Methotrexate Analogues. 10. Direct Coupling of Methotrexate and Diethyl L-Glutamate in the Presence of Peptide Bond-Forming Reagents

Andre Rosowsky* and Cheng-Sein Yu

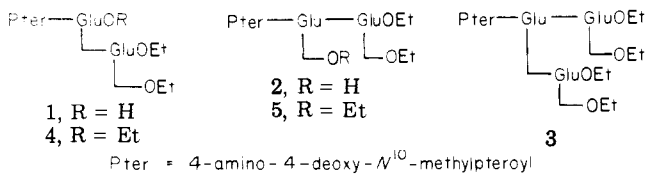
The Sidney Farber Cancer Institute and Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115. *Received September 19, 1977*

The reaction of methotrexate (4-amino-4-deoxy-N^{10}-methylpteroyl-L-glutamic acid, MTX) with diethyl L-glutamate in the presence of peptide bond-forming reagents such as *N,N'*-dicyclohexylcarbodiimide (DCC) or diphenylphosphoryl azide gives methotrexate γ-L-glutamate diethyl ester (1), methotrexate α-L-glutamate diethyl ester (2), and methotrexate α,γ-bis(L-glutamate tetraethyl ester) (3). The major monosubstituted product is the α-isomer 2. With excess diethyl L-glutamate the bis-adduct 3 can be made to predominate, and this compound is also accessible from 2 by reaction with a second molar equivalent of diethyl L-glutamate or via a direct mixed anhydride condensation between 4-amino-4-deoxy-N^{10}-methylpteroic acid and tetraethyl α,γ-bis(L-glutamate). Esterification of 1 and 2 yielded the corresponding triethyl esters 4 and 5, which could also be obtained via mixed anhydride coupling from 4-amino-4-deoxy-N^{10}-methylpteroic acid and triethyl γ-L-glutamyl-L-glutamate or triethyl α-L-glutamyl-L-glutamate, respectively. Compounds 4 and 5 were also prepared from methotrexate α-monoethyl ester and methotrexate γ-monoethyl ester by reaction with diethyl L-glutamate. TLC and dry column silica gel chromatographic evidence indicates that partial racemization of the glutamate side chain occurs in MTX when either DCC or diphenylphosphoryl azide is used as the coupling reagent. This finding is explainable on the basis that *N*-aroyl type substitution on the glutamate side chain of MTX favors racemization via a resonance-stabilized oxazolone intermediate.

Polyglutamates of methotrexate (MTX, 4-amino-4-deoxy-N^{10}-methylpteroylglutamic acid) have aroused considerable interest because of growing evidence that they play a larger role in the clinical pharmacology of this antitumor drug than has heretofore been realized. It has now been shown in several laboratories[1-4] that the liver and kidneys of animals treated with MTX accumulate significant amounts of monoglutamate and diglutamate adducts, MTX (G_1) and MTX (G_2), whose structures have been established unequivocally by chemical synthesis.[5] Higher polyglutamates of MTX are not produced in measurable amount in mammalian tissues, in contrast to the higher polyglutamates of reduced folates,[6-8] which are formed readily in animal as well as bacterial cells and possess a number of very interesting biological properties.[8-13] It has also been observed recently that MTX (G_1) and MTX (G_2) are active inhibitors of dihydrofolate reductase and that MTX (G_1) is approximately as effective as MTX in preventing the growth of L1210 leukemic cells in vitro and in vivo.[14,15]

We reported briefly in 1975 that the monosodium salt of MTX (G_1) diethyl ester shows significant activity against L1210 mouse leukemia in vivo.[16] When administered by ip injection at a dose of 120 mg/kg (q3d 1, 4, 7) to L1210 leukemic mice, this compound elicited an increase in median survival of +77%. Our results were consistent with subsequently published data on MTX (G_1) itself,[14] and we were therefore encouraged to extend our investigation in the direction of other ester modifications of methotrexate polyglutamates. An important consideration in our program was that the strongly lipophilic character of such esters might have a profound influence on their pharmacologic distribution and perhaps also on their uptake by cells.

In this paper we report the direct synthesis of methotrexate γ-L-glutamate diethyl ester (1), methotrexate α-L-glutamate diethyl ester (2), and methotrexate α,γ-bis(L-glutamate tetraethyl ester) (3) from MTX and diethyl L-glutamate. Further esterification of 1 and 2 with ethanolic HCl[17] led to the triesters 4 and 5, respectively.



Compounds 4 and 5 were also obtained from methotrexate α- and γ-monoethyl esters[18] by condensation with diethyl L-glutamate, and compound 5 was obtained from 4-amino-4-deoxy-N^{10}-methylpteroic acid[19] by reaction with triethyl α-L-glutamyl-L-glutamate. Unequivocal alternative routes provided additional support for the assignment of structure of these compounds.

Three products were formed when MTX was allowed to react with excess diethyl L-glutamate after prior activation of the amino ester with diethyl phosphorochloridite.[20] The principal product, methotrexate α-L-glutamate diethyl ester (2), was obtained in 20% yield. Smaller amounts of methotrexate γ-L-glutamate diethyl ester (1) and methotrexate α,γ-bis(L-glutamate tetraethyl ester) (3) were also isolated. Since compound 3 lacked a free COOH group and was much less polar than 1 and 2, it could be separated readily from them by $CHCl_3$ extraction. The diesters 1 and 2, on the other hand, were soluble in *n*-butyl alcohol and could be separated from unchanged MTX by means of this solvent. Separation of the diesters 1 and 2 from one another was achieved by column chromatography on *deactivated* silica gel.[21] The faster moving component of the mixture, both on TLC plates (cf. Table I) and on the column, was compound 2. The observed order of elution was consistent with our structural assignment, since one would expect the less acidic and therefore more easily eluted isomer to be the one with a free γ-COOH group. Some difference in solubility of the two compounds in dilute $NaHCO_3$ was noted, the faster moving isomer being the less soluble one. However, the difference was too small to allow effective separation of the isomers by alkaline extraction.[22]

Qualitatively similar results were observed when the coupling of MTX and diethyl L-glutamate was effected in the presence of DCC, though the yield of each product was higher. When equimolar proportions of the reactants were used, the γ- and α-monoglutamates 1 and 2 were isolated in yields of 17 and 31%, respectively, and the α,γ-diglutamate 3 was obtained in 11% yield. When MTX, diethyl L-glutamate, and DCC were combined in a 1:2:2 ratio in order to favor α,γ-disubstitution, the yield of compound 3 was increased to 39%. A fourth product was also detected, however, whose lack of solubility in dilute base and whose high R_f value indicated the absence of a free COOH group. The infrared spectrum contained an ester carbonyl stretching peak at 1740 cm^{-1}, but the height of this peak was only half that of the corresponding peak

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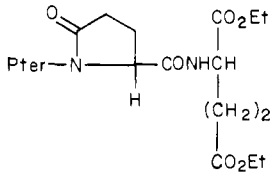
Table I. Methotrexate Mono- and Bis(L-glutamate) Adducts

Compd	Method[a]	Mp, °C[b]	TLC solvent systems and R_f values: C_6H_6-MeOH-AcOH[c] 45:30:1	9:6:1	9:3:1	$CHCl_3$-MeOH[d] 1:1	3:1	6:1	Formula	Analyses[e]
1	A	127-138	0.44	0.35	0.20	0.23	0.02	0.01	$C_{29}H_{37}N_9O_8 \cdot 2.2H_2O$	C, H, N
2	A	125-135	0.58	0.46	0.31	0.28	0.10	0.04	$C_{29}H_{37}N_9O_8 \cdot 0.5H_2O$	C, H, N
3 (mixture of LLL and DLL isomers	A	106-113	0.82	0.68	0.47	0.82	0.77	0.46 0.40	$C_{38}H_{52}N_{10}O_{11} \cdot 0.5H_2O$	C, H, N
3 (LLL isomer)	B	80-105				0.82	0.78	0.46	$C_{38}H_{52}N_{10}O_{11} \cdot 0.5H_2O$	C, H, N
3 (DLL isomer)	B	80-107				0.82	0.77	0.43	$C_{38}H_{52}N_{10}O_{11} \cdot 0.5H_2O$	C, H, N
4	C	92-105				0.79	0.76	0.44	$C_{31}H_{41}N_9O_8 \cdot 0.5H_2O$	C, H, N
5	C	99-110				0.79	0.73	0.40	$C_{31}H_{41}N_9O_8 \cdot 0.5H_2O$	C, H, N

[a] A = MTX + diethyl L-glutamate-$(EtO)_2POCl$; B = 1 + diethyl L-glutamate-$(PhO)_2PON_3$; C = EtOH-HCl esterification of 1 or 2. [b] Melting points are those of the analytical sample; values may vary from one experiment to another depending on the physical state of the specimen. [c] Analtech GF silica gel plates. [d] Analabs GF silica gel plates. [e] C, H, and N analyses were within ±0.4% of calculated values.

in compound 3. We considered the possibility that DCC had brought about intramolecular amide bond formation to give structures 6 or 7. However, this was rejected on



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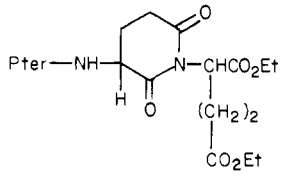

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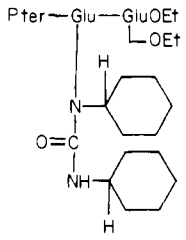

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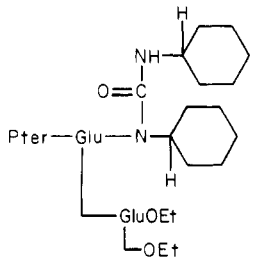

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the basis of the NMR spectrum, which revealed a larger number of CH_2 protons than could be accommodated by these structures. On the basis of the spectral evidence and microanalytical data we concluded that the fourth product of the DCC reaction was an acylurea, i.e., 8 and/or 9.

Advantageous use of the coupling reagent diphenylphosphoryl azide[23] was likewise made during this work. Reaction of MTX with diethyl L-glutamate and diphenylphosphoryl azide (2.4 molar equiv each; i.e., a 20% excess over the stoichiometric amount) led to formation of the α,γ-bis(L-glutamate) 3 in 30% yield. A similar yield of 3 was obtained by replacing MTX with the α-L-glutamate 2 and adjusting the molar ratio of reactants from 2.4:1 to 1.2:1. When the α-monoethyl and γ-monoethyl esters of MTX[18] were employed, the triesters 4 and 5 were isolated in yields of 20 and 55%, respectively. The fact that a substantially higher yield was produced from the γ- than from the α-monoethyl ester was of interest, since it supported the view that the α-COOH group in MTX is the one most readily activated by peptide bond-forming reagents such as DCC and diphenylphosphoryl azide.

An alternative method of synthesis of compounds 4 and 5 consisted of esterifying adducts 1 and 2 with 1% HCl in absolute ethanol at room temperature,[17] and it was demonstrated that the product of esterification of compound 1 was identical with the product derived from methotrexate α-monoethyl ester and diethyl L-glutamate, whereas the product of esterification of compound 2 was identical with the one obtained from methotrexate γ-monoethyl ester.

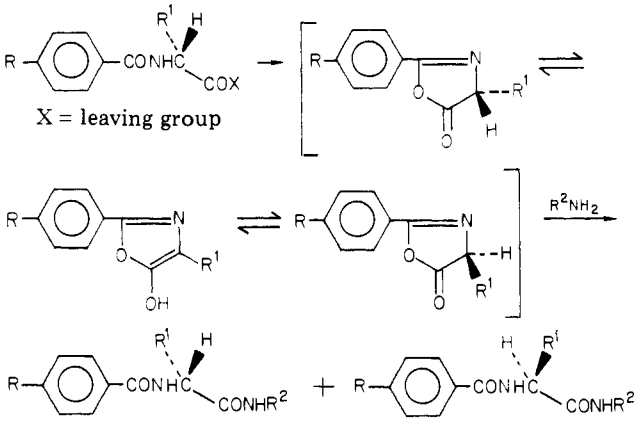
As expected, the triethyl esters 4 and 5 were insoluble in dilute base and were much less polar than 1 and 2. Infrared spectra of the triesters were distinguishable from those of the diesters on the basis of the relative intensities of the ester and amide C=O peaks at 1740 and 1615–1625 cm^{-1}, respectively. Whereas the ester/amide peak height ratio was about 1:2 in the diesters 1 and 2, it was close to 1:1 in the triesters 4 and 5. Compounds 4 and 5 were distinguishable from each other, on the other hand, by TLC (cf. Table I) and on the basis of two characteristic patterns of infrared peaks in the fingerprint region at 1300–1400 and 1100–1140 cm^{-1}.

Alternative synthetic routes were developed which left no doubt about the position of substitution in compounds 1 and 2. Triethyl α-L-glutamyl-L-glutamate was obtained from L-glutamic acid γ-monoethyl ester by protection of the amino group with benzyl chloroformate at pH 9 (87% yield), activation of the α-COOH group with isobutyl chloroformate and *N*-methylmorpholine, coupling to diethyl L-glutamate (42% yield), and catalytic hydrogenolysis over 10% Pd/C in the presence of HCl (ca. 100% yield). The resultant dipeptide ester HCl salt could not be induced to crystallize but was pure enough to be added directly to 4-amino-4-deoxy-N^{10}-methylpteroic acid which had been activated as the isobutyl mixed anhydride.[19] Though the yield in the last step was only about 10%, it was apparent from TLC and infrared spectral comparison that the product of this sequence and the compound obtained from 2 by esterification, or from methotrexate γ-monoethyl ester and diethyl L-glutamate in the presence of diphenylphosphoryl azide, were one and the same. In a similar vein, condensation of *N*-carbobenzoxy-L-glutamic acid α-monobenzyl ester with diethyl L-glutamate in the presence of diphenylphosphoryl azide (63% yield), catalytic hydrogenolysis in the presence of 10% Pd/C (71% yield), and direct coupling of the resultant dipeptide with the isobutyl mixed anhydride of 4-amino-4-deoxy-N^{10}-methylpteroic acid (23% yield) gave a product which was indistinguishable from the slower moving monoadduct (1) isolated from the reaction of MTX and diethyl L-glutamate.

Careful analysis of the α,γ-bispeptide 3 by TLC revealed that this material actually consisted of two compounds with identical melting points and virtually superimposable infrared and NMR spectra. The mixture could not be resolved on a preparative scale by conventional silica gel column chromatography, but by using the "dry column" technique we did succeed in isolating the two components in pure form. Since they gave microanalyses which were identical within experimental error, we concluded that the

glutamate moiety in MTX had undergone partial racemization during coupling, i.e., that we were dealing with a mixture of LLL and DLL diastereomers. In order to determine which compound was the LLL isomer, we prepared tetraethyl α,γ-bis(L-glutamyl-L-glutamate) from *N*-carbobenzoxy-L-glutamic acid by condensation with 2 equiv of diethyl L-glutamate in the presence of DCC and *N*-hydroxysuccinimide, followed by hydrogenolysis in the presence of 10% Pd/C, and then coupled the tripeptide tetraester to 4-amino-4-deoxy-N^{10}-methylpteroic acid via the mixed anhydride method.[19] On the basis of TLC comparison it was clear that the slower moving diastereomer of compound **3** was the one with the LLL configuration.

It is important to note that all three of the peptide bond-forming reagents used in this work led to partial racemization of the glutamate moiety in MTX. We believe that the observed loss of chirality in the presence of DCC or diphenylphosphoryl azide is due to the fact that substitution on the glutamate nitrogen is of the *N*-aroyl type, which would be expected to favor racemization via a resonance-stabilized cyclic intermediate as shown below. Classical peptide bond-forming reagents such as carbodiimides have been used in several laboratories to form macromolecular conjugates of MTX[24-29] and, in one instance, a fluorescein-coupled derivative.[30] Macromolecular conjugates of MTX have been used for such diverse purposes as the purification of dihydrofolate reductase via affinity column chromatography,[25] the preparation of specific antibodies for radioimmunoassay,[26,27] and the treatment of experimental animal tumors.[28,29] To our knowledge, the potential adverse effect of partial side-chain racemization on the antitumor activity of MTX macromolecular conjugates, or of the MTX released from them by chemical or enzymatic hydrolysis in vivo, has not received much consideration despite the fact that the D enantiomer of MTX is known to be less potent against L1210 mouse leukemia on a molar basis than the L enantiomer.[31]



Biological Activity. Compounds 1–3 were tested for antitumor activity against L1210 murine leukemia according to a standard procedure.[32,33] Compound **3**, being highly insoluble in water, was suspended in 10% Tween 80. Compounds 1 and **2** were dissolved in 0.005 M sodium phosphate buffer, pH 7.4, and the solutions were freshly prepared just before use. Injections were made ip on days 1, 4, and 7 following tumor implantation (10^5 cells ip). Doses ranged from 20 to 160 mg/kg for compounds 1 and **2** and from 50 to 400 mg/kg for compound **3**. MTX was used as a positive control and was administered as the disodium salt in aqueous solution. Five animals were used in each test group, and 15 were used as controls. The median survival time of untreated mice was 9–10 days.

Table II. Growth Inhibition of Human Lymphoblastic Leukemia Cells (CCRF-CEM) in Culture[a]

Compd	ID_{50}, μg/mL
1	0.88
2	10+
3 (LLL)	3.8
3 (DLL)	9.0
4	1.8
5	0.85

[a] Data kindly furnished by Dr. Herbert Lazarus, Sidney Farber Cancer Institute, Boston, Mass. The use of CCRF-CEM cells has been described previously.[34,35] The ID_{50} value for MTX in this system is 0.003 μg/mL.

The increase in median life span in a typical experiment with MTX on the q3d (1, 4, 7) schedule was +60% at a dose of 15 mg/kg. In the same experiment compound 1, i.e., MTX (G_1) diethyl ester,[16] and compound **2** produced increases in median life span of 40 and 10%, respectively, at a dose of 160 mg/kg. Since there was some weight loss indicating toxicity at this dose, and even at 80 mg/kg, higher concentrations of drug were not tested.

The growth-inhibitory activity of compounds 1–**5** was also assayed in vitro against human lymphoblastic leukemia cells (CCRF-CEM) in culture. As indicated in Table II, the γ-monoadduct 1 was at least ten times more active than the α-monoadduct **2**. This was consistent with, and even more striking than, the difference in activity between these compounds in vivo and was also in accord with data we have obtained for the α- and γ-monoethyl esters of MTX.[18] Interestingly, conversion of compound **2** to the triester **4** produced a marked enhancement in activity, but similar modification of compound 1 had the opposite effect. It is also noteworthy that the LLL diastereomer of bis-adduct **3** was 2.4 times more active than the DLL diastereomer, as one might expect from published data on MTX and its D-glutamate analogue.[31]

Experimental Section

IR spectra were obtained on a Perkin-Elmer Model 137B double-beam recording spectrophotometer, and NMR spectra were measured by means of a Varian T-60A with Me_4Si as the reference. TLC was performed on 250-μ Anasil GF or OF silica gel plates (Analabs, Inc., North Haven, Conn.), and spots were visualized in a UV box at 254 nm. Unless otherwise specified, column chromatography was carried out on Baker 5-3405 silica gel (60–200 mesh). Where deactivated silica gel was used, it was prepared as described in the literature.[21] "Dry column" chromatography was performed on Woelm activity grade III/30 mm silica gel (ICN Pharmaceuticals, Inc., Cleveland, Ohio). As used here, the term "dry column" means only that the column was *packed without solvent*. The material to be purified was either applied to the column in a minimum of solvent or preadsorbed on silica gel which was then packed on top of the column. In all other respects, the elution procedure was the same as in conventional chromatography. Melting points were measured in Pyrex capillary tubes in a Mel-Temp apparatus (Laboratory Devices, Inc., Cambridge, Mass.) and are uncorrected. Microanalyses were performed by Galbraith Laboratories, Knoxville, Tenn., and were within ±0.4% of the calculated C, H, and N values.

Direct Coupling of MTX and Diethyl L-Glutamate. A. Diethyl L-glutamate hydrochloride salt (1.8 g, 0.0075 mol) was suspended in Et_2O (50 mL) containing Et_3N (2 mL) and the mixture was stirred magnetically at 0 °C while a solution of diethyl phosphorochloridite (1.2 g, 0.0075 mol) in Et_2O (50 mL) was added dropwise over a 30-min period. After another 30 min of stirring at room temperature, the Et_3N·HCl was filtered (2.1 g, 100% yield) and the filtrate was concentrated to a colorless oil (2.3 g, 95% yield) under reduced pressure. A solution of MTX free acid (2.2 g, 0.005 mol) in dry DMF (30 mL) was heated on a steam bath while a solution of the oily phosphite amide in dry DMF (20 mL) was added gradually, with occasional agitation, over a period of 4.5 h. The reaction mixture was allowed to stand at room

temperature overnight and then reheated on the steam bath while a second portion of phosphite amide (4.0 g, 0.015 mol) in DMF (40 mL) was added with occasional shaking. After another 4 h of heating, the DMF was evaporated. Water (500 mL) was added to the amber-colored syrup, and the aqueous mixture was extracted with $CHCl_3$ (4 × 200 mL) and then H_2O–saturated *n*-butyl alcohol (4 × 500 mL). The combined $CHCl_3$ extracts were washed with H_2O (160 mL), dried, and evaporated to a brown oil. Trituration with Et_2O produced a yellow solid (0.67 g) which was purified by column chromatography on silica gel. The column was eluted with $CHCl_3$–MeOH mixtures ranging in composition from 99:1 to 95:5. Compound **3** was detected by TLC in the 99:1 and 98:2 eluates. Pooling of appropriate tubes and solvent removal left compound **3** as a bright yellow solid (0.18 g, 4.6% yield): IR 1740 (ester C=O), 1625 cm^{-1} (amide C=O); peak height ratio ca. 1:1. The combined *n*-butyl alcohol extracts were washed with H_2O (600 mL) and concentrated to dryness. Residual H_2O was removed by repeatedly adding fresh *n*-butyl alcohol and evaporating under reduced pressure. Trituration of the solid with Et_2O produced a crude product (1.5 g) which was purified further by column chromatography on deactivated silica gel (40 g)[21] with 85:15 $CHCl_3$–MeOH as the eluent. Individual tubes containing 15–20 mL were monitored by TLC and pooled. Evaporation of tubes 7–23 gave compound **2** as a yellow-orange solid (0.63 g, 20% yield), and evaporation of tubes 57–73 gave compound 1 as a yellow-orange solid (0.13 g, 4.3% yield). NMR spectra of the monopeptide adducts (Me_2SO-d_6) contained in each instance peaks at τ 8.8 (CH_2CH_3) and 6.8 (NCH_3) whose integrated areas had the expected 2:1 ratio for compounds containing two ester groups.

B. Methotrexate free acid (0.55 g, 0.0012 mol) was dissolved in hot DMF (10 mL), and the solution was cooled to room temperature. Diethyl L-glutamate hydrochloride salt (0.30 g, 0.0012 mol) and tri-*n*-butylamine (0.3 mL) were added, the solution was stirred briefly, and DCC (0.29 g, 0.0014 mol) in dry DMF (2 mL) was added. After 41 h of stirring, the mixture was filtered in order to remove the *N,N'*-dicyclohexylurea (0.24 g, 75% yield), and the filtrate was concentrated to dryness under reduced pressure. Trituration of the semisolid residue with Et_2O dissolved the remaining unchanged DCC and left a yellow-orange solid (1.0 g) whose TLC showed several spots. A solution of this material in a minimum volume of 2:1 $CHCl_3$–MeOH was applied to a column of deactivated silica gel (40 g)[21] which was eluted successively with 95:5 (160 × 10 mL), 90:10 (50 × 10 mL), and 85:15 (200 × 10 mL) $CHCl_3$–MeOH. Individual tubes were monitored by TLC and pooled into appropriate fractions. Evaporation of tubes 19–60 gave 0.12 g (11% yield) of compound **3** as a yellow-orange solid, evaporation of tubes 125–157 gave 0.24 g (31% yield) of compound **2** as an orange solid, and evaporation of tubes 185–280 gave 0.13 g (17% yield) of compound 1 as a yellow-orange solid: IR 1740 (ester C=O), 1610 cm^{-1} (amide C=O); peak height ratio ca. 1:1 for compound **3** and 1:2 for compounds 1 and **2**.

C. A solution of methotrexate free acid (1.7 g, 0.0037 mol) in dry DMF (30 mL) was cooled to 0–5 °C, and to it were added successively diethyl L-glutamate hydrochloride salt (1.8 g, 0.0074 mol), tri-*n*-butylamine (1.8 mL), and after 5 min a solution of DCC (1.7 g, 0.008 mol) in dry DMF (10 mL). Stirring was continued at 0–5 °C for 3 h and then at room temperature for 39 h, whereupon the white precipitate of *N,N'*-dicyclohexylurea was filtered (yield 1.5 g, 80%), and the filtrate worked up as in the preceding experiment. The semisolid yellow-orange residue was taken up in a minimum volume of $CHCl_3$ and applied to a column of silica gel (40 g, "column A") which was eluted with $CHCl_3$–MeOH mixtures ranging in composition from 100:0 to 80:20. Individual tubes (400 × 20 mL) were monitored by TLC and the contents pooled into three fractions: A_1 (tubes 93–240), A_2 (tubes 241–290), and A_3 (tubes 291–390). Fractions A_1 and A_3 were combined and rechromatographed on silica gel (40 g, "column B"). The column was eluted with $CHCl_3$–MeOH mixtures ranging in composition from 95:5 to 70:30, and individual tubes (25 × 60 mL) were analyzed by TLC and pooled into four fractions: B_1 (tube 2 only), B_2 (tubes 3–19), B_3 (tubes 20 and 21), and B_4 (tubes 22–25). Fractions A_2 and B_2, which were judged to contain the largest amount of compound **3**, were combined and applied to a third column of silica gel (150 g, "column C") which was eluted with 98:2 (25 × 20 mL), 97:3 (25 × 20 mL), 96:4 (25 × 20 mL), 95:5 (100 × 20 mL), 94:6 (75 × 20 mL), 93:7 (25 × 20 mL), and 90:10 (75 × 20 mL) $CHCl_3$–MeOH. Individual tubes were pooled into fractions: C_1 (tubes 71–100), 0.14 g; C_2 (tubes 101–130), 0.30 g; C_3 (tubes 131–156), 0.26 g; C_4 (tubes 157–190), 0.26 g; C_5 (tubes 191–200), 0.059 g; C_6 (tubes 201–210), 0.10 g; C_7 (tubes 211–215), 0.039 g; C_8 (tubes 216–255), 0.37 g; and C_9 (tubes 256–290), 0.12 g. Fractions C_3–C_9 (combined yield 1.2 g, 39%) all gave a single spot (R_f 0.77) on silica gel when the solvent was 3:1 $CHCl_3$–MeOH but a pair of close-moving spots (R_f 0.46 and 0.40) when the solvent was 6:1 $CHCl_3$–MeOH. IR and NMR spectra of all the fractions were essentially indistinguishable. Fractions C_3 and C_8, which were judged to be the most nearly homogeneous by TLC, had mp 87–101 and 85–100 °C, respectively. The microanalytical data were identical within experimental error and indicated that we were dealing with a mixture of LLL and DLL diastereomers of compound **3**. Fractions B_1 and C_1 (combined weight 0.28 g) were TLC homogeneous (R_f 0.82, silica gel, 6:1 $CHCl_3$–MeOH) and had mp 125–140 °C. Their infrared spectra differed from those of compound **3**, and the NMR spectrum (Me_2SO-d_6) revealed more protons in the τ 8.15–9.20 region than would be expected in a tetraethyl ester. These data and the microanalytical results suggested that this fast-moving compound is probably a monoacylurea (structure **8** or **9**).

Methotrexate α,γ-Bis(L-glutamate tetraethyl ester) (3). A. Synthesis from 4-Amino-4-deoxy-N^{10}-methylpteroic Acid and Tetraethyl α,γ-Bis(L-glutamyl-L-glutamate) via Mixed Anhydride Coupling. To a solution of the isobutyl mixed anhydride of 4-amino-4-deoxy-N^{10}-methylpteroic acid (0.34 g, 0.0008 mol)[19] in dry DMF (5 mL) was added a suspension of tetraethyl α,γ-bis(L-glutamyl-L-glutamate) hydrochloride (0.55 g, 0.001 mol) in DMF (5 mL) containing Et_3N (0.12 g, 0.001 mol). The mixture was heated to 70–80 °C on the steam bath for 45 min and then evaporated to dryness under reduced pressure. The brown residue was dissolved in CH_2Cl_2 (50 mL), and the solution was washed successively with H_2O (20 mL), cold 0.1 N $NaHCO_3$ (3 × 20 mL), and cold 0.1 N HCl (2 × 20 mL), rinsed again with H_2O (20 mL), and finally dried and evaporated. The semisolid residue was taken up in a minimum volume of 3:1 $CHCl_3$–MeOH and chromatographed on silica gel (40 g) with 100:0, 99:1, 98:2, 97:3, 96:4, and 95:5 $CHCl_3$–MeOH (200 mL of each) as eluents. The product was recovered from the 95:5 eluates as a yellow solid (0.07 g, 11% yield): mp 87–102 °C; IR 1740 (ester C=O), 1620 cm^{-1} (amide C=O); peak height ratio ca. 1:1. This material was TLC homogeneous and had the same mobility (R_f 0.36, silica gel, 6:1 $CHCl_3$–MeOH) as the slower moving of the two bis-adducts (i.e., fraction "C_8") from the reaction of MTX with 2 equiv of diethyl L-glutamate in the presence of DCC.

B. Synthesis from Compound 2 via Diphenylphosphoryl Azide Coupling. To an ice-cold solution of compound **2** (0.10 g, 0.00016 mol) and diethyl L-glutamate hydrochloride salt (0.045 g, 0.00019 mol) in dry DMF (1.5 mL) were added diphenylphosphoryl azide (0.052 g, 0.00019 mol) in dry DMF (1.0 mL) and then Et_3N (0.035 g, 0.00034 mol). After being stirred at 0 °C for 3 h and room temperature for 41 h, the mixture was concentrated to dryness in vacuo. The amber-colored residue was taken up in CH_2Cl_2 (30 mL) and the solution washed with H_2O (10 mL), 0.1 N $NaHCO_3$ (3 × 10 mL), and finally H_2O (10 mL). Drying of the organic layer and evaporation left a yellow-orange solid which was dissolved in a minimum of $CHCl_3$ and applied to a silica gel dry column (10 g). The column was eluted with 99:1 (22 × 9 mL), 98:2 (22 × 9 mL), and 90:10 (22 × 9 mL) $CHCl_3$–MeOH. Individual tubes were monitored by TLC and pooled into fractions: tubes 34–35, 0.01 g; tubes 36–40, 0.018 g; tubes 41–48, 0.006 g; and tubes 49–54, 0.025 g. The total yield was 0.041 g (36%). The yellow solid obtained from tubes 34–35 was TLC homogeneous (R_f 0.40, silica gel, 6:1 $CHCl_3$–MeOH) and had mp 80–105 °C. The yellow solid from tubes 41–48 was likewise TLC homogeneous (R_f 0.36) and had mp 80–107 °C. Infrared spectra of the two solids were practically superimposable and could not be distinguished from those of the bispeptide prepared via DCC coupling. Thus, it appears that diphenylphosphoryl azide leads to partial racemization, just as DCC does, and that the bis-peptide synthesized by either procedure consists of a mixture of LLL and DLL diastereomers.

Methotrexate γ-L-Glutamate Diethyl Ester (1). Synthesis from 4-Amino-4-deoxy-N^{10}-methylpteroic Acid via Mixed

Anhydride Coupling. To a solution of 4-amino-4-deoxy-N^{10}-methylpteroyl isobutyl mixed anhydride (0.064 g, 0.00015 mol)[19] in dry DMF (3 mL) was added a mixture of diethyl γ-L-glutamyl-L-glutamate hydrochloride salt (0.097 g, 0.00029 mol) and 1,1,3,3-tetramethylguanidine (0.039 g, 0.0003 mol) in dry DMF (3 mL). After being heated for 20 min on the steam bath (80–90 °C), the reaction mixture was evaporated to dryness under reduced pressure and the residue was triturated with Et_2O until a gummy orange-brown solid was obtained. This was applied in a minimum volume of 3:1 $CHCl_3$–MeOH to a silica gel column (25 g) which was developed with 95:5 (22 × 9 mL), 90:10 (22 × 9 mL), 80:20 (22 × 9 mL), and 75:25 (111 × 9 mL) $CHCl_3$–MeOH. Tubes 114–149 were pooled and evaporated to obtain 0.02 g (23% yield) of compound 1 as a TLC homogeneous yellow-orange solid. IR spectra of this material and of the slow-moving monoadduct from the coupling of MTX and diethyl L-glutamate in the presence of DCC were indistinguishable, melting points of the two compounds were identical, and a mixture could not be resolved by TLC (silica gel, 9:6:1 C_6H_6–MeOH–AcOH).

Methotrexate γ-L-Glutamate Triethyl Ester (4). A. Synthesis from Methotrexate α-Monoethyl Ester via Diphenylphosphoryl Azide Coupling. To an ice-cold solution of methotrexate α-monoethyl ester (0.14 g, 0.00029 mol)[18] and diethyl L-glutamate hydrochloride salt (0.094 g, 0.004 mol) in dry DMF (2 mL) were added diphenylphosphoryl azide (0.11 g, 0.0004 mol) in dry DMF (1 mL) and then Et_3N (0.064 g, 0.0006 mol). After being stirred at 0 °C for 5 h and room temperature for another 14 h, the mixture was concentrated to dryness under reduced pressure. The amber-colored semisolid was taken up in CH_2Cl_2 (40 mL) and the solution was washed with H_2O (10 mL), 0.1 N $NaHCO_3$ (3 × 10 mL), and finally H_2O (10 mL). Drying and solvent evaporation left a yellow-orange solid which was purified by passage through a column of silica gel (8 g) with 99:1 (13 × 8 mL), 98:2 (13 × 8 mL), 97:3 (13 × 8 mL), 96:4 (13 × 8 mL), and 94:6 (13 × 8 mL) $CHCl_3$–MeOH as eluents. Tubes 44–61, which contained only a single TLC spot, were pooled and evaporated to a bright yellow solid (0.039 g, 20% yield).

B. Synthesis from Methotrexate γ-L-Glutamate Diethyl Ester. The diester 1 (0.05 g) was dissolved in absolute EtOH (10 mL) containing 0.5% dry HCl gas, the mixture was stirred at room temperature for 24 h, the solvent was evaporated under reduced pressure, and the residue was dissolved in a minimum of $CHCl_3$ and passed through a column of silica gel (10 g) with $CHCl_3$ as the eluent. Evaporation gave a yellow-orange solid (0.025 g, 48% yield): IR 1740 (ester C=O), 1615 cm^{-1} (amide C=O); peak height ratio ca. 1:1. The infrared spectra of material prepared by procedures A and B were superimposable.

Methyltrexate α-L-Glutamate Triethyl Ester (5). A. Synthesis from Methotrexate γ-Monoethyl Ester via Diphenylphosphoryl Azide Coupling. To an ice-cold solution of methotrexate γ-monoethyl ester (0.070 g, 0.00014 mol)[18] and diethyl L-glutamate hydrochloride salt (0.048 g, 0.0002 mol) in dry DMF (1 mL) were added diphenylphosphoryl azide (0.055 g, 0.0002 mol) in dry DMF (0.5 mL) and then Et_3N (0.032 g, 0.0003 mol). After being stirred at 0 °C for 4.5 h and room temperature for 41 h, the mixture was concentrated to dryness under reduced pressure. The orange glass was dissolved in CH_2Cl_2 (20 mL), and the solution was washed with H_2O (6 mL), 0.1 N $NaHCO_3$ (3 × 6 mL), and finally H_2O (6 mL). Drying and evaporation of the organic layer gave a yellow solid which was dissolved in a minimum of 3:1 $CHCl_3$–MeOH and passed through a silica gel dry column (10 g). Elution with 99:1 (22 × 9 mL) and 98:2 (22 × 9 mL) $CHCl_3$–MeOH and evaporation of the pooled contents of tubes 26–36 gave a yellow solid (0.051 g, 55% yield).

B. Synthesis from Methotrexate α-L-Glutamate Diethyl Ester. The diester 2 (0.1 g) was dissolved in absolute EtOH (20 mL) containing 1% dry HCl gas, the solution was stirred at room temperature for 19 h, and the solvent was evaporated. The residue was dissolved in a minimum of $CHCl_3$ and passed through a column of silica gel with $CHCl_3$ as the eluent. Solvent removal left a yellow solid (0.085 g) which was insoluble in 1 N $NaHCO_3$. A portion of this material (0.03 g) was purified further by passage through a silica gel dry column (10 g) using $CHCl_3$ (7 × 7 mL), 99:1 $CHCl_3$–MeOH (7 × 7 mL), and 98:2 $CHCl_3$–MeOH (7 × 7 mL) as eluents. Tubes 4–15 yielded 0.022 g (70% recovery) of analytically pure product: IR 1740 (ester C=O), 1615 cm^{-1} (amide C=O); peak height ratio ca. 1:1.

C. Synthesis from 4-Amino-4-deoxy-N^{10}-methylpteroic Acid and Triethyl α-L-Glutamyl-L-glutamate via Mixed Anhydride Coupling. Isobutyl chloroformate (0.24 g, 0.0018 mol) was added to a mixture of 4-amino-4-deoxy-N^{10}-methylpteroic acid (0.55 g, 0.0015 mol, based on the formula $C_{15}H_{15}N_7O_2 \cdot 0.5HCl \cdot 1.5H_2O$)[19] and Et_3N (0.3 g, 0.003 mol) in dry DMF (40 mL). After 15 min of stirring at room temperature, a suspension of triethyl α-L-glutamyl-L-glutamate hydrochloride salt (1.0 g, 0.0026 mol) in dry DMF (5 mL) containing Et_3N (0.3 g, 0.003 mol) was added. Stirring was continued at room temperature for 30 min and at 70–75 °C (steam bath) for another 30 min, and the solvent was removed in vacuo. Trituration of the semisolid residue with Et_2O produced a yellow-orange solid. Further trituration with 0.2 N $NaHCO_3$ (300 mL), extraction with $CHCl_3$ (5 × 100 mL), washing of the combined organic layers with H_2O (100 mL), 0.1 N HCl (200 mL), and finally H_2O (200 mL), drying, and evaporation afforded a yellow solid (0.14 g) which was dissolved in a minimum of $CHCl_3$ and chromatographed on silica gel (20 g) with 100:0, 99:1, 98:2, 97:3, 96:4, and 95:5 $CHCl_3$–MeOH (200 mL each) as eluents. The product was obtained on evaporation of the 95:5 $CHCl_3$–MeOH eluate as a bright yellow solid (0.07 g). The infrared spectrum was identical with the spectra of the products isolated in the preceding two experiments. Moreover, clear differences were seen in IR spectra of triesters 4 and 5 in the 1300–1400- and 1100–1140-cm^{-1} fingerprint regions. The NMR spectrum of compound 5 ($CDCl_3$) showed signals at τ 8.85 (m, 9 H, CH_3CH_2O), 7.2–8.4 (br m, 8 H, glutamyl CH_2CH_2), 6.93 (s, 3 H, NMe), 5.0–6.4 (m, 10 H, CH_3CH_2O, NHCH, NCH_2), 1.1–4.3 (complex m, 11 H, 2- and 4-NH_2, CONH protons, phenyl and C_7-pteridine protons). Careful neutralization of the 0.1 N HCl wash (vide supra) with 1 N NaOH and extraction with $CHCl_3$ (2 × 100 mL) gave an additional 0.04 g of product: total 0.11 g (9.5% yield). Acidification of the 0.2 N $NaHCO_3$ wash (vide supra) led to precipitation of unchanged 4-amino-4-deoxy-N^{10}-methylpteroic acid (0.13 g, 24% recovery).

Diethyl γ-L-Glutamyl-L-glutamate Hydrochloride Salt. To a stirred mixture of *N*-benzyloxycarbonyl-L-glutamic acid α-monobenzyl ester (1.11 g, 0.003 mol) and diethyl L-glutamte hydrochloride salt (0.864 g, 0.0036 mol) in dry DMF (6 mL) at 0 °C was added an ice-cold solution of diphenylphosphoryl azide (0.99 g, 0.0036 mol) and Et_3N (0.67 g, 0.0066 mol) in dry DMF (3 mL). After 3.5 h of stirring at 0 °C, the reaction mixture was filtered and the DMF evaporated under reduced pressure to obtain a light brown semisolid. This was taken up in EtOAc (100 mL), a small amount of undissolved white solid was removed, and the solution was washed successively with H_2O (20 mL), 0.1 N $NaHCO_3$ (2 × 20 mL), 0.1 N HCl (2 × 20 mL), and H_2O (20 mL). Drying and evaporation yielded a syrup from which crystalline material was obtained by dissolving it in a small volume of EtOAc, adding petroleum ether (bp 40–50 °C), and allowing the solution to stand overnight at room temperature. Two crops totaling 1.14 g (68% yield) were obtained: mp 90–91 °C (EtOAc–petroleum ether); R_f 0.68 (silica gel, 25:1 $CHCl_3$–MeOH). Anal. ($C_{29}H_{36}N_2O_9$) C, H, N.

The protected dipeptide (1.13 g, 0.002 mol) and 1 mL of 15% HCl in aqueous EtOH were added to a suspension of prereduced 10% Pd/C (0.2 g) in glacial AcOH in a Parr hydrogenation bottle. After 2.5 h of shaking under 3 atm of hydrogen, the catalyst was filtered and the solvent evaporated under reduced pressure to obtain an amber-colored syrup which could not be made to crystallize from a variety of solvents. Column chromatography on silica gel (40 g) with 90:10 (40 × 10 mL), 85:15 (40 × 10 mL), and 80:20 (60 × 10 mL) $CHCl_3$–MeOH as eluents gave 0.48 g (71% yield) of ninhydrin-positive colorless solid whose TLC contained a single major spot (R_f 0.35, silica gel, 1:1 $CHCl_3$–MeOH) and two trace impurities (R_f 0.51 and 0.71): mp 95–97 °C. This material was coupled directly to 4-amino-4-deoxy-N^{10}-methylpteroic acid (vide supra) to obtain compound 1.

Triethyl α-L-Glutamyl-L-glutamate Hydrochloride Salt. A solution of L-glutamic acid γ-monoethyl ester (3.5 g, 0.02 mol) in H_2O (40 mL) was adjusted to pH 9 with a few drops of 5 N NaOH and diluted with acetone (15 mL). To this solution was added dropwise with stirring (20 min) a solution of benzyl chloroformate (4.5 g, 0.025 mol) in acetone (20 mL). The pH of the solution was kept at ca. 9 by occasional addition of 5 N NaOH

(20 mL total) and the temperature was maintained at 20–25 °C by means of a cooling bath. After 2 h at room temperature the reaction mixture was cooled in ice while the pH was adjusted to 2 with 12 N HCl. The product which precipitated was extracted into EtOAc (4 × 50 mL) and the combined extracts were washed with H_2O (2 × 50 mL), dried overnight, and evaporated to a colorless syrup which was kept in a P_2O_5 desiccator overnight to remove the last traces of moisture: yield 5.4 g (87%). This noncrystalline *N*-benzyloxycarbonyl derivative (3.1 g, 0.01 mol) was dissolved directly in a mixture of dry THF (30 mL) and *N*-methylmorpholine (1.1 g, 0.011 mol), the solution was cooled to about −20 °C, and isobutyl chloroformate (1.4 g, 0.01 mol) was added. The mixture was stirred at −15 °C for 30 s, and then a suspension of diethyl L-glutamate hydrochloride salt (2.4 g, 0.01 mol) in dry THF (20 mL) containing *N*-methylmorpholine (1.1 g, 0.011 mol) was added. After 30 min at 0 °C and 21 h at room temperature, the reaction mixture was poured into 5% $NaHCO_3$ (250 mL) and the product extracted into EtOAc (4 × 100 mL). The combined extracts were washed with H_2O (100 mL), 0.1 N HCl (3 × 80 mL), and finally H_2O (100 mL), and the organic layer was dried overnight. Evaporation left a white solid (3.8 g) which was purified by recrystallization from EtOAc–petroleum ether (bp 40–50 °C): yield 2.1 g (42%); mp 98.5–100 °C. Anal. ($C_{24}H_{34}N_2O_9$) C, H, N.

The protected dipeptide (1.3 g, 0.0026 mol) and 1 mL of 15% HCl in aqueous EtOH were added to a Parr hydrogenation bottle containing absolute EtOH (170 mL) and 0.2 g of previously hydrogen-saturated 10% Pd/C catalyst. The mixture was shaken under 3 atm of hydrogen for 4 h, the catalyst was removed, and the solvent was evaporated under reduced pressure. The resultant straw-colored syrup was TLC homogeneous and gave a positive ninhydrin test and was therefore coupled directly to 4-amino-4-deoxy-N^{10}-methylpteroic acid (vide supra) without further purification in order to obtain compound **5**.

Tetraethyl α,γ-Bis(L-glutamyl-L-glutamate) Hydrochloride Salt. Dicyclohexylcarbodiimide (1.67 g, 0.008 mol) was added to an ice-cold solution of *N*-benzyloxycarbonyl-L-glutamic acid (1.14 g, 0.004 mol) and *N*-hydroxysuccinimide (0.95 g, 0.008 mol) in dry DMF (10 mL). The mixture was allowed to stand in the refrigerator for 20 h, at which time a suspension of diethyl L-glutamate hydrochloride salt (1.92 g, 0.008 mol) in dry DMF (4 mL) containing Et_3N (0.81 g, 0.008 mol) was added. The reaction mixture was stirred at room temperature for 2 h and then filtered to remove the dicyclohexylurea and triethylammonium chloride. Evaporation of the filtrate under reduced pressure left a light brown semisolid which was taken up in EtOAc (100 mL). A small amount of white solid which remained undissolved was filtered off, and the EtOAc solution was washed consecutively with H_2O (25 mL), 0.1 N $NaHCO_3$ (3 × 25 mL), 0.1 N HCl (3 × 25 mL), and H_2O (25 mL). The organic layer was then dried and concentrated to a volume of 25 mL. Overnight standing at room temperature yielded another small crop of dicyclohexylurea which was removed by filtration. Further reduction in volume and addition of petroleum ether (bp 40–50 °C) gave a colorless solid (1.51 g, 58% yield): mp 142–143 °C (EtOAc–petroleum ether); R_f 0.42 (silica gel, 25:1 $CHCl_3$–MeOH). Anal. ($C_{31}H_{45}N_3O_{12}$) C, H, N.

The protected tripeptide (1.3 g, 0.002 mol) was hydrogenolyzed in EtOH (150 mL) containing 15% HCl by weight in the presence of 10% Pd/C as described in the preceding experiment. The catalyst was removed with the aid of Celite and the filtrate evaporated to a pale yellow syrup whose TLC revealed a single major product (R_f 0.67, silica gel, 6:1 $CHCl_3$–MeOH) and only one minor contaminant (R_f 0.15). Both spots were ninhydrin-positive, and there was evidence of unreacted starting material. Attempts to recrystallize the tripeptide from various solvent mixtures were unsuccessful, and it was therefore coupled to 4-amino-4-deoxy-N^{10}-methylpteroic acid (vide supra) without additional purification in order to obtain the LLL diastereomer of compound **3**.

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