(16) Baltimore Biological Laboratory, Inc., Baltimore, Md. 21218.
(17) Spectronic 20 colorimeter/spectrophotometer, Bausch and Lomb, Rochester, N.Y. 14602.
(18) Modified Diamond's medium: trypticase (BBL), 24.0 g; yeast extract (Difco), 12.0 g; maltose, 6.0 g; L-cysteine hydrochloride, 1.2 g; L-ascorbic acid, 0.24 g; K_2HPO_4, 0.96 g; KH_2PO_4, 0.96 g; agar, 0.60 g; distilled water, 1080 mL. The pH was adjusted to 7.1 with 1 N NaOH. Following sterilization at 121 °C for 15 min, 5% of Bacto Dubos horse serum was added.
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Methotrexate Analogues. 11. Unambiguous Chemical Synthesis and in Vitro Biological Evaluation of α- and γ-Monoesters as Potential Prodrugs

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Several α- and γ-monoesters of methotrexate (MTX) were synthesized chemically and evaluated as inhibitors of cultured human lymphoblastic leukemia (CCRF-CEM) cells and purified dihydrofolate reductase (DHFR) from rabbit liver. Chemical methods included direct HCl-catalyzed half-esterification of MTX, partial cleavage of methotrexate diesters in the presence of base, and mixed anhydride coupling from 4-amino-4-deoxy-N^{10}-methylpteroic acid. ID_{50} values obtained for methotrexate γ-monobutyl ester against CCRF-CEM cells and rabbit liver DHFR were 0.76×10^{-6} and 1.7×10^{-8} mol/L, respectively. In vitro incubation of methotrexate dibutyl ester in whole human serum at 37 °C for 48 h produced only 12% cleavage to monobutyl esters and <1% cleavage to free MTX, in contrast to similar incubation in mouse serum which gave 93% free MTX. HPLC analysis of the monobutyl ester fraction from serum incubation revealed a γ/α isomer ratio of approximately 85:15, indicating that serum esterase cleavage is regioselective. The results of this study suggest that methotrexate monoesters may have a significant role in the pharmacology of methotrexate diesters in nonrodent species and should be viewed as potential therapeutic agents on their own merit.

Diesters of the widely used anticancer drug methotrexate (MTX, 4-amino-4-deoxy-N^{10}-methylpteroyl-L-glutamic acid) have been the subject of several chemical and biological investigations in this laboratory[1-7] and elsewhere.[8-12] Our interest in these very lipophilic derivatives was kindled initially by the concept that they might enter cells via passive diffusion instead of active transport. In this respect the mode of uptake of these compounds would resemble that of "small molecule" folic acid antagonists, despite the fact that they still contain most of the structural features of classical antifolates. In addition to the possibility that the diesters might be distributed selectively in tissues with a special affinity for lipophilic molecules, such as the liver or CNS, these compounds were of special interest because of their potential use against MTX-resistant tumors, which represent a major unsolved clinical problem in antifolate chemotherapy. At the same time it was recognized that the diesters might function as prodrugs,[2] free MTX being released as a result of cleavage by nonspecific esterases in the plasma or other physiologic fluids.[8-11] A prodrug mechanism is, in fact, probably the predominant mode of action of methotrexate diesters in mice and other rodents in view of the very high esterase levels in the serum of these experimental animals.[13,14] On the other hand, in species whose serum esterase levels are low (e.g., primates)[13,14] methotrexate diesters may have a biological effect of their own or at least a composite effect involving both inherent activity and prodrug activity. Preliminary support for an inherent effect was provided by studies of [^{3}H]-TdR incorporation into the DNA of L1210 mouse leukemia and CCRF-CEM human lymphoblastic leukemia cells in short-term serum-free culture.[5,6] Whereas MTX itself either caused a slight increase in [^{3}H]-TdR incorporation or had no effect, the diesters brought about a marked decrease in DNA labeling. Moreover, the inhibitory effect of methotrexate diesters was only partly reversed on addition of leucovorin. Thus it appears that the biochemical mode of action of methotrexate diesters is more complex than had been indicated earlier by in vivo studies in rodents.[2,8-11]

As a prelude to further studies in nonrodent mammalian systems we required authentic samples of some α- and γ-monoesters of MTX, a class of derivatives not heretofore described in detail in the literature. Apart from the obvious fact that the monoesters represent potential metabolites of the diesters or impurities in their chemical synthesis, these compounds are inherently attractive as MTX prodrugs because they retain a free COOH group and should therefore be intermediate in lipophilicity between the diesters and methotrexate free acid. This paper reports the synthesis of several monoesters of MTX and their effects on cultured leukemic cells and purified mammalian dihydrofolate reductase. A monoester of 3′,5′-dichloromethotrexate (DCM) is also described. The structures and physical constants of the monoesters are listed in Table I, and their biological activities are summarized in Table II. Unequivocal evidence is also presented for the first time that human serum converts methotrexate dibutyl ester predominantly to methotrexate γ-monobutyl ester while yielding only a negligible amount of free MTX even after 72 h of incubation at 37 °C.

Chemistry. Several chemical approaches to the synthesis of methotrexate α- and γ-monoesters were investigated during this work. The first (method A) was a modification of the HCl-catalyzed esterification process developed earlier in this laboratory as a means of obtaining diesters directly from MTX.[2] Instead of the usual large excess of HCl, the reaction was conducted in the presence of only 1.5 molar equiv of HCl per mole of methotrexate free acid. After solvent evaporation the monoesters were separated from the diesters, which were always preponderant regardless of the quantity of HCl in the reaction

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Table I. Physical Constants of Methotrexate and 3',5'-Dichloromethotrexate Monoesters

Compd	Method[a]	Yield, %	Mp, °C dec	TLC, R_f[b] 1	2	3	Formula	Analyses[c]
MTX γ-monomethyl ester	D	23	170–180	0.10	0.31	0.63	$C_{21}H_{24}N_8O_5 \cdot 2.25H_2O$	C, H, N
MTX α-monoethyl ester	A	6	157–172		0.26	0.60	$C_{22}H_{26}N_8O_5 \cdot 0.15CHCl_3$	C, H, N
MTX γ-monoethyl ester	A	30	192–204		0.26	0.60	$C_{22}H_{26}N_8O_5 \cdot 0.67CHCl_3$	C, H, N
	C	60						
	D	23						
MTX α-monobutyl ester	A	11	135–145	0.02	0.26	0.62	$C_{24}H_{30}N_8O_5 \cdot 0.5H_2O$	C, H, N
MTX γ-monobutyl ester	A	21	166–180	0.05	0.26	0.62	$C_{24}H_{30}N_8O_5 \cdot 0.3CHCl_3$	C, H, N
	B	39						
	D	15						
DCM γ-monoethyl ester	C	75	171-182		0.41	0.48	$C_{22}H_{24}Cl_2N_8O_5 \cdot C_2H_5OH$	C, H, Cl, N

[a] A, HCl-catalyzed esterification; B, partial cleavage of diester with NaOH; C, partial cleavage of diester with piperidine; D, mixed anhydride coupling. [b] System 1, silica gel, 3:1 $CHCl_3$–MeOH; system 2, silica gel, 1:1 $CHCl_3$–MeOH; system 3, cellulose, pH 7.4 phosphate buffer. [c] C, H, and N (and Cl for DCM γ-monoethyl ester) were all within ±0.4% of theoretical values.

Table II. In Vitro Biological Activity of Methotrexate Monoesters and Diesters

Compd	Human lymphoblastic leukemia cells (CCRF-CEM) in culture, ID_{50}, mol/L[a]	Rabbit dihydrofolate reductase, ID_{50}, mol/L[b]
MTX γ-monomethyl ester	0.43×10^{-6}	
MTX dimethyl ester	0.40×10^{-6}	
MTX α-monoethyl ester	6.2×10^{-6}	
MTX γ-monoethyl ester	0.58×10^{-6}	
MTX diethyl ester	0.012×10^{-6}	
MTX α-monobutyl ester	2.0×10^{-6}	1.7×10^{-8}
MTX γ-monobutyl ester	0.76×10^{-6}	1.7×10^{-8}
MTX dibutyl ester	0.057×10^{-6}	1.4×10^{-7}
MTX	0.006×10^{-6}	9.0×10^{-9}

[a] See ref 23 for details of the assay procedure. [b] See ref 24 and the Experimental Section for details of the assay procedure.

mixture, by extraction with alkaline phosphate buffer. The base-soluble fraction was then chromatographed on a silica gel dry column in order to separate the α- and γ-monoesters from each other and from unreacted MTX which was also sometimes present. In some instances it was convenient to also include DEAE-cellulose chromatography in the purification scheme. The need to use a silica gel *dry column* in the purification of the monoesters should be stressed, since chromatography on a conventional silica gel column does not bring about acceptable separation of these compounds.

It is of interest to note that for any pair of α- and γ-monoesters, the isomer migrating more rapidly on a silica gel dry column was the one with a free γ-COOH group, i.e., the α-monoester. This order of elution was consistent with the relative acidity of α- and γ-COOH groups in simple glutamic acid derivatives and resembled the behavior of other α- and γ-monosubstituted derivatives of MTX studied in this laboratory.[15] Two other differences were noted which may be useful in characterizing these isomeric compounds: (1) the yields of γ-monoesters were three to five times higher than those of α-monoesters, and (2) the melting points of γ-monoesters were 20–30 °C higher than those of α-monoesters.

A second approach to the chemical synthesis of monoesters involved partial saponification of the diesters. Although both α- and γ-monoesters might be expected to form via this route, when methotrexate dibutyl ester was treated with 1 molar equiv of NaOH in 2:1 MeOH–H_2O at room temperature for 1 h (method B), the only monoester isolated, in addition to some free MTX, was the γ-monobutyl isomer (39% yield). Thus the reaction appeared to be "regioselective", though one cannot rule out the possibility that some α-monoester was formed but underwent rapid cleavage to MTX or base-catalyzed α → γ rearrangement, a process which is well known for derivatives of glutamic acid.[16]

A novel variant of this approach was discovered during a study of the synthesis of methotrexate bisamides from methotrexate diethyl ester.[7] A wide assortment of primary amines was found to react with the diester in the absence of solvent, giving good yields of the corresponding bisamides. The secondary amine pyrrolidine behaved similarly, but the use of piperidine resulted in a complex mixture which was found to contain little or none of the desired bisamide. The major product could be shown to contain a COOH group on the basis of its slow migration on silica gel and its solubility in dilute base. The IR spectrum exhibited characteristic ester C=O absorption at 1740 cm^{-1}, but the intensity of this peak was only about half the intensity of the ester peak in the starting material. Comparison with the two isomeric monoethyl esters derived from MTX by direct esterification, and with the authentic γ-monoethyl ester obtained by unequivocal synthesis as described below, established that the product of the piperidine reaction was, in fact, methotrexate γ-monoethyl ester. By conducting the reaction in 95% EtOH solution (method C) instead of in piperidine alone, we succeeded in obtaining a 60% yield of methotrexate γ-monoethyl ester from methotrexate diethyl ester and a 75% yield of 3',5'-dichloromethotrexate γ-monoethyl ester from 3',5'-dichloromethotrexate diethyl ester. Two other nitrogenous bases whose action caused partial cleavage to a monoester in preference to bisamide formation were *N*-aminopyrrolidine and *N*,*N*-dimethylhydrazine. Precedents for the use of such reagents to cleave diesters to monoesters exist in the literature but are few in number.[17] The marked contrast between pyrrolidine and piperidine in the reaction with methotrexate diesters[7] may be explained on the basis that the nucleophilicity of the ring nitrogen in the latter compound is diminished because of steric factors.

Unequivocal assignment of structure to the γ-monoethyl and γ-monobutyl esters of MTX was achieved by condensing the isobutyl mixed anhydride of 4-amino-4-deoxy-N^{10}-methylpteroic acid[3] with L-glutamic acid γ-ethyl and γ-butyl esters, respectively (method D). In the case

Table III. Percent Distribution of Radioactivity in TLC Fractions Obtained after in Vitro Incubation of [^{3}H]Methotrexate Dibutyl Ester in Mouse, Monkey, and Human Serum

Time, h	Percent of total recovered label after TLC[a]								
	Mouse[b]			Monkey			Human		
	DBMTX	MBMTX	MTX	DBMTX	MBMTX	MTX	DBMTX	MBMTX	MTX
2	2	55	43	98	2	<1	98	2	<1
6	2	52	46	96	4	<1	96	4	<1
24	2	9	89	93	7	<1	86	13	1
48	<1	6	93	71	27	2	88	12	<1

[a] MBMTX, α- and γ-monobutyl ester mixture; DBMTX, unchanged methotrexate dibutyl ester. [b] Results in rat serum were virtually identical.

of the γ-ethyl ester, the α-COOH group was left unprotected and the coupling step (23% yield) was conducted in the presence of 1,1,3,3-tetramethylguanidine.[18] L-Glutamic acid γ-butyl ester, a previously undescribed derivative, was synthesized from *N*-carbobenzyloxy-L-glutamic acid α-benzyl ester in two steps by treatment of the cesium salt with *n*-butyl bromide,[19] followed by catalytic hydrogenolysis in the presence of 10% Pd/C (overall yield 81%). For the synthesis of the γ-monobutyl ester of MTX, L-glutamic acid γ-butyl ester was converted into a trimethylsilyl derivative by treatment with hexamethyldisilazane,[20] and the protected monoester was condensed with 4-amino-4-deoxy-N^{10}-methylpteroic acid via a mixed anhydride coupling reaction in 1:1 Me_2SO–THF. Following evaporation of the solvents, alkaline phosphate buffer (pH 8.5) was added in order to effect desilylation, and the deprotected product (15% yield) was purified by DEAE-cellulose chromatography. A 36% yield of unchanged 4-amino-4-deoxy-N^{10}-methylpteroic acid was also recovered. The coupling procedures described here were chosen purposely over more conventional methods involving COOH protection via alkyl esters because we felt that they might lessen the chance of racemization of the L-glutamate side chain during deprotection.[21]

Biological Activity. As indicated in Table II, the methotrexate γ-monoesters tested during this study were consistently more active as growth inhibitors of human leukemic cells in vitro than the isomeric α-monoesters. The difference between the α- and γ-monoethyl esters was about tenfold, whereas the difference between the longer α- and γ-monobutyl esters was only 2.5-fold. Whether or not the activity of methotrexate α-monoesters in this assay system continues to increase as a function of alkyl chain length remains to be determined. It is of considerable interest to note that the methotrexate monoethyl and monobutyl esters were less inhibitory than the corresponding diesters. This was especially apparent with the diethyl ester, which was 516 times and 48 times more active than the α- and γ-monoethyl analogues, respectively. Less pronounced but nonetheless significant was the difference between the dibutyl and monobutyl derivatives, the dibutyl ester being 35 times more active than the α-monobutyl ester and 13 times more active than the γ-monobutyl isomer. The dimethyl ester, in contrast, was only as active as the γ-monomethyl analogue.[22] Thus the activities of the lower γ-monoalkyl ester of MTX appear to decrease as a function of alkyl group size up to at least C_4 in this particular bioassay system, whereas the activities of the corresponding diesters seem to obey a parabolic relationship with a maximum at C_2 or C_3.

Since it was our intention, ultimately, to study the pharmacology of methotrexate diesters in animals other than mice, it was necessary to carry out a comparative study of the effect of incubation of these compounds in serum derived from several different species. Accordingly, [^{3}H]methotrexate dibutyl ester was synthesized by the usual HCl-catalyzed esterification method[2] and incubated in whole mouse, rat, monkey, and human serum for 48 h. Aliquots were withdrawn at periodic intervals and analyzed by TLC on cellulose, with pH 7.4 phosphate buffer as the developing solvent. In this system the dibutyl ester remained near the origin, whereas free MTX migrated rapidly and the α- and γ-monoesters had intermediate R_f values. The monoesters were not separable under these conditions, so that radioactivity in the intermediate zone had to be assumed to represent either or both isomers. Spots were cut out and analyzed by scintillation counting, and the radioactivity in each spot was expressed as a percentage of total recovered label. As shown in Table III, after 2 h of incubation in mouse serum only 2% of the total counts represented unchanged dibutyl ester, whereas free MTX and the monoester(s) accounted for 43 and 55%, respectively. There was little change after 6 h, but at the end of 24 h the proportion of free MTX had risen to 89%. Essentially the same results were obtained in rat serum (data not shown) but not in monkey or human serum. In monkey serum unchanged dibutyl ester accounted for 93% of the radioactivity even after 24 h, whereas free MTX represented less than 1%. Even after 48 h, the dibutyl ester was still the predominant species (71%), although some monoester(s) could now be seen (27%). In human serum there was once again very little free MTX even after 48 h and apparently even less monoester(s) than in monkey serum (12% after 48 h vs. 27%). These results support earlier findings[9,10] in respect to the in vivo fate of methotrexate diesters in mice. On the other hand, the slow cleavage of methotrexate dibutyl ester that we observed in monkey and human serum in vitro suggests that the pharmacologic action of this compound may be quite different in primates than in rodents. Indeed our data suggest that, since the dibutyl ester remains essentially intact in human serum for up to 48 h, this compound ought to undergo widespread tissue distribution in man before being cleaved to MTX, in marked contrast to the mouse where MTX formation is almost instantaneous.

In order to compare the relative rate of cleavage of the α- and γ-ester groups under the influence of serum esterases, a 150-mg sample of methotrexate dibutyl ester was incubated in fresh human serum for 72 h. The proteins were precipitated by chilling to 0 °C and diluting threefold with MeOH, and the supernatant was clarified by membrane filtration. The filtrate was then passed rapidly through a DEAE-cellulose column with pH 7.4 phosphate buffer as the eluent in order to remove unchanged diester. The pooled eluates afforded 15 mg of a mixture whose HPLC analysis is shown in Figure 1. Comparison with the clearly resolvable authentic mixture of MTX and its α- and γ-monoesters revealed that the preponderant product was the γ-monoester. A small amount of α-monoester was also visible, the γ-monoester/α-monoester ratio being about 85:15 according to the relative areas under the HPLC peaks. Although there were some ad-

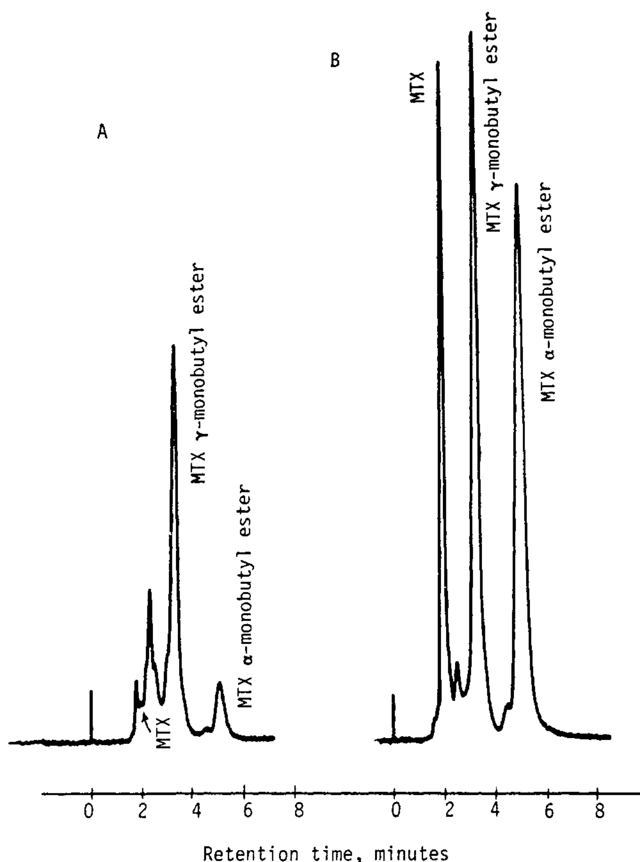

Figure 1. HPLC separation of MTX and its α-monobutyl and γ-monobutyl esters: A, incubation of methotrexate dibutyl ester in whole human serum (37 °C, 72 h); B, synthetic mixture of MTX and monoesters.

ditional peaks that represented nonprecipitable components of the serum, it was apparent that very little free MTX was formed. These findings are the first unequivocal evidence that cleavage of methotrexate diesters by serum esterase occurs preferentially at the α position and follows the same course as chemical cleavage in the presence of piperidine.

The variations in growth-inhibitory activity against cultured human leukemic cells that we observed between the α- and γ-monoesters of MTX could be due to differences in ease of passage across the plasma membrane, differences in affinity for dihydrofolate reductase, or differences in ease of cleavage to MTX. In order to help clarify this question, the mono- and dibutyl esters of MTX were assayed as inhibitors of a partially purified dihydrofolate reductase from rabbit liver. As indicated in Table II, the α- and γ-monobutyl esters were equiactive against this particular enzyme but were approximately eight times more inhibitory than the dibutyl ester, being in fact almost as active as MTX itself. Thus the inhibitory effect of these compounds against cultured human leukemic cells increased in the order α- and γ-monobutyl esters < dibutyl ester < free acid, whereas in the enzyme inhibition assay they followed the order dibutyl ester < α- and γ-monobutyl esters < free acid. Additional studies will be required in order to explain this lack of correlation between cytotoxicity and dihydrofolate reductase inhibition.

In summary, we have established the following facts in this study: (1) the action of primate serum on methotrexate dibutyl ester in vitro is much slower than that of rodent serum and yields methotrexate γ-monobutyl ester as the major metabolite, with a smaller amount of methotrexate α-monobutyl ester and even less methotrexate free acid; and (2) though the α- and γ-monoesters are less cytotoxic to cultured leukemic cells than either the dibutyl ester or MTX itself, they nonetheless can bind strongly to purified mammalian dihydrofolate reductase in a cell-free assay system. Our results indicate that the monoesters of MTX should be expected to play a significant role in the pharmacology of the diesters in nonrodent species, such as the dog, monkey, or human. Whether the monoesters themselves deserve consideration as potential chemotherapeutic agents on their own merit cannot be assessed until additional structure–activity studies have been carried out. Such studies are currently in progress in this laboratory.

Experimental Section

Melting points (uncorrected) were determined in Pyrex capillary tubes by means of a Mel-Temp apparatus (Laboratory Devices, Inc., Cambridge, Mass.) at a heating rate of approximately 2 °C/min. 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. TLC analysis was performed on Anasil OF silica gel plates (250-μ thickness, New England Nuclear Corp., Boston, Mass.) and on Eastman 13181 silica gel or Eastman 13254 cellulose sheets with fluorescent indicator. Spots were visualized under laboratory light or at 254 nm in a viewing chamber. Conventional column chromatography was carried out on Baker 5-3405 silica gel (60–200 mesh), and *dry column* chromatography was performed on Woelm activity grade III/30 mm silica gel (ICN Pharmaceuticals, Inc., Cleveland, Ohio) as described elsewhere.[15] Ion-exchange chromatography was carried out on DEAE-cellulose (Whatman DE-52 preswollen powder). Liquid chromatographic (HPLC) analyses were performed on a Waters instrument (Model ALC202) equipped with a U6K injector, dual Model 6000 pumps, Model 660 solvent programmer, and standard 254-nm ultraviolet absorbance detector. A Schoeffel Spectroflow Monitor Model SF770 was also connected to the system in order to record the absorbance simultaneously at 270 nm. A Waters μ Bondapak CN column (3.9 mm i.d. × 30 cm) was used, with 0.005 M ammonium phosphate buffer, pH 5.0, containing 30% EtOH as the eluent. Fresh buffer was prepared daily, degassed by boiling for at least 15 min, and passed through a 0.20-μ filter membrane (Gelman Metricel Type GA-8). Solvents used for mixed anhydride coupling were dried over Linde 4A molecular sieves, and other routine drying of organic solutions prior to rotary evaporation was carried out over anhydrous Na_2SO_4. Microanalyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn., and were within ±0.4% of the calculated value except where otherwise indicated. L-Glutamic acid γ-monomethyl and γ-monoethyl esters were purchased from Fox Chemical Co., Los Angeles, Calif., and Bachem, Inc., Torrance, Calif., respectively. Both monoesters were confirmed to be pure single isomers, free of MTX, by TLC prior to use. *N*-Carbobenzyloxy-L-glutamic acid and hexamethyldisilazane were purchased from Aldrich Chemical Co., Milwaukee, Wis., and cesium carbonate was obtained from Alfa Division/Ventron Co., Beverly, Mass. Methotrexate and 3′,5′-dichloromethotrexate diesters were prepared by HCl-catalyzed esterification as previously described.[2] The DCM employed for this purpose was a generous gift from Lederle Laboratories, Pearl River, N.Y. [^{3}H]-MTX was kindly furnished by Dr. Harry Wood, Division of Cancer Treatment, National Cancer Institute. [^{3}H]Folic acid was purchased from Amersham Corp., Arlington Heights, Ill.

Synthesis of Monoesters by Partial Esterification. Methotrexate α-Monoethyl and γ-Monoethyl Esters (Method A). Anhydrous HCl in absolute EtOH (1% solution, 36 mL, 0.0099 mol) was added to a suspension of methotrexate free acid (3 g, 0.0066 mol) in absolute EtOH (600 mL), and the mixture was stirred at room temperature for 16 h. A homogeneous solution was obtained after about 20 min. At the end of this time the excess HCl was removed by bubbling nitrogen gas through the solution for 15 min. Rotary evaporation (30–35 °C) gave an orange solid which was suspended in pH 7.4 phosphate buffer. The pH was adjusted to ca. 8 with 5 N NaOH and the insoluble portion, consisting of methotrexate diethyl ester (1.5 g, 59% yield), was

filtered off. The filtrate was lyophilized, the residue was dissolved in a minimum of water, and the pH of the solution was adjusted to ca. 5 with 12 N HCl. The mixture was allowed to stand in the refrigerator for 30 min and filtered, and the solid was washed with water and dried in vacuo over P_2O_5. The crude solid (1.7 g) was dissolved in 3:1 $CHCl_3$–MeOH, a small amount of silica gel (Woelm activity grade III, suitable for dry column use) was added, and the solvent was removed under reduced pressure. The powder was then added to the top of a silica gel dry column (140 g) which was eluted with 95:5 (1000 mL), 90:10 (2000 mL), 85:15 (1000 mL), 80:20 (1000 mL), and 75:25 (1000 mL) $CHCl_3$–MeOH. Five fractions were collected: (1) methotrexate diethyl ester (0.18 g); (2) methotrexate α-monoethyl ester (0.19 g); (3) methotrexate α-monoethyl ester and methotrexate γ-monoethyl ester mixture (0.072 g); (4) methotrexate γ-monoethyl ester (0.96 g); and (5) methotrexate free acid. The ratio of γ- and α-monoethyl isomers isolated in this experiment was 5:1. Although the two isomers could not be distinguished by TLC using a number of different solvent systems, there were characteristic differences in IR absorption in the 1450–1650-cm^{-1} fingerprint region. On the basis of these differences it could be determined that the slow-moving isomer and the compound obtained from 4-amino-4-deoxy-N^{10}-methylpteroic acid (cf. method D) were one and the same.

Synthesis of Monoesters by Partial Cleavage of Diesters with NaOH (Method B). Methotrexate γ-Monobutyl Ester. A solution of NaOH (0.0078 g, 0.00019 mol) in water (3 mL) was added to a solution of methotrexate dibutyl ester (0.1 g, 0.00018 mol),[2] and the mixture was allowed to stand at room temperature for 1 h. Evaporation under reduced pressure left an orange glass which was taken up in water (3 mL) to which was then added 3 drops of 1 N HCl to bring the pH down to ca. 5. Filtration of the precipitated solid, washing with water, and drying in vacuo over P_2O_5 gave a yellow solid (0.075 g) which was passed through a silica gel dry column (15 g, Woelm activity grade III) with 95:5 (25 × 8 mL) and 80:20 (175 × 8 mL) $CHCl_3$–MeOH as eluents. Individual tubes were monitored by TLC and pooled into appropriate fractions. Tubes 6–12 contained mainly unchanged methotrexate dibutyl ester (0.0045 g), tubes 64–183 contained methotrexate γ-monobutyl ester (0.035 g), and the higher tubes contained mainly methotrexate free acid. The TLC, HPLC, and IR properties of this material and of the product obtained from 4-amino-4-deoxy-N^{10}-methylpteroic acid (cf. method D) were identical.

Synthesis of Monoesters by Partial Cleavage of Diesters with Piperidine (Method C). 3′,5′-Dichloromethotrexate γ-Monoethyl Ester. A mixture of 3′,5′-dichloromethotrexate diethyl ester (0.2 g, 0.00035 mol),[2] 95% EtOH (16 mL), and piperidine (0.4 mL) was stirred under reflux for 47 h. After rotary evaporation, the glassy residue was taken up in a minimum volume of 9:1 $CHCl_3$–EtOH and the solution was applied to a silica gel column (10 g) which was eluted successively with 9:1 $CHCl_3$–EtOH (10 × 10 mL), 8:2 $CHCl_3$–EtOH (20 × 10 mL), and 7:3 $CHCl_3$–EtOH (20 × 10 mL). The γ-monoester (0.14 g) was isolated from tubes 9–12 as a bright yellow solid which was soluble in pH 7.4 phosphate buffer and exhibited a C=O ester peak of medium intensity of 1740 cm^{-1}.

Synthesis of Monoesters by Mixed Anhydride Coupling (Method D). A. Methotrexate γ-Monoethyl Ester. Isobutyl chloroformate (0.55 g, 0.004 mol) was added at room temperature to a stirred solution of 4-amino-4-deoxy-N^{10}-methylpteroic acid (0.93 g, 0.0025 mol, based on the formula $C_{15}H_{17}N_7O_2 \cdot 0.5HCl \cdot 1.5H_2O$)[3] in dry DMF (100 mL). After being stirred for 20 min, the mixture was concentrated to dryness (30 °C) with the aid of a rotary evaporator connected to a vacuum pump (dry ice–acetone trap), and the amber-colored residue was triturated twice with pH 7.4 phosphate buffer, rinsed with water, and dried in vacuo over P_2O_5 to obtain the crude mixed anhydride as a yellowish brown solid (1.0 g, 91% yield). To a solution of this anhydride (0.51 g, 0.00012 mol) in dry THF (35 mL) was added a suspension of L-glutamic acid γ-ethyl ester (0.38 g, 0.0019 mol) in dry THF (10 mL) containing triethylamine (0.19 g, 0.0019 mol), and the mixture was stirred under reflux for 2 h. Since TLC analysis showed that the reaction was not proceeding under these conditions, the THF and triethylamine were evaporated under reduced pressure and replaced with dry DMF (20 mL) containing 1,1,3,3-tetramethylguanidine (0.22 g, 0.001 mol), and the mixture was stirred at 80–90 °C for 25 min. Evaporation of the DMF yielded an amber-colored oil which was dissolved by suspending it in a small volume of pH 7.4 phosphate buffer and adjusting the pH to 8 with 5 N NaOH. The solution was applied to a column of DEAE-cellulose (90 g) which was eluted with pH 8 phosphate buffer (80 × 20 mL, 120 mL/h). Individual tubes were monitored by TLC (cellulose, pH 7.4 phosphate buffer) Fractions 27–52 were pooled and lyophilized, the residue was dissolved in a minimum of water, and the pH was adjusted to ca. 4 with 12 N HCl. Filtration of the solid, washing with water, and drying in vacuo over P_2O_5 afforded two crops (0.14 g total, 23% yield). A portion (0.05 g) of this solid was purified further by passage through a silica gel dry column (6 g) with 95:5 (10 × 20 mL) and 80:20 (60 × 20 mL) $CHCl_3$–MeOH as eluents. The pure γ-monoethyl ester was obtained by evaporation of the pooled 80:20 $CHCl_3$–MeOH eluates as a yellow-orange solid (0.04 g, 80% recovery): IR (KCl) 1740 (ester C=O), 1610 cm^{-1} (amide C=O).

B. Methotrexate γ-Monobutyl Ester. To a suspension of L-glutamic acid γ-butyl ester (0.122 g, 0.0006 mol) in hexamethyldisilazane (33 mL) was added a mixture of dry benzene (10 mL) and concentrated H_2SO_4 (0.072 g). After 2 h under reflux (drying tube), the mixture was cooled and triethylamine (1.1 mL) was added dropwise. After an additional 5 min of refluxing, the solvent was removed at 40 °C with the aid of a rotary evaporator (water aspirator followed by vacuum pump). The colorless residue was allowed to dry in vacuo over P_2O_5 for 1 h before being resuspended in 2:1 Me_2SO–THF (30 mL). To this suspension was then added a solution of the isobutyl mixed anhydride of 4-amino-4-deoxy-N^{10}-methylpteroic acid (0.128 g, 0.0003 mol)[2] in 1:1 Me_2SO–THF (5 mL). After 16 h of stirring at room temperature, the mixture was heated on a steam bath for 5 min. Rotary evaporation (vacuum pump, 50 °C) gave an amber-colored syrup which was taken up in pH 8.5 phosphate buffer and chromatographed on a column of DEAE-cellulose (50 g) with pH 7.4 phosphate buffer (0.005 M) as the eluent (9-mL fractions, 120 mL/h). Fractions 31–70 were pooled and lyophilized, the residue was redissolved in a minimum of water, and the pH was adjusted to 5 by careful acidification with 12 N HCl. After 2 h at 2 °C, the precipitated solid was filtered, washed with ice-cold water, and dried in vacuo over P_2O_5 to obtain pure methotrexate γ-monobutyl ester as a yellow-orange powder (0.023 g): IR (KCl) 1745 (ester C=O), 1610 cm^{-1} (amide C=O). A small amount of unchanged 4-amino-4-deoxy-N^{10}-methylpteroic acid (0.046 g, 36% recovery) was obtained from fractions 81–128. TLC and HPLC comparison of this material with the slower moving product from the HCl-catalyzed esterification reaction (cf. method A) established their identity, as did the fingerprint regions of their IR spectra which were superimposable.

***N*-Benzyloxycarbonyl-L-glutamic Acid α-Benzyl γ-Butyl Ester.** A solution of *N*-benzyloxycarbonyl-L-glutamic acid α-benzyl ester (1.0 g, 0.00269 mol) in a mixture of MeOH (15 mL) and H_2O (1.5 mL) was adjusted to ca. pH 8 by adding 20% aqueous cesium carbonate (3.4 mL). After removal of the solvent under reduced pressure, the residue was dried by repeated addition of dry DMF and rotary evaporation at 45 °C with the aid of a vacuum pump and dry ice–acetone trap. The white residue was stirred with *n*-butyl bromide (0.48 g, 0.0034 mol) in dry DMF (10 mL) at room temperature for 19 h. Filtration of the reaction mixture and rotary evaporation of the filtrate (vacuum pump, 45 °C) yielded a colorless semisolid which was triturated with H_2O (50 mL) and then extracted with EtOAc (40, 25, and 25 mL). The combined EtOAc extracts were dried and evaporated to a yellow oil which was chromatographed on a conventional silica gel column (25 g) with C_6H_6, 1:1 C_6H_6–$CHCl_3$, 99:1 $CHCl_3$–MeOH, and 98:2 $CHCl_3$–MeOH as eluents (all 22 × 9 mL). Fractions 17–68 were pooled and rechromatographed on dry silica gel (30 g) using $CHCl_3$ (44 × 9 mL) as the eluent. Fractions 2–22, which contained a single spot on TLC (R_f 0.68, silica gel, 99:1 $CHCl_3$–MeOH), yielded a colorless oil (0.97 g, 84% yield): IR (neat) 3420, 1745, 698 cm^{-1}; NMR ($CDCl_3$) τ 9.11 (unresolved t, 3 H, CH_3), 8.56 (m, 4 H, butyl CH_2CH_2), 8.13–7.50 (m, 4 H, glutamyl CH_2CH_2), 5.98 (t, 2 H, butyl OCH_2, J = 6 Hz), 5.63 (m, 1 H, NHC*H*), 4.91 (s, 2 H, benzyloxycarbonyl CH_2), 4.88 (s, 2 H, α-benzyl ester CH_2), 4.50 (br d, 1 H, NH, J = 8 Hz), 2.67 (s, 10 H, aromatic protons). Anal. ($C_{24}H_{29}NO_6$) C, H, N.

L-Glutamic Acid γ-Butyl Ester. To a glass pressure bottle (500 mL) containing absolute EtOH (60 mL) and 10% Pd/C (0.15 g, prereduced for 30 min in a Parr shaker apparatus) were added in succession a solution of the *N*-benzyloxycarbonyl derivative (0.89 g, 0.002 mol) in absolute EtOH (10 mL) and a mixture of concentrated HCl (0.15 mL) and absolute EtOH (0.25 mL). After hydrogenation for 2 h, the reaction mixture was filtered through Celite. Evaporation of the filtrate and trituration of the light brown residue gave a colorless solid (0.41 g, 97% yield) which was TLC homogeneous (R_f 0.11, silica gel, 1:1 $CHCl_3$–MeOH): mp 163–165.6 °C dec. The product was readily soluble in 1 N $NaHCO_3$ and 1 N HCl and gave a positive reaction with ninhydrin TLC spray. The analytical sample was prepared by recrystallization from absolute EtOH: mp 183–184.5 °C dec; IR (KCl) 3505, 2500–2900, 1700, 1752 cm^{-1}; NMR (CF_3COOH) τ 9.02 (unresolved t, 3 H, CH_3), 8.8–7.9 (m, 4 H, butyl CH_2CH_2), 7.8–6.8 (m, 4 H, glutamyl CH_2CH_2), 5.73 (t, 2 H, OCH_2, J = 6 Hz), 6.0–5.2 (m, 1 H, NHC*H*), 2.25 (m, 3 H, NH_3^+). Anal. ($C_9H_{17}NO_4{\cdot}0.8H_2O$) C, N; H: calcd, 8.61; found, 8.11.

Incubation of Methotrexate Dibutyl Ester with Human Serum. A solution of methotrexate dibutyl ester (0.15 g, 0.00026 mol) in Me_2SO (5 mL) was added to four centrifuge tubes containing a total of 95 mL of freshly prepared human serum, and the tubes were stoppered and kept at 37 °C in a rocking platform incubator for 72 h, at which time TLC analysis revealed a single moving spot (R_f 0.55, cellulose, pH 7.4 phosphate buffer) along with unchanged starting material which remained at the origin. Free MTX was barely detectable (an authentic sample spotted as a control migrated with an R_f value of 0.80). Ice-cold MeOH (190 mL) was added to the pooled incubation mixtures in order to terminate the reaction, and the precipitated proteins were spun down in a refrigerated centrifuge. The supernatant was filtered through a membrane filter (0.2-μ diameter) and applied to the top of a DEAE-cellulose column (90 g) which was eluted rapidly with pH 7.4 phosphate buffer. The entire yellow band which passed through the column was collected as a single fraction. The solution was concentrated to a volume of 20 mL by rotary evaporation, and glacial AcOH was added dropwise at 0 °C until the pH was brought down to 4.0. Centrifugation of the precipitated solid, decantation, washing to neutrality with ice–water, transfer to small filter with ether, and drying in vacuo at 50 °C for 48 h gave a bright yellow powder (15 mg). HPLC analysis revealed that the main product in this fraction was methotrexate γ-monobutyl ester, with smaller amounts of methotrexate α-monobutyl ester and a trace of free MTX also present. A deliberate mixture of MTX and the γ- and α-monobutyl esters gave clearly resolvable peaks with retention times of 2.0, 3.2, and 5.1 min, respectively (Waters μ Bondapak CN column, 3.9 mm i.d. × 30 cm, 0.005 M ammonium phosphate, pH 5, containing 30% EtOH, 1.5 mL/min). The approximate ratio of γ- to a α-monobutyl ester was 85:15 as measured on the basis of relative peak areas.

Species Differences in the Serum Esterase Cleavage of Methotrexate Dibutyl Ester. [^{3}H]Methotrexate dibutyl ester (0.006 g, sp act. 10 mCi/mmol) was prepared via the standard HCl-catalyzed esterification method,[2] dissolved in Me_2SO (0.1 mL), and added to whole serum (0.9 mL). The mixture was incubated at 37 °C and at designated times an aliquot (0.2 mL) was removed, cooled to 0 °C, diluted with MeOH (0.3 mL), and spun down to obtain a clear supernatant. Multiple 10-μL samples were spotted on cellulose TLC sheets which were developed with pH 7.4 phosphate buffer. Spots were visualized under UV light, cut out with scissors, and analyzed by scintillation counting. The radioactivity measured at the origin and in the mobile spots at R_f 0.55–0.60 and 0.75–0.80 was taken to represent unchanged dibutyl ester, α- and/or γ-monobutyl esters, and free acid, respectively. The relative abundance of each fraction was expressed as a percentage. The results for mouse, monkey, and human serum after incubation for 2, 6, 24, and 48 h are shown in Table III.

Dihydrofolate Reductase Inhibition Studies. A modification of the method of Rothenberg[24] was used. The reaction mixture contained 0.02 M citrate buffer, pH 4.8; 7 μM TPNH; 15 μM 2-mercaptoethanol; 1.8×10^{-9} M [^{3}H]folic acid (sp act. 61 Ci/mmol); and 0.100 mL of dihydrofolate reductase prepared from rabbit liver.[25] The total volume was 1.0 mL. The mixture was incubated at 37 °C for 10 min and quenched by rapid cooling and addition of 0.4 mL of 1.3×10^{-2} M unlabeled folic acid. Unreduced folic acid was precipitated by adding 0.4 mL of 0.088 M zinc sulfate, and after centrifugation an aliquot of supernatant was counted in 10 mL of Biofluor scintillation medium (New England Nuclear Corp., Boston, Mass.) in a Searle Mark III scintillation counter. ID_{50} values were obtained graphically and are listed in Table II.

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(22) Although we have succeeded in preparing methotrexate α-monomethyl ester by HCl-catalyzed esterification, as well as by mixed anhydride coupling from 4-amino-4-deoxy-N^{10}-methylpteroic acid and L-glutamic acid α-methyl ester, this compound is considerably less stable than the γ-monomethyl isomer and was therefore not included in the biological studies. Even the γ-monomethyl ester has a tendency to decompose to MTX on storage (a sample kept

for 10 months in a tightly capped amber bottle was found to contain approximately 50% MTX). Although the longer chain monoesters are much more stable than the methyl derivatives, they should be stored at low temperature, preferably in a desiccator, and their homogeneity should be checked periodically by TLC.

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Effects of Molecular Modification on Hypocholesteremic Activity of 1,3-Bis(substituted phenoxy)-2-propanones and Related Derivatives

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A series of 1,3-bis(substituted phenoxy)-2-propanones, long-chain ketones, and related derivatives has been synthesized, and it has been found that certain analogues produce significant lowering of serum cholesterol levels in Sprague–Dawley rats. These compounds possess no estrogenic properties and are nontoxic at 10 mg/kg/day. Physical studies on these compounds include an attempt to correlate hypocholesteremic activity with the lipophilic, electronic, and steric properties of the compounds. The 1-octanol–H_2O partition coefficient was measured for certain derivatives and the π constant for the substituents was calculated. Hammett's σ constants for aromatic substituents were obtained from the literature. The only correlation found to exist is between hypocholesteremic activity and steric size and position of the aromatic substituents in the propanones.

Previous work has revealed that certain analogues of a series of 1,3-bis(substituted phenoxy)-2-propanones[1] and long-chain ketones[2] possess excellent hypocholesteremic activity at dose levels of 10 mg/kg/day. Both series of compounds are nontoxic and nonestrogenic, possessing none of the antifertility properties seen in the 2,8-dibenzylcyclooctanones.[3] The most probable hypocholesteremic mechanism lies in the inhibition of HMG-CoA reductase by 1,3-bis(*p*-methylphenoxy)-2-propanone and 2-hexadecanone, while 2-hexadecanone also inhibits acetyl-CoA synthetase.[4] 1,3-Bis(*p*-methylphenoxy)-2-propanone has also been shown to produce significant lowering of serum triglyceride levels at 10 mg/kg/day in correlation with the inhibition of *sn*-glycerol-3-phosphate acyltransferase and phosphatidate phosphohydrolase in vitro.[5] The work reported here extends the propanone series in order to elucidate more completely the effects of molecular modification on hypocholesteremic activity.

Experimental Section

Chemical Synthesis. All melting points were corrected and obtained using a Thomas-Hoover melting point apparatus. Purity of intermediates not subjected to elemental analysis was ascertained by thin-layer chromatography. Micro-thin-layer chromatography was performed using silica gel G coated microslides with chloroform as the eluting solvent. All column chromatography was performed using either silica gel 60 (70–230 mesh) or Florisil. Infrared spectra were obtained on a Perkin-Elmer 257 infrared spectrophotometer. All chemicals were used as received from manufacturers. Elemental analyses (±0.4%) were performed on all biologically tested derivatives by either Atlantic Microlab, Atlanta, Ga., or M-H-W Laboratories, Garden City, Mich. Infrared spectral data are included for those oils only that were column chromatographed and, therefore, not identified by their respective boiling points.

General Procedure for Preparation of 1-(Substituted phenoxy)-2-propanols 1 and 2. One equivalent each of the appropriate phenol and sodium hydroxide was dissolved in 1,4-dioxane (30 mL per 0.3 mol of phenol) at 98–101 °C. To this solution was added 1.0 equiv of propylene oxide dropwise over a 10-min period, and the reaction mixture was stirred for at least 5 h. The 1,4-dioxane was removed in vacuo and the residue dissolved in ether, extracted with 10% sodium hydroxide, and dried over Na_2SO_4. Filtration and removal of the ether afforded the crude product which was purified by either distillation or column chromatography (Table I).

General Procedure for Preparation of 1,3-Bis(substituted aryloxy)-2-propanols 3–6. These derivatives, except **28**, were prepared according to the general procedure for the preparation of 1,3-bis(substituted phenoxy)-2-propanols[1] (Table I).

General Procedure for Preparation of 1-(Substituted phenoxy)-3-(substituted benzoxy)-2-propanols 7 and 8. Dry pyridine (50 mL) and 0.10 mol of the appropriate 3-(substituted phenoxy)-1,2-propandiol were dissolved in 400 mL of dry chloroform, and the solution was cooled to 0 °C. To this solution was added dropwise 0.10 mol of the appropriate benzoyl chloride at 0 °C with stirring over a 1-h period. After the addition, the reaction temperature was allowed to slowly come to room temperature and was stirred an additional 3 h. The chloroform and pyridine were removed in vacuo, and the residue was dissolved in sufficient ether and extracted with water (1 × 150 mL), 1.0 N H_2SO_4 (3 × 150 mL), water (1 × 150 mL), 5% $NaHCO_3$ (3 × 100 mL), and water (1 × 100 mL). The ether solution was dried over Na_2SO_4 and filtered, and the ether was removed to afford the crude ester which was then purified by chromatography or recrystallization (Table I).

General Oxidation Procedure for 9–23. All ketones reported in this paper (except **35** and **36**) were obtained from the corresponding hydroxy derivatives using dicyclohexylcarbodimide (DCC), dimethyl sulfoxide (Me_2SO), pyridine, and trifluoroacetic acid as previously described[1] (Table II).

1-(*p*-Methylphenoxy)-2-hexadecanol (24). *p*-Cresol (10.1 g, 0.093 mol) and 3.7 g (0.093 mol) of NaOH were dissolved in 40 mL of 1,4-dioxane at 90–95 °C. To this solution was added 15.0 g (0.062 mol) of 1,2-epoxyhexadecane dropwise, and the reaction was stirred at 100 °C for 4 h. The dioxane was removed in vacuo, the residue was taken up in 400 mL of chloroform, extracted with water (2 × 100 mL) and 10% NaOH, dried over Na_2SO_4, and filtered, and the chloroform was removed to give a crude, brown solid. Recrystallization from 2-propanol afforded 15.7 g (73%) of crystals: mp 86–88 °C; R_f 0.68.

1-(*p*-Methylphenoxy)-2,3-epoxypropane (25). The general procedure for the synthesis of phenoxypropylene oxides described by Britton and Slagh was followed.[6] Sodium hydroxide (7.4 g, 0.185 mol) and 20.0 g (0.185 mol) of *p*-cresol were dissolved in 50 mL of water at 98–100 °C. To this solution was added 34.2 g (0.369 mol) of epichlorohydrin, and the reaction was stirred overnight. The solvents were removed in vacuo, the residue was taken up in ether, extracted with water (2 × 100 mL), dried over Na_2SO_4, and filtered, and the ether was removed to give 42.1 g

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