). Method F. A mixture of MTX α-*tert*-butyl ester (1.07 g, 2 mmol), Cs_2CO_3 (0.65 g, 2 mmol), and *n*-hexadecyl bromide (1.22 g, 4 mmol) in dry DMF (20 mL) was stirred at room temperature overnight. The DMF was removed by rotary evaporation, and the residue was taken up in $CHCl_3$. The $CHCl_3$ solution was washed with 5% $NaHCO_3$, dried, and evaporated, and the residue was chromatographed on a silica gel column, which was eluted successively with $CHCl_3$ and 95:5 $CHCl_3$–MeOH. Appropriate TLC-homogeneous fractions were pooled and evaporated to obtain a bright-yellow solid (1.04 g): IR (KBr) ν 3450, 2940, 1740 (ester C=O), 1635, 1610 cm^{-1}.

MTX γ-*n*-Hexadecyl Ester (6). Method C. A solution of the diester **15** (156 mg, 0.2 mmol) in a mixture of MeCN (4 mL) and DMF (4 mL) was treated with tetrabutylammonium chloride (167 mg, 0.6 mmol) and $KF{\cdot}2H_2O$ (79 mg, 0.84 mmol) and stirred at 48 °C (bath temperature) for 2 days. The solvents were removed by rotary evaporation and replaced with $CHCl_3$, and the $CHCl_3$ solution was washed thoroughly with H_2O and concentrated to dryness. The residue was triturated with 10% AcOH, filtered, air-dried (2 h), and chromatographed on silica gel with 4:1 $CHCl_3$–MeOH as the eluent: yield 72 mg; IR (KBr) ν 3450, 2940, 1720, (ester C=O), 1600–1635 cm^{-1}. This material and the product obtained by direct esterification of MTX (method A) were spectroscopically and chromatographically indistinguishable.

MTX γ-*n*-Hexadecyl Ester (6). Method D. A solution of the diester **16** (367 mg, 0.5 mmol) in dry benzene (50 mL) containing *p*-toluenesulfonic acid dihydrate (475 mg, 2.5 mmol) was refluxed 45 min and then evaporated to dryness. The residue was triturated with dilute ammonia, the pH was adjusted to 4 with AcOH, and the solid was collected, dried, and chromatographed on a silica gel column using 4:1 $CHCl_3$–MeOH as the eluent: yield 132 mg. This product and the one obtained in the preceding experiment (method C) were spectroscopically and chromatographically indistinguishable.

DNA Synthesis Inhibition. Logarithmically growing CEM or CEM/MTX cells (2×10^5/tube) were suspended in 2 mL of serum-free Earle's balanced salt solution (EBSS) containing various concentrations of **6** (10^{-7}–10^{-4} M). The cells were incubated at 37 °C under a 5% CO_2 atmosphere for 1 h, and either [^{3}H]Thd or [^{3}H]dUrd (45 μL of 1 mCi/mL stock solution) was added. After another 0.5 h at 37 °C, 10 mL of cold EBSS was added, and the cells were centrifuged at 4 °C, washed three times with 3 mL of cold EBSS, and lysed by adding 0.5 mL of cold 1% sodium dodecyl sulfate (SDS) buffer. Three drops of SDS buffer containing yeast tRNA as a nonradioactive carrier were then added, followed by 5 mL of 0.5 N $HClO_4$ at 0 °C. The acid-insoluble material was filtered on a glass-fiber disk (Whatman), which was then rinsed with ice-cold 5% trichloroacetic acid and transferred to a scintillation vial containing 10 mL of Biofluor (New England Nuclear, Boston, MA) for counting. Results were expressed in counts per minute/10^5 cells (Table III).

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